universität wien

# DISSERTATION

Titel der Dissertation

## Development of diagnostic PCR assays for typing and subtyping of the highly pathogenic bacterial taxa *Brucella*, *Francisella* and *Yersinia pestis*

angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr. rer. nat.)


| | |
|---|---|
| Verfasserin: | MMag. Birgit Huber |
| Matrikel-Nummer: | 9720369 |
| Dissertationsgebiet: | Genetik - Mikrobiologie |
| Betreuer: | Univ.-Doz. Dr. Hans-Jürgen Busse |


Wien, im September 2010

# Acknowledgements

The work presented here was carried out between October 2005 and August 2008 at the Institute of Bacteriology, Mycology and Hygiene at the University of Veterinary Medicine in Vienna under the supervision of Univ.-Doz. Dr. Hans-Jürgen Busse.

I want to thank Hans-Jürgen Busse for giving me the opportunity to work on this thesis, as well as for his amicable mentoring and excellent guidance. I am especially grateful for his straightforwardness and ceaseless support throughout my studies.

I also thank Univ.-Prof. Dr. Dr. Renate Rosengarten for warmly welcoming me in her department.

Furthermore, I want to thank all my lab colleagues for their continuous help, vivid and fruitful discussions as well as many cheerful moments. Special thanks in this respect go to Stefan Langer for his assistance and friendship.

I thank all members of the IBMH for their help and constructive comments, their contribution to the good working atmosphere and for three unforgettable years.

In remembrance of the late Dr. Wolfgang Jechlinger, I would like to add that I am deeply grateful for his crucial help and valuable advice.

Last but most, I wish to thank my family and Clemens for their uncompromising support, encouragement and patience.

# Abstract

The present study was conducted in order to develop rapid diagnostic assays on the basis of conventional PCR which allow detection and discrimination of species, subspecies and biovars of the potential bioterrorist agents *Francisella*, *Brucella* and *Yersinia* not targeted by the currently established gel-based PCR assays. Reference and field strains were analysed using RAPD and rep-PCR in order to find regions of genetic heterogeneity in the genomes of the closely related species, subspecies and biovars for each of the different genera. A number of promising differences could be found in the resulting RAPD fingerprints. Distinctive bands were cloned in *E. coli* and sequenced. Differences on the molecular level found through analysis of the obtained sequence data as well as genome comparisons, together with expedient information taken from previous external studies were used to develop specific and robust PCR assays for typing and subtyping of the taxa *Brucella* and *Francisella* as well as an assay that can identify atypical *Yersinia pseudotuberculosis* strains.

In addition to the commonly used PCR assays like the AMOS-PCR and some variants thereof, the developed *Brucella* assay specifically identified *Brucella neotomae*, *Brucella pinnipedialis*, *Brucella ceti*, and *Brucella microti*. Furthermore, it differentiated *Brucella abortus* biovars 1, 2, 4 from biovars 3, 5, 6, 9, as well as between *Brucella suis* biovar 1, biovars 3, 4, and biovars 2, 5. The *Francisella* multiplex-PCR developed in the course of this study distinguished between the novel *Francisella* species *Francisella hispaniensis*, representatives of all other *Francisella* species and subspecies, clades A.I and A.II of *Francisella tularensis* subsp. *tularensis* and *Francisella tularensis* subsp. *holarctica* biovar japonica. The novel *Yersinia* triplex assay allowed for a clear distinction between *Yersinia pestis* and *Yersinia pestis*-like *Yersinia pseudotuberculosis* strains and distinguished them from typical strains of *Y. pseudotuberculosis*.

For a molecular comparison of strains and to assure the specificity of the developed PCR assays, it was indispensible to determine unambiguously the taxonomic status of field strains using the available methods. In collaboration with colleagues from other institutes, a great number of field strains of *Brucella* and other representatives of the family *Brucellaceae,* including *Ochrobactrum, Pseudochrobactrum* and *Paenochrobactrum,* as well as strains of *Francisella* were

therefore classified applying classical microbiological and chemotaxonomical methods, analyses of housekeeping genes (*rrs*, *recA*, *gyrB*, *groEL*), RAPD-PCR and partly also DNA-DNA hybridization. Strains that could not be assigned to a recognized species were proposed as novel species, leading to the description of two *Brucella* species (*B. microti*, *B. inopinata*), five *Ochrobactrum* species (*O. pseudogrignonense*, *O. pituitosum*, *O. rhizosphaerae*, *O. haematophilum*, *O. thiophenivorans*), two *Pseudochrobactrum* species (*Ps. kiredjianiae*, *Ps. lubricantis*), one *Paenochrobactrum* species (*Paenochrobactrum gallinarii*) and a novel *Francisella* species (*F. hispaniensis*).

# Zusammenfassung

Die vorliegende Studie wurde durchgeführt, um diagnostische Schnelltests auf der Basis konventioneller PCR (Polymerase Kettenreaktion) zu entwickeln, die die Detektion und Unterscheidung jener Arten, Unterarten und Biovare der potentiell bioterroristisch nutzbaren Bakterien der Gattungen *Francisella*, *Brucella* und *Yersinia* erlauben, die noch nicht durch gängige Gel-basierte PCR-Tests erkannt werden. Referenzstämme und Feldisolate wurden mittels RAPD (randomly amplified polymorphic DNA) und rep-PCR (gegen repetitive, nichtkodierende, intercistronische Abschnitte gerichtet) analysiert, um Regionen genetischer Variabilität zwischen den eng verwandten Arten, Unterarten und Biovaren der einzelnen Gattungen ausfindig zu machen. Mittels dieses Ansatzes wurden in den resultierenden RAPD Mustern spezifische PCR-Produkte gefunden. Diese Produkte wurden in *E. coli* kloniert und dann sequenziert. Sequenzunterschiede, die im Vergleich zu in Datenbanken hinterlegten Sequenzen identifiziert werden konnten, zusammen mit Informationen aus diversen Veröffentlichungen, wurden verwendet, um spezifische und robuste PCR Schnelltests zur Typisierung und Subtypisierung der Taxa *Brucella* und *Francisella* zu entwickeln. Weiters wurde ein PCR-Test entwickelt, um *Yersinia pestis*-ähnliche *Yersinia pseudotuberculosis* Stämme zu identifizieren.

Zusätzlich zu den allgemein gebräuchlichen PCR Tests wie der AMOS-PCR und einigen ihrer Varianten kann der neu entwickelte *Brucella* Ansatz *Brucella neotomae*, *Brucella pinnipedialis*, *Brucella ceti* und *Brucella microti* spezifisch detektieren. Darüber hinaus konnte der Test die *Brucella abortus* Biovare 1,2,4 von den Biovaren 3,5,6 und 9, sowie auch *Brucella suis* Biovar 1 von den Biovaren 3,4 und 2,5 unterscheiden. Ein im Rahmen dieser Arbeit entwickelter *Francisella* Multiplex-Ansatz differenzierte zwischen der neuen Art *Francisella hispaniensis*, Repräsentanten aller anderer *Francisella*-Arten und Unterarten, *F. tularensis* subsp. *tularensis* A.I und A.II Stämmen und Biovar japonica der Unterart *F. tularensis* subsp. *holarctica*. Der neu entwickelte *Yersinia* Triplex-Ansatz ermöglichte eine spezifische Unterscheidung von *Y. pestis* und atypischen *Y. pestis*-ähnlichen *Y. pseudotuberculosis* Stämmen und unterschied sie auch gleichzeitig von anderen Stämmen der Spezies *Y. pseudotuberculosis*.

Zur Evaluierung der entwickelten Tests war es unerlässlich, die taxonomische Stellung einer Reihe von Feldisolaten zu bestimmen. Im Laufe dieser Studie wurden deshalb zusammen mit Kollegen anderer Institute eine große Zahl an Feldisolaten von *Brucella* und anderen Vertretern der Familie *Brucellaceae* (*Ochrobactrum, Pseudochrobactrum* und *Paenochrobactrum*) wie auch von *Francisella* charakterisiert, wobei klassische mikrobiologische und chemotaxonomische Methoden sowie die Analyse konservierter Gene (*rrs*, *recA*, *gyrB*, *groEL*) und RAPD-PCR Anwendung fanden. In einigen Fällen wurden auch DNA-DNA Hybridisierungen durchgeführt, um den Speziesstatus von Stämmen eindeutig zu klären. Stämme, die keiner etablierten Art zugeordnet werden konnten, wurden dann als neue Art beschrieben; fünf *Ochrobactrum* Arten (*O. haematophilum*, *O. rhizosphaerae*, *O. pituitosum*, *O. pseudogrignonense*, *O. thiophenivorans*), zwei *Pseudochrobactrum* Arten (*Ps. kiredjianiae*, *Ps. lubricantis*), und eine *Paenochrobactrum* Art (*Paenochrobactrum gallinarii*), zwei *Brucella* Arten (*B. microti*, *B. inopinata*) und eine *Francisella* Art (*F. hispaniensis*).

# List of Publications

**Major parts of this thesis are published:**

**Huber, B.**, Escudero, R., Busse, H.-J., Seibold, E., Scholz, H. C., Anda, P., Kämpfer, P. & Splettstoesser, W. D. (2010). Description of *Francisella hispaniensis* sp. nov., isolated from human blood, reclassification of *Francisella novicida* (Larson *et al.* 1955) Olsufiev *et al.* 1959 as *Francisella tularensis* subsp. *novicida* comb. nov., and emended description of the genus *Francisella. Int J Syst Evol Microbiol* 60, 1887-1896.

**Huber, B.**, Scholz, H. C., Kämpfer, P., Falsen, E., Langer, S. & Busse, H.-J. (2010). *Ochrobactrum pituitosum* sp. nov., isolated from an industrial environment. *Int J Syst Evol Microbiol* 60, 321-326.

**Huber, B.**, Scholz, H. C., Lucero, N. & Busse, H.-J. (2009). Development of a PCR assay for typing and subtyping of *Brucella* species. *Int J Med Microbiol* 299, 563-573.

Kämpfer, P., Martin, E., Lodders, N., Jäckel, U., **Huber, B. E.**, Schumann, P., Langer, S., Busse, H.-J. & Scholz, H. C. (2010). *Paenochrobactrum gallinarii* gen. nov., sp. nov., isolated from the air of a duck barn and reclassification and emendation of *Pseudochrobactrum glaciei* as *Paenochrobactrum glaciei* comb. nov. *Int J Syst Evol Microbiol* 60, 1493-1498.

Scholz, H. C., Nöckler, K., Göllner, C., Bahn, P., Vergnaud, G., Tomaso, H., Al-Dahouk, S., Kämpfer, P., Cloeckaert, A., Maquart, M., Zygmunt, M. S., Whatmore, A. M., Pfeffer, M., **Huber, B.**, Busse, H.-J. & De, B. K. (2010). *Brucella inopinata* sp. nov., isolated from a breast implant infection. *Int J Syst Evol Microbiol* 60, 801-808.

Kämpfer, P., **Huber, B.**, Lodders, N., Warfolomeow, I., Busse, H.-J. & Scholz, H. C. (2009). *Pseudochrobactrum lubricantis* sp. nov., isolated from a metal-working fluid. *Int J Syst Evol Microbiol* 59, 2464-2467.

Kämpfer, P., Sessitsch, A., Schloter, M., **Huber, B.**, Busse, H.-J. & Scholz, H. C. (2008). *Ochrobactrum rhizosphaerae* sp. nov. and *Ochrobactrum thiophenivorans* sp. nov., isolated from the environment. *Int J Syst Evol Microbiol* 58, 1426-1431.

Scholz, H. C., Hubalek, Z., Sedlácek, I., Vergnaud, G., Tomaso, H., Al Dahouk, S., Melzer, F., Kämpfer, P., Neubauer, H., Cloeckaert, A., Maquart, M., Zygmunt, M. S., Whatmore, A. M., Falsen, E., Bahn, P., Göllner, C., Pfeffer, M., **Huber, B.**, Busse, H.-J. & Nöckler, K. (2008). *Brucella microti* sp. nov., isolated from the common vole *Microtus arvalis*. *Int J Syst Evol Microbiol* 58, 375-382.

Kämpfer, P., Scholz, H. C., **Huber, B.**, Falsen, E. & Busse, H.-J. (2007). *Ochrobactrum haematophilum* sp. nov. and *Ochrobactrum pseudogrignonense* sp. nov., isolated from human clinical specimens. *Int J Syst Evol Microbiol* 57, 2513-2518.

Kämpfer, P., Scholz, H., **Huber, B.**, Thummes, K., Busse, H.-J., Maas, E. W. & Falsen, E. (2007). Description of *Pseudochrobactrum kiredjianiae* sp. nov. *Int J Syst Evol Microbiol* 57, 755-760.

**Conference contributions**

- 6$^{th}$ International Conference on Tularemia, September 2009 (Berlin, Germany):
  Huber, B., Busse, H.-J., Seibold, E., Scholz, H. C., Kämpfer, P. & Splettstoesser, W. D. Description of '*Francisella novicida*' FSC454$^{T}$ as a novel species of the genus *Francisella*. Abstract S 4-4.

- Joint Annual Conference of the Association for General and Applied Microbiology and the German Society for Biochemistry and Molecular Biology, March 2008 (Frankfurt, Germany):
  Huber, B., Scholz, H. C. & Busse, H.-J. A novel multiplex PCR assay for the identification and differentiation of established *Brucella* species and selected biovars. Poster abstract. BioSpektrum Tagungsband 2008:197.

- Annual Conference of the Association for General and Applied Microbiology, April 2007 (Osnabrück, Germany):
  Huber, B. E., Scholz, H. C. & Busse, H.-J. Use of *gyrB* and *groEL* sequences for classification of *Brucella* and *Brucella*-like strains. Poster abstract. BioSpektrum Tagungsband 2007:155.

**Further publications, not integrated into this thesis:**

Kämpfer, P., Lodders, N., **Huber, B.**, Falsen, E. & Busse, H.-J. (2008). *Deinococcus aquatilis* sp. nov., isolated from water. *Int J Syst Evol Microbiol* 58, 2803-2806.

Kämpfer, P., **Huber, B.**, Buczolits, S., Thummes, K., Grun-Wollny, I. & Busse, H.-J. (2008). *Streptomyces specialis* sp. nov. *Int J Syst Evol Microbiol* 58, 2602-2606.

Fahrbach, M., Kuever, J., Remesch, M., **Huber, B. E.**, Kämpfer, P., Dott, W. & Hollender, J. (2008). *Steroidobacter denitrificans* gen. nov., sp. nov., a steroidal hormone-degrading gammaproteobacterium. *Int J Syst Evol Microbiol* 58, 2215-2223.

Young, C. C., Kämpfer, P., Ho, M. J., Busse, H.-J., **Huber, B. E.**, Arun, A.B., Shen, F. T., Lai, W. A. & Rekha, P. D. (2007). *Arenimonas malthae* sp. nov., a gammaproteobacterium isolated from an oil-contaminated site. *Int J Syst Evol Microbiol* 57, 2790-2793.

Albert, R. A., Archambault, J., Lempa, M., Hurst, B., Richardson, C., Gruenloh, S., Duran, M., Worliczek, H. L., **Huber, B. E.**, Rosselló-Mora, R., Schumann, P. & Busse, H.-J. (2007). Proposal of *Viridibacillus* gen. nov. and reclassification of *Bacillus arvi*, *Bacillus arenosi* and *Bacillus neidei* as *Viridibacillus arvi* gen. nov., comb. nov., *Viridibacillus arenosi* comb. nov. and *Viridibacillus neidei* comb. nov. *Int J Syst Evol Microbiol* 57, 2729-2737.

Kämpfer, P., Rosselló-Mora, R., Hermansson, M., Persson, F., **Huber, B.**, Falsen, E. & Busse, H.-J. (2007). *Undibacterium pigrum* gen. nov., sp. nov., isolated from drinking water. *Int J Syst Evol Microbiol* 57, 1510-1515.

Kämpfer, P., **Huber, B.**, Buczolits, S., Thummes, K., Grün-Wollny, I. & Busse, H.-J. (2007). *Nocardia acidivorans* sp. nov., isolated from soil of the island of Stromboli. *Int J Syst Evol Microbiol* 57, 1183-1187.

Kämpfer, P., **Huber, B.**, Thummes, K., Grün-Wollny, I. & Busse, H.-J. (2007). *Actinoplanes couchii* sp. nov. *Int J Syst Evol Microbiol* 57, 721-724.

Lai, W. A., Kämpfer, P., Arun, A. B., Shen, F. T., **Huber, B.**, Rekha, P. D. & Young, C. C. (2006). *Deinococcus ficus* sp. nov., isolated from the rhizosphere of *Ficus religiosa* L. *Int J Syst Evol Microbiol* 56, 787-791.

# Abbreviations

| | |
|---|---|
| acc. no. | accession number |
| AFLP | amplified fragment length polymorphism |
| AMOS PCR | *Brucella abortus*/*melitensis*/*ovis*/*suis* differentiating PCR |
| BOX | repetitive genomic element |
| bp | base pair(s) |
| BWC | Biological Weapons Convention |
| CDC | Centers for Disease Control and Prevention (http://www.cdc.gov) |
| cfu | colony forming unit |
| DIG | digoxigenine |
| DNA | deoxyribonucleic acid |
| dNTPs | deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP) |
| ELISA | enzyme-linked immunosorbent assay |
| EM | electron microscopy |
| ERIC | enterobacterial repetitive intergenic consensus |
| FESLF | Far East scarlet-like fever |
| IFA | immunofluorescent assay |
| IRS-PCR | infrequent restriction site PCR |
| IS | insertion sequence |
| kb | kilobase pair(s) |
| MLST | multilocus sequence typing |
| MLVA | multilocus variable number tandem repeat (VNTR) analysis |
| PCR | polymerase chain reaction |
| PFGE | pulsed-field gel electrophoresis |
| RAPD | random amplified polymorphic DNA |
| REP | repetitive extragenic palindromic |
| rep-PCR | interspersed repetitive element (REP, ERIC, BOX) PCR |
| RFLP | restriction fragment length polymorphism |
| RT | room temperature |
| SNP | single nucleotide polymorphism |
| spp. | species (plural) |
| subsp. | subspecies |

| | |
|---|---|
| TAE | Tris/acetic acid/EDTA |
| U | unit(s) |
| VNTR | variable number tandem repeat |
| 16S rRNA gene | 16S ribosomal ribonucleic acid gene (*rrs*) |

# Table of Contents

# 1. Introduction

## 1.1. Bioterrorism and biothreat agents

Bioterrorism has been a subject of increasing worldwide concern. In the last century, especially between World War I and the early Cold War, there was active research for biological weapons regardless of the prohibition of their use by the 1925 Geneva Protocol. However, the anticipated growing danger for all parties led to an expanded ban on the development, production, trading and use of microbial or other biological and toxin weapons imposed by the 1972 Biological Weapons Convention (BWC) which has hitherto been signed by more than 170 states. (Biological and Toxin Weapons Convention website, http://www.opbw.org) However, policy makers are currently seeking to strengthen the BWC because of the ongoing proliferation of biological weapons and biotechnologies as well as the apprehended danger of bioterrorist acts (Christopher et al., 1997; Eubanks et al., 2007). Additionally, there is the fear that molecular biology and biotechnology could intentionally be used for the manipulation of microbes to fit homicidal purposes which would add a new dimension to warfare and terrorist threat in future (Cannons et al., 2006).

Among previous incidents of bioterrorist activities (Tucker, 1999), the most recent anthrax attacks of 2001 in the U.S. directed political and scientific attention to the problem of bioterrorism (Bossi et al., 2004a; Murphy, 2004; Schuler, 2004) and once more emphasised the importance of epidemiological studies and of diagnostic tools that can quickly and accurately identify infectious agents to facilitate a rapid response in both, a bioterroristic event or naturally occurring outbreak. The diagnosis of the classic bacterial agents of biological warfare (*Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, *Brucella* spp., *Burkholderia mallei*, *Burkholderia pseudomallei*) and other biothreat agents listed by the CDC and the U.S. Department of Health and Human Services has thus become a very important subject of research over the last decade. Various molecular diagnostic assays rapidly detecting and confirming biothreat agents have already been developed (Oggioni et al., 2002; Ellerbrok et al., 2002; Bell et al., 2002; Loïez et al., 2003; Lundquist et al., 2005; Wilson et al., 2005; Ulrich et al., 2006; Larsson et al., 2007; Van Ert et al., 2007; Das et al., 2008; Yang

et al., 2008; Wang et al., 2009; Yang et al., 2009; Deshpande et al., 2010; Matero et al., 2010).

### 1.2. Outline and methodology of the study

In the face of the recent terrorist attacks involving a biological threat agent, *Bacillus anthracis*, research in the area of biodefense was also intensified in Europe. The present study, supported by a Contract-Research Project of the German Ministry of Defence (Grant No. M-SAB1-5-A005), was conducted in order to develop differentiating PCR assays for rapid and accurate typing and subtyping of three highly pathogenic proteobacterial taxa, namely *Francisella*, *Brucella* and *Yersinia*, causing zoonotic diseases in humans and considered to have bioterroristic potential (for their clinical implications see Table 1). *Francisella tularensis* and *Yersinia pestis* are classified by the CDC as category (A) pathogens and *Brucella* spp. as category (B) pathogens and thus are recognised as biological threat agents (Rotz et al., 2002). None of these organisms are known to have been used in bioterrorist actions so far but they have been thoroughly studied or applied as bioweapons and hence are important subjects of research in the area of biodefense (Christopher et al., 1997).
So far, the major focus of diagnostic biodefense research has been on the development and optimization of sensitive, specific and reproducible immunoassays, real-time PCR assays, DNA microarrays and biochip arrays for immunological detection, flow cytometry, and chemical and physical biosensors that can, often

**Table 1.** The clinical implications of *Brucella* spp., *Yersinia pestis* and *Francisella tularensis* (adapted from Franz et al., 1997)

| Agent | Infective dose (Aerosol) | Incubation period | Diagnostic samples | Clinical diagnostic assay | Patient isolation precautions |
|---|---|---|---|---|---|
| ***Brucella* spp.** causing brucellosis | 10-100 organisms | 5-60 days | blood, bone marrow, serum | culture<br>serology: agglutination | standard precautions, contact isolation if draining lesions present |
| ***Yersinia pestis*** causing plague | 100-500 organisms | 2-3 days | blood, sputum, lymph node aspirate | Gram or Wright-Giemsa stain, antigen-ELISA, culture, serology: ELISA, IFA | pneumonic: droplet precautions until patient treated for 3 days |
| ***Francisella tularensis*** causing tularemia | 10-50 organisms | 2-10 days | blood, sputum, serum, EM of tissue | culture,<br>serology: agglutination | standard precautions |

simultaneously, detect a range of different biothreat agents at the species or genus level (Lim et al., 2005). But, since especially taxa like *Francisella tularensis* with several subspecies and biovars, the genetically and phenotypically highly similar *Brucella* species with their biovars and other members of the family *Brucellaceae* and *Yersinia pestis* with its biovars and being a clone of *Y. pseudotuberculosis* (Achtman et al., 1999) can internally only be separated with difficulty and conventional typing methods are risky for the lab personnel, the development of PCR-based assays that primarily aim at subtyping those highly pathogenic taxa was a major reason for carrying out this study.

The technology of choice for the diagnostic assays to be developed was conventional gel-based PCR, on the one hand, because of the actual laboratory equipment and expertise and, on the other hand, because it has become a worldwide standard procedure and the assays are cost-effective and easy to reproduce without any sophisticated know-how. The core strategy towards the development of the assays for the studied taxa was to find genetically unique or highly variable regions in the respective genomes via genomic fingerprinting (RAPD and rep-PCR).

Despite their high level of genomic similarity, there is a range of different methods for the identification of group-specific variations in brucellae, *F. tularensis* and *Y. pestis*/*pseudotuberculosis* that have shown the potential to identify useful biomarkers. These include IS-anchored arbitrary PCR (Ouahrani-Bettache et al., 1996), infrequent restriction site-PCR and restriction maps (Cloeckaert et al., 2003; Michaux-Charachon et al., 1997), multilocus sequencing (Whatmore et al., 2007), ribotyping (Verger et al., 2000), suppression subtractive hybridization (Radnedge et al., 2001; Radnedge et al., 2002; Wang et al., 2006; Molins et al., 2008), comparative whole-genome hybridization and DNA microarray analyses (Rajashekara et al., 2004; Zhou et al., 2004b; Broekhuijsen et al., 2003; Zhou et al., 2004d) and whole-genome sequence comparisons (Halling et al., 2005; Chain et al., 2005; Ratushna et al., 2006; Chain et al., 2006). RAPD-profiles have been used to identify species-specific DNA fragments in order to develop PCR assays for the identification of species within bacterial taxa other than *Brucella*, *Francisella* and *Yersinia* (Manulis et al., 1994; Guillot and Mouton, 1997; Teng et al., 2000; Brown et al., 2004). Since this method is technically less demanding than others and has the potential to reveal not only species-specific but also subgroup-specific differences within the conserved genomes of the studied taxa (Fekete et al., 1992; Tcherneva et al., 1996; 2000;

Mercier et al., 1996; Johansson et al., 2000; De La Puente-Redondo et al., 2000), the approach of using RAPD and rep-PCR fingerprinting for the identification of biomarkers seemed promising.

Successful detection of clearly differentiating bands was to be followed by excision of the bands from the gel and cloning, sequencing and analysis of the DNA fragments. The sequence information on differences within the conserved genomes of *Brucella* species and biovars, *Francisella tularensis* subspecies and biovars and strains of the *Yersinia pestis/pseudotuberculosis* group obtained through RAPD and rep-PCR fingerprinting and subsequent analyses was to be used to design specifc primers for the diagnostic assays which should then be able to differentiate highly pathogenic members within *Brucella*, *Francisella* and *Yersinia* from less pathogenic or non-pathogenic members and that do not only allow for a specific identification on the species level, but also enable a differential detection of subspecies, biovars and other subgroups, many of which had so far not been included in previously published assays.

Also important was the often neglected exact determination of the taxonomic status of the field strains to be used to determine the specificity of the novel assays. Cultivation of *Francisella*, *Brucella* and *Yersinia pestis* strains and standard biotyping of field strains was to be done in a biosafety level 3 laboratory (InstMikroBioBw TE010) by our collaborators of the Bundeswehr Institute of Microbiology, Neuherbergstraße 11, 80937 Munich. Other *Yersinia* strains and the *Brucella*-like strains of the closely related genera *Ochrobactrum* and *Pseudochrobactrum* could be cultivated without such special safety measures. In order to confirm the preliminary affiliation of field strains determined in Munich, DNA-DNA hybridization, the gold standard for species delineation, was to be carried out between the respective type and field strains of the studied bacterial taxa. Whereas this method is not applicable to *Brucella* species, which have to be discriminated via a set of conventional diagnostic tests because of their high DNA homology (Verger et al., 1985; 2000), it is very important in regard to *Brucella*-like strains which are – due to their close relationship – sometimes misidentified as *Brucella* and vice versa (Scholz et al., 2008a). Thus, the taxonomic status of a large number of *Brucella*-like strains was to be determined by DNA reassociation experiments, classical microbiological and chemotaxonomic methods, analyses of housekeeping genes and RAPD-PCR before their use as negative controls in the *Brucella*-specific assay. *Francisella* test strains

were also to be examined as to their taxonomic position before their use in subsequent experiments. Strains identified as novel species were to be taxonomically characterised and formally described.

### 1.3. Aims of the study

The main objectives of the present thesis are summarized as follows:

- identification of taxon or group specific genomic regions
- development of rapid, specific and sensitive taxon or group specific PCR assays for typing and subtyping of the studied bacterial pathogens
- analysis of the taxonomic status of field isolates
- description of thereby detected novel species

# 2. Genus *Brucella*

## 2.1. Introduction

### 2.1.1. Brucellosis

The different *Brucella* species are of medical importance because they can cause the disease brucellosis in animals and humans. *Brucella* infection frequently leads to abortion, lowered fertility and milk yield in productive livestock and is therefore responsible for considerable economic losses (Collier et al., 1998; Wright, 1987). At least the species *B. abortus* (host: cattle and bison), *B. melitensis* (host: goats and cattle), *B. suis* (host: swine) and *B. canis* (host: dogs) are known as the causative agents of human brucellosis (also named Mediterranean fever, Gibraltar fever, Malta fever, Cyprus fever, undulant fever and typhomalarial fever; Young, 1995). *B. melitensis* is the most pathogenic species for humans, followed by *B. suis*, *B. abortus* and *B. canis* (Young, 1995). The disease can be acquired by inhalation of aerosols, consumption of unsterilized dairy products or meat from infected animals or by handling of infected animals but is rarely transmitted from human to human (Young, 1995). For marine brucellae, the disease neurobrucellosis in humans was described (Brew et al., 1999; Sohn et al., 2003). Symptoms of brucellosis are rather unspecific, including arthralgia, inconstant fevers, anaemia, weakness, headache, sweating, depression and irritability (Young, 1995; Iserson, 2002). The disease often becomes chronic and sometimes leads to death of mostly immunocompromised hosts, usually because of *B. melitensis* endocarditis or meningitis (Bossi et al., 2004b).

*Brucella* species were of interest for bioweapon research because the agent can be transmitted via the air and 10-100 organisms are enough to cause the disease in humans (see Table 1). *B. suis* was the first bioweapon agent developed to be used in bomblets by the USA (Endicott and Hagerman, 1998). Although *Brucella* species can survive well in aerosols and resist desiccation, the weaponized agent proved to be too ineffective (Bossi et al., 2004b). The incubation period can be long (see Table 1), mortality is low and *Brucella* can be inactivated by exposure to heat and most disinfectants (Bossi et al., 2004b). Nevertheless, the use as a bioterrorist agent

cannot be ruled out since the effects of brucellosis, especially when inhaled, are quite debilitating on a large scale.

### 2.1.2. Classification

*Brucella* strains have been isolated from a range of different terrestrial and marine mammals (including humans) and recently also from a human breast implant wound and a lung biopsy of a pneumonia patient (Scholz et al., 2010; Tiller et al., 2010). The genus comprises now the six classical *Brucella* species *Brucella melitensis* (host: goats), *Brucella abortus* (host: cattle), *Brucella suis* (host: mostly pigs and hares), *Brucella neotomae* (host: desert wood rat), *Brucella canis* (host: dogs), *Brucella ovis* (host: sheep), and the more recently described species *Brucella ceti, Brucella pinnipedialis* (both from marine mammals), and two species described with contributions from the present study, *Brucella microti* (host: common vole) and *Brucella inopinata* (human). *B. melitensis, B. abortus* und *B. suis* are further divided into biovars. Marine *Brucella* species have recently also been shown to contain significant subtypes which might be assigned biovar status or even species status in the future (Dawson et al., 2008; Maquart et al., 2009).

DNA-DNA hybridization studies showed that, according to the common taxonomic rules (DNA homology >70%, Wayne et al., 1987) the classical species only represent one species (Verger et al., 1985; 2000) and therefore should be combined into the single genomospecies *Brucella melitensis.* Nevertheless, to avoid confusion, the "Subcommittee on the taxonomy of *Brucella"* proposed to keep the nomen-species (Osterman and Moriyon, 2006). Classification into the six classical species is mainly based on the differences in phenotypic characteristics, pathogenicity and host preference (Corbel and Brinley-Morgan, 1984). Also the novel marine species occur in specific hosts. *B. ceti* is predominant in cetacean species like dolphins, minke whales and porpoises and *B. pinnipedialis* is commonly isolated from pinnipeds like hooded seals and other seal species. *B. microti* was originally isolated from common voles but has recently been reported to infect red foxes as well (Scholz et al., 2009). *B. inopinata* was currently only isolated from immunocompromised patients (Scholz et al., 2010; Tiller et al., 2010).

*Brucella* is genetically closely related to the genus *Ochrobactrum* (Velasco et al., 1998). The taxon *Ochrobactrum* currently contains fifteen species, *O. anthropi* (Holmes et al., 1988), *O. cytisi* (Zurdo-Piñeiro et al., 2007), *O. gallinifaecis* (Kämpfer et al., 2003), *O. grignonense* (Lebuhn et al., 2000), *O. haematophilum* (Kämpfer et al., 2007b), *O. intermedium* (Velasco et al., 1998), *O. lupini* (Trujillo et al., 2005), *O. oryzae* (Tripathi et al., 2006), *O. pituitosum* (Huber et al., 2010), *O. pseudintermedium* (Teyssier et al., 2007), *O. pseudogrignonense* (Kämpfer et al., 2007b), *O. rhizosphaerae* (Kämpfer et al., 2008a), *O. thiophenivorans* (Kämpfer et al., 2008a), *O. tritici* (Lebuhn et al., 2000) and *O. ciceri* (Imran et al., 2010). Descriptions of species belonging to this genus have recently been prolific; ten novel species were described in the last five years and *O. pituitosum*, *O. haematophilum*, *O. pseudogrignonense*, *O. rhizosphaerae* and *O. thiophenivorans* with contributions of the here presented thesis.

The genus contains both, non-pathogenic soil and rhizoplane bacteria and the facultative pathogenic species *O. anthropi* and *O. intermedium*. The pathogens cause infections in humans that are usually immunocompromised but some strains were found to cause severe *Brucella*-like infections in previously healthy humans (Kettaneh et al., 2003; Ozdemir et al., 2006; Vaidya et al., 2006). Another species, *Ochrobactrum pseudintermedium*, was isolated from a patient with severe health problems (Teyssier et al., 2007) and is presumably pathogenic for humans too. Scholz et al. (2008b) pointed at the probability of other potential human pathogens within species like *O. tritici*, *O. haematophilum* and *O. pseudogrignonense*.

In phylogenetic analyses based on 16S rRNA gene and *recA* sequences, *Brucella* strains grouped within the genus *Ochrobactrum* which means that clear demarcation criteria for both genera have to be defined (Scholz et al., 2006; Scholz et al., 2008b). Because of their close relationship and similar genetic properties, *Ochrobactrum* species are frequently designated "*Brucella*-like" and it has been discussed to combine *Brucella* and *Ochrobactrum* into one genus, *Brucella*, but there is a strong tendency to keep the names as they are in order to avoid confusion concerning such an important pathogen. Considering their close relationship and sometimes even similar disease pattern, it is of great importance to have specific diagnostic tools at hand that can unambiguously identify pathogenic brucellae and differentiate them from the less pathogenic and non-pathogenic *Brucella*-like species.

### 2.1.3. Diagnosis

Classical differentiation between the different *Brucella* species and biovars is based on phage typing, sensibility to dyes, oxidative metabolic profiles, $CO_2$ requirements, $H_2S$ production and agglutination with monospecific antisera (Alton et al., 1988). These typing methods are time-consuming, often subjective and a risk for the laboratory personnel; that is why methodological improvements are desirable. Current epidemiological and diagnostic *Brucella* research, also stimulated by the bioterrorism debate, has been exploiting novel molecular typing methods to develop more rapid, reliable and safe diagnostic tools.

Despite the high genetic similarities, a range of different PCR techniques for differentiation of *Brucella* species and to some extent also biovars have already been established. These molecular approaches include analysis of outer-membrane genes and PCR-RFLP (Ficht et al., 1989; 1996; Cloeckaert et al., 1995; 1996; 2001; Vizcaino et al., 1997; 2004; Garcia-Yoldi et al., 2005; Al Dahouk et al., 2005), locus specific conventional and real-time PCR assays targeting different polymorphic regions (Fekete et al., 1990; Bricker and Halling, 1994; Redkar et al., 2001; Ferrao-Beck et al., 2006; Garcia-Yoldi et al., 2006; Ratushna et al., 2006), arbitrary primed and rep-PCR (Mercier et al., 1996; Tcherneva et al., 1996; Fekete et al., 1992; Tcherneva et al., 2000), 'infrequent restriction site' (IRS)-PCR (Cloeckaert et al., 2003), multilocus sequence typing (Whatmore et al., 2007), multilocus variable number tandem-repeat analysis (Bricker et al., 2003; Le Fleche et al., 2006; Whatmore et al., 2006; Garcia-Yoldi et al., 2007; Al Dahouk et al., 2007), AFLP (Whatmore et al., 2005), and SNP-PCR (Marianelli et al., 2006; Scott et al., 2007; Fretin et al., 2008; Foster et al., 2008).

Several studies describe PCR assays that make use of the specific occurrence of the multiple insertion element IS*711* which was described by Halling et al. (1993) and is stable in number and position in the *Brucella* chromosomes (Bricker and Halling 1994; 1995; Bricker et al., 2000; Cloeckaert et al., 2000; 2003; Ohishi et al., 2004; Ocampo-Sosa et al., 2005). So, it was shown with a limited set of reference strains that a multiplex-PCR could identify and differentiate some biovars of *B. abortus*, *B. melitensis*, *B. ovis* and *B. suis* biovar 1 and also two vaccine strains. In another study, the majority (>90%) of the tested *B. abortus* and *B. melitensis* strains could be correctly identified in a real-time multiplex assay, employing only one reverse primer

(binding IS*711* of *B. abortus* and *B. melitensis*) and two specific forward primers (targeting the neighbouring *alkB* and BMEI1162 gene, respectively) (Probert et al., 2004). In a similar approach, a real-time PCR assay was developed that could differentiate between *B. abortus*, *B. melitensis* and *B. suis* biovar 1 (Redkar et al., 2001). Further analyses showed that marine and terrestrial isolates can be differentiated by the size of a specific PCR product that is amplified using primers that bind the *bp26* gene and the IS*711* palindromic element (Cloeckaert et al., 2000). Also the novel species *B. inopinata* can be differentiated from other *Brucella* species by a specific IS*711* occurrence (Scholz et al., 2010). A comprehensive approach was made by Hinic et al. (2008) who used unique genetic loci of the six classical species to develop seven individual reactions for detection of the *Brucella* genus and the differentiation between the six species to be used in conventional as well as real-time PCR. Scholz et al. (2008a) described an assay targeting the conserved gene *recA* that detects and differentiates *O. anthropi*, *O. intermedium* and *Brucella* by conventional PCR in a single reaction. A differentiation between some of the classical *Brucella* species was achieved by the use of the relatively time-consuming 'infrequent restriction site' (IRS)-PCR and even characteristic IRS-PCR patterns for *B. suis* biovar 2 could be found (Cloeckaert et al., 2003). In another study, DNA polymorphisms in the genes for the different outer membrane proteins (OMPs; *omp2*, *omp25*, *omp31*) were examined and tested for differentiation purposes. So it was shown that strains of *B. melitensis* are characterized by the lack of the signature sequence for the restriction enzyme *Eco*RV in the *omp25* PCR product and *B. ovis* strains by a 50 bp deletion in the same gene. Furthermore, in the restriction patterns for the *omp2* PCR product, species- and also biovar-specific features were found (Cloeckaert et al., 1995). A high variability was also found for the restriction patterns of this PCR product in marine brucellae which does not only hint at the existence of different species but also of different biovars (Cloeckaert et al., 2001). The target sequence of another PCR based diagnosis of human brucellosis is *omp31*, encoding the 31-kDa *Brucella* outer membrane protein (Casañas et al., 2001). In this assay, which has already been adapted to a real-time PCR (Queipo-Ortuño et al., 2005), *Brucella* species cannot be differentiated from all members of the closely related genus *Ochrobactrum.* SNPs were used in real-time applications to identify *B. suis* and differentiate between its biovars (Fretin et al., 2008) and Marianelli et al. (2006)

reported that the *rpoB* gene contains enough DNA polymorphisms for the identification of *Brucella* spp.

A comprehensive microarray study by Rajashekara et al. (2004) revealed several differences in the genomes of the classical *Brucella* species most of which were insertions or deletions. Genomic fingerprinting is another approach to differentiate between *Brucella* species, biovars and strains. REP- and ERIC-PCR (rep-PCR) were used to examine whether these assays could be used for a differential typing of brucellae on the basis of the resulting banding patterns (genomic fingerprints) (Mercier et al., 1996; Techerneva et al., 1996). The two studies yielded inconsistent results and rep-PCR assays proved to be difficult to reproduce and not the method of choice for reliable differentiation of species and strains. However, both groups showed that by the one or the other technique a range of different banding patterns could be obtained and that even *B. canis* strains could be differentiated from the other species by their banding patterns after REP-PCR (Tcherneva et al., 1996). Complex banding patterns were also found after PCR analyses of *Brucella* when short (10mer) and randomly designed primers were used for amplification (RAPD-PCR) (Fekete et al., 1992; Tcherneva et al., 2000). A fingerprinting method with a DIG-labeled IS*711* probe was successfully used to differentiate between terrestrial and marine *Brucella* strains (Ouahrani-Bettache et al., 1996; Bricker et al., 2000). A similarly high variability in genomic fingerprints was detected in the typing of *Brucella* strains after a ‘multi-locus variable number tandem repeats’ analysis. However, the results showed neither species- nor biovar specificity (Bricker et al., 2003).

The high genomic similarities between *Brucella* species is a major reason for the difficulty to differentiate between them on the basis of molecular techniques. However, studies aimed at typing brucellae into species and biovars indicate that, despite the close genomic similarity between the different species, there is sufficient variability to be able to develop a PCR method that cannot only differentiate between the different species but also the biovars. Especially the studies carried out by Fekete et al. (1992) and Tcherneva et al. (1996, 2000) inspired us to use the different rep-PCR and RAPD fingerprinting techniques as the key strategy towards the identification of regions of variability in the *Brucella* genomes.

## 2.2. Development of a PCR assay for typing and subtyping of *Brucella* species

Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
International Journal of Medical Microbiology 299 (2009) 563–573
IJMM
www.elsevier.de/ijmm

# Development of a PCR assay for typing and subtyping of *Brucella* species

Birgit Huber[a], Holger C. Scholz[b], Nidia Lucero[c], Hans-Jürgen Busse[a,*]

[a]*Institute of Bacteriology, Mycology and Hygiene, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Vienna, Austria*
[b]*Bundeswehr Institute of Microbiology, Neuherbergstraße 11, 80937 Munich, Germany*
[c]*National Laboratories and Institutes of Health Administration (ANLIS), 'Dr. C.G. Malbrán', Avda. Velez Sarsfield 563, 1281 Buenos Aires, Argentina*

Received 14 August 2008; received in revised form 25 April 2009; accepted 19 May 2009

## Abstract

In the course of this study, examinations were carried out to develop a PCR-based test which allows discrimination of *Brucella* species and biovars not targeted by the currently established gel-based PCR assays. Appropriate primers were designed based on specific deletions and insertions in the different *Brucella* genomes as determined by RAPD-PCR and whole-genome comparisons. After testing the specificity of the primers with a set of 22 *Brucella* reference strains of all species and biovars, they were used to supplement the existing PCR assays resulting in a 19-primer multiplex PCR. In addition to the commonly used PCR assays, the developed assay specifically identified *B. neotomae*, *B. pinnipedialis*, *B. ceti*, and *B. microti*. Furthermore, it differentiated *B. abortus* biovars 1, 2, 4 from biovars 3, 5, 6, 9, as well as between *B. suis* biovar 1, biovars 3, 4, and biovars 2 and 5. When tested in the multiplex assay, all *Brucella* type and reference strains and the majority of 118 field strains examined could be accurately identified by their respective banding patterns according to their previous typing. *B. canis* strains were subdivided into 2 groups, one exhibiting a unique pattern and the other one a banding pattern shared with *B. suis* biovars 3 and 4. Species of the closely related genus *Ochrobactrum* and several other clinically relevant bacteria showed no amplification product. Hence, the developed PCR assay is useful for rapid identification of *Brucella* at the species and at the biovar level.


***Keywords:*** *Brucella*; Multiplex PCR; Molecular diagnostics; Detection; Identification; Genetic fingerprinting

## Introduction

The alphaproteobacterial genus *Brucella* comprises a group of facultative intracellular pathogens that can infect a variety of animal species and cause the zoonotic disease brucellosis in humans (Corbel, 1997). Because of their high infectious potential, *Brucella* species are listed in National Institute of Allergy and Infectious Diseases (NIAID) category B priority pathogens and are considered to have bioterroristic potential. At present, *Brucella* consists of 9 different species: *B. abortus*, *B. melitensis*, *B. suis*, *B. canis*, *B. ovis*, *B. neotomae* (Osterman and Moriyon, 2006), and the recently described species *B. microti* (Scholz et al., 2008b) as well as the marine mammals-infecting species *B. ceti* and *B. pinnipedialis* (Foster et al., 2007). Based on

*Corresponding author. Tel.: +43 1 25077 2128; fax: +43 1 25077 2190.
*E-mail address:* Hans-Juergen.Busse@vu-wien.ac.at (H.-J. Busse).

1438-4221/$ - see front matter © 2009 Elsevier GmbH. All rights reserved.
doi:10.1016/j.ijmm.2009.05.002

phenotypic traits, i.e. $CO_2$ requirement, $H_2S$ production, dye-sensitivity, oxidative metabolic profile, susceptibility to specific bacteriophages and agglutination with monospecific antisera (Corbel and Banai, 2005), *B. abortus*, *B. suis*, and *B. melitensis* have been subtyped into biovars. The genus can be considered monospecific if the gold standard in bacterial classification, DNA-DNA hybridization, is applied (Verger et al., 1985). Comparative whole-genome studies have confirmed this notion (Paulsen et al., 2002; Rajashekara et al., 2004; Halling et al., 2005; Ratushna et al., 2006). Hence, restricted genomic polymorphisms complicate the discrimination between the various species and biovars at the molecular level. Nevertheless, several studies were carried out in recent years in order to develop PCR-based tests for the discrimination among species and biovars of brucellae. Bricker and Halling (1994) were the first to develop a multiplex PCR (AMOS) that differentiates strains of *B. abortus* biovars 1, 2, and 4, *B. melitensis*, *B. ovis*, and *B. suis* biovar 1 by specific PCR products based on unique chromosomal loci of the mobile genetic element *IS711* in their genomes. Ocampo-Sosa et al. (2005) improved the AMOS PCR by adding a specific primer for the identification of *B. abortus* biovars 5, 6, 9 and genotype 3b of biovar 3. A 4-primer-set multiplex PCR approach to discriminate among *B. suis* biovars 1, 2 and 3 was developed by Ferrao-Beck et al. (2006), but still some inconsistencies with conventional field strain identification were observed. A more comprehensive approach was made by Garcia-Yoldi et al. (2006) who used species- and strain-specific genetic differences to differentiate among *Brucella* species, including isolates from marine mammals, and 3 vaccine strains. This PCR, however, cannot discriminate among *B. ceti* and *B. pinnipedialis* and the various *Brucella* biovars, and does not detect *B. microti* as a separate species. In the present study, we have used RAPD-PCR (random amplified polymorphic DNA) and available information on specific polymorphisms in *Brucella* genomes in order to develop a multiplex PCR that uses the AMOS primers, additional specific loci of the insertion element *IS711*, and other unique insertions and deletions (Bricker and Halling, 1994; Cloeckaert et al., 2000; Rajashekara et al., 2004; Ratushna et al., 2006; Scholz et al., 2008b). This novel PCR assay differentiates between all presently recognized *Brucella* species, including the recently described species *B. ceti* (formerly named '*Brucella maris*' or '*Brucella cetaceae*'), *B. pinnipedialis* (formerly named '*Brucella maris*' or '*Brucella pinnipediae*'), and *B. microti*, including some more recently described strains of the latter species (Scholz et al., 2008c, 2009), and also allows accurate differentiation of certain biovars of *B. abortus* and *B. suis*.

## Materials and methods

### Bacterial strains

Strains examined in this study are listed in Table 1. *Brucella* isolates were classified using the standard microbiological methods described by Alton et al. (1988) and AMOS PCR (Bricker and Halling, 1994) where applicable.

### DNA extraction

PCR template DNA was either prepared from pure cultures as described previously (Scholz et al., 2008a) or by 3 cycles of freeze-thawing (Wieser and Busse, 2000) from heat-inactivated biomass (bacteria were heated at 99 °C for 10 min and centrifuged for 2 min at 13,000 × g). 0.5–2 µl of the supernatant were used in PCR. DNA concentration was estimated by the chemical method according to Richards (1974). In short, about 2 µg DNA in a final volume of 50 µl, consisting of 1 × SSC buffer and 1.5 N $HClO_4$, were mixed with 30 µl of diphenylamine-reagent (glacial acetic acid, 0.01% paraldehyde, 4% diphenylamine). After incubation for 20–24 h at room temperature, absorption at 600 nm was measured. Under the same conditions, a calibration curve was generated with calf thymus DNA (0–4 µg).

### RAPD analysis

Primers used for fingerprinting and their sequences are listed in Table 2. Amplifications were carried out in a total reaction volume of 25 µl containing 1 × Green GoTaq® Flexi Buffer (Promega, Mannheim, Germany), 2 mM $MgCl_2$, 200 µM of each dNTP, 0.6 U of GoTaq® DNA polymerase (Promega), 8 µM RAPD primer (HPLC quality; VBC Biotech, Vienna, Austria), and approximately 5 ng of DNA. Reactions were performed using an initial 5-min denaturation step at 95 °C, followed by 45 cycles of 1 min at 95 °C, 1 min at 36 °C, 2 min at 72 °C, and a final extension step of 5 min at 72 °C. The resolution of banding patterns was improved by using a ramp rate of 0.3 °C $sec^{-1}$. Amplification products were separated by 1.5% (w/v) agarose gel electrophoresis in 1 × Tris/acetate/EDTA running buffer at a constant voltage of 110 V for at least 90 min. Fragments were visualized with UV light after staining with ethidium bromide. Interesting bands were excised from the gel, and DNA was eluted with the QIAquick® Gel Extraction Kit according to the instructions of the manufacturer (Qiagen, Hilden, Germany). The purified PCR products were cloned using the Promega pGEM®-T Easy Vector System and *Escherichia coli* strain DH10B according to the manufacturer's manual.

**Table 1.** Strains analysed in this study, source and multiplex PCR pattern.

| Strain | Source | Species and biovar | Multiplex PCR result (bp) |
|---|---|---|---|
| ***Brucella abortus*** | | | |
| NCTC 10093$^{T}$ | Cattle | *B. abortus* biovar 1 | 498, 370 |
| IMB 91 | Bovine | *B. abortus* biovar 1 | 498, 370 |
| IMB 92 | Unknown | *B. abortus* biovar 1 | 498, 370 |
| IMB 94 | Unknown | *B. abortus* biovar 1 | 498, 370 |
| IMB 95 | Rodent | *B. abortus* biovar 1 | 498, 370 |
| IMB 96 | Bovine | *B. abortus* biovar 1 | 498, 370 |
| IMB 98 | Unknown | *B. abortus* biovar 1 | 498, 370 |
| IMB 99 | Unknown | *B. abortus* biovar 1 | 498, 370 |
| IMB 100 | Unknown | *B. abortus* biovar 1 | 498, 370 |
| NCTC 10501$^{ref}$ | Bovine | *B. abortus* biovar 2 | 498, 370 |
| IMB 2518 | Unknown | *B. abortus* biovar 2 | 498, 370 |
| NCTC 10502$^{ref}$ | Human | *B. abortus* biovar 3 | 370 |
| IMB 101 | Bovine | *B. abortus* biovar 3 | 370 |
| IMB 102 | Bovine | *B. abortus* biovar 3 | 370 |
| IMB 103 | Bovine | *B. abortus* biovar 3 | 370 |
| IMB 106 | Bovine | *B. abortus* biovar 3 | 370 |
| IMB 112 | Bovine | *B. abortus* biovar 3 | 370 |
| NCTC 10503$^{ref}$ | Bovine | *B. abortus* biovar 4 | 498, 370 |
| IMB 120 | Human | *B. abortus* biovar 4 | 498, 370 |
| NCTC 10504$^{ref}$ | Bovine | *B. abortus* biovar 5 | 370 |
| NCTC 10505$^{ref}$ | Bovine | *B. abortus* biovar 6 | 370 |
| IMB 7 | Dairy milk | *B. abortus* biovar 6 | 370 |
| IMB 121 | Unknown | *B. abortus* biovar 6 | 370 |
| IMB 122 | Bovine | *B. abortus* biovar 6 | 370 |
| IMB 124 | Bovine | *B. abortus* biovar 6 | 370 |
| IMB 2523 | Bovine | *B. abortus* biovar 6 | 498, 370 |
| NCTC 10506 | Unknown | *B. abortus* former bv. 7 | 370 |
| NCTC 10507$^{ref}$ | Bovine | *B. abortus* biovar 9 | 370 |
| IMB 2524 | Bovine | *B. abortus* biovar 9 | 370 |
| IMB 2525 | Bovine | *B. abortus* biovar 9 | 370 |
| ***Brucella canis*** | | | |
| RM6/66$^{T}$ | Dog | *B. canis* | 887, 436, 217 |
| Hoy 1066 | Dog | *B. canis* | 887, 436, 217 |
| 315 | Dog | *B. canis* | 887, 436, 217 |
| IMB 130 | Unknown | *B. canis* | 887, 436, 217 |
| IMB 131 | Unknown | *B. canis* | 887, 436, 217 |
| IMB 132 | Dog | *B. canis* | 887, 436, 217 |
| IMB 911 | Dog | Atypical *B. canis*$^{a}$ | 436, 217 |
| IMB 921 | Dog | Atypical *B. canis*$^{b}$ | 436, 217 |
| IMB 951 | Dog | Atypical *B. canis*$^{b}$ | 436, 217 |
| IMB 954 | Dog | Atypical *B. canis*$^{b}$ | 436, 217 |
| IMB 1004 | Dog | Atypical *B. canis*$^{a}$ | 436, 217 |
| IMB 1019 | Dog | Atypical *B. canis*$^{a}$ | 436, 217 |
| IMB 1035 | Human | Atypical *B. canis*$^{a}$ | 436, 217 |
| IMB 1245 | Dog | Atypical *B. canis*$^{b}$ | 436, 217 |
| IMB 1246 | Dog | Atypical *B. canis*$^{a}$ | 436, 217 |
| ***Brucella melitensis*** | | | |
| ATCC 23456$^{T}$ | Goat | *B. melitensis* biovar 1 | 731, 436, 321 |
| IMB 13 | Human | *B. melitensis* biovar 1 | 731, 436 |
| IMB 14 | Human | *B. melitensis* biovar 1 | 731, 436, 321 |
| IMB 22 | Human | *B. melitensis* biovar 1 | 731, 436 |
| IMB 23 | Goat | *B. melitensis* biovar 1 | 731, 436, 321 |
| IMB 27 | Human | *B. melitensis* biovar 1 | 731, 436, 321 |
| NCTC 10508$^{ref}$ | Goat | *B. melitensis* biovar 2 | 731, 436 |
| IMB 31 | Human | *B. melitensis* biovar 2 | 731, 436, 321 |

**Table 1.** (*continued*)

| Strain | Source | Species and biovar | Multiplex PCR result (bp) |
|---|---|---|---|
| IMB 40 | Human | *B. melitensis* biovar 2 | 731, 436 |
| IMB 41 | Human | *B. melitensis* biovar 2 | 731, 436 |
| IMB 51 | Sheep | *B. melitensis* biovar 2 | 731, 436 |
| NCTC 10509[ref] | Goat | *B. melitensis* biovar 3 | 731, 436, 321 |
| IMB 70 | Human | *B. melitensis* biovar 3 | 731, 436, 321 |
| IMB 72 | Human | *B. melitensis* biovar 3 | 731, 436 |
| IMB 76 | Human | *B. melitensis* biovar 3 | 731, 436, 321 |
| IMB 80 | Human | *B. melitensis* biovar 3 | 731, 436 |
| ***Brucella neotomae*** | | | |
| ATCC 23459[T] | Desert wood rat | *B. neotomae* | 1370, 436, 321 |
| ***Brucella microti*** | | | |
| CCM 4915[T] | Common vole | *B. microti* | 666, 436, 321, 217 |
| CCM4916 | Common vole | *B. microti* | 666, 436, 321, 217 |
| IMB 257 | Wild red fox | *B. microti* | 666, 436, 321, 217 |
| IMB 284 | Wild red fox | *B. microti* | 666, 436, 321, 217 |
| BMS 17 | Soil | *B. microti* | 666, 436, 321, 217 |
| ***Brucella ovis*** | | | |
| ATCC 25840[T] | Sheep | *B. ovis* | 976, 569, 436 |
| IMB 172 | Unknown | *B. ovis* | 976, 569, 436 |
| IMB 173 | Unknown | *B. ovis* | 976, 569, 436 |
| IMB 174 | Unknown | *B. ovis* | 976, 569, 436 |
| IMB 175 | Unknown | *B. ovis* | 976, 569, 436 |
| IMB 176 | Unknown | *B. ovis* | 976, 569, 436 |
| IMB 177 | Unknown | *B. ovis* | 976, 569, 436 |
| IMB 179 | Unknown | *B. ovis* | 976, 569, 436 |
| IMB 180 | Unknown | *B. ovis* | 976, 569, 436 |
| IMB 2535 | Ovine | *B. ovis* | 976, 569, 436 |
| ***Brucella suis*** | | | |
| NCTC 10316[T] | Wild boar | *B. suis* biovar 1 | 436, 285, 217 |
| IMB 2526 | Human | *B. suis* biovar 1 | 436, 285, 217 |
| NCTC 10510[ref] | Swine | *B. suis* biovar 2 | 436, 321 |
| IMB 146 | Wild boar | *B. suis* biovar 2 | 436, 321 |
| IMB 149 | Swine | *B. suis* biovar 2 | 436, 321 |
| IMB 153 | Wild boar | *B. suis* biovar 2 | 436, 321 |
| IMB 162 | Wild boar | *B. suis* biovar 2 | 436, 321 |
| IMB 165 | Wild boar | *B. suis* biovar 2 | 436, 321 |
| IMB 166 | Wild boar | *B. suis* biovar 2 | 436, 321 |
| IMB 167 | Wild boar | *B. suis* biovar 2 | 436, 321 |
| IMB 171 | Wild boar | *B. suis* biovar 2 | 436, 321 |
| IMB 2527 | Human | *B. suis* biovar 2 | 436, 321 |
| NCTC 10511[ref] | Swine | *B. suis* biovar 3 | 436, 217 |
| NCTC 11364[ref] | Reindeer | *B. suis* biovar 4 | 436, 217 |
| NCTC 11996 [ref] | Rodent | *B. suis* biovar 5 | 436, 321 |
| **Marine *Brucella* species formerly named '*Brucella maris*'** | | | |
| ***Brucella ceti*** (formerly also named '*Brucella cetaceae*') | | | |
| NCTC 12891[T] | Harbour porpoise | *B. ceti* | 1140, 436, 321, 217 |
| 7763/2 | Bottlenose dolphin | *B. ceti* | 1140, 436, 321, 217 |
| B14/94 | Common dolphin | *B. ceti* | 1140, 436, 321 |
| M13/05/1 | Striped dolphin | *B. ceti* | 1140, 436, 321 |
| M61/05/1 | White-sided dolphin | *B. ceti* | 1140, 436, 321 |
| M452/97/2 | Common dolphin | *B. ceti* | 1140, 436, 321, 217 |
| M644/93/1 | Common dolphin | *B. ceti* | 1140, 436, 321 |
| M654/99/1 | Striped dolphin | *B. ceti* | 1140, 436, 321 |
| M870/97/1 | White-beaked dolphin | *B. ceti* | 1140, 436, 321, 217 |

**Table 1.** (*continued*)

| Strain | Source | Species and biovar | Multiplex PCR result (bp) |
|---|---|---|---|
| M997/94/2 | White-sided dolphin | *B. ceti* | 1140, 436, 321, 217 |
| M1570/94/1 | Porpoise | *B. ceti* | 1140, 436, 321, 217 |
| M2438/95/1 | White-sided dolphin | *B. ceti* | 1140, 436, 321, 217 |
| ***Brucella pinnipedialis*** (formerly also named '*Brucella pinnipediae*') | | | |
| NCTC 12890$^T$ | Common seal | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2412 | Seal | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2417 | Seal | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2420 | Grey seal | *B. pinnipedialis* | 1140, 569, 436, 321, 217 |
| IMB 2424 | Seal | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2428 | Seal | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2430 | Seal | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2431 | Seal$^+$ | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2432 | Seal$^+$ | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2433 | Seal$^+$ | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2434 | Seal$^+$ | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2435 | Seal$^+$ | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2436 | Seal$^+$ | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2437 | Seal$^+$ | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2442 | Seal | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2453 | Seal | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2461 | Seal$^{++}$ | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2465 | Seal$^{++}$ | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2466 | Seal$^{++}$ | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2476 | Seal$^{+++}$ | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2477 | Seal$^{+++}$ | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2481 | Seal$^{++++}$ | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2482 | Seal$^{++++}$ | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2483 | Seal$^{++++}$ | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2484 | Seal$^{++++}$ | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2485 | Seal$^{++++}$ | *B. pinnipedialis* | 1140, 569, 436, 321 |
| IMB 2486 | Seal$^{++++}$ | *B. pinnipedialis* | 1140, 569, 436, 321 |
| **Untyped *Brucella* strains** | | | |
| IMB 2423 | Porpoise | *Untyped* | 1140, 569, 436, 321 |
| AGES1 | Cattle | Untyped | 498, 370 |
| AGES2 | Swine | Untyped | 436, 321 |
| AGES3 | Hare | Untyped | 436, 321 |
| SP 1582 | Hare | Untyped | 436, 321 |
| SP 346a | Hare | Untyped | 436, 321 |
| SP 346b | Hare | Untyped | 436, 321 |
| SP 886 | Hare | Untyped | 436, 321 |
| ***Ochrobactrum*** | | | |
| LMG 3301$^T$ | Human blood | *O. intermedium* | – |
| LMG 3331$^T$ | Sewage plant | *O. anthropi* | – |
| Oga9A$^T$ | Soil | *O. grignonense* | – |
| LMG 18957$^T$ | Wheat rhizoplane | *O. tritici* | – |
| Lup21$^T$ | *Lupinus albus* | *O. lupini* | – |
| Iso196$^T$ | *Gallus gallus* | *O. gallinifaecis* | – |
| DSM 17490$^T$ | Human | *O. pseudintermedium* | – |
| DSMZ 17471$^T$ | *Oryza sativa* | *O. oryzae* | – |
| CCUG 38531$^T$ | Human blood | *O. haematophilum* | – |
| CCUG 30717$^T$ | Human blood | *O. pseudogrignonense* | – |
| DSM 7216$^T$ | Waste water | *O. thiophenivorans* | – |
| PR17$^T$ | Potato rhizosphere | *O. rhizosphaerae* | – |
| Esc1$^T$ | *Cytisus scoparius* | *O. cytisi* | – |

**Table 1.** (*continued*)

| Strain | Source | Species and biovar | Multiplex PCR result (bp) |
|---|---|---|---|
| **Other bacterial strains** | | | |
| Y 784 | Human | *Yersinia pestis* | – |
| Y 111$^T$ | Turkey | *Y. pseudotuberculosis* | – |
| ATCC 6223$^T$ | Human lymph node | *Francisella tularensis* subsp. *tularensis* | – |
| ATCC 29684 | Water rat | *F. tularensis* subsp. *holarctica* | – |
| DSM 1103 | Clinical isolate | *Escherichia coli* | – |

+, ++, +++, ++++ indicating the same seal individuals.
[a]susceptibility to penicillin, weaker growth on media supplemented with thionin than RM6/66.
[b]in addition: weak growth on media with basic fuchsin, pyronine, and malachite green, RM6/66 (–).

**Table 2.** Oligonucleotides used in this study.

| Primer designation | Sequence of primer (5′-3′) | Source | Amplicon size (bp) |
|---|---|---|---|
| IS711 | TGC CGA TCA CTT AAG GGC CTT CAT | Bricker and Halling, 1994 | |
| B_ab_spec | GAC GAA CGG AAT TTT TCC AAT CCC | Bricker and Halling, 1994 | 498 with IS711 |
| B_mel_spec | AAA TCG CGT CCT TGC TGG TCT GA | Bricker and Halling, 1994 | 731 with IS711 |
| B_ov_spec | CGG GTT CTG GCA CCA TCG TCG | Bricker and Halling, 1994 | 976 with IS711 |
| B_su1_spec | GCG CGG TTT TCT GAA GGT TCA GG | Bricker and Halling, 1994 | 285 with IS711 |
| B_mic_spec | GAC AAC ACC ACT TCC GCT CC | This work | 666 with IS711 |
| 26A | GCC CCT GAC ATA ACC CGC TT | Cloeckaert et al., 2000 | 1140 with IS711 |
| B_neo_f | CAT TGA GCC TGA GAT TCG GG | This work | 1065 |
| B_neo_r | GTG ATC CCC GAA ACC AAC TG | This work | |
| B_ov_pin_f | ATC AGT GCG TTG GTG AAC TTC C | This work | 569 |
| B_ov_pin_r | GGC GTC TCC AAT TTC ATC AGT C | This work | |
| B_ab_f | GTC TTC CTG ATT GCG GCT GG | This work | 370/436 |
| B_ab_r | ACC ACC AGC GAC AGG AAC ATC | This work | |
| B_can_f | GGC TTC CTC ACG ACA TAC CAG | This work | 887 |
| B_can_r | CTG AAC AGG ATG CAA TGA CGC | This work | |
| B_su134_f | CCA CGA ACG AGC GAA CAG C | This work | 217 |
| B_su134_r | CGG TAA AGC GTT GAT TTC CAT G | This work | |
| B_su25_f | CTT TAC GCC CGT GTA TCG AC | Ratushna et al., 2006 | 321 |
| B_su25_r | CAT GGG GTC CTG TGT TGA G | Ratushna et al., 2006 | |
| RAPD1a | GTG CCG ACG G | This work | – |
| RAPD1b | GTG CCG ACG GC | This work | – |
| RAPD2a | ATA GCC GAC G | This work | – |
| RAPD2b | ATA GCC GAC GC | This work | – |
| RAPD3a | TCT GAC CAC C | This work | – |
| RAPD3b | TCT GAC CAC CG | This work | – |
| RAPD4 | CAG GCT TCC C | This work | – |
| RAPD5 | CGG CAA CGG G | This work | – |
| RAPD6 | GAC TGC GCA C | This work | – |
| RAPD7 | GCG TCA GGA G | This work | – |
| RAPD8 | CCC TTA TGC G | This work | – |
| RAPD9 | AAC AGG GCG G | This work | – |
| RAPD10 | GGA CGC GGT GC | This work | – |
| RAPD11 | GCA CGC ATT GG | This work | – |
| RAPD12 | CTG AGG CTC CC | This work | – |
| RAPD13 | CCA CCT CGC A | This work | – |
| RAPD14 | AAA GGA TCG GG | This work | – |
| RAPD15 | TGT GGA GCC C | This work | – |
| RAPD16 | GTT CGT GCG GT | This work | – |
| 27f | AGA GTT TGA TCM TGG CTC AG | Lane, 1991 | ~1465 |
| 1492r | TAC GGY TAC CTT GTT ACG ACT T | Lane, 1991 | |

Plasmid insert sequences were subjected to database searches (EMBL and GenBank) and compared to *Brucella* genome sequences accessible at the time of the study (acc. nos.: AE017223/4, AE014291/2, AE008917/8, and CP000708/9).

### Design of specific primers

Primers were designed using GeneRunner 3.05 (Hastings Software, Inc.), and interaction of primers was analysed with the online tool OligoAnalyzer 3.0 provided by IDT Integrated DNA Technologies. Primer sequences are listed in Table 2.

### Multiplex PCR for *Brucella* typing

The 25-µl reaction mixture consisted of 0.8 × Green GoTaq® Flexi Buffer (Promega), 2 mM $MgCl_2$, 200 µM (each) of the 4 deoxynucleoside triphosphates (dNTPs), 0.6 U GoTaq® DNA polymerase (Promega), and the 19-primer (HPLC purified; provided by VBC Biotech) cocktail (0.28 µM of B_ab_spec, B_su1_spec, B_mel_spec, B_mic_spec, 0.2 µM of B_ab_f, B_ab_r, 0.53 µM of B_ov_spec, 0.8 µM of IS711, B_su25_f, B_su25_r, 1.52 µM of 26A, B_neo_f, B_neo_r, 0.48 µM of B_can_f, B_can_r, 1.07 µM of B_su134_f, B_su134_r, 0.27 µM of B_ov_pin_f, B_ov_pin_r). Approximately 20 ng of template DNA was added. A negative control containing no target DNA and a second one containing *O. intermedium* LMG 3301$^T$ DNA were included in the assay. Reactions were performed in an Eppendorf Mastercycler using an initial 5-min denaturation step at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 67 °C, 2 min 30 s at 72 °C, and a final extension step of 5 min at 72 °C. The products (7 µl of each reaction mixture) were separated and visualized as described above. Assay performance was assessed using different polymerases (GE Healthcare illustra™ puReTaq Ready-To-Go PCR Beads, Bioron DFS-Taq DNA Polymerase, Fermentas Taq DNA Polymerase) as well as different thermocyclers (MWG-Biotech Primus, Perkin Elmer GeneAmp PCR System 2400).

### Results and discussion

The intention of the present study was to develop a single multiplex PCR to distinguish among *Brucella* species and also between their biovars. In order to detect highly discriminating genetic polymorphisms, RAPD-PCR was applied. Based on the knowledge that arbitrary primers had already been successfully used to differentiate among *Brucella* strains (Fekete et al., 1992; Tcherneva et al., 2000), the reference strains of all *Brucella* species and biovars were subjected to RAPD-PCR using different 10- or 11-mer random primers (Table 2). In the majority of RAPD analyses, examined *Brucella* strains showed identical banding patterns, and useful differences were rarely found, confirming genomic homogeneity within the genus *Brucella* (Verger et al., 1985; Gandara et al., 2001; Gee et al., 2004). However, specific polymorphisms were detected using the primers RAPD3a and RAPD7. RAPD3a-PCR produced a unique band of 446 bp for all *B. abortus* strains and RAPD7-PCR a band of 488 bp specific to *B. ovis* and *B. pinnipedialis* (results not shown). Cloning and analysing the RAPD3a product revealed a 66-bp deletion specific to *B. abortus* chromosome I (ORF in the region of deletion in *B. melitensis* genome sequence acc. no. AE009593: BMEI1581). The RAPD7 product of *B. ovis* was found to be located on chromosome II in a transposase-rich region (corresponding ORF in *B. ovis* genome sequence acc. no. CP000709: BOV_A0492). Sequence comparisons in public databases (GenBank and EMBL Nucleotide Sequence Database) neither showed identities with any other *Brucella* genome sequence nor significant identities with sequences of other organisms. Hence, both genomic sites were considered to be suitable for designing specific primer pairs designated B_ab_f/r and B_ov_pin_f/r, respectively. Primer pair B_ov_pin_f/r was designed to produce an amplicon of 569 bp for *B. ovis* and *B. pinnipedialis* only and primer pair B_ab_f/r an amplicon of 370 bp with DNA of all *B. abortus* strains and a 436-bp fragment in all other *Brucella* strains. Since PCR amplification resulted in a 370-bp and a 436-bp fragment, respectively, with DNA of all *Brucella* strains but not with DNA of the closely related *Ochrobactrum* nor other non-*Brucella* species, the latter primer pair is also useful as an internal, genus-specific marker.

Rajashekara et al. (2004) stated that the species-specific deletions in the different *Brucella* spp., as identified by microarray analysis, proved to be highly conserved. Hence, specific primers for the differentiation of *B. canis* (B_can_f/r, 887-bp product) and *B. neotomae* (B_neo_f/r, 1065-bp product) from each other as well as from other *Brucella* strains were designed based on deletions in the 2 genomes (Rajashekara et al., 2004): a 976-bp deletion in chromosome I of *B. canis* and a 1055-bp deletion in chromosome I of *B. neotomae*. Testing these primers with DNA of *B. canis* and *B. neotomae* revealed the expected PCR products whereas other *Brucella* species gave no amplification (results not shown). Occasionally, 2 faint bands of 1863 bp and 2120 bp were observed with species other than *B. canis* or *B. neotomae.* These bands resulted from the corresponding amplicons from strains which do not possess the above-mentioned deletions (Rajashekara et al., 2004) but identical primer target sequences. It would be highly desirable to examine the specificity of the *B. neotomae* primers which was not done in the course of this study due to lack of access to additional strains of

the species. Another deletion (Rajashekara et al., 2004) specific to *B. suis* and *B. canis* (20883 bp, in chromosome I) was used to design primers B_su134_f/r. Applying them in PCR, these primers resulted in a 217-bp product with *B. suis* biovars 1, 3, and 4, *B. microti*, *B. canis*, and *B. ceti*. An *IS711* element between a putative membrane protein-coding gene and *cboL* specifically in *B. microti* (Scholz et al., 2008b) was used to design a primer that amplifies a 666-bp product with the *IS711* AMOS reverse primer. This *B. microti*-specific primer performed as expected (not shown). Tested in individual PCRs, the 5 AMOS primers (Bricker and Halling, 1994), primer 26A (Cloeckaert et al., 2000), and primers B_su25_f/r (Ratushna et al., 2006; primer pair number 5) generated the specific PCR products of expected size (results not shown).

All primers were then integrated into a single multiplex assay. Results of application of the new multiplex PCR with DNAs of all type strains of *Brucella* species and reference strains of the different biovars are shown in Fig. 1, and the banding pattern of each strain examined in our study is specified in Table 1. All *B. abortus* strains exhibited a unique PCR product of 370 bp and could be distinguished into 2 groups based on another unique band of 498 bp characteristic of the reference strains of biovars 1, 2, and 4 (Fig. 1). All other *Brucella* species showed a band of 436 bp. *B. suis* strains were distinguished into 3 groups. One group, only consisting of the reference strain for biovar 1, exhibited

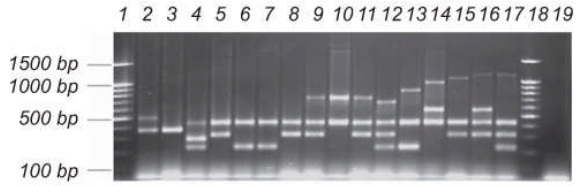


**Fig. 1.** Specificity testing of the multiplex PCR for differentiation of *Brucella* strains using DNAs of all type strains of *Brucella* species and reference strains of biovars. Lanes 1 and 18, 100-bp + 1.5-kb marker; lane 2, *B. abortus* biovar 1 NCTC 10093$^{T}$ (identical banding patterns were obtained with DNA of *B. abortus* biovar 2 NCTC 10501$^{ref}$ and biovar 4 NCTC 10503$^{ref}$); lane 3, *B. abortus* biovar 3 NCTC 10502$^{ref}$ (identical banding patterns were obtained with DNA of *B. abortus* biovar 5 NCTC 10504$^{ref}$, biovar 6 NCTC 10505$^{ref}$, former biovar 7 NCTC 10506$^{ref}$, and biovar 9 NCTC 10507$^{ref}$); lane 4, *B. suis* biovar 1 NCTC 10316$^{T}$; lane 5, *B. suis* biovar 2 NCTC 10510$^{ref}$; lane 6, *B. suis* biovar 3 NCTC 10511$^{ref}$; lane 7, *B. suis* biovar 4 NCTC 11364$^{ref}$; lane 8, *B. suis* biovar 5 NCTC 11996$^{ref}$; lane 9, *B. melitensis* biovar 1 ATCC 23456$^{T}$; lane 10, *B. melitensis* biovar 2 NCTC 10508$^{ref}$; lane 11, *B. melitensis* biovar 3 NCTC 10509$^{ref}$; lane 12, *B. microti* CCM 4915$^{T}$; lane 13, *B. canis* ATCC 23365$^{T}$; lane 14, *B. ovis* ATCC 25840$^{T}$; lane 15, *B. neotomae* ATCC 23459$^{T}$; lane 16, *B. pinnipedialis* NCTC 12890$^{T}$; lane 17, *B. ceti* NCTC 12891$^{T}$; lane 19, *O. intermedium* LMG 3301$^{T}$.

2 additional bands of 217 bp and 285 bp; the second group, consisting of reference strains for biovars 3 and 4, only displayed the band of 217 bp; the third group, represented by the reference strains for biovars 2 and 5, showed a band of 321 bp. Strains of *B. melitensis* showed a unique band of 731 bp in addition to the band of 436 bp. Furthermore, the reference strains of biovars 1 and 3 showed a band of 321 bp which was lacking in the reference strain for biovar 2. The type strains of the marine mammal brucellae could be distinguished by a band of 569 bp only in *B. pinnipedialis* and one of 217 bp only in *B. ceti*. Each of the remaining type strains of *Brucella* species showed a unique band distinguishing it from all other species.

The novel multiplex PCR was put to the test by applying it to a number of *Brucella* field strains (Table 1). They could be successfully typed into species except for some strains of *B. canis*. In contrast to the type strain of *B. canis*, several atypical isolates obtained as strains of *B. canis* (Table 1) showed the same banding pattern as *B. suis* biovars 3 and 4. This observation indicates that these strains lack the 976-bp deletion which was used to design the *B. canis*-specific primers. However, this result once more reflects the problems in differentiation between *B. canis* and *B. suis* biovars 3 and 4. In a recent real-time PCR approach to *B. suis* identification, Fretin et al. (2008) faced the problem that *B. canis* and *B. suis* biovars 4 displayed the same SNP allele profiles in their typing scheme. Also Hinic et al. (2008), who developed specific primer pairs for the differentiation among the classical *Brucella* species, could not distinguish between *B. canis* and *B. suis* biovar 4. VNTR and MLST studies showed their close relatedness as demonstrated by clustering of *B. canis* among *B. suis* biovars (Whatmore et al., 2006, 2007). It can be questioned whether a taxonomy based on host specificity, a major criterion in the *Brucella* species concept, makes sense if it is contradicted by phylogenetic relatedness.

The suggested ability to distinguish *B. melitensis* biovar 1 and 3 strains from biovar 2 strains lacking the amplicon of 321 bp was not confirmed by examination of *B. melitensis* biovar 1, 2, and 3 field strains. Several biovar 1 and 3 field strains lacked the band of 321 bp, and one strain of biovar 2 showed the band of 321 bp (Table 1). Therefore, the 321-bp band is not useful for the differentiation of biovar 2 strains from the other 2 biovars. This result is not surprising because biovar affiliation is based only on reaction with 2 monospecific antisera targeting different O-chains of the LPS (A- and M-specific epitopes). It is worth mentioning here that strains containing predominantly A-epitopes (biovar 2) always contain small proportions of M-epitopes and strains containing predominantly M-epitopes contain small amounts of A-epitopes (biovar 1), whereas biovar 3 strains exhibit high

amounts of both epitopes (Corbel and Banai, 2005). Hence, biovar affiliation is apparently based on varying regulation of expression of corresponding genes. The impossibility of grouping *B. melitensis* in accordance with biovar affiliation has also been demonstrated based on multi-locus sequencing (Whatmore et al., 2007). *B. abortus* strain IMB 2523, received as a strain of biovar 6, was grouped with *B. abortus* biovars 1, 2, and 4. This result may indicate a drawback of our PCR approach but it also appears to be possible that the strain was misclassified as a biovar 6 strain since differentiation between biovars 1 and 6 is only based on a single trait, the ability to grow on media containing thionin (Corbel and Banai, 2005). However, our PCR assay grouped the majority of biovar 6 strains correctly (Table 1). Also a strain of *B. suis* designated IMB 2529, which was received as biovar 3, did not group according to its classification but showed the banding pattern of *B. suis* biovar 1. Fretin et al. (2008) stated that in their analysis most *B. suis* biovar 3 field strains matched the allelic profile of biovar 1 rather than that of the reference strain and that these findings were in accordance with the results of other genetic typing methods. Hence, our result reflects a problem discussed in literature. These inconsistent results may point to a general problem in the development of a classification system for biotypes applying molecular methods. It can be suspected that this goal can only be achieved if genes directly corresponding to the phenotypic characteristics that determine the various biotypes are targeted.

For the typing of strains from marine mammals, only the presence or absence of the 569-bp band proved to be relevant. The 569-bp band was amplified from all *B. pinnipedialis* isolates as well as from an untyped isolate from a porpoise (IMB 2423) but from none of the tested *B. ceti* isolates. The amplification of the 217-bp band, on the other hand, was no stable characteristic of all *B. ceti* isolates. Cloeckaert et al. (2003) had already demonstrated by specific PCR that the marine brucellae now classified as *B. ceti* are divided into 2 groups. This observation is in accordance with results from other studies. Also oxidative metabolic profiles subdivide marine brucellae of the *B. ceti* group into 2 groups (Jacques et al., 2007), and Groussaud et al. (2007) showed that marine brucellae of the *B. ceti* group fall into 2 clusters when subjected to variable number tandem repeat (VNTR) and multi-locus sequence analysis (MLSA). Strains of the *B. ceti* group (M1570/94/1, 7763/2, M452/97/2 and M644/93/1, M13/05/1, M654/99/1, M61/05/1, respectively) distinguished by Groussaud et al. (2007) also exhibited different multiplex profiles in our study. Hence, our data support the view that *B. ceti* harbours 2 genomic groups, one of which might be proposed as a third marine *Brucella* species.

With the exception of strain IMB 2423, all untyped strains exhibited amplification products placing them in the species which was suggested by their sources of isolation (Corbel and Banai, 2005). The isolate from cattle, AGES1, showed the banding pattern characterising *B. abortus* biovars 1, 2, and 4, and all field strains from swine and hare were correctly identified as *B. suis*, showing the banding patterns of biovars 2 and 5. Since only biovar 2 strains have been isolated from hares and pigs, they may be placed in this biovar.

The specificity of the multiplex PCR was tested with all currently recognized *Ochrobactrum* species, the closest relatives of the *Brucella* group (Velasco et al., 1998), *Escherichia coli* DSM 1103, *Francisella tularensis* strains ATCC 6223$^T$ and ATCC 29684, and representatives of 2 *Yersinia* species (Table 1). Whereas the 16S rRNA gene could be amplified from template DNAs of all control strains with universal primers published by Lane (1991), demonstrating that quantity and quality were suitable for PCR amplification and no PCR inhibitors were present, the same template DNAs showed no amplification products in the multiplex assay under the standard conditions described above (results not shown).

In order to test the robustness of the developed multiplex PCR, influences of several parameters were examined. The PCR showed good performance when different thermocyclers (Eppendorf Mastercycler, MWG-Biotech Primus, Perkin Elmer GeneAmp PCR System 2400) were used. Two DNA polymerases performed well (Promega GoTaq DNA Polymerase, GE Healthcare illustra$^{TM}$ puReTaq Ready-To-Go PCR Beads), whereas Bioron DFS-Taq DNA Polymerase and Fermentas Taq DNA Polymerase resulted in incomplete banding patterns. The reproducibility of our optimised multiplex PCR was excellent as demonstrated by more than 10 repetitions with reference strains for each multiplex type using our standard equipment. Furthermore, the multiplex PCR was also successfully applied in a second laboratory using the Qiagen® Multiplex PCR kit (Cat. No. 206143). The lower limit of DNA concentration for obtaining reliable results was determined to be approximately 5 ng DNA in a 25 µl reaction volume, corresponding to $10^6$ genome equivalents.

Concerning sensitivity and rapidness, a conventional PCR like our multiplex PCR assay cannot compete with a real-time PCR as developed by Foster et al. (2008) for specific detection of 7 major *Brucella* clades. However, for identification of a sample of unknown source, 7 different real-time PCRs have to be run in parallel to reach this goal. Furthermore, the approach of Foster et al. (2008) does not distinguish between biovars of certain species or between the marine brucellae according to current taxonomy, nor does it identify *B. microti*.

The relatively low analytical sensitivity does not allow detection of *Brucella* DNA directly from clinical samples

without subcultivation. However, the developed assay provides a simple and rapid tool to differentiate between currently recognized *Brucella* species and certain biovars in one step. The assay requires no expensive equipment and only little expertise, and the unambiguous banding patterns can be easily identified.

## Acknowledgements

The authors wish to thank A. Cloeckaert and K. Noeckler for providing DNA of *B. ceti* and *B. pinnipedialis*. This work was supported by a Contract-Research-Project for the Bundeswehr Medical Service.

## References

Alton, G.G., Jones, L.M., Angus, R.D., Verger, J.M., 1988. Techniques for the Brucellosis Laboratory. INRA, Paris, pp. 13–61.

Bricker, B.J., Halling, S.M., 1994. Differentiation of Brucella abortus bv. 1, 2, and 4, Brucella melitensis, Brucella ovis, and Brucella suis bv. 1 by PCR. J. Clin. Microbiol. 32, 2660–2666.

Cloeckaert, A., Grayon, M., Grepinet, O., 2000. An IS711 element downstream of the *bp26* gene is a specific marker of *Brucella* spp. isolated from marine mammals. Clin. Diagn. Lab. Immunol. 7, 835–839.

Cloeckaert, A., Grayon, M., Grepinet, O., Bourmedine, K.S., 2003. Classification of *Brucella* strains isolated from marine mammals by infrequent restriction site-PCR and development of specific PCR identification tests. Microbes Infect. 5, 593–602.

Corbel, M.J., 1997. Brucellosis: an overview. Emerg. Infect. Dis. 3, 213–221.

Corbel, M.J., Banai, M., 2005. Genus I. *Brucella* Meyer and Shaw 1920, 173AL. In: Brenner, D.J., Krieg, N.R., Staley, J.T., Garrity, G.M. (Eds.), Bergey's Manual of Systematic Bacteriology, Vol. 2. Springer, New York, pp. 370–386.

Fekete, A., Bantle, J.A., Halling, S.M., Stich, R.W., 1992. Amplification fragment length polymorphism in *Brucella* strains by use of polymerase chain reaction with arbitrary primers. J. Bacteriol. 174, 7778–7783.

Ferrao-Beck, L., Cardoso, R., Munoz, P.M., de Miguel, M.J., Albert, D., Ferreira, A.C., Marin, C.M., Thiebaud, M., Jacques, I., Grayon, M., Zygmunt, M.S., Garin-Bastuji, B., Blasco, J.M., Inacia Sa, M., 2006. Development of a multiplex PCR assay for polymorphism analysis of *Brucella suis* biovars causing brucellosis in swine. Vet. Microbiol. 115, 269–277.

Foster, G., Osterman, B.S., Godfroid, J., Jacques, I., Cloeckaert, A., 2007. *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. Int. J. Syst. Evol. Microbiol. 57, 2688–2693.

Foster, J.T., Okinaka, R.T., Svensson, R., Shaw, K., De, B.K., Robison, R.A., Probert, W.S., Kenefic, L.J., Brown, W.D., Keim, P., 2008. Real-time PCR assays of single-nucleotide polymorphisms defining the major *Brucella* clades. J. Clin. Microbiol. 46, 296–301.

Fretin, D., Whatmore, A.M., Al Dahouk, S., Neubauer, H., Garin-Bastuji, B., Albert, D., Van Hessche, M., Menart, M., Godfroid, J., Walravens, K., Wattiau, P., 2008. *Brucella suis* identification and biovar typing by real-time PCR. Vet. Microbiol. 131, 376–385.

Gandara, B., Merino, A.L., Rogel, M.A., Martinez-Romero, E., 2001. Limited genetic diversity of *Brucella* spp. J. Clin. Microbiol. 39, 235–240.

Garcia-Yoldi, D., Marin, C.M., de Miguel, M.J., Munoz, P.M., Vizmanos, J.L., Lopez-Goni, I., 2006. Multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. Clin. Chem. 52, 779–781.

Gee, J.E., De, B.K., Levett, P.N., Whitney, A.M., Novak, R.T., Popovic, T., 2004. Use of 16S rRNA gene sequencing for rapid confirmatory identification of *Brucella* isolates. J. Clin. Microbiol. 42, 3649–3654.

Groussaud, P., Shankster, S.J., Koylass, M.S., Whatmore, A.M., 2007. Molecular typing divides marine mammal strains of *Brucella* into at least three groups with distinct host preferences. J. Med. Microbiol. 56, 1512–1518.

Halling, S.M., Peterson-Burch, B.D., Bricker, B.J., Zuerner, R.L., Qing, Z., Li, L.-L., Kapur, V., Alt, D.P., Olsen, S.C., 2005. Completion of the genome sequence of *Brucella abortus* and comparison to the highly similar genomes of *Brucella melitensis* and *Brucella suis*. J. Bacteriol. 187, 2715–2726.

Hinic, V., Brodard, I., Thomann, A., Cvetnic, Z., Frey, J., Abril, C., 2008. Novel identification and differentiation of *Brucella melitensis, B. abortus, B. suis, B. ovis, B. canis*, and *B. neotomae* suitable for both conventional and real-time PCR systems. J. Microbiol. Meth. 75, 375–378.

Jacques, I., Grayon, M., Verger, J.-M., 2007. Oxidative metabolic profiles of *Brucella* strains isolated from marine mammals: contribution to their species classification. FEMS Microbiol. Lett. 270, 245–249.

Lane, D.J., 1991. 16S/23S sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. Wiley, Chichester, pp. 115–175.

Ocampo-Sosa, A.A., Agüero-Balbin, J., Garcia-Lobo, J.M., 2005. Development of a new PCR assay to identify *Brucella abortus* biovars 5, 6 and 9 and the new subgroup 3b of biovar 3. Vet. Microbiol. 110, 41–51.

Osterman, B., Moriyon, I., 2006. International Committee on Systematics of Prokaryotes. Subcommittee on the taxonomy of *Brucella*. Minutes of the meeting, 17 September 2003, Pamplona, Spain. Int. J. Syst. Evol. Microbiol. 56, 1173–1175.

Paulsen, I.T., Seshadri, R., Nelson, K.E., Eisen, J.A., Heidelberg, J.F., Read, T.D., Dodson, R.J., Umayam, L., Brinkac, L.M., Beanan, M.J., Daugherty, S.C., Deboy, R.T., Durkin, A.S., Kolonay, J.F., Madupu, R., Nelson, W.C., Ayodeji, B., Kraul, M., Shetty, J., Malek, J., Van Aken, S.E., Riedmuller, S., Tettelin, H., Gill, S.R., White, O., Salzberg, S.L., Hoover, D.L., Lindler, L.E., Halling, S.M., Boyle, S.M., Fraser, C.M., 2002. The *Brucella suis* genome reveals fundamental similarities between animal

and plant pathogens and symbionts. Proc. Natl. Acad. Sci. USA 99, 13148–13153.

Rajashekara, G., Glasner, J.D., Glover, D.A., Splitter, G.A., 2004. Comparative whole-genome hybridization reveals genomic islands in *Brucella* species. J. Bacteriol. 186, 5040–5051.

Ratushna, V.G., Sturgill, D.M., Ramamoorthy, S., Reichow, S.A., He, Y., Lathigra, R., Sriranganathan, N., Halling, S.M., Boyle, S.M., Gibas, C.J., 2006. Molecular targets for rapid identification of *Brucella* spp. BMC Microbiol. 6, 13.

Richards, G.M., 1974. Modifications of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. Anal. Biochem. 57, 369–376.

Scholz, H.C., Pfeffer, M., Witte, A., Neubauer, H., Al Dahouk, S., Wernery, U., Tomaso, H., 2008a. Specific detection and differentiation of *Ochrobactrum anthropi*, *Ochrobactrum intermedium* and *Brucella* spp. by a multiprimer PCR that targets the *recA* gene. J. Med. Microbiol. 57, 64–71.

Scholz, H.C., Hubalek, Z., Sedlácek, I., Vergnaud, G., Tomaso, H., Al Dahouk, S., Melzer, F., Kämpfer, P., Neubauer, H., Cloeckaert, A., Maquart, M., Zygmunt, M.S., Whatmore, A.M., Falsen, E., Bahn, P., Göllner, C., Pfeffer, M., Huber, B., Busse, H.-J., Nöckler, K., 2008b. *Brucella microti* sp. nov., isolated from the common vole *Microtus arvalis*. Int. J. Syst. Evol. Microbiol. 58, 375–382.

Scholz, H.C., Hubalek, Z., Nesvadbova, J., Tomaso, H., Vergnaud, G., Le Fleche, P., Whatmore, A.M., Dahouk, S., Krüger, M., Lodri, C., Pfeffer, M., 2008c. Isolation of *Brucella microti* from soil. Emerg. Infect. Dis. 14, 1316–1317.

Scholz, H.C., Hofer, E. Vergnaud, G., Le Fleche, P., Whatmore, A.M., Al Dahouk, S., Pfeffer, M., Krüger, M., Cloeckaert, A., Tomaso, H., 2009. Isolation of *Brucella microti* from mandibular lymph nodes of red foxes, *Vulpes vulpes*, in lower Austria. Vector-Borne Zoonotic Dis., in press (2008, Oct. 30; epub ahead of print), doi:10.1089/vbz.2008.0036.

Tcherneva, E., Rijpens, N., Jersek, B., Herman, L.M.F., 2000. Differentiation of *Brucella* species by random amplified polymorphic DNA analysis. J. Appl. Microbiol. 88, 69–80.

Velasco, J., Romero, C., Lopez-Goni, I., Leiva, J., Diaz, R., Moriyon, I., 1998. Evaluation of the relatedness of *Brucella* ssp. and *Ochrobactrum anthropi* and description of *Ochrobactrum intermedium* sp. nov., a new species with a closer relationship to *Brucella* ssp. Int. J. Syst. Bacteriol. 48, 759–768.

Verger, J.-M., Grimont, F., Grimont, P.A.D., Grayon, M., 1985. *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. Int. J. Syst. Bacteriol. 35, 292–295.

Whatmore, A.M., Shankster, S.J., Perrett, L.L., Murphy, T.J., Brew, S.D., Thirlwall, R.E., Cutler, S.J., MacMillan, A.P., 2006. Identification and characterization of variable-number tandem-repeat markers for typing of *Brucella* spp. J. Clin. Microbiol. 44, 1982–1993.

Whatmore, A.M., Perrett, L.L., MacMillan, A.P., 2007. Characterisation of the genetic diversity of *Brucella* by multilocus sequencing. BMC Microbiol. 7, 34.

Wieser, M., Busse, H.-J., 2000. Rapid identification of Staphylococcus epidermidis. Int. J. Syst. Evol. Microbiol. 50, 1087–1093.

## 2.3. *Brucella microti* sp. nov., isolated from the common vole *Microtus arvalis*

*International Journal of Systematic and Evolutionary Microbiology* (2008), **58**, 375–382 DOI 10.1099/ijs.0.65356-0

# *Brucella microti* sp. nov., isolated from the common vole *Microtus arvalis*

Holger C. Scholz,[1] Zdenek Hubalek,[2] Ivo Sedláček,[3] Gilles Vergnaud,[4,5] Herbert Tomaso,[1] Sascha Al Dahouk,[1]† Falk Melzer,[6] Peter Kämpfer,[7] Heinrich Neubauer,[6] Axel Cloeckaert,[8] Marianne Maquart,[8] Michel S. Zygmunt,[8] Adrian M. Whatmore,[9] Enevold Falsen,[10] Peter Bahn,[11] Cornelia Göllner,[11] Martin Pfeffer,[1] Birgit Huber,[12] Hans-Jürgen Busse[12] and Karsten Nöckler[11]

Correspondence
Holger C. Scholz
Holger1Scholz@bundeswehr.org

[1]Bundeswehr Institute of Microbiology, Neuherbergstrasse 11, D-80937 Munich, Germany

[2]Institute of Vertebrate Biology, Academy of Sciences, Kvetna 8, CZ-60365 Brno, Czech Republic

[3]Czech Collection of Microorganisms, Faculty of Science, Masaryk University, Brno, Czech Republic

[4]Centre d'Etudes du Bouchet, BP3, 91710 Vert le Petit, France

[5]Université Paris-Sud, Institut de Génétique et Microbiologie, Orsay, F-91405, France; CNRS, Orsay F-91405, France

[6]Friedrich Loeffler Institute, Institute of Bacterial Infections and Zoonoses, German Reference Center for Brucellosis, Jena, Germany

[7]Institute for Applied Microbiology, Justus-Liebig-Universität Giessen, IFZ, Heinrich-Buff- Ring 26-32, D-35392 Giessen, Germany

[8]INRA, UR1282, Infectiologie Animale et Santé Publique, IASP, Nouzilly, F-37380, France

[9]Veterinary Laboratories Agency, Woodham Lane, Addlestone KT15 3NB, UK

[10]Culture Collection, Department of Clinical Bacteriology, University of Göteborg, Guldhedsg. 10 S-41346 Göteborg, Sweden

[11]Federal Institute for Risk Assessment, Diedersdorfer Weg 1, D-12277 Berlin, Germany

[12]Institut für Bakteriologie, Mykologie und Hygiene, Veterinärmedizinische Universität, A-1210 Wien, Austria

Two Gram-negative, non-motile, non-spore-forming, coccoid bacteria (strains CCM 4915$^T$ and CCM 4916), isolated from clinical specimens of the common vole *Microtus arvalis* during an epizootic in the Czech Republic in 2001, were subjected to a polyphasic taxonomic study. On the basis of 16S rRNA (*rrs*) and *recA* gene sequence similarities, both isolates were allocated to the genus *Brucella*. Affiliation to *Brucella* was confirmed by DNA–DNA hybridization studies. Both strains reacted equally with *Brucella* M-monospecific antiserum and were lysed by the bacteriophages Tb, Wb, F1 and F25. Biochemical profiling revealed a high degree of enzyme activity and metabolic capabilities not observed in other *Brucella* species. The *omp2a* and *omp2b* genes of isolates CCM 4915$^T$ and CCM 4916 were indistinguishable. Whereas *omp2a* was identical to *omp2a* of brucellae from certain pinniped marine mammals, *omp2b* clustered with *omp2b* of terrestrial brucellae. Analysis of the *bp26* gene downstream region identified strains CCM 4915$^T$ and CCM 4916 as *Brucella* of terrestrial origin. Both strains harboured five to six copies of the insertion element IS*711*, displaying a unique banding pattern as determined by Southern blotting. In comparative multilocus VNTR (variable-number tandem-repeat) analysis (MLVA) with 296 different genotypes, the two isolates grouped together, but formed a separate

†Present address: RWTH University of Aachen, Department of Internal Medicine III, Pauwelsstraße 30, D-52074 Aachen, Germany.

Abbreviations: MLST, multilocus sequence typing; MLVA, multilocus VNTR (variable-number tandem-repeat) analysis; RTD, routine test dilution.

The GenBank/EMBL/DDBJ accession numbers for the gene sequences *omp22*, *omp25*, *omp25b*, *omp31* and *omp31b* of strain CCM 4915$^T$ are AM712379, AM712381, AM712383, AM712385 and AM712387, respectively.

cluster within the genus *Brucella*. Multilocus sequence typing (MLST) analysis using nine different loci also placed the two isolates separately from other brucellae. In the IS*711*-based AMOS PCR, a 1900 bp fragment was generated with the *Brucella ovis*-specific primers, revealing that the insertion element had integrated between a putative membrane protein and *cboL*, encoding a methyltransferase, an integration site not observed in other brucellae. Isolates CCM 4915$^T$ and CCM 4916 could be clearly distinguished from all known *Brucella* species and their biovars by means of both their phenotypic and molecular properties, and therefore represent a novel species within the genus *Brucella*, for which the name *Brucella microti* sp. nov. with the type strain CCM 4915$^T$ (=BCCN 07-01$^T$=CAPM 6434$^T$) is proposed.

At the time of writing, the genus *Brucella* comprises the six species *Brucella melitensis*, *Brucella abortus*, *Brucella suis*, *Brucella canis*, *Brucella neotomae* and *Brucella ovis* (Corbel & Brinley-Morgan, 1984). Two potential novel species of marine mammal origin, *Brucella pinnipedialis* (formerly '*Brucella pinnipediae*') and *Brucella ceti* (formerly '*Brucella cetaceae*'), have been proposed recently (Cloeckaert *et al.*, 2001; Foster *et al.*, 2007). Due to the high DNA–DNA relatedness of >90 % among these species, it was suggested that the genus should comprise only one species, *B. melitensis*, with the six biovars Melitensis, Abortus, Canis, Neotomae, Ovis and Suis (Verger *et al.*, 1985). However, in 2003, the ICSP Subcommittee on the Taxonomy of *Brucella* agreed unanimously on a return to pre-1986 *Brucella* taxonomy and as a consequence to the reapproval of the six *Brucella* species with the recognized biovars (Osterman & Moriyón, 2006).

Historically, classification of *Brucella* species was based on natural host preference (main isolation source) and phenotypic characteristics, i.e. $CO_2$ requirement, $H_2S$ production, dye-sensitivity, oxidative metabolic profiles, lysis by *Brucella*-specific bacteriophages and agglutination with monospecific antisera. Indeed, the last proposal for minimal standards to describe novel *Brucella* species was made by Corbel & Brinley-Morgan (1975) and certainly has to be reviewed, in respect of the newly developed molecular typing techniques and evolutionary findings.

Because of the high DNA–DNA relatedness and phenotypic similarity among the different *Brucella* species, a battery of phenotypic and genotypic tests is required for species delineation and for characterization of potentially novel *Brucella* species. Natural host preference might be used as a further criterion if enough isolates are available.

Recently, molecular approaches, in particular analysis of DNA polymorphism of outer-membrane genes (Cloeckaert *et al.*, 1995, 2001; Vizcaíno *et al.*, 2004), a set of species-differentiating PCR (PCR-restriction fragment length polymorphism; PCR-RFLP) assays (Al Dahouk *et al.*, 2005; Garcia-Yoldi *et al.*, 2006; Ferrao-Beck *et al.*, 2006; Ratushna *et al.*, 2006), multilocus sequence typing (MLST) (Whatmore *et al.*, 2007) and multilocus VNTR (variable-number tandem-repeat) analysis (MLVA) typing assays (Le Flèche *et al.*, 2006; Whatmore *et al.*, 2006; Garcia-Yoldi *et al.*, 2007; Al Dahouk *et al.*, 2007) have been developed that allow differentiation of *Brucella* species and to some extent also at the biovar/biotype level.

Here we describe the classification of two strains, CCM 4915$^T$ and CCM 4916, which had been originally isolated on meat peptone agar (MPA) at 37 °C under aerobic conditions from clinical specimens of diseased wild common voles (*Microtus arvalis*) during a recent epizootic in the Břeclav district in South Moravia, Czech Republic (Hubalek *et al.*, 2007), as a novel species of the genus *Brucella*.

The phenotype of strains CCM 4915$^T$ and CCM 4916 was investigated according to growth behaviour on different media, $CO_2$ requirement, $H_2S$ production, growth in the presence of dyes (thionine and basic fuchsin), reaction with monospecific A and M antisera, bacteriophage typing (Alton *et al.*, 1988) and light and transmission electron microscopy. Metabolic activity in comparison with other *Brucella* species, *Ochrobactrum anthropi* LMG 3331$^T$ and *Ochrobactrum intermedium* LMG 3301$^T$ was assessed using the Merlin MICRONAUT system (Neubauer *et al.*, 2000). Polar lipids and quinone system were analysed as described by Tindall (1990a, b), Altenburger *et al.* (1996) and Stolz *et al.* (2007), and the polyamine pattern was analysed as described by Busse & Auling (1988) and Stolz *et al.* (2007), despite the fact that the biomass was not collected from liquid culture but cells were harvested from trypticase soy agar (TSA) and inactivated by acetone.

Molecular analyses comprised AMOS PCR (Bricker & Halling, 1994), multiplex PCR (Garcia-Yoldi *et al.*, 2006), sequence analysis of the outer membrane genes *omp2a*, *omp2b* (Cloeckaert *et al.*, 1995, 2001), *omp22*, *omp25*, *omp25b*, *omp31* and *omp31b* (Vizcaíno *et al.*, 2004), analysis of the *bp26* gene downstream region to distinguish between brucellae of marine mammal and terrestrial mammal origin (Cloeckaert *et al.*, 2000), IS*711* (IS*6501*)-typing by Southern blotting analysis (Clavareau *et al.*, 1998), MLVA (Le Flèche *et al.*, 2006; Whatmore *et al.*, 2006; Garcia-Yoldi *et al.*, 2007; Al Dahouk *et al.*, 2007), MLST (Whatmore *et al.*, 2007) and DNA–DNA hybridization studies (Ziemke *et al.*, 1998).

Both isolates grew well at 28 and 37 °C on MPA without supplementary $CO_2$, forming transparent to whitish

**Table 1.** Differentiating physiological reactions of *B. microti* sp. nov. in comparison with other *Brucella* and *Ochrobactrum* species.

Taxa: 1, *B. microti* CCM 4915$^T$; 2, *B. microti* CCM 4916; 3, *B. abortus* strains: 544$^T$ (=NCTC 10093$^T$) (bv. 1), 86/8/59 (=NCTC 10501) (bv. 2), Tulya (=NCTC 10502) (bv. 3), 292 (=NCTC 10503) (bv. 4), B3196 (=NCTC 10504) (bv. 5), 870 (=NCTC 10505) (bv. 6), 63175 (=NCTC 10506) (bv. 7), C68 (=NCTC 10507) (bv. 9); 4, *B. melitensis* strains: 16M$^T$ (=NCTC 10094$^T$) (bv. 1), 63/9 (=NCTC 10508) (bv. 2), Ether (=NCTC 10509) (bv. 3); 5, *B . suis* strains: 1330 (=NCTC 10316) (bv. 1), Thomsen (=NCTC 10510) (bv. 2), 686 (=NCTC 10511) (bv. 3), Reference 40 (bv. 4), Reference 513 (bv. 5); 6, *B. ovis* strain: 63/290$^T$ (=NCTC 10512$^T$); 7, *B. canis* strain: RM6/66$^T$ (=NCTC 10854$^T$); 8, *B. neotomae* strain: 5K33$^T$ (=NCTC 10084$^T$); 9, *B. pinnipedialis* NCTC 12890$^T$; 10, *B. ceti* NCTC 12891$^T$; 11, *O. anthropi* LMG 3331$^T$; 12, *O. intermedium* LMG 3301$^T$. oNP, *o*-nitrophenyl; pNP, *p*-nitrophenyl; +, positive ($QO_2N$ values >70); −, negative ($QO_2N$ values <50); v, variable ($QO_2N$ values >50 and <70).

| Compound | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Substrate class |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| L-Glutamine | + | + | − | − | − | − | − | − | − | − | + | + | Amino acid |
| L-Histidine hydrochloride | + | + | − | − | − | − | − | − | − | − | + | + | Amino acid |
| D-Histidine | + | + | − | − | − | − | − | − | − | − | + | + | Amino acid |
| L-Citrulline | + | + | − | − | − | − | − | − | − | − | + | + | Amino acid |
| DAPI | + | + | − | − | − | − | − | − | − | − | + | + | Amino acid |
| Sarcosine hydrochloride | + | + | − | − | − | − | − | − | − | − | + | + | Amino acid derivative |
| Fumaric acid | + | + | − | − | − | − | − | − | − | − | + | + | Organic acid |
| Succinic acid | + | + | − | − | − | − | − | − | − | − | + | + | Organic acid |
| α-Ketoglutaric acid | + | + | − | − | − | − | − | − | − | − | + | + | Organic acid |
| Itaconic acid | + | + | − | − | − | − | − | − | − | − | − | − | Organic acid |
| Mesaconic acid | + | + | − | − | − | − | − | − | − | − | − | − | Organic acid |
| Glutaric acid | + | + | − | − | − | − | − | − | − | − | + | + | Organic acid |
| Arginine *p*-nitroanilide | + | + | − | − | − | − | − | − | − | − | v | + | Amino peptidase |
| pNP *N*-acetyl-β-D-galactosaminide (pH 7.5) | + | + | − | − | − | − | − | − | − | − | v | + | Esterase |
| oNP *N*-acetyl-α-D-galactosaminide (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Esterase |
| oNP *N*-acetyl-β-D-galactosaminide (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Esterase |
| pNP *N*-acetyl-β-D-glucosaminide (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Esterase |
| pNP *N*-acetyl-L-thio-β-D-glucosaminide (pH 7.5) | v | + | − | − | − | − | − | − | − | − | + | + | Esterase |
| 4-nitro α-D-maltoheptaose-4,6,*o*-ethylidene (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Esterase |
| pNP *N*-acetyl-β-D-glucosamide (pH 5.5) | + | + | − | − | − | − | − | − | − | − | + | − | Esterase |
| pNP α-L-arabinopyranoside (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Glucosidase |
| oNP β-D-xylopyranoside (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Glucosidase |
| pNP L-thio-β-D-galactopyranoside (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Glucosidase |
| 2-Methoxy-4-(2-nitrovinyl)-phenyl β-D-galactopyranoside | + | + | − | − | − | − | − | − | − | − | + | + | Glucosidase |
| pNP β-L-fucopyranoside (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Glucosidase |
| pNP β-D-thiofucopyranoside (pH 7.5) | v | + | − | − | − | − | − | − | − | − | + | + | Glucosidase |
| pNP β-D-mannopyranoside (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Glucosidase |
| pNP β-D-maltoside (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Glucosidase |
| pNP β-D-lactopyranoside (pH 7.5) | v | + | − | − | − | − | − | − | − | − | + | + | Glucosidase |
| pNP β-D-glucuronide (pH 5.5) | + | + | − | − | − | − | − | − | − | − | + | − | Glucosidase |
| Aesculin hydrolysis | − | − | − | − | v | − | v | − | − | − | + | + | Classical reaction |
| Voges–Proskauer reaction | + | + | − | − | − | − | − | − | − | − | + | + | Classical reaction |
| oNP β-D-galactopyranoside-6-phosphate | + | + | − | − | − | − | − | − | − | − | + | + | Phosphatase |
| pNP phosphate-di(2-amino-2-ethyl-1,3-propanediol) (pH 5.5) | + | + | − | − | − | − | − | − | − | − | + | + | Phosphatase |

colonies, 1–2 mm in diameter, after 1–2 days of incubation. $H_2S$ was not produced. Good growth was also observed on blood agar and on standard nutrient agar at 28 and 37 °C. Colonies were slightly concave, smooth, with very light brown exopigment; their edges were continuous. After 72 h of growth at 37 °C, cells appeared as large colonies (6–9 mm) with noticeable brownish pigmentation. Cell morphology was examined using a Zeiss light microscope at ×1000 magnification with cells grown for 2 days at 37 °C on standard nutrient agar. Gram staining was performed as described by Gerhardt *et al.* (1994) with cells grown for 2 days at 28 °C on MPA. Cells were small (<1 μm) Gram-negative coccobacilli or sporadically formed short rods, arranged individually or in irregular clusters. Flagellation

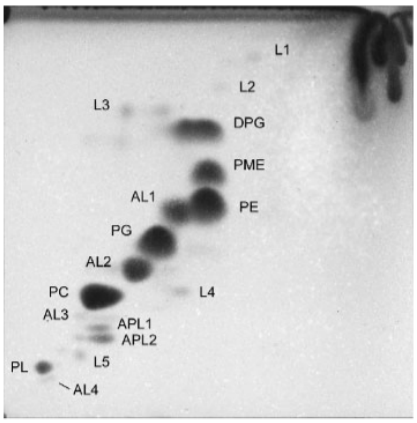


**Fig. 1.** Two-dimensional thin-layer chromatogram of total polar lipids of strain CCM 4915$^T$. PME, Phosphatidylmonomethylethanolamine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; AL1–4, unknown aminolipids; PL, unknown phospholipid; APL1 and 2, unknown aminophospholipids; L1–5, unknown polar lipids.

was determined by transmission electron microscopy (negative staining with 1 % uranyl acetate) using a JEM-1010 electron microscope at a magnification of ×40 000. Cells were unflagellated and morphologically indistinguishable from *B. melitensis* 16M$^T$, whereas flagella were clearly visible on *O. anthropi* LMG 3331$^T$ (data not shown), which is the type species of the phylogenetically closest genus.

Besides the classical biochemical reactions (Hubalek *et al.*, 2007), a total of 191 different carbohydrates, 190 substrates to determine enzymic activity and 191 different substrates for use as a nitrogen source were tested by using the Merlin MICRONAUT identification system (Neubauer *et al.*, 2000). Each reaction was carried out seven times. The results of the differentiating biochemical reactions of strains CCM 4915$^T$ and CCM 4916 in comparison with the other *Brucella* species and their biovars as well as with *Ochrobactrum* species are summarized in Table 1. The results demonstrate that both isolates exhibit outstanding metabolic capabilities with respect to all other brucellae, sharing a series of reactions with *O. anthropi* LMG 3331$^T$ and *O. intermedium* LMG 3301$^T$. It is remarkable that both strains were positive in the Voges–Proskauer reaction. Additional results of the phenotypic characterization of strains CCM 4915$^T$ and CCM 4916 by classical methods are given in the species description.

The two strains exhibited a quinone system with ubiquinone Q-10 (91–97 %) and Q-9 (3–9 %). A similar quinone system was detected in *B. melitensis*, with Q-10 (97 %) and Q-9 (3 %). Their polar lipid profiles consisted of the major compounds phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine and two unknown aminolipids (AL1 and 2), moderate amounts of an unknown phospholipid PL and small to trace amounts of additional unknown aminolipids (AL3 and 4), two unknown aminophospholipids (APL1 and 2) and five unknown polar lipids (Fig. 1). Two additional lipids, one staining positively with ninhydrin (aminolipid) and the other with molybdenum blue (phospholipid), may be present, showing almost the same chromatographic behaviour as AL1, but their presence could not unambiguously confirmed because of insufficient resolution from AL1 in the thin-layer chromatography. *B. abortus* NCTC 10093$^T$ and *B. melitensis* 16M$^T$ exhibited almost the same profile (results not shown), but unknown aminolipid AL2 was not detectable in these two strains and phosphatidylmonomethylethanolamine was present either in traces or lacking. The polyamine patterns of strains CCM 4915$^T$ and CCM 4916 contained the major compound spermidine (77.0–80.2 %), putrescine (14.0–16.8 %), 1,3-diaminopropane (3.4–5.0 %), *sym*-homospermidine (0.8–1.9 %) and spermine (0.4–0.5 %). Similar polyamine patterns were detected in *B. melitensis* 16M$^T$ (74.2 % spermidine, 18.6 % putrescine, 3.0 % 1,3-diaminopropane, 3.5 % *sym*-homospermidine and 0.4 % spermine) and representative species of the genus *Ochrobactrum*, including *O. intermedium* LMG 3301$^T$ (results not shown), the nearest phylogenetic relative of the genus *Brucella*. In contrast, species of the genus *Pseudochrobactrum* exhibit a polyamine pattern lacking *sym*-homospermidine (Kämpfer *et al.*, 2006, 2007).

In the IS*711*-based AMOS multiplex-PCR, a 1900 bp fragment, not amplified from other *Brucella* species, was generated. Using single species-specific primer pairs, it could be demonstrated that the fragment was amplified with the *B. ovis*-specific primers (Hubalek *et al.*, 2007). Sequencing of the PCR product and subsequent comparative BLASTN analyses revealed that the IS element has integrated between the coding sequences of a putative membrane protein (TIGR/KEGG locus tag BR1284/BMEI0717) and *cboL* (BR1285/BME0716), a methyltransferase gene (data not shown). At the *B. ovis* primer side the partial *radC* (BR1283/BMEI0718) gene, involved in DNA repair, is present. In none of the published *Brucella* genomes is IS*711* located at this position. In the multiplex PCR developed by Garcia-Yoldi *et al.* (2006), strains CCM 4915$^T$ and CCM 4916 were indistinguishable from *B. suis* (data not shown).

The entire open reading frames of the *omp22* (639 bp), *omp25* (642 bp) and *omp31* (752 bp) genes were identical to the corresponding genes of *Brucella suis* 1330 (GenBank accession no. AE014291.4), *Brucella ceti* B202R (AY484523.1) and *Brucella pinnipedialis* B2/94$^T$ (AY484522.1). Similarity values of 99 % (one to three substitutions) were observed with other brucellae. Within *omp31b* (786 bp), neither deletions, as reported for *B. melitensis*, nor premature stop codons, as observed for *B. canis* and *B. ovis* (Vizcaíno *et al.*, 2004), nor inversion of the *omp25b* gene (785 bp), as described for the majority of *B. ceti* strains (Vizcaíno *et al.*, 2004), were detected.

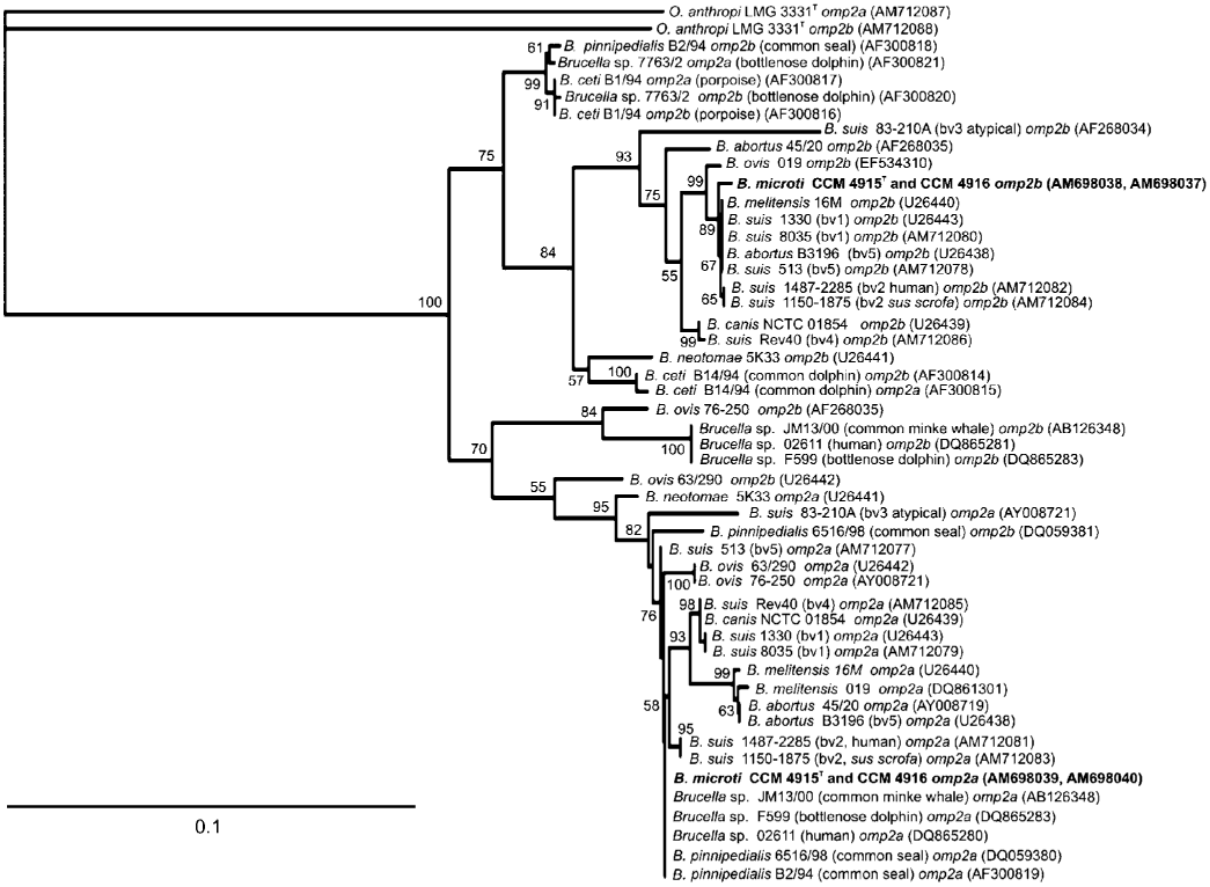


**Fig. 2.** Phylogenetic tree reconstructed with *omp2a* (1104 nt) and *omp2b* (1089 nt) sequences using CLUSTREE neighbour-joining analysis. The significance of each branch is indicated by a bootstrap percentage calculated for 1000 subsets. The tree was rooted by outgrouping sequence *omp2a* of *O. anthropi* LMG 3331$^T$. Bar, 0.1 divergent residues per site.

The *omp2a* genes were 100 % identical to *omp2a* of the marine *Brucella pinnipedialis* strain B2/94$^T$ (GenBank accession no. AF300819) and some other *Brucella* strains isolated from marine mammals, i.e. *Brucella pinnipedialis* strain 6516/9 (DQ059380) and *Brucella* sp. JM13/00 (AB126348). In contrast, the *omp2b* genes were mostly related to *omp2b* of *Brucella* strains from terrestrial mammals with one to three mismatches, (99–98 % similarity). The combination of *omp2a* from marine mammal and *omp2b* from terrestrial mammal origin has not yet been described.

A phylogenetic neighbour-joining tree constructed with *omp2a* and *omp2b* of strains CCM 4915$^T$ and CCM 4916, *B. suis* biovars (bv) 2, 4, 5 and sequences available from the EMBL nucleotide database is shown in Fig. 2. The tree was constructed using the online accessible bioinformatics tools of HUSAR, available from http://genius.embnet.dkfz-heidelberg.de/menu/w2h/w2hdkfz. It was constructed from the alignments obtained with CLUSTREE and the implemented Kimura two-parameter model. In order to assess probabilities of the nodes, 1000 bootstrap resamplings were performed. The tree was rooted with *omp2a* of *Ochrobactrum anthropi* LMG 3331$^T$ as the outgroup.

The PCR fragment size of the *bp26* gene downstream region was 1029 bp, which is characteristic for terrestrial mammal brucellae (data not shown).

The MLVA data obtained with strains CCM 4915$^T$ and CCM 4916 were compared with 424 *Brucella* isolates corresponding to 296 genotypes that have previously been analysed by Le Flèche *et al.* (2006), Garcia-Yoldi *et al.* (2007) and Al Dahouk *et al.* (2007). Cluster analysis was performed as described by Al Dahouk *et al.* (2007) using eight-minisatellite (panel 1) and seven-microsatellite VNTR markers (panel 2) and the categorical distance coefficient and the unweighted pair-group method using arithmetic averages (UPGMA) clustering method. Briefly, the seven microsatellites are divided in two groups, taking

into account their mutation rate (Garcia-Yoldi *et al.*, 2007). Panel 2A comprises the most stable microsatellites Bruce 18, 19, 21 and 30. Panel 2B comprises the highly variable markers Bruce 07, 09 and 16. In the clustering analysis, markers from panel 1, 2A and 2B were given an individual weight of respectively 2, 1 and 0.3. In this analysis, the two strains clustered together (and differed from each other by 4 out of 16 VNTR loci, including one marker from panel 2A and the three markers from panel 2B), but grouped separately from all other known *Brucella* species and their biovars (Fig. 3). Because of the large number of strains, clusters were condensed. *B. neotomae* reference strain 5K33 and *B. pinnipedialis* strain B2/94 appear as closest neighbours in this analysis, whereas the two *B. suis* biovar 5 strains are more distantly related.

In MLST analysis (Whatmore *et al.*, 2007), the two strains clustered separately from other *Brucella* strains most closely related to *B. suis* biovar 5 (ST19) and marine mammal ST25, from which they differ at two out of nine loci (Fig. 4). The MLST profile was 1-11-4-2-1-3-5-2-1. In the IS*711* insertion element typing analysis using the Southern blotting technique, the two isolates were indistinguishable from each other, but exhibited a unique banding pattern when compared with other *Brucella* species, corresponding to five to six copies (not shown).

Results from DNA–DNA hybridizations using labelled DNA of *B. melitensis* 16M$^{T}$ showed 84.1 % ± 0.7 DNA relatedness with strain CCM 4915$^{T}$. These results are in agreement with previous reports (Verger *et al.*, 1985) which showed that, if the 70 % DNA-relatedness threshold value is applied, species of this genus cannot be separated based on results from DNA–DNA hybridizations. The DNA relatedness to the phylogenetically closest neighbour, *O. intermedium* LMG 3301$^{T}$, was 55.5 %.

In summary, from the results of the phenotypic and genotypic analyses, it is evident that isolates CCM 4915$^{T}$ and CCM 4916 are members of the genus *Brucella*, but can be clearly differentiated from all established species of this genus, including their biovars. Hence, by applying the *Brucella* species concept suggested by the ICSP Subcommittee on the taxonomy of *Brucella* (Osterman & Moriyón, 2006), the two strains represent a novel species of the genus *Brucella*, for which we propose the name *Brucella microti* sp. nov.

Future investigations such as whole-genome sequencing and studies on host cell interactions will provide further insight in the evolution of brucellae and host–pathogen interactions.

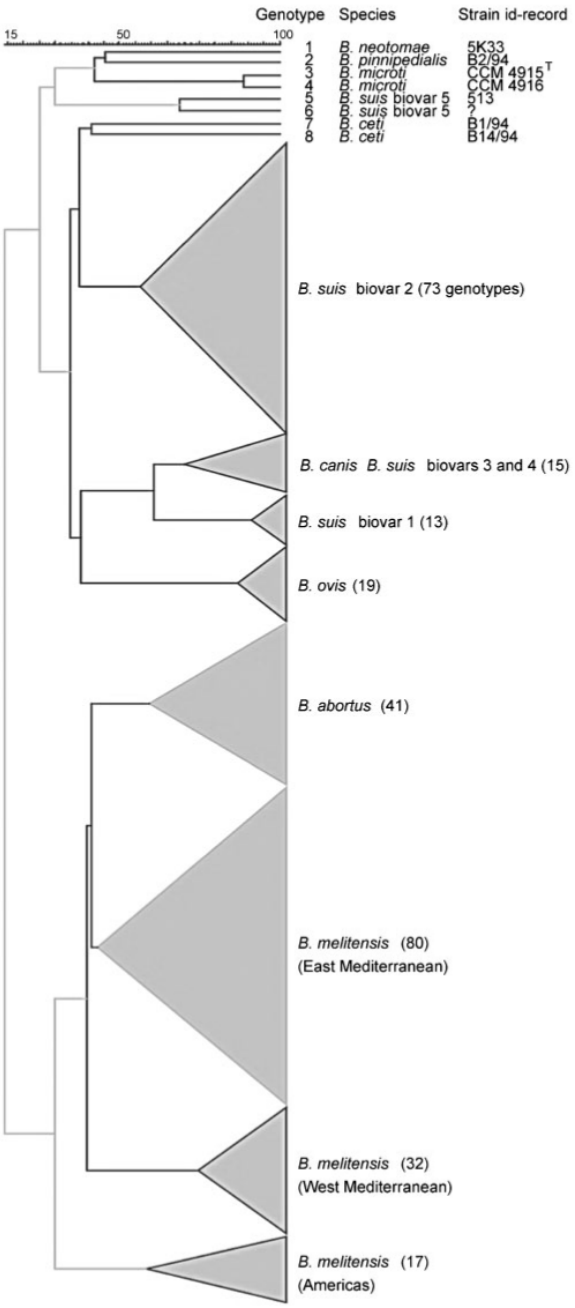


**Fig. 3.** Condensed dendrogram of clustered MLVA-16 genotypes obtained with 424 *Brucella* isolates corresponding to 296 different genotypes. Bar, percentage of sequence divergence.

## Description of *Brucella microti* sp. nov.

*Brucella microti* (mi.cro′ti. L. gen. n. *microti* of *Microtus*, after the vole genus from which the first strains of this species have been isolated).

Cocci, coccobacilli or short rods, 0.5–0.8 μm in diameter and 0.6–1.4 μm in length. Arranged individually or in irregular clusters. Gram-negative. Aerobic, non-fermentative, non-motile, non-spore-forming. Oxidase-, catalase- and urease-positive. Nitrate and nitrite are reduced (with gas formation from nitrate). No production of $H_2S$,

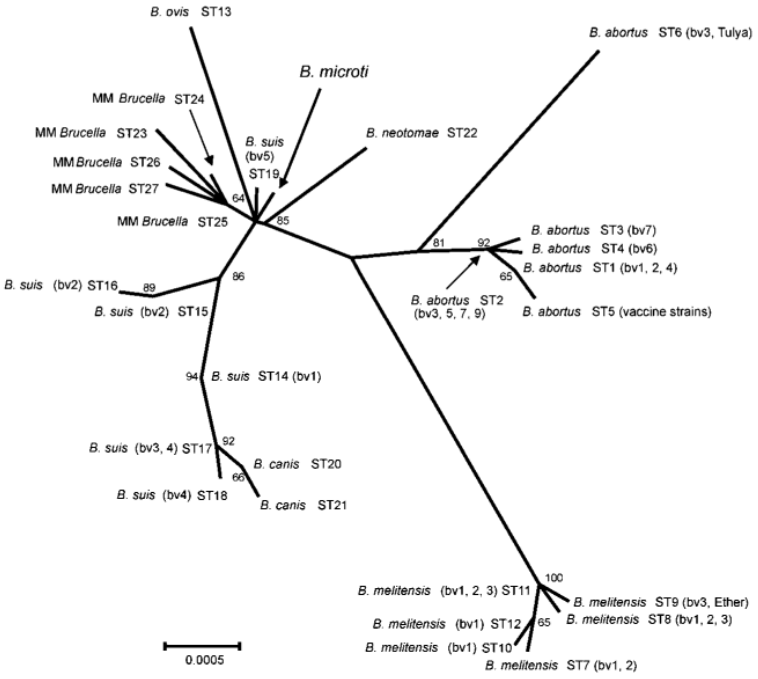


**Fig. 4.** Unrooted phylogenetic reconstruction of the relationships between sequence types (ST). This tree was constructed with the concatenated sequence data of the nine loci (4396 bp) using the neighbour-joining approach. The Jukes–Cantor model, which is based on the assumption that all nucleotide substitutions are equally likely, was used to determine genetic distances. Percentage bootstrap confidence levels of internal branches were calculated from 1000 resamplings of the original data. bv, Biovar; MM, marine mammals. Bar, 0.0005 substitutions per site.

arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, fluorescein (King's B medium). Negative on Simmons' citrate, malonate and acetamide and no growth in broth with 6.5 % NaCl. Positive (API 20NE) for D-glucose, maltose, L-arabinose, D-mannose, *N*-acetylglucosamine and adipic acid. Negative for D-mannitol, citric acid, gluconate, capric acid, malic acid and phenylacetic acid. Acid is produced from glucose, maltose, fructose and xylose in OF medium (Difco). Positive (API ZYM) for acid phosphatase, alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, $\beta$-glucosidase and esterase lipase. Enzymes absent on API ZYM are esterase, lipase, $\alpha$- and $\beta$-galactosidase, $\beta$-glucosidase, $\beta$-glucuronidase, *N*-acetyl-$\beta$-glucosaminidase, $\alpha$-mannosidase and $\alpha$-fucosidase. Starch, casein, DNA, tyrosine, aesculin, ONPG, Tween 80, lecithin and gelatin are not hydrolysed. Differentiating physiological reactions (MICRONAUT) of strains CCM 4915$^T$ and CCM 4916 with respect to other *Brucella* species and biovars and *Ochrobactrum anthropi* LMG 3331$^T$ are given in Table 1. Cells are sensitive to gentamicin, tobramycin, cotrimoxazole (trimethoprim plus sulphamethoxazole) and ofloxacin, but resistant to colistin, piperacillin, ceftazidime and tazobactam. Non-fastidious; good growth occurs on MPA, TSA, blood agar and standard nutrient agar at 25–42 °C. Beige, translucent to whitish colonies with light-brown exopigment and entire edges are formed within 24 h, with a diameter of approximately 2 mm. Good growth does not require $CO_2$, supplementary serum or blood. No haemolysis is observed. Isolates grow with thionine at dilutions of 1/25 000, 1/50 000 and 1/100 000, and with basic fuchsin at dilutions of 1/50 000 and 1/100 000. Cultures are lysed by Tb, F1 and F25 phages at routine test dilution (RTD) $10^4$ but not at RTD and by Weybridge phage (Wb) at both dilutions. Both strains agglutinate with monospecific anti-M serum up to a dilution of 1 : 80. The quinone system is composed of the major compound ubiquinone Q-10 and minor amounts of Q-9. Predominant polar lipids are phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine and two unknown aminolipids, moderate amounts of an unknown phospholipid PL and small to trace amounts of additional unknown aminolipids (AL3 and 4), two unknown aminophospholipids (APL1 and 2) and five unknown polar lipids. The polyamine pattern contains the major compound spermidine, moderate amounts of putrescine and small amounts of 1,3-diaminopropane, *sym*-homospermidine and spermine.

The type strain is CCM 4915$^T$ (=BCCN 07-01$^T$=CAPM 6434$^T$) isolated in 2000 from the liver of *Microtus arvalis*, South Moravia, Czech Republic. Another strain of this species is CCM 4916.

## Acknowledgements

We are grateful to Csilla Lodri, Stefan Schatz and Angelika Draeger for excellent technical assistance. We thank Philippe Le Flèche for the MLVA typing and Mark S. Koylass for MLST analysis of strains CCM 4915$^T$ and CCM 4916. The development of genotyping methods for the precise strain identification of dangerous pathogens as part of microbial forensics is supported by the French DGA (Délégation Générale pour l'Armement). The work of B. H. and H.-J. B. was

supported by a Contract-Research-Project for the Bundeswehr Medical Service.

## References

**Al Dahouk, S., Tomaso, H., Prenger-Berninghoff, E., Splettstoesser, W. D., Scholz, H. C. & Neubauer, H. (2005).** Identification of *Brucella* species and biotypes using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). *Crit Rev Microbiol* **31**, 191–196.

**Al Dahouk, S., Le Flèche, P., Noeckler, K., Jacques, I., Grayon, M., Scholz, H. C., Tomaso, H., Vergnaud, G. & Neubauer, H. (2007).** Evaluation of *Brucella* MLVA typing for human brucellosis. *J Microbiol Methods* **69**, 137–145.

**Altenburger, P., Kämpfer, P., Makristathis, A., Lubitz, W. & Busse, H.-J. (1996).** Classification of bacteria isolated from a medieval wall painting. *J Biotechnol* **47**, 39–52.

**Alton, G. G., Jones, L. M., Angus, R. D. & Verger, J. M. (1988).** *Techniques for the Brucellosis Laboratory (Techniques et Pratiques)*. Paris: INRA.

**Bricker, B. J. & Halling, S. M. (1994).** Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis, Brucella ovis* and *Brucella suis* bv. 1 by PCR. *J Clin Microbiol* **32**, 2660–2666.

**Busse, H. J. & Auling, G. (1988).** Polyamine pattern as a chemotaxonomic marker within the *Proteobacteria*. *Syst Appl Microbiol* **11**, 1–8.

**Clavareau, C., Wellemans, V., Walravens, K., Tryland, M., Verger, J.-M., Grayon, M., Cloeckaert, A., Letesson, J. J. & Godfroid, J. (1998).** Phenotypic and molecular characterization of a *Brucella* strain isolated from a minke whale (*Balaeonoptera acutorostrata*). *Microbiology* **144**, 3267–3273.

**Cloeckaert, A., Verger, J. M., Grayon, M. & Grepinet, O. (1995).** Restriction site polymorphism of the genes encoding the major 25 kDa and 36 kDa outer-membrane proteins of *Brucella*. *Microbiology* **141**, 2111–2121.

**Cloeckaert, A., Grayon, M. & Grepinet, O. (2000).** An IS*711* element downstream of the bp26 gene is a specific marker of *Brucella* spp. isolated from marine mammals. *Clin Diagn Lab Immunol* **7**, 835–839.

**Cloeckaert, A., Verger, J.-M., Grayon, M., Paquet, J. Y., Garin-Bastuji, B., Foster, G. & Godfroid, J. (2001).** Classification of *Brucella* spp. isolated from marine mammals by DNA polymorphism at the *omp2* locus. *Microbes Infect* **3**, 729–738.

**Corbel, M. J. & Brinley-Morgan, W. J. (1975).** Proposal for minimal standards for descriptions of new species and biotypes of the genus *Brucella*. *Int J Syst Bacteriol* **25**, 83–89.

**Corbel, M. J. & Brinley-Morgan, W. J. (1984).** Genus *Brucella*. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 377–388. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

**Ferrao-Beck, L., Cardoso, R., Munoz, P. M., de Miguel, M. J., Albert, D., Ferreira, A. C., Marin, C. M., Thiebaud, M., Jacques, I. & other authors (2006).** Development of a multiplex PCR assay for polymorphism analysis of *Brucella suis* biovars causing brucellosis in swine. *Vet Microbiol* **115**, 269–277.

**Foster, G., Osterman, B., Godfroid, J., Jacques, I. & Cloeckaert, A. (2007).** *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. *Int J Syst Evol Microbiol* **57**, 2688–2693.

**Garcia-Yoldi, D., Marin, C. M., de Miguel, M. J., Munoz, P. M., Vizmanos, J. L. & Lopez-Goni, I. (2006).** Multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains *Brucella* abortus S19 and RB51 and *Brucella melitensis* Rev1. *Clin Chem* **52**, 779–781.

**Garcia-Yoldi, D., Le Flèche, P., Marin, C. M., De Miguel, M. J., Munoz, P. M., Vergnaud, G. & Lopez-Goni, I. (2007).** Assessment of genetic stability of *Brucella melitensis* Rev 1 vaccine strain by multiple-locus variable-number tandem repeat analysis. *Vaccine* **25**, 2858–2862.

**Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (editors) (1994).** *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.

**Hubálek, Z., Scholz, H. C., Sedláček, I., Melzer, F., Sanogo, Y. & Nesvadbová, J. (2007).** Brucellosis of the common vole (*Microtus arvalis*). *Vector Borne Zoonotic Dis* **7**, 679–688.

**Kämpfer, P., Rosselló-Mora, R., Scholz, H. C., Welinder-Olsson, C., Falsen, E. & Busse, H.-J. (2006).** Description of *Pseudochrobactrum* gen. nov., with the two species *Pseudochrobactrum asaccharolyticum* sp. nov. and *Pseudochrobactrum saccharolyticum* sp. nov. *Int J Syst Evol Microbiol* **56**, 1823–1829.

**Kämpfer, P., Scholz, H., Huber, B., Thummes, K., Busse, H.-J., Maas, E. W. & Falsen, E. (2007).** Description of *Pseudochrobactrum kiredjianiae* sp. nov. *Int J Syst Evol Microbiol* **57**, 755–760.

**Le Flèche, P., Jacques, I., Grayon, M., Al Dahouk, S., Bouchon, P., Denoeud, F., Nockler, K., Neubauer, H., Guilloteau, L. A. & Vergnaud, G. (2006).** Evaluation and selection of tandem repeat loci for a Brucella MLVA typing assay. *BMC Microbiol* **6**, 9.

**Neubauer, H., Molitor, M., Rahalison, L., Aleksic, S., Backes, H., Chanteau, S. & Meyer, H. (2000).** A miniaturised semiautomated system for identification of *Yersinia* species within the genus *Yersinia*. *Clin Lab* **46**, 561–567.

**Osterman, B. & Moriyón, I. (2006).** International Committee on Systematics of Prokaryotes Subcommittee on the Taxonomy of *Brucella*. Minutes of the meeting, 17 September 2003, Pamplona, Spain. *Int J Syst Evol Microbiol* **56**, 1173–1175.

**Ratushna, V. G., Sturgill, D. M., Ramamoorthy, S., Reichow, S. A., He, Y., Lathigra, R., Sriranganathan, N., Halling, S. M., Boyle, S. M. & other authors (2006).** Molecular targets for rapid identification of *Brucella* spp. *BMC Microbiol* **6**, 13.

**Stolz, A., Busse, H.-J. & Kämpfer, P. (2007).** *Pseudomonas knackmussii* sp. nov. *Int J Syst Evol Microbiol* **57**, 572–576.

**Tindall, B. J. (1990a).** A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* **13**, 128–130.

**Tindall, B. J. (1990b).** Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199–202.

**Verger, J.-M., Grimont, F., Grimont, P. A. D. & Grayon, M. (1985).** *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. *Int J Syst Bacteriol* **35**, 292–295.

**Vizcaíno, N., Caro-Hernandez, P., Cloeckaert, A. & Fernandez-Lago, L. (2004).** DNA polymorphism in the *omp25/omp31* family of *Brucella* spp.: identification of a 1.7-kb inversion in *Brucella cetaceae* and of a 15.1-kb genomic island, absent from *Brucella ovis*, related to the synthesis of smooth lipopolysaccharide. *Microbes Infect* **6**, 821–834.

**Whatmore, A. M., Shankster, S. J., Perrett, L. L., Murphy, T. J., Brew, S. D., Thirlwall, R. E., Cutler, S. J. & MacMillan, A. P. (2006).** Identification and characterization of variable-number tandem-repeat markers for typing of *Brucella* spp. *J Clin Microbiol* **44**, 1982–1993.

**Whatmore, A. M., Perrett, L. L. & Macmillan, A. P. (2007).** Characterisation of the genetic diversity of *Brucella* by multilocus sequencing. *BMC Microbiol* **7**, 34.

**Ziemke, F., Höfle, M. G., Lalucat, J. & Rosselló-Mora, R. (1998).** Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.

## 2.4. *Brucella inopinata* sp. nov., isolated from a breast implant infection

*International Journal of Systematic and Evolutionary Microbiology* (2010), **60**, 801–808 DOI 10.1099/ijs.0.011148-0

# *Brucella inopinata* sp. nov., isolated from a breast implant infection

Holger C. Scholz,[1] Karsten Nöckler,[2] Cornelia Göllner,[2] Peter Bahn,[2] Gilles Vergnaud,[3,4] Herbert Tomaso,[1] Sascha Al Dahouk,[5] Peter Kämpfer,[6] Axel Cloeckaert,[7] Marianne Maquart,[7] Michel S. Zygmunt,[7] Adrian M. Whatmore,[8] Martin Pfeffer,[1] Birgit Huber,[9] Hans-Jürgen Busse[9] and Barun Kumar De[10]

Correspondence
Holger C. Scholz
Holger1Scholz@bundeswehr.org

[1]Bundeswehr Institute of Microbiology, Neuherbergstrasse 11, D-80937 Munich, Germany

[2]Federal Institute for Risk Assessment, Diedersdorfer Weg 1, D-12277 Berlin, Germany

[3]DGA/D4S – Mission pour la Recherche et l'Innovation Scientifique, 7, rue des Mathurins, 92220 Bagneux, France

[4]Université Paris-Sud 11, CNRS, UMR8621, Institut de Génétique et Microbiologie, 91405 Orsay, France

[5]RWTH Aachen University, Department of Internal Medicine III, Pauwelsstraße 30, D-52074 Aachen, Germany

[6]Institute for Applied Microbiology, Justus-Liebig-Universitat Giessen, IFZ, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany

[7]INRA, UR1282, Infectiologie Animale et Santé Publique, IASP, F-37380 Nouzilly, France

[8]Veterinary Laboratories Agency, Woodham Lane, Addlestone KT15 3NB, UK

[9]Institut für Bakteriologie, Mykologie und Hygiene, Veterinärmedizinische Universität, A-1210 Wien, Austria

[10]Centers for Disease Control and Prevention, 1600 Clifton Rd, Atlanta, GA 30333, USA

A Gram-negative, non-motile, non-spore-forming coccoid bacterium (strain BO1$^T$) was isolated recently from a breast implant infection of a 71-year-old female patient with clinical signs of brucellosis. Affiliation of strain BO1$^T$ to the genus *Brucella* was confirmed by means of polyamine pattern, polar lipid profile, fatty acid profile, quinone system, DNA–DNA hybridization studies and by insertion sequence *711* (IS*711*)-specific PCR. Strain BO1$^T$ harboured four to five copies of the *Brucella*-specific insertion element IS*711*, displaying a unique banding pattern, and exhibited a unique 16S rRNA gene sequence and also grouped separately in multilocus sequence typing analysis. Strain BO1$^T$ reacted with *Brucella* M-monospecific antiserum. Incomplete lysis was detected with bacteriophages Tb (Tbilisi), F1 and F25. Biochemical profiling revealed a high degree of enzymic activity and metabolic capabilities. In multilocus VNTR (variable-number tandem-repeat) analysis, strain BO1$^T$ showed a very distinctive profile and clustered with the other 'exotic' *Brucella* strains, including strains isolated from marine mammals, and *Brucella microti*, *Brucella suis* biovar 5 and *Brucella neotomae*. Comparative *omp2a* and *omp2b* gene sequence analysis revealed the most divergent *omp2* sequences identified to date for a *Brucella* strain. The *recA* gene sequence of strain BO1$^T$ differed in seven nucleotides from the *Brucella recA* consensus sequence. Using the *Brucella* species-specific multiplex PCR assay, strain BO1$^T$ displayed a unique banding pattern not observed in other *Brucella* species. From the phenotypic and molecular analysis it became evident that strain BO1$^T$ was clearly different from all other *Brucella* species, and therefore represents a novel species within the genus *Brucella*. Because of its unexpected isolation, the name *Brucella inopinata* with the type strain BO1$^T$ (=BCCN 09-01$^T$=CPAM 6436$^T$) is proposed.

The genus *Brucella* was constituted in 1920 by Meyer & Shaw (1920) with *Brucella melitensis*, the causative agent of Malta fever, as the type species and *Brucella abortus* causing abortion in cattle as the second species. Within 48 years four additional *Brucella* species, namely *Brucella suis* (Huddleson, 1929), *Brucella ovis* (Buddle, 1956), *Brucella neotomae* (Stoenner & Lackman, 1957) and *Brucella canis* (Carmichael & Bruner, 1968) were described. It took nearly another 40 years before two additional species, *Brucella pinnipedialis* and *Brucella ceti,* originating from marine mammals were published (Foster *et al.*, 2007). The most recently published description of a *Brucella* species is that for *Brucella microti*, isolated initially from common voles in the Czech Republic (Hubalek *et al.*, 2007; Scholz *et al.*, 2008a) and recently also from red foxes in Lower Austria (*Vulpes vulpes*) and directly from soil (Scholz *et al.*, 2009, 2008c).

Before the development of molecular techniques, differentiation of the various *Brucella* species and their biovars was solely based on phenotypic traits, i.e. $CO_2$ requirement, $H_2S$ production, dye sensitivity, metabolic profiles, lysis by *Brucella*-specific bacteriophages and agglutination with monospecific antisera. Another important criterion for the differentiation of the various *Brucella* species was the natural host preference, which is why species names were given in accordance with their preferred host. Today it is known that all *Brucella* species are highly related at the genetic level, exhibiting identical 16S rRNA (Gee *et al.*, 2004) and *recA* gene sequences (Scholz *et al.*, 2008b) as well as highly similar genomes in terms of sequence identity and gene synteny (Del Vecchio *et al.*, 2002; Paulsen *et al.*, 2002; Halling *et al.*, 2005). In the majority of housekeeping genes only single nucleotide polymorphisms are present among the various *Brucella* species (Whatmore *et al.*, 2007). Indeed, applying the gold standard for species delineation, DNA–DNA hybridization (Wayne *et al.*, 1987), all recognized *Brucella* species belong to a single species, as the DNA–DNA relatedness among all species is above 70 % (Verger *et al.*, 1985). Consequently, Verger *et al.* (1985) suggested *Brucella* as a monospecific genus with *B. melitensis* as the sole true species and the other species to be recognized as biovars. This perception was later confirmed by using data from comparative whole genome sequencing analysis of *B. melitensis* (Del Vecchio *et al.*, 2002), *B. suis* (Paulsen *et al.*, 2002), and more recently of *Brucella abortus* strain 9-941 (Halling *et al.*, 2005) and *B. abortus* vaccine strain S19 (Crasta *et al.*, 2008).

However, in 2003, the Subcommittee on the Taxonomy of *Brucella* agreed unanimously on a return to pre-1986 *Brucella* taxonomy and reapproval of the six *Brucella* nomenspecies and their biovars (Meeting in Pamplona, Spain in 2003), published in 2006 by Osterman & Moriyón (2006).

Following the currently used *Brucella* taxonomy we describe a novel *Brucella* species, *Brucella inopinata* sp. nov., with the type strain BO1$^T$, isolated from breast implant wound fluid and blood of a 71-year-old female patient with clinical symptoms consistent with brucellosis (De *et al.*, 2008).

*B. inopinata* sp. nov. is the first *Brucella* species that exhibits a unique 16S rRNA gene sequence and lower sequence similarities in various housekeeping genes and genes encoding outer-membrane proteins when compared with all other recognized *Brucella* species and hence represents the most unique species within this genus.

## Phenotypic analysis of strain BO1$^T$

Phenotypic analysis of strain BO1$^T$, i.e. growth behaviour on different media at various temperatures, $H_2S$ production, $CO_2$ requirement, agglutination with monospecific A- and M-antisera, cellular fatty acid analysis, lysis with bacteriophage Tb and growth in the presence of dyes (thionin and basic fuchsin), have been described previously (De *et al.*, 2008) and are given in the species description. The antimicrobial susceptibility pattern of strain BO1$^T$ was determined using the CLSI interpretive criteria for *Brucella* spp. (De *et al.*, 2008). Results are given in the species description. Additional phenotypic characterization of strain BO1$^T$ carried out in this study comprised other growth characteristics, standard biochemical characterization by using API 20E, API 20NE and API ZYM (bioMérieux), extended biochemical profiling using the MICRONAUT (MERLIN Diagnostika GmbH) system, electron microscopy, and lysis with additional *Brucella*-specific bacteriophages, F1 and F25, as described for *B. microti* (Scholz *et al.*, 2008a).

The growth characteristics of strain BO1$^T$ resembled those of *B. microti*, characterized by very fast growth at 37 °C on various standard media, such as sheep blood agar and standard nutrient agar (both from Oxoid). On *Brucella* agar (Merck), growth became visible within 6 h of incubation at 37 °C with or without supplementary $CO_2$ (data not shown). Single colonies of 1–2 mm were formed within 12–24 h. As determined by using transmission electron microscopy (negative staining with 1 % uranyl acetate) with a JEM-1010 electron microscope at a magnification of 40 000, cells were non-flagellated and arranged individually or in irregular clusters, exhibiting a

Abbreviations: MLST, multilocus sequence typing; MLVA, multilocus VNTR (variable-number tandem-repeat) analysis; RTD, routine test dilution.

The GenBank/EMBL/DDBJ accession numbers for the *omp2a*, *omp2b*, *omp25*, *omp31* and *recA* gene sequences of strain BO1$^T$ are FM177715, FM177716, FM177717, FM177718 and FM177719, respectively.

Figures showing lysis of strain BO1$^T$ on *Brucella* agar by bacteriophage Tb (Tbilisi), a two-dimensional thin-layer chromatogram of total polar lipids of strain BO1$^T$, IS*711* element fingerprinting by using Southern blotting and a condensed dendrogram of clustered MLVA-16 genotypes obtained with more than 470 *Brucella* isolates corresponding to 324 different genotypes are available as supplementary material with the online version of this paper.

mean diameter of 0.5 μm and a length of 1 μm (Fig. 1a and b). Strain BO1$^T$ agglutinated weakly with monospecific anti-M serum up to a dilution of 1 : 40 but not with anti-A serum. Incomplete lysis was observed with bacteriophages Tb, F1 and F25 at $10^4 \times$ routine test dilution ($10^4 \times$ RTD) but not at any other dilutions (Supplementary Fig. S1, in IJSEM Online). This finding is in contradiction to previous results (De *et al.*, 2008), in which no lysis could be detected with phage Tb. This can be explained by the rapid growth of strain BO1$^T$. In order to detect lysis, the standard protocol had to be modified in terms of bacteria quantities and incubation periods. Briefly, *Brucella* agar plates were flooded with 5 ml *Brucella* bouillon including 5 μl of a $10^{11}$ cell suspension of BO1$^T$ ml$^{-1}$. Medium in excess was removed immediately and the plates were dried at 37 °C for 2 h before the various phage dilutions were applied. The incubation time of the phage dilutions was 10 s. Plates were incubated at 37 °C and inspected for phage lysis after 12 h.

As described previously for *B. microti* (Scholz *et al.*, 2008a), strain BO1$^T$ exhibited outstanding metabolic capabilities in comparison with recognized *Brucella* species, sharing a series of reactions with *Ochrobactrum anthropi* LMG 3331$^T$ and *Ochrobactrum intermedium* LMG 3301$^T$. The results of the differential biochemical reactions of strain BO1$^T$ in comparison with other *Brucella* species and their biovars as well as with *Ochrobactrum* species are summarized in Table 1. Hence, *B. inopinata* sp. nov. is the second fast-growing *Brucella* species with a highly active metabolism.

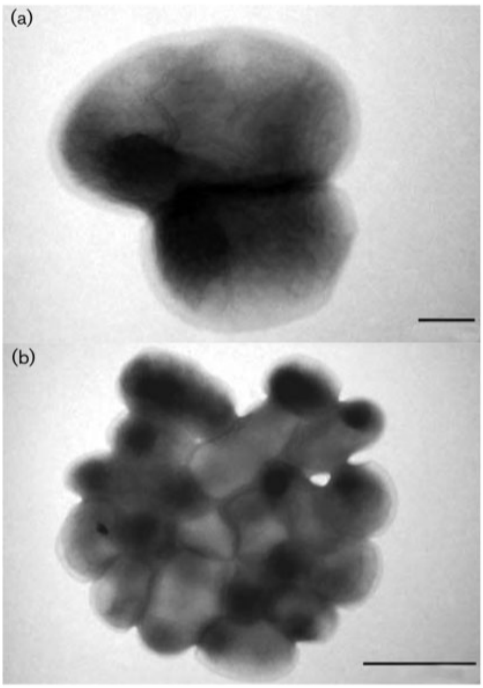


**Fig. 1.** Transmission electron micrographs (magnification of 40 000) showing two inactivated non-flagellated *B. inopinata* BO1$^T$ cells (a) and a cluster of *B. inopinata* BO1$^T$ cells (b). Bars, 0.2 μm (a) and 1 μm (b).

Using the API 20E and API 20NE test systems, strain BO1$^T$ was misidentified as *Ochrobactrum anthropi* with identification scores of 98 and 99 %, respectively. Detailed results of API 20E, API 20NE, and API ZYM test systems are given in the species description.

Quinones and polar lipids were extracted and analysed as described by Tindall (1990a, b) and Altenburger *et al.* (1996). The quinone system consisted of the major compound ubiquinone Q-10 (99 %) and ubiquinone Q-11 (1 %). The polar lipid profile consisted of five major compounds: phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine and one unknown aminolipid (Supplementary Fig. S2, in IJSEM Online). The unknown aminolipid exhibited the same chromatographic behaviour as the unknown aminolipid AL1, which was also reported to be present in the type strains of *B. microti*, *B. melitensis* and *B. abortus* (Scholz *et al.*, 2008a). In addition, moderate amounts of phosphatidylmonomethylethanolamine and minor to trace amounts of an unknown aminophospholipid and an unknown phospholipid corresponding to APL2 and PL, respectively, which were detected in *B. microti*, as well as four unknown polar lipids were detected. One of these unknown polar lipids showed the same chromatographic behaviour as the lipid L4 present in *B. microti* but not in other *Brucella* species (Scholz *et al.*, 2008a). Unknown aminolipid AL2 found in *B. microti* (Scholz *et al.*, 2008a) as a major compound was not detectable. Hence, the polar lipid profile clearly distinguished strain BO1$^T$ from *B. microti* as well as from *B. melitensis* and *B. abortus* based on the presence/absence of unknown aminolipid AL2 and unknown polar lipid L4. The polyamine pattern contained the major compounds spermidine (45 %) and putrescine (43 %) and small amounts of 1,3-diaminopropane (7 %), *sym*-homospermidine (1 %) and spermine (1 %). This polyamine pattern is in good agreement with those of other *Brucella* species (Scholz *et al.*, 2008a).

The whole-cell cellular fatty acid profile of strain BO1$^T$ was similar to those of *B. abortus, B. melitensis, B. neotomae, B. ovis* and *B. suis*, characterized by the presence of major amounts (5–46 %) of $C_{19:0}$ cyclo 11-12, $C_{18:1}\omega7c$, $C_{18:0}$ and $C_{16:0}$ and smaller amounts (1–4 %) of Br-$C_{19:1}$. The antimicrobial susceptibility pattern of strain BO1$^T$ was comparable with profiles of other *Brucella* species described by Jevitt *et al.* (2005), with the exception of resistance to various cephalosporins (cefoxitin, cephotiam and cefuroxim). Details are given in the species description.

## Molecular analyses of strain BO1$^T$

Previous molecular analyses of strain BO1$^T$ comprised 16S rRNA gene sequencing, DNA–DNA hybridization experi-

**Table 1.** Differential physiological reactions of *B. inopinata* sp. nov. BO1$^T$ in comparison with other *Brucella* and *Ochrobactrum* species

Taxa: 1, *B. microti* CCM 4915$^T$; 2, *B. inopinata* sp. nov. BO1$^T$; 3, *B. abortus* strains: 544$^T$ (=NCTC 10093$^T$) (bv. 1), 86/8/59 (=NCTC 10501) (bv. 2), Tulya (=NCTC 10502) (bv. 3), 292 (=NCTC 10503) (bv. 4), B3196 (=NCTC 10504) (bv. 5), 870 (=NCTC 10505) (bv. 6), 63175 (=NCTC 10506) (bv. 7), C68 (=NCTC 10507) (bv. 9); 4, *B. melitensis* strains: 16M$^T$ (=NCTC 10094$^T$) (bv. 1), 63/9 (=NCTC 10508) (bv. 2), Ether (=NCTC 10509) (bv. 3); 5, *B . suis* strains: 1330$^T$ (=NCTC 10316$^T$) (bv. 1), Thomsen (=NCTC 10510) (bv. 2), 686 (=NCTC 10511) (bv. 3), reference 40 (bv. 4), reference 513 (bv. 5); 6, *B. ovis* 63/290$^T$ (=NCTC 10512$^T$); 7, *B. canis* RM6/66$^T$ (=NCTC 10854$^T$); 8, *B. neotomae* 5K33$^T$ (=NCTC 10084$^T$); 9, *B. pinnipedialis* NCTC 12890$^T$; 10, *B. ceti* NCTC 12891$^T$; 11, *O. anthropi* LMG 3331$^T$; 12, *O. intermedium* LMG 3301$^T$. oNP, *o*-Nitrophenyl; pNP, *p*-nitrophenyl; $QO_2N$, mean of 10 repetitions per reaction. +, Positive ($QO_2N$ values >70); −, negative ($QO_2N$ values <50); v, variable ($QO_2N$ values >50 and <70).

| Compound | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Substrate class |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| L-Glutamine | + | + | − | − | − | − | − | − | − | − | + | + | Amino acid |
| L-Histidine hydrochloride | + | + | − | − | − | − | − | − | − | − | + | + | Amino acid |
| D-Histidine | + | + | − | − | − | − | − | − | − | − | + | + | Amino acid |
| L-Citrulline | + | + | − | − | − | − | − | − | − | − | + | + | Amino acid |
| DAPI | + | + | − | − | − | − | − | − | − | − | + | + | Amino acid |
| Sarcosine hydrochloride | + | + | − | − | − | − | − | − | − | − | + | + | Amino acid derivative |
| Fumaric acid | + | + | − | − | − | − | − | − | − | − | + | + | Org. acid |
| Succinic acid | + | + | − | − | − | − | − | − | − | − | + | + | Org. acid |
| α-Ketoglutaric acid | + | + | − | − | − | − | − | − | − | − | + | + | Org. acid |
| Itaconic acid | + | + | − | − | − | − | − | − | − | − | − | − | Org. acid |
| Mesaconic acid | + | + | − | − | − | − | − | − | − | − | − | − | Org. acid |
| Glutaric acid | + | + | − | − | − | − | − | − | − | − | + | + | Org. acid |
| Arginine *p*-nitroanilide | + | + | − | − | − | − | − | − | − | − | v | + | Amino peptidase |
| L-Pyroglutamic acid β-naphthylamide | − | − | − | − | − | − | − | − | − | − | + | + | Amino peptidase |
| pNP *N*-Acetyl-β-D-galactosaminide (pH 7.5) | + | + | − | − | − | − | − | − | − | − | v | + | Esterase |
| oNP *N*-Acetyl-α-D-galactosaminide (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Esterase |
| oNP *N*-Acetyl-β-D-galactosaminide (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Esterase |
| pNP *N*-Acetyl-β-D-glucosaminide (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Esterase |
| pNP *N*-Acetyl-1-thio-β-D-glucosaminide (pH 7.5) | v | + | − | − | − | − | − | − | − | − | + | + | Esterase |
| 4-Nitrophenyl-α-D-maltoheptaoside-4,6,*O*-ethylidene (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Esterase |
| pNP *N*-Acetyl-β-D-glucosamide (pH 5.5) | + | + | − | − | − | − | − | − | − | − | + | − | Esterase |
| pNP *N*-α-L-Arabinopyranoside (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Glucosidase |
| oNP *N*-β-D-Xylopyranoside (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Glucosidase |
| pNP *N*-1-thio-β-D-Galactopyranoside (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Glucosidase |
| 2-Methoxy-4-(2-nitrovinyl)-phenyl β-*D*-galactopyranoside | + | + | − | − | − | − | − | − | − | − | + | + | Glucosidase |
| pNP β-L-Fucopyranoside (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Glucosidase |
| pNP β-D-Thiofucopyranoside (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Glucosidase |
| pNP β-D-Maltoside (pH 7.5) | + | + | − | − | − | − | − | − | − | − | + | + | Glucosidase |
| pNP β-D-Lactopyranoside (pH 7.5) | v | + | − | − | − | − | − | − | − | − | + | + | Glucosidase |
| pNP β-D-Glucuronide (pH 5.5) | + | + | − | − | − | − | − | − | − | − | + | − | Glucosidase |
| Aesculin hydrolysis | − | − | − | − | v | − | v | − | − | − | + | + | Classical reaction |
| Voges–Proskauer reaction | + | + | − | − | − | − | − | − | − | − | + | + | Classical reaction |
| oNP β-D-Galactopyranoside-6-phosphate | + | + | − | − | − | − | − | − | − | − | + | + | Phosphatase |
| pNP Phosphate-di(2-amino-2-ethyl-1,3-propanediol) (pH 5.5) | + | + | − | − | − | − | − | − | − | − | + | + | Phosphatase |

ments, multilocus sequence typing (MLST) analysis and IS*711*-specific PCR (De *et al.*, 2008). DNA–DNA reassociation of >70 % between strain BO1$^T$ and the type strains of *B. melitensis* (80 %) and *B. suis* (78 %) as well as the presence of the *Brucella*-specific IS*711* element clearly demonstrated that strain BO1$^T$ is a member of the genus *Brucella*. However, the data from 16S rRNA gene sequencing and MLST analysis also revealed that strain BO1$^T$ was

markedly different from all recognized *Brucella* species and their biovars. For example, strain BO1$^T$ is characterized by a unique 16S rRNA gene sequence differing in five nucleotides when compared with the 16S rRNA gene consensus sequence of *Brucella* species and also exhibits a significantly lower level of sequence similarity in various housekeeping genes (De *et al.*, 2008).

In this study, we have additionally sequenced the *recA*, *omp2a/b*, *omp25* and *omp31* genes as described for *B. microti* (Scholz *et al.*, 2008a). As shown previously, all recognized *Brucella* species, including *B. microti*, are identical in their *recA* gene sequences (Scholz *et al.*, 2008b). However, the partial *recA* gene sequence of strain BO1$^T$ (GenBank no. FM177719) differed in seven of 631 nucleotides (98.9 % identity). These findings again emphasized the unique position of strain BO1$^T$ within *Brucella*. As in MLST analysis, the various *omp* gene sequences of strain BO1$^T$ also exhibited lower sequence similarities. The *omp31* gene (723 bp) was only 96 % identical to the respective sequences of other *Brucella* species. The *omp25* gene (642 bp) sequence of strain BO1$^T$ was most closely related to *omp25* of *B. suis* ATCC 23445 (GenBank no. CP000911) with a similarity of 99 %. In the phylogenetic analysis using the neighbour-joining method, the *omp2a* and *omp2b* gene sequences of BO1$^T$ were most closely related to *omp2a/b* of *Brucella* strain 83-210 (83/13), an isolate with uncertain affiliation (Fig. 2), and differed considerably (97–98 % sequence similarity) from *omp* gene sequences of other *Brucella* species.

In addition to sequencing of the above-mentioned genes, strain BO1$^T$ was characterized by using AMOS PCR (Bricker & Halling, 1994) and species-specific multiplex PCR (Garcia-Yoldi *et al.*, 2006). In multiplex PCR analysis a unique pattern of six PCR fragments (150 bp, 272 bp, 450 bp, 587 bp, 794 bp and 1682 bp), not observed in

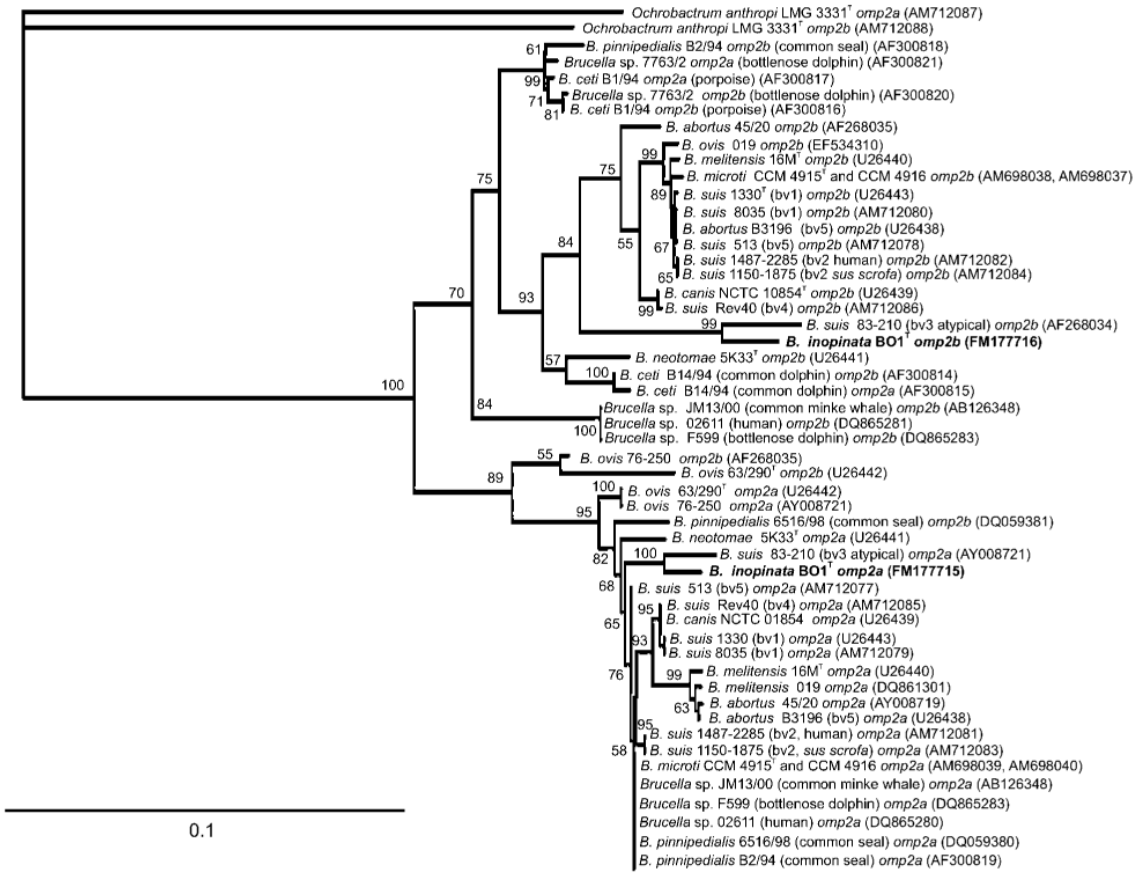


**Fig. 2.** Phylogenetic tree reconstructed with *omp2a* (1104 nt) and *omp2b* (1089 nt) sequences using CLUSTREE neighbour-joining analysis (Thompson *et al.*, 1994) and Kimura distances. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. The tree was rooted by outgrouping sequence *omp2a* of *O. anthropi* LMG 3331$^T$. Bar, 0.1 divergent residues per site.

other *Brucella* species, was generated. AMOS PCR was negative using all primer combinations (data not shown). Finally, strain BO1$^T$ was typed by using IS*711* fingerprinting using the Southern blotting technique and by multilocus VNTR (variable-number tandem-repeat) analysis (MLVA) using eight mini- and eight micro-satellite VNTR markers as described recently for *B. microti* (Scholz *et al.*, 2008a). Of the *Brucella*-specific insertion sequence IS*711*, four to five copies, displaying a unique banding pattern, were detected (Supplementary Fig. S3, in IJSEM Online). The MLVA typing pattern obtained was significantly different from those for previously analysed strains (Le Flèche *et al.*, 2006; Al Dahouk *et al.*, 2007). A new intermediate size allele was observed at locus Bruce42 (coded 2.5). No amplification product could be obtained at four loci, bruce07, bruce18, bruce21 and bruce30 (Le Flèche *et al.*, 2006), an exceptional behaviour not observed previously. All these loci are associated with an interspersed repeat element (containing one or two octameric tandem repeats) present in about 20 locations in *B. melitensis* 16M$^T$ strain as reported previously by Bricker *et al.* (2003). This suggests that these four loci are the result of recent spreading events of this presumably mobile element within the *Brucella* genus, after the occurrence of the last common ancestor to *B. inopinata* sp. nov. and to the other *Brucellae*. Alternatively, these four loci might have been lost or rearranged along the inopinata lineage as a result of their peculiar structure. MLVA cluster analysis using the categorical distance coefficient and the unweighted pair-group method with arithmetic averages (UPGMA) was carried out as described previously (Scholz *et al.*, 2008a). In these analyses, strain BO1$^T$ clustered together with the *B. microti, B. suis* biovar 5 and *B. neotomae* strains and three representative marine mammal strains (Supplementary Fig. S4, in IJSEM Online) in a very loosely connected group with long branches. The inopinata branch is in proportion markedly shorter than the relatively very long branch observed by using MLST (De *et al.*, 2008). This might be because of the inherent homoplasy at MLVA loci. Furthermore, relatively long evolutionary distances will be underestimated by MLVA as 16 markers were used in this MLVA assay, and the distance is the categorical coefficient; an MLVA distance between two strains cannot be more than 16. Another possibility is that VNTR markers do not evolve as fast in these presumably environmental species, in comparison with the highly pathogenic and host-adapted variants (the more classical *Brucellae*), which are under different stress conditions. However, as yet nothing is known about the natural reservoir or the host-range of this novel *Brucella* species.

From the phenotypic and molecular data it was obvious that strain BO1$^T$ belonged to the genus *Brucella* but also differed from all recognized species and their biovars. We therefore conclude that BO1$^T$ represents a novel species of the genus *Brucella*, for which the name *Brucella inopinata* sp. nov. is proposed.

### Description of *Brucella inopinata* sp. nov.

*Brucella inopinata* (in.o.pi.na′ta. L. fem. adj. *inopinata* unexpected).

Aerobic, non-fermentative, non-motile, non-spore-forming, Gram-negative coccobacilli or short rods, 0.5–0.7 μm in diameter and 0.6–1.2 μm in length arranged individually or in irregular clusters. Non-fastidious, fast growth (visible within 6 h) occurs on *Brucella* agar, trypticase soy agar (Becton Dickinson), sheep blood agar and standard nutrient agar at 25–42 °C. Positive for growth on MacConkey agar (Becton Dickinson). Smooth, opaque colonies are formed within 18 h, with a diameter of approximately 1–2 mm. Good growth does not require $CO_2$, supplementary serum or blood. No haemolysis is observed. Growth occurs in the presence of thionin at concentrations of 1/25 000, 1/50 000 and 1/100 000, and with basic fuchsin at concentrations of 1/50 000 and 1/100 000. Incomplete lysis occurs with bacteriophages Tb, F1 and F25 at $10^4 \times$ RTD, but not at other dilutions. Agglutinates with monospecific anti-M serum up to a dilution of 1 : 40. Oxidase- and catalase-positive. Fast urease reaction (<5 min). Growth occurs in nutrient broth with or without 6 % NaCl. Nitrate and nitrite are reduced (with gas formation from nitrate). Production of $H_2S$ and Voges–Proskauer reaction are positive. Negative for hydrolysis of aesculin, gelatin liquefaction, production of indole, citrate utilization, growth on cetrimide and *Salmonella–Shigella* (SS) agar. Acid production is observed in King's oxidation–fermentation base from D-glucose and D-xylose, whereas no acid production is observed in King's oxidation–fermentation base from mannitol, lactose, sucrose and maltose. Positive (API ZYM) for acid phosphatase, alkaline phosphatase, trypsin, leucine arylamidase and naphthol-AS-BI-phosphohydrolase. Negative (API ZYM) for esterase, esterase lipase, lipase, valine arylamidase, cystine arylamidase, α-chymotrypsin, α- and β-galactosidase, β-glucuronidase, α- and β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Positive (API 20NE) for D-glucose, maltose, L-arabinose, D-mannose, *N*-acetylglucosamine and adipic acid. Negative for D-mannitol, citric acid, gluconate, capric acid, malic acid and phenylacetic acid. In API 20E, positive for fermentation of L-arabinose. Additional differential physiological reactions (MICRONAUT) of strain BO1$^T$ in comparison with other *Brucella* species and biovars and *O. anthropi* LMG 3331$^T$ are given in Table 1. Susceptible to the following antimicrobial agents: doxycycline (0.12 μg ml$^{-1}$), tetracycline (0.25 μg ml$^{-1}$), streptomycin (2 μg ml$^{-1}$), gentamicin (1 μg ml$^{-1}$) and trimethoprim–sulfamethoxazole (<0.5 and 9.5 μg ml$^{-1}$). Major whole-cell cellular fatty acids (5–46 %) are $C_{19:0}$ cyclo 11-12, $C_{18:1}\omega7c$, $C_{18:0}$ and $C_{16:0}$, and smaller amounts (1–4 %) of Br-$C_{19:1}$. Quinone system is composed of the major compound ubiquinone Q-10 and minor amounts of Q-11. The polar lipid profile contains the major compounds phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine and one unknown

aminolipid, moderate amounts of phosphatidylmonomethylethanolamine and minor to trace amounts of an unknown aminophospholipid, an unknown phospholipid and two unknown polar lipids. The polyamine pattern consists of the major compounds spermidine and putrescine and small amounts of 1.3-diaminopropane, *sym*-homospermidine and spermine.

The type strain, BO1$^T$ (=BCCN 09-01$^T$=CPAM 6436$^T$), was isolated in 2005 from a breast implant infection of a 71-year-old female patient in the USA.

## Acknowledgements

We are grateful to Csilla Lodri, Stefan Schatz, Robert Schneider, and Angelika Draeger for excellent technical assistance. The development of genotyping methods for the precise strain identification of dangerous pathogens as part of microbial forensics is supported by the French DGA (Délégation Générale pour l'Armement). MRIS and BIM are members of the European Biodefense Laboratory Network (EBLN) supported by the European Defence Agency (EDA). We thank Philippe Le Flèche for the MLVA typing and Mark S Koylass for MLST analysis of strain BO1$^T$.

## References

**Al Dahouk, S., Fleche, P. L., Noeckler, K., Jacques, I., Grayon, M., Scholz, H. C., Tomaso, H., Vergnaud, G. & Neubauer, H. (2007).** Evaluation of *Brucella* MLVA typing for human brucellosis. *J Microbiol Methods* **69**, 137–145.

**Altenburger, P., Kämpfer, P., Makristathis, A., Lubitz, W. & Busse, H.-J. (1996).** Classification of bacteria isolated from a medieval wall painting. *J Biotechnol* **47**, 39–52.

**Bricker, B. J. & Halling, S. M. (1994).** Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis, Brucella ovis* and *Brucella suis* bv. 1 by PCR. *J Clin Microbiol* **32**, 2660–2666.

**Bricker, B. J., Ewalt, D. R. & Halling, S. M. (2003).** Brucella 'HOOF-Prints': strain typing by multi-locus analysis of variable number tandem repeats (VNTRs). *BMC Microbiol* **11**, 3–15.

**Buddle, M. B. (1956).** Studies on *Brucella ovis* (n. sp.), a cause of genital disease of sheep in New Zealand and Australia. *J Hyg (Lond)* **54**, 351–364.

**Carmichael, L. E. & Bruner, D. W. (1968).** Characteristics of a newly-recognized species of *Brucella* responsible for infectious canine abortions. *Cornell Vet* **48**, 579–592.

**Crasta, O. R., Folkerts, O., Fei, Z., Mane, S. P., Evans, C., Martino-Catt, S., Bricker, B., Yu, G., Du, L. & Sobral, B. W. (2008).** Genome sequence of *Brucella abortus* vaccine strain S19 compared to virulent strains yields candidate virulence genes. *PLoS One* **3**, e2193.

**De, B. K., Stauffer, L., Koylass, M. S., Sharp, S. E., Gee, J. E., Helsel, L. O., Steigerwalt, A. G., Vega, R., Clark, T. A. & other authors (2008).** Novel *Brucella* strain (BO1) associated with a prosthetic breast implant infection. *J Clin Microbiol* **46**, 43–49.

**DelVecchio, V. G., Kapatral, V., Redkar, R. J., Patra, G., Mujer, C., Los, T., Ivanova, N., Anderson, I., Bhattacharyya, A. & other authors (2002).** The genome sequence of the facultative intracellular pathogen *Brucella melitensis. Proc Natl Acad Sci U S A* **99**, 443–448.

**Foster, G., Osterman, B., Godfroid, J., Jacques, I. & Cloeckaert, A. (2007).** *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. *Int J Syst Evol Microbiol* **57**, 2688–2693.

**Garcia-Yoldi, D., Marin, C. M., de Miguel, M. J., Munoz, P. M., Vizmanos, J. L. & Lopez-Goni, I. (2006).** Multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. *Clin Chem* **52**, 779–781.

**Gee, J. E., De, B. K., Levett, P. N., Whitney, A. M., Novak, R. T. & Popovic, T. (2004).** Use of 16S rRNA gene sequencing for rapid confirmatory identification of *Brucella* isolates. *J Clin Microbiol* **42**, 3649–3654.

**Halling, S. M., Peterson-Burch, B. D., Bricker, B. J., Zuerner, R. L., Qing, Z., Li, L. L., Kapur, V., Alt, D. P. & Olsen, S. C. (2005).** Completion of the genome sequence of *Brucella abortus* and comparison to the highly similar genomes of *Brucella melitensis* and *Brucella suis. J Bacteriol* **187**, 2715–2726.

**Hubalek, Z., Scholz, H. C., Sedlaceck, I., Melzer, F., Sanogo, Y & Nesvadbova, J. (2007).** Brucellosis of the Common Vole (*Microtus arvalis*). *Vector Borne Zoonotic Dis* **7**, 679–687.

**Huddleson, I. F. (1929).** The differentiation of the species of the genus *Brucella. Michigan State College Agricultural Experimental Station Technical Bulletin* **100**, 1–16.

**Jevitt, L. A., Weigel, L. M., De, B., Popovic, T. & Patel, J. B. (2005).** Abstr. C-357. *Abstr. 105th Gen. Meet. Am. Soc. Microbiol.* Washington, DC: American Society for Microbiology.

**Le Flèche, P., Jacques, I., Grayon, M., Al Dahouk, S., Bouchon, P., Denoeud, F., Nockler, K., Neubauer, H., Guilloteau, L. A. & other authors (2006).** Evaluation and selection of tandem repeat loci for a Brucella MLVA typing assay. *BMC Microbiol* **9**, 6–9.

**Meyer, K. F. & Shaw, E. B. (1920).** A comparison of the morphologic, cultural and biochemical characteristics of *B. abortus* and *B. melitensis* from cattle. Studies on the genus *Brucella* nov. gen. *J Infect Dis* **27**, 173–184.

**Osterman, B. & Moriyón, I. (2006).** International Committee on Systematics of Prokaryotes; Subcommittee on the Taxonomy of Brucella: minutes of the meeting, 17 September 2003, Pamplona, Spain. *Int J Syst Evol Microbiol* **56**, 1173–1175.

**Paulsen, I. T., Seshadri, R., Nelson, K. E., Eisen, J. A., Heidelberg, J. F., Read, T. D., Dodson, R. J., Umayam, L., Brinkac, L. M. & other authors (2002).** The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts. *Proc Natl Acad Sci U S A* **99**, 13148–13153.

**Scholz, H. C., Hubalek, Z., Sedlácek, I., Vergnaud, G., Tomaso, H., Al Dahouk, S., Melzer, F., Kämpfer, P., Neubauer, H. & other authors (2008a).** *Brucella microti* sp. nov., isolated from the common vole *Microtus arvalis. Int J Syst Evol Microbiol* **58**, 375–382.

**Scholz, H. C., Al Dahouk, S., Tomaso, H., Neubauer, H., Witte, A., Schloter, M., Kämpfer, P., Falsen, E., Pfeffer, M. & Engel, M. (2008b).** Genetic diversity and phylogenetic relationships of bacteria belonging to the *Ochrobactrum-Brucella* group by *recA* and 16S rRNA gene-based comparative sequence analysis. *Syst Appl Microbiol* **31**, 1–16.

**Scholz, H. C., Hubalek, Z., Nesvadbova, J., Tomaso, H., Vergnaud, G., Le Flèche, P., Whatmore, A. M., Al Dahouk, S., Krüger, M. & other authors (2008c).** Isolation of *Brucella microti* from soil. *Emerg Infect Dis* **14**, 1316–1317.

**Scholz, H. C., Hofer, E., Vergnaud, G., Le Flèche, P., Whatmore, A. M., Al Dahouk, S., Pfeffer, M., Krüger, M., Cloeckaert, A. & Tomaso, H. (2009).** Isolation of *Brucella microti* from mandibular lymph nodes of red foxes, *Vulpes vulpes*, in Lower Austria. *Vector Borne Zoonotic Dis* **9**, 153–156.

**Stoenner, H. G. & Lackman, D. B. (1957).** A new species of *Brucella* isolated from the desert wood rat, *Neotoma lepida* Thomas. *Am J Vet Res* **18**, 947–951.

**Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994).** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

**Tindall, B. J. (1990a).** A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* **13**, 128–130.

**Tindall, B. J. (1990b).** Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199–202.

**Verger, J.-M., Grimont, F., Grimont, P. A. D. & Grayon, M. (1985).** *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. *Int J Syst Bacteriol* **35**, 292–295.

**Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987).** Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

**Whatmore, A. M., Perrett, L. L. & Macmillan, A. P. (2007).** Characterisation of the genetic diversity of *Brucella* by multilocus sequencing. *BMC Microbiol* **7**, 34.

## 2.5. *Ochrobactrum pituitosum* sp. nov., isolated from an industrial environment

*International Journal of Systematic and Evolutionary Microbiology* (2010), **60**, 321–326 DOI 10.1099/ijs.0.011668-0

# *Ochrobactrum pituitosum* sp. nov., isolated from an industrial environment

Birgit Huber,[1] Holger C. Scholz,[2] Peter Kämpfer,[3] Enevold Falsen,[4] Stefan Langer[1] and Hans-Jürgen Busse[1]

Correspondence
Hans-Jürgen Busse
hans-juergen.busse@vetmeduni.ac.at

[1]Institut für Bakteriologie, Mykologie und Hygiene, Veterinärmedizinische Universität, A-1210 Wien, Austria

[2]Bundeswehr Institute of Microbiology and Toxicology, D-80937 Munich, Germany

[3]Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Giessen, D-35392 Giessen, Germany

[4]CCUG – Culture Collection University of Göteborg, S-413 46 Göteborg, Sweden

Strain CCUG 50899, a Gram-negative, rod-shaped, non-spore-forming, motile bacterium isolated from industrial environment in Sweden and tentatively assigned to the species *Ochrobactrum anthropi*, was studied in order to clarify its taxonomic status. 16S rRNA gene sequence similarities placed the strain in the genus *Ochrobactrum*, sharing highest similarity with the type strains of *Ochrobactrum rhizosphaerae* (99.3 %), *Ochrobactrum thiophenivorans* (98.7 %), *Ochrobactrum pseudogrignonense* (98.6 %) and *Ochrobactrum grignonense* (98.5 %). The fatty acid profile of [*O. anthropi*] CCUG 50899 (major fatty acids $C_{18:1}\omega7c$ and $C_{19:0}$ cyclo $\omega8c$ and presence of $C_{18:1}$ 2-OH), the polar lipid profile (diphosphatidylglycerol, phosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, two unknown aminolipids and an unknown phospholipid), the presence of the quinone system ubiquinone Q-10 and a polyamine pattern with the major compounds putrescine and spermidine and moderate amounts of *sym*-homospermidine supported its affiliation to the genus *Ochrobactrum*. DNA–DNA reassociation experiments with the type strains of its closest relatives *O. rhizosphaerae*, *O. pseudogrignonense*, *O. thiophenivorans* and *O. grignonense* demonstrated that [*O. anthropi*] CCUG 50899 should be placed in a novel species, which is distinguishable from related species by a set of biochemical traits. Based on these data, reclassification of [*O. anthropi*] CCUG 50899 as the type strain of a novel species appears to be justified. Hence, we describe a novel species to accommodate this strain, for which we propose the name *Ochrobactrum pituitosum* sp. nov. The type strain is CCUG 50899$^T$ (=DSM 22207$^T$).

The genus *Ochrobactrum* is affiliated with the family *Brucellaceae* (Garrity *et al.*, 2005). 16S rRNA gene and *recA* sequence-based phylogenies both suggest that the genera *Brucella* and *Ochrobactrum* could be unified in a single genus (Kämpfer *et al.*, 2008; Scholz *et al.*, 2006, 2008). At the time of writing, the genus comprises 13 species: *Ochrobactrum anthropi* (Holmes *et al.*, 1988), *O. intermedium* (Velasco *et al.*, 1998), *O. tritici* and *O. grignonense* (Lebuhn *et al.*, 2000), *O. gallinifaecis* (Kämpfer *et al.*, 2003), *O. oryzae* (Tripathi *et al.*, 2006), *O. lupini* (Trujillo *et al.*, 2005), *O. pseudintermedium* (Teyssier *et al.*, 2007), *O. cytisi* (Zurdo-Piñeiro *et al.*, 2007), *O. haematophilum* and *O. pseudogrignonense* (Kämpfer *et al.*, 2007), *O. rhizosphaerae* and *O. thiophenivorans* (Kämpfer *et al.*, 2008). In a comprehensive study, Scholz *et al.* (2008) examined the phylogenetic relationships of numerous *Ochrobactrum* and *Brucella* strains including strain CCUG 50899, reported as a strain of *O. anthropi* according to the CCUG catalogue but which was shown to be phylogenetically distant from the type strain of *O. anthropi* on the basis of both 16S rRNA gene and *recA* phylogeny. In comparison to the majority of *Ochrobactrum* strains, [*O. anthropi*] CCUG 50899 shows an insertion of 47 nt (positions 124–170) in the 16S rRNA gene sequence (GenBank accession no. AM490609) which so far has only been reported for some strains of *O. intermedium* (Teyssier *et al.*, 2003; Scholz *et al.*, 2008). Corrected for the insertion sequence, [*O. anthropi*] CCUG

Abbreviations: pNA, *p*-nitroanilide; pNP, *p*-nitrophenyl.

The GenBank/EMBL/DDBJ accession numbers for the partial *groEL* and *gyrB* sequences of *O. rhizosphaerae* PR17$^T$, *Ochrobactrum* sp. CCUG 50899, *O. grignonense* OgA9a$^T$, *O. pseudogrignonense* CCUG 30717$^T$ and *O. thiophenivorans* DSM 7216$^T$ are respectively FM863819–FM863823 (*groEL*) and FM863818 and FM863814–FM863817 (*gyrB*).

50899 shares highest 16S rRNA gene sequence similarity with *O. rhizosphaerae* PR17$^T$ (99.3 %), *O. thiophenivorans* DSM 7216$^T$ (98.7 %), *O. pseudogrignonense* CCUG 30717$^T$ (98.6 %) and *O. grignonense* OgA9a$^T$ (98.5 %). Other *Ochrobactrum* species show lower similarity values. Based on partial *recA* sequence similarities, *O. grignonense* OgA9a$^T$ (93.3 %) and *O. rhizosphaerae* PR17$^T$ (91.4 %) are the closest relatives of [*O. anthropi*] CCUG 50899 (GenBank accession no. AM422871), with other members of *Ochrobactrum* sharing only 83.5–88.3 % similarity in the corresponding partial gene sequences. However, these similarity values demonstrate unambiguously the affiliation of [*O. anthropi*] CCUG 50899 to the genus *Ochrobactrum*. Here, we report the classification of [*O. anthropi*] CCUG 50899, applying biochemical, physiological, chemotaxonomic and genetic characterization and comparison to phylogenetic relatives identified recently based on 16S rRNA gene and *recA* phylogenies (Scholz *et al.*, 2006, 2008).

Recalculation of its 16S rRNA gene sequence-based phylogenetic position placed [*O. anthropi*] CCUG 50899 on a separate branch within *Ochrobactrum* consisting of the type strains of *O. grignonense*, *O. pseudogrignonense*, *O. thiophenivorans* and the closest related species *O. rhizosphaerae* with high bootstrap support (Fig. 1), which is in agreement with the relationship indicated by 16S rRNA gene sequence similarities.

Since 16S rRNA gene and *recA* sequence analyses had identified different *Ochrobactrum* species as the closest relatives, partial *gyrB* (1032 nt) and *groEL* (1169 nt) sequences of [*O. anthropi*] CCUG 50899 were analysed and compared to the corresponding sequences of selected reference strains, including *O. anthropi* LMG 3331$^T$, *O. grignonense* OgA9A$^T$, *O. rhizosphaerae* PR17$^T$, *O. thiophenivorans* DSM 7216$^T$, *O. pseudogrignonense* CCUG 30717$^T$ and *O. intermedium* LMG 3301$^T$. DNA was released by subjecting a cell suspension in sterile water to three cycles of freezing in liquid nitrogen and thawing at 60 °C. Released DNA in the supernatant obtained after a short centrifugation was used as the template in PCRs. Partial *gyrB* and *groEL* sequences were amplified with the primer pairs GyrB_f [5′-GATGATGATCTTGTG(AG)TA(AGC)-CGCAG-3′] and GyrB_r (5′-CGAGGTCATCATGACCCA-GCTTC-3′) and GroEL_f (5′-CGGCGAAGACCTGCTG-ATC-3′) and GroEL_r (5′-GCAACGATACCTTCTTCAA-CCG-3′), respectively. The 50 µl assay contained primers (0.5 pmol µl$^{-1}$), $MgCl_2$ (3 mM), dNTPs (0.2 mM each), 10 µl 5× GoTaq Flexi buffer and GoTaq DNA Polymerase enzyme (1.25 U; Promega), 1–2 µl DNA preparation and sterile water. For both primer pairs, PCR amplification was performed using the following conditions: 5 min initial denaturation at 95 °C followed by 35 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 64 °C and extension for 90 s at 72 °C with a final extension for 5 min at 72 °C. PCR products were excised from a 1.5 % agarose gel and then purified with the QIAquick gel extraction kit (Qiagen). Products were sequenced at VBC Genomics (Vienna, Austria). Primers GyrB_r, GyrB_int_544_r (5′-GACGCTGACCGGCGATGACTG-3′), GroEL_f and GroEL_r were used as sequencing primers. Multiple sequence alignment was made with the CLUSTAL_X software, version 1.81 (Thompson *et al.*, 1997), and a similarity matrix was calculated with the software BioEdit (Hall, 1999).

For the partial *groEL* gene sequences, similarities between the reference strains were in the range 91.3–96.0 % (Table 1). For the partial *gyrB* sequences, similarity values between the reference strains were significantly lower, ranging from 83.9 to 91.5 %. In both partial gene sequences, [*O. anthropi*] CCUG 50899 exhibited highest similarity with *O. grignonense* OgA9A$^T$ (97.9 % for *groEL*, 95.0 % for *gyrB*), suggesting this species as the closest relative and thus confirming the results from *recA* analyses (Scholz *et al.*, 2008). Similarity values with the reference

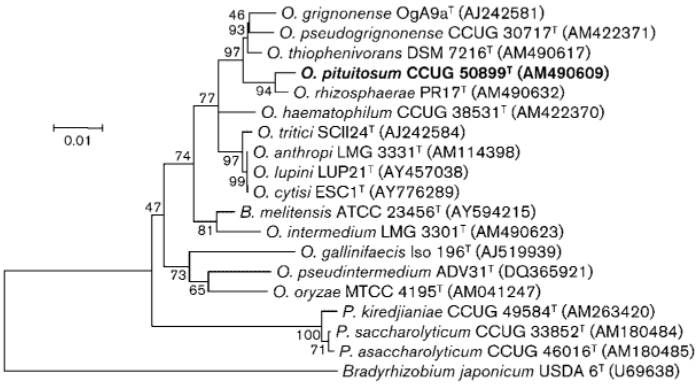


**Fig. 1.** Phylogenetic tree based on 16S rRNA gene sequences (1355–1394 bp) available from the EMBL database constructed after multiple alignment of data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to the maximum composite likelihood model) were calculated and clustering with the neighbour-joining method was performed using the software package MEGA version 4.0 (Tamura *et al.*, 2007). Bootstrap values based on 2000 replications are listed as percentages at branching points (the bootstrap consensus tree is shown). Bar, 0.01 substitutions per nucleotide position. *O.*, *Ochrobactrum*; *B.*, *Brucella*; *P.*, *Pseudochrobactrum*. The sequence of *Bradyrhizobium japonicum* USDA 6$^T$ was used as an outgroup.

**Table 1.** Similarities of partial *groEL* and *gyrB* sequences

*groEL* similarities are given as percentages above the diagonal, with percentage *gyrB* similarities below the diagonal. Sequences were aligned using CLUSTAL_X (Thompson *et al.*, 1997) and sequence similarities were determined using BioEdit (Hall, 1999). Values for [*O. anthropi*] CCUG 50899 are highlighted in bold.

| Strain | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| 1. *O. anthropi* LMG 3331$^T$ | – | 92.8 | **92.4** | 92.3 | 91.4 | 93.1 | 96.0 |
| 2. *O. grignonense* OgA9A$^T$ | 85.8 | – | **97.9** | 95.2 | 94.9 | 92.4 | 92.8 |
| 3. [*O. anthropi*] CCUG 50899 | **86.0** | **95.0** | **–** | **95.6** | **95.5** | **92.5** | **92.4** |
| 4. *O. rhizosphaerae* PR17$^T$ | 83.9 | 87.1 | **87.5** | – | 95.8 | 92.8 | 92.1 |
| 5. *O. thiophenivorans* DSM 7216$^T$ | 84.6 | 85.3 | **83.2** | 85.1 | – | 92.6 | 91.3 |
| 6. *O. pseudogrignonense* CCUG 30717$^T$ | 87.1 | 88.9 | **89.1** | 85.6 | 85.4 | – | 92.8 |
| 7. *O. intermedium* LMG 3301$^T$ | 91.5 | 87.2 | **86.8** | 85.2 | 84.8 | 87.7 | – |

strains were in the range 92.4–95.6 % (*groEL*) and 83.2–89.1 % (*gyrB*), respectively. Similarity of 92.4 % (*groEL*) and 86.0 % (*gyrB*) between [*O. anthropi*] CCUG 50899 and *O. anthropi* LMG 3331$^T$ does not suggest a relationship at the species level.

In order to clarify whether [*O. anthropi*] CCUG 50899 should be considered a member of a separate species, DNA–DNA hybridizations were carried out with DNAs of the nearest relatives *O. grignonense* OgA9a$^T$, *O. thiophenivorans* DSM 7216$^T$, *O. pseudogrignonense* CCUG 30717$^T$ and *O. rhizosphaerae* PR17$^T$. High-molecular-mass DNA was extracted and purified according to Pitcher *et al.* (1989). DNA concentrations were estimated chemically according to Richards (1974). DNA–DNA hybridizations were performed according to Ziemke *et al.* (1998) except that, for nick translation, 2 µg DNA was labelled during a 90 min incubation at 15 °C. In the hybridization experiments, DNA of [*O. anthropi*] CCUG 50899 exhibited 55 % (reciprocal 56 %) reassociation with *O. grignonense* OgA9a$^T$, 47 % (reciprocal 37 %) reassociation with *O. thiophenivorans* DSM 7216$^T$, 62 % (reciprocal 35 %) with *O. pseudogrignonense* CCUG 30717$^T$ and 43 % (reciprocal 46 %) reassociation with *O. rhizosphaerae* PR17$^T$. These data demonstrate that [*O. anthropi*] CCUG 50899 represents a novel species of the genus *Ochrobactrum*.

For phenotypic characterization, [*O. anthropi*] CCUG 50899 was subjected to the set of methods already employed for classification of *O. haematophilum*, *O. pseudogrignonense*, *O. rhizosphaerae* and *O. thiophenivorans* (Kämpfer *et al.*, 2007, 2008).

Polar lipids and quinones were extracted from biomass grown in PYE medium (0.3 % peptone from casein, 0.3 % yeast extract, pH 7.2) and analysed as described previously (Tindall, 1990a, b; Altenburger *et al.*, 1996; Stolz *et al.*, 2007). Polyamines were extracted and analysed as described by Busse & Auling (1988) and Stolz *et al.* (2007). Analysis of quinones indicated that [*O. anthropi*] CCUG 50899 contains a quinone system with ubiquinone Q-10 (99 %) predominating and minor amounts of ubiquinone Q-9 (1 %). The polar lipid profile of [*O. anthropi*] CCUG 50899 exhibited all the major compounds reported for other *Ochrobactrum* species, including the characteristic aminolipid AL2 (Kämpfer *et al.*, 2003, 2007, 2008). Differences in the presence of minor, quite hydrophilic components were detected (Fig. 2), but we do not want to give too much emphasis here to this observation because, so far, its significance has not been demonstrated by results from other strains of the same species. The polyamine pattern contained the major compounds putrescine and spermidine [34.2 and 27.6 µmol (g dry weight)$^{-1}$, respectively], moderate amounts of *sym*-homospermidine [4.6 µmol (g dry weight)$^{-1}$] and small amounts of spermine and 1,3-diaminopropane [0.9 and 0.4 µmol (g dry weight)$^{-1}$, respectively], which is characteristic of *Ochrobactrum*

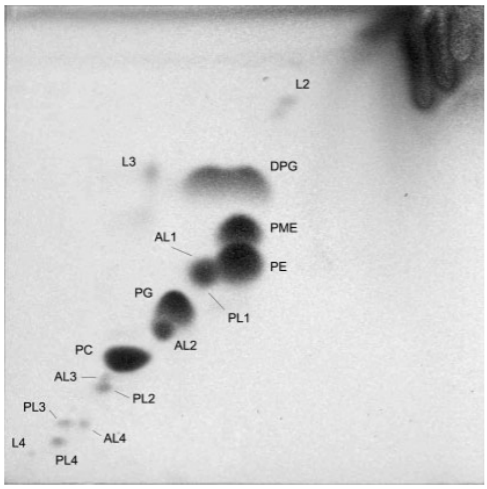


**Fig. 2.** Total polar lipid profile of [*O. anthropi*] CCUG 50899 after two-dimensional TLC and detection with molybdatophosphoric acid. DPG, Diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PME, phosphatidylmonomethylethanolamine; AL1–4, unknown aminolipids; L2–4, unknown polar lipids; PL1–4, unknown phospholipids.

species (Kämpfer *et al.*, 2007, 2008). Fatty acids were extracted and analysed according to the instructions of the Microbial Identification System (MIDI Inc.) as described before (Kämpfer & Kroppenstedt, 1996). The fatty acid profile of [*O. anthropi*] CCUG 50899 exhibited the characteristics also reported for other species of the genus (Table 2). The fatty acids $C_{18:1}\omega7c$ and $C_{19:0}$ cyclo $\omega8c$ were predominant and the hydroxy acid $C_{18:1}$ 2-OH was detected. Examination of the fatty acid profile from cells grown for only 24 h (all other analyses were carried out from cells grown for 48 h) showed a significantly increased amount of $C_{18:1}\omega7c$ and decreased amount of $C_{19:0}$ cyclo $\omega8c$, but the sum of the two amounts was almost the same as in cells grown for 48 h. This observation demonstrates that, for comparison of fatty acid profiles of *Ochrobactrum* strains, only the sum of the amounts of $C_{18:1}\omega7c$ and $C_{19:0}$ cyclo $\omega8c$ should be considered. In contrast to *O. rhizosphaerae* PR17$^T$ and *O. thiophenivorans* DSM 7216$^T$, small amounts of the hydroxy acid $C_{18:1}$ 3-OH were also detected.

**Table 2.** Fatty acid compositions of [*O. anthropi*] CCUG 50899 and the type strains of closely related species

Strains: 1, [*O. anthropi*] CCUG 50899; 2, *O. rhizosphaerae* PR17$^T$ (data from Kämpfer *et al.*, 2008); 3, *O. grignonense* DSM 13338$^T$ (Kämpfer *et al.*, 2007); 4, *O. pseudogrignonense* CCUG 30717$^T$ (Kämpfer *et al.*, 2007); 5, *O. thiophenivorans* DSM 7216$^T$ (Kämpfer *et al.*, 2008). All strains were grown on TSA at 28 °C for 48 h prior to fatty acid analysis; [*O. anthropi*] CCUG 50899 was also analysed for fatty acids after growth under the same conditions for 24 h (in parentheses). For unsaturated fatty acids, the position of the double bond is located by counting from the methyl ($\omega$) end of the carbon chain; *cis* isomers are indicated by the suffix *c*.

| Fatty acid | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Saturated fatty acids | | | | | |
| $C_{16:0}$ | 4.9 (2.7) | 8.3 | 2.9 | 9.7 | 7.3 |
| $C_{17:0}$ | 2.3 (1.6) | 2.8 | 1.7 | 1.6 | 1.4 |
| $C_{18:0}$ | 8.6 (7.7) | 7.6 | 7.2 | 4.9 | 2.9 |
| Unsaturated fatty acids | | | | | |
| $C_{13:1}$ at 12–13 | – | – | 0.7 | – | – |
| $C_{17:1}\omega6c$ | – | – | 0.5 | – | – |
| $C_{18:1}\omega7c$ | 22.5 (61.3) | 61.5 | 31.6 | 19.2 | 40.9 |
| 11-Methyl $C_{18:1}\omega7c$ | 0.9 (–) | – | 1.0 | 1.7 | 1.5 |
| $C_{20:1}\omega7c$ | 0.4 (0.7) | – | 0.8 | – | – |
| $C_{20:2}\omega6,9c$ | 0.6 (–) | – | 0.8 | 0.9 | 1.8 |
| Hydroxy fatty acids | | | | | |
| $C_{18:1}$ 2-OH | 0.6 (2.0) | 4.7 | 0.5 | 1.8 | 1.5 |
| $C_{18:1}$ 3-OH | 0.4 (–) | – | 0.5 | 0.5 | – |
| Summed feature 3* | 1.2 (1.5) | 4.6 | 1.0 | 1.4 | 5.8 |
| Cyclopropane acids | | | | | |
| $C_{17:0}$ cyclo | 1.0 | – | – | 1.0 | 2.1 |
| $C_{19:0}$ cyclo $\omega8c$ | 56.0 (20.8) | 11.2 | 50.2 | 57.0 | 34.9 |
| Unknown 13.957† | 0.6 | – | 0.2 | 0.3 | – |
| Unknown 14.959† | – (0.7) | – | 0.7 | – | – |

*Summed feature 3 contained $C_{15:0}$ iso 2-OH and/or $C_{16:1}\omega7c$. Since no other iso-branched fatty acids were detected, it is most likely that summed feature 3 in fact represents only $C_{16:1}\omega7c$.
†Unknown fatty acids not identified by the MIDI system. Values are equivalent chain-lengths.

Tolerance towards NaCl was examined on PYE agar supplemented with 1, 2, 3, 4, 5, 6, 7 and 10 % NaCl (w/v) and temperature tolerance was examined in an adjustable incubator. Other phenotypic characteristics were studied using the methods described by Kämpfer *et al.* (1991).

Physiological and biochemical traits are listed in Table 3 and the species description. Biochemical characterization of [*O. anthropi*] CCUG 50899 showed that this strain is distinguishable from the type strains of the closest related species including *O. rhizosphaerae* PR17$^T$, *O. grignonense* DSM 13338$^T$, *O. pseudogrignonense* CCUG 30717$^T$ and *O. thiophenivorans* DSM 7216$^T$ as well as from *O. anthropi* CIP 14970$^T$ based on a set of traits. These data support our view that strain CCUG 50899 is affiliated neither with *O. anthropi* nor with any of the other related species.

Phylogenetically, [*O. anthropi*] CCUG 50899 is affiliated unambiguously with the genus *Ochrobactrum*, and its chemotaxonomic traits such as quinone system, polar lipid profile, fatty acids and polyamine pattern are in agreement with this classification. Results from DNA–DNA hybridizations demonstrate that *Ochrobactrum* sp. CCUG 50899 is a representative of a novel species that differs in several phenotypic traits from phylogenetically closely related species. Hence, we here describe a novel species to accommodate strain CCUG 50899, for which we propose the name *Ochrobactrum pituitosum* sp. nov.

## Description of *Ochrobactrum pituitosum* sp. nov.

*Ochrobactrum pituitosum* (pi.tu.i.to′sum. L. neut. adj. *pituitosum* slimy, referring to the consistency of the colonies after extended incubation).

Cells stain Gram-negative and also show Gram-negative behaviour in the KOH and aminopeptidase tests. Aerobic. Highly motile in the early exponential growth phase but may become less motile or non-motile when approaching the stationary growth phase. Cells are non-spore-forming, pleomorphic rods (approx. 1.5–2.5 μm long). Good growth is observed on Columbia agar supplemented with sheep blood and on nutrient agar, TSA, PYE agar, R2A agar and MacConkey agar. Growth is observed on PYE agar supplemented with 6 % NaCl but not with 7 % NaCl (w/v). Optimal growth occurs at 25–30 °C; weak growth at 20 and 35 °C. No growth occurs at 4 °C or at temperatures above 35 °C on PYE agar. Small, beige, translucent and shiny colonies up to 1 mm in diameter are formed within 24 h on PYE agar. After extended incubation, colonies become slimy. Oxidase- and catalase-positive. The quinone system consists of the predominant ubiquinone Q-10 and minor amounts of Q-9. The polyamine pattern consists of the major compounds spermidine and putrescine, moderate amounts of *sym*-homospermidine and minor amounts of 1,3-diaminopropane and spermine. Predominant polar

**Table 3.** Physiological characteristics of [*O. anthropi*] CCUG 50899, *O. anthropi* CIP 14970$^T$ and type strains of four closely related *Ochrobactrum* species

Strains: 1, [*O. anthropi*] CCUG 50899; 2, *O. rhizosphaerae* PR17$^T$; 3, *O. grignonense* DSM 13338$^T$; 4, *O. pseudogrignonense* CCUG 30717$^T$; 5, *O. thiophenivorans* DSM 7216$^T$; 6, *O. anthropi* CIP 14970$^T$. +, Positive; −, negative; (+), weakly positive. All strains were positive for acid formation from D-glucose (in some cases, a weak positive result was observed). All strains were negative for acid formation from sucrose, D-mannitol, dulcitol, raffinose, maltose, D-arabitol, D-mannose, trehalose and methyl D-glucoside. All strains were positive for hydrolysis of L-alanine *p*-nitroanilide (pNA) and L-proline pNA and negative for hydrolysis of aesculin, *p*-nitrophenyl (pNP) *β*-D-galactopyranoside, pNP *β*-D-glucuronide, pNP phosphorylcholine and 2-deoxythymidine-5′-pNP phosphate. All strains were also positive for assimilation of *N*-acetyl-D-galactosamine, L-arabinose, D-fructose, D-galactose, D-glucose, D-mannose, D-ribose, D-xylose, inositol, D-sorbitol, 4-aminobutyrate, fumarate, glutarate, DL-3-hydroxybutyrate, DL-lactate, L-malate, oxoglutarate, L-alanine, *β*-alanine, L-aspartate, L-ornithine, L-proline and L-serine. All strains were negative for assimilation of *p*-arbutin, melibiose, salicin, putrescine, adipate, azelate, itaconate, mesaconate, suberate, L-phenylalanine, L-tryptophan, 3-hydroxybenzoate, 4-hydroxybenzoate and phenylacetate.

| Test | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **Acid production from:** | | | | | | |
| Lactose | − | − | (+) | − | − | − |
| L-Arabinose | + | + | + | + | (+) | − |
| Inositol | − | − | − | − | (+) | − |
| Rhamnose | − | − | (+) | (+) | − | − |
| D-Xylose | + | − | + | − | − | − |
| Cellobiose | − | − | + | − | − | − |
| Adonitol | − | − | − | − | − | (+) |
| Erythritol | (+) | − | + | (+) | (+) | (+) |
| Melibiose | − | − | (+) | − | − | − |
| **Hydrolysis of:** | | | | | | |
| pNP α-D-glucopyranoside, pNP *β*-D-glucopyranoside | (+) | − | − | − | − | − |
| pNP phenylphosphonate | + | + | − | (+) | (+) | − |
| L-Glutamate-*γ*-3-carboxy pNA | + | − | − | − | − | − |
| *bis*-pNP phosphate | − | (+) | (+) | (+) | (+) | (+) |
| **Assimilation of:** | | | | | | |
| Cellobiose, maltose, maltitol | + | + | − | − | − | + |
| Sucrose, trehalose, adonitol | + | + | − | + | − | + |
| Acetate, propionate, pyruvate | − | + | + | + | + | + |
| L-Rhamnose, *cis*-aconitate, citrate, L-histidine, L-leucine | + | + | + | + | − | + |
| *trans*-Aconitate | + | + | − | + | − | − |
| *N*-Acetyl-D-glucosamine | + | (+) | − | − | (+) | (+) |
| D-Gluconate | − | − | + | + | + | + |

lipids are phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol, phosphatidylcholine and diphosphatidylglycerol. Moderate amounts of two unidentified aminolipids (AL1, AL2) and a phospholipid (PL1) and traces of several unknown phospholipids, aminolipids and lipids that have not been characterized further are also present. The fatty acid profile contains mainly $C_{19:0}$ cyclo *ω8c* and $C_{18:1}$*ω7c* and the hydroxy acids $C_{18:1}$ 2-OH and $C_{18:1}$ 3-OH. Carbon source assimilation and hydrolysis of chromogenic substrates are listed in Table 3.

The type strain CCUG 50899$^T$ (=DSM 22207$^T$) was isolated from an industrial environment in Sweden.

## Acknowledgements

The work was supported by a Contract Research Project for the Bundeswehr Medical Service.

## References

**Altenburger, P., Kämpfer, P., Makristathis, A., Lubitz, W. & Busse, H.-J. (1996).** Classification of bacteria isolated from a medieval wall painting. *J Biotechnol* **47**, 39–52.

**Busse, H.-J. & Auling, G. (1988).** Polyamine pattern as a chemotaxonomic marker within the *Proteobacteria*. *Syst Appl Microbiol* **11**, 1–8.

**Garrity, G. M., Bell, J. A. & Lilburn, T. (2005).** Family III. *Brucellaceae* Breed, Murray and Smith 1957, 394$^{AL}$. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2, part C, p. 370. Edited by D. J. Brenner, N. R. Krieg, J. T. Staley & G. M. Garrity. New York: Springer.

**Hall, T. A. (1999).** BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.

**Holmes, B., Popoff, M., Kiredjian, M. & Kersters, K. (1988).** *Ochrobactrum anthropi* gen. nov., sp. nov. from human clinical specimens and previously known as group Vd. *Int J Syst Bacteriol* **38**, 406–416.

**Kämpfer, P. & Kroppenstedt, R. M. (1996).** Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42**, 989–1005.

**Kämpfer, P., Steiof, M. & Dott, W. (1991).** Microbiological characterization of a fuel-oil contaminated site including numerical identification of heterotrophic water and soil bacteria. *Microb Ecol* **21**, 227–251.

**Kämpfer, P., Buczolits, S., Albrecht, A., Busse, H.-J. & Stackebrandt, E. (2003).** Towards a standardized format for the description of a novel species (of an established genus): *Ochrobactrum gallinifaecis* sp. nov. *Int J Syst Evol Microbiol* **53**, 893–896.

**Kämpfer, P., Scholz, H. C., Huber, B., Falsen, E. & Busse, H.-J. (2007).** *Ochrobactrum haematophilum* sp. nov. and *Ochrobactrum pseudogrignonense* sp. nov., isolated from human clinical specimens. *Int J Syst Evol Microbiol* **57**, 2513–2518.

**Kämpfer, P., Sessitsch, A., Schloter, M., Huber, B., Busse, H.-J. & Scholz, H. C. (2008).** *Ochrobactrum rhizosphaerae* sp. nov. and *Ochrobactrum thiophenivorans* sp. nov., isolated from the environment. *Int J Syst Evol Microbiol* **58**, 1426–1431.

**Lebuhn, M., Achouak, W., Schloter, M., Berge, O., Meier, H., Barakat, M., Hartmann, A. & Heulin, T. (2000).** Taxonomic characterization of *Ochrobactrum* sp. isolates from soil samples and wheat roots, and description of *Ochrobactrum tritici* sp. nov. and *Ochrobactrum grignonense* sp. nov. *Int J Syst Evol Microbiol* **50**, 2207–2223.

**Pitcher, D. G., Saunders, N. A. & Owen, R. J. (1989).** Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* **8**, 151–156.

**Richards, G. M. (1974).** Modification of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. *Anal Biochem* **57**, 369–376.

**Scholz, H. C., Tomaso, H., Al Dahouk, S., Witte, A., Schloter, M., Kämpfer, P., Falsen, E. & Neubauer, H. (2006).** Genotyping of *Ochrobactrum anthropi* by *recA*-based comparative sequence, PCR-RFLP, and 16S rRNA gene analysis. *FEMS Microbiol Lett* **257**, 7–16.

**Scholz, H. C., Al Dahouk, S., Tomaso, H., Neubauer, H., Witte, A., Schloter, M., Kämpfer, P., Falsen, E., Pfeffer, M. & Engel, M. (2008).** Genetic diversity and phylogenetic relationships of bacteria belonging to the *Ochrobactrum-Brucella* group by *recA* and 16S rRNA gene-based comparative sequence analysis. *Syst Appl Microbiol* **31**, 1–16.

**Stolz, A., Busse, H.-J. & Kämpfer, P. (2007).** *Pseudomonas knackmussii* sp. nov. *Int J Syst Evol Microbiol* **57**, 572–576.

**Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007).** MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

**Teyssier, C., Marchandin, H., Siméon De Buochberg, M., Ramuz, M. & Jumas-Bilak, E. (2003).** Atypical 16S rRNA gene copies in *Ochrobactrum intermedium* strains reveal a large genomic rearrangement by recombination between *rrn* copies. *J Bacteriol* **185**, 2901–2909.

**Teyssier, C., Marchandin, H., Jean-Pierre, H., Masnou, A., Dusart, G. & Jumas-Bilak, E. (2007).** *Ochrobactrum pseudintermedium* sp. nov., a novel member of the family *Brucellaceae*, isolated from human clinical samples. *Int J Syst Evol Microbiol* **57**, 1007–1013.

**Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

**Tindall, B. J. (1990a).** A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* **13**, 128–130.

**Tindall, B. J. (1990b).** Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199–202.

**Tripathi, A. K., Verma, S. C., Chowdhury, S. P., Lebuhn, M., Gattinger, A. & Schloter, M. (2006).** *Ochrobactrum oryzae* sp. nov., an endophytic bacterial species isolated from deep-water rice in India. *Int J Syst Evol Microbiol* **56**, 1677–1680.

**Trujillo, M. E., Willems, A., Abril, A., Planchuelo, A. M., Rivas, R., Ludeña, D., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. (2005).** Nodulation of *Lupinus* by strains of the new species *Ochrobactrum lupini* sp. nov. *Appl Environ Microbiol* **71**, 1318–1327.

**Velasco, J., Romero, C., López-Goñi, I., Leiva, J., Díaz, R. & Moriyón, I. (1998).** Evaluation of the relatedness of *Brucella* spp. and *Ochrobactrum anthropi* and description of *Ochrobactrum intermedium* sp. nov., a new species with a closer relationship to *Brucella* spp. *Int J Syst Bacteriol* **48**, 759–768.

**Ziemke, F., Höfle, M. G., Lalucat, J. & Rosselló-Mora, R. (1998).** Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.

**Zurdo-Piñeiro, J. L., Rivas, R., Trujillo, M. E., Vizcaíno, N., Carrasco, J. A., Chamber, M., Palomares, A., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. (2007).** *Ochrobactrum cytisi* sp. nov. isolated from nodules of *Cytisus scoparius* in Spain. *Int J Syst Evol Microbiol* **57**, 784–788.

## 2.6. *Ochrobactrum haematophilum* sp. nov. and *Ochrobactrum pseudogrignonense* sp. nov., isolated from human clinical specimens

*International Journal of Systematic and Evolutionary Microbiology* (2007), **57**, 2513–2518 DOI 10.1099/ijs.0.65066-0

# *Ochrobactrum haematophilum* sp. nov. and *Ochrobactrum pseudogrignonense* sp. nov., isolated from human clinical specimens

Peter Kämpfer,[1] Holger C. Scholz,[2] Birgit Huber,[3] Enevold Falsen[4] and Hans-Jürgen Busse[3]

Correspondence
Peter Kämpfer
peter.kaempfer@agrar.uni-giessen.de

[1]Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Giessen, D-35392 Giessen, Germany

[2]Bundeswehr Institute of Microbiology, D-80937 Munich, Germany

[3]Institut für Bakteriologie, Mykologie und Hygiene, Veterinärmedizinische Universität, A-1210 Wien, Austria

[4]Culture Collection, University of Göteborg, Department of Clinical Bacteriology, SE-413 46 Göteborg, Sweden

Three Gram-negative, rod-shaped, non-spore-forming bacteria were isolated from clinical specimens between 1992 and 2000. On the basis of 16S rRNA gene sequence similarities, these strains (CCUG 30717$^T$, CCUG 43892 and CCUG 38531$^T$) were shown to belong to the *Alphaproteobacteria*, most closely related to *Ochrobactrum grignonense* (99.0 and 98.2 % similarity to the type strain). Chemotaxonomic data (major ubiquinone Q-10; major polyamines spermidine, *sym*-homospermidine and putrescine; major polar lipids phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol and phosphatidylcholine; major fatty acids $C_{18:1}\omega7c$ and $C_{19:0}$ cyclo $\omega8c$) supported the affiliation of the isolates to the genus *Ochrobactrum*. The results of DNA–DNA hybridization and physiological and biochemical tests allowed genotypic and phenotypic differentiation of the isolates from described *Ochrobactrum* species. Isolates CCUG 30717$^T$ and CCUG 43892 were closely related on the basis of DNA–DNA reassociation experiments and therefore represent one novel species, for which the name *Ochrobactrum pseudogrignonense* sp. nov. is proposed, with the type strain CCUG 30717$^T$ (=CIP 109451$^T$). Isolate CCUG 38531$^T$ was different from these strains and also from other *Ochrobactrum* species. For this strain, the name *Ochrobactrum haematophilum* sp. nov. is proposed, with the type strain CCUG 38531$^T$ (=CIP 109452$^T$).

The genus *Ochrobactrum* was introduced by Holmes *et al.* (1988) and, at the time of writing, the genus comprises the nine species *Ochrobactrum anthropi* (Holmes *et al.*, 1988), *O. intermedium* (Velasco *et al.*, 1998), *O. tritici* and *O. grignonense* (Lebuhn *et al.*, 2000), *O. gallinifaecis* (Kämpfer *et al.*, 2003b), *O. lupini* (Trujillo *et al.*, 2005), *O. oryzae* (Tripathi *et al.*, 2006), *O. pseudintermedium* (Zurdo-Piñeiro *et al.*, 2007) and *O. cytisi* (Teyssier *et al.*, 2007).

Strains CCUG 30717$^T$ and CCUG 38531$^T$ were isolated in Sweden in 1992 and 1997, respectively, from human blood and strain CCUG 43892 was isolated in Norway in 2000 from a human ear and the strains were presumptively identified as '*O. anthropi*-like'. All strains showed beige-coloured colonies on nutrient agar (Oxoid) at 37 °C. Subcultivation was done on tryptone soy agar (TSA) at 28 °C for 48 h.

Gram-staining was performed as described by Gerhardt *et al.* (1994). Cell morphology was observed under a Zeiss light microscope at ×1000, with cells grown for 3 days at 28 °C on TSA. The 16S rRNA gene was analysed as described by Kämpfer *et al.* (2003a). Strains CCUG 30717$^T$ and CCUG 43892 showed identical 16S rRNA gene sequences. Phylogenetic analysis was performed using the ARB software package (Ludwig *et al.*, 2004) and also the software package MEGA version 2.1 (Kumar *et al.*, 2001) after multiple alignment of data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura-2 model) were calculated and clustering with the neighbour-joining method and maximum-parsimony was performed by using bootstrap values based on 1000 replications. The almost-complete 16S rRNA gene sequences of the three strains were compared by sequence

Abbreviations: pNA, *p*-nitroanilide; pNP, *p*-nitrophenyl.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CCUG 30717$^T$ and CCUG 38531$^T$ are respectively AM422371 and AM422370.

similarity calculations after a neighbour-joining analysis. The results of these calculations indicated that the closest relative of all strains was *O. grignonense* (99.0 and 98.2 % similarity to the type strain). Lower sequence similarities were found with strains of all other species of the genus *Ochrobactrum* with validly published names. A phylogenetic tree is shown in Fig. 1.

In *recA*-based phylogenetic analyses including the type strains of some *Ochrobactrum* and *Brucella* species, strain CCUG 38531$^T$ clustered as a separate lineage most closely related to *Brucella* species (Scholz *et al.*, 2006). *recA* could not be amplified from strains CCUG 30717$^T$ and CCUG 43892, as described previously for the type strain of *O. grignonense* (Scholz *et al.*, 2006).

For quinone and polar lipid analysis, cells were grown on PYE medium (Busse *et al.*, 2005). Detailed results of chemotaxonomic analyses are given in the species description. The following analytical procedures were performed as described: respiratory quinones (Tindall, 1990; Altenburger *et al.*, 1996) but using an HPLC consisting of a JASCO PU 2080 Plus pump and JASCO UV-2075 Plus UV/Vis detector; polyamines (Busse & Auling, 1988; Busse *et al.*, 1997) using a JASCO PU 2080 Plus pump; polar lipids (Ventosa *et al.*, 1993); and fatty acids (Kämpfer & Kroppenstedt, 1996). The quinone system (Q-10) supports affiliation of the three strains to the *Alphaproteobacteria*, where the majority of species (including *Ochrobactrum* species) have Q-10 as the major quinone (Lechner *et al.*, 1995; Yokota *et al.*, 1992). The polyamine pattern, with the predominant compounds putrescine and spermidine, was in agreement with the patterns reported previously for two species, including *O. anthropi* (Lechner *et al.*, 1995; Hamana & Takeuchi, 1998), and is distinct from the polyamine patterns of members of the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*/*Ensifer*, *Aminobacter* and *Phyllobacterium*, which were shown to contain *sym*-homospermidine as the major compound in their polyamine patterns (Busse & Auling, 1988; Auling *et al.*, 1991; Hamana & Takeuchi, 1998), whereas spermidine is only present in minor to moderate amounts. In contrast, other relatives, such as species of *Pseudaminobacter*, exhibit polyamine patterns with the predominant compounds putrescine, spermidine and *sym*-homospermidine, and *Mycoplana bullata* shows the presence of spermidine as the major compound with moderate amounts of *sym*-homospermidine (Hamana & Takeuchi, 1998; Kämpfer *et al.*, 1999). However, in the three strains, we were able to detect significant amounts of *sym*-homospermidine [9–17 μmol (g dry weight)$^{-1}$]. Since the *Ochrobactrum* species examined so far have been reported to lack *sym*-homospermidine, we have reanalysed all type strains of *Ochrobactrum* species for their polyamine patterns, and every strain was found to contain at least moderate amounts [>5 μmol (g dry weight)$^{-1}$] of this polyamine (not shown). These results suggest that, under the standardized conditions for growth of biomass applied here (growth on PYE medium at 28 °C and harvesting the cells at approximately 70 % of the maximum optical density of the culture), *Ochrobactrum* species are characterized by a polyamine pattern containing the major compound spermidine and moderate to major amounts of putrescine and *sym*-homospermidine. Absence of *sym*-homospermidine in representatives of the closely related genus *Pseudochrobactrum* (Kämpfer *et al.*, 2006, 2007; B. Huber and H.-J. Busse, unpublished results) appears to be a distinguishing trait between the two genera. The polar lipid profiles of CCUG 30717$^T$ (Fig. 2), CCUG 43892 and CCUG 38531$^T$ were almost indistinguishable and exhibited a high degree of similarity to the profile of *O. gallinifaecis* (Kämpfer *et al.*, 2003b). Like this species, the strains showed the presence of aminolipid AL2, but its content in the profile of CCUG 38531$^T$ was lower than in the other two strains (not shown). Since AL2 is lacking in extracts of *Pseudochrobactrum* species (Kämpfer *et al.*, 2006, 2007), its presence is apparently another distinguishing trait between *Ochrobactrum* and *Pseudochrobactrum*. In this context, it is worth mentioning here that other *Ochrobactrum* species also contain this characteristic in their polar lipid profiles (B. Huber and H.-J. Busse, unpublished results).

The fatty acid profiles of the three strains are shown in Table 1. No significant differences in fatty acid profiles have been found for other *Ochrobactrum* species; however,

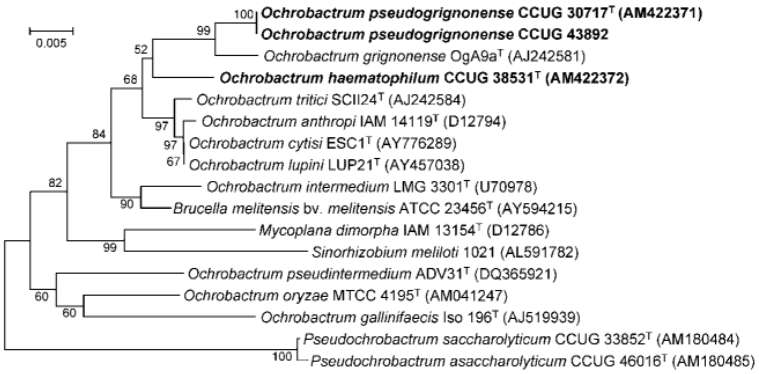


**Fig. 1.** Phylogenetic analysis based on 16S rRNA gene sequences available from the EMBL database (accession numbers in parentheses) constructed after multiple alignment of data by CLUSTAL_X (Thompson *et al.*, 1997). The tree was constructed as described in the text. Bootstrap values based on 1000 replications are listed as percentages at branching points. Bar, 0.005 substitutions per nucleotide position.

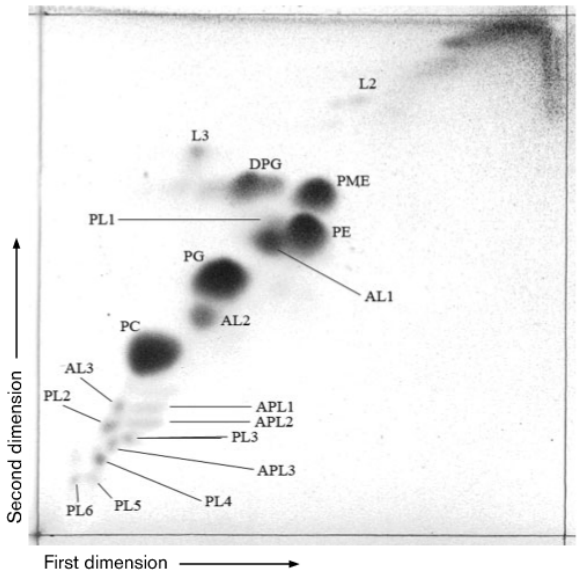


**Fig. 2.** Two-dimensional TLC of polar lipids of strain CCUG 30717$^T$. PME, Phosphatidylmonomethylethanolamine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; AL1–3, unknown aminolipids; PL1–6, unknown phospholipids; APL1–3, unknown aminophospholipids; L2–3, unknown polar lipids.

no hydroxylated fatty acids were detected in strain CCUG 38531$^T$.

Results of the physiological characterization are given in the species description and in Table 2 (differential characters), using previously described methods (Kämpfer *et al.*, 1991). DNA–DNA hybridization experiments were performed with all three strains and the type strains of all *Ochrobactrum* species using the method described by Ziemke *et al.* (1998), except that, for nick translation, 2 μg DNA was labelled during a 3 h incubation at 15 °C. Results for the novel strains and the four most closely related type strains are shown in Table 3.

Phylogenetic examinations based on almost-entire 16S rRNA gene sequences showed the affiliation of the three strains to the genus *Ochrobactrum*. They also shared the distinguishing characteristics of the genus, the presence of unknown aminolipid AL2 and a polyamine pattern containing significant amounts of *sym*-homospermidine in addition to spermidine and putrescine. The relationship of strains CCUG 30717$^T$ and CCUG 43892 at the species level as suggested by their identical 16S rRNA gene sequences was confirmed by the results of DNA–DNA hybridizations. In the phylogenetic tree, they were placed next to *O. grignonense* OgA9a$^T$, but their status as a distinct species was confirmed by DNA–DNA hybridizations with *O. grignonense* DSM 13338$^T$; *O. anthropi* CIP 14970$^T$, *O. tritici* LMG 18957$^T$ and *O. intermedium* LMG 3301$^T$. The

**Table 1.** Major fatty acids (%) of the type strains of species of the genus *Ochrobactrum* closely related to strains CCUG 30717$^T$, CCUG 43892 and CCUG 38531$^T$

Strains: 1, *O. pseudogrignonense* sp. nov. CCUG 30717$^T$; 2, *O. pseudogrignonense* sp. nov. CCUG 43892; 3, *O. haematophilum* sp. nov. CCUG 38531$^T$; 4, *O. grignonense* DSM 13338$^T$; 5, *O. gallinifaecis* Iso 196$^T$; 6, *O. intermedium* LMG 3301$^T$; 7, *O. anthropi* CIP 14970$^T$; 8, *O. tritici* LMG 18957$^T$. All strains were grown on trypticase soy broth agar at 28 °C for 48 h prior to fatty acid analysis. For unsaturated fatty acids, the position of the double bond is located by counting from the methyl ($\omega$) end of the carbon chain. *cis* and *trans* isomers are indicated by the suffixes *c* and *t*, respectively. –, Not detected.

| Fatty acid | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Saturated fatty acids | | | | | | | | |
| $C_{14:0}$ | – | – | – | – | 0.7 | – | – | – |
| $C_{15:0}$ | – | 0.3 | – | – | – | – | – | – |
| $C_{16:0}$ | 9.7 | 9.2 | 10.8 | 2.9 | 8.9 | 3.7 | 6.6 | 3.7 |
| $C_{17:0}$ | 1.6 | 2.1 | 2.5 | 1.7 | – | 3.1 | 1.4 | 0.9 |
| $C_{18:0}$ | 4.9 | 5.0 | 7.7 | 7.2 | 3.7 | 4.1 | 8.8 | 9.6 |
| Unsaturated fatty acids | | | | | | | | |
| $C_{13:1}$ at 12–13 | – | – | – | 0.7 | – | – | 0.6 | – |
| $C_{17:1}\omega6c$ | – | – | – | 0.5 | – | 1.1 | – | – |
| $C_{18:1}\omega7c$ | 19.2 | 24.2 | 32.7 | 31.6 | 28.8 | 25.8 | 45.6 | 77.9 |
| 11-Methyl $C_{18:1}\omega7t$ | 1.7 | 1.3 | – | 1.0 | 1.6 | 1.5 | 1.0 | – |
| $C_{20:1}\omega7c$ | – | 0.2 | – | 0.8 | – | – | – | – |
| $C_{20:2}\omega6,9c$ | 0.9 | 0.3 | 1.2 | 0.8 | 1.1 | 0.9 | 0.5 | – |
| Hydroxy fatty acids | | | | | | | | |
| $C_{18:1}$ 2-OH | 1.8 | 2.0 | – | 0.5 | 1.5 | 1.8 | 0.6 | 1.4 |
| $C_{18:0}$ 3-OH | 0.5 | 0.6 | – | 0.5 | – | – | – | – |
| Summed feature 3* | 1.4 | 1.7 | 1.8 | 1.0 | 3.7 | 0.7 | 1.1 | 0.7 |
| Cyclopropane acids | | | | | | | | |
| $C_{17:0}$ cyclo | 1.0 | 1.3 | – | – | 2.9 | – | 0.8 | – |
| $C_{19:0}$ cyclo $\omega8c$ | 57.0 | 50.8 | 43.2 | 50.2 | 47.2 | 57.4 | 32.7 | 5.9 |
| Unknown 13.957† | 0.3 | 0.2 | – | 0.2 | – | – | – | – |
| Unknown 14.959† | – | 0.3 | – | 0.7 | – | – | 0.3 | – |

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 contained $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH.
†Unknown fatty acids have no name listed in the peak library file of the MIDI system and therefore cannot be identified. Values are equivalent chain lengths.

results from DNA–DNA hybridizations also demonstrated that CCUG 38531$^T$ is a representative of another species of the genus *Ochrobactrum*. On the basis of unique phenotypic traits and genotypic characterization, we therefore describe two novel *Ochrobactrum* species.

### Description of *Ochrobactrum pseudogrignonense* sp. nov.

*Ochrobactrum pseudogrignonense* [pseu.do.gri.gno.nen′se. Gr. adj. *pseudes* false; N.L. neut. adj. *grignonense* a bacterial

**Table 2.** Physiological characteristics of the type strains of *Ochrobactrum* species closely related to strains CCUG 30717$^T$, CCUG 43892 and CCUG 38531$^T$

Strains: 1, *O. pseudogrignonense* sp. nov. CCUG 30717$^T$ and CCUG 43892; 2, *O. haematophilum* sp. nov. CCUG 38531$^T$; 3, *O. grignonense* DSM 13338$^T$; 4, *O. anthropi* CIP 14970$^T$; 5, *O. gallinifaecis* Iso 196$^T$; 6, *O. intermedium* LMG 3301$^T$; 7, *O. tritici* LMG 18957$^T$. +, Positive; −, negative; (+), weakly positive. All strains were positive for hydrolysis of L-alanine *p*-nitroanilide (pNA) and weak hydrolysis of bis-*p*-nitrophenyl (pNP) phosphate. All strains were negative for hydrolysis of aesculin[a]*, pNP β-D-galactopyranoside[a], pNP β-D-glucuronide, pNP α-D-glucopyranoside, pNP β-D-glucopyranoside, pNP phosphorylcholine and 2-deoxythymidine-5′-pNP phosphate. All strains were also positive for assimilation of L-arabinose[a], D-galactose[a], gluconate, D-glucose, D-mannose, D-ribose[a], D-xylose, acetate, propionate[a], fumarate[a], glutarate, DL-lactate, L-malate[a], oxoglutarate[a], pyruvate[a], L-alanine, L-proline, L-serine[a] and ornithine[a]. All strains were negative for assimilation of *p*-arbutin, salicin, putrescine, L-phenylalanine, L-tryptophan, 3-hydroxybenzoate, adipate[b], itaconate, mesaconate, phenylacetate[a], α-D-melibiose[a] and azelate.

| Test | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Hydrolysis of: | | | | | | | |
| pNP phenylphosphonate | (+) | − | − | − | − | − | (+) |
| L-Glutamate γ-3-carboxy-pNA | − | − | − | (+) | − | + | (+) |
| L-Proline pNA[b]* | + | + | + | + | + | (+) | (+) |
| Assimilation of: | | | | | | | |
| D-Fructose, *myo*-inositol[a], L-rhamnose[a], D-sorbitol[a], *cis*-aconitate, citrate[ab], DL-3-hydroxybutyrate[a] | + | + | + | + | − | + | + |
| 4-Aminobutyrate, β-alanine[a] | + | + | + | + | (+) | + | + |
| D-Maltose[ab], *N*-acetyl-D-glucosamine[a] | − | + | − | + | − | + | + |
| Adonitol[ac] | + | + | − | + | − | + | + |
| Sucrose[ab], trehalose | +† | + | − | + | − | − | + |
| Maltitol | − | + | − | + | − | − | − |
| *trans*-Aconitate[a] | + | + | − | − | − | (+) | − |
| D-Cellobiose[ac] | − | − | − | + | − | + | − |
| *N*-Acetyl-D-galactosamine | + | − | + | (+) | − | + | + |
| Suberate[a] | − | − | − | − | − | − | + |
| L-Aspartate[a] | + | + | + | + | + | + | − |
| 4-Hydroxybenzoate | − | + | − | (+) | − | (+) | + |
| L-Histidine[a] | + | + | + | + | + | + | (+) |
| L-Leucine[a] | + | + | + | + | − | + | (+) |

*Tests (based on different methods) were also performed in the following studies and gave congruent results: *a*, Holmes *et al.* (1988) with *O. anthropi*; *b*, Velasco *et al.* (1998) with *O. intermedium*; *c*, Lebuhn *et al.* (2000) with type strains of all four previously described species.
†Strain CCUG 30717$^T$ showed only weakly positive results.

species epithet; N.L. neut. adj. *pseudogrignonense* a false (*Ochrobactrum*) *grignonense*].

Cells are non-motile, non-spore-forming rods (approx. 2 μm in length). Gram-negative and oxidase-positive, showing an oxidative metabolism. Good growth occurs on R2A agar, TSA, nutrient agar and MacConkey agar at 25–30 °C. Growth is observed on PYE agar supplemented with 5 % NaCl (w/v) but different results are observed for the two known strains at 7 % NaCl. Growth is observed on PYE agar at 4 °C but not at 42 °C. Beige, translucent and shiny colonies with entire edges are formed within 24 h, with a diameter of approximately 2 mm. The quinone system consists of Q-10 (96–99 %) and Q-9 (2–4 %). The polyamine pattern [(g dry weight)$^{-1}$] consists of the major compounds spermidine (23–35 μmol) and putrescine (27–34 μmol), moderate amounts of *sym*-homospermidine (9–10 μmol) and minor amounts of 1,3-diaminopropane (1–2 μmol) and spermine (1 μmol). Predominant polar lipids are phosphatidylethanolamine, phosphatidylmonomethylethanolamine,

**Table 3.** Percentage DNA–DNA reassociation values between the novel strains and related *Ochrobactrum* type strains

| Strain | 1 | 2 | 3 | *O. grignonense* DSM 13338$^T$ | *O. anthropi* CIP 14970$^T$ | *O. tritici* LMG 18957$^T$ | *O. intermedium* LMG 3301$^T$ |
|---|---|---|---|---|---|---|---|
| 1. CCUG 30717$^T$ | 100 | 81 | 21 | 49 | 26 | 32 | 19 |
| 2. CCUG 43892 | 100 | 100 | 22 | 45 | 48 | 25 | 13 |
| 3. CCUG 38531$^T$ | 38 | 13 | 100 | 40 | 18 | 42 | 47 |

phosphatidylglycerol and phosphatidylcholine. Moderate amounts of phosphatidyldimethylethanolamine, diphosphatidylglycerol and two unidentified phosphorus-free aminolipids (AL1 and AL2) and small amounts of several unknown phospholipids, aminophospholipids and an aminolipid are also present. The fatty acid profile is composed largely of $C_{18:1}\omega7c$ (19.2–24.2 %) and $C_{19:0}$ cyclo $\omega8c$ (50.8–57.8 %). Carbon source utilization and hydrolysis of chromogenic substrates (including differentiating characters for all *Ochrobactrum* species) are indicated in Table 2.

The type strain, CCUG 30717$^T$ (=CIP 109451$^T$), was isolated in 1992 from blood of a 28-year-old man in Göteborg, Sweden. Strain CCUG 43892 was isolated in 2000 from the ear of a newborn in Tromsö, Norway.

### Description of *Ochrobactrum haematophilum* sp. nov.

*Ochrobactrum haematophilum* (hae.ma.to.phi'lum. Gr. n. *haema -atis* blood; Gr. adj. *philos* loving; N.L. neut. adj. *haematophilum* blood-loving).

Cells are non-motile, non-spore-forming rods (approx. 2 μm in length). Gram-negative and oxidase-positive, showing an oxidative metabolism. Good growth occurs on R2A agar, TSA, PYE agar, nutrient agar and MacConkey agar at 25–30 °C. Growth is observed on PYE agar supplemented with 5 % NaCl (w/v) but not with 7 %. No growth is observed on PYE agar at 4 or 42 °C. Beige, translucent and shiny colonies with entire edges form within 24 h, with a diameter of approximately 2 mm. The quinone system consists of Q-10 (98 %) and Q-9 (2 %). The polyamine pattern [(g dry weight)$^{-1}$] consists of the major compounds spermidine (49 μmol), putrescine (19 μmol) and *sym*-homospermidine (17 μmol) and minor amounts of 1,3-diaminopropane (3 μmol) and spermine (2 μmol). Predominant polar lipids are phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol and phosphatidylcholine. Moderate amounts of phosphatidyldimethylethanolamine, diphosphatidylglycerol and two unidentified phosphorus-free aminolipids (AL1 and AL2) and small amounts of several unknown phospholipids, aminophospholipids and an aminolipid are also present. The fatty acid profile is composed largely of $C_{18:1}\omega7c$ (32.7 %) and $C_{19:0}$ cyclo $\omega8c$ (43.2 %). Carbon source utilization and hydrolysis of chromogenic substrates (including differentiating characters for all *Ochrobactrum* species) are indicated in Table 2.

The type strain, CCUG 38531$^T$ (=CIP 109452$^T$), was isolated in 1997 from blood of a 79-year-old man in Falun, Sweden.

## Acknowledgements

The CCUG is grateful to the depositors and to the devoted staff Elisabeth Inganäs, Maria Ohlén, Sofia Wernersson and Geneviève Mosella.

## References

**Altenburger, P., Kämpfer, P., Makristathis, A., Lubitz, W. & Busse, H.-J. (1996).** Classification of bacteria isolated from a medieval wall painting. *J Biotechnol* **47**, 39–52.

**Auling, G., Busse, H.-J., Pilz, F., Webb, L., Kneifel, H. & Claus, D. (1991).** Rapid differentiation by polyamine analysis of *Xanthomonas* strains from phytopathogenic pseudomonads and other members of the class *Proteobacteria* interacting with plants. *Int J Syst Bacteriol* **41**, 223–228.

**Busse, H.-J. & Auling, G. (1988).** Polyamine pattern as a chemotaxonomic marker within the *Proteobacteria*. *Syst Appl Microbiol* **11**, 1–8.

**Busse, H.-J., Bunka, S., Hensel, A. & Lubitz, W. (1997).** Discrimination of members of the family *Pasteurellaceae* based on polyamine patterns. *Int J Syst Bacteriol* **47**, 698–708.

**Busse, H.-J., Hauser, E. & Kämpfer, P. (2005).** Description of two novel species, *Sphingomonas abaci* sp. nov. and *Sphingomonas panni* sp. nov. *Int J Syst Evol Microbiol* **55**, 2565–2569.

**Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (editors) (1994).** *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.

**Hamana, K. & Takeuchi, M. (1998).** Polyamine profiles as chemotaxonomic markers within alpha, beta, gamma, delta, and epsilon subclasses of class *Proteobacteria*: distribution of 2-hydroxyputrescine and homospermidine. *Microbiol Cult Collect* **14**, 1–14.

**Holmes, B., Popoff, M., Kiredjian, M. & Kersters, K. (1988).** *Ochrobactrum anthropi* gen. nov., sp. nov. from human clinical specimens and previously known as group Vd. *Int J Syst Bacteriol* **38**, 406–416.

**Kämpfer, P. & Kroppenstedt, R. M. (1996).** Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42**, 989–1005.

**Kämpfer, P., Steiof, M. & Dott, W. (1991).** Microbiological characterisation of a fuel-oil contaminated site including numerical identification of heterotrophic water and soil bacteria. *Microb Ecol* **21**, 227–251.

**Kämpfer, P., Müller, C., Mau, M., Neef, A., Auling, G., Busse, H.-J., Osborn, A. M. & Stolz, A. (1999).** Description of *Pseudaminobacter* gen. nov. with two new species, *Pseudaminobacter salicylatoxidans* sp. nov. and *Pseudaminobacter defluvii* sp. nov. *Int J Syst Bacteriol* **49**, 887–897.

**Kämpfer, P., Dreyer, U., Neef, A., Dott, W. & Busse, H.-J. (2003a).** *Chryseobacterium defluvii* sp. nov., isolated from wastewater. *Int J Syst Evol Microbiol* **53**, 93–97.

**Kämpfer, P., Buczolits, S., Albrecht, A., Busse, H.-J. & Stackebrandt, E. (2003b).** Towards a standardized format for the description of a novel species (of an established genus): *Ochrobactrum gallinifaecis* sp. nov. *Int J Syst Evol Microbiol* **53**, 893–896.

**Kämpfer, P., Rosselló-Mora, R., Scholz, H. C., Welinder-Olsson, C., Falsen, E. & Busse, H.-J. (2006).** Description of *Pseudochrobactrum* gen. nov., with the two species *Pseudochrobactrum asaccharolyticum* sp. nov. and *Pseudochrobactrum saccharolyticum* sp. nov. *Int J Syst Evol Microbiol* **56**, 1823–1829.

**Kämpfer, P., Scholz, H., Huber, B., Thummes, K., Busse, H.-J., Maas, E. W. & Falsen, E. (2007).** Description of *Pseudochrobactrum kiredjianiae* sp. nov. *Int J Syst Evol Microbiol* **57**, 755–760.

**Kumar, S., Tamura, K., Jakobsen, I.-B. & Nei, M. (2001).** MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–1245.

**Lebuhn, M., Achouak, W., Schloter, M., Berge, O., Meier, H., Barakat, M., Hartmann, A. & Heulin, T. (2000).** Taxonomic characterization of *Ochrobactrum* sp. isolates from soil samples and wheat roots, and description of *Ochrobactrum tritici* sp. nov. and *Ochrobactrum grignonense* sp. nov. *Int J Syst Evol Microbiol* **50**, 2207–2223.

**Lechner, U., Baumbach, R., Becker, D., Kitunen, V., Auling, G. & Salkinoja-Salonen, M. (1995).** Degradation of 4-chloro-2-methylphenol by an activated sludge isolate and its taxonomic description. *Biodegradation* **6**, 83–92.

**Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S. & other authors (2004).** ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.

**Scholz, H. C., Tomaso, H., Al Dahouk, S., Witte, A., Schloter, M., Kämpfer, P., Falsen, E. & Neubauer, H. (2006).** Genotyping of *Ochrobacterium anthropi* by *recA*-based comparative sequence, PCR-RFLP, and 16S rRNA gene analysis. *FEMS Microbiol Lett* **257**, 7–16.

**Teyssier, C., Marchandin, H., Jean-Pierre, H., Masnou, A., Dusart, G. & Jumas-Bilak, E. (2007).** *Ochrobactrum pseudintermedium* sp. nov., a novel member of the family *Brucellaceae*, isolated from man. *Int J Syst Evol Microbiol* **57**, 1007–1013.

**Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

**Tindall, B. J. (1990).** A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* **13**, 128–130.

**Tripathi, A. K., Verma, S. C., Chowdhury, S. P., Lebuhn, M., Gattinger, A. & Schloter, M. (2006).** *Ochrobactrum oryzae* sp. nov., an endophytic bacterial species isolated from deep-water rice in India. *Int J Syst Evol Microbiol* **56**, 1677–1680.

**Trujillo, M. E., Willems, A., Abril, A., Planchuelo, A. M., Rivas, R., Ludeña, D., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. (2005).** Nodulation of *Lupinus* by strains of the new species *Ochrobactrum lupini* sp. nov. *Appl Environ Microbiol* **71**, 1318–1327.

**Velasco, J., Romero, C., López-Goñi, I., Leiva, J., Díaz, R. & Moriyón, I. (1998).** Evaluation of the relatedness of *Brucella* spp. and *Ochrobactrum anthropi* and description of *Ochrobactrum intermedium* sp. nov., a new species with a closer relationship to *Brucella* spp. *Int J Syst Bacteriol* **48**, 759–768.

**Ventosa, A., Marquez, M. C., Kocur, M. & Tindall, B. J. (1993).** Comparative study of "*Micrococcus* sp." strains CCM 168 and CCM 1405 and members of the genus *Salinicoccus*. *Int J Syst Bacteriol* **43**, 245–248.

**Yokota, A., Akagawa-Matsushita, M., Hiraishi, A., Katayama, Y., Urakami, T. & Yamasato, K. (1992).** Distribution of quinone systems in microorganisms: Gram-negative Eubacteria. *Bull Jpn Fed Cult Coll* **8**, 136–171.

**Ziemke, F., Höfle, M. G., Lalucat, J. & Rosselló-Mora, R. (1998).** Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.

**Zurdo-Piñeiro, J. L., Rivas, R., Trujillo, M. E., Vizcaíno, N., Carrasco, J. A., Chamber, M., Palomares, A., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. (2007).** *Ochrobactrum cytisi* sp. nov. isolated from nodules of *Cytisus scoparius* in Spain. *Int J Syst Evol Microbiol* **57**, 784–788.

## 2.7. *Ochrobactrum rhizosphaerae* sp. nov. and *Ochrobactrum thiophenivorans* sp. nov., isolated from the environment

*International Journal of Systematic and Evolutionary Microbiology* (2008), **58**, 1426–1431 DOI 10.1099/ijs.0.65407-0

# *Ochrobactrum rhizosphaerae* sp. nov. and *Ochrobactrum thiophenivorans* sp. nov., isolated from the environment

Peter Kämpfer,[1] Angela Sessitsch,[2] Michael Schloter,[3] Birgit Huber,[4] Hans-Jürgen Busse[4] and Holger C. Scholz[5]

Correspondence
Peter Kämpfer
peter.kaempfer@umwelt.uni-giessen.de

[1]Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Giessen, D-35392 Giessen, Germany

[2]Austrian Research Centers GmbH, Department of Bioresources, A-2444 Seibersdorf, Austria

[3]Helmholtz Zentrum München, German Research Center for Environmental Health, Terrestrial Ecogenetics, Ingolstaedter Landstrasse 1, D-85764 Neuherberg, Germany

[4]Institut für Bakteriologie, Mykologie und Hygiene, Veterinärmedizinische Universität Wien, A-1210 Wien, Austria

[5]Bundeswehr Institute of Microbiology, D-80937 Munich, Germany

Two Gram-negative, rod-shaped, non-spore-forming bacteria, PR17$^T$ and DSM 7216$^T$, isolated from the potato rhizosphere and an industrial environment, respectively, were studied for their taxonomic allocation. By *rrs* (16S rRNA) gene sequencing, these strains were shown to belong to the *Alphaproteobacteria*, most closely related to *Ochrobactrum pseudogrignonense* (98.4 and 99.3 % similarity to the type strain, respectively). Chemotaxonomic data (major ubiquinone Q-10; major polyamines spermidine, *sym*-homospermidine and putrescine; major polar lipids phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol and phosphatidylcholine and the *Ochrobactrum*-specific unidentified aminolipid AL2; major fatty acids $C_{18:1}\omega7c$ and $C_{19:0}$ cyclo $\omega8c$) supported the genus affiliation. The results of DNA–DNA hybridization and physiological and biochemical tests allowed genotypic and phenotypic differentiation of the isolates from all hitherto-described *Ochrobactrum* species. Hence, both isolates represent novel species of the genus *Ochrobactrum*, for which the names *Ochrobactrum rhizosphaerae* sp. nov. (type strain PR17$^T$ =CCUG 55411$^T$ =CCM 7493$^T$ =DSM 19824$^T$) and *Ochrobactrum thiophenivorans* sp. nov. (type strain DSM 7216$^T$ =CCUG 55412$^T$ =CCM 7492$^T$) are proposed.

The genus *Ochrobactrum*, introduced by Holmes *et al.* (1988), is a genus of which several novel species have recently been proposed. At the time of writing, the genus comprises the 11 species *Ochrobactrum anthropi* (the type species; Holmes *et al.*, 1988), *O. intermedium* (Velasco *et al.*, 1998), *O. tritici* and *O. grignonense* (Lebuhn *et al.*, 2000), *O. gallinifaecis* (Kämpfer *et al.*, 2003), *O. lupini* (Trujillo *et al.*, 2005), *O. oryzae* (Tripathi *et al.*, 2006), *O. cytisi* (Zurdo-Piñeiro *et al.*, 2007), *O. pseudintermedium* (Teyssier *et al.*, 2007), *O. haematophilum* and *O. pseudogrignonense* (Kämpfer *et al.*, 2007b).

Abbreviations: pNA, *p*-nitroanilide; pNP, *p*-nitrophenyl.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains PR17$^T$ and DSM 7216$^T$ are respectively AM490632 and AM490617.

Strains PR17$^T$ and DSM 7216$^T$ were both isolated from the environment. Strain PR17$^T$ was isolated in Austria from the potato rhizosphere and strain DSM 7216$^T$ was isolated from wastewater in Germany. The latter strain was able to produce thiophene-2-carboxylate CoA esterase and utilizes thiophene 2-carboxylate as a sole source of carbon and sulfur (Kreimer, 1992). Both strains were presumptively identified as *O. anthropi*-like. They showed beige-coloured colonies on nutrient agar (Oxoid) at 37 °C. Subcultivation was done on tryptone soy agar (TSA) at 28 °C for 48 h.

Gram staining was performed as described by Gerhardt *et al.* (1994). Cell morphology was observed under a Zeiss light microscope at ×1000, with cells grown for 3 days at 28 °C on TSA. Partial 16S rRNA gene sequences (both 1387 bp) were studied as described by Kämpfer *et al.* (2003) and subsequent analysis was performed using the

software package MEGA version 2.1 (Kumar *et al.*, 2001) after multiple alignment of data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura-2 model) and clustering with the neighbour-joining and maximum-parsimony methods was performed by using bootstrap values based on 1000 replications. The 16S rRNA gene sequences of these two strains were compared to each other and to entries in the nucleotide collection databases (nr/nt) using the BLASTN search tool available at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi. The results of these calculations showed that the closest relative of strain PR17$^T$ was strain DSM 7216$^T$ (98.7 % similarity) and the type strain of *O. pseudogrignonense*, CCUG 30717$^T$ (98.4 % similarity). The closest relatives of strain DSM 7216$^T$ were *O. pseudogrignonense* CCUG 30717$^T$ (99.3 % similarity), strain PR17$^T$ (98.7 % similarity) and the type strain of *O. haematophilum*, CCUG 38531$^T$ (98.6 % similarity). Lower sequence similarities were found with all other species of the genus *Ochrobactrum*. An *rrs*-based tree is shown in Fig. 1.

In *recA*-based phylogenetic analysis including the type strains of some *Ochrobactrum* and *Brucella* species, both strains again grouped close to *O. grignonense* but formed separate branches (Scholz *et al.*, 2006; Fig. 2).

For polar lipid and quinone analyses, cells were grown on PYE medium (0.3 % peptone from casein, 0.3 % yeast extract, pH 7.2). Extraction and analyses were carried out as described previously (Tindall, 1990a, b; Altenburger *et al.*, 1996; Stolz *et al.*, 2007). Polyamines were extracted and analysed as described by Busse & Auling (1988) and Stolz *et al.* (2007). Detailed results of chemotaxonomic analyses are given in the species descriptions. Analysis of quinones revealed a spot that corresponded to ubiquinone-10 (Q-10). The quinone system supports the affiliation of the two strains to the *Alphaproteobacteria*, where the majority of species have Q-10 as the major quinone (Lechner *et al.*, 1995; Yokota *et al.*, 1992); other *Ochrobactrum* species and unnamed *Ochrobactrum* strains have been reported to exhibit Q-10 as the major quinone (Lechner *et al.*, 1995; Yokota *et al.*, 1992; Kämpfer *et al.*, 2003; Teyssier *et al.*, 2007; B. Huber and H.-J. Busse, unpublished results). The polyamine patterns, with the three predominant compounds putrescine, spermidine and *sym*-homospermidine, are in agreement with patterns reported previously for two species of this genus (Kämpfer *et al.*, 2007b) and with the polyamine patterns of several other species of the genus (B. Huber and H.-J. Busse, unpublished results). Polar lipid profiles of PR7$^T$ and DSM 7216$^T$ exhibited only slight quantitative differences and strongly resembled the profiles of *O. gallinifaecis* (Kämpfer *et al.*, 2003), *O. haematophilum*, *O. pseudogrignonense* (Kämpfer *et al.*, 2007b) and other *Ochrobactrum* strains (B. Huber and H.-J. Busse, unpublished results). Furthermore, the characteristic unknown aminolipid AL2 was detected, which is absent from polar lipid extracts of representatives of the closely related genus *Pseudochrobactrum* (Kämpfer *et al.*, 2006, 2007a).

Fatty acid methyl esters were prepared, separated and identified according to the instructions of the Microbial Identification System (MIDI Inc.) (Kämpfer & Kroppenstedt, 1996). The fatty acid profiles of the three strains are shown in Table 1. No significant differences in the fatty acid profiles were found compared with other *Ochrobactrum* species.

Results of physiological and biochemical tests are given in the species descriptions and in Table 2. Tests were performed with methods described previously (Kämpfer *et al.*, 1991). DNA–DNA hybridization experiments were performed with strains PR17$^T$ and DSM 7216$^T$ and the type strains of the most closely related *Ochrobactrum* species, *O. grignonense* and *O. pseudogrignonense*, using the method described by Ziemke *et al.* (1998) except that, for nick translation, 2 µg DNA was labelled during a 3 h incubation at 15 °C. The results are shown in Table 3.

Examinations based on almost entire *rrs* and *recA* gene sequences showed the affiliation of the two strains to the genus *Ochrobactrum*. In both trees (Figs 1 and 2), strains PR17$^T$ and DSM 7216$^T$ grouped close to *O. grignonense* OgA9a$^T$ and *O. pseudogrignonense* CCUG 30717$^T$.

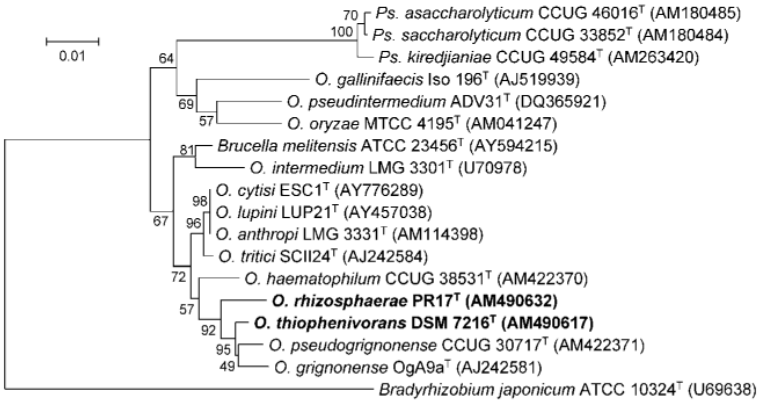


**Fig. 1.** Phylogenetic analysis based on 16S rRNA gene (1387 bp) sequences available from the EMBL database (accession numbers in parentheses) constructed after multiple alignment of data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura-2 model) and clustering with the neighbour-joining method was performed by using the software package MEGA version 2.1 (Kumar *et al.*, 2001). Bootstrap values based on 1000 replications are listed as percentages at branching points. Bar, 0.01 substitutions per nucleotide position. *Ps.*, *Pseudochrobactrum*.

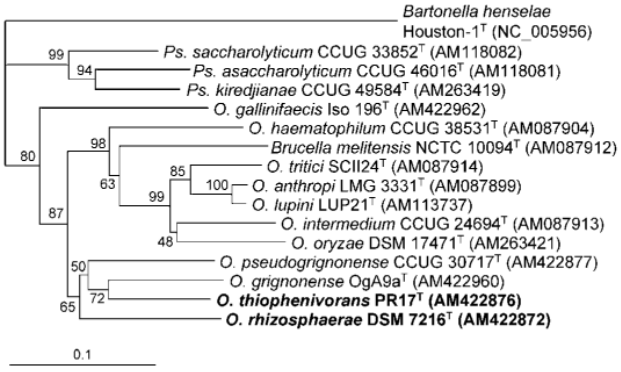


**Fig. 2.** Phylogenetic analysis based on *recA* (879 bp) gene sequences. See legend to Fig. 1 for further details. Bar, 0.1 substitutions per nucleotide position.

However, DNA–DNA hybridization studies (Table 3) clearly demonstrated that the two strains represent separate species distinct from *O. grignonense* and *O. pseudogrignonense*. On the basis of these results, we describe two novel *Ochrobactrum* species.

## Description of *Ochrobactrum rhizosphaerae* sp. nov.

*Ochrobactrum rhizosphaerae* [rhi.zo.sphae′rae. Gr. fem. n. *rhiza* root; L. fem. n. *sphaera -ae* (from Gr. fem. n. *sphaira -as*)

**Table 1.** Major fatty acids (%) of the novel strains and type strains of closely related species of the genus *Ochrobactrum*

Strains: 1, PR17$^T$ (*O. rhizosphaerae* sp. nov.); 2, DSM 7216$^T$ (*O. thiophenivorans* sp. nov.); 3, *O. pseudogrignonense* CCUG 30717$^T$; 4, *O. pseudogrignonense* CCUG 43892$^T$; 5, *O. haematophilum* CCUG 38531$^T$; 6, *O. grignonense* DSM 13338$^T$; 7, *O. gallinifaecis* Iso 196$^T$; 8, *O. intermedium* LMG 3301$^T$; 9, *O. anthropi* CIP 14970$^T$; 10, *O. tritici* LMG 18957$^T$; 11, *O. lupini* LUP21$^T$. All strains were grown on TSA at 28 °C for 48 h prior to fatty acid analysis. For unsaturated fatty acids, the position of the double bond is located by counting from the methyl ($\omega$) end of the carbon chain; *cis* and *trans* isomers are indicated by the suffixes *c* and *t*, respectively.

| Fatty acid | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Saturated fatty acids | | | | | | | | | | | |
| $C_{14:0}$ | | | | | | | 0.7 | | | | |
| $C_{15:0}$ | | | | 0.3 | | | | | | | |
| $C_{16:0}$ | 8.3 | 7.3 | 9.7 | 9.2 | 10.8 | 2.9 | 8.9 | 3.7 | 6.6 | 3.7 | 5.8 |
| $C_{17:0}$ | 2.8 | 1.4 | 1.6 | 2.1 | 2.5 | 1.7 | | 3.1 | 1.4 | 0.9 | 1.8 |
| $C_{18:0}$ | 7.6 | 2.9 | 4.9 | 5.0 | 7.7 | 7.2 | 3.7 | 4.1 | 8.8 | 9.6 | 3.5 |
| Unsaturated fatty acids | | | | | | | | | | | |
| $C_{13:1}$ at 12–13 | | | | | | 0.7 | | | 0.6 | | |
| $C_{17:1}\omega6c$ | | | | | | 0.5 | | 1.1 | | | |
| $C_{18:1}\omega7c$ | 61.5 | 40.9 | 19.2 | 24.2 | 32.7 | 31.6 | 28.8 | 25.8 | 45.6 | 77.9 | 57.3 |
| 11-Methyl $C_{18:1}\omega7t$ | | 1.5 | 1.7 | 1.3 | | 1.0 | 1.6 | 1.5 | 1.0 | | 2.0 |
| $C_{20:1}\omega7c$ | | | | 0.2 | | 0.8 | | | | | |
| $C_{20:2}\omega6,9c$ | | 1.8 | 0.9 | 0.3 | 1.2 | 0.8 | 1.1 | 0.9 | 0.5 | | |
| Hydroxy fatty acids | | | | | | | | | | | |
| $C_{18:1}$ 2-OH | 4.7 | 1.5 | 1.8 | 2.0 | | 0.5 | 1.5 | 1.8 | 0.6 | 1.4 | 1.8 |
| $C_{18:0}$ 3-OH | | | 0.5 | 0.6 | | 0.5 | | | | | |
| Summed feature 3* | 4.6 | 5.8 | 1.4 | 1.7 | 1.8 | 1.0 | 3.7 | 0.7 | 1.1 | 0.7 | 2.1 |
| Cyclopropane acids | | | | | | | | | | | |
| $C_{17:0}$ cyclo | | 2.1 | 1.0 | 1.3 | | | 2.9 | | 0.8 | | |
| $C_{19:0}$ cyclo $\omega8c$ | 11.2 | 34.9 | 57.0 | 50.8 | 43.2 | 50.2 | 47.2 | 57.4 | 32.7 | 5.9 | 23.8 |
| Unknown 13.957† | | | 0.3 | 0.2 | | 0.2 | | | | | |
| Unknown 14.959† | | | | 0.3 | | 0.7 | | | 0.3 | | 2.0 |

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 contained $C_{16:1}\omega7c$ and/or $C_{15:0}$ iso 2-OH.
†Unknown fatty acids have no name listed in the peak library file of the MIDI system and therefore cannot be identified.

**Table 2.** Physiological characteristics of the novel strains and type strains of closely related *Ochrobactrum* species

Strains: 1, PR17$^T$ (*O. rhizosphaerae* sp. nov.); 2, DSM 7216$^T$ (*O. thiophenivorans* sp. nov.); 3, *O. pseudogrignonense* CCUG 30717$^T$ and CCUG 43892; 4, *O. haematophilum* CCUG 38531$^T$; 5, *O. grignonense* DSM 13338$^T$; 6, *O. anthropi* CIP 14970$^T$; 7, *Ochrobactrum gallinifaecis* Iso 196$^T$; 8, *O. intermedium* LMG 3301$^T$; 9, *O. tritici* LMG 18957$^T$; 10, *O. lupini* LUP21$^T$; 11, *O. oryzae* DSM 17471$^T$ (data from Tripathi *et al.*, 2006). +, Positive; −, negative; (+), weakly positive; ND, no data available. All strains were positive for hydrolysis of L-alanine *p*-nitroanilide (pNA) and weak hydrolysis of bis-*p*-nitrophenyl (pNP) phosphate. All strains (except *O. oryzae* DSM 17471$^T$, for which not all tests were performed) were negative for hydrolysis of aesculin[a*], pNP *β*-D-galactopyranoside[a], pNP *β*-D-glucuronide, pNP α-D-glucopyranoside, pNP *β*-D-glucopyranoside, pNP phosphorylcholine and 2-deoxythymidine-5′-pNP phosphate. All strains were also positive for assimilation of L-arabinose[a], D-galactose[a], D-glucose, D-mannose, D-ribose[a], D-xylose, acetate, propionate[a], fumarate[a], glutarate, DL-lactate, L-malate[a], oxoglutarate[a], pyruvate[a], L-alanine, L-proline, L-serine[a] and ornithine[a]. All strains were negative for assimilation of *p*-arbutin, salicin, putrescine, L-phenylalanine, L-tryptophan, 3-hydroxybenzoate, adipate[b], itaconate, mesaconate, phenylacetate[a], melibiose[a] and azelate.

| Test | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Hydrolysis of: | | | | | | | | | | | |
| pNP phenylphosphonate | + | (+) | (+) | − | − | − | − | − | (+) | − | ND |
| L-Glutamate-γ-3-carboxy pNA | − | − | − | − | − | (+) | − | + | (+) | + | ND |
| L-Proline pNA[b*] | + | + | + | + | + | + | + | (+) | (+) | + | ND |
| Assimilation of: | | | | | | | | | | | |
| D-Fructose, *myo*-inositol[a], D-sorbitol[a], DL-3-hydroxybutyrate[a] | + | + | + | + | + | + | − | + | + | + | + |
| L-Rhamnose[a] | + | − | + | + | + | + | − | + | + | + | + |
| *cis*-Aconitate | + | − | + | + | + | + | − | + | + | − | + |
| Citrate[ab] | + | − | + | + | + | + | − | + | + | + | − |
| 4-Aminobutyrate, *β*-alanine[a] | + | + | + | + | + | + | (+) | + | + | + | ND |
| Maltose[ab] | + | − | − | + | − | + | − | + | + | + | + |
| D-Gluconate | − | + | + | + | + | + | + | + | + | (+) | + |
| *N*-Acetyl-D-glucosamine[a] | (+) | (+) | − | + | − | + | − | + | + | + | + |
| Adonitol[ac] | + | − | + | + | − | + | − | + | + | + | + |
| Sucrose[ab], trehalose | + | − | +† | + | − | + | − | − | + | + | + |
| Maltitol | + | − | − | + | − | + | − | − | − | + | ND |
| *trans*-Aconitate[a] | + | − | + | + | − | − | − | (+) | − | − | ND |
| Cellobiose[ac] | + | − | − | − | − | + | − | + | − | − | ND |
| *N*-Acetyl-D-galactosamine | + | + | + | − | + | (+) | − | + | + | + | ND |
| Suberate[a] | − | − | − | − | − | − | − | − | + | − | ND |
| L-Aspartate[a] | + | + | + | + | + | + | + | + | − | + | + |
| 4-Hydroxybenzoate | − | − | − | + | − | (+) | − | (+) | + | + | ND |
| L-Histidine[a] | + | − | + | + | + | + | + | + | (+) | + | + |
| L-Leucine[a] | + | − | + | + | + | + | − | + | (+) | + | + |

*Test (based on a different method) was also performed as follows and gave congruent results: *a*, Holmes *et al.* (1988) with *O. anthropi*; *b*, Velasco *et al.* (1998) with *O. intermedium*; *c*, Lebuhn *et al.* (2000) with type strains of all four previously described species.
†Strain CCUG 30717$^T$ showed only weakly positive results.

ball, any globe, sphere; N.L. gen. fem. n. *rhizosphaerae* of the rhizosphere].

Cells from the early exponential growth phase are stongly motile, but may become less motile or non-motile when approaching the stationary growth phase. Cells are non-spore-forming rods (approx. 2 μm long). Gram-negative and oxidase-positive, showing an oxidative metabolism. Good growth occurs on R2A agar, TSA, nutrient agar, PYE agar and MacConkey agar at 25–30 °C. Colonies are beige, translucent and shiny. Colonies with entire edges are formed within 24 h, with a diameter of

**Table 3.** DNA–DNA reassociation (%) between the novel strains and closely related *Ochrobactrum* type strains

| Strain | PR17$^T$ | DSM 7216$^T$ | *O. grignonense* DSM 13338$^T$ | *O. pseudogrignonense* CCUG 30717$^T$ |
|---|---|---|---|---|
| PR17$^T$ | 100 | – | 40.0 | 27.3 |
| DSM 7216$^T$ | 32.1 | 100 | 33.3 | 24.2 |

approximately 2 mm. The quinone system consists of Q-10 (99 %) and Q-9 (1 %). The polyamine pattern consists of the major compounds spermidine [28 μmol (g dry weight)$^{-1}$], putrescine [41 μmol (g dry weight)$^{-1}$] and *sym*-homospermidine [10 μmol (g dry weight)$^{-1}$] and minor amounts of 1,3-diaminopropane [1 μmol (g dry weight)$^{-1}$] and spermine [<1 μmol (g dry weight)$^{-1}$]. Predominant polar lipids are phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol, phosphatidylcholine and diphosphatidylglycerol. Moderate to minor amounts of phosphatidyldimethylethanolamine and two unidentified aminolipids AL1 and the *Ochrobactrum*-specific AL2 and traces of several unknown lipids are also present. The fatty acid profile is composed mainly of $C_{18:1}\omega7c$ (61.5 %) and $C_{19:0}$ cyclo $\omega8c$ (11.2 %). Carbon source utilization and hydrolysis of chromogenic substrates (including differentiating characters for all *Ochrobactrum* species) are indicated in Table 1.

The type strain PR17$^T$ (=CCUG 55411$^T$ =CCM 7493$^T$ =DSM 19824$^T$) was isolated from the potato rhizosphere in Austria.

### Description of *Ochrobactrum thiophenivorans* sp. nov.

*Ochrobactrum thiophenivorans* (thi′o.phe.ni.vo′rans. N.L. n. *thiophenum* thiophene; L. part. adj. *vorans* devouring; N.L. part. adj. *thiophenivorans* thiophene-devouring, referring to the ability to utilize thiophene 2-carboxylate as a sole source of carbon and sulfur).

Cells are non-motile, non-spore-forming rods (approx. 2 μm long). Gram-negative and oxidase-positive, showing an oxidative metabolism. Good growth occurs on R2A agar, TSA, PYE agar, nutrient agar and MacConkey agar at 25–30 °C. Forms beige, translucent and shiny colonies with entire edges within 24 h, with a diameter of approximately 2 mm. The quinone system consists of Q-10 (99 %) and Q-9 (1 %). The polyamine pattern consists of the major compounds spermidine [28 μmol (g dry weight)$^{-1}$], putrescine [19 μmol (g dry weight)$^{-1}$] and *sym*-homospermidine [9 μmol (g dry weight)$^{-1}$] and minor amounts of 1,3-diaminopropane [4 μmol (g dry weight)$^{-1}$] and spermine [2 μmol (g dry weight)$^{-1}$]. Predominant polar lipids are phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol, phosphatidylcholine, diphosphatidylglycerol and the unknown *Ochrobactrum*-specific aminolipid AL2. Moderate amounts of phosphatidyldimethylethanolamine and another unidentified aminolipid AL1 and small to trace amounts of several unknown lipids are also present. The fatty acid profile is composed mainly of $C_{18:1}\omega7c$ (19.2 %) and $C_{19:0}$ cyclo $\omega8c$ (57.0 %). Carbon source utilization and hydrolysis of chromogenic substrates (including differentiating characters for all *Ochrobactrum* species) are indicated in Table 2.

The type strain DSM 7216$^T$ (=CCUG 55412$^T$ =CCM 7492$^T$) was isolated from wastewater in Germany.

## Acknowledgements

We thank J. P. Euzéby for his help with the etymology of the specific epithets and Birgit Mitter for helpful discussions. The work of B. H. and H.-J. B. was supported by a Contract Research Project for the Bundeswehr Medical Service.

## References

**Altenburger, P., Kämpfer, P., Makristathis, A., Lubitz, W. & Busse, H.-J. (1996).** Classification of bacteria isolated from a medieval wall painting. *J Biotechnol* **47**, 39–52.

**Busse, H.-J. & Auling, G. (1988).** Polyamine pattern as a chemotaxonomic marker within the *Proteobacteria*. *Syst Appl Microbiol* **11**, 1–8.

**Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (editors) (1994).** *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.

**Holmes, B., Popoff, M., Kiredjian, M. & Kersters, K. (1988).** *Ochrobactrum anthropi* gen. nov., sp. nov. from human clinical specimens and previously known as group Vd. *Int J Syst Bacteriol* **38**, 406–416.

**Kämpfer, P. & Kroppenstedt, R. M. (1996).** Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42**, 989–1005.

**Kämpfer, P., Steiof, M. & Dott, W. (1991).** Microbiological characterisation of a fuel-oil contaminated site including numerical identification of heterotrophic water and soil bacteria. *Microb Ecol* **21**, 227–251.

**Kämpfer, P., Buczolits, S., Albrecht, A., Busse, H.-J. & Stackebrandt, E. (2003).** Towards a standardized format for the description of a novel species (of an established genus): *Ochrobactrum gallinifaecis* sp. nov. *Int J Syst Evol Microbiol* **53**, 893–896.

**Kämpfer, P., Rosselló-Mora, R., Scholz, H., Welinder-Olsen, C. & Busse, H.-J. (2006).** Description of *Pseudochrobactrum* gen. nov., with the two species *Pseudochrobactrum asaccharolyticum* sp. nov. and *Pseudochrobactrum saccharolyticum* sp. nov. *Int J Syst Evol Microbiol* **56**, 1823–1829.

**Kämpfer, P., Scholz, H., Huber, B., Thummes, K., Busse, H.-J., Maas, E. W. & Falsen, E. (2007a).** Description of *Pseudochrobactrum kiredjianiae* sp. nov. *Int J Syst Evol Microbiol* **57**, 755–760.

**Kämpfer, P., Scholz, H. C., Falsen, E. & Busse, H.-J. (2007b).** *Ochrobactrum haematophilum* sp. nov. and *Ochrobactrum pseudogrignonense* sp. nov., isolated from human clinical specimens. *Int J Syst Evol Microbiol* **57**, 2513–2518.

**Kreimer, S. (1992).** *Untersuchungen zum Stoffwechsel von Thiophen-2-carboxylat in Agrobacterium tumefaciens und Ochrobactrum anthropi und Reindarstellung einer CoA-Esterase*. Doctoral thesis, University Göttingen, Germany (in German).

**Kumar, S., Tamura, K., Jakobsen, I.-B. & Nei, M. (2001).** MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–1245.

**Lebuhn, M., Achouak, W., Schloter, M., Berge, O., Meier, H., Barakat, M., Hartmann, A. & Heulin, T. (2000).** Taxonomic characterization of *Ochrobactrum* sp. isolates from soil samples and wheat roots, and description of *Ochrobactrum tritici* sp. nov. and *Ochrobactrum grignonense* sp. nov. *Int J Syst Evol Microbiol* **50**, 2207–2223.

**Lechner, U., Baumbach, R., Becker, D., Kitunen, V., Auling, G. & Salkinoja-Salonen, M. (1995).** Degradation of 4-chloro-2-methylphenol by an activated sludge isolate and its taxonomic description. *Biodegradation* **6**, 83–92.

**Scholz, H. C., Tomaso, H., Al Dahouk, S., Witte, A., Schloter, M., Kämpfer, P., Falsen, E. & Neubauer, H. (2006).** Genotyping of *Ochrobactrum anthropi* by *recA*-based comparative sequence, PCR-RFLP, and 16S rRNA gene analysis. *FEMS Microbiol Lett* **257**, 7–16.

**Stolz, A., Busse, H.-J. & Kämpfer, P. (2007).** *Pseudomonas knackmussii* sp. nov. *Int J Syst Evol Microbiol* **57**, 572–576.

**Teyssier, C., Marchandin, H., Jean-Pierre, H., Masnou, A., Dusart, G. & Jumas-Bilak, E. (2007).** *Ochrobactrum pseudintermedium* sp. nov., a novel member of the family *Brucellaceae*, isolated from man. *Int J Syst Evol Microbiol* **57**, 1007–1013.

**Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

**Tindall, B. J. (1990a).** A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* **13**, 128–130.

**Tindall, B. J. (1990b).** Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199–202.

**Tripathi, A. K., Verma, S. C., Chowdhury, S. P., Lebuhn, M., Gattinger, A. & Schloter, M. (2006).** *Ochrobactrum oryzae* sp. nov., an endophytic bacterial species isolated from deep-water rice in India. *Int J Syst Evol Microbiol* **56**, 1677–1680.

**Trujillo, M. E., Willems, A., Abril, A., Planchuelo, A. M., Rivas, R., Ludeña, D., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. (2005).** Nodulation of *Lupinus* by strains of the new species *Ochrobactrum lupini* sp. nov. *Appl Environ Microbiol* **71**, 1318–1327.

**Velasco, J., Romero, C., López-Goñi, I., Leiva, J., Díaz, R. & Moriyón, I. (1998).** Evaluation of the relatedness of *Brucella* spp. and *Ochrobactrum anthropi* and description of *Ochrobactrum intermedium* sp. nov., a new species with a closer relationship to *Brucella* spp. *Int J Syst Bacteriol* **48**, 759–768.

**Yokota, A., Akagawa-Matsushita, M., Hiraishi, A., Katayama, Y., Urakami, T. & Yamasato, K. (1992).** Distribution of quinone systems in microorganisms: gram-negative Eubacteria. *Bull Jpn Fed Cult Coll* **8**, 136–171.

**Ziemke, F., Höfle, M. G., Lalucat, J. & Rosselló-Mora, R. (1998).** Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.

**Zurdo-Piñeiro, J. L., Rivas, R., Trujillo, M.E., Vizcaíno, N., Carrasco, J. A., Chamber, M., Palomares, A., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. (2007).** *Ochrobactrum cytisi* sp. nov. isolated from nodules of *Cytisus scoparius* in Spain. *Int J Syst Evol Microbiol* **57**, 784–788.

## 2.8. Description of *Pseudochrobactrum kiredjianiae* sp. nov.

*International Journal of Systematic and Evolutionary Microbiology* (2007), **57**, 755–760 DOI 10.1099/ijs.0.64714-0

# Description of *Pseudochrobactrum kiredjianiae* sp. nov.

Peter Kämpfer,[1] Holger Scholz,[2] Birgit Huber,[3] Kathrin Thummes,[1] Hans-Jürgen Busse,[3] Elizabeth W. Maas[4] and Enevold Falsen[5]

Correspondence
Peter Kämpfer
peter.kaempfer@agrar.uni-giessen.de

[1]Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Giessen, D-35392 Giessen, Germany

[2]Bundeswehr Institute of Microbiology, D-80937 Munich, Germany

[3]Institut für Bakteriologie, Mykologie und Hygiene, Veterinärmedizinische Universität, A-1210 Wien, Austria

[4]National Institute of Water & Atmospheric Research Ltd, Taihoro Nukurangi Greta Point, PO Box 14-901, Kilbirnie, Wellington, New Zealand

[5]Culture Collection University Göteborg, Department of Clinical Bacteriology, S-41346 Göteborg, Sweden

A Gram-negative, rod-shaped, oxidase-positive, non-spore-forming, non-motile bacterium (strain CCUG 49584$^T$), isolated from a seafood processing plant sample in New Zealand, was subjected to a polyphasic taxonomic study. On the basis of 16S rRNA and *recA* gene sequence similarities, the isolate was allocated to the genus *Pseudochrobactrum*. This was confirmed by fatty acid data (major fatty acids: $C_{18:1}\omega7c$ and $C_{19:0}$ cyclo $\omega8c$), a polar lipid profile exhibiting major characteristics of *Pseudochrobactrum* (phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine), quinone system Q-10 and a polyamine pattern with the predominant compounds spermidine and putrescine. DNA–DNA hybridization with the type strains of the two established species of *Pseudochrobactrum* and physiological and biochemical data clearly differentiated the isolate from established *Pseudochrobactrum* species. As a consequence, this organism represents a novel species, for which the name *Pseudochrobactrum kiredjianiae* sp. nov. is proposed, with the type strain CCUG 49584$^T$ (=CIP 109227$^T$).

The genus *Pseudochrobactrum* was proposed recently by Kämpfer *et al.* (2006), comprising the species *Pseudochrobactrum saccharolyticum* and *Pseudochrobactrum asaccharolyticum*. This genus could be clearly differentiated from *Ochrobactrum* and from *Brucella* species based on its phylogenetic position and the presence of a combination of certain phenotypic traits (Kämpfer *et al.*, 2006).

Strain CCUG 49584$^T$ was isolated in 1995 from stainless-steel vent covers from a seafood processing plant in Nelson, New Zealand, on standard plate count agar (Oxoid) at 30 °C. Subcultivation was done on tryptone soy agar (TSA) at 28 °C for 48 h. On this agar, the organism also grew at 15–45 °C, but not at 10 or 50 °C. Growth at 30 °C was also observed on MacConkey agar and R2A agar (all from Oxoid).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *recA* gene sequences of strain CCUG 49584$^T$ are AM263420 and AM263419, respectively.

Gram-staining was performed as described by Gerhardt *et al.* (1994). Cell morphology was observed under a Zeiss light microscope at ×1000, with cells grown for 3 days at 30 °C on nutrient agar. The 16S rRNA gene was analysed as described by Kämpfer *et al.* (2003). Phylogenetic analysis was performed using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 2.1 (Kumar *et al.*, 2001) after multiple alignment of data using CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura two-parameter model on the basis of 1360 bp) and clustering with the neighbour-joining method were performed by using bootstrap values based on 1000 replications (Fig. 1). The sequenced length of the 16S rRNA gene of strain CCUG 49584$^T$ was 1387 bp (GenBank accession no. AM263420). The sequence similarities with the two *Pseudochrobactrum* species were 99.4 % to *P. saccharolyticum* CCUG 33852$^T$ and 99.5 % to *P. asaccharolyticum* CCUG 46016$^T$. Nucleotide sequence similarities to all established species of the genus *Bartonella* ranged from 94.2 to 94.3 %. Sequence similarities to established species of

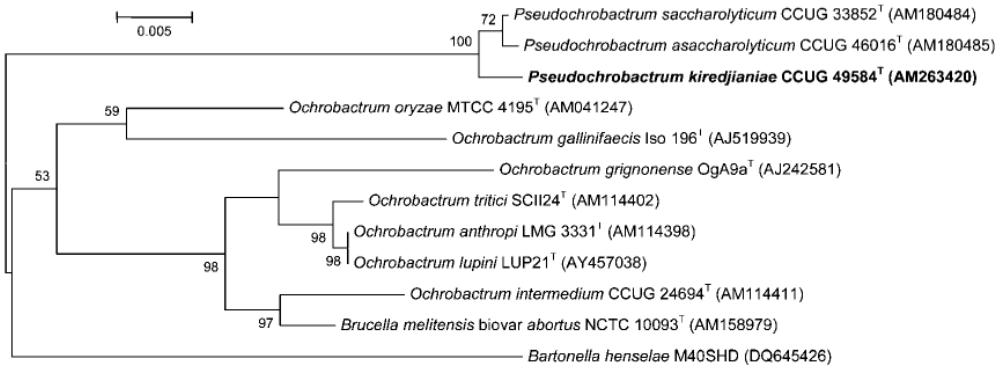


**Fig. 1.** Phylogenetic analysis based on 16S rRNA gene sequences available from GenBank/EMBL/DDBJ (accession numbers are given in parentheses) constructed after multiple alignment of data using CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining method were performed by using MEGA version 2.1 (Kumar *et al.*, 2001). Bootstrap percentages based on 1000 replications are given at branch points. Bar, 0.005 substitutions per nucleotide position.

*Brucella* and *Ochrobactrum* were 94.6–94.8 and 93.8–94.6 %, respectively. The 16S rRNA gene-based phylogenetic tree shown in Fig. 1 resulted from a neighbour-joining reconstruction using the Kimura two-parameter correction and 1000 resamplings for bootstrap analysis.

A partial *recA* sequence of strain CCUG 49584$^T$ was analysed according to Scholz *et al.* (2006). PCR was performed with primers RecA-PsOchro-f (5′-AAGGCTCTGGACGCGGC-ACT-3′) and RecA-PsOchro-r (5′-CGCAAGGTCAGTTC-AATCTCAT-3′). The similarities of *recA* between strain CCUG 49584$^T$ and the two other *Pseudochrobactrum* species were 90.5 % for *P. saccharolyticum* CCUG 33852$^T$ and 91.3 % for *P. asaccharolyticum* CCUG 46016$^T$ within 897 nt. Lower similarity values were observed with *Brucella* species (76.7 %), *Ochrobactrum* species (77.3–81.4 %), *Bartonella* (71.0 %) and *Rickettsia felis* (62.3 %) (Fig. 2). Strain CCUG 49584$^T$ showed several motifs within *recA* that are absent from *Bartonella*, *Brucella* and *Ochrobactrum* species, but are present in *Pseudochrobactrum* species (Kämpfer *et al.*, 2006).

Strain CCUG 49584$^T$ formed a separate branch in the phylogenetic tree of *recA* sequences (897 nt) (Fig. 2). The tree was constructed as described above for the 16S rRNA gene.

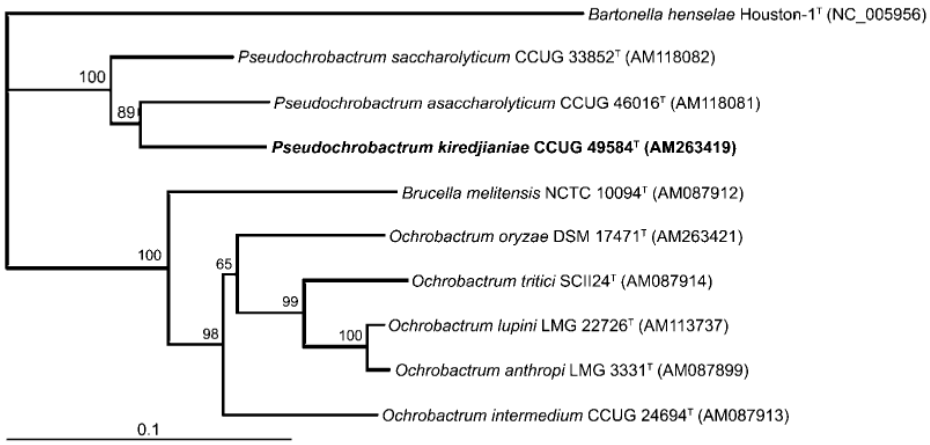


**Fig. 2.** Phylogenetic analysis based on *recA* gene sequences (909 nt) available from GenBank/EMBL/DDBJ (accession numbers are given in parentheses) constructed after multiple alignment of data using CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining method were performed by using MEGA version 2.1 (Kumar *et al.*, 2001). Bootstrap percentages based on 1000 replications are given at branch points. Bar, 0.1 substitutions per nucleotide position.

Results of the fatty acid analysis are shown in Table 1. Fatty acids were analysed according to Kämpfer & Kroppenstedt (1996). The fatty acid profile of strain CCUG 49584$^T$ mainly comprised $C_{19:0}$ cyclo $\omega 8c$ (53.0 %), $C_{18:1}\omega 7c$ (33.4 %), $C_{18:0}$ (7.7 %) and $C_{16:0}$ (2.8 %). A clear differentiation from the other *Pseudochrobactrum* species was possible based on more than 2-fold greater amounts of $C_{18:1}\omega 7c$ in the *Pseudochrobactrum* reference strains and more than 3.5-fold greater amounts of $C_{19:0}$ cyclo $\omega 8c$ in strain CCUG 49584$^T$. A quinone system with ubiquinone Q-10 predominant, which was detected after extraction and analysis as reported by Tindall (1990) and Altenburger *et al.* (1996), but using an HPLC-apparatus consisting of a JASCO PU 2080 Plus Pump and a JASCO UV-2075 Plus UV/VIS-Detector, was in agreement with the characteristics of the two established *Pseudochrobactrum* species. Polyamines were extracted and analysed by HPLC according to Busse & Auling (1988), employing JASCO PU 2080 Plus Pump and a JASCO model 821-FP spectrofluorometric detector. Similar to *P. asaccharolyticum* CCUG 46016$^T$, the polyamine pattern of strain CCUG 49584$^T$ consisted predominantly of spermidine and putrescine. Polar lipids were analysed according to Ventosa *et al.* (1993) from biomass that was grown overnight in PYE medium (0.3 % peptone from casein, 0.3 % yeast extract, pH 7.2). The polar lipid profile of strain CCUG 49584$^T$ shared the major characteristics reported recently for the two *Pseudochrobactrum* species (Kämpfer *et al.*, 2006). It consisted of major components phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine, moderate amounts of phosphatidylmonomethylethanolamine and an unknown aminolipid AL1, and small to trace amounts of three unknown polar lipids [L3, L4 and L5, which have been reported to be present in minor amounts in the two established *Pseudochrobactrum* species;

**Table 1.** Major fatty acid compositions (%) of strain CCUG 49584$^T$ and type strains of *Pseudochrobactrum* and *Ochrobactrum* species

Strains: 1, CCUG 49584$^T$ (*Pseudochrobactrum kiredjianiae* sp. nov.); 2, *P. asaccharolyticum* CCUG 46016$^T$; 3, *P. saccharolyticum* CCUG 33852$^T$; 4, *O. gallinifaecis* Iso 196$^T$; 5, *O. intermedium* LMG 3301$^T$; 6, *O. anthropi* CIP 14970$^T$; 7, *O. grignonense* DSM 13338$^T$; 8, *O. lupini* LUP21$^T$; 9, *O. tritici* LMG 18957$^T$. All strains were grown on trypticase soy broth agar at 28 °C for 48 h prior to fatty acid analysis. For unsaturated fatty acids, the position of the double bond is located by counting from the methyl ($\omega$) end of the carbon chain; *cis* and *trans* isomers are indicated by the suffixes *c* and *t*, respectively. −, Not detected.

| Fatty acid | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8* | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Saturated acids: | | | | | | | | | |
| $C_{12:0}$ | − | − | 0.7 | − | − | − | − | − | − |
| $C_{14:0}$ | − | − | − | 0.7 | − | − | − | − | − |
| $C_{16:0}$ | 2.8 | 5.5 | 1.9 | 8.9 | 3.7 | 6.6 | 2.9 | 4.2 | 3.7 |
| $C_{17:0}$ | − | − | − | − | 3.1 | 1.4 | 1.7 | 1.6 | 0.9 |
| $C_{18:0}$ | 7.7 | 11.0 | 7.4 | 3.7 | 4.1 | 8.8 | 7.2 | 3.0 | 9.6 |
| Unsaturated acids: | | | | | | | | | |
| $C_{13:1}$ at 12–13 | − | − | − | − | − | 0.6 | 0.7 | − | − |
| $C_{17:1}\omega 6c$ | − | − | − | − | 1.1 | − | 0.5 | 1.4 | − |
| $C_{18:1}\omega 7c$ | 33.4 | 74.9 | 74.6 | 28.8 | 25.8 | 45.6 | 31.6 | 70.8 | 77.9 |
| $C_{18:3}\omega 6c$ (6,9,12) | − | − | 0.5 | − | − | − | − | − | − |
| 11-Methyl-$C_{18:1}\omega 7t$ | 1.0 | − | − | 1.6 | 1.5 | 1.0 | 1.0 | − | − |
| $C_{20:1}\omega 7c$ | 0.8 | − | − | − | − | − | 0.8 | − | − |
| $C_{20:2}\omega 6,9c$ | 0.9 | − | − | 1.1 | 0.9 | 0.5 | 0.8 | − | − |
| Hydroxy acids: | | | | | | | | | |
| $C_{18:1}$ 2-OH | − | − | − | 1.5 | 1.8 | 0.6 | 0.5 | 6.2 | 1.4 |
| $C_{18:0}$ 3-OH | − | − | − | − | − | − | 0.5 | 1.0 | − |
| Summed feature 3† | − | 0.8 | − | 3.7 | 0.7 | 1.1 | 1.0 | 2.3 | 0.7 |
| Cyclopropane acids: | | | | | | | | | |
| $C_{17:0}$ cyclo | 0.5 | − | − | 2.9 | − | 0.8 | − | − | − |
| $C_{19:0}$ cyclo $\omega 8c$ | 53.0 | 7.3 | 14.3 | 47.2 | 57.4 | 32.7 | 50.2 | 4.3 | 5.9 |
| Unknown 13.957‡ | − | − | − | − | − | − | 0.2 | − | − |
| Unknown 14.959‡ | − | 0.6 | 0.9 | − | − | 0.3 | 0.7 | − | − |

*Data from Trujillo *et al.* (2005).

†Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 contains $C_{16:1}\omega 7c$ and/or $C_{15:0}$ iso 2-OH.

‡Unknown fatty acids have no name listed in the peak library file of the MIDI system and therefore cannot be identified; they are referred to by their equivalent chain length.

**Table 2.** Physiological characteristics of strain CCUG 49584$^T$ and type strains of *Pseudochrobactrum* and *Ochrobactrum* species

Strains: 1, CCUG 49584$^T$ (*Pseudochrobactrum kiredjianiae* sp. nov.), 2, *P. asaccharolyticum* CCUG 46016$^T$; 3, *P. saccharolyticum* CCUG 33852$^T$; 4, *O. gallinifaecis* Iso 196$^T$; 5, *O. intermedium* LMG 3301$^T$; 6, *O. anthropi* CIP 14970$^T$; 7, *O. grignonense* DSM 13338$^T$; 8, *O. lupini* LUP21$^T$; 9, *O. tritici* LMG 18957$^T$. +, Positive; −, negative; (+), weakly positive; ND, not determined; *p*NA, *p*-nitroanilide; *p*NP, *p*-nitrophenyl. All strains (except *O. lupini* LUP21$^T$, for which only the data given in the table are available) are positive for hydrolysis of L-alanine *p*NA and weakly positive for hydrolysis of bis-*p*NP phosphate. All strains (except *O. lupini* LUP21$^T$) are negative for hydrolysis of aesculin, *p*NP β-D-galactopyranoside, *p*NP β-D-glucuronide, *p*NP α-D-glucopyranoside, *p*NP β-D-glucopyranoside, *p*NP phosphoryl choline and 2-deoxythymidine-5′-*p*NP phosphate. All strains (except *O. lupini* LUP21$^T$) are positive for assimilation of acetate, oxoglutarate, L-alanine, L-proline and L-serine. All strains are negative for assimilation of *p*-arbutin, salicin, putrescine, L-phenylalanine, L-tryptophan, 3-hydroxybenzoate, adipate, itaconate, mesaconate, phenylacetate, α-D-melibiose and azelate. Hydrolysis of aesculin and *p*NP β-D-galactopyranoside and assimilation of oxoglutarate, L-serine, phenylacetate and α-D-melibiose were also tested using a different method by Holmes *et al.* (1998) with *O. anthropi*; results were in agreement with those of this study. Assimilation of adipate was also tested using a different method by Velasco *et al.* (1998) with *O. intermedium*; results were in agreement with those of this study.

| Characteristic* | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8† | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Hydrolysis of: | | | | | | | | | |
| *p*NP Phenylphosphonate | − | − | − | − | − | − | − | ND | (+) |
| L-Glutamate-γ-3-carboxy *p*NA | − | − | − | − | + | (+) | − | ND | (+) |
| L-Proline *p*NA[a] | + | + | + | + | (+) | + | + | ND | (+) |
| Assimilation of: | | | | | | | | | |
| Gluconate | − | − | − | + | + | + | + | + | + |
| L-Arabinose[b], D-mannose, D-xylose | − | − | + | + | + | + | + | + | + |
| D-Glucose | + | − | + | + | + | + | + | + | + |
| D-Fructose | − | − | + | − | + | + | + | − | + |
| DL-3-Hydroxybutyrate[b] | − | − | + | − | + | + | + | ND | + |
| D-Galactose[b] | (+) | − | + | + | + | + | + | − | + |
| Pyruvate[b], ornithine[b] | − | − | + | + | + | + | + | ND | + |
| D-Ribose[b], propionate[b], fumarate[b], glutarate, DL-lactate, L-malate[b] | + | − | + | + | + | + | + | ND | + |
| i-Inositol[b], D-sorbitol[b] | − | − | − | − | + | + | + | − | + |
| L-Rhamnose[b] | + | − | − | − | + | + | + | + | + |
| Citrate[a,b] | − | − | − | − | + | + | + | + | + |
| 4-Aminobutyrate, β-alanine[b] | + | − | + | (+) | + | + | + | ND | + |
| *cis*-Aconitate | − | + | + | − | + | + | + | ND | + |
| D-Maltose[a,b], adonitol[b,c] | − | − | − | − | + | + | − | + | + |
| *N*-Acetyl-D-glucosamine[b] | (+) | − | + | − | + | + | − | + | + |
| Sucrose[a,b] | − | − | − | − | − | + | − | ND | + |
| D-Trehalose | − | − | + | − | − | + | − | − | + |
| Maltitol | − | − | − | − | − | + | − | ND | − |
| D-Mannitol | − | − | − | − | − | + | + | + | + |
| *trans*-Aconitate[b] | − | − | − | − | (+) | − | − | ND | − |
| D-Cellobiose[b,c] | − | − | − | − | + | + | − | − | − |
| *N*-Acetyl-D-galactosamine | − | − | − | − | + | (+) | + | ND | + |
| Suberate[b] | − | − | − | − | − | − | − | ND | + |
| L-Aspartate[b] | (+) | − | + | + | + | + | + | ND | − |
| 4-Hydroxybenzoate | − | − | − | − | (+) | (+) | − | ND | + |
| L-Histidine[b] | − | − | + | + | + | + | + | ND | (+) |
| L-Leucine[b] | − | − | − | − | + | + | + | ND | (+) |

*Tested using a different method in another study; results were in agreement with those of this study. *a*, Velasco *et al.* (1998) with *O. intermedium*; *b*, Holmes *et al.* (1988) with *O. anthropi*; *c*, Lebuhn et al. (2000) with *O. anthropi*, *O. grignonense*, *O. tritici* and *O. intermedium*. †Data from Trujillo *et al.* (2005).

see Supplementary Fig. S2 of Kämpfer *et al.* (2006) in IJSEM Online]. In addition, trace amounts of an unknown aminolipid were detected, which was not reported to be present in the established *Pseudochrobactrum* species (Kämpfer *et al.*, 2006), and phosphatidyldimethylethanolamine, an unknown phospholipid and two unknown polar lipids, present in the reference species, were not detected. In contrast to Kämpfer *et al.* (2006), reanalysis of the polar lipid profile of *P. asaccharolyticum* CCUG 46016$^T$ from newly grown biomass, as for strain CCUG 49584$^T$, also revealed the absence of phosphatidyldimethylethanolamine, the unknown phospholipid, the two unknown polar lipids and the presence of trace amounts of the unknown aminolipid. These observations indicate that the presence/absence of phosphatidyldimethylethanolamine, the unknown phospholipid, the unknown aminolipid and the two unknown polar lipids may be related to the physiological conditions of the cells when harvested for polar lipid extraction. Based on these results, strain CCUG 49584$^T$ shares the polar lipid characteristics that differentiate *Pseudochrobactrum* species from *Ochrobactrum* and *Brucella* species (Kämpfer *et al.*, 2006).

Results of the physiological characterization are given in the species description and in Table 2. The methods used were the same as those described previously (Kämpfer *et al.*, 1991). The organism could be clearly differentiated from other *Pseudochrobactrum* and *Ochrobactrum* species on the basis of several tests.

DNA–DNA hybridization experiments were performed with strain CCUG 49584$^T$ and the type strains of the two established *Pseudochrobactrum* species using the method described by Ziemke *et al.* (1998), except that, for nick translation, 2 μg DNA was labelled during a 3 h incubation at 15 °C. Strain CCUG 49584$^T$ showed relatively low DNA–DNA relatedness to *P. asaccharolyticum* CCUG 46016$^T$ (37.8 %) and *P. saccharolyticum* CCUG 33852$^T$ (22.8 %).

From the results of 16S rRNA gene and *recA* sequencing, DNA–DNA hybridization data, fatty acid analyses and from the physiological characteristics it is evident that strain CCUG 49584$^T$ is different from the two established species of *Pseudochrobactrum*. Hence, strain CCUG 49584$^T$ represents a novel species of the genus *Pseudochrobactrum*, for which we propose the name *Pseudochrobactrum kiredjianiae*.

### Description of *Pseudochrobactrum kiredjianiae* sp. nov.

*Pseudochrobactrum kiredjianiae* (ki.red.ji.a′ni.ae. N.L. fem. gen. n. *kiredjianiae* of Kiredjian, named after Martine Kiredjian, a contemporary French microbiologist, for her numerous contributions to the taxonomy of *Ochrobactrum* and related organisms).

Shares all characteristics listed in the genus description. Good growth occurs on R2A agar, TSA, nutrient agar and MacConkey agar at 25–30 °C. Beige, translucent and shiny colonies with entire edges form within 24 h, with a diameter of approximately 2 mm. Quinone system is ubiquinone Q-10 (99 %) and Q-9 (1 %). The polar lipid profile consists of the major components phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and phosphatidylcholine, moderate amounts of phosphatidylmonomethylethanolamine and unknown aminolipid AL1 and trace amounts of three unknown lipids. Polyamine profile consists of spermidine [60.3 μmol (g dry weight)$^{-1}$], putrescine [42.7 μmol (g dry weight)$^{-1}$], 1,3-diaminopropane [2.0 μmol (g dry weight)$^{-1}$] and spermine [1.5 μmol (g dry weight)$^{-1}$]. Carbon source utilization and hydrolysis of chromogenic substrates (including characteristics that differentiate *Ochrobactrum* species) are given in Table 2.

The type strain is CCUG 49584$^T$ (=CIP 109227$^T$), which was isolated in 1995 from a stainless-steel vent cover, from a seafood processing plant in Nelson, New Zealand.

## Acknowledgements

We thank E. R. B. Moore for helpful discussions and Gundula Will and Maria Sowinsky for excellent technical assistance. The work of B. H. and H.-J. B. was supported by a Contract-Research-Project for the Bundeswehr Medical Service.

## References

**Altenburger, P., Busse, H.-J., Kämpfer, P., Lubitz, W. & Makristathis, A. (1996).** Classification of bacteria isolated from a medieval wall painting. *J Biotechnol* **47**, 39–52.

**Busse, H.-J. & Auling, G. (1988).** Polyamine pattern as a chemotaxonomic marker within the *Proteobacteria*. *Syst Appl Microbiol* **11**, 1–8.

**Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (editors) (1994).** *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.

**Holmes, B., Popoff, M., Kiredjian, M. & Kersters, K. (1988).** *Ochrobactrum anthropi* gen. nov., sp. nov. from human clinical specimens and previously known as Group Vd. *Int J Syst Bacteriol* **38**, 406–416.

**Kämpfer, P. & Kroppenstedt, R. M. (1996).** Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42**, 989–1005.

**Kämpfer, P., Steiof, M. & Dott, W. (1991).** Microbiological characterisation of a fuel-oil contaminated site including numerical identification of heterotrophic water and soil bacteria. *Microb Ecol* **21**, 227–251.

**Kämpfer, P., Dreyer, U., Neef, A., Dott, W. & Busse, H.-J. (2003).** *Chryseobacterium defluvii* sp. nov., isolated from wastewater. *Int J Syst Evol Microbiol* **53**, 93–97.

**Kämpfer, P., Rosselló-Mora, R., Scholz, H. C., Welinder-Olsson, C., Falsen, E. & Busse, H.-J. (2006).** Description of *Pseudochrobactrum* gen. nov., with the two species *Pseudochrobactrum asaccharolyticum* sp. nov. and *Pseudochrobactrum saccharolyticum* sp. nov. *Int J Syst Evol Microbiol* **56**, 1823–1829.

**Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001).** MEGA2: Molecular Evolutionary Genetics Analysis software. *Bioinformatics* **17**, 1244–1245.

**Lebuhn, M., Achouak, W., Schloter, M., Berge, O., Meier, H., Barakat, M., Hartmann, A. & Heulin, T. (2000).** Taxonomic characterization of *Ochrobactrum* sp. isolates from soil samples and wheat roots, and description of *Ochrobactrum tritici* sp. nov. and *Ochrobactrum grignonense* sp. nov. *Int J Syst Evol Microbiol* **50**, 2207–2223.

**Scholz, H. C., Tomaso, H., Al Dahouk, S., Witte, A., Schloter, M., Kämpfer, P., Falsen, E. & Neubauer, H. (2006).** Genotyping of *Ochrobacterium anthropi* by *recA*-based comparative sequence, PCR-RFLP, and 16S rRNA gene analysis. *FEMS Microbiol Lett* **257**, 7–16.

**Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

**Tindall, B. J. (1990).** A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* **13**, 128–130.

**Trujillo, M. E., Willems, A., Abril, A., Planchuelo, A.-M., Rivas, R., Ludena, D., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. (2005).** Nolulation of *Lupinus albus* by strains of *Ochrobactrum lupini* sp. nov. *Appl Environ Microbiol* **71**, 1318–1327.

**Velasco, J., Romero, C., López-Goñi, I., Leiva, J., Díaz, R. & Moriyón, I. (1998).** Evaluation of the relatedness of *Brucella* spp. and *Ochrobactrum anthropi* and description of *Ochrobactrum intermedium* sp. nov., a new species with a closer relationship to *Brucella* spp. *Int J Syst Bacteriol* **48**, 759–768.

**Ventosa, A., Marquez, M. C., Kocur, M. & Tindall, B. J. (1993).** Comparative study of "*Micrococcus* sp." strains CCM 168 and CCM 1405 and members of the genus *Salinicoccus*. *Int J Syst Bacteriol* **43**, 245–248.

**Ziemke, F., Höfle, M. G., Lalucat, J. & Rosselló-Mora, R. (1998).** Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.

## 2.9. *Pseudochrobactrum lubricantis* sp. nov., isolated from a metal-working fluid

*International Journal of Systematic and Evolutionary Microbiology* (2009), **59**, 2464–2467 DOI 10.1099/ijs.0.008540-0

# *Pseudochrobactrum lubricantis* sp. nov., isolated from a metal-working fluid

Peter Kämpfer,[1] Birgit Huber,[2] Nicole Lodders,[1] Isabel Warfolomeow,[3] Hans-Jürgen Busse[2] and Holger C. Scholz[4]

Correspondence
Peter Kämpfer
peter.kaempfer@umwelt.uni-giessen.de

[1]Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Giessen, D-35392 Giessen, Germany

[2]Institut für Bakteriologie, Mykologie und Hygiene, Veterinärmedizinische Universität, A-1210 Wien, Austria

[3]Berufsgenossenschaft Metall Nord Süd – BGM, D-55130 Mainz, Germany

[4]Bundeswehr Institute of Microbiology, D-80937 Munich, Germany

A Gram-negative, rod-shaped, oxidase-positive, non-spore-forming, non-motile bacterium (KSS 7.8$^T$) was isolated from a water-mixed metal-working fluid. On the basis of 16S rRNA gene and *recA* sequence similarities, the isolate was clearly grouped in the genus *Pseudochrobactrum*. This allocation was confirmed by fatty acid data (major fatty acids: $C_{18:2}\omega7c$ and $C_{19:0}$ cyclo $\omega8c$), polar lipid profile (major components: phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and phosphatidylcholine, plus moderate amounts of phosphatidylmonomethylethanolamine and unknown aminolipid AL1), quinone system (ubiquinone Q-10) and polyamine pattern (spermidine and putrescine predominant). DNA–DNA pairing with the most closely related *Pseudochrobactrum* species showed values ranging from 24.2 to 45.7 %, and physiological and biochemical data clearly differentiated this isolate from described *Pseudochrobactrum* species. This organism represents a novel species of the genus *Pseudochrobactrum*, for which the name *Pseudochrobactrum lubricantis* sp. nov. is proposed, with the type strain KSS 7.8$^T$ (=CCUG 56963$^T$=CCM 7581$^T$).

The genus *Pseudochrobactrum* was proposed by Kämpfer *et al.* (2006). At present, it comprises *Pseudochrobactrum saccharolyticum*, *Pseudochrobactrum asaccharolyticum*, *Pseudochrobactrum kiredjianiae* (Kämpfer *et al.*, 2006, 2007) and *Pseudochrobactum glaciei* (Romanenko *et al.*, 2008). Members of the genus can be clearly differentiated from members of the genera *Ochrobactrum* and *Brucella* on the basis of 16S rRNA gene sequence and *recA* sequence data, as well as chemotaxonomic data (Kämpfer *et al.*, 2006, 2007).

Strain KSS 7.8$^T$ was isolated from a metal-working fluid of a metal-processing company in Germany on nutrient agar at 30 °C. Subcultivation was done on tryptone soy agar at 28 °C for 48 h. On this agar, this organism grew also at 15–45 °C, but not at 10 or 50 °C. Growth at 30 °C was also observed on MacConkey agar and R2A agar (all from Oxoid).

Gram staining was performed as described by Gerhardt *et al.* (1994). Cell morphology was observed under a Zeiss light microscope at ×1000, with cells grown for 3 days at 30 °C on nutrient agar. The 16S rRNA gene was analysed as described previously (Kämpfer *et al.*, 2003). Sequence analysis and similarity calculations were carried out by using the software programs Bionumerics v. 4.0 (Applied Maths BVBA) and MEGA v. 3.1 (Kumar *et al.*, 2004). The sequenced length of the 16S rRNA gene was 1401 bp (GenBank accession no. FM209496) for strain KSS 7.8$^T$. Sequence similarities to the three most closely related *Pseudochrobactrum* species were 99.8 % to *P. saccharolyticum* CCUG 33852$^T$, 99.8 % to *P. asaccharolyticum* CCUG 46016$^T$ and 99.3 % to *P. kiredjianiae* CCUG 49584$^T$. A lower similarity of 96.2 % was found to *P. glaciei* KMM 3858$^T$. The 16S rRNA gene-based tree shown in Fig. 1 results from a neighbour-joining reconstruction using the Kimura two-parameter correction and 1000 resamplings for bootstrap analysis.

The partial *recA* sequence of strain KSS 7.8$^T$ was analysed as described previously (Scholz *et al.*, 2006). The PCR was performed with primers RecA-PsOchro-f (5′-AAGGCTC-TGGACGCGGCACT-3′) and RecA-PsOchro-r (5′-CGC-AAGGTCAGTTC AATCTCAT-3′). *recA* sequence similarity with *P. kiredjianiae* CCUG 49584$^T$ was 89.2 %; it was

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the *recA* sequence of strain KSS 7.8$^T$ are FM209496 and FM211811, respectively.

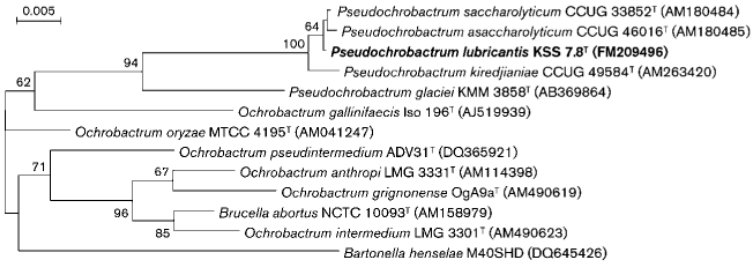


**Fig. 1.** 16S rRNA gene sequence-based phylogenetic neighbour-joining tree reconstruction, showing the affiliation of *Pseudochrobactrum lubricantis* KSS 7.8$^T$ to its closest relatives. The significance of each branch is indicated by a bootstrap value (%) calculated for 1000 subsets. *Bartonella henselae* M40SHD was used as outgroup to construct a pseudo-rooted tree. Bar, 0.005 divergent residues per site.

88.8 % with *P. saccharolyticum* CCUG 33852$^T$ and 91.7 % with *P. asaccharolyticum* CCUG 46016$^T$ within 743 nt. Lower similarity values were observed to *Brucella melitensis* NCTC 10094$^T$ (80.5 %) and *Ochrobactrum anthropi* LMG 3331$^T$ (82.2 %). Strain KSS 7.8$^T$ formed a separate branch in the phylogenetic tree of *recA* sequences (743 nt) (Fig. 2). The tree was constructed by using CLUSTREE neighbour-joining analysis (Higgins *et al.*, 1996). The *recA* gene of *P. glaciei* KMM 3858$^T$ could not be amplified with the primers described above and hence was not included in the analysis.

The results of the fatty acid analysis are shown in Table 1. Fatty acids were analysed according to Kämpfer & Kroppenstedt (1996). The fatty acid profile of strain KSS 7.8$^T$ was mainly composed of $C_{19:0}$ cyclo $\omega 8c$ (53.9 %), $C_{18:1}\omega 7c$ (29.1 %), $C_{18:0}$ (7.8 %) and $C_{16:0}$ (4.5 %) and showed a high similarity to the fatty acid profiles of all other *Pseudochrobactrum* species. Quinones and polar lipids were extracted by an integrated protocol as described previously (Tindall, 1990a, b; Altenburger *et al.*, 1996); quinone composition was analysed by HPLC as reported by Stolz *et al.* (2007). Polyamines were extracted and analysed as reported previously (Busse & Auling, 1988; Busse *et al.*, 1997; Stolz *et al.*, 2007). The quinone system of strain KSS 7.8$^T$ consisted of the major compound ubiquinone Q-10 (97 %) and minor amounts of Q-8, Q-9 and Q-11 (0.7–1.9 %). The polar lipid profile (results not shown) consisted of the major compounds phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and phosphatidylcholine, moderate amounts of phosphatidylmonomethylethanolamine and unknown aminolipid AL1, and small to trace amounts of three unknown polar lipids (L3, L4 and L5) corresponding to the lipids shown for *P. asaccharolyticum* CCUG 46016$^T$ (Kämpfer *et al.*, 2006). The polyamine pattern was as follows [in μmol (g dry weight)$^{-1}$]: spermidine, 63.8; putrescine, 43.9; 1,3-diaminopropane, 0.7; spermine, 2.3. Both polar lipid profile and polyamine pattern are quite similar to those reported for other *Pseudochrobactrum* species and are in good agreement with the characteristics of the genus description (Kämpfer *et al.*, 2006, 2007), although the unknown lipids L1 and L2 were not detectable in strain KSS 7.8$^T$.

Results of the physiological characterization are given in the species description and in Table 2. The methods used have been described previously (Kämpfer *et al.*, 1991). The organism can be clearly differentiated from the other *Pseudochrobactrum* species on the basis of several tests.

DNA–DNA hybridization experiments were performed with strain KSS 7.8$^T$ and type strains of the three most closely related *Pseudochrobactrum* species by using the method described by Ziemke *et al.* (1998), except that for nick translation, 2 μg DNA was labelled during a 3 h incubation at 15 °C. Strain KSS 7.8$^T$ showed relatively low DNA–DNA similarity to *P. asaccharolyticum* CCUG 46016$^T$ (24.8 %), *P. saccharolyticum* CCUG 33852 (45.7 %) and *P. kiredjianiae* CCUG 49584$^T$ (40.9 %).

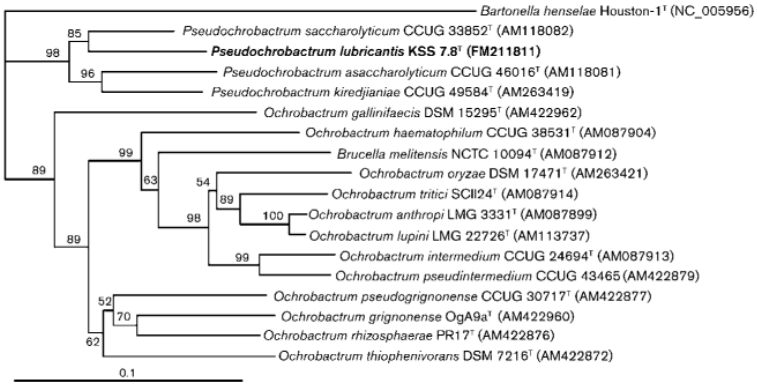


**Fig. 2.** Phylogenetic tree reconstruction with *recA* sequences (743 nt) using CLUSTREE neighbour-joining analysis (Higgins *et al.*, 1996). The significance of each branch is indicated by a bootstrap value (%) calculated for 1000 subsets. The tree was rooted by using the sequence of *Bartonella henselae* Houston-1$^T$ as an outgroup. Bar, 0.1 divergent residues per site.

**Table 1.** Major fatty acid compositions (%) of type strains of *Pseudochrobactrum* species

Taxa: 1, strain KSS 7.8$^T$; 2, *P. kiredjianiae* CCUG 49584$^T$; 3, *P. asaccharolyticum* CCUG 46016$^T$; 4, *P. saccharolyticum* CCUG 33852$^T$; 5, *P. glaciei* KMM 3858$^T$. All strains were grown on trypticase soy broth agar at 28 °C for 48 h prior to fatty acid analysis. Data were obtained in the present study except where indicated.

| Fatty acid | 1 | 2 | 3 | 4 | 5* |
|---|---|---|---|---|---|
| Saturated: | | | | | |
| $C_{12:0}$ | | | | 0.7 | |
| $C_{14:0}$ | | | | | 0.6 |
| $C_{16:0}$ | 4.5 | 2.8 | 5.5 | 1.9 | 8.9 (5.4) |
| $C_{18:0}$ | 7.8 | 7.7 | 11.0 | 7.4 | 5.5 (4.4) |
| Unsaturated†: | | | | | |
| $C_{18:1}\omega7c$ | 29.1 | 33.4 | 74.9 | 74.6 | 31.2 (41.5) |
| $C_{18:3}\omega6c$ (6,9,12) | | | | 0.5 | |
| 11-methyl-$C_{18:1}\omega7t$ | 1.8 | 1.0 | | | 1.0 |
| $C_{20:1}\omega7c$ | | 0.8 | | | |
| $C_{20:2}\omega6,9c$ | 1.4 | 0.9 | | | 0.9 |
| Hydroxy: | | | | | |
| $C_{18:0}$ 3-OH | | | | | 0.6 |
| Summed feature 3‡ | | | 0.8 | | 1.6 |
| Cyclo propane acids: | | | | | |
| $C_{17:0}$ cyclo | | 0.5 | | | 1.0 |
| $C_{19:0}$ cyclo $\omega8c$ | 53.9 | 53.0 | 7.3 | 14.3 | 47.9 (46.2) |
| Unknown (ECL 14.959)§ | 1.2 | | 0.6 | 0.9 | 0.9 |

*Data in parentheses are from Romanenko *et al.* (2008).
†The position of the double bond is located by counting from the methyl ($\omega$) end of the carbon chain.
‡Summed features are groups of fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 contains $C_{16:1}\omega7c$ and/or $C_{15:0}$ iso 2-OH.
§Unknown fatty acids have no name listed in the peak library file of the MIDI system and therefore cannot be identified. ECL, Equivalent chain length.

From the results of 16S rRNA gene and *recA* sequencing and from the physiological characteristics, it is evident that strain KSS 7.8$^T$ is different from previously described species of the genus *Pseudochrobactrum*. For this reason, *Pseudochrobactrum lubricantis* sp. nov. is proposed to accommodate strain KSS 7.8$^T$.

## Description of *Pseudochrobactrum lubricantis* sp. nov.

*Pseudochrobactrum lubricantis* [lu.bri.can′tis. L. v. *lubricare* to lubricate; N.L. n. *lubricans -antis* (from L. part. adj. *lubricans*) a lubricant; N.L. gen. n. *lubricantis* of/from a (coolant) lubricant].

Shares all characteristics listed in the genus description (Kämpfer *et al.*, 2006). In addition, good growth occurs on R2A agar, tryptone soy agar, nutrient agar and MacConkey agar at 25–30 °C. Beige, translucent and shiny colonies with entire edges form within 24 h, with a diameter of approximately 2 mm. Carbon-source utilization and hydrolysis of chromogenic substrates (including differentiating characters for all *Pseudochrobactrum* species) are indicated in Table 2. The major fatty acids are $C_{18:2}\omega7c$ and $C_{19:0}$ cyclo $\omega8c$. The polar lipid profile consists of the major compounds phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and phosphatidylcholine, moderate amounts of phosphatidylmonomethylethanolamine and unknown aminolipid AL1, and small to trace amounts of three unknown polar lipids. Ubiquinone Q-10 is the major quinone. The polyamine pattern consists of the major compounds spermidine and putrescine and minor amounts of 1,3-diaminopropane and spermine.

**Table 2.** Physiological characteristics of the type strains of *Pseudochrobactrum* species

Taxa: 1, strain KSS 7.8$^T$; 2, *P. kiredjianiae* CCUG 49584$^T$; 3, *P. asaccharolyticum* CCUG 46016$^T$; 4, *P. saccharolyticum* CCUG 33853$^T$; 5, *P. glaciei* KMM 3858$^T$. Data for strains 1 and 5 are from the present study; data for strains 2–4 are from Kämpfer *et al.* (2007), based on the same methods. +, Positive; −, negative; (+), weakly positive. All strains were positive for hydrolysis of L-alanine-*p*-nitroanilide and L-proline-*p*-nitroanilide and weak hydrolysis of bis-*p*-nitrophenylphosphate. All strains were negative for hydrolysis of aesculin, *p*-nitrophenyl-*β*-D-galactopyranoside, *p*-nitrophenyl-*β*-D-glucuronide, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-*β*-D-glucopyranoside, *p*-nitrophenylphosphorylcholine, *p*-nitrophenyl phenylphosphonate and 2-deoxythymidine-5′-*p*-nitrophenylphosphate. All strains were also positive for assimilation of acetate, oxoglutarate, L-alanine, L-proline and L-serine. All strains (*P. glaciei* KMM 3858$^T$ was not included in the tests) were negative for assimilation of adonitol, *p*-arbutin, cellobiose, D-gluconate, i-inositol, maltose, D-maltitol, D-mannitol, α-D-melibiose, D-sorbitol, salicin, trehalose, putrescine, L-phenylalanine, L-tryptophan, 3-hydroxybenzoate, *trans*-aconitate, adipate, citrate, itaconate, mesaconate, L-leucine, phenylacetate, suberate, azelate and 4-hydroxybenzoate.

| Characteristic | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Assimilation of: | | | | | |
| L-Arabinose | − | − | − | + | − |
| D-Xylose | − | − | − | + | + |
| D-Mannose | + | − | − | + | (+) |
| D-Glucose | + | + | − | + | (+) |
| D-Fructose | + | − | − | + | + |
| DL-3-Hydroxybutyrate | − | − | − | + | − |
| D-Galactose | + | (+) | − | + | + |
| Pyruvate, ornithine | + | - | − | + | − |
| D-Ribose, propionate, glutarate | + | + | − | + | − |
| Fumarate | + | + | − | + | − |
| DL-Lactate, L-malate | + | + | − | + | − |
| L-Rhamnose | − | + | − | − | − |
| 4-Aminobutyrate, *β*-alanine | + | + | − | + | − |
| *cis*-Aconitate | (+) | − | + | + | − |
| *N*-Acetyl-D-glucosamine | + | (+) | − | + | + |
| L-Aspartate | + | (+) | − | + | − |
| L-Histidine | + | − | − | + | + |

The type strain is KSS 7.8$^T$ (=CCUG 56963$^T$=CCM 7581$^T$), isolated from a water-mixed metal-working fluid.

## Acknowledgements

The study was supported by a grant to P. K. by the Berufsgenossenschaft Metall Nord Süd – BGM (formerly Berufsgenossenschaft Metall Süd). The work of B. H. and H.-J. B. was supported by a Contract Research Project for the Bundeswehr Medical Service. We thank Dr L. A. Romanenko for kindly providing the type strain of *Pseudochrobactrum glaciei*.

## References

**Altenburger, P., Kämpfer, P., Makristathis, A., Lubitz, W. & Busse, H.-J. (1996).** Classification of bacteria isolated from a medieval wall painting. *J Biotechnol* **47**, 39–52.

**Busse, H.-J. & Auling, G. (1988).** Polyamine pattern as a chemotaxonomic marker within the *Proteobacteria*. *Syst Appl Microbiol* **11**, 1–8.

**Busse, H.-J., Bunka, S., Hensel, A. & Lubitz, W. (1997).** Discrimination of members of the family *Pasteurellaceae* based on polyamine patterns. *Int J Syst Bacteriol* **47**, 698–708.

**Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (editors) (1994).** *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.

**Higgins, D. G., Thompson, J. D. & Gibson, T. J. (1996).** Using CLUSTAL for multiple sequence alignments. *Methods Enzymol* **266**, 383–402.

**Kämpfer, P. & Kroppenstedt, R. M. (1996).** Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42**, 989–1005.

**Kämpfer, P., Steiof, M. & Dott, W. (1991).** Microbiological characterization of a fuel-oil contaminated site including numerical identification of heterotrophic water and soil bacteria. *Microb Ecol* **21**, 227–251.

**Kämpfer, P., Dreyer, U., Neef, A., Dott, W. & Busse, H.-J. (2003).** *Chryseobacterium defluvii* sp. nov., isolated from wastewater. *Int J Syst Evol Microbiol* **53**, 93–97.

**Kämpfer, P., Rosselló-Mora, R., Scholz, H., Welinder-Olsen, C. & Busse, H.-J. (2006).** Description of *Pseudochrobactrum* gen. nov., with the two species *Pseudochrobactrum asaccharolyticum* sp. nov. and *Pseudochrobactrum saccharolyticum* sp. nov. *Int J Syst Evol Microbiol* **56**, 1823–1829.

**Kämpfer, P., Scholz, H., Huber, B., Thummes, K., Busse, H.-J., Maas, E. W. & Falsen, E. (2007).** Description of *Pseudochrobactrum kiredjianiae* sp. nov. *Int J Syst Evol Microbiol* **57**, 755–760.

**Kumar, S., Tamura, K. & Nei, M. (2004).** MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.

**Romanenko, L. A., Tanaka, N., Frolova, G. M. & Mikhailov, V. V. (2008).** *Pseudochrobactrum glaciei* sp. nov., a novel sea ice isolate from Peter the Great Bay of the Sea of Japan. *Int J Syst Evol Microbiol* **58**, 2454–2458.

**Scholz, H. C., Tomaso, H., Al Dahouk, S., Witte, A., Schloter, M., Kämpfer, P., Falsen, E. & Neubauer, H. (2006).** Genotyping of *Ochrobacterium anthropi* by *recA*-based comparative sequence, PCR-RFLP, and 16S rRNA gene analysis. *FEMS Microbiol Lett* **257**, 7–16.

**Stolz, A., Busse, H.-J. & Kämpfer, P. (2007).** *Pseudomonas knackmussii* sp. nov. *Int J Syst Evol Microbiol* **57**, 572–576.

**Tindall, B. J. (1990a).** Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199–202.

**Tindall, B. J. (1990b).** A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* **13**, 128–130.

**Ziemke, F., Höfle, M. G., Lalucat, J. & Rosselló-Mora, R. (1998).** Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.

## 2.10. *Paenochrobactrum gallinarii* gen. nov., sp. nov., isolated from air of a duck barn, and reclassification of *Pseudochrobactrum glaciei* as *Paenochrobactrum glaciei* comb. nov.

*International Journal of Systematic and Evolutionary Microbiology* (2010), 60, 1493–1498 DOI 10.1099/ijs.0.015842-0

# *Paenochrobactrum gallinarii* gen. nov., sp. nov., isolated from air of a duck barn, and reclassification of *Pseudochrobactrum glaciei* as *Paenochrobactrum glaciei* comb. nov.

Peter Kämpfer,[1] Elena Martin,[2] Nicole Lodders,[1] Udo Jäckel,[2] Birgit E. Huber,[3] Peter Schumann,[4] Stefan Langer,[3] Hans-Jürgen Busse[3] and Holger Scholz[5]

Correspondence
Peter Kämpfer
peter.kaempfer@umwelt.uni-giessen.de

[1]Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Giessen, D-35392 Giessen, Germany

[2]Bundesanstalt für Arbeitschutz und Arbeitsmedizin, D-10317 Berlin, Germany

[3]Institut für Bakteriologie, Mykologie und Hygiene, Veterinärmedizinische Universität, A-1210 Wien, Austria

[4]DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, D-38124 Braunschweig, Germany

[5]Bundeswehr Institute of Microbiology, D-80937 Munich, Germany

A Gram-negative, rod-shaped, oxidase-positive, non-spore-forming, non-motile bacterium (Sa25$^T$) was isolated from air of a duck barn. 16S rRNA gene and *recA* sequence analyses clearly placed the isolate in the vicinity of the *Brucella–Ochrobactrum–Pseudochrobactrum* group, with the closest relative being *Pseudochrobactrum glaciei* KMM 3858$^T$. This allocation was confirmed by analyses of the quinone system (ubiquinone Q-10), fatty acid data (major fatty acids $C_{18:1}\omega7c$ and $C_{19:0}$ cyclo $\omega8c$) and polar lipid profile (major components diphosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine and unknown aminolipid AL1; moderate amounts of three unknown polar lipids, L1–L3, an unknown aminolipid and an unknown aminophospholipid APL2). The polyamine pattern of Sa25$^T$ exhibited the major compound putrescine and moderate amounts of spermidine; a similar polyamine pattern with the major compound putrescine was also detected in *Pseudochrobactrum glaciei* KMM 3858$^T$. DNA–DNA hybridization of strain Sa25$^T$ with *Pseudochrobactrum glaciei* KMM 3858$^T$ and the type strains of the other *Pseudochrobactrum* species showed values ranging from 50.3 to 24.8 %, and physiological and biochemical data clearly differentiated this isolate from the described *Pseudochrobactrum* species. Since Sa25$^T$ and *Pseudochrobactrum glaciei* KMM 3858$^T$ form a distinct lineage in the 16S rRNA gene sequence-based phylogenetic tree, and this separate position is supported by unique characteristics of their polyamine patterns and polar lipid profiles, we propose the novel genus *Paenochrobactrum* gen. nov., with the type species *Paenochrobactrum gallinarii* sp. nov. (type strain Sa25$^T$ =CCUG 57736$^T$ =CCM 7656$^T$) and the reclassification of *Pseudochrobactrum glaciei* as *Paenochrobactrum glaciei* comb. nov. (type strain Pi26$^T$ =KMM 3858$^T$ =NRIC 0733$^T$ =JCM 15115$^T$).

The genus *Pseudochrobactrum* was proposed by Kämpfer *et al.* (2006). At present, it comprises the five species *Pseudochrobactrum saccharolyticum*, *Pseudochrobactrum asaccharolyticum*, *Pseudochrobactrum kiredjianiae*, *Pseudochrobactrum lubricantis* (Kämpfer *et al.*, 2006, 2007a, 2009) and *Pseudochrobactrum glaciei* (Romanenko *et al.*, 2008). The genus can be differentiated clearly from *Ochrobactrum* and *Brucella* on the basis of 16S rRNA gene and *recA*

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and partial *recA* sequence of strain Sa25$^T$ are FN391023 and FM995366, respectively.

A *recA* sequence-based neighbour-joining tree, the total lipid profile of strain Sa25$^T$ and a comparison of its fatty acid profile with those of related type strains are available as supplementary material with the online version of this paper.

sequence data, as well as chemotaxonomic properties (Kämpfer *et al.*, 2006, 2007a).

Strain Sa25$^T$ was isolated from air collected in a duck barn in Germany on tryptone soy agar (TSA) at 25 °C. The investigated barn accommodated 2500 ducks (each 14 months old). Bioaerosol samples were collected on gelatin filters by sampling 500 l air at a rate of 30 l min$^{-1}$. Subsequently, filters were dissolved and decimally diluted in isotonic NaCl (up to $10^{-8}$). For cultivation, TSA plates were inoculated in triplicate with 0.1 ml of each dilution. Strain Sa25$^T$ was isolated from a plate inoculated with fluid from the first decimal dilution. The concentration of Sa25$^T$ was approximately $2 \times 10^3$ c.f.u. per m$^3$ air.

Subcultivation was done on TSA at 28 °C for 48 h. On this agar, this organism grew at 15–45 °C, but not at 10 or 50 °C. Growth at 30 °C was also observed on MacConkey agar and R2A agar (all from Oxoid).

Gram-staining was performed as described by Gerhardt *et al.* (1994). Cell morphology was observed under a Zeiss light microscope at ×1000, with cells grown for 3 days at 30 °C on nutrient agar. Isolation of DNA for estimation of the G+C content was done according to Cashion *et al.* (1977) and the G+C content of the DNA was estimated according to Mesbah *et al.* (1989), resulting in a value of 49.7 mol%. The 16S rRNA gene was analysed as described by Kämpfer *et al.* (2003). DNA extraction was carried out using the GenElute plant genomic DNA kit (Sigma-Aldrich) according to the manufacturer's instructions. The 16S rRNA gene was amplified by PCR using the primers 27F (5′-GAGTTTGATCMTGGCTCAG) and 1492R (5′-ACGGYTACCTTGTTACGACTT) (Lane, 1991) and the following cycling conditions: 95 °C for 3 min, 28 cycles of 94 °C for 1 min, 57.3 °C for 45 s and 72 °C for 2 min and a final elongation step at 72 °C for 15 min. The PCR product was purified with the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions and sequenced with standard sequencing primers for the 16S rRNA gene. Phylogenetic analysis was performed using the ARB software package (December 2007 version; Ludwig *et al.*, 2004) and the corresponding SILVA SSURef 95 database (July 2008 version; Pruesse *et al.*, 2007). Trees were reconstructed using the maximum-likelihood method with fastDNAml (Olsen *et al.*, 1994) and a 30 % conservation filter (only alignment columns in which the frequency of the most abundant nucleotide is equal to or more than 30 % are included in the calculation). Tree topology was further tested with a 50 % filter and without filters. No significant differences could be detected between these trees regarding the grouping of strain Sa25$^T$. Trees reconstructed with the neighbour-joining and maximum-parsimony methods using the software package MEGA version 4.0 (Tamura *et al.*, 2007) showed similar results (not shown). The almost-complete 16S rRNA gene sequence (1345 bp) of strain Sa25$^T$ was compared by distance calculations (distance options according to the Kimura-2 model) using the software package MEGA version 4.0 (Tamura *et al.*, 2007). A high similarity of 99.5 % was found to *Pseudochrobactrum glaciei* KMM 3858$^T$. The sequence similarity to the type strains of three other *Pseudochrobactrum* species was 96.6 % (*Pseudochrobactrum saccharolyticum* CCUG 33852$^T$), 96.5 % (*Pseudochrobactrum asaccharolyticum* CCUG 46016$^T$) and 96.7 % (*Pseudochrobactrum kiredjianiae* CCUG 49584$^T$). A maximum-likelihood tree based on 16S rRNA gene sequences is shown in Fig. 1.

The partial *recA* sequence of strain Sa25$^T$ was amplified with degenerate primers *recA*-wob-f and *recA*-wob-r as described by Scholz *et al.* (2006), resulting in a 768 bp *recA* fragment that was compared to available *recA* sequences in the EMBL nucleotide database. The highest sequence similarity (92.7 %) within 678 bp was found with *Pseudochrobactrum glaciei* KMM 3858$^T$, supporting the results of the 16S rRNA gene sequence analysis. A neighbour-joining tree based on partial *recA* gene sequences is shown in Supplementary Fig. S1 (available in IJSEM Online). Markedly lower partial *recA* gene sequence similarities were detected in comparison with other *Pseudochrobactrum* species. The similarity in the *recA* sequence (within 678 nt) to *Pseudochrobactrum kiredjianiae* CCUG 49584$^T$, *Pseudochrobactrum saccharolyticum* CCUG 33852$^T$ and *Pseudochrobactrum asaccharolyticum* CCUG 46016$^T$ was 85.3, 84.2 and 83.9 %, respectively.

Together with *Pseudochrobactrum glaciei* KMM 3858$^T$, strain Sa25$^T$ formed a separate cluster in the phylogenetic tree of *recA* sequences apart from the other recognized *Pseudochrobactrum* species. Hence, both the 16S rRNA and *recA* gene sequence-based phylogenetic reconstructions support the separate position of *Pseudochrobactrum glaciei* KMM 3858$^T$ and strain Sa25$^T$ in comparison to the other recognized *Pseudochrobactrum* species.

For polyamine, polar lipid and quinone analyses, cells were grown on PYE medium (0.3 % peptone from casein, 0.3 % yeast extract, pH 7.2). Quinones and polar lipids were extracted by an integrated protocol as described previously (Tindall 1990a, b; Altenburger *et al.*, 1996); quinone composition was analysed by HPLC as reported by Stolz *et al.* (2007). Polyamines were extracted from biomass harvested at the late exponential growth phase and analysed as reported previously (Busse & Auling, 1988; Busse *et al.*, 1997; Stolz *et al.*, 2007). The quinone system of strain Sa25$^T$ consisted of the major compound ubiquinone Q-10 (98 %) and minor amounts of Q-8, Q-9 and Q-11 (0.2–1.2 %). The polar lipid profile of strain Sa25$^T$ consisted of the major compounds phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine, phosphatidylmonomethylethanolamine and unknown aminolipid AL1, moderate amounts of three unknown polar lipids, L1, L2 and L3, unknown aminolipid AL2 and an unknown aminophospholipid APL2 (Supplementary Fig. S2). In addition, an aminolipid that stretched over a long range in the first chromatographic dimension ('stretched aminolipid') was detected, which has not been reported to be present in the profile of

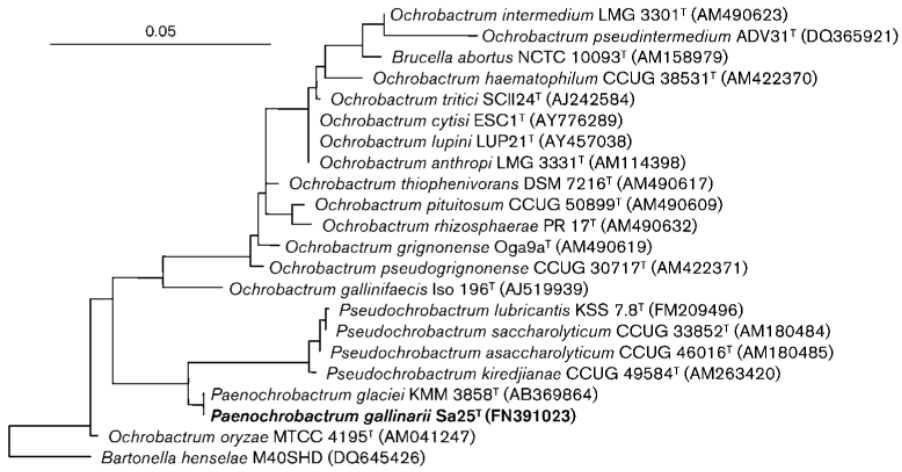


**Fig. 1.** Phylogenetic analysis based on 16S rRNA gene sequences available from the EMBL database (accession numbers in parentheses). The phylogenetic tree was constructed using the ARB software package (December 2007 version; Ludwig *et al.*, 2004) and the corresponding SILVA SSURef 95 database (July 2008 version; Pruesse *et al.*, 2007). Tree building was performed using the maximum-likelihood method with fastDNAml (Olsen *et al.*, 1994) and 30 % conservation filter. Bar, 0.05 substitutions per nucleotide position.

any established *Brucella*, *Ochrobactrum* or *Pseudochrobactrum* species. Minor amounts of other lipids were also detected. The polar lipid profile of *Pseudochrobactrum glaciei* KMM 3858$^T$ shared the major characteristics, including the presence of the stretched aminolipid, but the unknown lipids AL2 and APL2 were not detectable. Furthermore, in the polar lipid profile of *Pseudochrobactrum glaciei* KMM 3858$^T$, another unidentified aminolipid was detected, which exhibited the same $R_f$ value (i.e. the distance travelled by the analyte divided by the distance travelled by the solvent front) in the second dimension as AL1 but showed a slight smaller $R_f$ in the first dimension (not shown). However, these data demonstrate that the polar lipid profile distinguishes Sa25$^T$ and *Pseudochrobactrum glaciei* KMM 3858$^T$ from each other and both strains from all *Brucella*, *Ochrobactrum* and *Pseudochrobactrum* species examined so far. The polyamine pattern of Sa25$^T$ consisted of the major compounds putrescine [99.7 μmol (g dry weight)$^{-1}$] and spermidine [12.1 μmol (g dry weight)$^{-1}$] and minor amounts of spermine [1.8 μmol (g dry weight)$^{-1}$]. *Pseudochrobactrum glaciei* KMM 3858$^T$ exhibited a similar polyamine pattern, with the major compounds putrescine [56.7 μmol (g dry weight)$^{-1}$] and spermidine [33.9 μmol (g dry weight)$^{-1}$] and minor amounts of spermine [0.4 μmol (g dry weight)$^{-1}$]. This polyamine pattern distinguishes the two strains from *Pseudochrobactrum*, *Ochrobactrum* and *Brucella* species, which have been reported to contain high putrescine concentrations, but the major compound is spermidine (*Pseudochrobactrum*) or *sym*-homospermidine is present (*Ochrobactrum* and *Brucella*) (Kämpfer *et al.*, 2006, 2007a, b, 2008; Scholz *et al.*, 2008). Table 1 summarizes the major chemotaxonomic features in comparison with those of the genera *Pseudochrobactrum*, *Ochrobactrum* and *Brucella*.

The results of fatty acid analysis are shown in Supplementary Table S1. Fatty acids were analysed according to Kämpfer & Kroppenstedt (1996). The fatty acid profile of strain Sa25$^T$ was composed mainly of $C_{19:0}$ cyclo $\omega 8c$ (45.2 %), $C_{18:1}\omega 7c$ (29.5 %), $C_{18:0}$ (4.0 %) and $C_{16:0}$ (10.6 %) and showed high similarity to the fatty acid profiles of the type strains of *Pseudochrobactrum kiredjianiae* and *Pseudochrobactrum glaciei*. There were no clear similarities to the fatty acid profiles of the type strains of

**Table 1.** Characteristics that differentiate the genera *Paenochrobactrum* gen. nov., *Ochrobactrum*, *Pseudochrobactrum* and *Brucella*

Genera: 1, *Paenochrobactrum* gen. nov.; 2, *Ochrobactrum* (data from Huber *et al.*, 2010; Kämpfer *et al.*, 2007b, 2008; B. Huber and H.-J. Busse, unpublished results); 3, *Pseudochrobactrum* (Kämpfer *et al.*, 2006, 2007a, 2009); 4, *Brucella* (Scholz *et al.*, 2008, 2010).

| Characteristic | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| **Polyamine pattern** | | | | |
| *sym*-Homospermidine present | − | + | − | + |
| Major compound* | P | S or S and P | S | S |
| **Polar lipid profile** | | | | |
| Stretched aminolipid present | + | − | − | − |

*P, Putrescine; S, spermidine.

*Pseudochrobactrum asaccharolyticum* or *Pseudochrobactrum saccharolyticum*.

Results of the physiological characterization are given in the species description and in Table 2. Methods used were described previously (Kämpfer *et al.*, 1991). The organism can be differentiated clearly from *Pseudochrobactrum glaciei* and the other *Pseudochrobactrum* species on the basis of several tests.

DNA–DNA hybridization experiments were performed with Sa25$^T$ and *Pseudochrobactrum glaciei* KMM 3858$^T$ and also with the type strains of three other *Pseudochrobactrum* species using the method described by Ziemke *et al.* (1998) except that, for nick translation, 2 µg DNA was labelled during a 3 h incubation at 15 °C. Strain Sa25$^T$ showed moderate DNA–DNA relatedness to *Pseudochrobactrum glaciei* KMM 3858$^T$ (50.3 %) and lower DNA–DNA relatedness to *Pseudochrobactrum asaccharolyticum* CCUG 46016$^T$ (24.8 %), *Pseudochrobactrum saccharolyticum* CCUG 33852$^T$ (45.7 %) and *Pseudochrobactrum kiredjianiae* CCUG 49584$^T$ (40.9 %). Values given are means of two independent analyses.

**Table 2.** Physiological characteristics of strain Sa25$^T$, *Paenochrobactrum glaciei* comb. nov. KMM 3858$^T$ and type strains of *Pseudochrobactrum* species

Strains: 1, Sa25$^T$; 2, *Paenochrobactrum glaciei* comb. nov. KMM 3858$^T$; 3, *Pseudochrobactrum kiredjianiae* CCUG 49584$^T$; 4, *Pseudochrobactrum asaccharolyticum* CCUG 46016$^T$; 5, *Pseudochrobactrum saccharolyticum* CCUG 33853$^T$. Data were obtained in this study. +, Positive; −, negative; (+), weakly positive. All strains were positive for hydrolysis of L-alanine *p*-nitroanilide (pNA) and L-proline pNA and weak hydrolysis of bis-*p*-nitrophenyl (pNP) phosphate. All strains were negative for hydrolysis of aesculin, pNP *β*-D-galactopyranoside, pNP *β*-D-glucuronide, pNP α-D-glucopyranoside, pNP *β*-D-glucopyranoside, pNP phosphorylcholine, pNP phenylphosphonate and 2-deoxythymidine-5′-pNP phosphate. All strains were also positive for assimilation of acetate and L-alanine. All strains were negative for assimilation of adonitol, *p*-arbutin, cellobiose, D-gluconate, *myo*-inositol, maltose, maltitol, D-mannitol, α-melibiose, D-sorbitol, salicin, trehalose, putrescine, L-phenylalanine, L-tryptophan, 3-hydroxybenzoate, *trans*-aconitate, adipate, citrate, itaconate, mesaconate, L-leucine, phenylacetate, suberate, azelate and 4-hydroxybenzoate.

| Assimilation of: | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| L-Arabinose | − | − | − | − | + |
| D-Xylose | − | + | − | − | + |
| D-Mannose | − | (+) | − | − | + |
| D-Glucose | + | (+) | + | − | + |
| D-Fructose | − | + | − | − | + |
| DL-3-Hydroxybutyrate | − | − | − | − | + |
| D-Galactose | − | + | (+) | − | + |
| Pyruvate, ornithine | − | − | − | − | + |
| Propionate | + | − | + | − | + |
| D-Ribose, glutarate | − | − | + | − | + |
| Fumarate | − | − | + | − | + |
| DL-Lactate | + | − | + | − | + |
| L-Malate | − | − | + | − | + |
| L-Rhamnose | − | − | + | − | − |
| 4-Aminobutyrate, *β*-alanine | + | − | + | − | + |
| *cis*-Aconitate | − | − | − | + | + |
| *N*-Acetyl-D-glucosamine | − | + | (+) | − | + |
| L-Aspartate | − | − | (+) | − | + |
| L-Histidine | − | + | − | − | + |

From the results of 16S rRNA gene and *recA* sequencing and from the physiological characteristics, it is evident that strain Sa25$^T$ is different from *Pseudochrobactrum asaccharolyticum*, *Pseudochrobactrum saccharolyticum* and *Pseudochrobactrum kiredjianiae*. Furthermore, strain Sa25$^T$ shares with *Pseudochrobactrum glaciei* KMM 3858$^T$ a polyamine pattern and a polar lipid profile containing a stretched aminolipid that are unique among the *Brucellaceae*. For this reason, we propose the novel genus *Paenochrobactrum* gen. nov., with the type species *Paenochrobactrum gallinarii* sp. nov., and the reclassification of *Pseudochrobactrum glaciei* as a second species of this genus, *Paenochrobactrum glaciei* comb. nov.

## Description of *Paenochrobactrum* gen. nov.

*Paenochrobactrum* (Pae′no.chro.bac′trum. L. adv. *paene* nearly, almost; N.L. neut. n. *Ochrobactrum* a bacterial genus name; N.L. masc. n. *Paenochrobactrum* almost *Ochrobactrum*).

Cells are non-motile, non-spore-forming rods (approx. 2 µm long). Gram-negative and oxidase-positive, showing an oxidative metabolism. The polyamine pattern exhibits the major compound putrescine and moderate to large amounts of spermidine, while *sym*-homospermidine is absent. The polar lipids consist of the major compounds phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine, phosphatidylmonomethylethanolamine and unknown aminolipid AL1 and moderate or minor amounts of unknown polar lipids L1 and L2. In addition, an aminolipid that stretches over a long range in the first chromatographic dimension (stretched aminolipid) is detectable (Supplementary Fig. S2). The quinone system is ubiquinone Q-10. Major fatty acids are $C_{18:1}\omega7c$ and $C_{19:0}$ cyclo $\omega8c$. On the basis of 16S rRNA gene sequence analysis, most closely related to the genera *Pseudochrobactrum*, *Ochrobactrum* and *Brucella*. Member of the family *Brucellaceae*. The type species is *Paenochrobactrum gallinarii*.

## Description of *Paenochrobactrum gallinarii* sp. nov.

*Paenochrobactrum gallinarii* (gal.li.na′ri.i. L. n. *gallinarium* a coop; L. gen. n. *gallinarii* of a coop, referring to the isolation of the type strain from a duck barn).

Shares all characteristics listed in the genus description. Good growth occurs on R2A agar, TSA, nutrient agar and MacConkey agar at 25–30 °C. Beige, translucent and shiny colonies with entire edges form within 24 h, with a diameter of approximately 2 mm. Carbon source utilization and hydrolysis of chromogenic substrates (including differentiating characters for all *Paenochrobactrum* and *Pseudochrobactrum* species) are indicated in Table 2. In addition to the lipids listed in the genus description, moderate amounts of the polar lipid L3, unknown aminolipid AL2 and unknown aminophospholipid APL2 are present, and other lipids are present in minor to trace amounts. Quinone system and polyamine pattern are as listed in the genus description. In addition, the polyamine pattern contains small amounts of spermine. The G+C content of the DNA of the type strain is 49.7 mol%.

The type strain is Sa25$^T$ (=CCUG 57736$^T$ =CCM 7656$^T$), isolated from a duck barn in Germany.

### Description of *Paenochrobactrum glaciei* comb. nov.

Basonym: *Pseudochrobactrum glaciei* Romanenko *et al.* 2008.

The description of the species is that given in the description of *Pseudochrobactrum glaciei* provided by Romanenko *et al.* (2008) with the following additions. The polar lipid profile exhibits the characteristics listed in the genus description. Furthermore, the polar lipid profile contains an unidentified aminolipid, which exhibits the same $R_f$ as AL1 in the second dimension but a slightly lower $R_f$ in the first dimension. The quinone system and polyamine pattern are as listed in the genus description. In addition, the polyamine pattern contains small amounts of 1,3-diaminopropane and spermine.

The type strain is Pi26$^T$ (=KMM 3858$^T$ =NRIC 0733$^T$ =JCM 15115$^T$).

## Acknowledgements

We thank Dr I. Romanenko for kindly providing the type strain of *Pseudochrobactrum glaciei*, KMM 3858$^T$.

## References

**Altenburger, P., Kämpfer, P., Makristathis, A., Lubitz, W. & Busse, H.-J. (1996).** Classification of bacteria isolated from a medieval wall painting. *J Biotechnol* **47**, 39–52.

**Busse, H.-J. & Auling, G. (1988).** Polyamine pattern as a chemotaxonomic marker within the *Proteobacteria*. *Syst Appl Microbiol* **11**, 1–8.

**Busse, H.-J., Bunka, S., Hensel, A. & Lubitz, W. (1997).** Discrimination of members of the family *Pasteurellaceae* based on polyamine patterns. *Int J Syst Bacteriol* **47**, 698–708.

**Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977).** A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.

**Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (editors) (1994).** *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.

**Huber, B., Scholz, H. C., Kämpfer, P., Falsen, E., Langer, S. & Busse, H.-J. (2010).** *Ochrobactrum pituitosum* sp. nov., isolated from an industrial environment. *Int J Syst Evol Microbiol* **60**, 321–326.

**Kämpfer, P. & Kroppenstedt, R. M. (1996).** Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42**, 989–1005.

**Kämpfer, P., Steiof, M. & Dott, W. (1991).** Microbiological characterization of a fuel-oil contaminated site including numerical identification of heterotrophic water and soil bacteria. *Microb Ecol* **21**, 227–251.

**Kämpfer, P., Dreyer, U., Neef, A., Dott, W. & Busse, H.-J. (2003).** *Chryseobacterium defluvii* sp. nov., isolated from wastewater. *Int J Syst Evol Microbiol* **53**, 93–97.

**Kämpfer, P., Rosselló-Mora, R., Scholz, H., Welinder-Olsen, C. & Busse, H.-J. (2006).** Description of *Pseudochrobactrum* gen. nov., with the two species *Pseudochrobactrum asaccharolyticum* sp. nov. and *Pseudochrobactrum saccharolyticum* sp. nov. *Int J Syst Evol Microbiol* **56**, 1823–1829.

**Kämpfer, P., Scholz, H., Huber, B., Thummes, K., Busse, H.-J., Maas, E. W. & Falsen, E. (2007a).** Description of *Pseudochrobactrum kiredjianiae* sp. nov. *Int J Syst Evol Microbiol* **57**, 755–760.

**Kämpfer, P., Scholz, H. C., Falsen, E. & Busse, H.-J. (2007b).** *Ochrobactrum haematophilum* sp. nov. and *Ochrobactrum pseudogrignonense* sp. nov., isolated from human clinical specimens. *Int J Syst Evol Microbiol* **57**, 2513–2518.

**Kämpfer, P., Sessitsch, A., Schloter, M., Huber, B., Busse, H.-J. & Scholz, H. C. (2008).** *Ochrobactrum rhizosphaerae* sp. nov. and *Ochrobactrum thiophenivorans* sp. nov., isolated from the environment. *Int J Syst Evol Microbiol* **58**, 1426–1431.

**Kämpfer, P., Huber, B., Lodders, N., Warfolomeow, I., Busse, H.-J. & Scholz, H. C. (2009).** *Pseudochrobactrum lubricantis* sp. nov., isolated from a metal-working fluid. *Int J Syst Evol Microbiol* **59**, 2464–2467.

**Lane, D. J. (1991).** 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.

**Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S. & other authors (2004).** ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.

**Mesbah, M., Premachandran, U. & Whitman, W. B. (1989).** Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

**Olsen, G. J., Matsuda, H., Hagström, R. & Overbeek, R. (1994).** fastDNAml: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Comput Appl Biosci* **10**, 41–48.

**Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J. & Glöckner, F. O. (2007).** SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35**, 7188–7196.

**Romanenko, L. A., Tanaka, N., Frolova, G. M. & Mikhailov, V. V. (2008).** *Pseudochrobactrum glaciei* sp. nov., isolated from sea ice collected from Peter the Great Bay of the Sea of Japan. *Int J Syst Evol Microbiol* **58**, 2454–2458.

**Scholz, H. C., Tomaso, H., Al Dahouk, S., Witte, A., Schloter, M., Kämpfer, P., Falsen, E. & Neubauer, H. (2006).** Genotyping of *Ochrobactrum anthropi* by *recA*-based comparative sequence,

PCR-RFLP, and 16S rRNA gene analysis. *FEMS Microbiol Lett* **257**, 7–16.

**Scholz, H. C., Hubalek, Z., Sedláček, I., Vergnaud, G., Tomaso, H., Al Dahouk, S., Melzer, F., Kämpfer, P., Neubauer, H. & other authors (2008).** *Brucella microti* sp. nov., isolated from the common vole *Microtus arvalis*. *Int J Syst Evol Microbiol* **58**, 375–382.

**Scholz, H. C., Nöckler, K., Göllner, C., Bahn, P., Vergnaud, G., Tomaso, H., Al Dahouk, S., Kämpfer, P., Cloeckaert, A. & other authors (2010).** *Brucella inopinata* sp. nov., isolated from a breast implant infection. *Int J Syst Evol Microbiol* **60**, 801–808.

**Stolz, A., Busse, H.-J. & Kämpfer, P. (2007).** *Pseudomonas knackmussii* sp. nov. *Int J Syst Evol Microbiol* **57**, 572–576.

**Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007).** MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

**Tindall, B. J. (1990a).** Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199–202.

**Tindall, B. J. (1990b).** A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* **13**, 128–130.

**Ziemke, F., Höfle, M. G., Lalucat, J. & Rosselló-Mora, R. (1998).** Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.

## 2.11. Use of *groEL* and *gyrB* sequences for classification of *Brucella* and *Brucella*-like strains

### 2.11.1. Introduction

The alphaproteobacterial family *Brucellaceae* (Breed et al., 1957) includes the genera *Brucella* (Meyer and Shaw, 1920), *Mycoplana* (Gray and Thornton, 1928), *Ochrobactrum* (Holmes et al., 1988), *Pseudochrobactrum* (Kämpfer et al., 2006) and *Paenochrobactrum* (Kämpfer et al., 2010). Brucellae are major zoonotic pathogens that are found in a broad spectrum of animal reservoirs. Differentiation between *Brucella* species is mainly based on the different hosts they are infecting but phenotypic and genotypic interspecies variation is low. Applying the widely accepted species definition of Wayne et al. (1987), the genus *Brucella* would have to be considered to be monospecific with the only species *Brucella melitensis*. In conclusion, Verger et al. (1985) proposed reclassification of all species recognized at that time to subspecies of *B. melitensis*. However, because of the medical and economic importance of *Brucella* species, it was decided by the "Subcommittee on the Taxonomy of *Brucella*" that the nomenspecies should be retained in order to avoid confusion (Osterman and Moriyon, 2006).

Next related to the highly infectious genus *Brucella* is the genus *Ochrobactrum. Ochrobactrum* species, predominantly *O. anthropi* and *O. intermedium*, can cause a sometimes brucellosis-like disease in humans too (Young, 1995; Scholz et al., 2008a,b). During routine identification, *Brucella* isolates may be misclassified as members of *Ochrobactrum* and vice versa and hence, *Ochrobactrum* strains are often named *Brucella*-like. In fact, *Brucella* and *Ochrobactrum* must be considered a monophyletic taxon, as was shown in various studies via DNA-rRNA hybridization, whole-cell protein analysis, 16S rRNA gene, 16S/23S rDNA internal spacer region 1 (ITS1) and *recA* sequence comparisons (De Ley et al., 1987; Velasco et al., 1998; Lebuhn et al., 2006; Scholz et al., 2008b).

Several novel taxa have been assigned to the family *Brucellaceae* in the last few years and interest in *Brucella*-like species has grown due to the bioterroristic potential of *Brucella* species. Currently, *Brucella* consists of nine species, namely *B. melitensis*, *B. abortus*, *B. ovis*, *B. canis*, *B. suis*, *B. neotomae*, *B. pinnipedialis*, *B. ceti*, *B. microti* and *B. inopinata* (Meyer and Shaw, 1920; Buddle, 1956; Carmichael

and Bruner, 1968; Huddleson, 1929; Stoenner and Lackman, 1957; Foster et al., 2007; Scholz et al., 2008c; Scholz et al., 2010). Within the genus *Ochrobactrum*, fifteen species are recognized today: *O. anthropi*, *O. intermedium*, *O. grignonense*, *O. tritici*, *O. gallinifaecis*, *O. lupini*, *O. oryzae*, *O. cytisi*, *O. pseudintermedium*, *O. pseudogrignonense*, *O. haematophilum*, *O. thiophenivorans*, *O. rhizoshaerae*, *O. pituitosum* and *O. ciceri* (Holmes et al., 1988; Velasco et al., 1998; Lebuhn et al., 2000; Kämpfer et al., 2003; Trujillo et al., 2006; Tripathi et al., 2006; Zurdo-Piñeiro et al., 2007; Teyssier et al., 2007; Kämpfer et al., 2007b; Kämpfer et al., 2008; Huber et al., 2010; Imran et al., 2009). The genus *Pseudochrobactrum* contains four species, *Ps. kiredjianiae, Ps. asaccharolyticum*, *Ps. saccharolyticum* and *Ps. lubricantis* (Kämpfer et al., 2006; 2007a; 2009). A novel, closely related genus, *Paenochrobactrum*, with the two species *Paenochrobactrum gallinarii* and *Paenochrobactrum glaciei*, was recently proposed (Kämpfer et al., 2010).

In order to contribute to a stable classification system for the *Brucella*-*Ochrobactrum* group, we have subjected more than 40 *Brucella*, *Ochrobactrum* and several *Pseudochrobactrum* ("*Brucella*-like") strains to sequence analyses of two housekeeping genes coding for DNA gyrase subunit B (*gyrB*) and the chaperonin GroEL (*groEL*), respectively. The main aim of the study was to evaluate the discriminatory power of these genes in regard to a collection of *Brucella* and *Brucella*-like type and field strains and their suitability for a rapid taxonomic allocation.

## 2.11.2. Materials and methods

### *Bacterial strains*

A representative panel of 35 different *Ochrobactrum* strains representing all currently described species except for *O. ciceri* and the type strains of all ten *Brucella* species as well as three *Pseudochrobactrum* type strains were examined. The strains used in this study are listed in Table 2. *Ochrobactrum* and *Pseudochrobactrum* strains were grown on PYE medium (3 $g \cdot L^{-1}$ peptone, 3 $g \cdot L^{-1}$ yeast extract, pH 7.2) at RT. *Brucella* strains were grown on trypticase soy agar (TSA) at 37°C in a 5% $CO_2$ atmosphere.

**Table 2.** List of strains used for *gyrB* and *groEL* sequencing.

| Strain | Source | Geographic origin | Species |
|---|---|---|---|
| *Brucella* | | | |
| ATCC 25840$^T$ | sheep | New Zealand | *B. ovis* |
| NCTC 12891$^T$ | whale | n/a | *B. ceti* |
| NCTC 12890$^T$ | sea lion | n/a | *B. pinnipedialis* |
| CCM 4915$^T$ | common vole | Czech Republic | *B. microti* |
| BO1$^T$ | breast implant wound | USA | *B. inopinata* |
| ATCC 23459$^T$ | desert wood rat | USA | *B. neotomae* |
| ATCC 23365$^T$ | dog | USA | *B. canis* |
| *Ochrobactrum* | | | |
| LMG 3331$^T$ | human | France | *O. anthropi* |
| CCUG 50485 | human blood | Sweden | |
| CCUG 34461 | human tracheal secretion | Norway | |
| LMG 3305 | human blood | France | |
| LMG 3313 | vagina | France | |
| LMG 371 | sputum | USA | |
| LMG 5438 | blood culture | UK | |
| DSM 20150 | urine of leech, *Hirudo* sp. | Germany | |
| LUP21$^T$ (LMG 20667) | *Lupinus albus* | Spain | *O. lupini* |
| LUP23 | *Lupinus albus* | Spain | |
| Esc1$^T$ | *Cytisus scoparius* nodules | Spain | *O. cytisi* |
| Esc5 | *Cytisus scoparius* nodules | Spain | |
| LMG 18957$^T$ | wheat rhizoplane | France | *O. tritici* |
| CCUG 29689 | human blood | Sweden | |
| LMG 401 | cervix | USA | |
| TA93 | industrial environment | Austria | |
| LMG 3301$^T$ | human blood | France | *O. intermedium* |
| RMA 16994 | human pelvic abscess | USA | |
| CCUG 44770 | human sputum | Austria | |
| TA13 | industrial environment | Austria | |
| CCUG 39736 | human blood | Sweden | |
| LMG 5446 | bladder | USA | |
| CCUG 1838 | n/a | n/a | |
| DSM 17490$^T$ | human axillary swab | France | *O. pseudintermedium* |
| CCUG 43465 | paper mill | Sweden | |
| CCUG 34735 | water | Sweden | |
| CCUG 30717$^T$ | human blood | Sweden | *O. pseudogrignonense* |
| CCUG 43892 | human ear (newborn) | Norway | |
| DSMZ 17471$^T$ | *Oryza sativa* | India | *O. oryzae* |
| CCUG 38531$^T$ | human blood | Sweden | *O. haematophilum* |
| OgA9a$^T$ | soil | France | *O. grignonense* |
| CCUG 50899$^T$ | industrial environment | Sweden | *O. pituitosum* |
| PR17$^T$ | potatoe rhizospere | Austria | *O. rhizosphaerae* |
| DSM 7216$^T$ | waste water | Germany | *O. thiophenivorans* |
| Iso 196$^T$ | *Gallus gallus* faeces | Germany | *O. gallinifaecis* |
| *Pseudochrobactrum* | | | |
| CCUG 46016$^T$ | human knee aspirate | Sweden | *Ps. asaccharolyticum* |
| CCUG 33852$^T$ | industrial glue | Sweden | *Ps. sachharolyticum* |
| CCUG 49584$^T$ | seafood processing plant | New Zealand | *Ps. kiredjianiae* |

### *DNA preparation*

DNA was prepared from one loopful of cell material of one distinctive colony suspended in 100 µl of sterile water by 3 cycles of freeze-thawing (Wieser & Busse, 2000) or, in the case of the highly infectious *Brucella* species, from heat-inactivated

biomass (cell material was heated at 99°C for 10 min and centrifuged for 2 min at 13000×g) that was then treated in the same way. 0.5-1 µl of the cleared lysate were used as template in the PCR assays. High-molecular-mass DNA for the DNA-reassociation experiments was extracted from biomass according to Pitcher et al. (1989) and concentration of the purified DNA was estimated chemically according to Richards (1974).

*DNA-DNA hybridization*

In order to clarify the taxonomical status of the field strains used in this study, a majority of them was subjected to DNA-DNA hybridizations with DNAs of the type strains of their suspected nearest relatives. The hybridizations were performed according to Ziemke et al. (1998) except that, for nick translation, 2 µg DNA was labelled during a 90 min incubation at 15°C.

*Primer design and PCR amplification*

Primers are listed in Table 3. The partial *gyrB* and *groEL* sequences of *Ochrobactrum* and *Brucella* species were amplified by PCR with primers constructed using available *gyrB* and *groEL* sequence information in the NCBI nucleotide database. The *gyrB* primers used in PCR were designed with the help of an alignment of the (partial) *gyrB* sequences of *B. abortus* biovar 1 str. 9-941 (chromosome I, acc. no. AE017223), *B. suis* 1330$^{T}$ (acc. no. AE014291; *gyrB*: AAN29081; nucleotide position 140014 to 142446), *B. melitensis* bv. 1 str. 16M$^{T}$ (acc. no. AE008917), *O. anthropi* IAM 14119 (acc. no. AB014971) and *Phyllobacterium myrsinacearum* IAM 13587 (acc. no. AB014990). The primers GyrB_f binding to position 916-939 nt (relative to AAN29081) and GyrB_r binding to position 2118-2141 nt (relative to AAN29081) were employed to generate a 1226 bp fragment of the entire *gyrB* gene (AAN29081: 2433 bp). The *groEL* primers used in PCR were designed with the help of an alignment of the (partial) *groEL* sequences of *O. anthropi* ATCC 49188 (chromosome II, acc. no. CP000759), *B. abortus* S19 (acc. no. M82975), *B. melitensis* 16M (acc. no. AE008918) and B. suis 1330 (acc. no. AE014292). The primer pair GroEL_f binding to position -10-16 nt (relative to *groEL* part of M82975) and GyrB_r binding to position 1622-1640 nt (relative to *groEL* part of M82975) were used to generate a 1650 bp fragment including 1640 bp of the groEL gene (AAN29081: 1641 bp). In the actual *Brucella/Ochrobactrum/Pseudochrobactrum* alignments, *groEL* and *gyrB*

sequences were shortened to the same length, 1167 bp and 1023-1029 bp, respectively.

The 50 µl reaction mixture consisted of primers (0.5 pmol/µl), $MgCl_2$ (3 mM), dNTP's (0.2 mM each), 10 µl 5x buffer, and TaqDNA Polymerase enzyme (1.25 u, Promega), 1-2 µl DNA preparation and sterile water. Amplification was carried out in a Perkin Elmer GeneAmp PCR System 2400 or an Eppendorf Mastercycler. PCR amplification with all primer pairs was performed using the following conditions: 5 min initial denaturation at 95°C, followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 66°C and extension for 90 s at 72°C, final extension for 5 min at 72°C. Of each PCR reaction 5-7 µl were analyzed by agarose gel electrophoresis (1% w/v in TAE buffer) for the presence of a 1226 bp or 1650 bp product. Most *gyrB* PCR products were purified using the QIAquick PCR purification kit and *groEL* products had to be excised from a 1.7 % agarose gel and then purified with the QIAquick gel extraction kit (both QIAGEN) because of interfering by-products.

**Table 3.** Primers used for PCR and sequencing.

| **Primer*** | **Primer sequence (5' -> 3')** | **Position**[+] |
|---|---|---|
| GroEL_f (amp, s) | GGA GAG TAA AAT GGC TGC AAA AGA CG | 545 (→) |
| GroEL_r (amp, s) | GAA GTC CAT GCC GCC CAT G | 2194 (←) |
| Gro_int_f (s) | GAA GCC GAA ATC GGC AAG ATG | 1017 (→) |
| Gro_Pseud_f (amp, s) | CGG CGA AGA CCT GCT GAT C | 410 (→) |
| Gro_Pseud_r (amp, s) | GCA ACG ATA CCT TCT TCA ACC G | 1793 (←) |
| Gro31_32_33_f (s) | GTT GCT GCC GGA ATG AAC CC | 73 (→) |
| GyrB_f (amp) | GAT GAT GAT CTT GTG (AG)TA (AGC)CG CAG | 916 (→) |
| GyrB_r (amp, s) | CGA GGT CAT CAT GAC CCA GCT TC | 2141 (←) |
| GyrB_111206 (s) | CAA GGA CAA GCT TGT TTC CTC C | 1399 (←) |

*Abbreviations: amp, amplification primer; s, sequencing primer

[+]Positions are numbered relative to the *groEL/groES* sequence of *Brucella abortus* (acc. no. M82975) and *gyrB* sequence of *Brucella suis* (acc. no. AAN29081). Arrows indicate direction of primers (→, forward; ←, reverse).

*GyrB and groEL sequence analysis*

The nucleotide sequences of the *gyrB* and *groEL* fragments were determined by dideoxynucleotide sequencing of both strands with the PCR primers and, if necessary, also some internal sequencing primers to accomplish incomplete sequences (Table 3). Sequencing was partly carried out at VBC Genomics, Vienna and partly at AGOWA, Berlin. Nucleotide sequences were further analysed using the Chromas 1.45 program. Multiple sequence alignments of partial *gyrB* and *groEL* gene sequences were performed with Clustal X software, version 1.81 (Thompson et al., 1994) and further processed and analysed with BioEdit 7.0.5.3 (Hall, 1999). Nucleotide and protein sequence identities were also calculated with BioEdit. The phylogenetic *gyrB* (1023-1029 bp) and *groEL* (1167 bp) trees were obtained by using the maximum-likelihood method (PHYLIP version 3.66; Felsenstein, 2006). Trees were drawn with Treeview version 1.5 software (Page, 1996). 100 bootstrap resamplings were performed to assess the tree topology. *Bartonella henselae* Houston-1$^T$ (BX897699) was used as an outgroup.

The following *groEL* and *gyrB* sequences were taken from existing entries in the database: *O. rhizosphaerae* PR17$^T$ (FM863819, FM863814), *O. pituitosum* CCUG 50899$^T$ (FM863820, FM863815), *O. gignonense* OgA9a$^T$ (FM863821, FM863816), *O. pseudogrignonense* CCUG 30717$^T$ (FM863822, FM863817), *O. thiophenivorans* DSM 7216$^T$ (FM863823, FM863818), *B. suis* 1330$^T$ (AE014291), *B. melitensis* 16M$^T$ (AE008917), *Bartonella henselae* Houston-1 (BX897699) and all *recA* sequences used for the phylogenetic analysis (acc. nos. given in Fig. 3).

## 2.11.3. Results and discussion

*RecA*- and 16S rRNA gene-based phylogenetic analyses of *Brucella* and *Brucella*-like strains have already been successfully employed to separate different *Ochrobactrum* species and to distinguish them from the highly pathogenic *Brucella* species (Scholz et al., 2006; 2008b). However, the 16S rRNA gene showed a resolution too low for differentiation between very closely related species and at the infraspecies level. Since less conserved protein coding housekeeping genes like *recA* generally have a higher discriminatory potential than the 16S rRNA gene, which is in many cases useful for the discrimination of more distantly related species only,

they are often used in multilocus sequence typing (MLST) for population genetic purposes or in multilocus sequence analysis (MLSA) to discriminate between closely related species. Among others, partial *gyrB* and *groEL* sequences are often included in MLST/MLSA studies. The DNA gyrase subunit B gene (*gyrB*) codes for a type II topoisomerase responsible for the negative supercoiling of closed circular double-stranded DNA during genome replication. The chaperonin GroEL (*groEL*) is a crucial molecular chaperone that helps to fold proteins in the cell. In order to evaluate the usefulness of *gyrB* and *groEL* sequence analyses for classification of *Brucella* and *Brucella*-like strains, a number of type and field strains were examined. Unlike previous sequence typing studies, the present work used DNA-DNA hybridization to determine the species status of most field strains that were included and thus provided a stable basis for interpretation of the obtained sequence data.

The primers GyrB_f/r and GroEL_f/r, designed for amplification of the respective genes, could be successfully employed and GyrB_f/r yielded a specific product for all included *Brucella*, *Ochrobactrum* and *Pseudochrobactrum* strains. GroEL_f/r primers were not suitable to amplify an appropriate product from DNA of *Pseudochrobactrum* strains and more permissive primers had to be constructed for representatives of this genus. The obtained *gyrB* and *groEL* sequences, as well as *recA* sequences from the database were used to create multiple sequence alignments which were then subjected to phylogenetic analyses with the PHYLIP software package. Protein and nucleotide sequence identity values were calculated with the program BioEdit and evaluated for species demarcation criteria useful for a rapid taxonomic allocation of *Brucella* and *Brucella*-like strains.

The *gyrB* sequence proved to be the least conserved of the two examined genes and *recA*, as well (Scholz et al., 2006; 2008b), showing a higher resolution at the subspecies level, whereas *groEL* was the most conserved gene. Especially in the *groEL* phylogenetic maximum-likelihood dendrogram, the deeply branching lineages showed little statistical significance, indicated by bootstrap values lower than 70% (Fig. 2). However, the concatenated tree shows high bootstrap support for most branches which also confirms the comparable clustering of the dendrograms derived from only a single gene (Fig. 4). In the trees based on *groEL*, *gyrB* and *recA* sequences, respectively, species clustered according to their taxonomic affiliation (Figs. 1-3).

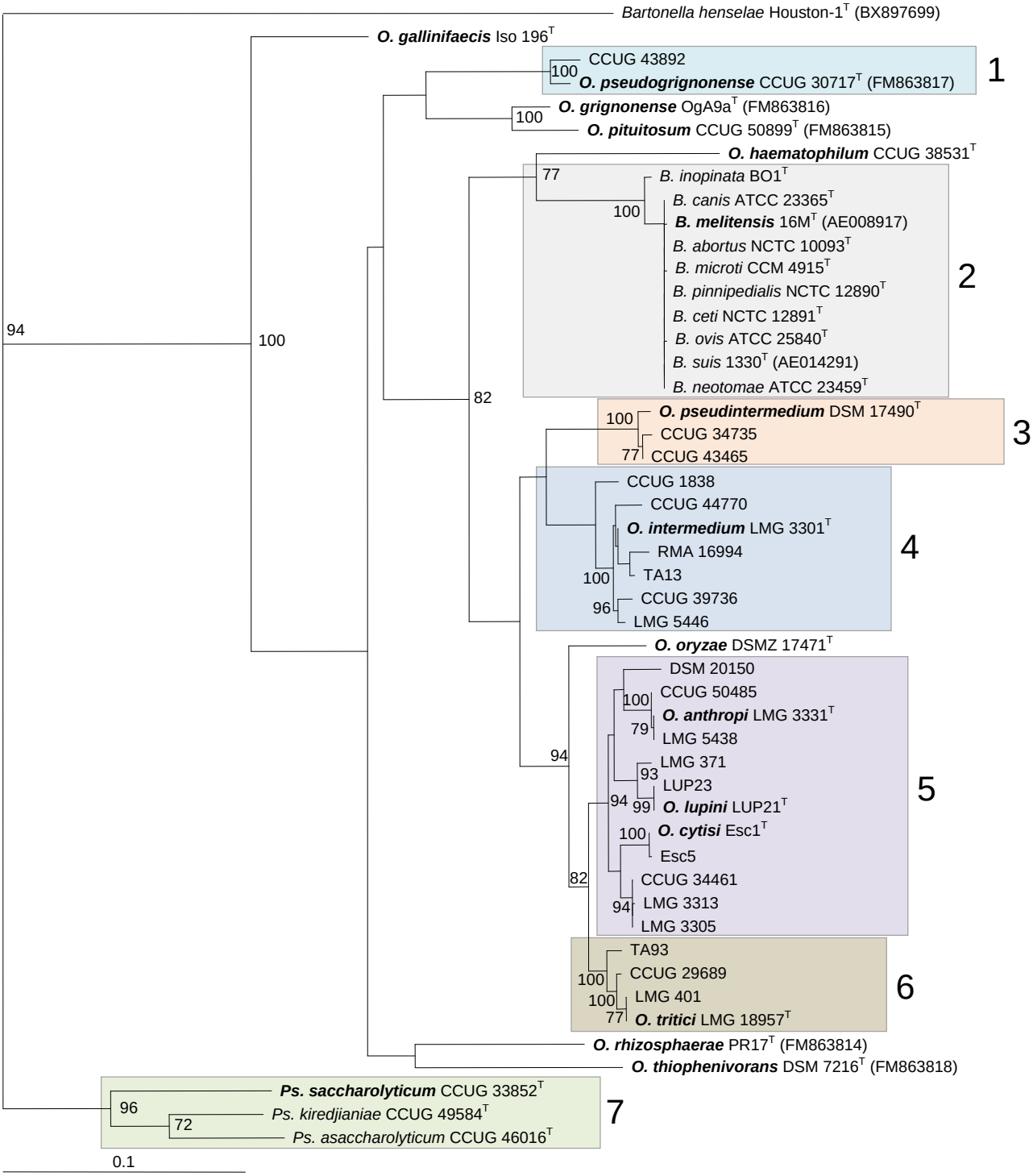


**Fig. 1. Maximum-likelihood tree based on the *gyrB* (1029 nt) nucleotide sequences.** The dendrogram was created from a Clustal X alignment using PHYLIP. Representatives of *Brucella* and *Brucella*-like groups are included (*O*, *Ochrobactrum*; *B*, *Brucella*; *Ps*, *Pseudochrobactrum*). The numbers at the nodes of the tree indicate bootstrap values for each node out of 100 bootstrap resamplings (values below 70% not shown). *Bartonella henselae* Houston-1 was used as outgroup. If available, accession numbers are listed beside the nominal designation of the strains. The scale bar indicates 10% divergence. The different clusters are indicated by the numbers 1-7.

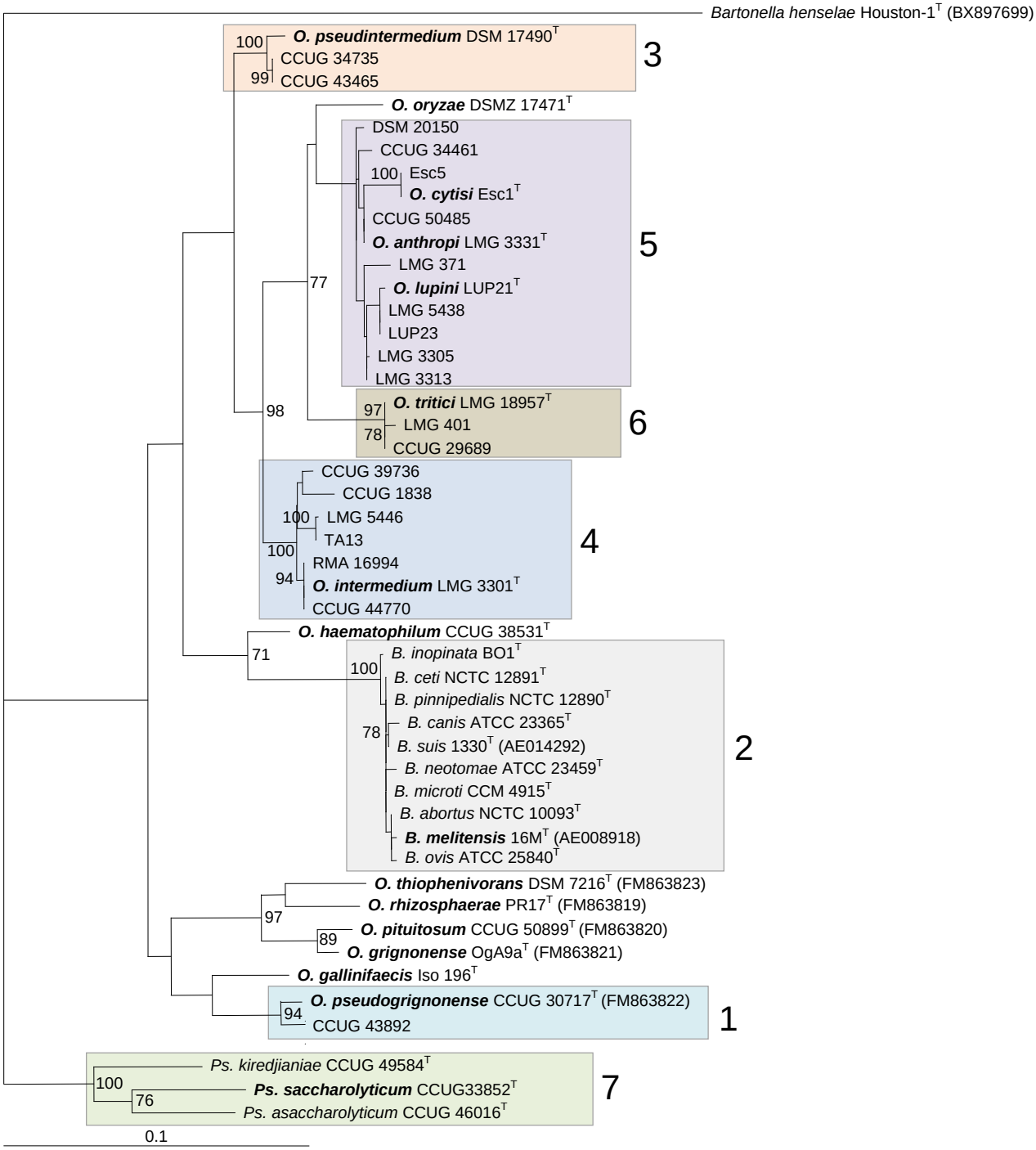


**Fig. 2. Maximum-likelihood tree based on the *groEL* (1167 nt) nucleotide sequences.** The dendrogram was created from a Clustal X alignment using PHYLIP. Representatives of *Brucella* and *Brucella*-like groups are included (*O*, *Ochrobactrum*; *B*, *Brucella*; *Ps*, *Pseudochrobactrum*). The numbers at the nodes of the tree indicate bootstrap values for each node out of 100 bootstrap resamplings (values below 70% not shown). *Bartonella henselae* Houston-1 was used as outgroup. If available, accession numbers are listed beside the nominal designation of the strains. The scale bar indicates 10% divergence. The different clusters are indicated by the numbers 1-7.

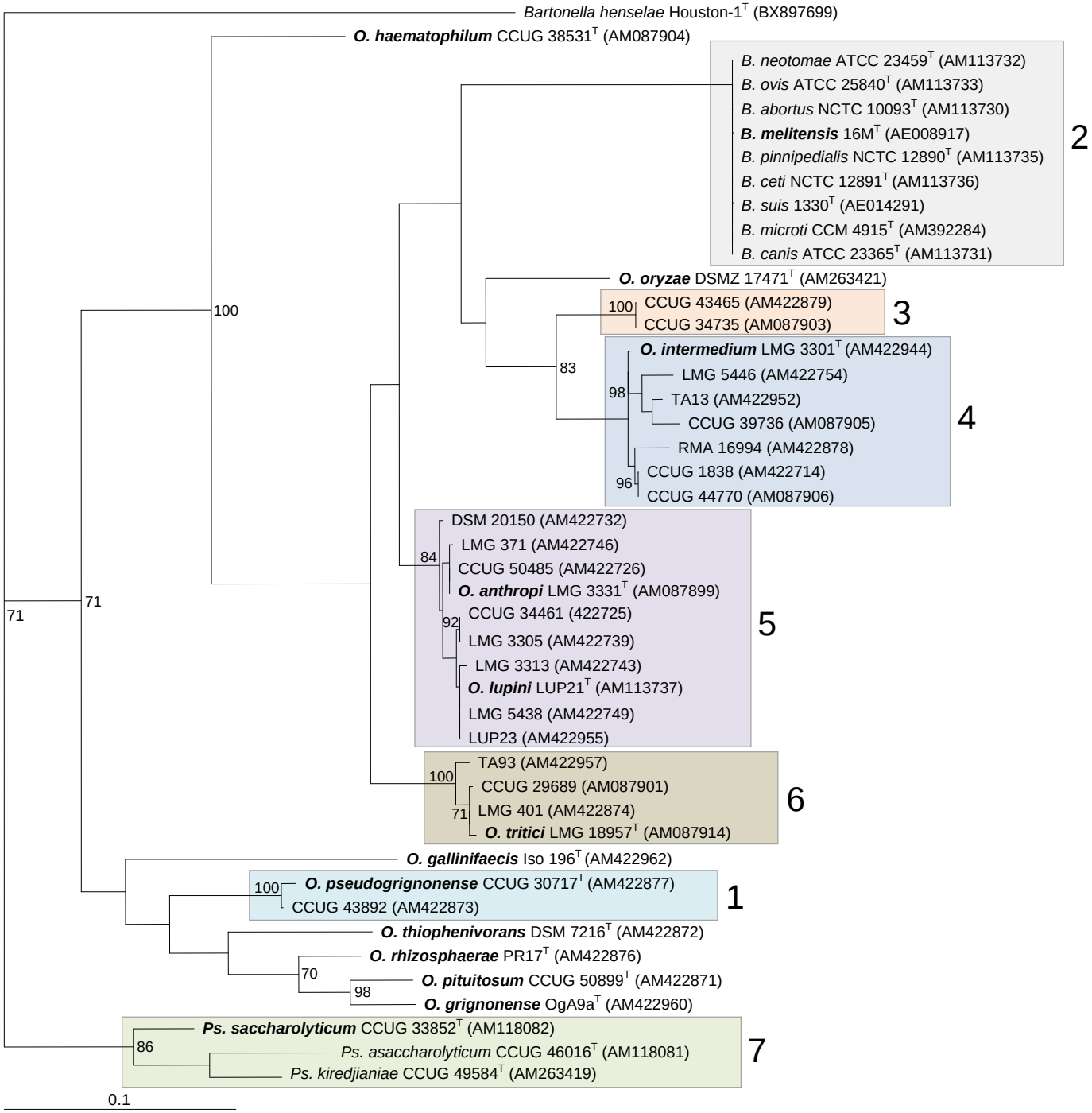


**Fig. 3. Maximum-likelihood tree based on the *recA* (684 nt) nucleotide sequences.** The dendrogram was created from a Clustal X alignment using PHYLIP. Representatives of *Brucella* and *Brucella*-like groups are included (*O*, *Ochrobactrum*; *B*, *Brucella*; *Ps*, *Pseudochrobactrum*). The numbers at the nodes of the tree indicate bootstrap values for each node out of 100 bootstrap resamplings (values below 70% not shown). *Bartonella henselae* Houston-1 was used as outgroup. If available, accession numbers are listed beside the nominal designation of the strains. The scale bar indicates 10% divergence. The different clusters are indicated by the numbers 1-7.

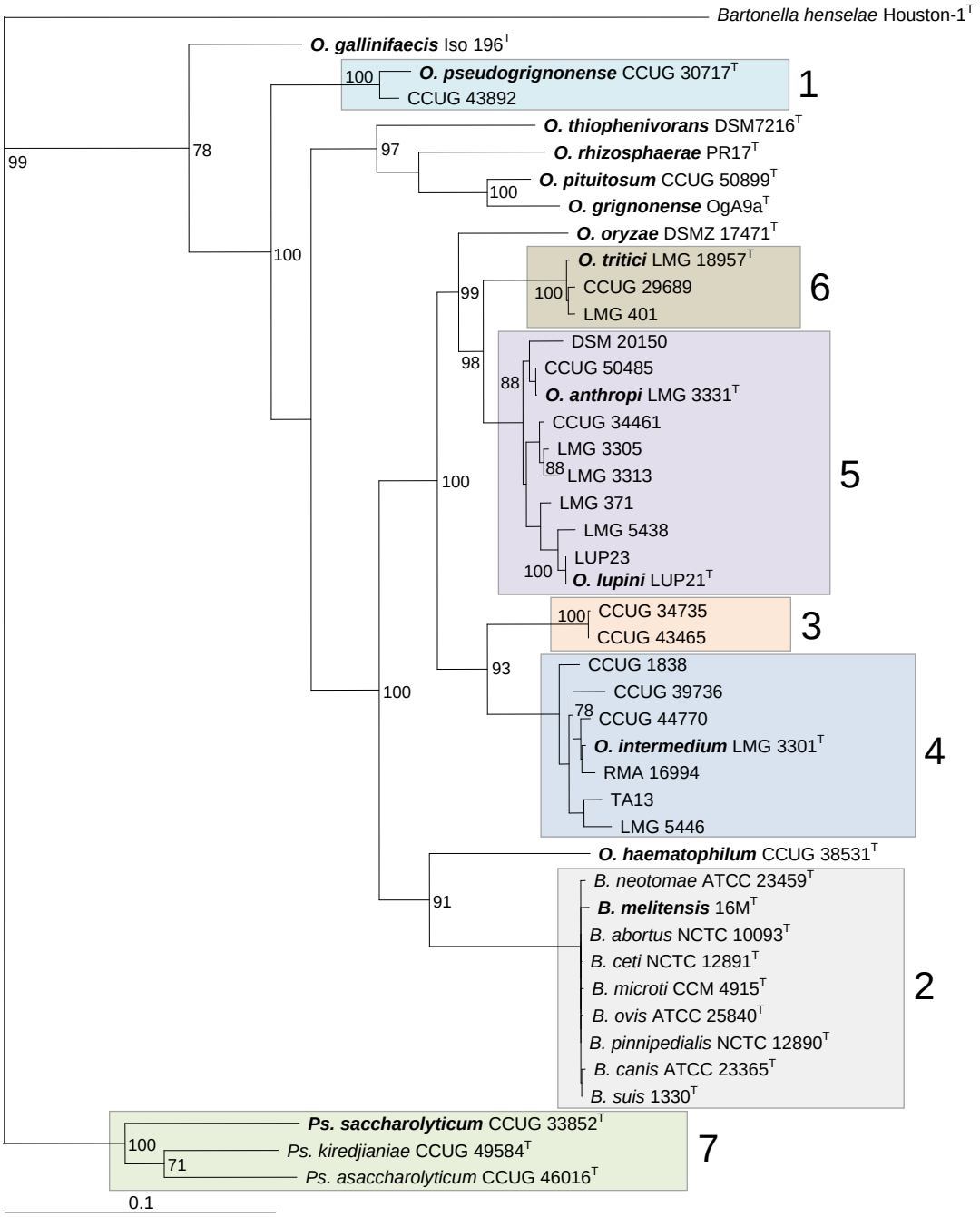


**Fig. 4. Maximum-likelihood dendrogram based on a concatenated dataset, containing the *gyrB*, *groEL* and *recA* gene sequences (2884 nt).** The tree was created from a Clustal X alignment using PHYLIP. Representatives of *Brucella* and *Brucella*-like groups are included (*O*, *Ochrobactrum*; *B*, *Brucella*; *Ps*, *Pseudochrobactrum*). The numbers at the nodes of the tree indicate bootstrap values for each node out of 100 bootstrap resamplings (values below 70% not shown). *Bartonella henselae* Houston-1 was used as outgroup. If available, accession numbers are listed beside the nominal designation of the strains. The scale bar indicates 10% divergence. The different clusters are indicated by the numbers 1-7.

Strains of *O. pseudogrignonense*, *Brucella*, *O. pseudintermedium*, *O. intermedium*, *O. anthropi*, *O. tritici* and *Pseudochrobactrum* formed recognizable clusters 1-7 in all trees including the concatenated tree calculated on the basis of *gyrB*, *groEL* and *recA* sequences. A comparable clustering was also shown by Scholz et al. (2008b) based on *recA* sequences only.

*Brucella* strains formed a distinct clade designated cluster 2 but showed very little interspecies variability. Compared to the *rrs* and *recA* sequences, which showed 100% identity (Scholz et al., 2008b), *gyrB* and *groEL* sequences of *Brucella* species showed at least some variability: 99.6-100% and 99.3-100%, respectively (excluding *B. inopinata*). However, only the strain *B. inopinata* BO1$^T$ clearly differed from the other species in its *gyrB* and *groEL* sequence. This is in agreement with other exceptional characteristics of this *Brucella* species (Scholz et al., 2010). According to the *groEL* nucleotide sequence, *O. haematophilum*, *O. intermedium*, *O. pseudintermedium* and *O. anthropi* are closest related to *Brucella*, according to *gyrB* *O. intermedium* and *O. pseudintermedium*. *Brucella* species can be clearly differentiated from these *Ochrobactrum* species in the phylogenetic dendrograms and by *groEL* identity values <94.6% (99.3-100% within the *Brucella* cluster) and *gyrB* identity values <88.8% (98.3-100% within the *Brucella* cluster). Thus, *groEL* sequence identities >99% and *gyrB* sequence identities >98% of a novel isolate could indicate that it belongs to the genus *Brucella*.

High sequence identities in both genes (*groEL*: ≥ 98.7%; *gyrB*: >96%) were found within cluster 5 comprising the strains *O. anthropi* LMG 3331$^T$, *O. anthropi* CCUG 50485, *O. anthropi* LMG 3305, *O. anthropi* LMG 3313, *O. anthropi* CCUG 34461, *O. anthropi* LMG 5438, *O. anthropi* LMG 371, *O. anthropi* DSM 20150, *O. lupini* LUP23 and *O. lupini* LUP21$^T$. These results reveal a close relationship between the *O. anthropi* strains and support the *recA*-based findings of Scholz et al. (2008b). The two *O. lupini* strains could not be distinguished from *O. anthropi* strains and, hence, were included in cluster 5, which emphasizes the general difficulty in differentiating between the two species. Moreover, a very close relationship of *O. cytisi* ESC1$^T$ and *O. cytisi* ESC5 to *O. anthropi* strains was demonstrated. So, they were also assigned to cluster 5. The close relationships between *O. anthropi*, *O. lupini* and *O. cytisi* strains are not surprising when considering the high DNA relatedness between the species that was found by Trujillo et al. (2005) and Zurdo-Piñeiro et al. (2007). The *O. lupini* type strain shows 68% DNA reassociation with *O. anthropi*, which is close to

the threshold value of 70%. *O. cytisi* resulted in 66% reassociation with *O. anthropi* and 70% with *O. lupini*. The identity values do not make a differentiation possible either, with *gyrB* nucleotide sequence identities of *O. lupini* being 96.1-98.9% and of *O. cytisi* being 95.4-97.9% to *O. anthropi* (within *O. anthropi*: 96-100%) and *groEL* sequence identities of *O. lupini* being 98.8-100% and of *O. cytisi* being 98.2-98.8% to *O. anthropi* (within *O. anthropi*: 98.7-100%). Strains of cluster 5 can be differentiated from their nearest neighbours by *groEL* identity values >98% (*O. tritici* 96-96.7%; *O. oryzae* 95.8-96.3%) and *gyrB* identity values >96% (*O. tritici* 94.2-95.7%; *O. oryzae* 92.2-92.8%).

The strains of cluster 6, *O. tritici* LMG 18957$^T$, *O. tritici* CCUG 39659 and LMG 401 showed almost identical *groEL* sequences (99.7-99.9%), while the identities to other species of the genus were <97%. These results are in excellent accordance with the data of Scholz et al. (2008b). Also the *gyrB* identity values >98% within cluster 6, including strain TA93, and <96% to other *Ochrobactrum* and *Brucella* species confirm the close relationship between the examined *O. tritici* strains and the separation from all other species of the genus.

The high nucleotide sequence identities of strains *O. pseudintermedium* CCUG 43465 und *O. pseudintermedium* CCUG 34735 in both genes (99-100%) indicate that they are actually members of this species. Similarly, the high sequence identities in regard to both genes between the cluster 4 strains *O. intermedium* TA13, *O. intermedium* CCUG 39736, *O. intermedium* CCUG 44770, *O. intermedium* CCUG 1838, *O. intermedium* RMA 16994, *O. intermedium* LMG 5446 and the type strain of the species *O. intermedium* LMG 3301$^T$ (*groEL*: >98.4%; *gyrB*: >96,7%), and slightly lower values to other species of the genus (*groEL*: <96.4%; *gyrB*: <92.6%) confirm their affiliation to this species.

All other *Ochrobactrum* species could also be clearly differentiated from each other by comparison of sequence identity values and clustering in the phylogenetic trees. *O. haematophilum* clustered next to the *Brucella* cluster and *O. oryzae* near the *O. anthropi* and *O. tritici* clusters in the *gyrB* and *groEL* based dendrograms (Figs. 1-2). *O. pseudintermedium* was always near the *O. intermedium* cluster (Figs. 1-4). *O. grignonense* and *O. pituitosum* were nearest to each other in all trees (Figs. 1-4).

In the dendrograms, *Pseudochrobactrum* species formed a distinct cluster remote from the two other examined genera. The three species were clearly separated from

each other by differences in *gyrB* and *groEL* sequences. *GroEL* sequence identities between the types of the three species were below 94% and *gyrB* sequence identities below 91%. A differentiation from all *Brucella* and *Ochrobactrum* species was also possible, the highest identity values being <89.0% in the *groEL* sequence and <81% in the *gyrB* sequence. Only in the *groEL* phylogenetic tree, *Ps. asaccharolyticum* clustered with *Ps. saccharolyticum* with a bootstrap support of 76%, whereas in the other dendrograms it clusters with *Ps. kiredjianiae* (Figs. 1-4).

The results from the sequence comparisons of the *gyrB* and *groEL* gene showed that both genes are of excellent value for a differentiation between the genera *Brucella, Ochrobactrum* and *Pseudochrobactrum* and suitable for discriminating between most *Ochrobactrum* and *Pseudochrobactrum* species. Like with *recA*, both genes could not be employed to differentiate between *Brucella* species. Maximum sequence differences of 1.3% seem not to be sufficient for use of *groEL* or *gyrB* as genetic markers in a *Brucella* typing system (Table 5). However, this result was not surprising, since the species concept of the genus *Brucella* is mainly based on their different hosts and the genetic background rather suggests the inclusion of all *Brucella* species into a single species.

For the strains DSM 20150, CCUG 50485, LMG 5438, LMG 371 and CCUG 34461, sequence comparisons showed that they are affiliated to *O. anthropi*. The results of the DNA-DNA hybridization experiments (Table 4) confirmed that they are actually representatives of the species *O. anthropi*. For strains LMG 401 and TA93 the *gyrB* sequence comparison suggested a close relationship with *O. tritici*. This was also substantiated by DNA-DNA hybridization results.

The strains DSM 1838, TA13, LMG 5446, RMA 16994, CCUG 44770 and CCUG 39736 clustered with the type strain of *O. intermedium* LMG 3301$^T$ in both, *gyrB* and *groEL*. The DNA-DNA hybridization results revealed their actual affiliation to this species (Table 4).

Sequence identities of 99.0% (*groEL*) and 99.3% (*gyrB*) suggested that strain CCUG 43465 is a member of the species *O. pseudintermedium.* Its affiliation to *O. pseudintermedium* could be confirmed by DNA-DNA hybridization experiments that additionally disproved the previous assignment of CCUG 43465 to *O. intermedium* (Table 4).

In the primary amino acid sequences, the discriminatory potential of the phylogenetic markers is drastically reduced (Table 6). Protein sequences showed a

maximum difference of 4.4% (GroEL) and 11.2% (GyrB) within the genus *Ochrobactrum*. Infraspecies variation decreased. In both, the *groEL* and *gyrB* sequences, *O. anthropi*, *O. tritici*, *O. cytisi* and *O. oryzae* can hardly be differentiated and there is only 0.5-1% difference to *O. intermedium* and *O. pseudintermedium* (Table 6). In contrast to nucleotide based comparisons, most *O. anthropi* strains had identical GroEL protein sequences, except for DSM 20150 and LUP21$^T$. GyrB retained a certain differentiating power in the protein sequence, except for the very closely related species of cluster 5.

Sequence comparisons and phylogenetic relationships clearly separated representatives of the studied genera and discriminated between *Ochrobactrum* species as well. On the other hand, high homogeneity in both genes among members of *Brucella* was observed, confirming the mono-specificity of the genus. The results of this study suggest that, based on sequence similarities of the two genes, *Brucella* strains can be reliably identified only at the genus level, whereas the discriminatory power for classification of *Ochrobactrum* strains is significantly higher. Together with *recA*, the two genetic markers could find application in order to reliably identify respective field strains in a multilocus sequencing approach.

**Table 4.** Results of DNA-DNA hybridization experiments.

| | *O. anthropi* LMG 3331$^T$ lab* | *O. intermedium* LMG 3301$^T$ lab* | CCUG 34735 lab* | *O. tritici* LMG 18957$^T$ lab* | *O. pseudintermedium* DSM 17490$^T$ lab* |
|---|---|---|---|---|---|
| LMG 371 | 97 % | - | - | - | - |
| CCUG 34461 | 77 % | - | - | - | - |
| DSM 20150 | 85 % | - | - | - | - |
| CCUG 50485 | 97 % | - | - | - | - |
| LMG 5438 | 98 % | - | - | - | - |
| LMG 3301$^T$ | - | - | 40 % | - | - |
| TA 13 | - | 96 % | - | - | - |
| LMG 5446 | - | 84 % | - | - | - |
| RMA 16994 | - | 93 % | - | - | - |
| CCUG 44770 | - | 75 % | - | - | - |
| CCUG 39736 | - | 73 % | - | - | - |
| CCUG 1838 | - | 81 % (↔) | - | - | - |
| LMG 401 | - | - | - | 82 % | - |
| TA 93 | - | - | - | 79 % | - |
| CCUG 43465 | - | - | - | - | 87 % |

*labelled DNA

**Table 5.** Sequence identity values derived from a *gyrB* and *groEL* nucleotide sequence alignment of different *Brucella* reference, *Ochrobactrum* and *Pseudochrobactrum* strains; calculated with the computer software BioEdit. *GyrB* identity values are given as percentages above the diagonal, percentage *groEL* values below the diagonal. **Bruce:** *Brucella* **interm:** *O. intermedium* **psint:** *O. pseudintermedium* **anthr:** *O. anthropi* **tritici:** *O. tritici* (*groEL* without TA93) **cytisi:** *O. cytisi* **oryzae:** *O. oryzae* **psgrig:** *O. pseudogrignonense* **grig:** *O. grignonense* **galli:** *O. gallinifaecis* **pitu:** *O. pituitosum* **rhizo:** *O. rhizosphaerae* **thio:** *O. thiophenivorans* **haem:** *O. haematophilum* **Psoch:** *Pseudochrobactrum*

| *gyrB* % / *groEL* % | Bruce | interm | psint | anthr | tritici | cytisi | oryzae | psgrig | grig | galli | pitu | rhizo | thio | haem | Psoch |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bruce | 98.3-100<br>99.3-100 | 87.3-88.8 | 87.7-88.5 | 87.0-88.0 | 87.4-88.0 | 86.7-87.0 | 87.1-87.4 | 84.1-85.0 | 84.3-84.6 | 83.7-84.1 | 84.5-84.8 | 83.8-84.0 | 83.0-83.3 | 86.2-86.4 | 78.0-78.8 |
| interm | 92.8-94.0 | 96.7-98.9<br>98.4-100 | 91.1-92.6 | 90.4-92.3 | 91.1-92.4 | 91.2-92.2 | 89.5-90.6 | 87.1-88.3 | 86.4-87.3 | 83.9-84.7 | 86.0-86.8 | 83.9-85.2 | 84.1-85.1 | 86.7-87.2 | 78.0-79.8 |
| psint | 93.0-93.6 | 96.1-96.4 | 99.1-99.8<br>99.0-100 | 90.3-91.2 | 90.0-91.2 | 90.9-91.4 | 90.3-90.6 | 86.5-87.5 | 85.3-85.5 | 84.7-84.9 | 85.1-85.6 | 84.7-85.2 | 84.1-84.4 | 85.8-86.0 | 78.2-79.2 |
| anthr | 93.2-94.0 | 94.8-96.0 | 94.6-95.2 | 93.7-100<br>98.7-100 | 94.2-95.7 | 95.4-97.9 | 92.2-92.8 | 86.6-87.6 | 85.5-85.9 | 84.7-85.4 | 85.0-86.0 | 83.8-84.2 | 84.5-85.1 | 85.6-86.3 | 78.2-80.5 |
| tritici | 92.6-93.2 | 94.3-94.9 | 94.3-94.6 | 96.0-96.7 | 98.1-99.9<br>99.7-99.9 | 95.2-95.8 | 92.9-93.6 | 86.3-87.3 | 85.1-85.3 | 84.0-84.6 | 84.8-85.0 | 83.6-83.7 | 84.8-85.0 | 85.7-86.3 | 78.0-80.0 |
| cytisi | 92.8-93.1 | 94.6-95.2 | 94.4 | 98.2-98.8 | 95.9-96.2 | 99.9<br>100 | 92.4-92.5 | 87.0-87.5 | 84.9-85.0 | 85.7-85.8 | 85.0-85.1 | 83.9-84.0 | 84.4-84.5 | 85.9-86.0 | 78.8-80.8 |
| oryzae | 92.4-92.8 | 94.9-95.2 | 93.9-94.0 | 95.8-96.3 | 95.0-95.2 | 96.1 | | 85.9 | 84.9 | 84.9 | 85.0 | 84.4 | 85.2 | 84.9 | 78.7-79.4 |
| psgrig | 91.0-91.6 | 92.4-93.2 | 92.8-93.3 | 92.8-93.4 | 92.3-92.9 | 92.8-93.2 | 92.3-92.7 | 97.8<br>98.6 | 88.9-89.5 | 85.8-86.4 | 89.1-89.2 | 85.4-85.7 | 85.6-85.8 | 81.8-82.5 | 77.7-79.1 |
| grig | 91.8-92.4 | 92.4-92.9 | 92.1-92.2 | 92.3-92.9 | 91.5-91.7 | 92.7 | 91.6 | 92.4 | | 85.1 | 95.0 | 87.0 | 85.3 | 82.5 | 76.4-77.7 |
| galli | 90.3-90.5 | 92.0-92.6 | 92.3-92.5 | 91.8-92.4 | 91.8-92.1 | 92.0 | 91.8 | 94.3-94.6 | 91.9 | | 84.4 | 84.4 | 83.9 | 82.5 | 79.0-80.4 |
| pitu | 91.8-92.2 | 91.9-92.4 | 91.8-91.9 | 91.9-92.4 | 91.5-91.7 | 92.4 | 91.6 | 92.5 | 97.9 | 91.8 | | 87.4 | 85.2 | 82.5 | 77.5-77.9 |
| rhizo | 91.1-91.6 | 91.4-92.2 | 91.3-91.4 | 91.7-92.2 | 91.6 | 91.8 | 91.4 | 92.8 | 95.2 | 92.3 | 95.6 | | 85.1 | 81.6 | 76.4-76.8 |
| thio | 90.1-90.5 | 90.9-91.4 | 90.9-91.0 | 90.8-91.4 | 90.3-90.4 | 91.2 | 90.8 | 92.5-92.6 | 94.9 | 91.7 | 95.5 | 95.8 | | 80.6 | 76.3-78.2 |
| haem | 94.2-94.6 | 94.1-94.7 | 94.3-94.6 | 94.3-94.6 | 93.4-93.7 | 94.1 | 93.4 | 93.4 | 93.0 | 92.7 | 92.9 | 92.9 | 92.0 | | 76.4-77.8 |
| Psoch | 84.0-85.5 | 86.4-87.1 | 86.8-87.9 | 86.4-87.5 | 85.5-86.2 | 86.7-87.3 | 85.7-86.9 | 88.1-88.6 | 87.0-87.7 | 87.6-88.3 | 86.1-87.2 | 86.7-88.0 | 85.8-86.8 | 86.9-87.4 | 86.6-97.8<br>93.4-93.6 |

**Table 6.** Sequence identity values derived from a *gyrB* and *groEL* protein sequence alignment of different *Brucella* reference, *Ochrobactrum* and *Pseudochrobactrum* strains; calculated with the computer software BioEdit. GyrB identity values are given as percentages above the diagonal, percentage GroEL values below the diagonal. **Bruce:** *Brucella* (without BO1$^{T}$) **interm:** *O. intermedium* **psint:** *O. pseudintermedium* (without DSM 17490$^{T}$) **anthr:** *O. anthropi* **tritici:** *O. tritici* **cytisi:** *O. cytisi* **oryzae:** *O. oryzae* **psgrig:** *O. pseudogrignonense* **grig:** *O. grignonense* **galli:** *O. gallinifaecis* **pitu:** *O. pituitosum* **rhizo:** *O. rhizosphaerae* **thio:** *O. thiophenivorans* **haem:** *O. haematophilum* **Psoch:** *Pseudochrobactrum*

| gyrB %<br>groEL % | Bruc | interm | psint | anthr | tritici | cytisi | oryzae | psgrig | grig | galli | pitu | rhizo | thio | haem | Psoch |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bruc | 98.8-100<br>98.9-100 | 95.3-96.1 | 95.3-96.1 | 95.6-97.0 | 95.8-97.0 | 95.6-96.7 | 95.3-96.1 | 94.1-95.0 | 92.6-93.5 | 93.8-94.7 | 93.2-94.1 | 93.2-93.8 | 94.4-95.3 | 91.2-91.8 | 87.4-88.8 |
| interm | 97.6-98.4 | 99.7-100<br>99.4-100 | 97.9-98.5 | 97.9-98.2 | 97.9-98.2 | 97.9-98.2 | 97.9 | 95.3-95.6 | 93.2-93.5 | 94.4 | 93.8-94.1 | 92.9-93.2 | 94.7 | 92.9-93.2 | 86.5-87.7 |
| psint | 97.6-98.4 | 99.4-100 | 99.7-100<br>99.7-100 | 97.6-98.2 | 97.6-98.2 | 97.6-98.2 | 97.6-97.9 | 94.7-95.0 | 92.6-92.9 | 94.7-95.0 | 93.2-93.5 | 92.0-92.3 | 94.7-95.0 | 92.9-93.2 | 86.2-87.4 |
| anthr | 96.9-97.6 | 98.4-98.9 | 98.4-98.9 | 99.1-100<br>99.4-100 | 99.1-100 | 99.1-100 | 98.5-99.1 | 95.3-95.6 | 92.9-93.2 | 94.4-95.0 | 93.5-93.8 | 92.6-92.9 | 95.0-95.6 | 92.3-92.9 | 86.5-87.7 |
| tritici | 97.1-97.9 | 98.7-99.2 | 98.7-99.2 | 99.2-99.7 | 99.4-100<br>99.7-100 | 99.4-99.7 | 98.8-99.1 | 95.3-95.6 | 92.9-93.2 | 94.7-95.0 | 93.5-93.8 | 92.6-92.9 | 95.3-95.6 | 92.3-92.6 | 86.5-88.3 |
| cytisi | 96.9-97.4 | 98.4-98.7 | 98.4-98.7 | 99.4-99.7 | 99.2-99.4 | 99.7<br>100 | 98.5-98.8 | 95.3-95.6 | 92.9-93.2 | 94.4-94.7 | 93.5-93.8 | 92.6-92.9 | 95.0-95.3 | 92.3-92.6 | 86.8-88.3 |
| oryzae | 97.4-97.9 | 98.9-99.2 | 98.9-99.2 | 98.9-99.2 | 99.2-99.4 | 98.9 | | 95.6 | 92.6 | 95.0 | 93.2 | 92.0 | 95.0 | 92.3 | 86.2-87.7 |
| psgrig | 96.6-97.1 | 97.6-97.9 | 97.6-97.9 | 97.9 | 97.9-98.2 | 98.2 | 97.6 | 100<br>100 | 96.1 | 95.6 | 96.7 | 94.1 | 94.4 | 90.9 | 85.9-87.1 |
| grig | 96.6-97.1 | 96.9-97.1 | 97.1 | 96.1-96.4 | 96.4-96.6 | 96.6 | 96.6 | 96.4 | | 93.8 | 99.4 | 95.0 | 93.5 | 88.8 | 85.0-85.9 |
| galli | 96.6-97.1 | 97.6-97.9 | 97.6-97.9 | 97.9-98.2 | 97.9-98.2 | 98.4 | 98.2 | 98.9 | 95.8 | | 94.4 | 93.8 | 95.3 | 89.7 | 85.9-87.4 |
| pitu | 96.9-97.4 | 97.1-97.4 | 97.4 | 96.4-96.6 | 96.6-96.9 | 96.9 | 96.9 | 97.1 | 99.2 | 96.1 | | 95.6 | 94.1 | 89.4 | 85.6-86.5 |
| rhizo | 96.4-96.9 | 96.6-96.9 | 96.9 | 96.4-96.6 | 96.4-96.6 | 96.9 | 96.6 | 96.6 | 98.2 | 96.4 | 98.9 | | 92.6 | 88.8 | 85.3-86.5 |
| thio | 95.8-96.4 | 96.1-96.4 | 96.4 | 95.6-95.8 | 95.6-95.8 | 96.1 | 95.8 | 95.8 | 97.9 | 95.6 | 98.7 | 98.9 | | 90.0 | 85.9-86.5 |
| haem | 98.2-98.7 | 98.4-98.7 | 98.4-98.7 | 97.6-97.9 | 97.9-98.2 | 97.6 | 98.2 | 97.1 | 96.6 | 97.1 | 96.9 | 96.4 | 95.8 | | 83.3-84.5 |
| Psoch | 94.8-96.6 | 95.3-96.9 | 95.6-96.9 | 95.3-96.9 | 95.3-96.6 | 95.3-96.6 | 95.3-96.9 | 95.1-95.8 | 94.3-95.3 | 94.8-96.4 | 94.6-95.6 | 94.3-95.6 | 93.5-95.5 | 95.8-97.1 | 95.0-97.3<br>96.9-98.4 |

# 3. Genus *Francisella*

## 3.1. Introduction

### 3.1.1. Tularemia

*Francisella tularensis* is a small, non-motile, Gram-stain negative, non-spore forming coccobacillus that causes the zoonotic disease tularemia (rabbit fever, deerfly fever) in humans and especially one of the four subspecies, *Francisella tularensis* subsp. *tularensis*, is generally highly virulent for humans and animals. It causes the severest form of the disease, the infectious dose being as few as 25 inhaled cells (Saslaw et al., 1961; Guryčivá, 1998). The other subspecies, *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *mediasiatica*, are of intermediate virulence, causing mostly non-lethal disease in humans (Ellis et al., 2002; Olsufjev and Meshcheryakova, 1982; 1983; Sandström et al., 1992). *F. tularensis* subsp. *novicida* infection is rare, normally occurs in immunocompromised hosts only and is linked to water-borne transmission (Ellis et al., 2002; Hollis et al., 1989). *F. tularensis* is found throughout the Northern Hemisphere and the natural reservoirs for the facultative intracellular bacterium in Europe are predominantly hares and rodents (Sjöstedt, 2007). The distribution of tularemia concurs with the distribution of holarctical animals (Sjöstedt, 2007). Arthropods such as ticks and mosquitoes are vectors that transmit *F. tularensis*. Humans are infected with the organism mainly through handling infected animals, consuming contaminated food or water, or inhaling aerosols (e.g. aerosolisation by using a lawn mower), but also through arthropod bites (Sjöstedt, 2005a).

Tularemia is an acute febrile invasive illness with rapid intracellular proliferation of bacteria, accumulation of phagocytes and tissue necrosis (Tärnvik and Chu, 2007). The typical period of incubation of the disease is three to five days after exposure or contact (Sjöstedt, 2007). The disease starts with unspecific symptoms like fever with chills, nausea and headache. According to the different clinical presentations, ulceroglandular and glandular (transmitted through skin or mucous membranes, >90% of European tularemia cases), oropharyngeal (through ingestion of contaminated food or water) and respiratory tularemia (through inhalation) are distinguished (Sjöstedt, 2007). Complications of a *F. tularensis* subsp. *tularensis*

infection include life-threatening pneumonia, septicemia, meningitis, endocarditis, hepatitis and renal failure; disease caused by *F. tularensis* subsp. *holarctica* can lead to nonfatal meningitis and septicemia (Tärnvik and Chu, 2007). A transmission of tularemia from human to human has not been proved yet (Sjöstedt, 2007).

Biodefense-associated research has shown much interest in *F. tularensis* since the 2001 bioterrorist attacks with anthrax in the U.S. because the biological agent is highly infectious and can be transmitted by aerosol, raising concerns about a potential use for biological warfare or terrorism (Sjöstedt, 2007; Keim et al., 2007). The usage of *F. tularensis* as a biological weapon was discovered shortly before and during World War II (Dennis et al., 2001). After the war, the U.S. developed techniques to employ aerosolized *F. tularensis* organisms as weapons until the BWC treaty came into force and stockpiled biological weapons had to be destroyed (Dennis et al., 2001). The former Soviet Union was also reported to have been engaged in the exploitation of *F. tularensis* as a biological weapon (Dennis et al., 2001; Alibek, 1999). In a bioterrorist attack, inhalation of aerosolized organisms or intake of contaminated water can result in fatal consequences with many casualties. Although a vaccine is available, it is not equally efficient for the different forms of tularemia and the two major pathogenic subspecies (Sjöstedt, 2007) and sufficient quantities may not be at hand in an actual attack because of the short incubation time. The CDC used a model developed by the World Health Organization (Anonymous, 1970) to predict the costs of a bioterrorist attack with *F. tularensis* (Sjöstedt, 2007). The outcome was that its extraordinary contagiousness, high morbidity and potential for large-scale dissemination would lead to very high costs. So, it is listed as a category A biothreat agent by the CDC, together with anthrax, *Clostridium botulinum* toxin, *Yersinia pestis*, smallpox and different viruses causing hemorrhagic fevers (Sjöstedt, 2007; Keim et al., 2007).

### 3.1.2. Classification

The genus *Francisella* belongs to the *Gammaproteobacteria* and is the only recognized genus in the family *Francisellaceae* exhibiting a unique cellular fatty acid composition (Sjöstedt, 2005a,b). Currently, the genus *Francisella* comprises the four species *Francisella tularensis*, *Francisella philomiragia*, *Francisella noatunensis* (syn.

*Francisella piscicida*), *Francisella hispaniensis* (proposed in the course of this thesis; Huber et al., 2010) and the recently proposed species *Francisella asiatica*. *F. tularensis* is divided into the subspecies *F. tularensis* subsp. *tularensis* (type A)*, F. tularensis* subsp. *holarctica* (former *palaearctica*, type B), *F. tularensis* subsp. *novicida* (former *F. novicida,* Huber et al., 2010) and *F. tularensis* subsp. *mediasiatica*. *F. tularensis* subsp. *holarctica* is divided into three biovars: biovar I $Ery^s$ (susceptible to erythromycin), biovar II $Ery^r$ (resistant to erythromycin) and biovar japonica (Hofer, 2002; Olsufjev and Meshcheryakova, 1983). *F. noatunensis* is divided into the subspecies *F. noatunensis* subsp. *noatunensis* and *F. noatunensis* subsp. *orientalis*.

The taxonomical classification of *F. tularensis* was based on the biochemical and genetic properties, virulence and geographical origin of strains (Sjöstedt, 2007). *F. tularensis* is endemic in the Northern Hemisphere and is especially common in North America, Russia and Japan (Ellis et al., 2002). However, outbreaks of tularemia were also reported from Spain, Switzerland, Turkey and Kosovo (Bachiller Luque et al., 1998; Wicki et al., 2000; Reintjes et al., 2002). Even in parts of Austria, the Czech Republic and Slovakia, *F. tularensis* could sporadically be isolated as the cause of human disease (Hofer et al., 1997). Generally, less virulent *F. tularensis* subsp. *holarctica* is predominantly isolated in Europe, Asia and North America, *Francisella tularensis* subsp. *mediasiatica* is mainly confined to Central Asia and *F. tularensis* subsp. *tularensis* is most abundant in North America (Ellis et al., 2002; Guryčivá, 1998). *F. tularensis* subsp. *novicida* is found rarely but worldwide and *F. philomiragia* is present in the Northern Hemisphere only (Keim et al., 2007). *F. philomiragia* was isolated from water samples and muskrats in Utah as well as from humans, *F. noatunensis* and *F. asiatica* were isolated from fish and *F. hispaniensis* from human blood and urine (Jensen et al., 1969; Hollis et al., 1989; Mikalsen et al., 2007; Mikalsen and Colquhoun, 2009; Escudero et al., 2010).

Different molecular typing methods identified two different phylogenetic groups within *F. tularensis* subsp. *tularensis*, A.I and A.II (Johansson et al., 2004; Garcia Del Blanco et al., 2002). The two groups are found in different geographic areas and altitudes; A.I is predominantly found in the eastern US and in California (same distribution as the tick vectors *Dermacentor variabilis* and *Amblyomma americanum* and the host *Sylvilagus floridanus*), A.II is found in the Rocky Mountain region of the western US (same distribution as the tick vectors *Dermacentor andersoni* and

*Chrysops discalis* and the host *Sylvilagus nuttallii*) (Farlow et al., 2005). Interestingly, A.I isolates were found to be of higher virulence to humans than A.II isolates (Staples et al., 2006).

### 3.1.3. Diagnosis

Diagnosis of tularemia is traditionally based on the detection of *F. tularensis*-specific antibodies in blood and does not discriminate between the pathogenic subspecies (Keim et al., 2007). The subspecies of *F. tularensis* are classically differentiated on the basis of their virulence, citrulline ureidase activity, glycerol and glucose fermentation (Ellis et al., 2002). The biovars I $Ery^s$, II $Ery^r$ and japonica of *F. tularensis* subsp. *holarctica* are distinguished based on their susceptibility to erythromycin and acid production from glycerol (Hofer, 2002). Their similarity in other characteristics like fatty acids (Jantzen et al., 1979), protein patterns (Sandström et al., 1988), expression of antigens (Nutter, 1971) and results of biochemical tests (Hollis et al., 1989) complicate the classification of *F. tularensis* field strains into different subspecies and biovars. Moreover, typing according to the phenotype is often inconsistent with modern genetic bacterial systematics and only partly applicable for the prediction of phylogenetic relationships (Keim et al., 2007). A further problem of traditional diagnosis is that the handling of human virulent strains poses a hazard and should be done at containment level 3 which is laborious and time-consuming. Therefore, rapid tools for identification and differentiation of *Francisella* based on molecular methods as well as highly discriminatory tools for epidemiological traceback are needed.

In the past decade, a range of different molecular typing methods with taxonomic ranges of discrimination from family to isolate level have been applied for *Francisella*: whole-genome DNA sequencing, 16S rRNA gene sequencing, DNA-DNA reassociation, ribotyping, whole-genome microarray, pulsed field gel electrophoresis (PFGE), insertion element-probed restriction fragment length polymorphism (RFLP), multilocus sequence typing (MLST), amplified fragment-length polymorphism (AFLP), multilocus variable number tandem repeat analysis (MLVA) and single nucleotide polymorphism (SNP) analysis (Keim et al., 2007). For differentiation between the species, subspecies, biovars and strains of *F. tularensis*, study of short-sequence tandem repeats (Johansson et al., 2002), insertion element based RFLP (Thomas et

al., 2003), automated ribotyping (Grif et al., 2003), MLST (Nübel et al., 2006), MLVA (Johansson et al., 2004; Farlow et al., 2001) and specific PCR-primers and probes (Forsman et al., 1994; Johansson et al., 2000; Broekhuijsen et al., 2003; Versage et al., 2003; Tomaso et al., 2006; Molins et al., 2009; Svensson et al., 2009) were employed. Although the high genomic similarity between the different subspecies of *F. tularensis* was frequently confirmed, studies based on DNA microarrays (Broekhuijsen et al., 2003; Samrakandi et al., 2004), proteom analysis (Hubálek et al., 2004) and a rep- and RAPD-PCR based typing approach (De La Puente-Redondo et al., 2000) have shown a certain degree of genomic heterogeneity that indicated the possibility to find specific markers for the development of rapid differentiating PCR-tests. These studies already showed interesting approaches to the identification of the examined *Francisella* strains, but the presented results do not convincingly allow for a rapid and reliable identification of *Francisella* field strains to the species, subspecies and biovar level. On the one hand, only a limited number of members of one or more of the different groups was included or representatives of one or more groups were left out in the studies, or, on the other hand, the methods were too time-consuming. Moreover, there was no clear indication of a reliable identification of the examined strains. Therefore, it is possible that the taxonomic status of the included strains was not adequately assured.

It was a goal of the present thesis to develop a PCR method considering all the aforementioned points of criticism and able to differentiate between all species, subspecies and biovars of *Francisella* in order to allow for a rapid typing of field strains and prevent misidentification of avirulent or low-virulent strains as virulent *F. tularensis*.

## 3.2. Description of *Francisella hispaniensis* sp. nov., isolated from human blood, reclassification of *Francisella novicida* (Larson et al. 1955) Olsufiev et al. 1959 as *Francisella tularensis* subsp. *novicida* comb. nov., and emended description of the genus *Francisella*

*International Journal of Systematic and Evolutionary Microbiology* (2010), 60, 1887–1896 DOI 10.1099/ijs.0.015941-0

# Description of *Francisella hispaniensis* sp. nov., isolated from human blood, reclassification of *Francisella novicida* (Larson *et al.* 1955) Olsufiev *et al.* 1959 as *Francisella tularensis* subsp. *novicida* comb. nov. and emended description of the genus *Francisella*

Birgit Huber,[1] Raquel Escudero,[2] Hans-Jürgen Busse,[1] Erik Seibold,[3] Holger C. Scholz,[3] Pedro Anda,[2] Peter Kämpfer[4] and Wolf D. Splettstoesser[3]

Correspondence
Hans-Jürgen Busse
hans-juergen.busse@vetmeduni.ac.at

[1]Institut für Bakteriologie, Mykologie und Hygiene, Veterinärmedizinische Universität, Veterinärplatz 1, A-1210 Wien, Austria

[2]Laboratorio de Espiroquetas y Patógenos Especiales, Centro Nacional de Microbiología Instituto de Salud Carlos III, Madrid, Spain

[3]Bundeswehr Institute of Microbiology, D-80937 Munich, Germany

[4]Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Giessen, D-35392 Giessen, Germany

Strain FhSp1$^T$, isolated from human blood in Spain in 2003, was studied for its taxonomic allocation. By 16S rRNA and *recA* gene sequencing, the strain was shown to belong to the genus *Francisella*. In the 16S rRNA gene sequence, *Francisella* sp. FhSp1$^T$ shared similarity of more than 99 % with strains of *Francisella tularensis* subspecies and *Francisella novicida* U112$^T$, 98 % with *Francisella piscicida* GM2212$^T$ and 98.4 % with *Francisella philomiragia* ATCC 25015$^T$. In the *recA* gene sequence, *Francisella* sp. FhSp1$^T$ exhibited 91.6–91.7 % similarity to strains of *F. tularensis* subspecies, 91.2 % to *F. novicida* U112$^T$ and 84 % to *F. philomiragia* ATCC 25017. The genus affiliation was supported by a quinone system typical of *Francisella* (Q-8 as the major component), a complex polar lipid profile similar to that of *F. tularensis* with the major components diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and an unknown aminophospholipid (APL4) and a fatty acid profile consisting mainly of $C_{10:0}$ (17.2 %), $C_{14:0}$ (11.2 %), $C_{16:0}$ (13.1 %), $C_{18:0}$ 3-OH (14.2 %) and $C_{18:1}\omega9c$ (7.1 %). DNA–DNA hybridization, which showed unambiguously that FhSp1$^T$ represents a novel species, and the results of biochemical tests allowed genotypic and phenotypic differentiation of the isolate from all hitherto-described *Francisella* species. A multiplex PCR developed in the course of this study discriminated FhSp1$^T$ from representatives of all other *Francisella* species and subspecies, clades A.I and A.II of *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* biovar japonica and also between these representatives of the genus. Therefore, we propose the name *Francisella hispaniensis* sp. nov., with the type strain FhSp1$^T$ (=FnSp1$^T$ =FSC454$^T$ =F62$^T$ =DSM 22475$^T$ =CCUG 58020$^T$). Furthermore, we formally propose the transfer of the species *Francisella novicida* to the species *Francisella tularensis* as *Francisella tularensis* subsp. *novicida* comb. nov. (type strain ATCC 15482$^T$ =CCUG 33449$^T$ =CIP 56.12$^T$). We also present an emended description of the genus *Francisella*.

*Francisella tularensis* is a facultatively intracellular bacterium, and all its subspecies cause the zoonotic disease tularaemia in humans and animals. *Francisella philomiragia* and *Francisella novicida* strains, although generally less virulent than strains of *F. tularensis*, may cause a tularaemia-like disease in immunocompromised patients (Hollis *et al.*, 1989).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *recA* sequences of strain FhSp1$^T$ are FN252413 and FN252412, respectively, and the accession number of the *recA* sequence of *F. tularensis* subsp. *tularensis* ATCC 6223$^T$ is FN545355.

The genus *Francisella*, together with *Wolbachia persica*, *Fangia hongkongensis* and *Caedibacter taeniospiralis*, represents one of, if not the deepest branch in the class *Gammaproteobacteria* (Forsman *et al.*, 1994; Lau *et al.*, 2007; Gao *et al.*, 2009). At the time of writing, the genus *Francisella* comprises four recognized species, *F. tularensis*, with the three subspecies *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *mediasiatica*, *F. novicida*, *F. philomiragia* and *F. noatunensis* (formerly *F. philomiragia* subsp. *noatunensis*), with the two subspecies *F. noatunensis* subsp. *noatunensis* and *F. noatunensis* subsp. *orientalis* (McCoy & Chapin, 1912; Larson *et al.*, 1955; Olsufiev *et al.*, 1959; Olsufjev & Meshcheryakova, 1983; Hollis *et al.*, 1989; Ottem *et al.*, 2009; Mikalsen *et al.*, 2007). A fifth species, *Francisella piscicida* (Ottem *et al.*, 2007), was proposed to represent a later heterotypic synonym of *F. noatunensis* subsp. *noatunensis* (Ottem *et al.*, 2009) based on high degrees of sequence similarities in certain housekeeping genes and results from DNA–DNA hybridizations slightly below the threshold (70 % reassociation) for species delineation. However, the reliability of these taxonomic rearrangements cannot be considered to be certain and, hence, we use the name *F. piscicida* in this contribution.

In several publications, including the chapter on *Francisella* in the second edition of *Bergey's Manual of Systematic Bacteriology* (Sjöstedt, 2005), *F. novicida* is named *Francisella tularensis* subsp. *novicida* (Sjöstedt, 2005), but this name is not formally correct; although Sjöstedt (2005) proposed the transfer of this species to *F. tularensis* as the subspecies '*F. tularensis* subsp. *novicida*', this name has never been validly published.

Strain FhSp1$^T$ was presumptively identified as a strain of *F. novicida* (Escudero *et al.*, 2003). However, recent analyses of five housekeeping genes and two genes encoding membrane proteins (Nübel *et al.*, 2006; Escudero *et al.*, 2010) as well as genomic fingerprinting using RAPD-PCR carried out in the course of our study (results not shown) revealed a clearly distant position of this strain from recognized *Francisella* species and subspecies. This uncertain taxonomic affiliation was further affirmed by the results of DNA typing methods employing 38 canonical insertion–deletion markers, in which strain FhSp1$^T$ failed in amplification of seven tested loci (Larsson *et al.*, 2007). Escudero *et al.* (2008) named strain FhSp1$^T$ an unusual *F. tularensis* strain. Here we report the classification of FhSp1$^T$ as a member of a novel species of *Francisella*, applying biochemical, physiological, chemotaxonomic and genetic characterization. Furthermore, we formally propose to transfer the species *F. novicida* to the species *F. tularensis* as *Francisella tularensis* subsp. *novicida* comb. nov.

FhSp1$^T$ showed distinct, convex, pale-white to grey colonies that reached maximum size (3–4 mm diameter) in 48 h on cysteine heart agar (HCA; BD) at 37 °C. Cysteine is not required for growth but enhances it. FhSp1$^T$ could also be grown on Columbia blood agar, whereas no growth occurred on MacConkey agar plates. Cell morphology was observed under an Axiostar microscope (Zeiss) at ×1000, with cells grown for 2 days at 37 °C on HCA. The pleomorphic and coccoid rods of FhSp1$^T$ stained faintly Gram-negative.

Amplification and sequencing of the 16S rRNA-encoding gene and *recA* were carried out as reported previously (Scholz *et al.*, 2006) using the primers listed in Table 1 (16S rRNA gene: 27f, 1492r, 341f, 518r, 926f; *recA*: recA_tul_f, recA_tul_r). Amplification of *recA* consisted of an initial denaturation step at 94 °C for 5 min and 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s and a final elongation step at 72 °C for 60 s. Primers recA_tul_f and recA_tul_r were constructed based on accessible genomic sequences of certain francisellae. 16S rRNA gene (1471 bp) and *recA* (965 bp) sequences of FhSp1$^T$ were compared to entries in the EMBL database using the FASTA33 search tool (Pearson & Lipman, 1988). Strain FhSp1$^T$ showed 99.7 % 16S rRNA gene sequence similarity to *F. novicida* U112$^T$ and *F. tularensis* subsp. *tularensis* SCHU S4 and strain FSC198, 99.5–99.6 % to other *F. tularensis* subspecies, 98.2–98.4 % to strains of *F. philomiragia*, including the type strain ATCC 25015$^T$, and 98.0 % to *F. piscicida* GM2212$^T$. The *recA* gene sequence of FhSp1$^T$ shared 91.2–91.7 % with the corresponding sequences of members of different *Francisella* subspecies, including *F. novicida* U112$^T$, and 84 % with that of *F. philomiragia* ATCC 25017 in 941 overlapping positions. *recA* sequences of recognized *Francisella* subspecies shared 99.9–100 % similarity with each other and 98.9–99.0 % with that of *F. novicida* U112$^T$. These data indicate a close relatedness of *F. novicida* to *F. tularensis* and a quite distant position of FhSp1$^T$ from recognized *Francisella* species. Subsequent analysis of the *recA* gene was performed using the software package MEGA version 4.0 (Tamura *et al.*, 2007) after multiple alignment of sequences by CLUSTAL_X (Thompson *et al.*, 1997). Distance options were according to the maximum composite likelihood model and clustering with the neighbour-joining method was performed by using bootstrap values based on 2000 replications. A *recA*-based tree is shown in Fig. 1. In further analyses, the relatedness of FhSp1$^T$ and *F. novicida* suggested by sequence similarities was confirmed.

For fatty acid, polar lipid and quinone analysis, cells were grown on HCA at 37 °C with 5 % $CO_2$ for 48 h, except *F. piscicida* DSM 18777$^T$, which was kept at 20 °C under ambient atmosphere, suspended in PBS, heat-killed and then lyophilized. Fatty acid methyl esters were prepared as described by Kämpfer & Kroppenstedt (1996).

The fatty acid profile of FhSp1$^T$ consisted of $C_{10:0}$ (17.2 %), $C_{18:0}$ 3-OH (14.2 %), $C_{16:0}$ (13.1 %), $C_{14:0}$ (11.2 %), $C_{18:1}\omega9c$ (7.1 %), $C_{24:1}$ (5.0 %), $C_{18:0}$ (3.8 %), $C_{16:0}$ 3-OH (2.8 %), $C_{10:0}$ 2-OH (2.7 %), $C_{22:0}$ (2.5 %), $C_{24:0}$ (2.5 %), $C_{20:0}$ (1.7 %), iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$ (1.4 %), anteiso-$C_{15:0}$ (1.6 %), iso-$C_{15:0}$ (1.1 %), $C_{12:0}$ (0.9 %), $C_{20:2}\omega6,9c$ (0.8 %), $C_{17:0}$ 3-OH

**Table 1.** Oligonucleotide primers used in this study and expected amplicon sizes when used in multiplex PCR

Primers were designed in this study unless indicated otherwise.

| Primer | Sequence (5′–3′) | Reference* | Amplicon size (bp) |
|---|---|---|---|
| **RAPD-PCR** | | | |
| RAPD1a | GTGCCGACGG | – | – |
| RAPD1b | GTGCCGACGGC | – | – |
| RAPD2a | ATAGCCGACG | – | – |
| RAPD2b | ATAGCCGACGC | – | – |
| RAPD3a | TCTGACCACC | – | – |
| RAPD3b | TCTGACCACCG | – | – |
| RAPD4 | CAGGCTTCCC | – | – |
| RAPD5 | CGGCAACGGG | – | – |
| RAPD6 | GACTGCGCAC | – | – |
| RAPD7 | GCGTCAGGAG | – | – |
| RAPD9 | AACAGGGCGG | – | – |
| RAPD10 | GGACGCGGTGC | – | – |
| RAPD11 | GCACGCATTGG | – | – |
| RAPD12-2 | GTTCGTGCGGT | – | – |
| RAPD13 | CCACCTCGCA | – | – |
| RAPD15 | TGTGGAGCCC | – | – |
| RAPD16 | GTTCGTGCGGT | – | – |
| RAPD17 | CGTTTCAATG | – | – |
| RAPD18 | GTTACATCAGTGC | – | – |
| RAPD19 | TCAACAAGGTG | – | – |
| RAPD20 | AAACATTATCTGC | – | – |
| RAPD21 | CAACCAGCTATAG | – | – |
| RAPD22 | CTTAAAAAGGTT | – | – |
| RAPD23 | AAGTTTGTTTGC | – | – |
| RAPD24 | CAAAGTTTGTTTGC | – | – |
| RAPD27 | AGTTTAGCTGG | – | – |
| RAPD28 | TTGCTGGGG | – | – |
| RAPD29 | AAGTTGTAGCTG | – | – |
| RAPD30 | CAGCACCAGC | – | – |
| **Multiplex PCR** | | | |
| RD1_new_f | GCACATACCTATTTTATACTTCCAACTTG | – | 629 (*F. tularensis* subsp. *holarctica*); 840 (*F. tularensis* subsp. *holarctica* bv. japonica); 1325 (*F. tularensis* subsp. *tularensis*) |
| RD1_r | GCCGAGTTTGATGCTGAAAA | 1 | |
| RD6_f | TGACCTGAGCCAGAGGTTAC | 3 | 369 |
| RD6_r | ACTTGCCAGCCTAATAATTAC | 3 | |
| F_nov_f | GCCATATAAAGGCAAAAGCTCCACTTC | – | 1395 |
| F_nov_r | GAGGGGATATGGCAATCATCTAGAGC | – | |
| F_med_f | GTCTTTGGTGGTCTTACGCCAATG | – | 1132 |
| F_med_r | GCTGGAATTACACTCTCTCTTAAATCCC | – | |
| F_philo_f | CGCACCCCAGTCTTTGCGATAG | – | 962 |
| F_philo_r | CGCTAAATAATTCTCTTCGTAGCGATTG | – | |
| F_pisc_f | GCGGTCTATCCTGTTGTCGAGAAG | – | 172 |
| F_pisc_r | TTGCTTGAAATATTGGAGAAGAAATGG | – | |
| F_62_f | GTTGATCGGCGATAGTACTTCATCT | – | 492 |
| F_62_r | CCAAAGATCACAAAGGCATTACCTAAG | – | |
| **Amplification and sequencing** | | | |
| *recA* | | | |
| recA_tul_f | CGCTAGAATCAGCCTTATCAC | – | 965 |
| recA_tul_r | TAAGCTCATCTTGAGTAACTGC | – | |
| 16S rRNA gene | | | |
| 27f | GCCCATTTGAGGGGGATACC | 2 | 1474 |
| 1492r | GGACTAAGAGTACCTTTTTGAGT | 2 | |

**Table 1.** cont.

| Primer | Sequence (5′–3′) | Reference* | Amplicon size (bp) |
|---|---|---|---|
| 341f | CCTACGGGAGGCAGCAG | 4 | – (Sequencing primer) |
| 518r | ATTACCGCGGCTGCTGG | 4 | – (Sequencing primer) |
| 926f | AAACTYAAAKGAATTGACGG | 2 | – (Sequencing primer) |

*1, Broekhuijsen *et al.* (2003); 2, Lane (1991); 3, Molins-Schneekloth *et al.* (2007); 4, Muyzer *et al.* (1993).

(0.7 %), anteiso-$C_{17:1}\omega9c$ (0.6 %), $C_{13:1}$ at 12–13 (0.5 %), anteiso-$C_{17:0}$ (0.4 %), $C_{9:0}$ (0.1 %) and an unidentified acid with an equivalent chain-length of 14.969. This fatty acid profile shares the majority of characteristics reported for different *F. tularensis* and *F. novicida* strains (Jantzen *et al.*, 1979; Hollis *et al.*, 1989), including relatively large amounts of $C_{10:0}$, $C_{14:0}$, $C_{16:0}$, $C_{24:1}$ and the long-chain hydroxy acid $C_{18:0}$ 3-OH and presence of significant amounts of $C_{10:0}$ 2-OH, $C_{16:0}$ 3-OH, $C_{20:0}$, $C_{22:0}$ and $C_{24:0}$. A fatty acid profile containing these components is unique among bacteria and, hence, it supports the assignment of FhSp1$^T$ to the genus *Francisella*.

Quinones were extracted and analysed by HPLC as described by Tindall (1990a) and Altenburger *et al.* (1996). The HPLC was equipped as described by Stolz *et al.* (2007). Strain FhSp1$^T$ exhibited a quinone system with the major compound ubiquinone Q-8 (98 %) and traces of Q-9 (2 %). The quinone system supports the affiliation of strain FhSp1$^T$ to the genus *Francisella*; several strains of the genus analysed for this trait also showed a quinone system with the major ubiquinone Q-8 (Hollis *et al.*, 1989). Our analysis of the quinone systems of strain FhSp1$^T$, *F. tularensis* subsp. *holarctica* strains F76 and ATCC 29684, *F. tularensis* subsp. *mediasiatica* F65, *F. tularensis* subsp. *tularensis* strains ATCC 6223$^T$ and SCHU S4, *F. tularensis* subsp. *novicida* ATCC 15482$^T$, *F. philomiragia* ATCC 25015$^T$ and *F. piscicida* DSM 18777$^T$ showed similar results. The main quinone was ubiquinone Q-8 (95–100 %), and minor amounts of ubiquinone Q-9 were also detected.

Polar lipids were extracted and analysed by two-dimensional TLC after Tindall (1990b). The polar lipid profile of strain FhSp1$^T$ was almost identical to that of *F. tularensis* subsp. *tularensis* ATCC 6223$^T$ (Fig. 2a), differing only by the absence of trace amounts of an unidentified aminophospholipid (APL2). The polar lipid profile of *F. tularensis* subsp. *tularensis* ATCC 6223$^T$ was composed of the major lipids phosphatidylethanolamine and diphosphatidylglycerol, moderate amounts of phosphatidylcholine and an unidentified aminophospholipid (APL4) and phospholipid (PL3) and minor to trace amounts of phosphatidylglycerol, unidentified phospholipids (PL1, PL2), an unidentified lipid with highly hydrophobic chromatographic behaviour (L) and unidentified aminophosphoglycolipids (APGL1–2), aminophospholipids (APL1–3) and an aminolipid (AL2). The polar lipid profile of *F. novicida* ATCC 15482$^T$ was indistinguishable from that of *F. tularensis* subsp. *tularensis* ATCC 6223$^T$ (not shown). The most striking difference in the polar lipid profile of *F. philomiragia* ATCC 25015$^T$ was the absence of aminophospholipid APL4 (Fig. 2b). Furthermore, aminophospholipid APL2 was not detectable, but minor amounts of an unidentified glycolipid (GL) and an unidentified aminolipid (AL1) were found. *F. piscicida* DSM 18777$^T$ exhibited a polar lipid profile most similar to that of *F. philomiragia* subsp. *philomiragia* ATCC 25015$^T$ but with trace amounts of APL2 and no detectable APGL2, AL1, AL2 and PL1 (not shown). These polar lipid profiles reflect quite well the close phylogenetic relationship between FhSp1$^T$ and *F. tularensis* subsp. *tularensis* on the one hand and between *F. philomiragia* ATCC 25015$^T$ and *F. piscicida* DSM 18777$^T$ on the other.

Metabolic fingerprinting was done using the Biolog MicroLog system (Gram-negative identification test panel GN2 MicroPlates) according to the instructions of the manufacturer. All strains were first grown on HCA at 37 °C

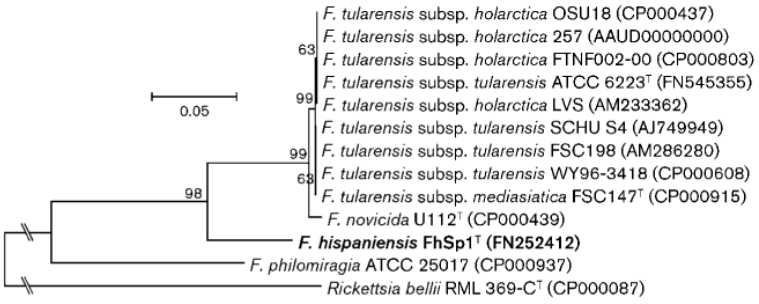


**Fig. 1.** Phylogenetic tree reconstruction with *recA* sequences (941–944 bp) using MEGA neighbour-joining analysis. Bar, 0.05 substitutions per nucleotide position. The significance of branches is indicated by bootstrap percentages calculated for 2000 subsets. The *recA* sequence of *Rickettsia bellii* RML 369-C$^T$ was used as an outgroup.

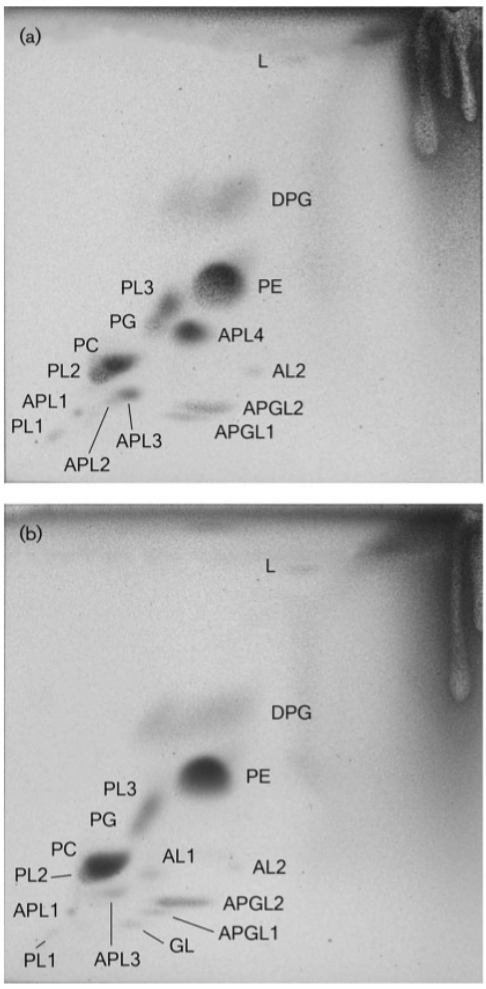


**Fig. 2.** Total polar lipid profiles of *F. tularensis* subsp. *tularensis* ATCC 6223$^T$ (a) and *F. philomiragia* ATCC 25015$^T$ (b) after two-dimensional TLC and detection with molybdatophosphoric acid. DPG, Diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; AL1–2, unknown aminolipids; PL1–3, unknown phospholipids; APL1–4, unknown aminophospholipids; APGL1–2, unknown aminophosphoglycolipids; GL, unknown glycolipid; L, unknown polar lipid.

with 5 % $CO_2$, except for *F. piscicida* DSM 18777$^T$, which was kept at 20 °C under ambient atmosphere. All strains were tested at least three times in independent test series. MICs of antimicrobial agents were determined by Etest (AB Biodisk; Tec-Laim) on HCA supplemented with 9 % chocolatized red blood cells (CHAB). From 48 h cultures on CHAB plates, cells of strain FhSp1$^T$ were resuspended in physiological saline to achieve a turbidity equivalent to that of a McFarland standard 0.5 and applied to the agar with the help of a sterile swab before application of Etest strips. MICs were read after 48 h of incubation at 37 °C in an atmosphere with 5 % $CO_2$. For quality assurance, each batch of plates was tested with the control strains *F. tularensis* subsp. *tularensis* ATCC 6223$^T$ and *F. tularensis* subsp. *holarctica* NCTC 10857$^T$.

Results of physiological and biochemical tests and susceptibility tests for antimicrobial agents are given in Table 2 or listed in the species description. Strain FhSp1$^T$ differed from *F. novicida* ATCC 15482$^T$ in 13 traits and from the other type strains of recognized *Francisella* species and subspecies in at least 12 traits.

For environmental studies aiming at identifying potential zoonotic reservoirs of *F. tularensis* or related species as well as in epidemiology, rapid differentiation of bacterial strains belonging to the genus *Francisella* is mandatory. Known differences in virulence between the distinct subspecies of *F. tularensis* may be critical for the clinical management of tularaemia patients. Moreover, the unusual natural resistance of strains affiliated to the genus *Francisella* against a wide range of antibiotics necessitates the rapid identification of these strains in critically ill people. There are several lines of evidence to show that immunocompromised patients in particular are prone to infections with uncommon *Francisella* strains that are not detected or identified by classical microbiological methods (Kugeler *et al.*, 2008) and which might even occur in regions that were not considered to be endemic for *F. tularensis* or related species (Leelaporn *et al.*, 2008).

PCR-based identification of *Francisella* strains at the genus, species and subspecies levels has been developed by Forsman *et al.* (1994) on the basis of differences in the 16S rRNA gene. Johansson *et al.* (2000) designed primers to differentiate between *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* isolates in a multiplex PCR that, however, needs further analyses in order to distinguish between these subspecies and *F. tularensis* subsp. *mediasiatica* and *F. novicida*. Broekhuijsen *et al.* (2003) developed a PCR based on the region of difference RD1, which was identified by a genome-wide DNA microarray analysis. This PCR allows discrimination between all three subspecies of *F. tularensis*, *F. novicida* and *F. tularensis* subsp. *holarctica* biovar japonica with one primer pair. However, it cannot discriminate between the two genotypes of *F. tularensis* subsp. *tularensis*, *F. philomiragia*, *F. piscicida* and FhSp1$^T$ and, since the characteristic amplicon that identifies *F. novicida* is larger than 3000 bp, the PCR assay is suboptimal. In the course of this study, examinations were carried out in order to develop a comprehensive multiplex-PCR assay that allows convenient and fast discrimination between *Francisella* subspecies and species, including FhSp1$^T$.

PCR template DNA was prepared by three cycles of freeze–thawing (Wieser & Busse, 2000) from heat-inactivated biomass. In order to find genomic differences suitable for the design of specific primers, comparative genomic fingerprinting was carried out with representatives of each

**Table 2.** Differential metabolic characteristics of strain FhSp1$^T$ and the type strains of other francisellae

Strains: 1, FhSp1$^T$; 2, *F. novicida* ATCC 15482$^T$; 3, *F. philomiragia* ATCC 25015$^T$; 4, *F. piscicida* DSM 18777$^T$; 5, *F. tularensis* subsp. *tularensis* ATCC 6223$^T$; 6, *F. tularensis* subsp. *mediasiatica* GIEM543; 7, *F. tularensis* subsp. *holarctica* GIEM503$^T$. The tests were carried out with the Biolog GN2 MicroPlate system; tests with *F. piscicida* DSM 18777$^T$ were carried out with 24 h of incubation at 20 °C; other tests were carried out for 24 h at 37 °C. +, Positive; −, negative; (+), weakly positive. All strains were positive for oxidation of D-fructose, L-serine, L-proline, L-glutamic acid, D-mannose, pyruvic acid methyl ester and α-D-glucose. All strains were negative for oxidation of water (control), α-cyclodextrin, Tweens 40 and 80, *N*-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, i-erythritol, L-fucose, gentiobiose, *myo*-inositol, lactose, lactulose, D-mannitol, melibiose, methyl *β*-D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, turanose, xylitol, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, *γ*-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, glucuronamide, L-histidine, L-leucine, L-phenylalanine, D-serine, DL-carnitine, urocanic acid, phenylethylamine, putrescine, 2-aminoethanol and 2,3-butanediol. Identification results according to Biolog were *F. philomiragia* for strains 1–4 and *F. tularensis* for strains 5–7.

| Test | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| **Activity of:** | | | | | | | |
| Catalase | (+) | (+) | + | + | + | + | (+) |
| Oxidase | + | − | + | − | − | − | − |
| **Oxidation of:** | | | | | | | |
| Dextrin | + | − | − | − | − | − | − |
| Glycogen | + | − | − | − | − | − | − |
| *N*-Acetyl-D-glucosamine | + | + | + | + | + | − | + |
| Cellobiose | + | + | − | − | − | − | − |
| D-Galactose | + | + | − | − | − | + | − |
| Maltose | − | − | + | − | − | − | − |
| Sucrose | + | + | + | − | − | − | − |
| Trehalose | + | − | + | − | − | − | − |
| Succinic acid monomethyl ester | + | + | + | + | − | + | + |
| Acetic acid | + | − | − | − | − | − | − |
| α-Hydroxybutyric acid | + | − | + | − | − | − | − |
| *β*-Hydroxybutyric acid | + | + | + | − | − | − | − |
| α-Ketobutyric acid | + | + | + | − | + | + | + |
| α-Ketoglutaric acid | + | − | − | − | − | − | − |
| DL-Lactic acid | + | + | + | − | − | − | + |
| Succinic acid | + | + | − | − | − | − | − |
| Bromosuccinic acid | + | − | − | − | − | − | − |
| Succinamic acid | − | − | + | − | − | − | − |
| L-Alaninamide | + | + | + | + | − | − | − |
| D-Alanine | + | + | + | − | − | − | − |
| L-Alanine | + | + | + | + | − | + | + |
| L-Alanyl glycine | + | + | + | + | − | − | − |
| L-Asparagine | + | + | + | + | − | − | + |
| L-Aspartic acid | + | + | + | − | − | − | + |
| Glycyl L-aspartic acid | + | − | − | − | − | − | − |
| Glycyl L-glutamic acid | + | + | + | − | − | − | − |

**Table 2.** cont.

| Test | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Hydroxy-L-proline | + | − | − | − | − | − | − |
| L-Ornithine | + | + | − | − | − | − | − |
| L-Pyroglutamic acid | + | + | + | − | − | − | − |
| L-Threonine | + | + | + | − | − | − | + |
| *γ*-Aminobutyric acid | + | − | − | − | − | − | − |
| Inosine | + | + | + | − | − | − | − |
| Uridine | + | + | + | − | − | − | + |
| Thymidine | + | + | + | − | − | − | + |
| Glycerol | + | + | + | − | + | + | − |
| DL-α-Glycerol phosphate | + | + | + | − | + | + | + |
| α-D-Glucose 1-phosphate | + | − | + | − | − | − | − |
| D-Glucose 6-phosphate | + | − | + | − | − | − | − |

*Francisella* taxon (results not shown). Primers used for RAPD-PCR fingerprinting and their sequences are listed in Table 1. Reactions were carried out in a final volume of 25 µl containing 1× Green GoTaq Flexi buffer, 2 mM $MgCl_2$, 200 µM each dNTP, 0.6 U GoTaq DNA polymerase (Promega), 8 µM RAPD primer and approximately 5 ng DNA. Reactions were performed using an initial 5 min denaturation step at 95 °C followed by 45 cycles of 1 min at 95 °C, 1 min at 32–38 °C and 2 min at 72 °C and a final extension step of 5 min at 72 °C. The resolution of bands was improved by using a ramp rate of 0.3 °C $s^{-1}$. Amplification products (5 µl each) were separated by 1.5 % (w/v) agarose gel electrophoresis in 1× TAE running buffer at a constant voltage of 110 V for at least 90 min. Fragments were visualized with UV light after staining with ethidium bromide. Bands of interest were excised from the gel and DNA was eluted with the QIAquick gel extraction kit according to the instructions of the manufacturer (Qiagen). PCR products were cloned using the Promega pGEM-T Easy vector system and *Escherichia coli* strain DH10B according to the manufacturer's manual. Plasmid insert sequences (sequencing was done at VBC-genomics, Vienna) were subjected to database searches and compared to genome sequences of *Francisella* strains (accession nos AM233362, CP000803, CP000437, AJ749949, CP000439, AM286280 and CP000608).

Primers used in the multiplex PCR were constructed based on comparative genomic fingerprinting and information from the literature. By means of RAPD-PCR, sequence fragments potentially suitable for the design of specific primers were identified and sequenced for strains of *F. piscicida* (RAPD12 band of 201 bp) and *F. philomiragia* (RAPD6 band of 1259 bp) and FhSp1$^T$ (RAPD13 band of 770 bp) (not shown). For *F. tularensis* subsp. *mediasiatica*, a specific primer pair was designed using a variable region that was detected after comparison of larger sequence regions of the *F. tularensis* subsp. *mediasiatica* genome sequence with other accessible *Francisella* genome sequences from the databases. The region of difference RD1 described by Broekhuijsen *et al.* (2003) provided a

primer pair that resulted in specific products for *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica*, including biovar japonica [the reverse primer was taken from Broekhuijsen *et al.* (2003) and the forward primer was redesigned], and was also used to design a primer pair specific for *F. novicida* (for amplicon sizes, see Table 3). The primer pair RD6_f/r (Molins-Schneekloth *et al.*, 2007) allowed differentiation between *F. tularensis* subsp. *tularensis* A.I and A.II by lack of the *F. tularensis* species-specific band of 369 bp in ATCC 6223$^T$ (A.II).

The primer pairs were tested in individual PCRs (results not shown) and then integrated into one multiplex-PCR assay. The 25 µl reaction mixture consisted of 5 µl 5× buffer, 2.5 µl $MgCl_2$, 0.5 µl dNTPs, 0.2 µl each of primers RD1_new_f, RD1_r, F_nov_f and F_nov_r, 0.1 µl each of primers RD6_f, RD6_r, F_pisc_f, F_pisc_r, F_philo_f, F_philo_r, F_62_f, F_62_r, F_med_f and F_med_r, 0.12 µl Promega GoTaq polymerase and 0.5 µl template DNA. Reactions were performed in an Eppendorf Mastercycler using an initial 5 min denaturation step at 95 °C followed by 35 cycles of 1 min at 95 °C, 1 min at 60 °C and 1 min 30 s at 72 °C and a final extension step of 5 min at 72 °C. The products were separated in a 1.5 % agarose gel as described above, stained with ethidium bromide and visualized with UV light. Applying the developed multiplex PCR, all test strains could be correctly identified (Table 3 and Fig. 3). The assay uses 14 primers

**Table 3.** *Francisella* strains examined in this study and their multiplex PCR profiles

| Strain | Taxon as received | Multiplex PCR profile (bp) |
|---|---|---|
| ***F. tularensis* subsp. *holarctica*** | | |
| F124$^T$ =GIEM503$^T$ | *F. tularensis* subsp. *holarctica* | 369, 629 |
| F1 | *F. tularensis* subsp. *holarctica* | 369, 629 |
| F4 | *F. tularensis* subsp. *holarctica* BVII | 369, 629 |
| F7 | *F. tularensis* subsp. *holarctica* BVII | 369, 629 |
| F9 | *F. tularensis* subsp. *holarctica* BVII | 369, 629 |
| F11 | *F. tularensis* subsp. *holarctica* BVII | 369, 629 |
| F12 | *F. tularensis* subsp. *holarctica* BVII | 369, 629 |
| F13 | *F. tularensis* subsp. *holarctica* BVII | 369, 629 |
| F17 | *F. tularensis* subsp. *holarctica* BVII | 369, 629 |
| F19 | *F. tularensis* subsp. *holarctica* BVII | 369, 629 |
| F33 | *F. tularensis* subsp. *holarctica* BVII | 369, 629 |
| F57 | *F. tularensis* subsp. *holarctica* | 369, 629 |
| F76 | *F. tularensis* subsp. *holarctica* | 369, 629 |
| F89 | *F. tularensis* subsp. *holarctica* BVI | 369, 629 |
| F88 | *F. tularensis* subsp. *holarctica* BVI | 369, 629 |
| F100 | *F. tularensis* subsp. *holarctica* | 369, 629 |
| F101 | *F. tularensis* subsp. *holarctica* | 369, 629 |
| F49 =ATCC 29684 | *F. tularensis* subsp. *holarctica* LVS | 369, 629 |
| F119 =FSC021 | *F. tularensis* subsp. *holarctica* bv. japonica | 369, 840 |
| F120 =FSC022 | *F. tularensis* subsp. *holarctica* bv. japonica | 369, 840 |
| ***F. tularensis* subsp. *tularensis*** | | |
| F66$^T$ =ATCC 6223$^T$ =GIEM Schu$^T$ | *F. tularensis* subsp. *tularensis*, avirulent; A.II | 1325 |
| FSC237 =SCHU S4 | *F. tularensis* subsp. *tularensis*; A.I | 369, 1325 |
| ***F. tularensis* subsp. *mediasiatica*** | | |
| F63$^T$ =GIEM543$^T$ | *F. tularensis* subsp. *mediasiatica* | 369, 1132 |
| F65 | *F. tularensis* subsp. *mediasiatica* | 369, 1132 |
| ***F. tularensis* subsp. *novicida*** | | |
| F61$^T$ =ATCC 15482$^T$ | *F. tularensis* subsp. *novicida* | 369, 1395 |
| F59 | *F. tularensis* subsp. *novicida* | 369, 1395 |
| F60 | *F. tularensis* subsp. *novicida* | 369, 1395 |
| ***F. hispaniensis* sp. nov.** | | |
| FhSp1$^T$ =FnSp1$^T$ =F62$^T$ =FSC454$^T$ =DSM 22475$^T$ =CCUG 58020$^T$ | *F. hispaniensis* | 492 |
| ***F. philomiragia*** | | |
| F51$^T$ =ATCC 25015$^T$ | *F. philomiragia* | 962 |
| F50 =ATCC 25017 | *F. philomiragia* | 962 |
| ***F. piscicida*** | | |
| F165$^T$ =DSM 18777$^T$ | *F. piscicida* | 172 |

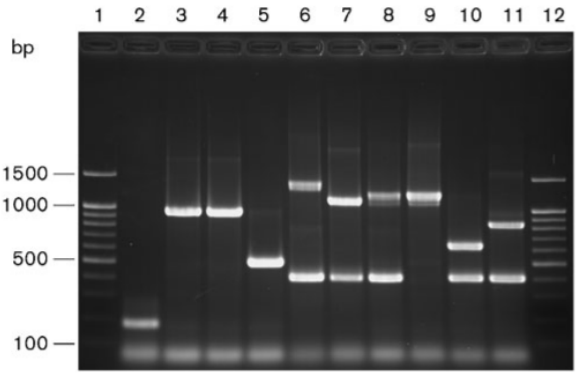


**Fig. 3.** Multiplex PCR results. Lanes: 1 and 12, 100 bp and 1.5 kb markers; 2, *F. piscicida* DSM 18777$^T$; 3, *F. philomiragia* ATCC 25017; 4, *F. philomiragia* ATCC 25015$^T$; 5, *F. hispaniensis* sp. nov. FhSp1$^T$; 6, *F. novicida* ATCC 15482$^T$; 7, *F. tularensis* subsp. *mediasiatica* GIEM543$^T$; 8, *F. tularensis* subsp. *tularensis* SCHU S4 (A.I); 9, *F. tularensis* subsp. *tularensis* ATCC 6223$^T$ (A.II); 10, *F. tularensis* subsp. *holarctica* GIEM503$^T$; 11, *F. tularensis* subsp. *holarctica* bv. japonica F119. Aliquots of 5 µl PCR product were loaded onto the 1.5 % agarose gel and visualized after staining with ethidium bromide.

and allows the differentiation of all *Francisella* species and subspecies including biovar japonica of *F. tularensis* subsp. *holarctica* and biovars A.I and A.II of *F. tularensis* subsp. *tularensis*.

Strain FhSp1$^T$ was shown to be distant from the other established *Francisella* species by sequence analyses of different housekeeping genes (Nübel *et al.*, 2006) as well as in RAPD-PCR and the developed multiplex PCR. Hence, these results indicate that this strain is not a representative of *F. novicida* or of any other recognized *Francisella* species, but a representative of a novel species of the genus *Francisella*.

In order to support the rank of a separate species, DNA reassociation experiments were carried out comparing FhSp1$^T$ with the type strains of two *F. tularensis* subspecies, *F. novicida* ATCC 15482$^T$, *F. philomiragia* ATCC 25015$^T$ and *F. piscicida* DSM 18777$^T$. DNA–DNA hybridization experiments were carried out according to Ziemke *et al.* (1998). Nick translation was done with 2 µg DNA for 90 min at 15 °C. High-molecular-mass DNA was extracted and purified according to Pitcher *et al.* (1989). DNA concentrations were estimated chemically according to Richards (1974). The results are means from at least six hybridizations. In hybridization experiments, strain FhSp1$^T$ exhibited the following levels of reassociation: 52 % (9.7 % SD), reciprocal 54 % (4.7 % SD), with *F. novicida* ATCC 15482$^T$; 61 % (8.4 % SD), reciprocal 35 % (3 % SD), with *F. tularensis* subsp. *mediasiatica* GIEM543$^T$; 57 % (7.3 % SD), reciprocal 38 % (7.5 % SD) with *F. tularensis* subsp. *holarctica* GIEM503$^T$; 39 % (7.7 % SD), reciprocal 43 % (5.9 % SD), with *F. philomiragia* ATCC 25015$^T$; and 47 % (3.4 % SD), reciprocal 28 % (6.2 % SD), with *F. piscicida* DSM 18777$^T$. DNA–DNA hybridization was not carried out successfully with the type strain of *F. tularensis* subsp. *tularensis* because DNA from repeated purifications of differently grown biomass was heavily fragmented. However, hybridizations between all other subspecies of *F. tularensis* and *F. novicida* have shown that the subspecies of *F. tularensis* and *F. novicida* exhibit reassociation values higher than 70 %, demonstrating that they belong to a single species (Hollis *et al.*, 1989). Hence, the strains of *F. tularensis* employed in our hybridization experiments are useful for reference and demonstrate unambiguously that FhSp1$^T$ can be considered as a representative of an as-yet undescribed species of *Francisella*.

The data presented here (polar lipids, quinone system, fatty acids) as well as molecular data from Nübel *et al.* (2006), Larsson *et al.* (2007) and Escudero *et al.* (2010) demonstrate that strain FhSp1$^T$ is a member of the genus *Francisella*. Results from DNA–DNA hybridization and unique physiological traits show that FhSp1$^T$ is a representative of a novel *Francisella* species. Therefore, we propose a novel *Francisella* species, *Francisella hispaniensis* sp. nov., to accommodate strain FhSp1$^T$.

The taxonomic status of *F. novicida* as one of four *Francisella* species has been questioned repeatedly. A range of different molecular data, including results of DNA–DNA hybridizations, has demonstrated a relatedness to *F. tularensis* at the species level. Sjöstedt (2005) discussed the matter in detail and proposed to reclassify *F. novicida* (Larson *et al.* 1955) Olsufiev *et al.* 1959 as '*F. tularensis* subsp. *novicida*'. This new combination is used in several papers; however, this combination has not been validly published by publication in the *International Journal of Systematic and Evolutionary Microbiology* or by inclusion in a Validation List to satisfy Rule 27 of the Bacteriological Code and, hence, has no standing in nomenclature.

Here, we propose to reclassify *F. novicida* as a subspecies of *F. tularensis*, *Francisella tularensis* subsp. *novicida* comb. nov., and therefore give the already widely adopted classification a formal basis. Furthermore, we provide an emended description of the genus *Francisella*.

### Emended description of the genus *Francisella* Dorofe'ev 1947

*Francisella* (Fran.ci.sel'la. N.L. dim. ending *-ella*; N.L. fem. n. *Francisella* named after Edward Francis, American bacteriologist, who extensively studied the aetiological agent and pathogenesis of tularaemia and is credited with naming the disease).

In addition to the description of the genus provided by Sjöstedt (2005), species of the genus are characterized by a complex polar lipid profile consisting of the major

components diphosphatidylglycerol and phosphatidylethanolamine, moderate to major quantities of phosphatidylcholine and minor amounts of phosphatidylglycerol, three unidentified phospholipids, an aminolipid, two aminophosphoglycolipids and two aminophospholipids. Additional lipids may be present. The quinone system is ubiquinone Q-8. The type species is *Francisella tularensis*.

### Description of *Francisella hispaniensis* sp. nov.

*Francisella hispaniensis* (his.pa.ni.en′sis. L. fem. adj. *hispaniensis* from Hispania, the Latin name for Spain, the country where the type strain was isolated).

Cells are pleomorphic or coccoid rods (approx. 0.5 × 1.5 μm). Gram-negative, oxidase-positive and showing weak catalase activity. Good growth occurs on HCA and Columbia blood agar at 20–42 °C with an optimum at 37 °C. Forms distinct, mucoid, pale-white to grey colonies within 24 h with a diameter of approx. 1–2 mm on HCA. Resistant to meropenem, amoxicillin plus clavulanic acid, ampicillin and aztreonam; susceptible to chloramphenicol, tetracycline, doxycycline, gentamicin, tobramycin, streptomycin, nalidixic acid, ciprofloxacin, levofloxacin, erythromycin and rifampicin. Does not produce indole or $H_2S$. Negative in the Voges–Proskauer test, nitrate reduction and growth on triple-sugar iron agar. Gelatin and aesculin are not hydrolysed. Negative for arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase, urease and β-galactosidase. The quinone system consists of Q-8 and traces of Q-9. Predominant polar lipids are phosphatidylethanolamine and phosphatidylcholine, as well as an unknown *F. tularensis*-specific aminophospholipid (APL4). Other polar lipids are diphosphatidylglycerol, phosphatidylglycerol, unknown phospholipids (PL1–3), an unknown lipid (L) and some unknown aminophosphoglycolipids (APGL1–2), aminophospholipids (APL1–2) and aminolipids (AL2). The fatty acid profile is mainly composed of the major compounds $C_{10:0}$, $C_{14:0}$, $C_{16:0}$ and the hydroxy acid $C_{18:0}$ 3-OH, as well as minor amounts of $C_{18:1}\omega 9c$, $C_{18:0}$, $C_{16:0}$ 3-OH and $C_{10:0}$ 2-OH. Results of biochemical tests are indicated in Table 2.

The type strain, FhSp1$^T$ (=FnSp1$^T$ =FSC454$^T$ =F62$^T$ =DSM 22475$^T$ =CCUG 58020$^T$), was isolated in Spain in 2003 from blood of a patient suffering severe septicaemia secondary to acute obstructive pyelonephritis (Escudero *et al.*, 2010).

### Description of *Francisella tularensis* subsp. *novicida* comb. nov.

*Francisella tularensis* subsp. *novicida* (no.vi.ci′da. L. adj. *novus* new; L. suff. *-cida* from L. v. *caedo* to cut, kill; N.L. n. *novicida* new killer).

Basonym: *Francisella novicida* (Larson *et al.* 1955) Olsufiev *et al.* 1959.

Other synonym: '*Pasteurella novicida*' Larson *et al.* 1955.

The species shares the characteristics listed in the emended genus description. Further characteristics were summarized by Larson *et al.* (1955) and Sjöstedt (2005). Biochemical characteristics determined with the Biolog GN2 MicroPlate are shown in Table 2.

The type strain, ATCC 15482$^T$ =CCUG 33449$^T$ =CIP 56.12$^T$, was isolated from a water sample from Ogden Bay, Utah, USA, in 1951 (Larson *et al.*, 1955).

## Acknowledgements

This study was supported by a Contract-Research Project of the German Ministry of Defence (grant no. M-SAB1-5-A005) and grant EM03/06 from Instituto de Salud Carlos III, Spain.

## References

**Altenburger, P., Kämpfer, P., Makristathis, A., Lubitz, W. & Busse, H.-J. (1996).** Classification of bacteria isolated from a medieval wall painting. *J Biotechnol* **47**, 39–52.

**Broekhuijsen, M., Larsson, P., Johansson, A., Byström, M., Eriksson, U., Larsson, E., Prior, R. G., Sjöstedt, A., Titball, R. W. & Forsman, M. (2003).** Genome-wide DNA microarray analysis of *Francisella tularensis* strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent *F. tularensis* subsp. *tularensis*. *J Clin Microbiol* **41**, 2924–2931.

**Escudero, R., Elía, M., Sáez-Nieto, J. A., Herrera, L., Galán, J. A., Ruiz, M., Menéndez, V., Royo, G. & Anda, P. (2003).** Preliminary characterization of a human isolate of *Francisella novicida*. In *Abstracts of the 4th International Conference on Tularemia*, 15–18 September 2003, Bath, UK, p. 30.

**Escudero, R., Toledo, A., Gil, H., Kovácsová, K., Rodríguez-Vargas, M., Jado, I., García-Amil, C., Lobo, B. & Bhide, M. (2008).** Molecular method for discrimination between *Francisella tularensis* and *Francisella*-like endosymbionts. *J Clin Microbiol* **46**, 3139–3143.

**Escudero, R., Elía, M., Sáez-Nieto, J. A., Menéndez, V., Toledo, A., Royo, G., Rodríguez-Vargas, M., Whipp, M. J., Gil, H. & other authors (2010).** A possible novel *Francisella* genomic species isolated from blood and urine of a patient with severe illness. *Clin Microbiol Infect* **16**, 1026–1030.

**Forsman, M., Sandstrom, G. & Sjöstedt, A. (1994).** Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. *Int J Syst Bacteriol* **44**, 38–46.

**Gao, B., Mohan, R. & Gupta, R. S. (2009).** Phylogenomics and protein signatures elucidating the evolutionary relationships among the *Gammaproteobacteria*. *Int J Syst Evol Microbiol* **59**, 234–247.

**Hollis, D. G., Weaver, R. E., Steigerwalt, A. G., Wenger, J. D., Moss, C. W. & Brenner, D. J. (1989).** *Francisella philomiragia* comb. nov. (formerly *Yersinia philomiragia*) and *Francisella tularensis* biogroup *novicida* (formerly *Francisella novicida*) associated with human disease. *J Clin Microbiol* **27**, 1601–1608.

**Jantzen, E., Berdal, B. P. & Omland, T. (1979).** Cellular fatty acid composition of *Francisella tularensis*. *J Clin Microbiol* **10**, 928–930.

**Johansson, A., Ibrahim, A., Göransson, I., Eriksson, U., Gurycova, D., Clarridge, J. E. & Sjöstedt, A. (2000).** Evaluation of PCR-based methods for discrimination of *Francisella* species and subspecies and development of a specific PCR that distinguishes the two major subspecies of *Francisella tularensis*. *J Clin Microbiol* **38**, 4180–4185.

**Kämpfer, P. & Kroppenstedt, R. M. (1996).** Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42**, 989–1005.

**Kugeler, K. J., Mead, P. S., McGowan, K. L., Burnham, J. M., Hogarty, M. D., Ruchelli, E., Pollard, K., Husband, B., Conley, C. & other authors (2008).** Isolation and characterization of a novel *Francisella* sp. from human cerebrospinal fluid and blood. *J Clin Microbiol* **46**, 2428–2431.

**Lane, D. J. (1991).** 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.

**Larson, C. L., Wicht, W. & Jellison, W. L. (1955).** A new organism resembling *P. tularensis* isolated from water. *Public Health Rep* **70**, 253–258.

**Larsson, P., Svensson, K., Karlsson, L., Guala, D., Granberg, M., Forsman, M. & Johansson, A. (2007).** Canonical insertion-deletion markers for rapid DNA typing of *Francisella tularensis*. *Emerg Infect Dis* **13**, 1725–1732.

**Lau, K. W. K., Ren, J., Fung, M.-C., Woo, P. C. Y., Yuen, K.-Y., Chan, K. K. M., Qian, P.-Y., Wong, P.-K. & Wu, M. (2007).** *Fangia hongkongensis* gen. nov., sp. nov., a novel gammaproteobacterium of the order *Thiotrichales* isolated from coastal seawater of Hong Kong. *Int J Syst Evol Microbiol* **57**, 2665–2669.

**Leelaporn, A., Yongyod, S., Limsrivanichakorn, S., Yungyuen, T. & Kiratisin, P. (2008).** *Francisella novicida* bacteremia, Thailand. *Emerg Infect Dis* **14**, 1935–1937.

**McCoy, G. W. & Chapin, C. W. (1912).** Further observations on a plague-like disease of rodents with a preliminary note on the causative agent, *Bacterium tularense*. *J Infect Dis* **10**, 61–72.

**Mikalsen, J., Olsen, A. B., Tengs, T. & Colquhoun, D. J. (2007).** *Francisella philomiragia* subsp. *noatunensis* subsp. nov., isolated from farmed Atlantic cod (*Gadus morhua* L.). *Int J Syst Evol Microbiol* **57**, 1960–1965.

**Molins-Schneekloth, C. R., Belisle, J. T. & Petersen, J. M. (2007).** Genomic markers for differentiation of *Francisella tularensis* subsp. *tularensis* A.I and A.II. *Appl Environ Microbiol* **74**, 336–341.

**Muyzer, G., de Waal, E. C. & Uitterlinden, A. G. (1993).** Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**, 695–700.

**Nübel, U., Reissbrodt, R., Weller, A., Grunow, R., Porsch-Özcürümez, M., Tomaso, H., Hofer, E., Splettstoesser, W., Finke, E.-J. & other authors (2006).** Population structure of *Francisella tularensis*. *J Bacteriol* **188**, 5319–5324.

**Olsufiev, N. G., Emelyanova, O. S. & Dunaeva, T. N. (1959).** Comparative studies of strains of *B. tularense* in the Old and New World and their taxonomy. *J Hyg Epidemiol Microbiol Immunol* **3**, 138–149.

**Olsufjev, N. G. & Meshcheryakova, I. S. (1983).** Subspecific taxonomy of *Francisella tularensis* McCoy and Chapin 1912. *Int J Syst Bacteriol* **33**, 872–874.

**Ottem, K. F., Nylund, A., Karlsbakk, E., Friis-Møller, A., Krossoy, B. & Knappskog, D. (2007).** New species in the genus *Francisella* (Gammaproteobacteria; Francisellaceae); *Francisella piscicida* sp. nov. isolated from cod (*Gadus morhua*). *Arch Microbiol* **188**, 547–550.

**Ottem, K. F., Nylund, A., Karlsbakk, E., Friis-Møller, A. & Kamaishi, T. (2009).** Elevation of *Francisella philomiragia* subsp. *noatunensis* Mikalsen *et al.* (2007) to *Francisella noatunensis* comb. nov. [syn. *Francisella piscicida* Ottem *et al.* (2008) syn. nov.] and characterization of *Francisella noatunensis* subsp. *orientalis* subsp. nov., two important fish pathogens. *J Appl Microbiol* **106**, 1231–1243.

**Pearson, W. R. & Lipman, D. J. (1988).** Improved tools for biological sequence comparison. *Proc Natl Acad Sci U S A* **85**, 2444–2448.

**Pitcher, D. G., Saunders, N. A. & Owen, R. J. (1989).** Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* **8**, 151–156.

**Richards, G. M. (1974).** Modifications of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. *Anal Biochem* **57**, 369–376.

**Scholz, H. C., Tomaso, H., Al Dahouk, S., Witte, A., Schloter, M., Kämpfer, P., Falsen, E. & Neubauer, H. (2006).** Genotyping of *Ochrobactrum anthropi* by *recA*-based comparative sequence, PCR-RFLP, and 16S rRNA gene analysis. *FEMS Microbiol Lett* **257**, 7–16.

**Sjöstedt, A. B. (2005).** Genus I. *Francisella* Dorofe'ev 1947, 176$^{AL}$. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2, part B, pp. 200–210. Edited by D. J. Brenner, N. R. Krieg, J. T. Staley & G. M. Garrity. New York: Springer.

**Stolz, A., Busse, H.-J. & Kämpfer, P. (2007).** *Pseudomonas knackmussii* sp. nov. *Int J Syst Evol Microbiol* **57**, 572–576.

**Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007).** MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

**Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

**Tindall, B. J. (1990a).** A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* **13**, 128–130.

**Tindall, B. J. (1990b).** Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199–202.

**Wieser, M. & Busse, H.-J. (2000).** Rapid identification of *Staphylococcus epidermidis*. *Int J Syst Evol Microbiol* **50**, 1087–1093.

**Ziemke, F., Höfle, M. G., Lalucat, J. & Rosselló-Mora, R. (1998).** Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.

# 4. Genus *Yersinia*

## 4.1. Introduction

### 4.1.1. Plague and yersiniosis

*Yersinia pestis* is the most prominent member of the genus, since it is the causative agent of one of the most ancient recognized human diseases, plague. It is a small, Gram-stain negative, non-motile, non-spore forming, facultative intracellular pathogen found in rodents and their fleas (Bottone et al., 2005). In humans the pathogen can cause three main forms of plague, bubonic, septicemic and pneumonic plague, which have a high mortality rate if untreated (Bossi et al., 2004c). Bubonic plague is the most common clinical form and characterised by swollen lymph nodes, the so-called buboes (Perry and Fetherston, 1997). After injection of *Y. pestis* via an infective flea bite, the microorganisms can multiply intracellularly in macrophages and spread in the host through the lymphatic system, accompanied by fever, headache, nausea and other unspecific symptoms (Pujol and Bliska, 2005; Perry and Fetherston, 1997). Then, the bacteria proliferate extracellularly at regional lymph nodes and cause their inflammation which leads to the characteristic buboes within 2-8 days (Bottone et al., 2005). The disease can turn into a severe secondary plague septicemia, pneumonia or meningitis and cause 40-60% lethality if not treated with antibiotics (Perry and Fetherston, 1997). Primary septicemic plague is a bacteremia without noticeable swelling of lymph nodes and symptoms resemble those of septicemias caused by other Gram-stain negative bacteria. In an advanced stage, septicemic plague may lead to necrotic fingers, toes, nose or lips (‘Black Death’) (Bossi et al., 2004c). The mortality rate for this form of plague is 30-50% because diagnosis is often very difficult (Perry and Fetherston, 1997). The third clinical form is primary pneumonic plague. The lungs are invaded by the microorganisms and the disease can spread from person to person or animals to humans through respiratory droplets (Bottone et al., 2005). This aggressive and deadly form of the disease can cause death in less than four days with a >90% lethality if untreated and thus a primary pneumonic plague epidemic would be devastating (Bottone et al., 2005; Perry and Fetherston, 1997).

In contrast, *Y. pseudotuberculosis*, although showing a strikingly high degree of DNA relatedness to *Y. pestis* (Bercovier et al., 1980a), and *Y. enterocolitica* cause much milder, enteric illnesses in humans that seldom lead to death and are mostly transmitted by the fecal-oral route. Like *Y. pestis*, *Y. pseudotuberculosis* is an intracellular parasite that reaches the lymphatic system (Bottone et al., 2005). Classical *Y. pseudotuberculosis* infection in humans causes gastroenteritis with mesenteric lymphadenitis that may be confused with appendicitis (Ochoa and Cleary, 2006). Symptoms include abdominal pain, fever, scarlatiniform skin rash, conjunctivitis and erythema nodosum, only sometimes diarrhea and enterocolitis (Ochoa and Cleary, 2006; Krober et al., 1983). In immunocompromised hosts, the infection can lead to a severe septicemia and other complications (Ochoa and Cleary, 2006). *Y. enterocolitica*, the third pathogenic *Yersinia* species, causes similar gastrointestinal manifestations in humans, including mesenteric lymphadenitis, pseudoappendicitis and also septicemia (Bercovier et al., 1980b; Wormser and Keusch, 1981). Acute enteritis in children is the most frequent representation (Bottone et al., 2005). The major animal reservoir for *Y. enterocolitica* strains that cause human illness is pigs, but strains of this species can also be isolated from many other animals (Hurvell, 1981). Infection is most often acquired by eating contaminated food or drinking contaminated unpasteurized milk or untreated water (Hurvell, 1981).

The pathogenicity of *Y. pestis* and the other human pathogenic *Yersinia* species is ascribed to virulence factors coded on the chromosome as well as on plasmids (Brubaker, 1991). A lot of virulence factors are coded on a 70 kb plasmid, pLCR/pYV/pYVe/pIB1 or pCD1 in *Y. pestis*, that is present in many but not all strains of human pathogenic *Yersinia* species (Ben-Gurion and Shafferman, 1981; Eppinger et al., 2007). It encodes the type III secretion system and effectors, the so-called Yops (*Yersinia* outer proteins) that are secreted into the host cell and hamper its phagocytic mechanisms and immune response (Trosky et al., 2008). *Y. pestis* contains two additional unique virulence plasmids, pMT1/pFra and pPCP1/pPst, or a chimeric plasmid pMT-PCP, combining the two in *Y. pestis* strain Angola (Eppinger et al., 2010), that contribute to the higher virulence of this species and are missing in the other pathogenic yersiniae (Filippov et al., 1990). The 100 kb plasmid pMT1 encodes different virulence factors, including the fraction 1 (F1) capsular antigen and murine toxin (Lindler, 2004). The second *Y. pestis*-specific 9.6 kb plasmid, pPCP1,

encodes the gene for the plasminogen activator Pla that is necessary for the spread of the bacterium in the host tissue (Sodeinde and Goguen, 1988; Huang et al., 2006).

The heightened virulence of *Y. pestis* for humans compared to *Y. pseudotuberculosis* and *Y. enterocolitica* is an important field of research and there has recently been facilitated a more thorough insight into *Yersinia* evolution and pathogenicity, since the genomes of several *Y. pestis* and *Y. pseudotuberculosis* strains were sequenced and compared (Parkhill et al., 2001; Deng et al., 2002; Chain et al., 2004; Song et al., 2004; Zhou et al., 2004a; Chain et al., 2006; Eppinger et al., 2007; Garcia et al., 2007; Zhang et al., 2009; Eppinger et al., 2010; Shen et al., 2010). DNA microarray analyses have also contibuted to a better understanding of *Yersinia* virulence (Hinchliffe et al., 2003; Zhou et al., 2004b; Han et al., 2005; Wang et al., 2007). A high degree of gene inactivation, gene loss, IS-mediated genome rearrangements and the acquisition of chromosomal regions uniquely conserved in *Y. pestis* strains might have led to changes that may also be responsible for the increased pathogenicity of this species (Chain et al., 2004). Especially the frequent genome rearrangements are thought to have provided a selective advantage for the adaption to host environments. Recent genome analyses have revealed a higher genetic diversity within the species *Y. pseudotuberculosis* and *Y. pestis* as previously thought (Eppinger et al., 2007; 2010). Eppinger et al. (2010) stated that “growing evidence suggests that the plasmid repertoire of *Y. pestis* is not restricted to the three classical virulence plasmids, and that its genetic inventory is partly shared, either due to common ancestry or lateral exchange, with other human and zoonotic bacterial pathogens, such as *Salmonella enterica* [...], *Klebsiella pneumoniae*, and *Streptococcus equi.*”

Whereas the two moderately pathogenic members of the genus, *Y. enterocolitica* and *Y. pseudotuberculosis*, cause infections with a low case-fatality rate and are not known to have been used in biological warfare, *Y. pestis* was probably already used against the enemy during the Middle Ages by throwing the corpses of plague victims over town walls (Wheelis, 1999). During World War II, Japanese used ceramic bombs with fleas carrying the bubonic plague and there are also accounts for water contamination and the distribution of food contaminated with plague bacteria (Harris, 1994; Barenblatt, 2004). *Y. pestis* appears to be a probable agent for a bioterrorist attack and is therefore listed as a CDC category A biological threat agent (http://www.bt.cdc.gov/agent/agentlist-category.asp). The release of aerosolised cells

could cause an outbreak of primary plague pneumonia (Bossi et al., 2004c). Although plague bacteria are not very resistant and only live for a short time when released in the air, they are highly virulent and can therefore infect humans as well as rodents effectively, the fatality rate being high, especially in regard to plague pneumonia (Bossi et al., 2004c; Rakin, 2003). Moreover, the worldwide extent of plague endemic-areas makes them easily accessible and great amounts can be cultured with minimal equipment (Rakin, 2003). The incubation period is short (1-7 days), suspensions can be easily aerosolised and infections show similar symptoms to other diseases like tuberculosis, melioidosis, tularemia or glanders (Rakin, 2003). Strains containing plasmids carrying multiple-antibiotic resistance (Galimand et al., 1997; Guiyoule et al., 2001) pose an additional threat in case of a bioterrorist event involving *Y. pestis*. The genetic diversity of *Y. pestis* could also be exploited for a bioterrorist usage in regard to heightened virulence or hindered diagnosis, treatment and prevention (Anisimov et al., 2004). An effective and broad plague immunization without side effects has not been developed yet but there is active research in this field (Titball and Williamson, 2001; Anisimov et al., 2004; Bubeck and Dube, 2007).

### 4.1.2. Classification

The genus *Yersinia* of the family *Enterobacteriaceae* currently consists of 14 species (Euzeby, 2010), *Y. aleksiciae* (Sprague and Neubauer, 2005), *Y. massiliensis* (Merhej et al., 2008) and *Y. similis* (Sprague et al., 2008) being the most recently described species. The three well-established pathogens *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* are known to cause the more or less severe diseases in humans described above and are therefore the most studied and prominent members of the genus. Because of their clinical relevance and the bioterroristic potential of *Y. pestis*, *Y. pseudotuberculosis* and *Y. pestis* were also a major focus of this thesis.

*Yersinia pestis* was originally described by Lehmann and Neumann (1896) as *Bacterium pestis*. Based on the phenotypical differences in glycerol fermentation, nitrate reduction and ammonium oxidation, Devignat (1951) divided this species into the three biovars Antiqua, Medievalis und Orientalis, a classification which has recently been challenged (Achtman et al., 2004b; Chain et al., 2006; Anisimov et al., 2004; Zhou et al., 2004c). Strains of biovar Antiqua were predominately isolated in

Asia, East and Central Africa, strains of biovar Medievalis in Central Asia and strains of biovar Orientalis worldwide. An atypical group of *Y. pestis* strains is named 'Pestoides'. It is different from the main group by its virulence to rodents and the ability to ferment rhamnose and melibiose (Martinevskij, 1969). Different molecular biological studies have shown that it is a heterogeneous group and that strains of this group are partly biovar Antiqua, Medievalis or not to be typed (Klevytska et al., 2001; Motin et al., 2002; Radnedge et al., 2001; 2002). Recently, the novel *Y. pestis* biovar Microtus was described which can be discriminated from the other groups because it cannot ferment arabinose, is exclusively pathogenic for small rodents and has a unique profile of deleted genes and pseudogenes (Zhou et al., 2004c).

Examinations in the field of population genetics (applying MLST, analysis of SNPs, MLVA, IS*100* typing, O-antigen typing) indicated that *Y. pestis* is a relatively young species that developed out of *Yersinia pseudotuberculosis* about 10000 – 40000 years ago (Achtman et al., 1999; Achtman, 2004a; Kotetishvili et al., 2005; Skurnik et al., 2000; Paradis et al., 2005). This close relationship between *Y. pestis* and *Y. pseudotuberculosis* had already been shown by Bercovier et al. (1980a) based on the results of DNA-DNA hybridization and by Ibrahim et al. (1993) and Trebesius et al. (1998) through analysis of the 16S rRNA gene sequences. According to the currently effective species concept, *Y. pestis* and *Y. pseudotuberculosis* are, in fact, members of a single species, since their DNAs reassociate at a level greater than 70% (Wayne et al., 1987).

### 4.1.3. Diagnosis

A classical laboratory diagnosis of plague is based on staining techniques (Gram, Giemsa, Wright or Wayson) subjected to strains isolated from blood, sputum, bubo aspirate, cerebrospinal fluid and scrapings from skin lesions, biochemical tests, antigen detection, IgM enzyme immunoassay, immunostaining, direct fluorescent antibody testing, antibiotic susceptibility testing, hemagglutination and specific phage lysis (Inglesby et al., 2000; Anisimov et al., 2004). Since these methods are often time-consuming and the handling of *Y. pestis* is hazardous for the laboratory personnel and has to be done in a safety level 3 laboratory, many studies have aimed at the development of rapid, specific and sensitive typing methods that require a minimum of infectious sample handling.

In addition to rather epidemiologically applied MLST, VNTR, MLVA, AFLP, RFLP, SNP and IS*100* analyses (Kotetishvili et al., 2005; Pourcel et al., 2004; Klevytska et al., 2001; Adair et al., 2000; Achtman et al., 1999; Radnedge et al., 2002; Chain et al., 2006; Motin et al., 2002), different PCR techniques for a reliable detection of *Y. pestis* were developed. These include PCR and nested PCR for the detection of the plasminogen activator (Pla) gene (Hinnebusch and Schwan, 1993; Engelthaler et al., 1999; Campbell et al., 1993), *yopB* (Khushiramani et al., 2006), *caf1* and *pla* (Norkina et al., 1994) and real-time assays targeting *pst* (Iqbal et al., 2000), *pla* (Higgins et al., 1998; Loïez et al., 2003) and *fur* (Gabitzsch et al., 2008), multiplex-PCR with different primer pairs that target the chromosomal DNA and the three virulence plasmids (Tsukano et al., 1996; Leal and de Almeida, 1999; Woron et al., 2006), separate PCR assays for detection of chromosomal DNA and the three virulence plasmids (Neubauer et al., 2000a), detection of *Y. pestis* signature genes for an unknown hypothetical protein, a putative prophage protein and methyltransferase (Zhou et al., 2004b), detection of a *Y. pestis*-specific 41.7 bp chromosomal region absent in *Y. pseudotuberculosis* (Radnedge et al., 2001) and a multiplex real-time PCR for simultaneous detection of chromosomal DNA and the *Y. pestis*-specific virulence plasmids, including an internal control for the PCR (Tomaso et al., 2003). Recently, two multiplex real-time PCR assays for the detection of a chromosomal target and all three virulence plasmids were developed (Woron et al., 2006; Stewart et al., 2008). Increasing the possibility of false-negative results, most PCR assays mentioned can not unambiguously detected DNA of *Y. pseudotuberculosis*, except for the ones developed by Radnedge et al. (2001), Chase et al. (2005) and Matero et al. (2009). In only one study, the atypical group Pestoides was considered (Tomaso et al., 2003). None of the studies included specific markers for the novel *Y. pestis* biovar Microtus or atypical strains of *Y. pseudotuberculosis*, e.g. strains that cause FESLF in humans. This was the impulse for a closer examination of three atypical *Y. pseudotuberculosis* strains showing major metabolic characteristics of *Y. pestis* in the course of this thesis. The goal was to develop a PCR assay to differentiate between these atypical *Y. pestis*-like strains and *Y. pestis* in order to rapidly and reliably rule out highly pathogenic *Y. pestis* strains and subsequent risk for human health.

## 4.2. A triplex PCR for differential detection of *Yersinia pestis*-like atypical strains of *Yersinia pseudotuberculosis*

### 4.2.1. Abstract

The aim of this study was to develop a gel-based PCR test that can identify a group of atypical *Yersinia pseudotuberculosis* strains, sharing physiological characteristics with *Yersinia pestis*. A set of *Y. pestis* and *Y. pseudotuberculosis* strains was examined for genomic polymorphisms employing rep- and RAPD-PCRs. Genomic differences possibly suitable for the design of specific PCR primers were detected by comparison of genomic fingerprints and examined by excising differentiating PCR products from the gel, cloning them in *E. coli*, sequencing and comparing them with available *Yersinia* genome sequences from the databases. Applying this strategy, three pairs of novel PCR primers could be developed which were integrated into a single assay that proved to be specific and robust. Hence, the newly developed triplex PCR assay is a useful tool to rapidly and reliably detect the atypical *Y. pestis*-like strains of *Y. pseudotuberculosis* and simultaneously differentiate them from *Y. pestis* and other *Y. pseudotuberculosis* strains.

### 4.2.2. Introduction

*Yersinia pestis* and *Yersinia pseudotuberculosis* are pathogenic for both, animals and humans. Whereas *Y. pseudotuberculosis* is a less severe, mostly enteric and rarely fatal pathogen, *Y. pestis* is the causative agent of plague (Bottone et al., 2005) and is of special current interest due to its bioterrorist potential. DNA reassociation experiments showed that *Y. pestis* and *Y. pseudotuberculosis* can be considered to be members of a single species applying the threshold of 70% in DNA-reassociation (Wayne et al., 1987) and combining the two species in a single species as *Y. pseudotuberculosis* subsp. *pseudotuberculosis* and *Y. pseudotuberculosis* subsp. *pestis* was proposed (Bercovier et al., 1980a). Based on practical concerns for human welfare, it was later decided to maintain *Y. pestis* and *Y. pseudotuberculosis* as separate nomenclatural species (Judicial Commission of the International Committee on Systematic Bacteriology, 1985). The close relationship could also be confirmed by results from multi-locus sequence typing as reported by Achtman et al.

(1999) who then concluded that *Y. pestis* was a clone of *Y. pseudotuberculosis.* Close relatedness is also reflected by only few physiological differences between the two species useful for discrimination. Isolates are typically differentiated based on motility at 28°C, urease activity, acid production from L-rhamnose, melibiose and their growth rate on nutrient agar (Bercovier et al., 1980a; Bottone et al., 2005).

Recently, we examined three *Y. pseudotuberculosis* strains (Y716, Y718, Y722) which exhibited major metabolic characteristics of *Y. pestis* when tested using the miniaturized *Yersinia* identification system (Merlin) as described in Neubauer et al. (2000b). Like *Y. pestis,* but unlike *Y. pseudotuberculosis,* these biochemically atypical *Y. pseudotuberculosis* strains were not able to ferment L-rhamnose at 28°C, a determinant feature at this temperature, and were negative in tests for L-pyroglutamic acid-β-naphthylamide. 35 out of 36 tested strains of *Y. pseudotuberculosis* fermented melibiose at 28°C unlike *Y. pestis* biogroups Antiqua and Orientalis and the atypical *Y. pseudotuberculosis* strains. However, in contrast to the *Y. pestis*-like biochemical profile, MLST analysis (Mark Achtman, genes: adk, argA, aroA, glnA, thrA, tmk, trpE, http://mlst.ucc.ie/mlst/dbs/Ypseudotuberculosis) confirmed strains Y716, Y718 and Y722 as *Y. pseudotuberculosis* with sequence types ST19, ST2 and ST19, respectively, whereas *Y. pestis* always shows the profile 1-2-1-2-5-2-10 (H.C. Scholz, unpublished data). Growth rate on nutrient agar is also typical for *Y. pseudotuberculosis*.

Several PCR-based techniques have been developed in the past which allow identification of *Y. pestis* (Campbell et al., 1993; Hinnebusch and Schwan, 1993; Norkina et al., 1994; Tsukano et al., 1996; Leal and de Almeida, 1999; Engelthaler et al., 1999; Neubauer et al., 2000a; Radnedge et al., 2001; Zhou et al., 2004b; Khushiramani et al., 2006) and more recently this set of methods was supplemented by real-time PCR techniques (Higgins et al., 1998; Iqbal et al., 2000; Tomaso et al., 2003; Loïez et al., 2003; Chase et al., 2005; Woron et al., 2006; Gabitzsch et al., 2008; Stewart et al., 2008; Matero et al., 2009). Hence, the atypical *Y. pseudotuberculosis* strains were also examined by real-time PCR as described in Tomaso et al. (2003) to rule out the presence of *Y. pestis*-specific virulence factors. As a matter of fact, the most characteristic *Y. pestis* virulence associated genes *pla* on plasmid pPCP1 (encoding the plasminogen activator) and *caf1* on plasmid pMT1 (encoding the fraction 1 capsular antigen) could not be detected in this analysis (H.C. Scholz, unpublished data). However, a negative response does not completely rule

out the risk of false-negative results since plasmids are sometimes lost. Also the more stable chromosomal markers for the identification of *Y. pestis* do not entirely eliminate the possibility of getting a false-negative result if there is PCR inhibition or malfunction and even internal amplification controls are not always totally reliable.

Hence, facing their deviant physiological characteristics, which may lead to misidentification as *Y. pestis* in a preliminary approach, a reliable and rapid method for a correct identification of these atypical *Y. pseudotuberculosis* strains and their simultaneous differentiation from the severe pathogen *Y. pestis* and the other *Y. pseudotuberculosis* strains is desirable. Therefore, we carried out examinations in order to develop a cost-effective, simple and rapid PCR assay that allows differential detection of the *Y. pestis*-like atypical strains of *Y. pseudotuberculosis*.

### 4.2.3. Materials and methods

*Bacterial strains and culture conditions*

The *Yersinia* strains used in this study are listed in Table 7. All except *Y. pestis* strains were cultivated on 3.3x PYE agar (10 $g \cdot L^{-1}$ peptone, 10 $g \cdot L^{-1}$ yeast extract) at 28°C. *Y. pestis* strains were grown on Columbia agar at 28°C. Strains Y716, Y718 and Y722 were kindly provided by Dr. Alexander Rakin (Max v. Pettenkofer Institut, München).

*Preparation of DNA*

DNA was prepared from pure cultures of *Y. pestis* as described previously (Scholz et al., 2008a). DNA of *Y. pseudotuberculosis* and the other *Yersinia* species was released subjecting a cell suspension in sterile water to three cycles of freezing in liquid nitrogen and thawing at 65°C (Wieser and Busse, 2000). Released DNA in the supernatant obtained after short centrifugation was used as template in PCR.

*Genomic fingerprinting and identification of molecular markers*

Since previous studies employing rep-PCR and RAPD analysis of *Yersinia* strains showed differences in the fingerprinting patterns down to the strain level (Kim et al., 2003; Makino et al., 1994), we decided to examine our set of *Yersinia* strains with these methods in order to identify signature regions useful for differentiation. The

RAPD primers used in this study are listed in Table 8 and in Huber et al. (2009). RAPD reactions were carried out in a final volume of 25 µl containing 1x Green GoTaq® Flexi Buffer, 2 mM $MgCl_2$ (Promega), 200 µM of each dNTP (Bio Products), 0.6 U of GoTaq® DNA polymerase (Promega), 8 µM primer (4 µM each when two primers were used), and approximately 5 ng of DNA. Reactions were performed using an initial 5 min denaturation step at 95°C, followed by 45 cycles of 1 min at 95°C, 1 min at an appropriate annealing temperature, 2 min at 72°C, and a final extension step of 5 min at 72°C. The annealing temperature was varied according to the melting temperatures of the primers (32-42°C). The resolution of banding patterns was improved by using a ramp rate of 0.3°C $sec^{-1}$. Repetitive sequence-based PCRs with ERIC and REP primers as well as BOXA1R (Table 8) were carried out as described in Louws et al. (1994).

**Table 7.** *Yersinia* strains used in this study, species affiliation and resulting banding patterns after triplex PCR

| Strain | Species | PCR result (bp) |
|---|---|---|
| Y784 | *Y. pestis* | 275, 364 |
| NCTC 2028 | *Y. pestis* | 275, 364 |
| NCTC 8775 | *Y. pestis* | 275, 364 |
| NCTC 8779 | *Y. pestis* | 275, 364 |
| NCTC 10029 | *Y. pestis* | 275, 364 |
| Kuma | *Y. pestis* bv. Antiqua | 275, 373 |
| Yokohama | *Y. pestis* bv. Antiqua | 275, 373 |
| M23 | *Y. pestis* bv. Orientalis | 275, 364 |
| TS | *Y. pestis* bv. Orientalis | 275, 364 |
| G8786 | *Y. pestis* bv. Pestoides | 275, 364 |
| Y111$^T$ = ATCC 29833$^T$ | *Y. pseudotuberculosis* | 364, 490 |
| Y117 | *Y. pseudotuberculosis* | 364, 490 |
| Y262 | *Y. pseudotuberculosis* | 364, 490 |
| Y36 | *Y. pseudotuberculosis* | 364, 490 |
| Y716 = PTB22 = 79WA486 | atypical *Y. pseudotuberculosis* | 355 |
| Y718 = H706/86 | atypical *Y. pseudotuberculosis* | 355 |
| Y722 = 776/88 | atypical *Y. pseudotuberculosis* | 355 |
| DSM 13030$^T$ | *Y. enterocolitica* subsp. *palearctica* | - |
| ATCC 29473$^T$ | *Y. ruckeri* | - |
| ATCC 33638$^T$ | *Y. kristensenii* | - |
| DSM 14987$^T$ | *Y. aleksiciae* | - |
| CCUG 52882$^T$ | *Y. similis* | - |

Amplification products (5 µl from each reaction mixture) were separated by 1.5% (w/v) agarose gel electrophoresis in 1x Tris/acetic acid/EDTA running buffer at a constant voltage of 110 V for at least 90 min. Fragments were visualized with UV light after staining with ethidium bromide. Interesting bands were excised from the gel and DNA was eluted with the QIAquick® Gel Extraction Kit according to the manufacturer's instructions (Qiagen). PCR products were cloned using the Promega pGEM®-T Easy Vector System and *Escherichia coli* strain DH10B according to the manufacturers' manual. Inserts were sequenced at VBC-Genomics (Vienna) and resulting sequences were subjected to database searches (EMBL and GenBank) and compared to the available *Yersinia* genome sequences (acc. nos.: CP000720, CP000308, AL590842, CP000950, AE017042, CP001048, BX936398, CP000668, CP000305, AE009952, CP000901).

**Table 8.** List of oligonucleotide primers used for PCR amplification in the novel triplex assay and for genomic fingerprinting. Primers that produced decisive differences in genomic DNA fingerprints are indicated in bold.

| Primer | Oligonucleotide sequence (5'→3') | Reference |
|---|---|---|
| Y_pspest_f | CATGTACTCGCAATGGAATG | this study |
| Y_pspest_r | ACATCCGTGACAAGGTGTG | this study |
| Y_pest_f | GCATTGATGAGCCTAGTATTTATAAGG | this study |
| Y_pest_r | TTCAAAGCCATAGTTAAAGTG | this study |
| Y_pstub_f | CACGCTAAGTTACAGTAGCG | this study |
| Y_pstub_r | CTCAGATTCGTCAATATCC | this study |
| ERIC2 | AAG TAA GTG ACT GGG GTG AGC G | Versalovic et al. (1991) |
| ERIC1R | ATG TAA GCT CCT GGG GAT TCA C | Versalovic et al. (1991) |
| REP2-I | ICG ICT TAT CIG GCC TAC | Versalovic et al. (1991) |
| REP1R-I | III ICG ICG ICA TCI GGC | Versalovic et al. (1991) |
| **BOXA1R** | **CTACGGCAAGGCGACGCTGACG** | Versalovic et al. (1994) |
| RAPD17 | CGT TTC AAT G | this study |
| RAPD18 | GTT ACA TCA GTG C | this study |
| **RAPD19** | **TCAACAAGGTG** | this study |
| RAPD20 | AAA CAT TAT CTG C | this study |
| RAPD21 | CAA CCA GCT ATA G | this study |
| RAPD22 | CTT AAA AAG GTT | this study |
| RAPD23 | AAA TTT GTT TGC | this study |
| RAPD24 | CAA AGT TTG TTT GC | this study |
| RAPD25 | AGT TTG TTT GC | this study |
| RAPD27 | AGT TTA GCT GG | this study |
| RAPD28 | TTG CTG GGG | this study |
| RAPD29 | AAG TTG TAG CTG | this study |
| RAPD30 | CAG CAC CAG C | this study |

*Triplex polymerase chain reaction assay and cycling conditions*

Primers are listed in Table 8. Each 25 µl PCR standard reaction contained 1× GoTaq® Flexi Buffer (Promega) with 2 mM $MgCl_2$ (Promega), 200 µM dNTPs (Bio Products), 0.75 U Promega GoTaq® polymerase, 5 ng *Yersinia* DNA and 0.5 µM of the primers Y_pspest_f, Y_pspest_r and 1 µM of the primers Y_pest_f, Y_pest_r, Y_pstub_f, Y_pstub_r. A negative control containing no template DNA was included in the set of reactions. Amplification was carried out in an Eppendorf Mastercycler with an initial denaturation step of 95°C for 3 min, followed by 35 cycles of 95°C for 30 sec, 59°C for 30 sec and 72°C for 30 sec, and a final extension step of 72°C for 5 min. Aliquots of 3-5 µl of PCR product were analyzed by electrophoresis in 1.5% (w/v) agarose gels that were stained with ethidium bromide.

*Analytical sensitivity and specificity of the triplex PCR assay*

The analytical sensitivity of the PCR assay was evaluated after the procedure described in del Cerro et al. (2002) using *Y. pseudotuberculosis* type strain Y111$^T$ and the atypical *Y. pseudotuberculosis* strain Y716. One milliliter of a PYE stationary-phase culture of each strain was centrifuged for 5 min at 12000×g, the pellet was resuspended in 50 µl of distilled water and cells were lysed by three cycles of freeze-thawing (Wieser and Busse, 2000). The cell debris was removed by centrifugation for 5 min at 12000×g, and 3 µl of serial tenfold dilutions of the supernatant were used in triplex reactions to estimate the detection limit. To enumerate the bacteria, serial dilutions of the same pure cultures were prepared and 100 µl aliquots of the respective dilutions were spread onto PYE agar in duplicates. The specificity of the assay was tested with cell lysates of several *Y. pestis* and *Y. pseudotuberculosis* strains as well as other *Yersinia* species (Table 7). Performance of the assay was assessed by using different thermocyclers (Eppendorf Mastercycler, MWG-Biotech Primus, Perkin Elmer GeneAmp PCR System 2400) and different polymerases (Promega GoTaq® DNA polymerase, GE Healthcare illustra™ puReTaq Ready-To-Go PCR Beads, Bio Products Taq Polymerase One).

4.2.4. Results

*Genomic fingerprinting, analysis of sequences and primer design*

In the course of this study, 33 different 9- to 15-mer RAPD primers and combinations out of them were tested, as well as repetitive sequence-based BOXA1R, ERIC and REP primers (for sequences and references see Table 8). The results of genomic fingerprinting confirmed the close relationship of *Y. pestis* and *Y. pseudotuberculosis*, since major differences in the banding patterns were moderate among the two species. However, minor strain specific differences could often be observed, but it was difficult to find specific marker bands for a coherent group of strains that distinguished it from another group. Only primers BOXA1R and RAPD19 showed bands that could successfully be exploited to clearly distinguish *Y. pestis* from *Y. pseudotuberculosis* and the atypical *Y. pestis*-like strains from both these species, respectively (Fig. 5).

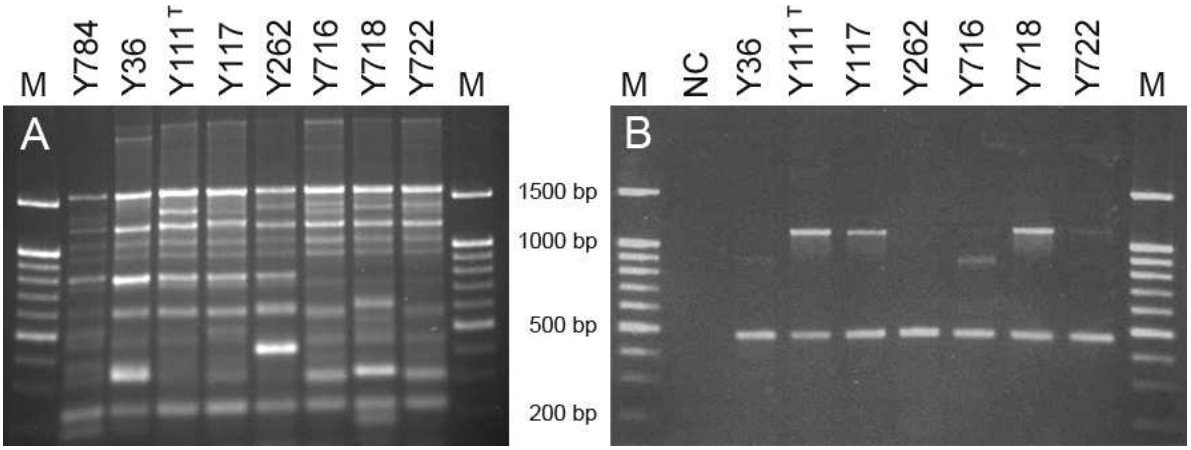


**Fig. 5.** Ethidium bromide-stained agarose gels showing the results of genomic fingerprinting using BOXA1R (A) and RAPD19 (B) primers with *Y. pestis* Y784, *Y. pseudotuberculosis* strains (Y36, $Y111^T$, Y117, Y262) and the atypical *Y. pseudotuberculosis* strains (Y716, Y718, Y722). **A** The 800 bp band of Y784 and the slightly smaller band of Y36 were excised, cloned and sequenced. **B** A slight difference in length between the ca. 460 bp bands of *Y. pseudotuberculosis* and the atypical strains was perceived and confirmed in a second and longer electrophoresis run (not shown). The respective bands of Y262 and Y722 were excised, cloned and sequenced. *M* 100 bp + 1,5 kbp DNA ladder size standard; *NC* negative control/sample without template DNA.

After BOXA1R, a product of approximately 800 bp specific of *Y. pestis* strains was identified. A slightly smaller band was observed in the *Y. pseudotuberculosis* patterns which was clearly missing in the BOXA1R patterns of the three atypical *Y. pseudotuberculosis* strains (Fig. 5). Cloning and analysing the *Y. pestis* PCR product revealed a 15 bp deletion in the *Y. pseudotuberculosis* genome relative to *Y. pestis* (deletion coordinates based on *Y. pestis* CO92 genome sequence AL590842: 4109578-4109592). The insert sequence showed 61.8% identity (620 of 762 bp overlap) to the insecticidal toxin complex subunit *tcaC* gene of *Yersinia enterocolitica* strain W22703 (AJ920332) (Bresolin et al., 2006).

RAPD19-PCR fingerprints showed a band of approximately 460 bp unique for the three atypical strains of *Y. pseudotuberculosis* since it was slightly smaller than the band observed in the other *Y. pseudotuberculosis* patterns (Fig. 5). The minor difference in size was confirmed in a longer electrophoresis run of the Y262 and Y716 samples (not shown). Cloning and sequencing of this fragment and a subsequent BLAST database search revealed a variable-number tandem repeat region in the genomes of *Y. pseudotuberculosis* and *Y. pestis* strains (position based on *Y. pseudotuberculosis* IP 31758 genome sequence CP000720: 1015661-1016127). Whereas the atypical *Y. pseudotuberculosis* strain Y722 and *Y. pseudotuberculosis* IP 31758 (acc. no. CP000720) possessed no AGCTAATTG repeat, the other *Y. pseudotuberculosis* strains had one (acc. nos. AL590842, CP000950, CP001048, BX936398) and *Y. pestis* strains had one to three repeats (one: Antiqua CP000308, Microtus AE017042, Pestoides F CP000668; two: Nepal516 CP000305, KIM AE009952; three: Angola CP000901).

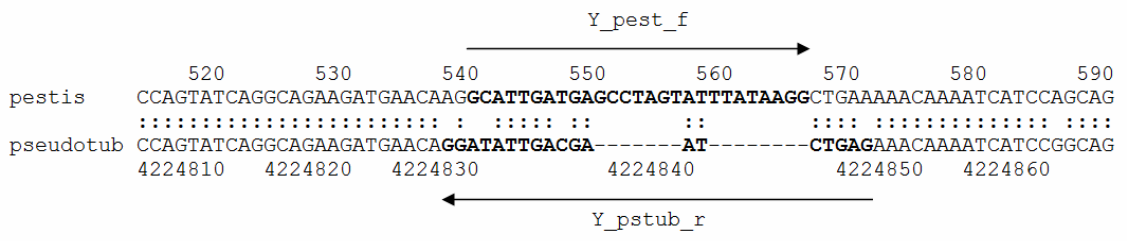


**Fig. 6.** Design of the *Y. pestis*- and *Y. pseudotuberculosis*-specific primers Y_pstub_r and Y_pest_f based on the 15 bp deletion in the genome of *Y. pseudotuberculosis*. Shown is part of the alignment of the *Y. pestis* Y784 761 bp BOXA1R-PCR fragment (*pestis*) and the *Y. pseudotuberculosis* IP32953 sequence from the database (acc. no. BX936398; *pseudotub*), resulting from an EMBL-EBI Fasta33 search with the *Y. pestis* fragment.

Exploiting the 15 bp deletion detected by BOXA1R-PCR, a *Y. pestis*-specific forward primer (Y_pest_f) and the *Y. pseudotuberculosis*-specific reverse primer (Y_pstub_r) were designed as indicated in Fig. 6. The respective reverse and forward primers of the two primer pairs were set up- and downstream of the deletion, respectively. The primers were tested in individual PCR assays and Y_pstub_f/r gave a 490 bp fragment with all *Y. pseudotuberculosis* strains except for the atypical isolates and no product with *Y. pestis* strains. The primer pair Y_pest_f/r gave a 275 bp product with all *Y. pestis* strains tested but no product with *Y. pseudotuberculosis* strains (results not shown).

Another primer pair (Y_pspest_f/r) was designed based on the region of the tandemly repeated sequence described above to give a 355 bp product with the atypical *Y. pseudotuberculosis* strains and a 364 bp product with *Y. pseudotuberculosis* and most *Y. pestis* strains (373 bp with *Y. pestis* Nepal and KIM, 382 bp with *Y. pestis* Angola). When tested in individual PCR, the primer pair Y_pspest_f/r resulted in the expected amplicons for the examined strains of *Y. pestis*, *Y. pseudotuberculosis* as well as the atypical *Y. pseudotuberculosis* strains (results not shown).

*Triplex PCR assay*

The three developed primer pairs were integrated into a single PCR assay. Results of application of the novel PCR with DNA samples of *Y. pestis*, *Y. pseudotuberculosis* and the three atypical *Y. pseudotuberculosis* strains are shown in Fig. 7, and amplicon sizes of all strains examined in our study are specified in Table 7. The atypical *Y. pseudotuberculosis* strains showed a single band of 355 bp, all *Y. pseudotuberculosis* strains exhibited two bands of 364 bp and 490 bp, and *Y. pestis* strains showed two bands of 275 bp and 364 bp (majority of strains) or 373 bp (strains Kuma and Yokohama). The negative control without template DNA showed no amplification products (results not shown). A faint product of approximately 190 bp detected in several *Y. pseudotuberculosis* and *Y. pestis* strains but not in Y716, Y718 and Y722, which probably resulted from an imperfect second binding site of the Y_pest_r or Y_pstub_f primer, does not hamper the interpretability of PCR results (Fig. 7). The primers Y_pest_r and Y_pstub_f could theoretically produce a 748 bp product in *Y. pestis* strains but this is not promoted by the PCR cycling conditions and was never observed.

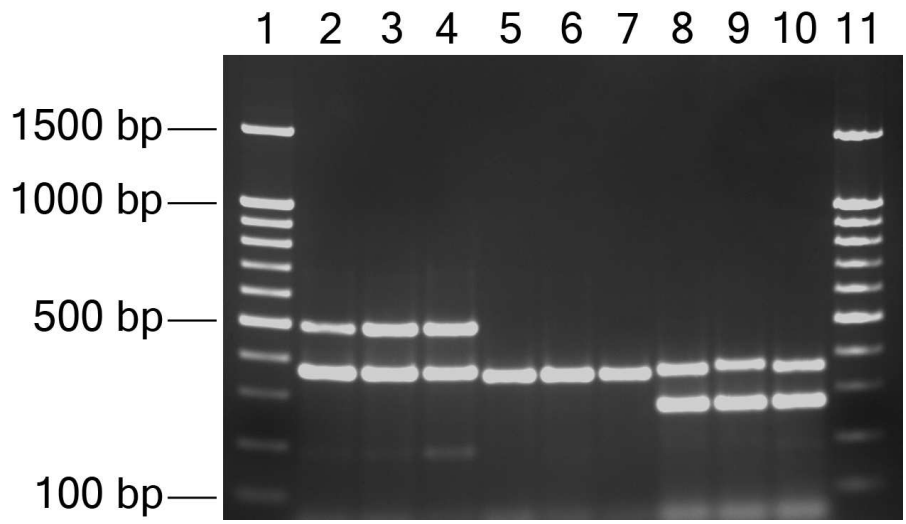


**Fig. 7.** Ethidium bromide-stained 1.5% agarose gel of triplex PCR products. *Lanes 1 and 11* 100 bp + 1.5 kbp DNA ladder size standard; *lane 2 Y. pseudotuberculosis* Y111$^{T}$; *lane 3 Y. pseudotuberculosis* Y117; *lane 4 Y. pseudotuberculosis* Y262; *lane 5* atypical *Y. pseudotuberculosis* Y716; *lane 6* atypical *Y. pseudotuberculosis* Y718; *lane 7* atypical *Y. pseudotuberculosis* Y722; *lane 8 Y. pestis* TS; *lane 9 Y. pestis* Yokohama; *lane 10 Y. pestis* G8786.

These data demonstrate that the atypical *Y. pestis*-like strains of *Y. pseudotuberculosis* can unambiguously be distinguished from *Y. pestis* based on a single band of 355 bp, whereas representatives of both, *Y. pestis* and *Y. pseudotuberculosis* showed an additional band clearly separated from the diagnostic band of the atypical strains (Fig. 7).

*Analytical sensitivity, specificity and performance of the triplex PCR*

The limits of detection of the novel PCR assay determined for *Y. pseudotuberculosis* Y716 and *Y. pseudotuberculosis* Y111$^{T}$ were $4{,}6 \cdot 10^{3}$ cfu·ml$^{-1}$ and $7{,}7 \cdot 10^{4}$ cfu·ml$^{-1}$ (equivalent to 14 and 231 cfu per PCR), respectively. Since only inactivated material was available, the sensitivity could not be assessed for *Y. pestis*. The specificity of the novel assay was verified by examining DNA of other *Yersinia* species (Table 7). By performing a standard PCR targeting the 16S rRNA gene (Lane, 1991) before using the samples in the triplex assay, it was demonstrated that quantity and quality of the DNAs were suitable for PCR amplification. The same template DNA samples did not give amplification products in the triplex assay under the conditions described above (results not shown). In order to examine the robustness of the developed PCR,

influences of several parameters were examined. The PCR showed good performance when different thermocyclers (Eppendorf Mastercycler, MWG-Biotech Primus, Perkin Elmer GeneAmp PCR System 2400) and polymerases (Promega GoTaq® DNA polymerase, GE Healthcare illustra™ puReTaq Ready-To-Go PCR Beads, Bio Products Taq Polymerase One) were used (results not shown).

### 4.2.5. Discussion

The genomes of *Y. pestis* and *Y. pseudotuberculosis* are highly similar and only few physiological traits are on hand to distinguish between the two taxa, which makes conventional diagnostic cumbersome (Bottone et al., 2005). Neubauer et al. (2000b) developed a miniaturised system that detects the different *Yersinia* species, including *Y. pseudotuberculosis* and *Y. pestis*, on the basis of their metabolic characteristics with a relatively high test sensitivity. But, recently, three strains affiliated to *Y. pseudotuberculosis*, Y716, Y718 and Y722, defied unambiguous typing with this system in showing some reactions determinant for *Y. pestis* and thus attracted our attention.

Because of these exceptional *Y. pestis*-like features, we developed a cost-effective, simple and rapid triplex PCR assay the major advantage of which is that a suspicious isolate, which cannot unambiguously be differentiated from *Y. pestis* employing standard diagnostics, is not only classified as non-*Y. pestis* but it also allows its identification as an atypical *Y. pseudotuberculosis* strain. To our knowledge, this is the first gel-based PCR assay specifically detecting members of an atypical *Y. pseudotuberculosis* lineage. Additionally, a distinction between *Y. pestis* and classical *Y. pseudotuberculosis* strains can also be made employing this assay.

The possibility of false-negative results is minimized since the assay was designed to differentiate between isolates that are already suspected to belong to the *Y. pestis*/*pseudotuberculosis* group, members of which are all positively detected showing at least one band. Additionally, the band of about 360 bp slighty differing in size and observed in *Y. pestis* and all strains of *Y. pseudotuberculosis* functions as an internal amplification control. In case of a negative result for the specific product of a *Y. pseudotuberculosis* (490 bp) or *Y. pestis* isolate (275 bp) and only a 364 bp (or slightly larger) product is present, the isolate is not an atypical *Y. pseudotuberculosis*

strain and the PCR should be repeated to clarify whether it belongs to *Y. pestis* or classical *Y. pseudotuberculosis*. No product at all indicates that the PCR did most probably not work and should also be repeated. DNA of *Y. pseudotuberculosis* cannot be positively and unambiguously detected in most of the PCR assays mentioned in the introduction except for the conventional PCR assays developed by Radnedge et al. (2001) and the real-time PCR assays by Chase et al. (2005) and Matero et al. (2009). Another gain is the rapidness of the novel triplex PCR assay which takes less than 100 min, including the time needed for gel electrophoreses. Due to the reduced sensitivity inherent in multiplex reactions, this triplex assay is most useful in the confirmation and characterization of culture isolates.

Recently, a much greater genomic diversity within the species *Y. pseudotuberculosis* was revealed than previously presumed (Eppinger et al., 2007). The three atypical strains examined in the present work might be related to a lineage of divergent *Y. pseudotuberculosis* strains causing Far East scarlet-like fever, a specific clinical form of *Y. pseudotuberculosis* infection (Somov, 1976). In a BLAST search of the *Y. pestis* Y784 DNA fragment sequenced in our study, the genome sequence of one *Y. pseudotuberculosis* strain (IP 31758, CP000720; Eppinger et al., 2007) showed a much lower identity (86.5%) than all the other *Y. pseudotuberculosis* strains in the database (>98% identity). Due to the low sequence identity, the *Y. pseudotuberculosis*-specific primers developed in this study would not target the published IP 31758 sequence, as it was the case with the three atypical *Y. pseudotuberculosis* strains that did not give a product with these primers in the triplex PCR. Moreover, the genome sequence of IP 31758 is the only one in the database that shows no repeat of the AGCTAATTG sequence just like the atypical strains in our study and would give a 355 bp PCR product with the primers Y_pspest_f/r. This observation suggests that strains Y716, Y718 and Y722 are closely related to *Y. pseudotuberculosis* strain IP 31758. Unfortunately, strain IP 31758 or other strains known to cause FESLF were not accessible to be included in this study.

In any case, employing the newly developed triplex assay, it is possible to reliably disprove a preliminary physiology-based misidentification of an atypical *Y. pestis*-like isolate as *Y. pestis* and therefore to rapidly – and with a minimum risk of false-negative results – rule out *Y. pestis* strains and subsequent high risk for human health.

# 5. Comprehensive summary of the papers which are part of this thesis

The development of improved PCR-based diagnostic methods for the three taxa *Brucella*, *Francisella* and *Yersinia,* containing highly human pathogenic members, was central to this thesis. The aim was to develop comprehensive PCR assays to complement an already existing set of diagnostic conventional and real-time multiplex PCR assays for biothreat agents insofar as they should not only enable a typing of species within these three taxonomical groups, but also of not yet detected subspecies, biovars and other so far unconsidered but significant subgroups. The exact identification of the pathogen in question beyond the species level can help health authorities to launch the most appropriate medical treatment and may further understanding of the disease especially in case of an epidemic. In this context, taxonomic assignment of so far unclassified strains, including work with a collection of available reference strains, was necessary.

**5.1. Huber, B.**, Scholz, H. C., Lucero, N. & H.-J. Busse (2009). Development of a PCR assay for typing and subtyping of *Brucella* species. *Int J Med Microbiol* 299, 563-573.

A reliable and rapid tool for discrimination between *Brucella* species and also biovars is desirable in order to distinguish less pathogenic or non-pathogenic members of *Brucella* from the pathogens with a greater medical impact as well as to avoid laborious conventional typing of the strains, requiring high biosafety laboratory precautions. The differential PCR detection of biovars has only partly been achieved by several studies (Bricker and Halling, 1994; Ocampo-Sosa et al., 2005; Ferrao-Beck et al., 2006; Fretin et al., 2008). *Ochrobactrum* species, which are closely related to *Brucella* and therefore could be confused in a preliminary diagnosis, have often been neglected as control organisms in previous *Brucella*-specific PCR assays or cross-reactions with them have been ignored (Casañas et al., 2001; Queipo-Ortuño et al., 2005; Hinic et al., 2008; Fretin et al., 2008). Thus, examinations were carried out in order to develop a single gel-based multiplex PCR assay allowing not only for the typing of all known *Brucella* species at the time of publication but also of *Brucella* biovars and does not cross-react with DNA of *Brucella*-like strains.

Although RAPD-PCR analyses mostly resulted in very similar banding patterns, they led to the detection of discriminating genetic polymorphisms in regard to three *Brucella* species. RAPD3a-PCR produced a unique band of 446 bp for all *B. abortus* strains and a slightly larger fragment with DNA of the other *Brucella* type and reference strains. Sequencing of this product revealed a 66 bp deletion in *B. abortus* relative to the other species. This difference was used to design genus-specific primers that simultaneously discriminate *B. abortus* strains from other brucellae. RAPD7-PCR produced a 488 bp band in *B. ovis* and *B. pinnipedialis* only. The 488 bp fragment was found to be located in a genomic region characteristic of these two species and therefore suitable to design specific primer. Primers specific for *B. neotomae*, *B. canis* and *B. suis* biovars 1,3,4 were designed on the basis of deletions (Rajashekara et al., 2004). Primers for *B. suis* biovars 2 and 5 were taken from Ratushna et al. (2006) and all other primers made use of the specific location of the insertion element IS*711* (Scholz et al., 2008c; Bricker and Halling, 1994; Cloeckaert et al., 2000). All primers were then integrated to constitute one single assay.

The novel 19-primer multiplex assay allowed for a specific detection of *Brucella* via an internal genus-specific marker resulting in either a 370 bp or a 436 bp band and did not amplify any PCR products with DNA of the tested *Brucella*-like species or other clinically relevant bacteria. In addition to the common species *B. abortus*, *B. melitensis, B. ovis* and *B. suis*, it specifically identified *B. neotomae*, *B. pinnipedialis*, *B. ceti* and *B. microti*. All *B. abortus* strains exhibited a unique PCR product of 370 bp. *B. melitensis* strains, which could not be typed according to biovars, were discriminated from the other species by a specific 731 bp band and *B. ovis* strains by a specific 976 bp band. *B. neotomae* and *B. microti* were characterized by a 1370 bp and a 666 bp band, respectively. *B. pinnipedialis* and *B. ceti* both showed a 1140 bp band and could be distinguished by an additional 569 bp band in the former only. For the specific detection of *B. canis*, primers were designed to amplify a 887 bp fragment. In regard to subtyping feasibilities, the assay can differentiate *Brucella abortus* biovars 1, 2, and 4 from biovars 3, 5, 6 and 9 via the presence of a specific 498 bp fragment amplified in the former three biovars. *Brucella suis* strains were distinguished into three groups. *B. suis* biovar 1 is characterized by a specific 285 bp fragment, biovars 3 and 4 by the presence of only a 217 bp fragment in addition to the genus-specific 436 bp fragment, and biovars 2 and 5 by an additional 321 bp fragment.

When tested in the multiplex assay, all *Brucella* type and reference strains and the majority of 118 field strains examined could be accurately identified by their respective banding patterns according to their previous typing. *B. canis* strains were subdivided into two groups, one exhibiting a unique pattern and the other one, consisting of several strains with atypical physiological features, a banding pattern shared with *B. suis* biovars 3 and 4. This result reflects the general difficulty to differentiate between *B. canis* and *B. suis* biovars 3 and 4 (Fretin et al., 2008; Hinic et al., 2008; Whatmore et al., 2006; 2007). The other encountered mistypings of *B. melitensis* biovars, one *B. abortus* bv. 6 strain and one *B. suis* bv. 3 strain are a minor drawback. They may point to a general problem in the development of a classification system for biotypes applying molecular methods, since the phenotypic characteristics exploited in conventional diagnostics generally do not correspond to the markers chosen for the molecular diagnostic approach and therefore do not reflect the specific characteristics that determine the respective biotypes.

Hence, the developed PCR assay is useful for rapid identification of *Brucella* at the species and at the biovar level. Concerning sensitivity and rapidness, a conventional PCR like this cannot compete with a real-time approach. However, real-time assays can only be multiplexed with certain restrictions and rarely have considered biovar typing so far (Fretin et al., 2008). Furthermore, real-time PCR equipment is much more expensive than a usual PCR apparatus, which is hence easier accessible. A major advantage of the *Brucella* multiplex PCR assay is that it can differentiate between all *Brucella* species known at the time of the study and a number of biovars. This was a clear enhancement to comparable assays at the time of publication. However, the newly-described species *Brucella inopinata* could not be included in the assay and it was later shown that this species results in the same characteristic PCR product as *Brucella microti* (unpublished results).

**5.2.** Kämpfer, P., Martin, E., Lodders, N., Jäckel, U., **Huber, B. E.**, Schumann, P., Langer, S., Busse, H.-J. & Scholz, H. (2010). *Paenochrobactrum gallinarii* gen. nov., sp. nov., isolated from air of a duck barn, and reclassification of *Pseudochrobactrum glaciei* as *Paenochrobactrum glaciei* comb. nov. *Int J Syst Evol Microbiol* 60, 1493-1498.

Scholz, H. C., Nöckler, K., Göllner, C., Bahn, P., Vergnaud, G., Tomaso, H., Al Dahouk, S., Kämpfer, P., Cloeckaert, A., Maquart, M., Zygmunt, M. S., Whatmore, A. M., Pfeffer, M., **Huber, B.**, Busse, H.-J. & De B. K. (2010). *Brucella inopinata* sp. nov., isolated from a breast implant infection. *Int J Syst Evol Microbiol* 60, 801-808.

**Huber, B.**, Scholz, H. C., Kämpfer, P., Falsen, E., Langer, S. & Busse, H.-J. (2010). *Ochrobactrum pituitosum* sp. nov., isolated from an industrial environment. *Int J Syst Evol Microbiol* 60, 321-326.

Kämpfer, P., **Huber, B.**, Lodders, N., Warfolomeow, I., Busse, H.-J. & Scholz, H. C. (2009). *Pseudochrobactrum lubricantis* sp. nov., isolated from a metal-working fluid. *Int J Syst Evol Microbiol* 59, 2464-2467.

Kämpfer, P., Sessitsch, A., Schloter, M., **Huber, B.**, Busse, H.-J. & Scholz, H. C. (2008). *Ochrobactrum rhizosphaerae* sp. nov. and *Ochrobactrum thiophenivorans* sp. nov., isolated from the environment. *Int J Syst Evol Microbiol* 58, 1426-1431.

Scholz, H. C., Hubalek, Z., Sedlácek, I., Vergnaud, G., Tomaso, H., Al Dahouk, S., Melzer, F., Kämpfer, P., Neubauer, H., Cloeckaert, A., Maquart, M., Zygmunt, M. S., Whatmore, A. M., Falsen, E., Bahn, P., Göllner, C., Pfeffer, M., **Huber, B.**, Busse, H.-J. & Nöckler, K. (2008). *Brucella microti* sp. nov., isolated from the common vole *Microtus arvalis*. *Int J Syst Evol Microbiol* 58, 375-382.

Kämpfer, P., Scholz, H. C., **Huber, B.**, Falsen, E. & Busse, H.-J. (2007). *Ochrobactrum haematophilum* sp. nov. and *Ochrobactrum pseudogrignonense* sp. nov., isolated from human clinical specimens. *Int J Syst Evol Microbiol* 57, 2513-2518.

Kämpfer, P., Scholz, H., **Huber, B.**, Thummes, K., Busse, H.-J., Maas, E. W. & Falsen, E. (2007). Description of *Pseudochrobactrum kiredjianiae* sp. nov. *Int J Syst Evol Microbiol* 57, 755-760.

As already stated above, *Ochrobactrum* and *Brucella* strains are closely related and *Ochrobactrum* strains can hence be considered *Brucella*-like strains. In fact, *Brucella* species always cluster within *Ochrobactrum* in gene-based phylogenetic analyses (Scholz et al., 2006; 2008b; chapter 2.11.). Moreover, several *Ochrobactrum* species infect humans as well and may have been underestimated as pathogens. It was previously thought that *Ochrobactrum* strains can only cause infections in immunocompromised hosts, but recent publications have also reported infections in healthy humans (Kettaneh et al., 2003; Ozdemir et al., 2006; Vaidya et al., 2006).

Scholz et al. (2008b) pointed out the pathogenic potential of not only *O. intermedium* and *O. anthropi* but also other *Ochrobactrum* species isolated from humans. Because of their similar properties, strains of *Brucella* and *Ochrobactrum* could be confused in clinical practice, where especially commercial identification tests based on biochemical features may result in a wrong typing (Scholz et al., 2008a). However, *Brucella*-like strains must not be misidentified as *Brucella*, species of which can cause brucellosis and are listed as important biothreat agents.

Over the last few years, *Brucella*-like strains have thus gained in importance and, in the course of this thesis, a closer examination of *Brucella* and *Brucella*-like taxa was carried out in order to detect genomic differences by use of genomic fingerprinting and to provide an accurate framework for the specificity test of the later developed PCR assay. *Brucellaceae* field strains were characterized applying biochemical, physiological, chemotaxonomical and genetic methods. Their taxonomic position was unambiguously determined by DNA-DNA hybridization. As a side effect, several novel species of the family *Brucellaceae* were formally described in the course of this thesis, which notably expanded the concept of this family. With the proposal of *Ochrobactrum pituitosum* as a novel species and major contributions to the description of nine other novel members of the *Brucellaceae* (*Brucella microti*, *Brucella inopinata*, *Ochrobactrum rhizosphaerae*, *Ochrobactrum haematophilum*, *Ochrobactrum thiophenivorans*, *Ochrobactrum pseudogrignonense*, *Ochrobactrum pituitosum*, *Pseudochrobactrum kiredjianiae*, *Pseudochrobactrum lubricantis*, *Paenochrobactrum gallinarii*), we greatly contributed to the awareness that the family contains more species and that the genus *Brucella* is more diverse than previously thought.

The two novel *Brucella* species, *B. microti* and *B. inopinata*, are both exceptional in comparison to other brucellae concerning their highly active metabolism. Affiliation to *Brucella*, however, was confirmed by DNA-DNA hybridization as well as by means of polyamine analysis, their polar lipid profile, fatty acid profile and quinone system. *B. inopinata* was found to be the most outstanding of all currently described *Brucella* species. Among other differences, it showed a unique *Brucella*-specific insertion element IS*711* banding pattern, exhibited a unique 16S rRNA gene sequence, significantly lower sequence similarities in various housekeeping genes and in genes encoding outer-membrane proteins. Moreover, *B. inopinata* could be differentiated from other brucellae via its polar lipid profile, based on the absence of an unknown

aminolipid AL2 (present in *B. microti* and the genus *Ochrobactrum*) and the presence of an unknown polar lipid L4 (present in *B. microti* but not in *B. abortus* and *B. melitensis*).

The *Ochrobactrum* isolates described as novel species all showed similar chemotaxonomic results (major ubiquinone Q-10; major polyamines spermidine, putrescine and *sym*-homospermidine; major polar lipids phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine and the characteristic aminolipid AL2; major fatty acids $C_{18:1}\omega7c$ and $C_{19:0}$ cyclo $\omega8c$). However, the results of DNA-DNA hybridization and physiological and biochemical tests enabled a genotypic and phenotypic differentiation of the isolates from all other described *Ochrobactrum* species.

*Pseudochrobactrum* species showed some major chemotaxonomical differences to *Brucella* and *Ochrobactrum*. They exhibited a polyamine pattern lacking *sym*-homospermidine and the polar lipid profile is missing AL2 which is useful for differentiating the genus from *Ochrobactrum*. However, the examined *Brucella* type strains of *B. abortus*, *B. melitensis* and *B. inopinata* also lack AL2. 16S rRNA gene and *recA* sequence similarities to strains of *Brucella* and *Ochrobactrum*, together with chemotaxonomic data allow for a clear differentiation from these genera. In phylogenetic dendrograms derived from *gyrB* and *groEL* sequence alignments, *Pseudochrobactrum* species cluster separately and also the calculated identity values clearly separate them (chapter 2.11.).

A novel genus, *Paenochrobactrum*, with the type species *Paenochrobactrum gallinarii* was described on the basis of characteristic polyamine patterns (major compound is putrescine) and polar lipid profiles (especially the presence of a “stretched aminolipid”) and because its members form a distinct lineage in the 16S rRNA gene sequence-based phylogenetic tree.

**5.3. Huber, B.**, Escudero, R., Busse, H.-J., Seibold, E., Scholz, H. C., Anda, P., Kämpfer, P. & Splettstoesser, W. D. (2010). Description of *Francisella hispaniensis* sp. nov., isolated from human blood, reclassification of *Francisella novicida* (Larson et al. 1955) Olsufiev et al. 1959 as *Francisella tularensis* subsp. *novicida* comb. nov. and emended description of the genus *Francisella*. Int *J Syst Evol Microbiol* 60, 1887-1896.

*Francisella tularensis* is an important pathogen, but not all subspecies are of equal pathogenicity. *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* frequently cause infections in humans that can be very severe, especially in the case of the highly contagious *F. tularensis* subsp. *tularensis*, but even within this subspecies two genetically distinct populations (A.I and A.II), which exhibit different manifestations of the disease, were recognized (Farlow et al., 2005; Staples et al., 2006). These known differences in virulence between subspecies and genotypes of *F. tularensis* and a detected inherent resistance to antibiotics of some uncommon *Francisella* strains (Kugeler et al., 2008) may be critical for the clinical management of tularemia patients. Therefore, as well as for environmental studies, aiming at identification of potential zoonotic reservoirs of *F. tularensis* and in epidemiology, rapid typing and subtyping of bacterial strains belonging to the genus *Francisella* is crucial.

Since previously published assays did not include the species *F. philomiragia*, *F. piscicida* (= *F. noatunensis*) and *F. hispaniensis* and cannot discriminate between all *F. tularensis* subspecies, bio- and genotypes in one step, examinations were carried out in order to develop a comprehensive multiplex assay that comes up to all these needs.

RAPD-PCR analysis identified a unique 201 bp band for *F. piscicida*, a 1259 bp band for *F. philomiragia* and a 770 bp band for *F. hispaniensis*. Based on the sequence information obtained through cloning and sequencing of the RAPD generated fragments, three different primer pairs for a specific detection of the respective species could be designed. For *F. tularensis* subsp. *mediasiatica*, a specific primer pair was designed on the basis of a variable genomic region detected in the published genome sequence of this subspecies in the course of database searches. The RD1 region of difference (Broekhuijsen et al., 2003) was used to design an appropriate forward primer for the specific detection of *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica* and its biovar japonica (reverse primer borrowed from Broekhuijsen et al.) and a primer pair specific for *F. tularensis* subsp.

*novicida*. An additional primer pair taken from Molins-Schneekloth et al. (2007) enabled a further distinction between *F. tularensis* subsp. *tularensis* genotypes A.I and A.II.

The primers were integrated to comprise the first diagnostic PCR assay to detect all *Francisella* species, subspecies and several biovars in a single reaction. *F. piscicida*, *F. philomiragia* and *F. hispaniensis* were characterized by one specific product each, a 172 bp band, a 962 bp band and a 462 bp band, respectively. All *F. tularensis* subspecies, except for *F. tularensis* subsp. *tularensis* A.II, showed a characteristic 369 bp band which was also the feature to discriminate A.II strains from the A.I genotype. *F. tularensis* subsp. *novicida* had an additional band of 1395 bp, *F. tularensis* subsp. *mediasiatica* one of 1132 bp, *F. tularensis* subsp. *tularensis* one of 1325 bp, *F. tularensis* subsp. *holarctica* one of 629 bp and biovar japonica one of 840 bp. A further species, *Francisella asiatica*, has only recently been proposed and could not be considered in the assay.

All type and reference strains and the 24 examined field strains could be successfully typed according to their previously determined affiliations. The taxonomical status of several strains, which were used for the evaluation of the novel 14-primer assay, was determined to the species level by DNA-DNA hybridization. This adds to the accuracy of the assay, while it is still not the standard to assure the taxonomical position of test strains with this method.

A strain isolated from human blood and affiliated to the genus *Francisella* according to 16S rRNA and *recA* gene sequencing was confirmed to be a novel *Francisella* species by DNA-DNA hybridization and the name *F. hispaniensis* was proposed. *F. hispaniensis* showed a quinone system typical of francisellae (mainly ubiquinone Q-8), a polar lipid profile with the major components phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylcholine and an unknown aminophospholipid (APL4) and phospholipid (PL3) and a typical fatty acid profile, primarily consisting of $C_{10:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ 3-OH and $C_{18:1}\omega 9c$. Biochemical tests allowed a phenotypic differentiation of the novel species from other members of the genus.

The polar lipid profile of *F. hispaniensis* was almost indistinguishable from the one of *F. tularensis* subsp. *tularensis* type strain. Also the other three *F. tularensis* subspecies showed most similar profiles. *F. philomiragia* and *F. piscicida* (= *F.*

*noatunensis*) type strains, on the contrary, lacked a major aminophospholipid (APL4), which can be used for their discrimination from *F. hispaniensis* and *F. tularensis*.

The proposed species, *Francisella hispaniensis*, together with four other species, *F. piscicida* (= *F. noatunensis* comb. nov.), *F. tularensis* (subspecies *tularensis*, *holarctica*, *mediasiatica* and *novicida*), *F. philomiragia* and *F. asiatica*, currently comprises the remote standing but medically important genus *Francisella*. Chemotaxonomical examinations of members of *Francisella* also led to an emended description of the genus, which had so far lacked inclusion of data on the polar lipid profile and quinone system. The proposed reclassification of *F. novicida* as a fourth subspecies of *F. tularensis*, mainly on the basis of DNA relatedness values (>85%), chemotaxonomical characteristics (fatty acid and polar lipid profiles) and an almost complete 16S rRNA gene sequence similarity to *F. tularensis*, was objected to by Johansson et al. (2010). Their major arguments for keeping the species separate were “differences in phenotype including chemotaxonomic markers, distinct ecological roles, different clinical and epidemiological characteristics, and differing abilities and modes of invasion and mechanisms of tissue damage in mammals” as well as practical considerations and recent genomic evidence for the evolution of *F. tularensis* “as a population lineage disconnected from *F. novicida*” (Johansson et al., 2010). A response to the objections was then written by Busse et al. (2010), in which most arguments were put into perspective or refuted.

## **5.4.** A triplex PCR for differential detection of *Yersinia pestis*-like atypical strains of *Yersinia pseudotuberculosis*

Gel-based PCR methods to type pathogenic *Yersinia* strains beyond the species level are rare (e.g. Bogdanovich et al., 2003). In the course of this thesis, DNA samples of *Y. pestis* and *Y. pseudotuberculosis* isolates, including biovars and atypical strains, were subjected to RAPD- and rep-PCR analyses in order to detect genomic differences usable for PCR typing methods. Whereas no appreciable differences were found within *Y. pestis* (e.g. for biovars Pestoides and Microtus), a RAPD-PCR assay produced a differentiating band for three atypical strains of *Y. pseudotuberculosis* that show major metabolic characteristics of *Y. pestis*, which was then cloned, sequenced and analysed. Facing the deviant metabolic characteristics

of these strains, which may lead to misidentification as *Y. pestis* in a preliminary approach, the detected genomic variation was used for the development of a PCR assay to differentiate between these atypical *Y. pestis*-like strains and *Y. pestis* in order to rapidly rule out the presence of highly pathogenic *Y. pestis* strains.

The 460 bp product unique for the three atypical *Y. pseudotuberculosis* strains Y716, Y718 and Y722 revealed one to three 9 bp repeats in *Y. pestis* and other *Y. pseudotuberculosis* strains relative to the atypical strains. This 9 bp to 27 bp difference was exploited for the design of primers to amplify a small product (355 - 382 bp) including the variable-number tandem repeat region in order to enable a differentiation of strains. A BOXA1R PCR product was found to be unique for *Y. pestis* strains. The analysis of this fragment showed a 15 bp deletion in the *Y. pseudotuberculosis* genome relative to *Y. pestis*, which could be used to design both, a *Y. pestis* and *Y. pseudotuberculosis* specific primer pair to give a 275 bp product with *Y. pestis* strains and a 490 bp fragment with strains of *Y. pseudotuberculosis*.

The three developed primer pairs were then integrated to form a diagnostic triplex PCR assay for a differential detection of the atypical *Y. pseudotuberculosis* strains. The primers for differentiation of Y716, Y718 and Y722 produced the expected 355 bp product with these three strains, as well as a 364 bp band in all tested *Y. pseudotuberculosis* and *Y. pestis* strains, except for strains Kuma and Yokohama (373 bp resulting from two repeats). Additionally, typical *Y. pseudotuberculosis* strains produced a unique band of 490 bp and *Y. pestis* strains one of 275 bp.

The determined detection limits for the triplex assay revealed a moderate sensitivity and performance was robust. So the assay is most useful for the characterization of culture isolates. Other *Yersinia* type species were tested negative in the novel PCR assay, confirming its specificity. A clear strength of the *Yersinia* triplex assay is, that a suspicious isolate, which cannot unambiguously be differentiated from *Y. pestis* employing standard diagnostics, is not only classified as non-*Y. pestis* but it also allows its identification as an atypical *Y. pseudotuberculosis* strain. Additionally, the assay is cost-effective, rapid and easy to perform. This is the first PCR assay presented that allows for a specific detection of members of an atypical *Y. pseudotuberculosis* lineage and its impact might increase if the three atypical strains, that show some striking parallels with a *Y. pseudotuberculosis* strain causing Far East scarlet-like fever, actually prove to be related to these unusual pathogens. However, this will be left to further analyses.

# 6. Conclusion

In science fiction stories, tools for an ultra-rapid medical diagnosis, like Star Trek's "Medical Tricorder", a fantastic real-time analyser which can accurately detect the trigger responsible for a disease in vivo, are frequently imagined future innovations. Actually, scientists all over the world are engaged in steadily improving the potential of diagnostic tools, especially for bacterial pathogens and viruses, in order to make medical diagnosis easier, more accurate and faster and therefore increase human chances of recovery. Rapid, specific and sensitive identification of biothreat agents has become an important field of research over the last decade because of the heightened awareness regarding bioterrorist issues. For these diagnostic purposes, PCR was an invaluable innovation, since PCR-based techniques can replace much of the often cumbersome and hazardous handling of pathogenic microorganisms in the laboratory, are usually less time-consuming and the interpretation of results is less subjective.

The gel-based PCR assays for the highly pathogenic taxa *Brucella*, *Francisella* and *Yersinia pestis/pseudotuberculosis* developed in this study are important novel methods, especially in regard to their discriminatory power. Apart from recently described *Brucella inopinata* and proposed *Francisella asiatica*, the rapid and specific *Brucella* and *Francisella* diagnostic assays have the power to identify a suspected *Brucella* or *Francisella* isolate to the species, subspecies and in many cases also to the biovar level, which made them the most comprehensive diagnostic tools at the time of publication. The novel *Yersinia* assay can identify members of an atypical *Y. pseudotuberculosis* lineage and differentiate them from other *Y. pseudotuberculosis* and most closely related *Y. pestis* strains. Hence, the assays minimize contact with the pathogens in the laboratory, provide information for taking the appropriate medical measures in case of an infection with one of the strains since they can distinguish the highly pathogenic from the less or non-pathogenic members of the taxa. A major strength of all the novel assays, apart from their highly differentiating potential, is the detection of all known members of the taxa via at least one distinctive band, avoiding the risk of false negative results which could be fatal in case of these severe pathogens.

All assays developed in this study employ conventional gel-based PCR that is usually – and especially if multiplexed – not as sensitive as modern real-time PCR. However, real-time equipment is more expensive and requires more know-how than conventional PCR. This is of importance if considering that brucellosis and plague are diseases primarily affecting developing nations where expensive technology might not be present in hospitals and laboratories. Furthermore, the number of targets is still more restricted in multiplex real-time PCR assays than in conventional PCR (Bahrdt et al. 2010).

A negative aspect of the chosen strategy of applying arbitrary PCR for the detection of biomarkers was that it was often not possible to assign the detected regions of difference to a specific known gene or another previously well studied genomic region. So, little could be found out about the genetic stability and other features of the target, the usefulness of which for differential detection could only be evaluated by testing the specificity of the designed primer pairs with all available field strains. Several primers had to be modified or discarded because they did not work as expected with all tested members of a group. Only primers that gave clear, unambiguous results were further integrated into the multiplex assays. For some species or subgroups, e.g. *Brucella neotomae* and *Francisella tularensis* subsp. *tularensis*, it was difficult to get a satisfactory set of different test strains since they are not very common and only few strains have been isolated worldwide or require special safety measures and are therefore rarely cultured and passed on. Thus, the assays will have to stand the final test in other laboratories that have access to more field strains. None of the developed assays was evaluated regarding performance with clinical samples. As moderate sensitivity is inherent to most multiplex reactions, the detection limit is often a bit higher than for assays employing only one primer pair. The evaluation of the assays using clinical and environmental samples as well as testing them with a wider range of field strains will be left to further analyses.

To enhance the power of the presented novel assays, especially for clinical diagnostic purposes that seek to avoid culture of pathogenic bacteria, further studies could take the results of these thesis as a basis for a comprehensive real-time – though restricted in multiplex use – or DNA chip approach, possibly intended to detect a range of different bacterial biothreat agents beyond the species level in one go. The developed assays could also contribute to an approach employing the modern Lab-on-a-Chip technologies that are especially attractive for biomedical

applications as well as defence research projects for portable biowarfare agent detection systems. This innovative method allows for laboratory functions and processes like sample preparation, DNA-amplification and electrophoresis to be carried out on a miniaturized chip which can be made of different types of material, like polymers, glass or silicon (Herold and Rasooly, 2009). Fluids can flow through tiny channels in a controlled way and appropriate sensors, e.g. optical or electrochemical sensors, can be mounted on this device. Such chips, when technically fully matured, are definitely a step towards science-fiction tools like the “Medical Tricorder”.

# 7. References

Achtman, M., Zurth, K., Morelli, G., Torrea, G., Guiyoule, A. & Carniel, E. (1999). *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. *Proc Natl Acad Sci USA* 96, 14043-14048.

Achtman, M. (2004a). Age, descent and genetic diversity within *Yersinia pestis*. In: Carniel, E., Hinnebusch, B. J. (eds.), Molecular and Cellular Biology of *Yersinia*. Wymondham: Horizon Scientific Press.

Achtman, M., Morelli, G., Zhu, P., Wirth, T., Diehl, I., Kusecek, B., Vogler, A. J., Wagner, D. M., Allender, C. J., Easterday, W. R. & other authors (2004b). Microevolution and history of the plague bacillus, *Yersinia pestis*. *Proc Natl Acad Sci USA* 101, 17837-17842.

Adair, D. M., Worsham, P. L., Hill, K. K., Klevytska, A. M., Jackson, P. J., Friedlander, A. M. & Keim, P. (2000). Diversity in a variable-number tandem repeat from *Yersinia pestis*. *J Clin Microbiol* 38, 1516-1519.

Al Dahouk, S., Tomaso, H., Prenger-Berninghoff, E., Splettstoesser, W. D., Scholz H. C. & Neubauer, H. (2005). Identification of *Brucella* species and biotypes using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). *Crit Rev Microbiol* 31, 191-196.

Al Dahouk, S., Le Flèche, P., Nöckler, K., Jacques, I., Grayon, M., Scholz, H. C., Tomaso, H., Vergnaud, G. & Neubauer, H. (2007). Evaluation of *Brucella* MLVA typing for human brucellosis. *J Microbiol Methods* 69, 137-145.

Alibek, K. (1999). Biohazard. New York: N.R. & House, pp. 29-38.

Alton, G. G., Jones, L. M., Angus, R. D. & Verger, J. M. (1988). Techniques for the brucellosis laboratory. Paris: Institute National de la Recherche Agronomique.

Anisimov, A. P., Lindler, L. E. & Pier, G. B. (2004). Intraspecific diversity of *Yersinia pestis*. *Clin Microbiol Rev* 17, 434-464.

Anonymous (1970). Health aspects of chemical and biological weapons. Geneva, Switzerland: World Health Organization.

Bachiller Luque, P., Perez Castrillon, J. L., Martin Luquero, M., Mena Martin, F. J., de la Lama Lopez-Areal, J., Perez Pascual, P., Mazon, M. A. & Herreros Guuillarte, V. (1998). Preliminary report of an epidemic tularemia outbreak in Valladolid. *Rev Clin Esp* 198, 789-793.

Bahrdt, C., Krech, A. B., Wurz, A. & Wulff, D. (2010). Validation of a newly developed hexaplex real-time PCR assay for screening for presence of GMOs in food, feed and seed. *Anal Bioanal Chem* 396, 2103-2112.

Barenblatt, D. (2004). A plague upon humanity: The secret genocide of axis Japan's germ warfare operation. New York: HarperCollins, p. 220-221.

Bell, C. A., Uhl, J. R., Hadfield, T. L., David, J. C., Meyer, R. F., Smith, T. F. & Cockerill, F. R. (2002). Detection of *Bacillus anthracis* DNA by LightCycler PCR. *J Clin Microbiol* 40, 2897-2902.

Ben-Gurion, R. & Shafferman, A. (1981). Essential virulence determinants of different *Yersinia* species are carried on a common plasmid. *Plasmid* 5, 183-187.

Bercovier, H., Mollaret, H. H., Alonso, J. M., Brault, J., Ursing, J., Fanning, G. R., Steigerwaldt, A. G. & Brenner, D. J. (1980a). Intra- and interspecies relatedness of *Yersinia pestis* by DNA hybridization and its relationship to *Yersinia pseudotuberculosis*. *Curr Microbiol* 4, 225-229.

Bercovier, H., Brenner, D. J., Ursing, J., Steigerwalt, A. G., Fanning, G. R., Alonso, J. M., Carter, G. P. & Mollaret, H. H. (1980b). Characterization of *Yersinia enterocolitica* sensu stricto. *Curr Microbiol* 4, 201-206.

Bogdanovich, T. M., Carniel, E., Fukushima, H. & Skurnik, M. (2004). Use of O-antigen gene cluster-specific PCRs for the identification and O-genotyping of *Yersinia pseudotuberculosis* and *Yersinia pestis. J Clin Microbiol* 41, 5103-5112.

Bossi, P., van Loock, F., Tegnell, A. & Gouvras, G. (2004a). Bichat clinical guidelines for bioterrorist agents. *Euro Surveill* 9(12).

Bossi, P., Tegnell, A., Baka, A., Van Loock, F., Hendriks, J., Werner, A., Maidhof, H. & Gouvras, G. (2004b). Bichat guidelines for the clinical management of brucellosis and bioterrorism-related brucellosis. *Euro Surveill* 9(12).

Bossi, P., Tegnell, A., Baka, A., Van Loock, F., Hendriks, J., Werner, A., Maidhof, H. & Gouvras, G. (2004c). Bichat guidlines for the clinical management of plague and bioterrorism-related plague. *Euro Surveill* 9(12).

Bottone, E. J., Bercovier, H. & Mollaret, H. H. (2005). Genus XLI. *Yersinia* Van Loghem 1944, 15AL. In: Brenner, D. J., Krieg, N. R., Staley, J. T., Garrity, G. M. (eds.), Bergey's Manual of Systematic Bacteriology, vol. 2, 2nd edn. New York: Springer, pp. 838-848.

Breed, R. S., Murray, E. G. D. & Smith, N. R. (1957). Bergey's Manual of Determinative Bacteriology, 7th edn., The Williams & Wilkins Co, Baltimore, p. 394.

Bresolin, G., Morgan, J. A. W., Ilgen, D., Scherer, S. & Fuchs, T. M. (2006). Low temperature-induced insecticidal activity of *Yersinia enterocolitica*. *Mol Microbiol* 59, 503-512.

Brew, S. D., Perrett, L. L., Stack, J. A., MacMillan, A. P. & Staunton, N. J. (1999). Human exposure to *Brucella* recovered from a sea mammal. *Vet Rec* 24, 483.

Bricker, B. J. & Halling, S. M. (1994). Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis*, *Brucella ovis*, and *Brucella suis* bv. 1 by PCR. *J Clin Microbiol* 32, 2660-2666.

Bricker, B. J. & Halling, S. M. (1995). Enhancement of the *Brucella* AMOS PCR assay for differentiation of *Brucella abortus* vaccine strains S19 and RB51. *J Clin Microbiol* 33, 1640-1642.

Bricker, B. J., Ewalt, D. R., MacMillan, A. P., Foster, G. & Brew, S. (2000). Molecular characterization of *Brucella* strains isolated from marine mammals. *J Clin Microbiol* 38, 1258-1262.

Bricker, B. J., Ewalt, D. R. & Halling, S. M. (2003). *Brucella* 'HOOF-Prints': strain typing by multi-locus analysis of variable number tandem repeats (VNTRs). *BMC Microbiol* 3:15.

Broekhuijsen, M., Larsson, P., Johansson, A., Byström, M., Eriksson, U., Larsson, E., Prior, R. G., Sjöstedt, A., Titball, R. W. & Forsman, M. (2003). Genome-wide DNA microarray analysis of *Francisella tularensis* strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent *F. tularensis* subsp. *tularensis*. *J Clin Microbiol* 41, 2924-2931.

Brown, J. M., Pham, K. N., McNeil, M. M. & Lasker, B. A. (2004). Rapid identification of *Nocardia farcinica* clinical isolates by a PCR assay targeting a 314-base-pair species-specific DNA fragment. *J Clin Microbiol* 42, 3655-3660.

Brubaker, R. R. (1991). Factors promoting acute and chronic diseases caused by yersiniae. *Clin Microbiol Rev* 4, 309-324.

Bubeck, S. S. & Dube, P. H. (2007). *Yersinia pestis* CO92 delta yopH is a potent live-attenuated plague vaccine. *Clin Vaccine Immunol* 14, 1235-1238.

Buddle, M. B. (1956). Studies on *Brucella ovis* (n. sp.), a cause of genital disease of sheep in New Zealand and Australia. *J Hyg* 54, 351-364.

Busse, H.-J., Huber, B., Anda, P., Escudero, R., Scholz, H. C., Seibold, E., Splettstoesser, W. D. & Kämpfer, P. (2010). Objections to the transfer of *Francisella novicida* to the subspecies rank of *Francisella tularensis* – response to Johansson *et al*. *Int J Syst Evol Microbiol* 60, 1718-1720.

Campbell, J., Lowe, J., Walz, S. & Ezzel, J. (1993). Rapid and specific identification of *Yersinia pestis* by using a nested polymerase chain reaction procedure. *J Clin Microbiol* 31, 758-759.

Cannons, A., Amuso, P. & Anderson, B. (2006). Biotechnology and the public health response to bioterrorism. In: Anderson, B., Friedman, H., Bendinelli, M. (eds.), Microorganisms and bioterrorism. New York: Springer, p. 2.

Carmichael, L. E. & Bruner, D. W. (1968). Characteristics of a newly recognized species of *Brucella* responsible for infectious canine abortions. *Cornell Veterinarian* 58, 579-592.

Casañas, M. C., Queipo-Ortuño, M. I., Rodriguez-Torres, A., Orduña A., Colmenero, J. D. & Morata, P. (2001). Specificity of a polymerase chain reaction assay of a target sequence ond the 31-kilodalton *Brucella* antigen DNA used to diagnose human brucellosis. *Eur J Microbiol Infect Dis* 20, 127-131.

Chain, P. S., Carniel, E., Larimer, F. W., Lamerdin, J., Stoutland, P. O., Regala, W. M., Georgescu, A. M., Vergez, L. M., Land, M. L., Motin, V. L. & other authors (2004). Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. *Proc Natl Acad Sci USA* 101, 13826-13831.

Chain, P. S. G., Comerci, D. J., Tolmasky, M. E., Larimer, F. W., Malfatti, S. A., Vergez, L. M., Aguero, F., Land, M. L., Ugalde, R. A. & Garcia, E. (2005). Whole-genome analyses of speciation events in pathogenic brucellae. *Infect Immun* 73, 8353-8361.

Chain, P. S., Hu, P., Malfatti, S. A., Radnedge, L., Larimer, F., Vergez, L. M., Worsham, P., Chu, M. C. & Andersen, G. L. (2006). Complete genome sequence of *Yersinia pestis* strains Antiqua and Nepal516: evidence of gene reduction in an emerging pathogen. *J Bacteriol* 188, 4453-4463.

Chase, C. J., Ulrich, M. P., Wasieloski, L. P., Kondig, J. P., Garrison, J., Lindler, L. E. & Kulesh, D. A. (2005). Real-time PCR assays targeting a unique chromosomal sequence of *Yersinia pestis. Clin Chem* 51, 1778-1785.

Christopher, G. W., Cieslak, T. J., Pavlin, J. A. & Eitzen, E. M. (1997). Biological Warfare: A Historical Perspective. *JAMA* 278, 412-417.

Cloeckaert A., Verger, J. M., Grayon, M. & Grepinet, O. (1995). Restriction site polymorphism of the genes encoding the major 25 kDa and 36 kDa outer-membrane proteins of *Brucella*. *Mirobiology* 141, 2111-2121.

Cloeckaert, A., Verger, J.-M., Grayon, M. & Vizcaino, N. (1996). Molecular and immunological characterization of the major outer membrane proteins of *Brucella*. *FEMS Microbiol Lett* 145, 1-8.

Cloeckaert, A., Grayon, M. & Grepinet, O. (2000). An IS*711* element downstream of the *bp26* gene is a specific marker of *Brucella* spp. isolated from marine mammals. *Clin Diagn Lab Immunol* 7, 835-839.

Cloeckaert, A., Verger, J.-M., Grayon, M., Paquer, J.-Y., Garin-Bastuji, B., Foster, G. & Godfroid, J. (2001). Classification of *Brucella* spp. isolated from marine mammals by DNA polymorphism at the *omp2* locus. *Microbes Infect* 3, 729-738.

Cloeckaert, A., Grayon, M., Grépinet, O. & Boumedine, K. S. (2003). Classification of *Brucella* strains isolated from marine mammals by infrequent restriction site-PCR and development of specific PCR identification test. *Microbes Infect* 5, 593-602.

Collier, L., Balows, A. & Sussman, M. (1998). Topley & Wilson's microbiology and microbial infections, vol. 3., 9th edn., London: Oxford University Press.

Corbel, M. J. & Brinley-Morgan, W. J. (1984). Genus *Brucella* Meyer and Shaw 1920, 173AL. In: Krieg, N. R., Holt, J. G. (eds.), Bergey's Manual of Systematic Bacteriology, vol. 1, Baltimore: The Williams & Wilkins Co., pp. 377-388.

Das, R., Hammamieh, R., Neill, R., Ludwig, G. V., Eker, S., Lincoln, P., Ramamoorthy, P., Dhokalia, A., Mani, S., Mendis, C. & other authors (2008). Early indicators of exposure to biological threat agents using host gene profiles in peripheral blood mononuclear cells. *BMC Infect Dis* 8:104.

Dawson, C. E., Stubberfield, E. J., Perrett, L. L., King, A. C., Whatmore, A. M., Bashiruddin, J. B., Stack, J. A. & Macmillan, A. P. (2008). Phenotypic and molecular characterisation of *Brucella* isolates from marine mammals. *BMC Microbiol* 8:224.

De La Puente-Redondo, V. A., García Del Blanco, N., Gutiérrez-Martin, C. B., García-Pena, F. J. & Rodríguez Ferri, E. F. (2000). Comparison of different PCR approaches for typing of *Francisella tularensis* strains. *J Clin Microbiol* 38, 1016-1022.

del Cerro, A., Marquez, I. & Guijarro, J. A. (2002). Simultaneous detection of *Aeromonas salmonicida*, *Flavobacterium psychrophilum*, and *Yersinia ruckeri*, three major fish pathogens, by multiplex PCR. *Appl Environ Microbiol* 68, 5177-5180.

De Ley, J., Mannheim, P., Segers, P., Lievens, A., Denijn, M., Vanhoucke, M. & Gillis, M. (1987). Ribosomal ribonucleic acid cistron similarities and taxonomic neighborhood of *Brucella* and CDC group Vd. *Int J Syst Bacteriol* 37, 35-42.

Deng, W., Burland, V., Plunkett, G., Boutin, A., Mayhew, G. F., Liss, P., Perna, N. T., Rose, D. J., Mau, B., Zhou, S. & other authors (2002). Genome sequence of *Yersinia pestis* KIM. *J Bacteriol* 184, 4601-4611.

Dennis, D. T., Inglesby, T. V., Henderson, D. A., Bartlett, J. G., Bartlett, J. G., Ascher, M. S., Eitzen, E., Fine, A. D., Friedlander, A. M., Hauer, J., Layton, M. & other authors (2001). Tularemia as a biological weapon. Medical and public health management. *JAMA* 285, 2763-2773.

Deshpande, A., Gans, J., Graves, S. W., Green, L., Taylor, L., Kim, H. B., Kunde, Y. A., Leonard, P. M., Li, P. E., Mark, J. & other authors (2010). A rapid multiplex assay for nucleic acid-based diagnostics. *J Microbiol Methods* 80, 155-163.

Devignat, R. (1951). Varieties of *Pasteurella pestis*; new hypothesis. *Bull World Health Organ* 4, 247-263.

Eini, F., Fallah Raoufi, M., Soleimani, M., Azarifard, F., Jamshidiyan, E. & Majidzadeh, K. (2010). Design of improved polymerase chain reaction (PCR) method containing internal positive control (IPC) for molecular detection of *Y. pestis*. *Int J Infec Dis* 14, e362-e363.

Ellerbrok, H., Nattermann, H., Ozel, M., Beutin, L., Appel, B. & Pauli, G. (2002). Rapid and sensitive identification of pathogenic and apathogenic *Bacillus anthracis* by real-time PCR. *FEMS Microbiol Lett* 214, 51-59.

Ellis, J., Oyston, P. C., Green, M. & Titball, R. W. (2002). Tularemia. *Clin Microbiol Rev* 15, 631-646.

Endicott, S. L. & Hagerman, E. (1998). The United States and Biological Warfare: Secrets from the Early Cold War and Korea. Bloomington: Indiana University Press, p. 274

Engelthaler, D. M., Gage, K. L., Montenieri, J. A., Chu, M. & Carter, L. G. (1999). PCR detection of *Yersinia pestis* in fleas: comparison with mouse inoculation. *J Clin Microbiol* 37, 1980-1984.

Eppinger, M., Rosovitz, M. J., Fricke, W. F., Rasko, D. A., Kokorina, G., Fayolle, C., Lindler, L. E., Carniel, E. & Ravel, J. (2007). The complete genome sequence of *Yersinia pseudotuberculosis* IP31758, the causative agent of Far East scarlet-like fever. *PLoS Genet* 3, E142.

Eppinger, M., Worsham, P. L., Nikolich, M. P., Riley, D. R., Sebastian, Y., Mou, S., Achtman, M., Lindler, L. E. & Ravel, J. (2010). Genome sequence of the deep-rooted *Yersinia pestis* strain Angola reveals new insights into the evolution and pangenome of the plague bacterium. *J Bacteriol* 192, 1685-1699.

Escudero, R., Elía, M., Sáez-Nieto, J. A., Menéndez, V., Toledo, A., Royo, G., Rodríguez-Vargas, M., Whipp, M. J., Gil, H. & other authors (2010). A possible novel *Francisella* genomic species isolated from blood and urine of a patient with severe illness. *Clin Microbiol Infect* 16, 1026-1030.

Eubanks, L. M., Dickerson, T. J. & Janda, K. D. (2007). Technological advancements for the detection of and protection against biological and chemical warfare agents. *Chem Soc Rev* 36, 458-470.

Euzeby, J. P. (2010). List of Prokaryotic names with Standing in Nomenclature. Genus *Yersinia*. http://www.bacterio.cict.fr/xz/yersinia.html, 6.8.2010

Farlow, J., Smith, K. L., Wong, J., Abrams, M., Lytle, M. & Keim, P. (2001). *Francisella tularensis* strain typing using multiple-locus, variable-number tandem repeat analysis. *J Clin Microbiol* 39, 3186-3192.

Farlow, J., Wagner, D. M., Dukerich, M., Stanley, M., Chu, M., Kubota, K., Petersen, J. & Keim, P. (2005). *Francisella tularensis* in the United States. *Emerg Infect Dis* 11, 1835-1841.

Fekete, A., Bantle, J. A., Halling, S. M. & Sanborn, M. R. (1990). Preliminary development of a diagnostic test for *Brucella* using polymerase chain reaction. *J Appl Bacteriol* 69, 216-227.

Fekete A., Bantle, J. A., Halling, S. M. & Stich, R. W. (1992). Amplification fragment length polymorphism in *Brucella* strains by use of polymerase chain reaction with arbitrary primers. *J Bacteriol* 174, 7778-7783.

Felsenstein, J. (2006). PHYLIP (Phylogeny Inference Package) version 3.66. *Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.*

Ferrao-Beck, L., Cardoso, R., Munoz, P.M., de Miguel, M.J., Albert, D., Ferreira, A.C., Marin, C.M., Thiebaud, M., Jacques, I. & other authors (2006). Development of a multiplex PCR assay for polymorphism analysis of *Brucella suis* biovars causing brucellosis in swine. *Vet Microbiol* 115, 269-277.

Ficht, T. A., Bearden, S. W., Sowa, B. A. & Adams, L. G. (1989). DNA sequence and expression of the 36-kilodalton outer membrane protein gene of *Brucella abortus*. *Infect Immun* 57, 3281-3291.

Ficht, T. A., Husseinen, H. S., Derr, J. & Bearden, S. W. (1996). Species-specific sequences at the omp2 locus of *Brucella* type strains. *Int J Syst Bacteriol* 46, 329-331.

Filippov, A. A., Solodovnikov, N. S., Kookleva, L. M. & Protsenko, O. A. (1990). Plasmid content in *Yersinia pestis* strains of different origin. *FEMS Microbiol Lett* 55, 45-48.

Forsman, M., Sandstrom, G. & Sjöstedt, A. (1994). Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. *Int J Syst Bacteriol* 44, 38-46.

Foster, G., Osterman, B. S., Godfroid, J., Jacques, I. & Cloeckaert, A. (2007). *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. *Int J Syst Evol Microbiol* 57, 2688-2693.

Foster, J. T., Okinaka, R. T., Svensson, R., Shaw, K., De, B. K., Robison, R. A., Probert, W. S., Kenefic, L. J., Brown, W. D. & Keim, P. (2008). Real-time PCR assays of single-nucleotide polymorphisms defining the major *Brucella* clades. *J Clin Microbiol* 46, 296-301.

Franz, D. R., Jahrling, P. B., Friedlander, A. M., McClain, D. J., Hoover, D. L., Bryne, W. R., Pavlin, J. A., Christopher, G. W. & Eitzen, E. M. (1997). Clinical recognition and management of patients exposed to biological warfare agents. *JAMA* 278, 399-411.

Fretin, D., Whatmore, A. M., Al Dahouk, S., Neubauer, H., Garin-Bastuji, B., Albert, D., Van Hessche, M., Ménart, M., Godfroid, J., Walravens, K. & Wattiau, P. (2008). *Brucella suis* identification and biovar typing by real-time PCR. *Vet Microbiol* 131, 376-385.

Gabitzsch, E. S., Vera-Tudela, R., Eisen, R. J., Bearden, S. W., Gage, K. L. & Zeidner, N. S. (2008). Development of a real-time quantitative PCR assay to enumerate *Yersinia pestis* in fleas. *Am J Trop Med Hyg* 79, 99-101.

Galimand, M., Guiyoule, A., Gerbaud, G., Rasoamanana, B., Chanteau, S., Carniel, E. & Courvalin, P. (1997). Multidrug resistance in *Yersinia pestis* mediated by a transferable plasmid. *N Engl J Med* 337, 677-680.

Garcia Del Blanco, N., Dobson, M. E., Vela, A. I., De La Puente, V. A., Gutiérrez, C. B., Hadfield, T. L., Kuhnert, P., Frey, J., Domínguez, L. & Rodríguez Ferri, E. F. (2002). Genotyping of *Francisella tularensis* strains by pulsed-field gel electrophoresis, amplified fragment length polymorphism fingerprinting, and 16S RNA gene sequencing. *J Clin Microbiol* 40, 2964-2972.

Garcia, E., Worsham, P., Bearden, S., Malfatti, S., Lang, D., Larimer, F., Lindler, L. & Chain, P. (2007). Pestoides F, an atypical *Yersinia pestis* strain from the former Soviet Union. *Adv Exp Med Biol* 603, 17-22.

Garcia-Yoldi, D., Marin, C. M. & Lopez-Goni, I. (2005). Restriction site polymorphisms in the genes encoding new members of group 3 outer membrane protein family of *Brucella* spp. *FEMS Microbiol Lett* 245, 79-84.

Garcia-Yoldi, D., Marin, C. M., de Miguel, M. J., Munoz, P. M., Vizmanos, J. L. & Lopez-Goni, I. (2006). Multiplex PCR assay fort he identification and differentiation of all *Brucella* species and the vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. *Clin Chem* 52, 779-781.

Garcia-Yoldi, D., Le Flèche, P., Marin, C. M., De Miguel, M. J., Munoz, P. M., Vergnaud, G. & Lopez-Goni, I. (2007). Assessment of genetic stability of *Brucella melitensis* Rev 1 vaccine strain by multiple-locus variable-number tandem repeat analysis. *Vaccine* 25, 2858-2862.

Gray, P. H. H. & Thornton, H. G. (1928). Soil bacteria that decompose certain aromatic compounds. *Zentralblatt für Bakteriologie*, *Parasitenkunde*, *Infektionskrankheiten und Hygiene* **73**, 74-96.

Grif, K., Dietrich, M. P., Much, P., Hofer, E. & Allerberger, F. (2003). Identifying and subtyping species of dangerous pathogens by automated ribotyping. *Diagn Microbiol Inf Dis* 47, 313-320.

Guiyoule, A., Gerbaud, G., Buchrieser, C., Galimand, M., Rahalison, L., Chanteau, S., Courvalin, P. & Carniel, E. (2001). Transferable plasmid-mediated resistance to streptomycin in a clinical isolate of *Yersinia pestis*. *Emerg Infect Dis* 7, 43-48.

Guillot, E. & Mouton, C. (1997). PCR-DNA probe assays for identification and detection of *Prevotella intermedia* sensu stricto and *Prevotella nigrescens*. *J Clin Microbiol* 35, 1876-1882.

Guryčivá, D. (1998). First isolation of *Francisella tularensis* subsp. *tularensis* in Europe. *Eur J Epidemiol* 14, 797-802.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser* 41, 95-98.

Halling, S. M, Tatum, F. M. & Bricker, B. J. (1993). Sequence and characterization of an insertion sequence, IS*711*, from *Brucella ovis*. *Gene* 133, 123-127.

Halling, S. M., Peterson-Burch, B. D., Bricker, B. J., Zuerner, R. L., Qing, Z., Li, L. L., Kapur, V., Alt, D. P. & Olsen, S. C. (2005). Completion of the genome sequence of *Brucella abortus* and comparison to the highly similar genomes of *Brucella melitensis* and *Brucella suis*. *J Bacteriol* 187, 2715-2726.

Han, Y., Zhou, D., Pang, X., Zhang, L., Song, Y., Tong, Z., Bao, J., Dai, E., Wang, J., Guo, Z. & other authors (2005). DNA microarray analysis of the heat- and cold-shock stimulons in *Yersinia pestis*. *Microbes Infect* 7, 335-348.

Harris, S. H. (1994). Factories of death: Japanese biological warfare 1932-45 and the American cover-up. New York: Routledge.

Herold, K. & Rasooly, A. (2009). Preface. In: Herold, K., Rasooly, A. (eds.), Lab on a chip technology, vol. 1. Norfolk, UK: Caister Academic Press, pp. xi-xiv.

Higgins, J. A., Ezzell, J., Hinnebusch, B. J., Shipley, M., Henchal, E. A. & Ibrahim, M. S. (1998). 5' Nuclease PCR assay to detect *Yersinia pestis*. *J Clin Microbiol* 36, 2284-2288.

Hinchliffe, S. J., Isherwood, K. E., Stabler, R. A., Prentice, M. B., Rakin, A., Nichols, R. A., Oyston, P. C., Hinds, J., Titball, R. W. & Wren, B. W. (2003). Application of DNA microarrays to study the evolutionary genomics of *Yersinia pestis* and *Yersinia pseudotuberculosis*. *Genome Res* 13, 2018-2029.

Hinic, V., Brodard, I., Thomann, A., Cvetnić, Ž., Makaya, P.V., Frey, J. & Abril, C. (2008). Novel identification and differentiation of *Brucella melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* suitable for both conventional and real-time PCR systems. *J Microbiol Methods* 75, 375-378.

Hinnebusch, J. & Schwan, T. G. (1993). New method for plague surveillance using polymerase chain reaction to detect *Yersinia pestis* in fleas. *J Clin Microbiol* 31, 1511-1514.

Hofer, E., Schildorfer, H., Flatscher, J. & Müller, M. (1997). Zum Nachweis der Tularämie bei Feldhasen (*Lepus europaeus*) in Österreich. *Wien Tierärztl Mschr* 81, 301-308.

Hofer, E. (2002). Epidemiologie, Klinik und mikrobiologische Diagnostik der Tularämie. http://www.antibiotikamonitor.at/12_02/12_02_05.htm

Hollis, D. G., Weaver, R. E., Steigerwaldt, A. G., Wenger, J. D., Moss, C. W. & Brenner, D. J. (1989). *Francisella philomiragia* comb. nov. (formerly *Yersinia philomiragia*) and *Francisella tularensis* biogroup novicida (formerly *Francisella novicida*) associated with human disease. *J Clin Microbiol* 27, 1601-1608.

Holmes, B., Popoff, M., Kiredjian, M. & Kersters, K. (1988). *Ochrobactrum anthropi* gen. nov., sp. nov. from human clinical specimens and previously known as group Vd. *Int J Syst Bacteriol* 38, 406-416.

Huang, X.-Z., Nikolich, M. P. & Lindler, L. E. (2006). Current trends in plague research: from genomics to virulence. *Clin Med Res* 4, 189-199.

Hubálek, M., Hernychová, L., Brychta, M., Lenčo, J., Zechovská, J. & Stulik, J. (2004). Comparative proteome analysis of cellular proteins extracted from highly virulent *Francisella tularensis* ssp. *tularensis* and less virulent *F. tularensis* ssp. *holarctica* and *F. tularensis* ssp. *mediasiatica*. *Proteomics* 4, 3048-3060.

Huber, B., Scholz, H. C., Lucero, N. & Busse, H.-J. (2009). Development of a PCR assay for typing and subtyping of *Brucella* species. *Int J Med Microbiol* 299, 563-573.

Huber, B., Scholz, H. C., Kämpfer, P., Falsen, E., Langer, S. & Busse, H.-J. (2010). *Ochrobactrum pituitosum* sp. nov., isolated from an industrial environment. *Int J Syst Evol Microbiol* 60, 321-326.

Huddleson, I. F. (1929). The differentiation of the species of the genus *Brucella*. *Michigan State College Agricultural Experimental Station Tech Bull* 100, 1-16.

Hurvell, B. (1981). Zoonotic *Yersinia enterocolitica*, infections, host range, clinical manifestations and transmission between man and animals. In: *Yersinia enterocolitica*. Bottone, E. J. (ed.), Boca Raton: CRC Press, pp. 145-159.

Ibrahim, A., Goebel, B. M., Liesack, W., Griffiths, M. & Stackebrandt, E. (1993). The phylogeny of the genus *Yersinia* based on 16S rDNA sequences. *FEMS Microbiol Lett* 114, 173-177.

Imran, A., Hafeez, F. Y., Frühling, A., Schumann, P., Malik, K. A. & Stackebrandt, E. (2010). *Ochrobactrum ciceri* sp. nov., isolated from nodules of *Cicer arietinum*. *Int J Syst Evol Microbiol* 60, 1548-1553.

Inglesby, T. V., Dennis, D. T., Henderson, D. A., Bartlett, J. G., Ascher, M. S., Eitzen, E., Fine, A. D., Friedlander, A. M., Hauer, J., Koerner, J. F. & other authors (2000). Plague as a biological weapon: medical and public health management. *JAMA* 283, 2281-2290.

Iqbal, S. S., Chambers, J. P., Goode, M. T., Valdes, J. J. & Brubaker, R. R. (2000). Detection of *Yersinia pestis* by pesticin fluorogenic probe-coupled PCR. *Mol Cell Probes* 14, 109-114.

Iserson, K. V. (2002). Demon doctors: physicians as serial killers. Tuscon: Galen Press, pp. 441.

Jantzen, E., Berdal, B. P. & Omland, T. (1979). Cellular fatty acid composition of *Francisella tularensis*. *J Clin Microbiol* 10, 928-930.

Jensen, W. I., Owen, C. R. & Jellison, W. L. (1969). *Yersinia philomiragia* sp. n., a new member of the *Pasteurella* group of bacteria, naturally pathogenic for the muskrat (*Ondatra zibethica*). *J Bacteriol* 100, 1237-1241.

Johansson, A., Ibrahim, A., Göransson, I., Eriksson, U., Gurycova, D., Clarridge, J. E. & Sjöstedt, A. (2000). Evaluation of PCR-based methods for discrimination of *Francisella* species and subspecies and development of a specific PCR that distinguishes the two major subspecies of *Francisella tularensis*. *J Clin Microbiol* 38, 4180-4185.

Johansson, A., Göransson, I., Larsson P. & Sjöstedt, A. (2002). Extensive allelic variation among *Francisella tularensis* strains in a short-sequence tandem repeat region. *J Clin Microbiol* 39, 3140-3146

Johansson, A., Farlow, J., Larsson, P., Dukerich, M., Chambers, E., Byström, M., Fox, J., Chu, M., Forsman, M., Sjöstedt, A. & Keim, P. (2004). Worldwide genetic relationships among *Francisella tularensis* isolates determined by multiple-locus variable-number tandem repeat analysis. *J Bacteriol* 186, 5808-5818.

Johansson, A., Celli, J., Conlan, W., Elkins, K. L., Forsman, M., Keim, P. S., Larsson, P., Manoil, C., Nano, F. E., Petersen, J. M. & Sjöstedt, A. (2010). Objections to the transfer of *Francisella novicida* to the subspecies rank of *Francisella tularensis*. *Int J Syst Evol Microbiol* 60, 1717-1718.

Judicial Commission of the International Committee on Systematic Bacteriology (1985). Opinion 60. Rejection of the name *Yersinia pseudotuberculosis* subsp. *pestis* (van Loghem) Bercovier et al. 1981 and conservation of the name *Yevsinia pestis* (Lehmann and Neumann) van Loghem 1944 for the plague bacillus. *Int J Syst Bacteriol* 35, 540.

Kämpfer, P., Buczolits, S., Albrecht, A., Busse, H.-J. & Stackebrandt, E. (2003). Towards a standardized format for the description of a novel species (of an established genus): *Ochrobactrum gallinifaecis* sp. nov. *Int J Syst Evol Microbiol* 53, 893-896.

Kämpfer, P., Rossello-Mora, R., Scholz, H. C., Welinder-Olsson, C., Falsen, E. & Busse, H.-J. (2006). Description of *Pseudochrobactrum* gen. nov., with the two species *Pseudochrobactrum asaccharolyticum* sp. nov. and *Pseudochrobactrum saccharolyticum* sp. nov. *Int J Syst Evol Microbiol* 56, 1823-1829.

Kämpfer, P., Scholz, H., Huber, B., Thummes, K., Busse, H.-J., Maas, E. W. & Falsen, E. (2007a). Description of *Pseudochrobactrum kiredjianiae* sp. nov. *Int J Syst Evol Microbiol* 57, 755-760.

Kämpfer, P., Scholz, H. C., Huber, B., Falsen, E. & Busse, H.-J. (2007b). *Ochrobactrum haematophilum* sp. nov. and *Ochrobactrum pseudogrignonense* sp. nov., isolated from human clinical specimens. *Int J Syst Evol Microbiol* 57, 2513-2518.

Kämpfer, P., Sessitsch, A., Schloter, M., Huber, B., Busse, H.-J. & Scholz, H. C. (2008). *Ochrobactrum rhizosphaerae* sp. nov. and *Ochrobactrum thiophenivorans* sp. nov., isolated from the environment. *Int J Syst Evol Microbiol* 58, 1426-1431.

Kämpfer, P., Martin, E., Lodders, N., Jäckel, U., Huber, B. E., Schumann, P., Langer, S., Busse, H.-J. & Scholz, H. (2010). *Paenochrobactrum gallinarii* gen. nov., sp. nov., isolated from air of a duck barn, and reclassification of *Pseudochrobactrum glaciei* as *Paenochrobactrum glaciei* comb. nov. *Int J Syst Evol Microbiol* 60, 1493-1498.

Keim, P., Johansson, A. & Wagner, D. M. (2007). Molecular epidemiology, evolution, and ecology of *Francisella*. *Ann NY Acad Sci* 1105, 30-66.

Kettaneh, A., Weill, F. X., Poilane, I., Fain, O., Thomas, M., Herrmann, J. L. and Hocqueloux, L. (2003). Septic shock caused by *Ochrobactrum anthropi* in an otherwise healthy host. *J Clin Microbiol* 41, 1339-1341.

Khushiramani, R., Shukla, J., Tuteja, U. & Batra, H. V. (2006). *Yersinia* outer-membrane protein B (YopB): a tool for identification of *Yersinia pestis* isolates. *J Med Microbiol* 55, 467-469.

Kim, W., Song, M. O., Song, W., Kim, K. J., Chung, S. I., Choi, C. S. & Park, Y. H. (2003). Comparison of 16S rDNA analysis and rep-PCR genomic fingerprinting for molecular identification of *Yersinia pseudotuberculosis*. *Antonie Van Leeuwenhoek* 83, 125-133.

Klevytska, A. M., Price, L. B., Schupp, J. M., Worsham, P. L., Wong, J. & Keim, P. (2001). Identification and characterization of variable-number tandem repeats in the *Yersinia pestis* genome. *J Clin Microbiol* 39, 3179-3185.

Kotetishvili, M., Kreger, A., Wauters, G., Morris, J. G., Sulakvelidze, A. & Stine, O. C. (2005). Multilocus sequence typing for studying genetic relationships among *Yersinia* species. *J Clin Microbiol* 43, 2674-2684.

Krober, M. S., Bass, J. W. & Barcia, P. J. (1983). Scarlatiniform rash and pleural effusion in a patient with *Yersinia pseudotuberculosis* infection. *J Pediatr* 102, 879-881.

Kugeler, K. J., Mead, P. S., McGowan, K. L., Burnham, J. M., Hogarty, M. D., Ruchelli, E., Pollard, K., Husband, B., Conley, C. & other authors (2008). Isolation and characterization of a novel *Francisella* sp. from human cerebrospinal fluid and blood. *J Clin Microbiol* 46, 2428-2431.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (eds.), Nucleic Acid Techniques in Bacterial Systematics. Chichester: Wiley, pp. 115-175.

Larsson, P., Larsson, P., Svensson, K., Karlsson, L., Guala, D., Granberg, M., Forsman, M. & Johanssont, A. (2007). Canonical insertion-deletion markers for rapid DNA typing of *Francisella tularensis*. *Emerg Infect Dis* 13, 1725-1732.

Leal, N. C. & de Almeida, A. M. P. (1999). Diagnosis of plague and identification of virulence markers in *Yersinia pestis* by multiplex-PCR. *Rev Inst Med Trop S Paulo* 41, 339-342.

Lebuhn, M., Achouak, W., Schloter, M., Berge, O., Meier, H., Barakat, M., Hartmann, A. & Heulin, T. (2000). Taxonomic characterization of *Ochrobactrum* sp. isolates from soil samples and wheat roots, and description of *Ochrobactrum tritici* sp. nov. and *Ochrobactrum grignonense* sp. nov. *Int J Syst Evol Microbiol* 50, 2207-2223.

Le Flèche, P., Jacques, I., Grayon, M., Al Dahouk, S., Bouchon, P., Denoeud, F., Nöckler, K., Neubauer, H., Guilloteau, L. A. & Vergnaud, G. (2006). Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. *BMC Microbiol* 6:9.

Lehmann, K. B. & Neumann, R. (1896). Atlas und Grundriss der Bakteriologie und Lehrbuch der speziellen bakteriologischen Diagnostik, 1st edn., München: J.F. Lehmann.

Lim, D. V., Simpson, J. M., Kearns, E. A. & Kramer, M. F. (2005). Current and developing technologies for monitoring agents of bioterrorism and biowarfare. *Clin Microbiol Rev* 18, 583-607.

Lindler, L. E. (2004). *Yersinia* spp. In: Fuchs, J., Podda, M. (eds.), Encyclopedia of medical genomics and proteomics. New York: Marcel Dekker Ltd, pp. 1–5.

Loïez, C., Herwegh, S., Wallet, F., Armand, S., Guinet, F. & Courcol, R. J. (2003). Detection of *Yersinia pestis* in sputum by real-time PCR. *J Clin Microbiol* 41, 4873-4875.

Louws, F. J., Fulbright, D. W., Stephens, C. T. & de Bruijn, F. J. (1994). Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Appl Environ Microbiol* 60, 2286-2295.

Lundquist, M., Caspersen, M. B., Wikström, P. & Forsman, M. (2005). Discrimination of *Francisella tularensis* subspecies using surface enhanced laser desorption ionization mass spectrometry and multivariate data analysis. *FEMS Microbiol Lett* 243, 303-310.

Makino, S., Okada, Y., Maruyama, T., Kaneko, S. & Sasakawa, C. (1994). PCR-based random amplified polymorphic DNA fingerprinting of *Yersinia pseudotuberculosis* and its practical applications. *J Clin Microbiol* 32, 65-69.

Manulis, S., Valinsky, L., Lichter, A. & Gabriel, D. W. (1994). Sensitive and specific detection of *Xanthomonas campestris* pv. *pelargonii* with DNA primers and probes identified by random amplified polymorphic DNA analysis. *Appl Environ Microbiol* 60, 4094-4099.

Maquart, M., Le Flèche P., Foster G., Tryland M., Ramisse F., Djønne B., Al Dahouk S., Jacques I., Neubauer H., Walravens K. & other authors (2009). MLVA-16 typing of 295 marine mammal *Brucella* isolates from different animal and geographic origins identifies 7 major groups within *Brucella ceti* and *Brucella pinnipedialis*. *BMC Microbiol* 9:145.

Marianelli, C., Ciuchini, F., Tarantino, M., Pasquali, P. & Adone, R. (2006). Molecular characterization of the *rpoB* gene in *Brucella* species: new potential molecular markers for genotyping. *Microbes Infect* 8, 860-865.

Martinevskij, I. L. (1969). Biology and genetic features of plague and plague-related microbes. Moscow, USSR: Meditsina Press.

Matero, P., Pasanen, T., Laukkanen, R., Tissari, P., Tarkka, E., Vaara, M. & Skurnik, M. (2009). Real-time multiplex PCR assay for detection of *Yersinia pestis* and *Yersinia pseudotuberculosis*. *APMIS* 117, 34-44.

Matero, P., Hemmilä, H., Tomaso, H., Piiparinen, H., Rantakokko-Jalava, K., Nuotio, L. & Nikkari, S. (2010). Rapid Field Detection Assays for *Bacillus anthracis*, *Brucella* spp., *Francisella tularensis* and *Yersinia pestis*. *Clin Microbiol Infect* (in press)

Mercier, E., Jumas-Bilak, E., Allardet-Servent, A., O'Callaghan, D. & Ramuz, M. (1996). Polymorphism in *Brucella* strains detected by studying distribution of two short repetitive DNA elements. *J Clin Microbiol* 34, 1299-1302.

Merhej, V., Adekambi, T., Pagnier, I., Raoult, D. & Drancourt, M. (2008). *Yersinia massiliensis* sp. nov., isolated from fresh water. *Int J Syst Evol Microbiol* 58, 779-784.

Meyer, K. F. & Shaw, E. B. (1920). A comparison of the morphologic, cultural and biochemical characteristics of *B*. *abortus* and *B*. *melitensis* from cattle. Studies on the genus *Brucella* nov. gen. *Journal of Infectious Diseases* 27, 173-184.

Michaux-Charachon, S., Bourg, G., Jumas-Bilak, E., Guigue-Talet, P., Allardet-Servent, A., O'Callaghan, D. & Ramuz, M. (1997). Genome structure and phylogeny in the genus *Brucella*. *J Bacteriol* 179, 3244-3249.

Mikalsen, J. & Colquhoun, D. J. (2009). *Francisella asiatica* sp. nov. isolated from farmed tilapia (*Oreochromis* sp.) and elevation of *Francisella philomiragia* subsp. *noatunensis* to species rank as *Francisella noatunensis* comb. nov., sp. nov. *Int J Syst Evol Microbiol*, doi:10.1099/ijs.0.002139-0

Mikalsen, J., Olsen, A. B., Tengs, T. & Colquhoun, D. J. (2007). *Francisella philomiragia* subsp. *noatunensis* subsp. nov., isolated from farmed Atlantic cod (*Gadus morhua* L.). *Int J Syst Evol Microbiol* 57, 1960-1965.

Molins-Schneekloth, C. R., Belisle, J. T. & Petersen, J. M. (2008). Genomic markers for differentiation of *Francisella tularensis* subsp. *tularensis* A.I and A.II. *Appl Environ Microbiol* 74, 336-341.

Molins, C. R., Carlson, J. K., Coombs, J. & Petersen, J. M. (2009). Identification of *Francisella tularensis* subsp. *tularensis* A1 and A2 infections by real-time polymerase chain reaction. *Diagn Microbiol Infect Dis* 64, 6-12.

Moreno, E., Cloeckaert, A. & Moriyon, I. (2002). *Brucella* evolution and taxonomy. *Vet Microbiol* 90, 209-227.

Motin, V. L., Georgescu, A. M., Elliott, J. M., Hu, P., Worsham, P. L., Ott, L. L., Slezak, T. R., Sokhansanj, B. A., Regala, W. M., Brubaker, R. R. & other authors (2002). Genetic variability of *Yersinia pestis* isolates as predicted by PCR-based IS*100* genotyping and analysis of structural genes encoding glycerol-3-phosphate dehydrogenase (*glpD*). *J Bacteriol* 184, 1019-1027.

Murphy, J. K. (2004). After 9/11: priority focus areas for bioterrorism preparedness in hospitals. *J Health Manag* 49, 227-235.

Neubauer, H., Meyer, H., Prior, S., Aleksic, S., Hensel, A. & Splettstösser, W. (2000a). A combination of different polymerase chain reaction (PCR) assays for the presumptive identification of *Yersinia pestis*. *J Vet Med B Infect Dis Vet Public Health* 47, 573-580.

Neubauer, H., Molitor, M., Rahalison, L., Aleksic, S., Backes, H., Chanteau, S. & Meyer, H. (2000b). A miniaturised semiautomated system for identification of *Yersinia* species within the genus *Yersinia*. *Clin Lab* 46, 561-567.

Norkina, O. V., Kulichenko, A. N., Gintsburg, A. L., Tuchkov, I. V., Popov, Yu. A., Aksenov, M. U. & Drosdov, I. G. (1994). Development of a diagnostic test for *Yersinia pestis* by the polymerase chain reaction. *J Appl Bacteriol* 76, 240-245.

Nübel, U., Reissbrodt, R., Weller, A., Grunow, R., Porsch-Özcürümez, M., Tomaso, H., Hofer, E., Splettstoesser, W., Finke, E.-J., Tschäpe, H. & W. Witte (2006). Population structure of *Francisella tularensis*. *J Bacteriol* 188, 5319-5324.

Nutter, J. E. (1971). Antigens of *Pasteurella tularensis*: preparative procedures. *Appl Microbiol* 22, 44-48.

Ocampo-Sosa, A. A., Agüero-Balbin, J. & Garcia-Lobo, J. M. (2005). Development of a new PCR assay to identify *Brucella abortus* biovars 5, 6 and 9 and the new subgroup 3b of biovar 3. *Vet Microbiol* 110, 41-51.

Ochoa, T. J. & Cleary, T. G. (2006). *Yersinia pseudotuberculosis*. In: McMillan, J. A., Feigin, R. D., DeAngelis, C. D., Jones, M. D. (eds.), Oski's Pediatrics. Principles and practice. Philadelphia: Lippincott Williams & Wilkins, pp. 1161-1162.

Oggioni, M. R., Meacci, F., Carattoli, A., Ciervo, A., Orru, G., Cassone, A. & Pozzi, G. (2002). Protocol for real-time PCR identification of anthrax spores from nasal swabs after broth enrichment. *J Clin Microbiol* 40, 3956-3963.

Ohishi, K., Takishita, K., Kawato, M., Zenitani, R., Bando, T., Fujise, Y., Goto, Y., Yamamoto, S. & Maruyama, T. (2004). Molecular evidence of new variant *Brucella* in North Pacific common minke whales. *Microbes Infect* 6, 1199-1204.

Olsufjev, N. G. & Meshcheryakova, I. S. (1982). Infraspecific taxonomy of tularemia agent *Francisella tularensis* McCoy and Chapin. *J Hyg Epidemiol Microbiol Immunol* 26, 291-299.

Olsufjev, N. G. & Meshcheryakova, I. S. (1983). Subspecific taxonomic of *Francisellla tularensis*. *Int J Syst Bacteriol* 33, 872-874.

Osterman, B. & Moriyon, I. (2006). International Committee on Systematics of Prokaryotes. Subcommittee on the taxonomy of *Brucella*. Minutes. *Int J Syst Evol Microbiol* 56, 1173-1175.

Ouahrani-Bettache, S., Soubrier, M.P. & Liautard, J.P. (1996). IS*6501*-anchored PCR for the detection and identification of *Brucella* species and strains. *J Appl Bacteriol* 81, 154-160.

Ozdemir, D., Soypacaci, Z., Sahin, I., Bicik, Z. & Sencan, I. (2006). *Ochrobactrum anthropi* endocarditis and septic shock in a patient with no prosthetic valve or rheumatic heart disease: a case report and review of literature. *Jpn J Infect Dis* 59, 264-265.

Page, R. D. M. (1996). TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* 12, 357-358.

Paradis, S., Boissinot, M., Paquette, N., Belanger, S. D., Martel, E. A., Boudreau, D. K., Picard, F. J., Ouellette, M., Roy, P. H. & Bergeron, M. G. (2005). Phylogeny of the *Enterobacteriaceae* based on genes encoding elongation factor Tu and F-ATPase beta-subunit. *Int J Syst Evol Microbiol* 55, 2013-2025.

Parkhill, J., Wren, B. W., Thomson, N. R., Titball, R. W., Holden, M. T., Prentice, M. B., Sebaihia, M., James, K. D., Churcher, C., Mungall, K. L. & other authors (2001). Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature* 413, 523-527.

Perry, R. D. & Fetherston, J. D. (1997). *Yersinia pestis* - etiologic agent of plague. *Clin Microbiol Rev* 10, 35-66.

Pitcher, D. G., Saunders, N. A. & Owen, R. J. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* 8, 151-156.

Pourcel, C., Andre-Mazeaud, F., Neubauer, H., Ramisse, F. & Vergnaud, G. (2004). Tandem repeats analysis for the high resolution phylogenetic analysis of *Yersinia pestis*. *BMC Microbiol* 4:22.

Probert, W. S., Schrader, K. N., Khuong, N. Y., Bystrom, S. L. & Graves, M. H. (2004). Real-time multiplex PCR assay for detection of *Brucella* spp., *B. abortus*, and *B. melitensis*. *J Clin Microbiol* 42, 1290-1293.

Pujol, C. & Bliska, J. B. (2005). Turning *Yersinia* pathogenesis outside in: subversion of macrophage function by intracellular yersiniae. *Clin Immunol* 114, 216-226.

Queipo-Ortuño, M., Colmenero, J.D., Baeza, G. & Morata, P. (2005). Comparison between LightCycler real-time polymerase chain reaction (PCR) assay with serum and PCR-enzyme-linked immunosorbent assay with whole blood samples for the diagnosis of human brucellosis. *Clin Infect Dis* 40, 260-264.

Radnedge, L., Gamez-Chin, S., McCready, P. M., Worsham, P. L. & Andersen, G. L. (2001). Identification of nucleotide sequences for the specific and rapid detection of *Yersinia pestis. Appl Environ Microbiol* 67, 3759-3762.

Radnedge, L., Agron, P. G., Worsham, P. L. & Andersen, G. L. (2002). Genome plasticity in *Yersinia pestis*. *Microbiology* 148, 1687-1698.

Rakin, A. (2003). *Yersinia pestis*. Eine Bedrohung für die Menschheit. Bundesgesundheitsblatt - Gesundheitsforschung - Gesundheitsschutz 46, 949-955.

Rajashekara, G., Glasner, J. D., Glover, D. A. & Splitter, G. A. (2004). Comparative whole-genome hybridization reveals genomic islands in *Brucella* species. *J Bacteriol* 186, 5040-5051.

Ratushna, V. G., Sturgill, D. M., Ramamoorthy, S., Reichow, S. A., He, Y., Lathigra, R., Sriranganathan, N., Halling, S. M., Boyle, S. M. & Gibas, C. J. (2006). Molecular targets for rapid identification of *Brucella* spp. *BMC Microbiol* 6:13.

Redkar, R., Rose, S., Bricker, B. & DelVecchio, V. (2001). Real-time detection of *Brucella abortus*, *Brucella melitensis* and *Brucella suis*. *Mol Cell Prob* 15, 43-52.

Reintjes, R., Dedushaj, I., Gjini, A., Jorgensen, T. R., Cotter, B., Lieftucht, A., D'Ancona, F., Dennis, D. T., Kosoy, M. A., Mulliqi-Osmani, G., Grunow, R., Kalaveshi, A., Gashi, L. & Humolli, I. (2002). Tularemia outbreak investigation in Kosovo: case control and environmental studies. *Emerg Infect Dis* 8, 69-73.

Richards, G. M. (1974). Modification of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. *Anal Biochem* 57, 369-376.

Rotz, L. D., Khan, A. S., Lillibridge, S. R., Ostroff, S. M. & Hughes, J. M. (2002). Public Health Assessment of Potential Biological Terrorism Agents. *Emerg Infect Dis* 8, 225-230.

Samrakandi, M. M., Zhang, C., Zhang, M., Nietfeldt, J., Kim, J., Iwen, P. C., Olson, M. E., Fey, P.D., Duhamel, G. E., Hinrichs, S. H., Cirillo, J. D. & Benson, A. K. (2004). Genome diversity among regional populations of *Francisella tularnesis* subspecies *tularnesis* and *Francisella tularnesis* subspecies *holarctica* isolated from the US. *FEMS Microbiol Lett* 237, 9-17.

Sandström, G., Löfgren, S. & Tärnvik, A. (1988). A capsule deficient mutant of *Francisella tularensis* LVS exhibits enhanced sensitivity to killing by serum but diminished sensitivity to killing by polymorphonuclear leukocytes. *Infect Immun* 56, 1194-1202.

Sandström, G., Sjöstedt, A., Forsman, M., Pavlovich, N. V. & Mishan'kin, B. N. (1992). Characterization and classification of strains of *Francisella tularensis* isolated in the central Asian focus of the Soviet Union and in Japan. *J Clin Microbiol* 30, 172-175.

Saslaw, S., Engelsbach, H. T., Prior, J. A., Wilson, H. E. & Carhart, S. (1961). Tularemia vaccine study. II. Respiratory challenge. *Arch Intern Med* 107, 702-714.

Scholz, H. C., Tomaso, H., Dahouk, S. A., Witte, A., Schloter, M. & Kämpfer, P. (2006). Genotyping of *Ochrobactrum anthropi* by recA-based comparative sequence, PCR-RFLP, and 16S rRNA gene analysis. *FEMS Microbiol Lett* 257, 7-16.

Scholz, H. C., Pfeffer, M., Witte, A., Neubauer, H., Al Dahouk, S., Wernery, U. & Tomaso, H. (2008a). Specific detection and differentiation of *Ochrobactrum anthropi*, *Ochrobactrum intermedium* and *Brucella* spp. by a multi-primer PCR that targets the *recA* gene. *J Med Microbiol* 57, 64-71.

Scholz, H. C., Al Dahouk, S., Tomaso, H., Neubauer, H., Witte, A., Schloter, M., Kämpfer, P., Falsen, E., Pfeffer, M. & Engel, M. (2008b). Genetic diversity and phylogenetic relationships of bacteria belonging to the *Ochrobactrum-Brucella* group by by recA and 16S rRNA gene-based comparative sequence analysis. *Syst Appl Microbiol* 31, 1-16.

Scholz, H. C., Hubalek, Z., Sedlácek, I., Vergnaud, G., Tomaso, H., Al Dahouk, S., Melzer, F., Kämpfer, P., Neubauer, H., Cloeckaert, A. & other authors (2008c). *Brucella microti* sp. nov., isolated from the common vole *Microtus arvalis*. *Int J Syst Evol Microbiol* 58, 375-382.

Scholz, H. C., Hofer, E., Vergnaud, G., Le Fleche, P., Whatmore, A. M., Al Dahouk, S., Pfeffer, M., Krüger, M., Cloeckaert, A. & Tomaso, H. (2009). Isolation of *Brucella microti* from mandibular lymph nodes of red foxes, *Vulpes vulpes*, in lower Austria. *Vector Borne Zoonotic Dis* 9, 153-156.

Scholz, H. C., Nöckler, K., Göllner, C., Bahn, P., Vergnaud, G., Tomaso, H., Al-Dahouk, S., Kämpfer, P., Cloeckaert, A., Maquart, M., Zygmunt, M. S., Whatmore, A. M., Pfeffer, M., Huber, B., Busse, H.-J. and De Kumar, B. (2010). *Brucella inopinata* sp. nov., isolated from a breast implant infection. *Int J Syst Evol Microbiol* 60, 801-808.

Schuler, A. (2004). Billions for biodefense: federal agency biodefense funding, FY2001-FY2005. *Biosecur Bioterror* 2, 86-96.

Scott, J. C., Koylass, M. S., Stubberfield, M. R. & Whatmore, A. M. (2007). Multiplex assay based on single-nucleotide polymorphisms for rapid identification of *Brucella* isolates at the species level. *Appl Eviron Microbiol* 73, 7331-7337.

Shen, X., Wang, Q., Xia, L., Zhu, X., Zhang, Z., Liang, Y., Cai, H., Zhang, E., Wei, J., Chen, C. & other authors (2010). Complete genome sequences of *Yersinia pestis* from natural foci in China. *J Bacteriol* 192, 3551-3552.

Sjöstedt, A. B. (2005a). Genus I. *Francisella* Dorofe'ev 1947, 176$^{AL}$. In: Brenner, D. J., Krieg, N. R., Staley, J. T., Garrity, G. M. (eds.), Bergey's Manual of Systematic Bacteriology, vol. 2, 2nd edn., New York: Springer, pp. 200-210.

Sjöstedt, A. B. (2005b). Family III. *Francisellaceae* fam. nov. In: Brenner, D. J., Krieg, N. R., Staley, J. T., Garrity, G. M. (eds.), Bergey's Manual of Systematic Bacteriology, vol. 2, 2nd edn., New York: Springer, pp. 199-200.

Sjöstedt, A. (2007). Tularemia: history, epidemiology, pathogen physiology, and clinical manifestations. *Ann NY Acad Sci* 1105, 1-29.

Skurnik, M., Peippo, A. & Ervela, E. (2000). Characterization of the O-antigen gene clusters of *Yersinia pseudotuberculosis* and the cryptic O-antigen gene cluster of *Yersinia pestis* shows that the plague bacillus is most closely related to and has evolved from *Y. pseudotuberculosis* serotype O:1b. *Mol Microbiol* 37, 316-330.

Sodeinde, O. A. & Goguen, J. D. (1988). Genetic analysis of the 9.5-kilobase virulence plasmid of *Yersinia pestis*. *Infect Immun* 56, 2743-2748.

Sohn, A. H., Probert, W. S., Glaser, C. A., Gupta, N., Bollen, A. W., Wong, J. D., Grace, E. M. & McDonald, W. C. (2003). Human neurobrucellosis with intracerebral granuloma caused by a marine mammal *Brucella*. spp. *Emerg Infect Dis* 9, 485-488.

Somov, G. P. (1976). Far Eastern scarlatina-like fever (human pseudotuberculosis). *Zh Mikrob Epidemiol Immunol* 4, 103-108.

Song, Y., Tong, Z., Wang, J., Wang, L., Guo, Z., Han, Y., Zhang, J., Pei, D., Zhou, D., Qin, H. & other authors (2004). Complete genome sequence of *Yersinia pestis* strain 91001, an isolate avirulent to humans. *DNA Research* 11, 179-197.

Sprague, L. D. & Neubauer, H. (2005). *Yersinia aleksiciae* sp. nov. *Int J Syst Evol Microbiol* 55, 831-835.

Sprague, L. D., Scholz, H. C., Amann, S., Busse, H.-J. & Neubauer, H. (2008). *Yersinia similis* sp. nov. *Int J Syst Evol Microbiol* 58, 952-958.

Staples, J. E., Kubota, K. A., Chalcraft, L. G., Mead, P. S. & Petersen, J. M. (2006). Epidemiologic and molecular analysis of human tularemia, United States, 1964-2004. *Emerg Infect Dis* 12, 1113-1118.

Stewart, A., Satterfield, B., Cohen, M., O'Neill, K. & Robinson, R. (2008). A quadruplex real-time PCR assay for the detection of *Yersinia pestis* and its plasmids. *J Med Microbiol* 57, 324-331.

Stoenner, H. G. & Lackman, D. B. (1957). A new species of *Brucella* isolated from the desert wood rat, *Neotoma lepida* Thomas. *Am J Vet Res* 18, 947-951.

Svensson, K., Granberg, M., Karlsson, L., Neubauerova, V., Forsman, M. & Johansson, A. (2009). A real-time PCR array for hierarchical identification of *Francisella* isolates. *PLoS One* 4, e8360.

Tärnvik, A. & Chu, M. C. (2007). New approaches to diagnosis and therapy of tularemia. *Ann NY Acad Sci* 1105, 378-404.

Tcherneva, E., Rijpens, N., Naydensky, C. & Herman, L. M. (1996). Repetitive element sequence based polymerase chain reaction for typing of *Brucella* strains. *Vet Microbiol* 51, 169-178.

Tcherneva, E., Rijpens, N., Jersek, B. & Herman, L. M. (2000). Differentiation of *Brucella* species by random amplified polymorphic DNA analysis. *J Appl Microbiol* 88, 69-80.

Teng, L.-J., Hsueh, P.-R., Tsai, J.-C., Chiang, F.-L., Chen, C.-Y., Ho, S.-W. & Luh, K.-T. (2000). PCR assay for species-specific identification of *Bacteroides thetaiotaomicron*. *J Clin Microbiol* 38, 1672-1675.

Teyssier, C., Marchandin, H., Jean-Pierre, H., Masnou, A., Dusart, G. & Jumas-Bilak, E. (2007). *Ochrobactrum pseudintermedium* sp. nov., a novel member of the family *Brucellaceae*, isolated from human clinical samples. *Int J Syst Evol Microbiol* 57, 1007-1013.

Thomas, R., Johansson, A., Neeson, B., Isherwood, K., Sjöstedt, A., Ellis, J. & Titball, R. W. (2003). Discrimination of human pathogenic subspecies of *Francisella tularensis* using restriction fragment length polymorphism. *J Clin Microbiol* 41, 50-57.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gappenalties and weight matrix choice. *Nucleic Acids Res* 22, 4673-4680.

Tiller, R. V., Gee, J. E., Lonsway, D. R., Gribble, S., Bell, S. C., Jennison, A. V., Bates, J., Coulter, C., Hoffmaster, A. R. & De, B. K. (2010). Identification of an unusual *Brucella* strain (BO2) from a lung biopsy in a 52-year old patient with chronic destructive pneumonia. *BMC Microbiol* 10:23.

Titball, R. W. & Williamson, E. D. (2001). Vaccination against bubonic and pneumonic plague. *Vaccine* 19, 4175-4184.

Tomaso, H., Reisinger, E. C., Al Dahouk, S., Frangoulidis, D., Rakin, A., Landt, O. & Neubauer, H. (2003). Rapid detection of *Yersinia pestis* with multiplex real-time PCR assay using fluorescent hybridisation probes. *FEMS Immunol Med Microbiol* 38, 117-126.

Tomaso, H., Scholz, H. C., Neubauer, H., Al Dahouk, S., Seibold, E., Landt, O., Forsman, M. & Splettstoesser, W. D. (2006). Real-time PCR using hybridization probes fort he rapid and specific identification of *Francisella tularensis* subspecies *tularensis*. *Mol Cell Probes* 21, 12-16.

Trebesius, K., Harmsen, D., Rakin, A., Schmelz, J. & Heesemann, J. (1998). Development of rRNA-targeted PCR and in situ hybridization with fluorescently labelled oligonucleotides for detection of *Yersinia* species. *J Clin Microbiol* 36, 2557-2564.

Tripathi, A. K., Verma, S. C., Chowdhury, S. P., Lebuhn, M., Gattinger, A. & Schloter, M. (2006). *Ochrobactrum oryzae* sp. nov., an endophytic bacterial species isolated from deep-water rice in India. *Int J Syst Evol Microbiol* 56, 1677-1680.

Trosky, J. E., Liverman, A. D. B. & Orth, K. (2008). *Yersinia* outer proteins: Yops. *Cell Microbiol* 10, 557-565.

Trujillo, M. E., Willems, A., Abril, A., Planchuelo, A. M., Rivas, R., Ludeña, D., Mateos, P. F., Martinez-Molina, E. & Velazquez, E. (2005). Nodulation of *Lupinus albus* by strains of *Ochrobactrum lupini* sp. nov. *Appl Environ Microbiol* 71, 1318-1327. Validation List No 110 (2006). *Int J Syst Evol Microbiol* 56, 1459-1460.

Tsukano, H., Itoh, K., Suzuki, S. & Watanabe, H. (1996). Detection and identification of *Yersinia pestis* by polymerase chain reaction (PCR) using multiplex primers. *Microbiol Immunol* 40, 773-775.

Tucker, J. B. (1999). Historical Trends Related to Bioterrorism: An Empirical Analysis. *Emerg Infect Dis* 5, 498-504.

Ulrich, R. L., Ulrich, M. P., Schell, M. A., Kim, H. S. & DeShazer, D. (2006). Development of a polymerase chain reaction assay for the specific identification of *Burkholderia mallei* and differentiation from *Burkholderia pseudomallei* and other closely related *Burkholderiaceae*. *Diagn Microbiol Infect Dis* 55, 37-45.

Vaidya, S. A., Citron, D. M., Fine, M. B., Murakami, G. & Goldstein, E. J. (2006). Pelvic abscess due to *Ochrobactrum anthropi* in an immunocompetent host: report case and review of literature. *J Clin Microbiol* 44, 1184-1186.

Van Ert, M. N., Easterday, W. R., Simonson, T. S., U'Ren, J. M., Pearson, T., Kenefic, L. J., Busch, J.D., Huynh, L. Y., Dukerich, M., Trim, C. B. & other authors (2007). Strain-specific single-nucleotide polymorphism assays for the *Bacillus anthracis* Ames strain. *J Clin Microbiol* 45, 47-53.

Velasco, J., Romero, C., Lopez-Goñi, I., Leiva, J., Diaz, R. & Moriyon, I. (1998). Evaluation of the relatedness of *Brucella* spp. And *Ochrobactrum anthropi* and description of *Ochrobactrum intermedium* sp. nov., a new species with a closer relationship to *Brucella* spp. *Int J Syst Bacteriol* 48, 759-768.

Verger, J.-M., Grimont, F., Grimont, P. A. D. & Grayon, M. (1985). *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. *Int J Syst Bacteriol* 35, 292-295.

Verger, J.-M., Grayon, M., Cloeckaert, A., Lefèvre, M., Ageron, E. & Grimont, F. (2000). Classification of *Brucella* strains isolated from marine mammals using DNA-DNA hybridization and ribotyping. *Res Microbiol* 151, 797-799.

Versage, J. L., Severin, D. D. M., Chu, M. C. & Petersen, J. M. (2003). Development of a multitarget real-time TaqMan PCR assay for enhanced detection of *Francisella tularensis* in complex specimens. *J Clin Microbiol* 41, 5492-5499.

Versalovic, J., Koeuth, T. & Lupski, J. R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* 19, 6823-6831.

Versalovic, J., Schneider, M., de Bruijn, F. J. & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol Cell Biol* 5, 25-40.

Vizcaino, N., Verger, J.-M., Grayon, M., Zygmunt, M. S. & Cloeckaert, A. (1997). DNA polymorphism at the omp-31 locus of *Brucella* spp.: evidence for a large deletion in *Brucella abortus*, and other species-specific markers. *Microbiology* 143, 2913-2921.

Vizcaíno, N., Caro-Hernández, P., Cloeckaert, A. & Fernández-Lago, L. (2004). DNA polymorphism in the omp25/omp31 family of *Brucella* spp.: identification of a 1.7-kb inversion in *Brucella cetaceae* and of a 15.1-kb genomic island, absent from *Brucella ovis*, related to the synthesis of smooth lipopolysaccharide. *Microbes Infect* 6, 821-834.

Wang, X., Han, Y., Li, Y., Guo, Z., Song, Y., Tan, Y., Du, Z., Rakin, A., Zhou, D. & Yang, R. (2007). *Yersinia* genome diversity disclosed by *Yersinia pestis* genome-wide DNA microarray. *Can J Microbiol* 53, 1211-1221.

Wang, J., Yang, Y., Zhou, L., Wang, J., Jiang, Y., Hu, K., Sun, X., Hou, Y., Zhu, Z., Guo, Z., Ding, Y. and Yang, R. (2009). Simultaneous detection of five biothreat agents in powder samples by a multiplexed suspension array. *Immunopharmacol Immunotoxicol* 31, 417-427.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* 37, 463-464.

Whatmore, A. M., Murphy, T. J., Shankster, S.,Young, E., Cutler, S. J. & Macmillan, A. P. (2005). Use of amplified fragment length polymorphism to identify and type *Brucella* isolates of medical and veterinary interest. *J Clin Microbiol* 43, 761-769.

Whatmore, A. M., Shankster, S. J., Perrett, L. L., Murphy, T. J., Brew, S. D., Thirlwall, R. E., Cutler, S. J. & MacMillan, A. P. (2006). Identification and characterization of variable-number tandem-repeat markers for typing of *Brucella* spp. *J Clin Microbiol* 44, 1982-1993.

Whatmore, A. M., Perrett, L. L. & MacMillan, A. P. (2007). Characterisation of the genetic diversity of *Brucella* by multilocus sequencing. *BMC Microbiol* 7:34.

Wheelis, M. (1999). Biological warfare before 1914. In: Geissler E., Moon JE. v. C. (eds.), Biological and toxin weapons: research, development and use from the Middle Ages to1945. London: Oxford University Press, p. 8-34.

Wicki, R., Lauter, P., Mettler, C., Natsch, A., Enzler, T., Pusterla, N., Kuhnert, P., Egli, G., Bernasconi, M., Lienhard, R., Lutz, H. & Leutenegger, C. M. (2000). Swiss Army survey in Switzerland to determine the prevalence of *Francisella tularensis*, members of the *Ehrlichia phagocytophila* genogroup, *Borrelia burgdorferi* sensu lato, and tick-borne encephalitis virus in ticks. *Eur J Clin Microbiol Infect Dis* 19, 427-432.

Wilson, W. J., Erler, A. M., Nasarabadi, S. L., Skowronski, E. W. & Imbro, P. M. (2005). A multiplexed PCR-coupled liquid bead array for the simultaneous detection of four biothreat agents. *Mol Cell Probes* 19, 137-144.

Wormser, G. P. & Keusch, G. T. (1981). *Yersinia enterocolitica*: clinical observations. In: Bottone, E. J. (ed.), *Yersinia enterocolitica*. Boca Raton: CRC Press, pp. 83-93.

Woron, A. M., Nazarian, E. J., Egan, C., McDonough, K. A., Cirino, N. M., Limberger, R. J. & Musser, K. A. (2006). Development and evaluation of a 4-target multiplex real-time polymerase chain reaction assay for the detection and characterization of *Yersinia pestis*. *Diagn Microbiol Infect Dis* 56, 261-268.

Wright, P. W. (1987). Brucellosis. *Am Fam Physician* 35, 155-159.

Yang, S., Rothman, R. E., Hardick, J., Kuroki, M., Hardick, A., Doshi, V., Ramachandran, P. & Gaydos, C. A. (2008). Rapid polymerase chain reaction-based screening assay for bacterial biothreat agents. *Acad Emerg Med* 15, 388-392.

Yang, S., Ramachandran, P., Rothman, R., Hsieh, Y. H., Hardick, A., Won, H., Kecojevic, A., Jackman, J. & Gaydos, C. (2009). Rapid identification of biothreat and other clinically relevant bacterial species by use of universal PCR coupled with high-resolution melting analysis. *J Clin Microbiol* 47, 2252-2255.

Young, E. J. (1995). An overview of human brucellosis. *Clin Infect Dis* 21, 283-290.

Zhang, Z., Hai, R., Song, Z., Xia, L., Liang, Y., Cai, H., Liang, Y., Shen, X., Zhang, E., Xu, J., Yu, D. & Yu, X. J. (2009). Spatial variation of *Yersinia pestis* from Yunnan Province of China. *Am J Trop Med Hyg* 81, 714-717.

Zhou, D., Han, Y., Song, Y., Huang, P. & Yang, R. (2004a). Comparative and evolutionary genomics of *Yersinia pestis*. *Microbes Infect* 6, 1226-1234.

Zhou, D., Han, Y., Dai, E., Pei, D., Song, Y., Zhai, J., Du, Z., Wang, J., Guo, Z. & Yang, R. (2004b). Identification of signature genes for rapid and specific characterization of *Yersinia pestis*. *Microbiol Immunol* 48, 263-269.

Zhou, D., Tong, Z., Song, Y., Han, Y., Pei, D., Pang, X., Zhai, J., Li, M., Cui, B., Qi, Z. & other authors (2004c). Genetics of metabolic variations between *Yersinia pestis* biovars and the proposal of a new biovar, microtus. *J Bacteriol* 186, 5147-5152.

Zhou, D., Han, Y., Song, Y., Tong, Z., Wang, J., Guo, Z., Pei, D., Pang, X., Zhai, J., Li, M. & other authors (2004d). DNA microarray analysis of genome dynamics in *Yersinia pestis*: insights into bacterial genome microevolution and niche adaptation. *J Bacteriol* 186, 5138-5146.

Ziemke, F., Höfle, M. G., Lalucat, J. & Rossello-Mora, R. (1998). Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* 48, 179-186.

Zurdo-Piñeiro, J. L., Rivas, R., Trujillo, M. E., Vizcaino, N., Carrasco, J. A., Chamber, M., Palomares, A., Mateos, P. F., Martinez-Molina, E. & Velazquez, E. (2007). *Ochrobactrum cytisi* sp. nov., isolated from nodules of *Cytisus scoparius* in Spain. *Int J Syst Evol Microbiol* 57, 784-788.

# Curriculum vitae

## Persönliche Daten

| | |
|---|---|
| Name: | Birgit Huber |
| geboren am: | 3. Februar 1979 in Steyr |
| Familienstand: | ledig |
| wohnhaft in: | 4312 Ried/Riedmark, Loitzenberg 1<br>Tel.: 0650/8430320 |

## Schulausbildung und Studium

| | |
|---|---|
| 09/89 – 07/93 | Hauptschule Ried/Riedmark |
| 09/93 – 07/97 | Bundesoberstufenrealgymnasium Perg, naturwissenschaftlicher Zweig |
| 10/97 – 06/03 | Studium der Biologie an der Paris-Lodron-Universität Salzburg<br>Diplomarbeitsthema: „Towards the characterization of a bacterial endosymbiont of the pennate freshwater diatom *Pinnularia* cf. '*mesogongyla*'" |
| 10/00 – 06/08 | Lehramtsstudium Englisch und Biologie und Umweltkunde |
| 10/05 – 08/08 | Dissertation an der Universität Wien (Studienzweig Genetik/ Mikrobiologie) unter der Betreuung von Univ.-Doz. Dr. Hans-Jürgen Busse |

## Studienbegleitende Tätigkeiten

| | |
|---|---|
| 2000 | Neugestaltung und Wartung der Homepage des Instituts für Didaktik der Naturwissenschaften an der Universität Salzburg |
| 1999 – 2005 | Tutorien an den Instituten für Pflanzenphysiologie und Genetik |
| 2003 – 2005 | Museumspädagogische Tätigkeit im *Haus der Natur* in Salzburg |
| 2005 – 2007 | Tutorien am Institut für Mikrobiologie und Genetik der Universität Wien |

## Berufspraxis

| | |
|---|---|
| 09/08 bis 09/09 | Unterrichtspraktikum am BRG Hamerlingstraße in Linz (Fächer: Englisch, Biologie und Umweltkunde) |
| seit 09/09 | Tätigkeit als Lehrerin für Englisch, Biologie und Chemie an der HLW Freistadt |