universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„Optimization of a DNA microarray for global transcription analysis of *Protochlamydia amoebophila*“

Verfasser

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angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag.rer.nat.)

Wien, 2009

Studienkennzahl lt. Studienblatt:	A 441
Studienrichtung lt. Studienblatt:	Genetik – Mikrobiologie (Stzw)
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B. Introduction

B.1. Chlamydiae

B.1.1. The order *Chlamydiales*

Chlamydiae are a group of obligate intracellular bacteria that constitute the single order *Chlamydiales* in the phylum *Chlamydiae*. A hallmark of all chlamydiae is their unique biphasic developmental cycle (Ward 1988; Moulder 1991; Abdelrahman et al. 2005). Until the identification of *Chlamydia*-like endosymbionts in acanthamoebae in the 1990s (Fritsche et al. 1993; Amann et al. 1997; Birtles et al. 1997; Fritsche et al. 2000; Horn et al. 2000), the only known family in the order *Chlamydiales* were the *Chlamydiaceae*, a family exclusively comprised of clinically relevant human and animal pathogens. Since then, numerous representatives of new chlamydial families were discovered and a total of seven new families of so-called environmental chlamydiae has been described (Kuo et al. 2008).

B.1.2. Clinical chlamydiae

Members of the family *Chlamydiaceae* are often referred to as clinical chlamydiae, since all species exhibit pathogenic potential in humans or animals. The family consists of two genera, *Chlamydia* and *Chlamydophila* (Everett et al. 1999). Species of the genus *Chlamydia* seem to be restricted to mammals as host organisms, while species of the genus *Chlamydophila* do not exhibit such a strict host specificity as they were also shown to infect amphibians, reptiles and birds, as well as mammalian species (Corsaro et al. 2004). The family *Chlamydiaceae* includes two of the most widespread human pathogens, *Chlamydia trachomatis* and *Chlamydophila pneumoniae*. *C. trachomatis* is the most common cause of bacterial sexually transmitted infections, with over 90 million new cases per year (WHO 2001). It is also the causative agent of trachoma that affects the inner upper eyelid and cornea and is one of the leading infectious causes of blindness (WHO 2009). *C. pneumoniae* is a widely distributed respiratory pathogen that accounts for an estimated 2 to 43% of community-acquired pneumonia and 5% of bronchitis and sinusitis cases (Kuo et al. 1995; Wellinghausen et al. 2006). Furthermore, it is possibly associated with several chronic diseases of unknown cause, such as atherosclerosis, multiple sclerosis, asthma and Alzheimer's disease (Mahony et al. 2003). Another major concern for public health is the zoonotic potential of *Chlamydophila psittaci* and *Chlamydophila abortus*. *C. psittaci* is the cause of avian psittacosis in birds, but infection can also be transferred to humans, where it leads to psittacosis with severe clinical

symptoms (Elder et al. 1999; Gaede et al. 2008). *C. abortus*, the causative agent of enzootic abortions in sheep (Aitken 2000; Longbottom et al. 2003), can also be transferred to humans, posing a threat especially to pregnant women (Longbottom et al. 2003).

B.1.3. Environmental chlamydiae

The term 'environmental chlamydiae' (Horn et al. 2001) describes the novel *Chlamydia*-like bacteria that were identified in the last years that do not belong to the family *Chlamydiaceae*. Their discovery has shown that the phylogenetic diversity is substantially greater than expected and has lead to the description of seven additional families in the order *Chlamydiales*: *Parachlamydiaceae*, *Simkaniaceae* (Everett et al. 1999), *Waddliaceae* (Rurangirwa et al. 1999), *Piscichlamydiaceae* (Draghi et al. 2004), *Rhabdochlamydiaceae* (Kostanjsek et al. 2004; Corsaro et al. 2007), *Criblamydiaceae* (Thomas et al. 2006), and *Clavochlamydiaceae* (Karlsen et al. 2008) (Figure 1). However, the actual diversity is expected to be even larger, indicated by the detection of phylogenetically diverse rRNA sequences related to recognized chlamydiae in a great number of different habitats (Ossewaarde et al. 1999; Corsaro et al. 2001; Horn et al. 2001; Corsaro et al. 2002; Horn 2008).

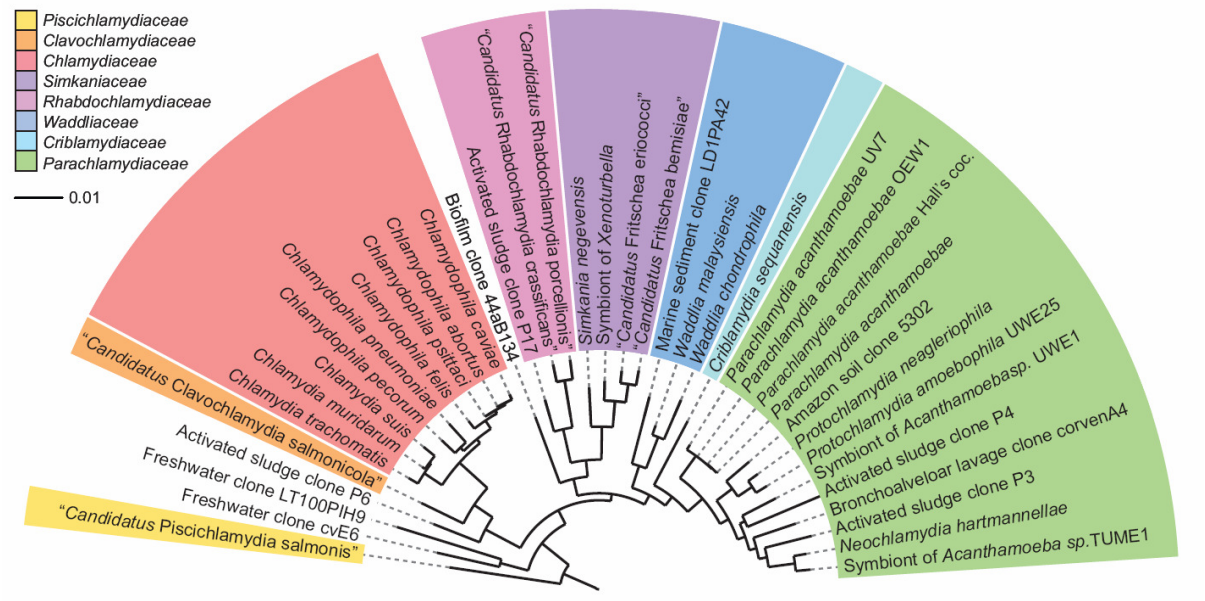

Figure 1. Phylogenetic 16S rRNA tree showing the diversity in the order *Chlamydiales*. The eight families described so far are indicated in different colours. From Horn 2008.

The majority of environmental chlamydiae identified so far belong to the *Parachlamydiaceae* and they were found mainly as symbionts of free-living amoebae (Fritsche et al. 1993; Amann et al. 1997; Birtles et al. 1997; Fritsche et al. 2000; Horn et al. 2000). Members of the other families, however, exhibit a host spectrum of remarkable phylogenetic diversity, with host organisms ranging from vertebrates including mammals, amphibians, birds, reptiles, and fish over insects to crustaceans (Horn 2008). The clinical relevance of environmental chlamydiae is still being discussed. There are some indications that environmental chlamydiae might be involved in human diseases (Lieberman et al. 1997; Marrie et al. 2001; Greub et al. 2002; Friedman et al. 2003; Corsaro et al. 2006; Baud et al. 2007; Haider et al. 2008), but proof of a causal relationship between environmental chlamydiae and human disease is still missing.

B.2. Free-living amoebae as hosts for bacterial endosymbionts

Free-living amoebae are ubiquitous protozoa that play an important ecological role in regulating microbial populations as major predators of bacteria, fungi, algae, and other protozoa (Rodriguez-Zaragoza 1994). Microorganisms are internalized by phagocytosis generally followed by digestion in phagolysosomes. Some bacteria, however, have evolved strategies to survive internalization and proliferate within free-living amoebae (Proca-Ciobanu et al. 1975; Fritsche et al. 1998; Greub et al. 2004). These amoeba-resistant bacteria either use free-living amoebae transiently as alternate hosts or are able to maintain an evolutionary stable association as endosymbionts (Birtles et al. 1996; Amann et al. 1997; Greub et al. 2004). Previously identified evolutionary lineages of bacterial endosymbionts of free-living amoebae are affiliated with the *Alphaproteobacteria*, *Betaproteobacteria*, *Bacteroidetes*, and *Chlamydiae*, a distribution that strongly suggests that symbiosis between bacteria and amoebae was developed several times independently during evolution (Horn et al. 2004). In case of a transient relationship the bacteria merely exploit the amoebae as a vessel for replication and dispersal. Many important human pathogens have been reported to deploy this strategy, the best studied example being *Legionella pneumophila*, where interaction with amoebae is considered to be central to its pathogenesis and ecology and a prerequisite for the infection of humans (reviewed in Molmeret et al. 2005). It has been suggested that interaction with free-living amoebae helps intracellular pathogens to develop and evolve mechanisms for a pathogenic lifestyle in higher eukaryotes, giving birth to the 'protozoan gym' hypothesis according to which free-living amoebae act as training grounds for intracellular bacterial

pathogens (Harb et al. 2000; Molmeret et al. 2005). Additionally, the ability to survive intracellularly in amoebae offers protection from harsh environmental conditions, making free-living amoebae clinically relevant as reservoirs and vectors of bacterial pathogens. For this reason, they are also referred to as 'Trojan Horses of the microbial world' (Barker et al. 1994).

B.3. *Protochlamydia amoebophila*

Protochlamydia amoebophila UWE25 is an environmental chlamydia recovered as an endosymbiont of an *Acanthamoeba* sp. isolated from a soil sample from western Washington State, USA (Fritsche et al. 1993; Fritsche et al. 2000). It belongs to the family *Parachlamydiaceae* and was proposed to constitute a new genus, since the 16S rRNA and 23S rRNA genes showed sequence identities of only 92.9% and 90.3%, respectively, to its closest relative within the *Parachlamydiaceae* (Collingro et al. 2005). The complete genome sequence of *P. amoebophila* UWE25 is available and it is the first and so far the only completely sequenced genome of a member of the environmental chlamydiae (Horn et al. 2004). Its genome is about twice as large as the genomes of any of the pathogenic chlamydia species investigated to date (Stephens et al. 1998; Kalman et al. 1999; Read et al. 2000; Shirai et al. 2000; Read et al. 2003; Carlson et al. 2005; Thomson et al. 2005; Azuma et al. 2006; Thomson et al. 2008), indicating a less advanced reduction of genomic content (Horn et al. 2004). In contrast to the *Chlamydiaceae*, *P. amoebophila* has retained several key features of the last common chlamydial ancestor, like a complete tricarboxylic acid cycle, but it still possesses major virulence mechanisms of present-day pathogenic chlamydiae, including a type three secretion system (Horn et al. 2004). Using the genome sequence, it was possible to design a microarray chip with oligonucleotide probes covering all open reading frames (ORFs) of the *P. amoebophila* genome, with the aim of studying the expression of temporally regulated genes during the chlamydial developmental cycle (Haider, unpublished).

B.4. The chlamydial developmental cycle

Chlamydiae are characterized by a unique biphasic developmental cycle that includes morphologically and physiologically distinct forms, the elementary body (EB) and the

reticulate body (RB) (Ward 1988; Hatch 1999; Abdelrahman et al. 2005). The EB is the infectious stage. It is metabolically inactive and adapted for survival in extracellular environments by an osmotically stable cell envelope (Hatch 1996) while the RB represents the reproductive, intracellular stage that is metabolically active but non-infectious. At the beginning of an infection cycle an EB attaches to the host cell and is taken up by a process originally termed 'parasite-specified endocytosis' (Byrne et al. 1978). However, in vitro studies could show that chlamydiae employ a variety of different attachment and entry mechanisms (Soderlund et al. 1983; Ward et al. 1984; Hodinka et al. 1986; Hodinka et al. 1988; Prain et al. 1989; Wyrick et al. 1989; Schramm et al. 1995; Escalante-Ochoa et al. 2000), and it is quite likely that they have evolved several, possibly redundant mechanisms of entry to guarantee their uptake (Scidmore 2006). Once internalized (Figure 2, step A), the chlamydiae remain inside a host-derived membrane vesicle termed chlamydial inclusion (Hackstadt et al. 1997), where the differentiation from EB to RB and subsequent replication take place (Figure 2, step B). Following this period of rapid cell division, RBs re-differentiate to EBs (Figure 2, step C) and leave the host cell either by a process called extrusion (Figure 2, step D) or via lysis (Figure 2, step E) (Hybiske et al. 2007).

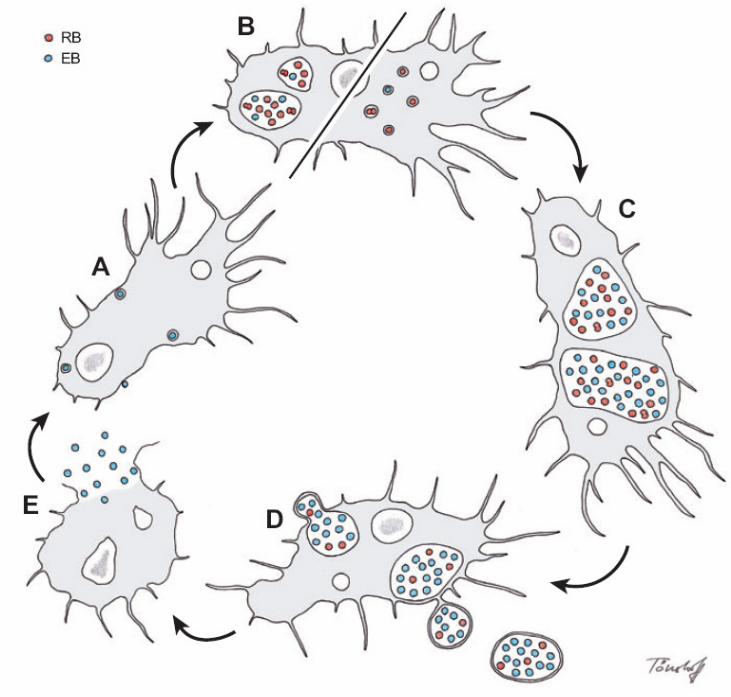

Figure 2. The developmental cycle of chlamydiae in free-living amoebae. Extracellular elementary bodies (EBs, shown in blue) are internalized (A) and differentiate to reticulate bodies (RBs, shown in red), which start multiplying and form large or single-cell inclusions (B). At some point, RBs re-differentiate to EBs (C) and are released within vesicles (D) or by lysis of the amoebae (E) to complete the infection cycle. Modified from Horn 2008.

Since the developmental stages are morphologically distinct (Ward 1988), the developmental cycle of several clinical but also environmental chlamydiae could be thoroughly described by electron microscopy (Matsumoto 1988; Ward 1988; Miyashita et al. 1993; Kahane et al. 2001; Greub et al. 2002; Kahane et al. 2002). The regulatory mechanisms that trigger these morphological changes are of great interest and need to be investigated on the molecular level. Chlamydiae are genetically inaccessible and molecular investigations are additionally complicated by their obligate intracellular growth, therefore whole genome DNA microarrays are a valuable tool to study the complex interactions between chlamydiae and their host cells during the developmental cycle.

B.5. DNA microarrays

DNA microarrays consist of a serial array of microscopic spots of DNA oligonucleotides that are displayed on the solid surface of a glass slide (reviewed in Watson et al. 1998; Duggan et al. 1999; Graves 1999). The DNA spots, commonly representing single genes, are immobilized on the slide by covalent attachment to a chemical matrix. One chip can contain spots of DNA segments corresponding to all the genes of an organism, thus permitting global analysis of transcription by hybridization with samples of fluorescently labeled cDNA. The immobilized oligonucleotides are called probes and the labeled samples are referred to as targets (Phimister 1999). In the last years, microarrays have become an important tool in microbiology (Dharmadi et al. 2004), especially due to a combination of features, namely high throughput, parallelism, miniaturization, speed, and automation (Gupta et al. 1999).

DNA microarrays have already been used to study the global expression of temporally regulated genes during the chlamydial developmental cycle (Shaw et al. 2000; Mahony 2002; Belland et al. 2003; Nicholson et al. 2003; Mäurer et al. 2007). According to their temporal expression pattern the genes of *C. trachomatis* and *C. pneumoniae* were assigned to three major clusters, the early-, the mid- and the late-cycle genes (Shaw et al. 2000; Mahony 2002). Early-cycle genes function to initiate the synthesis of macromolecules and to establish the intracellular niche, mid-cycle genes are coding for enzymes of intermediary metabolism and structural proteins and late-cycle genes are involved in the terminal differentiation of RBs back to EBs (Shaw et al. 2000; Mahony 2002). Another class of so-called tardy genes has

been suggested to account for genes that are mainly coding for mRNA that is carried over to EBs (Mäurer et al. 2007).

B.6. The *P. amoebophila* whole genome microarray

The *P. amoebophila* whole genome microarray (Haider, unpublished) consists of 2,031 oligonucleotide probes corresponding to all putative ORFs. The program OligoWiz 2.0 (Nielsen et al. 2003) was used to design the 50-mer (±5 bases) oligonucleotides. A 20 dATP spacer (A-spacer) was added to the 5'-end of each oligonucleotide in order to increase the on-chip accessibility of spotted probes to target DNA (Shchepinov et al. 1997; Southern et al. 1999). The oligonucleotides were synthesized by Microsynth (Microsynth GmbH, Balgach, Switzerland) in 384-well microtiter plates with a C6-amino linker modification at the 5'-end and provided in concentrations of 100 µM in 50% dimethyl sulfoxide (DMSO) to prevent evaporation during storage (Hegde et al. 2000). Oligonucleotides as well as several negative and positive controls are arrayed in triplicates, resulting in a total of 6132 spots per microarray chip. Using this microarray, it was possible to verify the transcription of 61% of the genes in the *P. amoebophila* genome during infection and thus obtain first insights into its gene expression profile (Haider, unpublished).

B.7. Aims of the diploma thesis

The objective of my diploma thesis was the optimization of the whole-genome microarray described above and the establishment of a procedure suitable for studying the gene expression of *P. amoebophila*. *P. amoebophila* is an obligate intracellular *Chlamydia*-like bacterium infecting amoebal cells and, like pathogenic chlamydiae, possesses a unique biphasic life cycle. The whole genome microarray should provide new insights about genes important for the differentiation stages and lead to a broader understanding of the chlamydial developmental cycle. When I started my diploma thesis, the design of the microarray and the evaluation of the hybridization were already completed and preliminary results had been obtained about the global gene expression profile of *P. amoebophila* during asynchronous infection (Haider, unpublished). The microarray procedure that was used to obtain these

results, however, was not performing well at that time and more efforts for optimization of the method were necessary.

The hitherto undiscovered diversity of chlamydiae in the environment is expected to be huge (Ossewaarde et al. 1999; Corsaro et al. 2001; Horn et al. 2001; Corsaro et al. 2002; Horn 2008). Therefore, another aim of my diploma thesis was the isolation of free-living amoebae from environmental samples in order to look for previously unknown symbioses between free-living amoebae and environmental chlamydiae or other bacterial endosymbionts.

C. Materials and Methods

C.1. Software

Table 1. Software used

Software	Company
Argus X1 V.4	biostep GmbH, Jahnsdorf, Germany
AxioVision 4	Carl Zeiss MicroImaging GmbH, Jena, Germany
FinchTV V.1.4.0	Geospiza Inc., Seattle, WA, USA
GenePix Pro 5.1	Molecular Devices GmbH, Ismaning/München, Germany
Microsoft Office 2003	Microsoft Corporation, Redmond, WA, USA

C.2. Technical equipment

Table 2. Technical equipment used

Equipment	Company
5804 R centrifuge	Eppendorf AG, Hamburg, Germany
ABI 3130x Genetic Analyzer	Applied Biosystems, Austin, TX, USA
Analytical Plus AP110 analytical balance	Ohaus Corporation, Pine Brooks, NJ, USA
BioView UV transillumator	biostep GmbH, Jahnsdorf, Germany
BL3100 balance	Sartorius AG, Göttingen, Germany
CCD camera AxioCam HRc	Carl Zeiss MicroImaging GmbH, Jena, Germany
Confocal laser scanning microscope LSM 510 Meta	Carl Zeiss MicroImaging GmbH, Jena, Germany
Epifluorescence microscope Axioplan 2 imaging	Carl Zeiss MicroImaging GmbH, Jena, Germany
FastPrep FP120 homogenizer	Bio 101/Savant, Farmingdale, NY, USA
GenePix 4100 microarray scanner	Molecular Devices GmbH, Ismaning/München, Germany
GFL type 1004 water bath	GFL - Ges. f. Labortechnik mbH, Burgwedel, Germany
iCycler thermal cycler	Bio-Rad Laboratories, Hercules, CA, USA
IKAMAG RCT basic magnetic stirrer	IKA Werke GmbH & Co.KG, Staufen, Germany
Incubator	Binder GmbH, Tuttlingen, Germany
inoLab pH Level 1 pH meter	WTW GmbH & Co.KG, Weilheim, Germany
Inverse microscope Axiovert 25	Carl Zeiss MicroImaging GmbH, Jena, Germany
Laminar flow hood Holten LaminAir model 1.2	Holten, Jouan Nordic, Allerød, Denmark
Laminar flow hood Holten LaminAir model 1.8	Holten, Jouan Nordic, Allerød, Denmark
Mastercycler gradient thermal cycler	Eppendorf AG, Hamburg, Germany
*Micro*Grid 610/TAS microarray spotter	BioRobotics Ltd, Cambridge, UK
Mikro 22R centrifuge	Hettich AG, Bäch, Switzerland
Milli-Q Biocel water purification system	Millipore GmbH, Vienna, Austria

Mixing Block MB-102	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
NanoDrop ND-1000 spectrophotometer	PEQLAB Biotechnologie GmbH, Erlangen, Germany
Neubauer counting chamber	Marienfeld GmbH & Co.KG, Lauda-Königshofen, Germany
OptimaTM L-100 XP ultracentrifuge	Beckman Coulter, Inc., Palo Alto, CA, USA
PowerPac Basic power supply	Bio-Rad Laboratories, Hercules, CA, USA
Ribonucleic acid, type VI from Torula Yeast	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Rotina 35R centrifuge	Hettich AG, Bäch, Switzerland
SD 220 VAC micro centrifuge	Carl Roth GmbH & Co.KG, Karlsruhe, Germany
SONOPLUS HD2070 sonicator	BANDELIN electronic GmbH & Co. KG, Berlin, Germany
Sub-Cell GT agarose gel electrophoresis system	Bio-Rad Laboratories, Hercules, CA, USA
SW41 Ti ultracentrifuge rotor	Beckman Coulter, Inc., Palo Alto, CA, USA
Thermo Haake DC10 water bath	Thermo Haake GmbH, Burgwedel, Germany
ThermoTWISTER comfort	Quantifoil Instruments GmbH, Jena, Germany
Vacufuge Concentrator 5301	Eppendorf AG, Hamburg, Germany
Varioclav 135 S H+P steam sterilizer	H+P Labortechnik GmbH, Oberschleißheim, Germany
Varioclav 25 T H+P table top steam sterilizer	H+P Labortechnik GmbH, Oberschleißheim, Germany
Vortex-Genie 2	Scientific Industries, Inc., Bohemia, NY, USA
VWR Ultrasonic Cleaner USC100T	VWR International bvba/sprl, Leuven, Belgium

C.3. Kits and expendable items

Table 3. Kits used

Kit	Company
DecaLabel DNA Labeling Kit	Fermentas GmbH, St.Leon-Rot, Germany
DNeasy Blood and Tissue Kit	QIAGEN, Valencia, CA, USA
illustra GenomiPhi V2 DNA Amplification Kit	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
MICROB*Enrich* Kit	Applied Biosystems/Ambion, Austin, TX, USA
RNEasy Mini Kit	QIAGEN, Valencia, CA, USA
QIAquick Nucleotide Removal Kit	QIAGEN, Valencia, CA, USA
QIAquick PCR Purification Kit	QIAGEN, Valencia, CA, USA

Table 4. Expendable items used

Expendable item	Company
Bead beater tubes (2 mL)	Bio 101/Savant, Farmingdale, NY, USA
Culture flasks 10 mL (25 cm²), sterile	Iwaki Europe GmbH, Willich, Germany
Culture flasks 150 mL (500 cm²), sterile	Iwaki Europe GmbH, Willich, Germany
Glass beads (0.75-1.0 mm)	Carl Roth GmbH & Co.KG, Karlsruhe, Germany
Greiner tubes (15 mL, 50 mL), sterile	Greiner Bio-One GmbH, Frickenhausen, Germany
HybriWell FL Sealing Systems	Grace Bio-Labs, Bend, OR, USA

Microarray slides VSS-25C, silylated, clear	CEL Associates, Los Angeles, CA, USA
Microcentrifuge tubes (1.5 mL)	GenXpress Service & Vertrieb GmbH, Wr. Neudorf, Austria
Microcentrifuge tubes (2 mL)	Greiner Bio-One GmbH, Frickenhausen, Germany
Microscopic slides, black epoxy resin colour mask, 10 reaction wells, Ø 6 mm	Marienfeld GmbH & Co.KG, Lauda-Königshofen, Germany
MicroSpot 2500 split pins	Zinsser Analytic GmbH, Frankfurt, Germany
Omnifix 5 mL syringe, single use, sterile	B. Braun Melsungen AG, Melsungen, Germany
Omnifix 50 mL syringe, single use, sterile	B. Braun Melsungen AG, Melsungen, Germany
PCR tubes (0.2 mL)	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Petri Dishes, 90 mm, triple vents, sterile	Sterilin Ltd, Aberbargoed, UK
Pipette tips, various sizes	Carl Roth GmbH & Co.KG, Karlsruhe, Germany
Plastic pipettes (10 mL), single use, sterile	Sterilin Ltd, Aberbargoed, UK
Sterican Needles (Ø 0.45 x 25 mm), sterile	B. Braun Melsungen AG, Melsungen, Germany
Syringe filter, single use, sterile, 0.20 µm pore size	Asahi Techno Glass Corporation, Funabashi-City, Japan
Syringe filter, single use, sterile, 1.20 µm pore size	Sartorius AG, Göttingen, Germany
Ultracentrifuge tubes	Beckham Coulter, Inc., Palo Alto, CA, USA

C.4. Chemicals and enzymes

Table 5. Chemicals used

Chemicals	Company
Agar	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Ammonium acetate (CH_3COONH_4)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Bacto Peptone	Becton, Dickinson and Company, Sparks, MD, USA
Biozym LE Agarose	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Blocking reagent	Roche Diagnostics GmbH, Mannheim, Germany
Bovine serum albumine (BSA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Boric acid (H_3BO_3)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Calcium chloride dihydrate ($CaCl_2*2H_2O$)	Mallinckrodt Baker B.V., Deventer, Netherlands
Chloroform/Isoamylalcohol (24:1, vol/vol)	Carl Roth GmbH & Co.KG, Karlsruhe, Germany
Cetyl trimethylammonium bromide (CTAB)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Citifluor	Citifluor Ltd, Leicester, UK
CyDye Post Labeling Reactive Dyes (Cy3/Cy5)	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
Diethylpyrocarbonate (DEPC)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Disodiumhydrogenphosphate dihydrate ($Na_2HPO_4*2H_2O$)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Dithiothreitol (DTT)	Invitrogen Corporation, Carlsbad, USA
EDTA disodium dihydrate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethanol absolute	AustrAlco Österr. AlkoholhandelsgmbH, Spillern, Austria

Ethanol denatured	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethidium bromide (EtBr)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2*6H_2O$)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Fetal Bovine Serum	MedPro GesmbH, Vienna, Austria
Folic acid (Pteroylglutamic acid)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Formaldehyde	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Glucose	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Glutamic acid	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Glycogen	Fermentas GmbH, St.Leon-Rot, Germany
HEPES (free acid)	Promega, Madison, WI, USA
Hydrochloric acid (HCl)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Hydroxylamine hydrochloride (NH_2OH-HCl)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Isopropyl alcohol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Magnesium sulfate heptahydrate ($MgSO_4*7\ H_20$)	Merck KGaA, Darmstadt, Germany
N-lauryl sarcosine	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Peptone	Oxoid Ltd., Basingstoke, Hampshire, England
Potassiumchloride (KCl)	Merck KGaA, Darmstadt, Germany
PerfectHyb Hybrididzation Buffer	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Potassiumdihydrogenphosphate (KH_2PO_4)	Merck KGaA, Darmstadt, Germany
Random nonamer primers	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
Sheared salmon sperm DNA	Eppendorf AG, Hamburg, Germany
SlideHyb Glass Array Hybridization Buffer #1	Applied Biosystems/Ambion, Austin, TX, USA
Sodium acetate trihydrate ($NaCH_3COO*3H_2O$)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium borohydride ($NaBH_4$)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Sodium bicarbonat ($NaHCO_3$)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium hydroxide (NaOH)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Soidumdihydrogenphosphate monohydrate ($NaH_2PO_4*H_2O$)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sucrose	Merck KGaA, Darmstadt, Germany
SYBR Green II	Cambrex Bio Science Rockland, Rockland, ME, USA
Trichloromethane (Chloroform)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Trishydroxymethylaminomethane (Tris)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Tris/HCl	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Trisodium citrate dihydrate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
TRIzol	Invitrogen Corporation, Carlsbad, USA
Trypticase Soy Broth	Oxoid Ltd., Basingstoke, Hampshire, England
Yeast extract	Oxoid Ltd., Basingstoke, Hampshire, England

Table 6. Enzymes used

Enzyme	Company
BsuRI (*HaeIII*) restriction enzyme	Fermentas GmbH, St.Leon-Rot, Germany
DNase I, Amplification Grade	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
RiboLock RNase Inhibitor	Fermentas GmbH, St.Leon-Rot, Germany
SuperScript II RT	Invitrogen Corporation, Carlsbad, USA
VspI restriction enzyme	Fermentas GmbH, St.Leon-Rot, Germany

C.5. Media and buffers

All media and buffers were prepared with purified double distilled water (ddH_2O) produced using a Milli-Q Biocel System (Millipore GmbH, Vienna, Austria) and if not stated otherwise were autoclaved for 20 min at 121°C and 1.013 x 10^5 Pa using a Varioclav 135 S H+P steam sterilizer.

- **TSY - Trypticase soy broth with yeast extract (Visvesvara 1999)**

  Trypticase Soy Broth 30 g L^{-1}
  Yeast Extract 10 g L^{-1}
  pH 7.3

- **10x PAS – Page's amoebic saline (Page 1988)**

  NaCl 1.2 g L^{-1}
  $MgSO_4 \cdot 7\ H_2O$ 0.04 g L^{-1}
  $CaCl_2 \cdot 2\ H_2O$ 0.04 g L^{-1}
  $Na_2HPO_4 \cdot 2\ H_2O$ 1.78 g L^{-1}
  KH_2PO_4 1.36 g L^{-1}

- **SPG – Sucrose-phosphate-glutamate buffer** – autoclaved at 110°C (15 min)

  Sucrose 75 g L^{-1}
  KH_2PO_4 1.104 g L^{-1}
  $Na_2HPO_4 * 2\ H_2O$ 1.306 g L^{-1}
  Glutamic acid 0.15 g L^{-1}

- **1x TBE – Tris-borate-EDTA buffer**

Tris	10.8 g L^{-1}
Boric acid	5.5 g L^{-1}
EDTA (disodium salt)	0.93 g L^{-1}
pH 8.0	

- **1x TAE – Tris-acetate-EDTA buffer**

Tris	4.84 g L^{-1}
Acetic acid	1.142 mL L^{-1}
EDTA	0.372 g L^{-1}
pH 8.0	

- **1x TE – Tris-EDTA buffer**

Tris-HCl	1.211 g L^{-1}
EDTA	0.372 g L^{-1}
pH 8.0	

- **20x SSC – Sodium carbonate-sodium citrate buffer**

NaCl	175.3 g L^{-1}
Sodium citrate	88.2 g L^{-1}
pH 7.0	

- **1x PBS – Phosphate buffered saline**

NaCl	8 g L^{-1}
KCl	0.2 g L^{-1}
NaH_2PO_4	1.44 g L^{-1}
KH_2PO_4	0.24 g L^{-1}
pH 7.4	

- **NNA – Non-nutrient agar**

10x PAS	100 mL L^{-1}
Agar	15 g L^{-1}

- **PYG – Peptone-yeast-glucose medium (Holdeman et al. 1977)**

Peptone	20 g L^{-1}
Glucose	18 g L^{-1}
Yeast extract	2 g L^{-1}
Sodium citrate	1 g L^{-1}
MgSO4*7 H_2O	0.98 g L^{-1}
Na_2HPO_4*7 H_2O	0.355 g L^{-1}
KH_2PO_4	0.34 g L^{-1}
$Fe(NH_4)_2(SO_4)_2$*6 H_2O	0.02 g L^{-1}
pH 6.5	

- **Remodified Chang medium (Chang 1971, modiefied by Lagkouvardos)**

Tryptone	10 g L^{-1}
Yeast extract	5 g L^{-1}
Na_2HPO_4*2 H_2O	1.66 g L^{-1}
KH_2PO_4	0.8 g L^{-1}
Glucose	3 g L^{-1}
Liver digest	4 g L^{-1}
Fetal bovine serum	100 mL L^{-1}

- **Modified PYNFH – Peptone-yeast-nucleic acid-folic acid-hemin (ATCC 1034)**

Bacto Peptone	10 g L^{-1}
Ribonucleic acid, type VI from Torula Yeast	1 g L^{-1}
Folic acid	0.015 g L^{-1}
Hemin	0.001 g L^{-1}
Buffer solution, pH 6.5 *(18.1 g L^{-1} KH_2PO_4, 25.0 g L^{-1} Na_2HPO_4*2 H_2O)*	20 mL L^{-1}
Fetal bovine serum	100 mL L^{-1}
pH 6.5	

C.6. Cell culture

C.6.1. Cultivation of *P. amoebophila*

P. amoebophila UWE25 was grown axenically at a constant temperature of 20°C in a continuous culture with *Acanthamoeba castellanii* Neff as host cells. Cell cultures were incubated in 150 mL TSY liquid medium in 500-cm² culture flasks. Medium was exchanged at least once a week to achieve optimal cell growth and the state of the cultures was routinely monitored using an inverse microscope.

C.6.2 Cell harvesting

Amoebae were detached from the bottom surface of the culture flask by vigorous shaking. The suspended cells were collected in 50 mL Greiner tubes and pelleted by centrifugation (7,323 x g, 5 min, RT). Fresh TSY medium was added to the empty culture flasks to enable growth of the remaining amoebae.

C.7. Total RNA isolation

All solutions used during RNA extraction were prepared with double-distilled water treated with 0.1% DEPC to prevent RNA degradation.

C.7.1. Phenol-chloroform-extraction

The simultaneous RNA isolation from *P. amoebophila* and its host was generally carried out using a phenol-chloroform extraction method. Infected amoebae were pelleted by short centrifugation (6,300 x g, 3 min, RT), resuspended in 1x PAS and counted using a Neubauer counting chamber. Approximately 10^7 cells were transferred into a microcentrifuge tube, pelleted again (6,300 x g, 3 min, RT) and immediately lysed by addition of 1.5 mL of the phenol and guanidine isothiocyanate containing reagent TRIzol that maintains the RNA integrity during tissue homogenization. DNA was sheared by pipetting up and down approximately 30 times and the TRIzol solution was transferred to a bead beater tube containing only one big bead and homogenized for 10 sec at a speed of 4.5 with a FastPrep homogenizer. Cell debris was centrifuged (13,600 x g, 5 min, RT) and the supernatant was transferred to a clean microcentrifuge tube and incubated at RT (5-10 min). Nucleic acids were extracted by addition of 0.2 volumes of chloroform to the supernatant. After vigorous

shaking by hand for 15 sec and incubation for 5 min at RT, the mixture was centrifuged (10,520 x g, 15 min, 4°C) in order to separate the phases. The aqueous phase containing the extracted RNA was transferred to a fresh tube and RNA was precipitated by adding 0.5 volumes of isopropyl alcohol related to the amount of supernatant after initial homogenization, 0.12 volumes of 5M ammonium acetate and 1 µL of a 20 mg/mL glycogen solution. After incubation for 15 min at -20°C the RNA was pelleted by centrifugation (21,250 x g, 15 min, 4°C), washed with 1.5 mL 75% ethanol to remove salts, centrifuged again for 10 min and the pellet air dried for 5 min and then dissolved in 50 µL RNase-free water. The concentration of the RNA samples, as well as the ratio of absorbance at the wavelengths of 260 nm and 280 nm (ratio 260/280) and at wavelengths of 260 nm and 230 nm (ratio 260/230), were spectrophotometrically determined. The ratio 260/280 is used as an easy way to assess the purity of RNA. Pure RNA should have a ratio of ~2.0. The ratio 260/230 is an additional measure of nucleic acid purity and should be >2.0 for pure RNA. DNA was digested using 1U Sigma DNase 1 Amplification Grade per 2 µg of RNA for 1 h at RT and the reaction was stopped by addition of 1 µL Stop Solution. RNA was precipitated for 30 min at -80°C after addition of 3 volumes of absolute ethanol, 0.1 volume of 3M sodium acetate (pH 5.2), and 0.01 volumes of glycogen. Then, the RNA was pelleted, washed and air dried as described before, and resolved in RNase-free water. RNA concentration, as well as the ratio 260/280 and the ratio 260/230, were determined using a NanoDrop spectrophotometer and the RNA was stored in the presence of RiboLock RNase Inhibitor. RNA quality was checked on a 1% agarose gel (as described in section C.13.) and a control PCR was conducted in order to verify the absence of DNA in the RNA samples (section C.7.3.).

C.7.2. RNeasy Mini Kit

As an alternative method for total RNA isolation the RNeasy Mini Kit (QIAGEN) was employed. Amoebal cells were pelleted (6,300 x g, 3 min, RT), resuspended in 1x PAS and counted using a Neubauer counting chamber. Approximately 10^7 cells were transferred into a microcentrifuge tube and pelleted again (6,300 x g, 3 min, RT). The pellet was resolved in 600 µL Buffer RLT and transferred to a bead beater tube containing only one big bead and homogenized for 10 sec at a speed of 4.5 with a FastPrep homogenizer and 600 µL of 75% ethanol were added. Successively, the first 700 µL and the remaining 500 µL of the sample were applied to the same RNeasy Mini spin column and centrifuged (8,000 x g, 15 sec, RT). Then the column was washed with 700 µL Buffer RW1 (8,000 x g, 15 sec, RT) and 500 µL

Buffer RPE (8,000 x g, 15 sec, RT). The flow-through was discarded after each centrifugation. The column was transferred into a new collection tube and centrifuged again (21,250 x g, 1 min, RT). Then it was placed in an empty 1.5 mL collection tube and eluted twice with 30 µL RNase-free water (8,000 x g, 1 min, RT). RNA concentration, as well as the ratio 260/280 and and the ratio 260/230, were determined using a NanoDrop spectrophotometer and the RNA was stored in the presence of RiboLock RNase Inhibitor. RNA quality was checked on a 1% agarose gel (as described in section C.13.).

C.7.3. Polymerase chain reaction

Polymerase chain reaction (PCR) was performed in order to verify the absence of DNA from the RNA samples. For this purpose the primer set PanF/R (Table 7) that is specific for the 16S rRNA gene of members of the order *Chlamydiales* was used.

Table 7. *Chlamydiales* specific primer set PanF/R used for 16S rDNA amplification, their annealing temperature (T_a) and the size of the amplicon (Corsaro et al. 2002).

Primer name	Sequence	T_a	Amplicon size
PanF	5´-GTC ATC RGC CYY ACC TTV SRC RYY TCT-3´	65°C	~1 445 bp
PanR	5´-CGT GGA TGA GGC ATG CRA GTC G-3´		

One PCR reaction (50 µL) was constituted of following components and 1 µL of RNA sample:

PCR Reaction (50 µL)

$MgCl_2$	4 µL
10x Reaction Buffer	5 µL
dNTP mix	5 µL
Taq polymerase	0.2 µL
Forward primer (100 pmol μL^{-1})	0.25 µL
Reverse primer (100 pmol μL^{-1})	0.25 µL
ddH_2O	35.3 µL

A positive and a negative control were included in each PCR. Following PCR program was used for the amplification of 16S rRNA gene fragments:

PCR Program for 16S PCRs (PanF/R)

Cycle 1 (1x):		
Step 1:	95.0ºC	for 03:00
Cycle 2 (30x):		
Step 1:	95.0ºC	for 00:30
Step 2:	65.0ºC	for 00:30
Step 3:	72.0°C	for 01:30
Cycle 3 (1x):		
Step 1:	72.0ºC	for 07:00
Cycle 4 (1x):		
Step 1:	22.0°C	HOLD

C.8. Bacterial RNA enrichment

The MICROB*Enrich* Kit was deployed to enrich the isolated total RNA sample in chlamydial RNA. The kit removes undesired eukaryotic RNA – both mRNA and rRNA – by capturing them with complementary oligonucleotide probes that are themselves captured by magnetic beads and removed from the solution using a magnet.

At first, the total RNA was precipitated (3 volumes absolute ethanol, 0.1 volume 3M sodium acetate, 5 µg glycogen) for 30 min at -80°C or over night at -20°C and pelleted by centrifugation (21,250 x g, 30 min, 4°C). The pellet was washed in 75% ethanol (21,250 x g, 10 min, 4°C), air dried and resuspended in 35 µL 1x TE buffer. 5 µL were put aside for agarose gel electrophoresis. The RNA sample was mixed with 300 µL Binding Buffer and 2 µL of Capture Oligo Mix per 5 µg RNA and incubated for 10 min at 70°C and 1 h at 37°C. During the incubation time the Oligo MagBeads were prepared (25 µL Oligo MagBeads per 5 µg of input RNA). They were captured on the magnetic stand, washed with nuclease-free water, washed with Binding Buffer, and kept on ice until 5 min before use when they were placed at RT. After incubation of the sample the beads were added and the mixture was incubated for 15 min at 37°C. The beads were captured on the magnetic stand and the supernatant was transferred to a collection tube on ice. 100 µL Wash Solution (prewarmed to 37°C) were added to the beads and after 5 min of incubation at 37°C, the beads were captured again and the supernatant was pooled with the RNA already in the collection tube. Subsequently the enriched bacterial RNA was precipitated (2.5 volumes absolute ethanol, 0.1

volume 3M sodium acetate, 20 µg glycogen) at -20°C for 1 h, centrifuged (21,250 x g, 30 min, 4°C), washed with 750 µL ice cold 75% ethanol, centrifuged again for 10 min and the pellet was air dried and resuspended in 35 µL RNase free water. Again, 5 µL were put aside for the gel, the RNA concentration was measured and the 5 µL samples taken before and after the MICROB*Enrich* procedure were subjected to agarose gel electrophoresis to confirm the successful enrichment. The Oligo MagBeads were regenerated to be used a second time. Regeneration was done by washing the beads with 2 volumes Regeneration Solution 1 and washing twice with Regeneration Solution 2. The beads were stored in 1 volume Regeneration Solution 2. All solutions that were not provided with the kit were prepared with double-distilled water treated with 0.1% DEPC to prevent RNA degradation.

C.9. Aminoallyl labeling

In order to measure gene expression with a microarray, the RNA sample has to be reversely transcribed into cDNA labelled with a fluorescent dye. This can be achieved by initially incorporating aminoallyl nucleotides during reverse transcription into the cDNA to which the fluorescent dye is then coupled in a second step. Indirect labeling of total RNA was performed based on a protocol described in Mäurer et al. 2007.

C.9.1. Reverse transcription

To an RNA sample containing 20 µg of total RNA or 5 µg of enriched RNA 1 µL of random nonamer primers was added and filled up with DEPC-treated ddH_2O to a volume of 11 µL (reaction volume increased with low RNA concentrations). To allow annealing of the primers, the reaction was incubated at 65°C for 10 min and cooled down for 5 min at RT and for 5 min on ice. Then a reverse transcription master mix was added, containing 4 µL 5x First Strand Buffer, 2 µL 0.1M DTT, 2 µL nucleotide mix (Table 8) and 1.5 µL SuperScript II RT 200U/µL per 9.5 µL. Reverse transcription reaction took place on 42°C over night. The remaining RNA was hydrolyzed by addition of 5 µL 2.5M sodium hydroxide and incubation for 15 min at 37°C. 10 µL of 2M HEPES (pH 7) were added to neutralize the pH and the cDNA was purified by ethanol precipitation (addition of 150 µL absolute ethanol and 6 µL 3M sodium acetate) at -20°C o/n. The precipitated aa-cDNA was pelleted (21,250 x g, 30 min, 4°C), washed with 75% ethanol, centrifuged (21,250 x g, 10 min, 4°C) and the pellet air

dried and resuspended in 17.5 µL 0.1M sodium bicarbonate buffer (pH 9) and the DNA concentration was measured.

Table 8. Different nucleotide mixes used for reverse transcription.

Nucleotide mix	Concentration of dATP/dCTP/dGTP	Concentration of dTTP	Concentration of aa-dUTP
Preliminary nucleotide mix	6.0 mM	0.8 mM	5.0 mM
1:2-nucleotide mix	6.0 mM	2.0 mM	4.0 mM
2:1-nucleotide mix	6.0 mM	4.0 mM	2.0 mM
3:2-nucleotide mix	6.0 mM	1.8 mM	1.2 mM
4:3-nucleotide mix	6.0 mM	1.7 mM	1.3 mM
1:1-nucleotide mix	6.0 mM	3.0 mM	3.0 mM

C.9.2. Coupling of aminoallyl-cDNA to the fluorescent dye

The aa-cDNA was coupled to a CyDye ester (Cy3 or Cy5) by mixing the resuspended cDNA in one tube CyDye and incubating in the dark. After 2 h, 7.5 µL 4M hydroxylamine were added, and the incubation was continued for 15 min.

C.9.3. Purification of labelled cDNA

Finally, the labelled cDNA was purified using the PCR Purification Kit. 5x reaction volume Buffer PB was added to the sample and the mixture was transferred to a QIAquick column placed in a 2 mL collection tube. After centrifugation (18,890 x g, 1 min, RT) the column was washed with 750 µL Buffer PE (18,890 x g, 1 min, RT) and dried by centrifugation (21,250 x g, 1 min, RT). The column was placed in a new 1.5 mL tube and the DNA was eluted twice with 30 µL ddH_2O (18,890 x g, 1 min, RT) to an end volume of 60 µL.

C.9.4. Calculation of labeling parameters

The samples were then photospectrometrically measured. From the absorption maxima at wavelengths of 550 nm for Cy3 or 650 nm for Cy5 and 260 nm for DNA, the amount of CyDye and cDNA, as well as the frequency of incorporation (FOI) that describes the number of incorporated labeled nucleotides per 1000 nucleotides of cDNA, were calculated.

The formula used for calculating the amount of CyDye in the sample is derived from the Beer-Lambert equation ($A = E \times b \times c$, where A is the absorbance, E the extinction coefficient [L mol^{-1} cm^{-1}], b the path length [cm], and c the analyte concentration [mol L^{-1}]):

$$CyDye\ (pmol) = \frac{A \times 10^6 \times v}{E \times b}$$

A… absorbance in absorbance units at 550 nm for Cy3, at 650 nm for Cy5
v … volume in µL
E… extinction coefficient in [L mol^{-1} cm^{-1}]; E = 150,000 for Cy3, E = 250,000 for Cy5
b … path length in cm (NanoDrop measurements are normalized to a path length of 0.1 cm)

For nucleic acid quantification, the Beer-Lambert equation is modified to use an extinction coefficient with units of [ng cm ml^{-1}]:

$$cDNA\ (\mu g) = \frac{A \times e \times v}{b}$$

A… absorbance in absorbance units at 260 nm
e … extinction coefficient in [µg cm mL^{-1}]; e = 37 for Cy-labelled cDNA
v … volume in mL
b … path length in cm (NanoDrop measurements are normalized to a path length of 0.1 cm)

From these two values, the FOI can be calculated:

$$FOI = \frac{CyDye\,[pmol] \times 324.5\,[g\ mol^{-1}] \times 1{,}000}{cDNA\,[\mu g]}$$

324.5 g mol^{-1} … average molecular weight of a deoxynucleotide

C.10. cDNA amplification and direct labeling

In order to enhance the performance of RNA samples enriched with MICROB*Enrich* (see section C.8.) an amplification step was introduced before the fluorescent labeling of the cDNA. For this purpose the enriched RNA samples were reversely transcribed as described in section C.9.1 with the exception that a dNTP mix (6mM dATP/dCTP/dGTP/dTTP) without aminoally-dNTPs was used and the cDNA was finally resuspended in 17.5 µL ddH_2O instead of sodium bicarbonate.

C.10.1. cDNA amplification

The illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare) was used for cDNA amplification. The kit utilizes the DNA polymerase of bacteriophage Phi29 to exponentially amplify linear DNA templates by a strand displacement reaction.

As a template, 1 µL of cDNA was mixed with 9 µL of sample buffer (containing random hexamers that nonspecifically prime Phi29 DNA polymerase catalyzed polymerization). The

sample was then denatured at 95°C for 3 min and cooled to 4°C on ice. In the meantime the GenomiPhi Kit reaction mix was prepared by combining 9 µL of reaction buffer (containing salts and deoxynucleotides) with 1 µL of enzyme mix. The reaction mix was then added to the cool sample and the mixture was incubated on 30°C over night (16 h). After amplification the polymerase was heat-inactivated at 65°C for 10 min.

C.10.2. Restriction enzyme digestion of amplified cDNA

Most DNA generated by GenomiPhi is of high molecular weight and therefore not suitable for microarray hybridization. Thus, the amplified cDNA was subjected to a restriction enzyme digestion with the enzyme BsuRI. BsuRI is a 4 base pair cutter that cuts at the recognition sequence 5'-GG^CC-3', thereby generating fragments of an average length of 256 base pairs.

The restriction digestion reaction, consisting of 17.5 µL of the GenomiPhi-amplified cDNA, 3.0 µL of the restriction enzyme BsuRI (10 U μL^{-1}), 3.0 µL of 10x Buffer R, and 6.5 µL ddH_2O, was incubated for 4 h on 37°C. The reaction was stopped by heat inactivation at 80°C for 20 min.

C.10.3. Purification of amplified cDNA

Before labeling, the amplified cDNA has to be purified to remove all the components of the amplification and the restriction enzyme digestion. For this purpose the QIAquick Nucleotide Removal Kit was used. 5 volumes of Buffer PN were added to the sample containing the amplified cDNA. The mixture was applied to a QIAquick spin column in a 2 mL collection tube. After centrifugation (3,824 x g, 1 min, RT), the flow-through was discarded, the column washed with 750 µL of Buffer PE (3,824 x g, 1 min , RT) and the flow-through was again discarded. Subsequently the column was dried by centrifugation (18,890 x g, 1 min, RT) and placed into a clean 1.5 µL microcentrifuge tube. The DNA bound to the column was eluted two times with 25 µL ddH_2O (18,890 x g, 1 min, RT).

C.10.4. Direct labeling of amplified cDNA

For labeling of the amplified cDNA the same protocol was used as for the labeling of genomic DNA (described in section C.12.).

C.11. Genomic DNA isolation

Genomic DNA was isolated according to a protocol described in (Zhou et al. 1996). Cells were harvested (7,232 x g, 5 min, RT), washed with 1x PAS and centrifuged as before. The pellet was resuspended in 250 µL TE buffer. 675 µL DNA extraction buffer (100mM Tris/HCl, 100mM EDTA, 100mM sodium phosphate, 1.5mM sodium chloride, 1% CTAB, 200 µg mL^{-1} proteinase K) were added and after incubation at 37°C for 30 min 75 µL of 20% SDS were added and incubation was continued at 65°C for 2 h, inverting the sample gently every 20 min. Then the sample was mixed with an equal volume of chloroform/isoamylalcohol, centrifuged (10,600 x g, 10 min, RT) and the aqueous phase was transferred into a new microcentrifuge tube. Nucleic acids were precipitated by addition of 0.6 volumes of isopropanol for 1 h at RT and pelleted (16,000 x g, 20 min, RT). The pellet was washed with ice-cold 75% ethanol (21,250 x g, 5 min, RT), resuspended in ddH_2O and he concentration was measured using NanoDrop. Subsequently the DNA was fragmented by sonication and fragment sizes were mainly ranging between 500 bp and 1,500 bp.

C.12. Direct labeling of genomic DNA

The protocol utilizes the DecaLabel DNA Labeling Kit (Fermentas) but with several modifications. For one labeling reaction 2 µg of template DNA were combined with 10 µL of 5x reaction buffer (containing decanucleotides for random priming) and filled with ddH_2O to 45 µL. The sample was denatured at 95°C for 10 min and immediately put on ice. Then 1 µL of dNTP mix (2mM dCTP, 5mM dATP/dTTP/dGTP), 2 µL of Cy-dCTP (1mM) and 0.8 µL of Klenow (50 U μL^{-1}) were added. The labeling reaction was incubated for 2.5 hours at 37°C. After 90 min, 4 µL of the dNTP mix delivered with the kit were added. After the incubation the labeling was purified using the QIAquick Nucleotide Removal Kit as described in section C.10.3. and measured with NanoDrop. Since the genomic DNA labeled according to this protocol consists of both labeled and unlabeled sense and antisense strands, meaningful incorporation rates could not be calculated.

C.13. Agarose gel electrophoresis

1% agarose gels were poured after dissolving 1 g of Biozym LE Agarose in 100 mL of TBE or TAE buffer for DNA or RNA gels, respectively, by short heating in the microwave. For 2.5% agarose gels, 2.5 g agarose were used. The gels were run using Sub-Cell GT Systems in combination with PowerPac Basic Power Supplies for approximately 1-2 h on 100-120 Volts. DNA gels were stained with ethidium bromide, RNA gels with SYBR Green II for 1 h. The staining was visualized using a BioView UV transilluminator with the ArgusX1 V.4 software.

C.14. Microarray fabrication

The microarray was constructed from 5'-amino modified oligonucleotide probes obtained from Microsynth (Microsynth GmbH, Balgach, Switzerland) that were printed onto silylated amine-aldehyde glass slides (VSS-25C, CEL Associates) using a *Bio*Robotics *Micro*Grid 610/TAS system equipped with 16 MicroSpot 2500 split pins (Zinsser Analytic GmbH). A maximum of 80 spots were printed per source visit, including 20 spots on pre-spotting slides to avoid printing excess material from the external surface of the pin onto the actual microarray slides. Spots after pre-spotting were about 150 µm in diameter and spot centres were about 300 µm apart. To minimize carry-over effects the pins were subjected to 6 wash cycles between each oligonucleotide spotted. The relative humidity within the spotting compartment was set to 50% during the spotting.

C.15. Post processing of microarray slides

The spotted slides were allowed to rest for one day at 100% humidity to increase covalent coupling between the aminated 5'-ends of the oligonucleotides to the aldehyde groups on the slide surface. To deactivate uncoupled aldehyde groups and to remove salts and unbound DNA the slides were washed twice under vigorous agitation in 0.2% SDS for 2 min and twice in double-distilled water for 2 min. After air drying for 5 min the slides were incubated in freshly prepared sodium borohydride solution (0.6 g $NaBH_4$ in 200 mL PBS and 60 mL of absolute ethanol) for 5 min to further strengthen the coupling linkages and to convert reactive aldehyde groups into non-reactive alcohol counterparts thereby reducing non-specific attachment of molecules to the microarray surface during hybridization (Schena 2003). The

reaction was stopped by dipping in ice cold absolute ethanol and the slides were washed twice under vigorous agitation in 0.2% SDS for 1 min and twice in double-distilled water for 1 min. Finally the slides were dried by centrifugation (289 x g, 2 min, RT) and stored in the dark. All steps were carried out at room temperature.

C.16. Spotting quality assessment

Hybridization with labeled poly-dTTP probes 12 nucleotides in length targeting the A-spacers of the printed oligonucleotides was used as an assessment of the spotting quality to detect spotting failures and as a fast and inexpensive way to investigate the effects of altered post-processing or hybridization conditions. Per hybridization 15 µL of a 1pM poly-dTTP probe solution were resuspended in 400 µL of hybridization buffer (5x SSC, 0.02% SDS, 0.1% N-lauryl sarcosine; 0.1% blocking reagent). The mixture was applied to the spotted slides using sealed coverslips and incubated at 30°C for 1 h under vigorous shaking at 400 rpm. To stop the hybridization, slides were rinsed two times in ice-cold double-distilled water for 2 min and dried by centrifugation (289 x g, 2 min, RT).

C.17. Prehybridization

To block and inactivate non-specific binding sites in order to reduce the background in following hybridizations the slides were incubated in a sterile-filtrated and preheated prehybridization solution (5x SSC, 0.1% SDS, and 1% bovine serum albumin [BSA]) immediately prior to hybridization for at least 1 h at 42°C (Hegde et al. 2000). Slides were washed at RT, two times in double-distilled water for 2 min, followed by washing in isopropanol for another 2 min, and dried by centrifugation (289 x g, 2 min, RT).

C.18. Hybridization and washing conditions

The key to successful hybridization results is the balance between signal intensity and specificity. The optimal stringency during hybridization and washing was experimentally determined in order to maximize the signal intensity and minimize cross-hybridization

(Haider, unpublished). Stringency can be adjusted by the formamide concentration in the hybridization buffer, hybridization temperature, salt concentration in the wash buffers, and washing temperature (Wildsmith et al. 2001).

C.18.1. Hybridization buffers

For most hybridizations in this study a self-made hybridization buffer was used that was prepared as follows:

		final concentration
20x SSC	250 µL	5x SSC
10% SDS	10 µL	0.1% SDS
0.1M DTT	1 µL	100 µM DTT
10% N-laurylsarcosine	10 µL	0.1 % N-laurylsarcosine
10% blocking reagent	100 µL	1% blocking reagent
formamide	350 µL	35% formamide
ddH_2O	279 µL	ad 1 mL with ddH_2O

The hybridization solution was filtered through a 0.45 µm filter and 15 µL sheared salmon sperm DNA were added to block unspecific binding positions in order to reduce unspecific signals. Before application to the microarray slide, the hybridization solution was mixed with the labeled sample and heated to 95°C for 10 min to completely denature the cDNA and remove any secondary structures.

Alternatively, two commercially available hybridization buffers were tested: PerfectHyb Hybridization Buffer obtained from Sigma and SlideHyb Glass Array Hybridization Buffer #1 obtained from Ambion. Both buffers were used according to manufacturer's instruction.

C.18.2. Hybridization system

Hybridization was performed using HybriWell FL Sealing Systems. 400 µL of hybridization buffer containing the labeled sample were applied to the prehybridized slide via the HybriWell, or, when the PerfectHyb buffer was used, directly onto the slide, due to the buffer's high viscosity. Slides were incubated at 42°C for 16 h under constant shaking using a ThermoTWISTER comfort (Quantifoil Instruments GmbH).

C.18.3. Washing procedures

After hybridization, the slides were washed with buffers of increasing stringencies that were preheated to 42°C. Stringencies were adjusted by the concentration of SSC and SDS in the wash buffers. Washing was performed by putting up to two slides into one Greiner tube containing 50 mL of the respective wash buffer and shaking it constantly for 2.5 min, then quickly changing the slides into the next Greiner tube and so on. After the washing steps the slides were dipped briefly in ice cold water and dried by centrifugation (289 x g, 2 min, RT). Different washing procedures were used for different hybridization buffers.

Standard washing procedure (for self-made hybridization buffer):

2x low stringency wash buffer:	2x SSC, 0.1% SDS
2x medium stringency wash buffer:	0.1x SSC, 0.1% SDS
2x high stringency wash buffer:	0.1x SSC

More stringent washing procedure (for PerfectHyb buffer):

2x low stringency wash buffer:	0.2x SSC, 0.1% SDS
2x medium stringency wash buffer:	0.1x SSC
2x high stringency wash buffer:	0.05x SSC

Static washing procedure (for SlideHyb buffer):

No shaking was involved in this procedure. The slides were just put in tubes with the washing buffers and incubated for 15 min in the water bath on 42°C.

2x low stringency wash buffer:	2x SSC, 0.5% SDS
2x high stringency wash buffer:	0.5x SSC, 0.5% SDS

C.19. Microarray scanning, signal quantification and data analysis

Microarrays were scanned using a GenePix 4100 (Axon Instruments). Initial visualization and data analysis was carried out using the GenePix Pro 5.0 software. A circle was drawn around each spot and the mean intensity of signal within the spot and of the local background area was calculated. The signal-to-noise ratio (SNR) that was used to discriminate true signals from noise was calculated as follows:

SNR = (signal mean – background mean) / (background standard deviation).

The commonly accepted criterion for the minimum signal (threshold) that can be accurately quantified is SNR≥3 (Verdnik et al. 2002) and this threshold was also used in this study. For further analysis, microarray data were transferred to Microsoft Excel.

C.20. Amoeba isolation

Free-living amoebae are ecologically important protozoa that are ubiquitously abundant in the environment and can be readily isolated from almost any soil samples.

C.20.1. Sample acquisition

Samples were taken in Feldkirch (Vorarlberg) in August 2008 in sterile 50 mL Greiner tubes:

Sample A: Soil taken from a molehill in my garden
Sample B: Sediment from the shores of a quarry lake

Samples were stored aerobically at 4°C for 2 days until start of isolation.

C.20.2. Extraction of amoebae on non-nutrient agar

For each sample, three replicate extractions were performed. Amoebae were extracted by applying sample to the middle of non-nutrient agar (NNA) plates seeded with living *Escherichia coli* K12. After a few days, amoebae had moved from the soil sample deposited in the middle of the plate towards the periphery, feeding on the bacterial cells. A piece of agar occupied with amoebae was cut out and under sterile conditions transferred to new NNA plates with living *E. coli*, a process that was repeated every 3 to 6 days. After 5 rounds of plate transfer the living *E. coli* cells were replaced with dead *E. coli* (heat inactivation for 1 h at 95°C) as a food source in order to cautiously prepare the amoebae for axenization. After three rounds on NNA plates with dead *E. coli*, cut out pieces of agar were inoculated in 25 cm^2 culture flasks containing 10 mL of PYG medium. When the amoebal cells were attached to the surface of the flask, the medium was replaced by 10 mL 1x PAS to trigger encystation by starvation. When most amoebae had formed cysts, the PAS was replaced by 0.2M hydrochloric acid (HCl), triggering the amoebae to excyst and at the same time killing all potential extracellular bacterial contaminations. After HCl-treatment for 10 min, the flasks were washed with 10 mL 1x PAS and then incubated with 10 mL PYG and supplementary

administration of dead *E. coli*. The medium in the culture flasks was regularly replaced and the amount of dead *E. coli* was reduced each time until none were added at all. Since the amoebae did not grow well, PYG was replaced by the richer media Chang and PYNFH later on.

C.20.3. Staining with 4',6-diamidino-2-phenylindole (DAPI)

In order to detect intracellular bacteria the amoeba cells were stained with 4',6-diamidino-2-phenylindole (DAPI). DAPI is a fluorescent dye that binds strongly to AT-rich sequences in ribonucleic acids. Its absorption maximum is at 365 nm and its emission maximum is at 418 nm. 15 µL of an amoeba solution were applied on a microscopic slide. After allowing the attachment of the amoebae on the surface, the cells were stained with 10 µL of a DAPI working solution (0.1 µg/mL). After 5 min incubation in the dark they were washed with MQ, air dried, and embedded in Citifluor and the staining was visualized with an epifluorescence microscope.

C.20.4. Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) was conducted to verify the presence of endosymbionts within the isolated amoebae. 10 mL amoeba culture was harvested (7,232 x g, 5 min, RT), washed with 500 µL and resuspended in 200 µL 1x PAS. The amoeba suspension was applied to a microscopic slide (20 µL per reaction well). After attachment of the amoebae to the surface, the supernatant was aspirated off and the cells were fixed with 10 µL 4% formaldehyde for 10 min. Then the cells were washed with 20 µL ddH_2O and air dried. 1 mL of hybridization buffer (180 µL 5M NaCl, 20 µL 1M Tris/HCl, 599 µL ddH_2O, 200 µL formamide, 1 µL 10% SDS) was prepared and 10 µL of buffer were applied to each well. The hybridization buffer contains 20% formamide to adjust the stringency level required for specific binding of the probes. Then 1 µL of each of the oligonucleotide probe solutions (Table 9) was added and the slide was placed horizontally into a Greiner tube containing a tissue paper soaked in the remaining hybridization buffer. This hybridization chamber was incubated at 46°C for 90 min. After hybridization the slide was washed in a Greiner tube containing 50 mL washing buffer (2.15 mL 5M NaCl, 1mL 1M Tris/HCl, 0.5 mL 0.5M EDTA, 46.35 mL ddH_2O; preheated to 48°C) for 15 min at 48°C. The concentration of sodium chloride in the washing buffer confers stringency upon the washing procedure, equivalent to formamide used during hybridization. After washing, the slide was dipped

briefly into ice-cold ddH_2O, dried as quickly as possible using compressed air and embedded in Citifluor. The cells were visualized and pictures were taken on a confocal laser scanning microscope (CLSM).

Table 9. FISH probes used. EUB338 (Amann et al. 1990), Chls-0523 (Poppert et al. 2002), BET42a (Manz et al. 1992), and ALF968 (Neef 1997) were used to detect the domain *Bacteria*, the order *Chlamydiales*, and the classes *Betaproteobacteria* and *Alphaproteobacteria*, respectively. To exclude unspecific binding the nonsense probe NONEUB (Wallner et al. 1993) was used. Oligonucleotide sequences and target groups (including coverage) are shown. ProbeBase (Loy et al. 2007) accession numbers are pB-00159 (EUB338), pB-00049 (Chls-0523), pB-00034 (BET42a), pB-00021 (ALF968) and pB-00243 (NONEUB). All probes were used at a formamide concentration of 20%.

Probe name	Sequence	Target group
EUB338	5'- GCT GCC TCC CGT AGG AGT -3'	Most bacteria (90% coverage)
Chls-0523	5'- CCT CCG TAT TAC CGC AGC -3'	*Chlamydiales* (93% coverage)
BET42a	5'- GCC TTC CCA CTT CGT TT -3'	*Betaproteobacteria* (93% coverage)
ALF968	5'- GGT AAG GTT CTG CGC GTT -3'	*Alphaproteobacteria* (79% coverage)
NONEUB	5'- ACT CCT ACG GGA GGC AGC -3'	Control complementary to EUB338

C.20.5. DNA isolation

DNA was isolated using the DNeasy Blood and Tissue Kit (QIAGEN). The cells were harvested by centrifugation (7,232 x g, 5 min, RT), the pellet was washed with 1x PAS (7,232 x g, 5 min, RT) and then resuspended in 180 µL lysis Buffer ATL. 20 µL of proteinase K were added and the sample was thoroughly mixed by vortexing and incubated at 56°C for 1 h under constant shaking. To the viscous lysate 200 µL Buffer AL and 200 µL of absolute ethanol were added and vortexed until a homogeneous solution was obtained. This mixture was pipetted into a DNeasy Mini spin column placed in a 2 mL collection tube. After centrifugation (6,940 x g, 1 min, RT), the column was washed with 500 µL Buffer AW1 (6,940 x g, 1 min, RT) and 500 µL Buffer AW2 (21,250 x g, 3 min, RT). Flow-through and collection tube were discarded after each centrifugation step. Then the column was placed into a clean 1.5 mL microcentrifuge tube and the DNA was eluted twice (6,940g, 1 min, RT) with 100 µL ddH_2O to an end volume of 200 µL. The concentration of the isolated DNA was determined using NanoDrop.

C.20.6. Polymerase chain reaction

Polymerase chain reaction (PCR) was used for amplification of 18S and 16S rRNA genes in order to identify the isolated amoebae and their endosymbionts, respectively. One PCR reaction (50 µL) was constituted of following components and 1-2 µL of template DNA:

PCR reaction (50 µL)	
$MgCl_2$	4 µL
10x Reaction Buffer	5 µL
dNTP mix	5 µL
Taq polymerase	0.2 µL
Forward primer, 100 (50) pmol μL^{-1}	0.25 (0.5 µL)
Reverse primer, 100 (50) pmol μL^{-1}	0.25 (0.5 µL)
ddH_2O	35.3 (34.8 µL)

The PCR programs that were used for amplification with 16S primers (Table 10) and 18S primers (Table 11) are shown below:

PCR Program for 16S PCRs (PanF/R)

Cycle 1 (1x):			
	Step 1:	95.0ºC	for 03:00
Cycle 2 (30x):			
	Step 1:	95.0ºC	for 00:30
	Step 2:	65.0ºC	for 00:30
	Step 3:	72.0°C	for 01:30
Cycle 3 (1x):			
	Step 1:	72.0ºC	for 07:00
Cycle 4 (1x):			
	Step 1:	22.0°C	HOLD

PCR Program for 18S PCRs (SSU1, SSU2, 18S-786f, 18S-893r)

Cycle 1 (1x):			
	Step 1:	95.0ºC	for 03:00
Cycle 2 (30x):			
	Step 1:	95.0ºC	for 00:30
	Step 2:	50.0ºC	for 00:30
	Step 3:	72.0°C	for 01:30
Cycle 3 (1x):			
	Step 1:	72.0ºC	for 07:00
Cycle 4 (1x):			
	Step 1:	22.0°C	HOLD

Table 10. *Chlamydiales* specific primer set PanF/R used for 16S rDNA amplification, their annealing temperature (T_a) and the size of the amplicon (Corsaro et al. 2002)

Primer name	Sequence	T_a	Amplicon size
PanF	5´-GTC ATC RGC CYY ACC TTV SRC RYY TCT-3´	65°C	~1 445 bp
PanR	5´-CGT GGA TGA GGC ATG CRA GTC G-3´		

Table 11. *Acanthamoeba* specific primers used for 18S rDNA amplification, annealing temperatures (T_a) and respective amplicon sizes (Gast et al. 1994; Fahrni et al. 2003)

Primer name	Sequence	T_a	Amplicon size
SSU1	5'-AAC CTG GTT GAT CCT GCC AGT-3´	50°C	~1400 bp
18S-893r	5'-AAG TTT CAG CCT TGC GAC CA-3´		
18S-786f	5'-GAT YAG ATA CCG TCG TAG TC-3´	50°C	~1000 bp
SSU2	5'-GAT CCT TCT GCA GGT TCA CCT AC-3´		

C.20.7. Sequencing of PCR products

The PCR products were purified using the QIAquick PCR Purification Kit as described in section C.9.3. and the purified PCR products were sequenced with an ABI 3130x Genetic Analyzer (Applied Biosystems). The same primers were used for sequencing as were used for amplification with one exception: no viable sequence could be obtained using primer PanR, so it was replaced with primer 781F (5'-AACAGGATTAGATAC-3') for the 16S rRNA gene sequencing.

C.20.8. Sequence analysis

Sequences were manually corrected using the FinchTV software and identified using the basic local alignment search tool BLAST (Altschul et al. 1990) to compare them with sequences in the database of the National Center for Biotechnology Information (NCBI). The alignment of the obtained 16S rRNA and 18S rRNA sequences, as well as the calculation of phylogenetic trees was performed using the ARB software package (Ludwig et al. 2004).

D. Results

D.1. RNA isolation

In order to obtain large amounts of RNA for the optimization of the microarray, 20 culture flasks (500 cm²) containing *A. castellanii* Neff infected with *P. amoebophila* UWE25 were harvested (section C.6.2.) and the total RNA was isolated using phenol-chloroform-extraction (section C.7.1.).

The concentration of the RNA samples, as well the ratio 260/280 and the ratio 260/230, were spectrophotometrically determined (Table S2). Before DNase digestion, the 260/280 values of the isolated RNA were ranging between 1.05 and 2.06 ($\bar{x}$ = 1.56), the 260/230 values between 0.92 and 1.39 ($\bar{x}$ = 1.20), indicating the presence of considerable amounts of contaminants, probably phenol and other carryover from the isolation procedure. To remove potential contaminating DNA, DNase digestion was carried out. The DNA digestion protocol also contains an additional purification step that should help to remove phenol and other contaminants.

Spectrophotometric measurements of the samples were taken again after DNase digestion (Table S2). A total of 4,565 µg RNA was extracted from 20 culture flasks (228 µg per flask). The 260/280 ratios were ranging between 2.06 and 2.14 ($\bar{x}$ = 2.12) and the 260/230 ratios were ranging between 2.26 and 2.42 ($\bar{x}$ = 2.36), indicating sufficient RNA purity. RNA quality was assessed on an agarose gel (section C.13., Figure 3A) and a control PCR (section 7.3.) was performed to verify the absence of DNA after digestion and no product was obtained for any of the isolated RNA samples (Figure 3B).

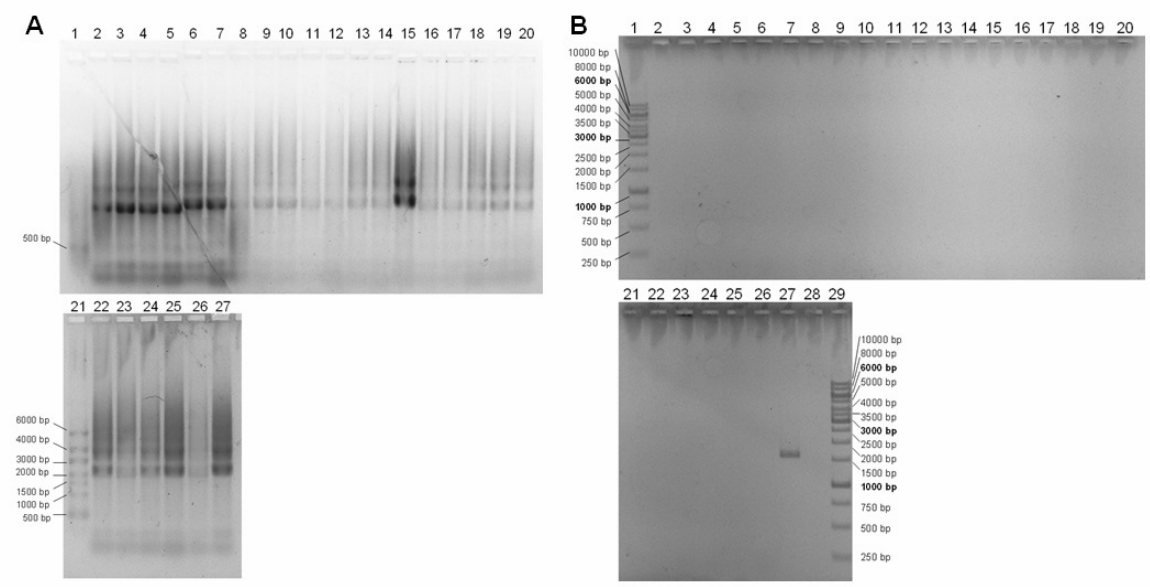

Figure 3. (A) Agarose gel of isolated RNA samples. Lane 1 and 21: RiboRuler RNA ladder, high range; lanes 2-20, 22-27: DNase digested RNA samples (DD01-DD25). Strong bands that are visible correspond to ribosomal RNA, the fastest running fragments correspond to small RNAs and possibly degraded RNA, mRNA populations are visible only as a smear. **(B) Control PCR with isolated RNA samples and PanF/R primers.** Lane 1 and 29: GeneRuler 1kb; lanes 2-26: DNase digested RNA samples (DD01-DD25); lane 27: positive control (DNA from UWE25 from the American Type Culture Collection ATCC, accession number PRA-7); lane 28: negative control (ddH_2O). No bands are present except for the PCR product obtained from the positive control.

RNA from this isolation was used for most experiments conducted in this study. For the cDNA amplification experiment, however, additional RNA was isolated. There, the TRIzol-chloroform-extraction (section C.7.1.) was compared to the RNA isolation with the RNeasy Mini Kit (section C.7.2.). Four large culture flasks containing *A. castellanii* Neff infected with *P. amoebophila* UWE25 were harvested (section C.6.2.) and 2 x 10^7 cells were used in each protocol. The yield of the TRIzol-chloroform-extraction protocol was higher than that of the RNeasy Kit (Table 12) and small RNA fragments were considerably more abundant (Figure 4), since with the RNeasy procedure, only RNA molecules longer than 200 nucleotides are purified and small RNAs, such as 5.8S rRNA, 5S rRNA, and tRNAs, are selectively excluded. A PCR to verify the DNA digestion was not performed since small amounts of residual DNA would not interfere with the experiment.

Table 12. RNA isolated with TRIzol-chloroform and RNeasy Kit.

Sample	Concentration	Volume	RNA amount	Ratio 260/280	Ratio 260/230
RNA-TRI	3382.3 ng/µL	200 µL	676 µg	2.02	2.01
RNA-easy	1353.4 ng/µL	200 µL	271 µg	2.14	1.73

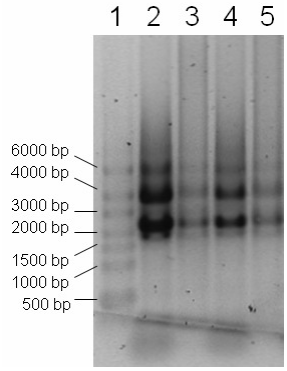

Figure 4. Agarose gel of isolated RNA samples. Lane 1: RiboRuler RNA ladder, high range; lane 2: RNA-TRI, before DNase digestion; lane 3: RNA-easy, before DNase digestion; lane 4: RNA-TRI, after DNase digestion; lane 5: RNA-easy, after DNase digestion. The concentration is clearly higher in RNA samples obtained with TRIzol-chloroform-extraction. Small RNA fragments <200 bp are less abundant after RNeasy Kit RNA isolation.

D.2. Bacterial RNA enrichment

A major challenge in studying the gene expression of intracellular bacteria is the low abundance of bacterial mRNA in comparison to the RNA of the host organism and the ribosomal RNA fraction, because all non-target RNA species are labeled alongside the target RNA and contribute to high background and low signal intensities. Separation of bacterial RNA from the total RNA population might therefore be advantageous and was carried out using the MICROB*Enrich* Kit. This kit removes polyadenylated eukaryotic mRNA as well as 18S and 28S rRNA by capturing them with complementary oligonucleotide probes that are themselves captured by magnetic beads and removed from the solution using a magnet (section C.8.). After application of the kit the samples were considerably depleted in overall RNA (Figure 5) and presumably enriched in chlamydial RNA. The protocol reduced the overall amount of RNA to an average of 16.4% of total starting RNA (Table S3).

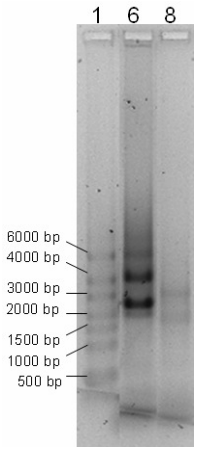

Figure 5. RNA before and after enrichment with MICROB*Enrich*. Lane 1: RiboRuler RNA ladder, high range; lane 6: total RNA; lane 8: enriched bacterial RNA. Note that after the enrichment only the ribosomal RNA bands that run lower on the gel are visible, indicating that the larger eukaryotic rRNAs were successfully removed from the mixture.

D.3. Aminoallyl labeling

One of the crucial steps in microarray analysis is the labeling of the RNA to turn it into a suitable target for hybridization. For this purpose, a reverse transcriptase enzyme is used to produce cDNA from RNA templates while incorporating aminoallyl dUTP nucleotides, the aminoallyl groups of which are then used to couple a fluorescent dye to the cDNA (section C.9.). In the start-up phase of the microarray, the CyScribe Post Labeling Kit had been successfully used for labeling until it stopped working suddenly without explainable reasons (Haider, unpublished). The procedure was subsequently changed to another labeling method (section C.9.) that was selected due to superior performance in a comparison of three different aminoallyl labeling protocols.

Initial labelings that were used to establish the new protocol were carried out using the preliminary nucleotide mix (section C.9.1., Table 8) and are shown in detail in Table S4. In this first labeling series, the protocol yielded an average of 2.1 µg of fluorescently labeled cDNA per 20 µg of total RNA. The incorporated amount of CyDye per labeling was on average 445 pmol, resulting in an incorporation rate (FOI, frequency of incorporation) of 54 for Cy5 and 69 for Cy3. Initial labelings with 5 µg enriched bacterial RNA as a templates yielded an average of 1.7 µg of labeled cDNA per 5 µg template and showed lower FOIs for Cy5 ($\bar{x}$ = 34) and comparable FOIs for Cy3 ($\bar{x}$ = 62).

The purification columns that were used to purify the labeling product have a maximum capacity of 10 µg, the amount of cDNA to be purified from a single labeling, however, is slightly higher. To avoid exceeding the maximum capacity of the purification columns, one

labeling reaction was split to two columns for purification. This measure increased the efficiency of the labeling procedure that now yielded an average of 3.2 µg of labeled cDNA per 20 µg of input total RNA, an increase of 52%. Detailed results of the second labeling series using the preliminary nucleotide mix are shown in Table S5. An average of 620 pmol CyDye was incorporated, resulting in an average FOI of 63, whereas the FOI for Cy3 ($\bar{x}$ = 70) was slightly higher than for Cy5 ($\bar{x}$ = 60) (Table S5). When bacterial RNA enrichment was performed, 5 µg of template RNA produced comparatively higher amounts of labeled cDNA (Table S5).

It is generally recommended to use FOI values between 20 and 50 for successful microarray hybridizations, since lower FOI values will generate weak signals from poor incorporation, whereas a higher FOI might lead to quenching between the fluorophores (GE Healthcare 2008). Since the FOI values of the initial hybridizations were located outside this area, the nucleotide mix used for reverse transcription was adjusted in order to achieve FOI values of 35 in order to tolerate some variation in both directions. Different ratios of dTTP and aa-dUTP in the nucleotide mix were tested and detailed results are supplied in Table S6. The preliminary nucleotide mix contained dTTP and aa-dUTP in a ratio of 1:6.25. In order to decrease the FOI, the amount of aa-dUTP was decreased. A dTTP:aa-dUTP ratio of 1:2 produced average FOI values of 60, at a ratio of 1:1 the FOI decreased to 34, at 2:1 to 29.

For further hybridizations a nucleotide mix with a dTTP:aa-dUTP ratio of 1:1 (6mM dATP/dCTP/dGTP, 3mM dTTP, 3mM aa-dUTP) was used. Labelings with this nucleotide mix performed reasonably well for Cy5, where the FOI values were stable between 22 and 41. Labelings using Cy3, in contrast, subsequently showed higher FOIs (up to an FOI of 57) with the 1:1-nucleotide mix, and further optimization of a separate nucleotide mix for labeling with Cy3 was carried out (Table S7). The 4:3-nucleotide mix worked best (average FOI of 41), 2:1- and 3:2-nucleotide mixes lead to FOIs that were slightly too low (average FOIs of 26 and 28, respectively).

Nevertheless, labelings using the Cy3 fluorophore did not perform as well as Cy5-labelings, the background after hybridizations was always considerably higher, thus reducing the signal-to-noise ratios. For this reason and since the hybridization of genomic DNA labeled in Cy3 was working, further cDNA labelings were conducted with Cy5, while Cy3-labeled genomic DNA was used for normalization. The aminoallyl labeling with Cy5 subsequently produced

stable results (Table S8) with incorporation ratios between 22 and 41 ($\bar{x}$ = 33). One labeling reaction (20 µg template RNA) produced an average amount of 3.2 µg labeled cDNA with 336 pmol of incorporated Cy5.

D.4. cDNA amplification and direct labeling

D.4.1. cDNA amplification

The bacterial RNA enrichment using MICROB*Enrich* did not lead to improved hybridization results (section D.7.3.). In order to enhance the performance of enriched samples, an amplification step was introduced before the fluorescent labeling of cDNA (section C.10.). The enriched RNA was reversely transcribed and amplification was performed using the illustra GenomiPhi V2 DNA Amplification Kit, thereby producing a large amount of DNA of high molecular weight from a very small amount of cDNA (Figure 6). The kit provided stable amplification independent of the amount of template cDNA that varied between 50 ng and 1 µg with an average amplification yield of 16.6 µg of DNA. However, the quantitation of unpurified amplification products using Nanodrop is inaccurate due to presence of protein and other kit components (GE Healthcare 2003) and presumably the amount of DNA is seriously overestimated.

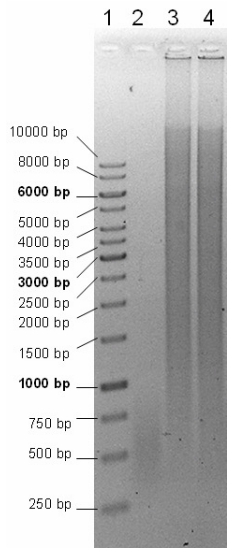

Figure 6. cDNA amplification using GenomiPhi DNA Amplification Kit. A very small amount of enriched cDNA (lane 2) produces large amounts of DNA of high molecular weight (lane 3 and 4). Lane 1: GeneRuler 1kb.

D.4.2. Restriction enzyme digestion of amplified cDNA

The GenomiPhi Kit produces DNA of high molecular weight not suitable for microarray hybridization. Therefore the amplification product was digested with the restriction enzyme

BsuRI (Figure 7). BsuRI is a 4 base pair cutter that generates fragments of an average length of 256 base pairs by cutting at the recognition sequence 5'GG^CC-3'. The fact that most fragments visible in Figure 7 are much larger may be attributed to insufficient incubation time.

D.4.3. Purification of amplified cDNA

Before labeling, the amplified cDNA was purified using the QIAquick Nucleotide Removal Kit in order to remove all the components of the amplification and the restriction enzyme digestion. This lead to a great reduction in the amount of DNA that was measured (an average of 2.1 µg of purified DNA remained for an average of 16.6 µg of unpurified amplified DNA), partly due to actual loss during the purification procedure (Figure 7) and partly due to NanoDrop measurement overestimating the amount of DNA after amplification.

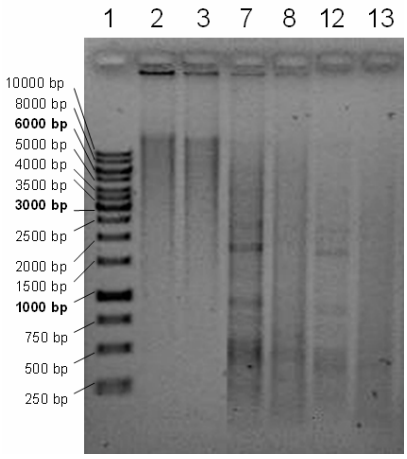

Figure 7. Restriction digestion of amplified cDNA. Lane 1: GeneRuler 1kb; lane 2: amplified control DNA (Lambda, 10 ng/µL); lane 3: amplified cDNA; lane 7: restricted amplified control DNA; lane 8: restricted amplified cDNA; lane 12: purified restricted amplified control DNA; lane 13: purified restricted amplified cDNA.

D.4.4. Direct labeling of amplified cDNA

For labeling of the amplified cDNA a protocol for labeling of genomic DNA was used (section C.12.). After purification, labelings of amplified cDNA showed results similar to genomic DNA labelings, without purification the labeling procedure was less efficient (Table S9).

D.5. Hybridization with poly-dTTP spacer probes

Poly-dTTP spacer probes (12 nucleotides) targeting the A-spacer of printed oligonucleotides were used to assess the spotting quality. Every first slide of a spotting series was subjected to a poly-dTTP hybridization and if not all spots were present the target height of the spotting

pins was adjusted. The microarray consists of three replicated blocks (Figure 8A), each block, composed of 16 subgrids, contains probes targeting all 2,031 putative open reading frames of *P. amoebophila*, as well as a probe for the *folA* gene encoding dihydrofolate reductase (a gene found on a contig of *P. amoebophila* which could not be correctly assembled so far) and several positive and negative controls (Figure 8B). The four probes used as negative controls are derived from phylogenetically unrelated bacteria *Desulfotomaculum thermoacetoxidans* DSM 5813 (*Firmicutes*), *Thermodesulfovibrio islandicus* DSM 12570 (*Nitrospira*), *Desulfovibrio profundus* DSM 11384 (*Deltaproteobacteria*) and *Thermodesulfobacterium thermophilum* DSM 1276 (*Thermodesulfobacteria*) and do not show cross-hybridization to sample RNA.

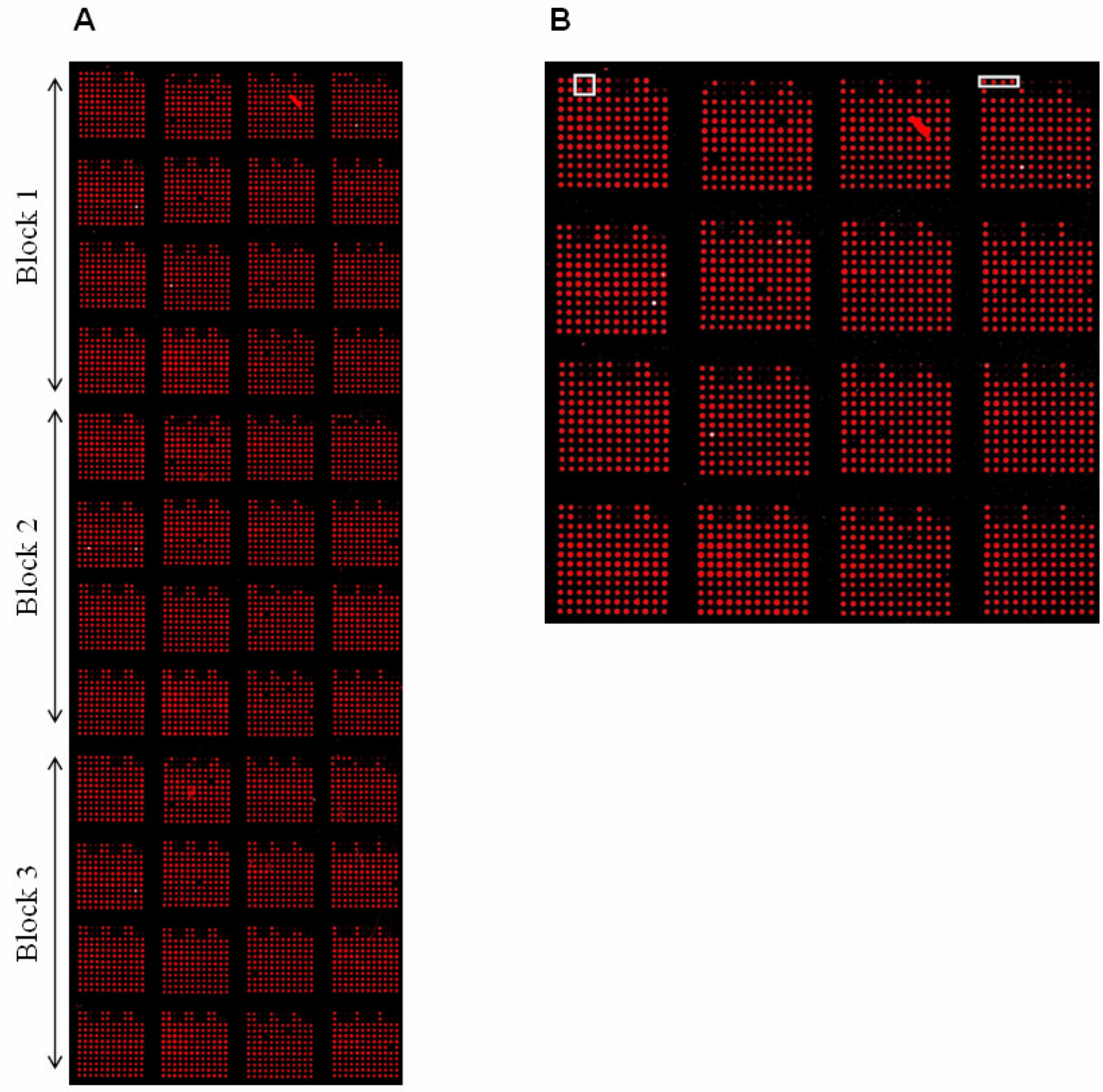

Figure 8. The *P. amoebophila* microarray after hybridization with a poly-dTTP spacer probe. (A) The microarray consists of three replicated blocks (B) A single block, composed of 16 subgrids, contains probes targeting all 2,032 annotated genes of *P. amoebophila* as well as several positive and negative controls. Negative controls are highlighted by white squares.

Since this poly-dTTP hybridization provides a cheap and easy means to conduct hybridizations that are at least to some degree comparable to cDNA hybridizations, it was also used to evaluate modifications to post-processing of the microarray slides and hybridization conditions.

When problems with the reproduction of successful hybridizations were experienced (section D.7.5.), poly-dTTP hybridizations were performed to determine the reason for the poor reproducibility. A slide subjected to the standard hybridization procedure was compared to a slide from the same spotting series (spotting series #2110) but with all the chemicals and solutions replaced by new ones and to a slide from an older spotting series (spotting series #0807) (Table 13). No significant improvement could be observed when the chemicals were replaced, but the signal-to-noise ratio was remarkably higher when a slide from an older spotting series was used due to strongly increased signal intensities above a comparable background.

Table 13. Poly-dTTP hybridization comparing replacement of slides and chemicals

Hybridization	Mean signal intensity values	Mean background intensity values	Mean signal-to-noise ratio
Spotting series #2110	449	303	**2**
Spotting series #2110, new chemicals	473	277	**3**
Spotting series #0807	1464	280	**19**

From then on, all the new spotting series were compared via a poly-dTTP hybridization to spotting series #2110 that served as a kind of negative control considered not suitable for hybridization (Table 14). Figure 9 shows relative signal-to-noise ratios standardized to spotting series #2110. Series #0711 had a signal-to-noise ratio that was clearly superior to #2110. Unfortunately, all subsequent spotting series (#2411, #2811, #0212) were comparable to spotting series #2110. The results of the simple hybridizations with poly-dTTP probes are consistent with the performances of the respective spotting series in cDNA hybridizations (see section D.7.).

Table 14. Comparison of new spotting series to spotting series #2110.

Comparison*	Mean signal intensity values	Mean background intensity values	Mean signal-to-noise ratio
spotting series #2110	11111	91	**1278**
spotting series #0711	35982	164	**2672**
spotting series #2110	4958	35	**1987**
spotting series #2411	13725	93	**1492**
spotting series #2110	16472	70	**2153**
spotting series #2811	26549	123	**2322**
spotting series #2110	3557	32	**1624**
spotting series #0212	3906	40	**1438**

*only slides from the same hybridization are comparable due to large variation in signal intensity between different hybridizations

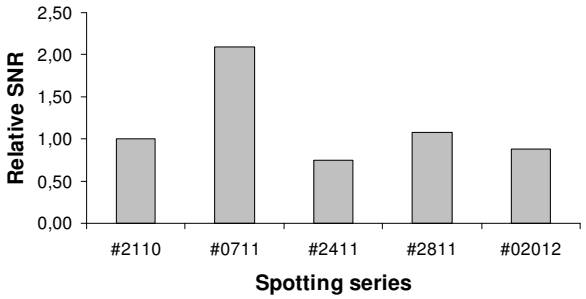

Figure 9. Signal-to-noise ratios relative to spotting series #2110.

It is hard to come up with a good explanation for the varying quality of the different spotting series, but it was suspected from observation, that longer reduction times in sodium borohydride might not only reduce the background intensities by inactivation of the slide surface, but also have a detrimental effect on the signal intensities, thereby decreasing the signal-to-noise ratio. Therefore, a test using poly-dTTP spacer probes and different lengths of reduction in sodium borohydride was performed (Figure 10).

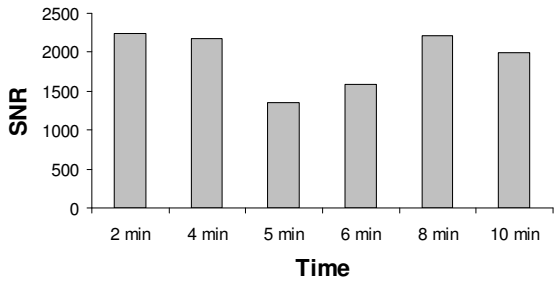

Figure 10. Comparison of different times of exposure to sodium borohydride.

D.6. Hybridization with genomic DNA

To adjust for effects which arise from variation in the microarray technology rather than from biological differences and to obtain quantitative results it is necessary to normalize the data. Normalization in cDNA microarray experiments can be conducted using genomic DNA. The number of detected genes was always ≥99%, when 1 or 2 µg of fragmented and labeled genomic DNA was hybridized to the microarray (Figure 11). When less genomic DNA was used, the number of genes detected was decreasing (97% - 0.5 µg, 77% - 0.25 µg). The negative controls were always below the signal threshold.

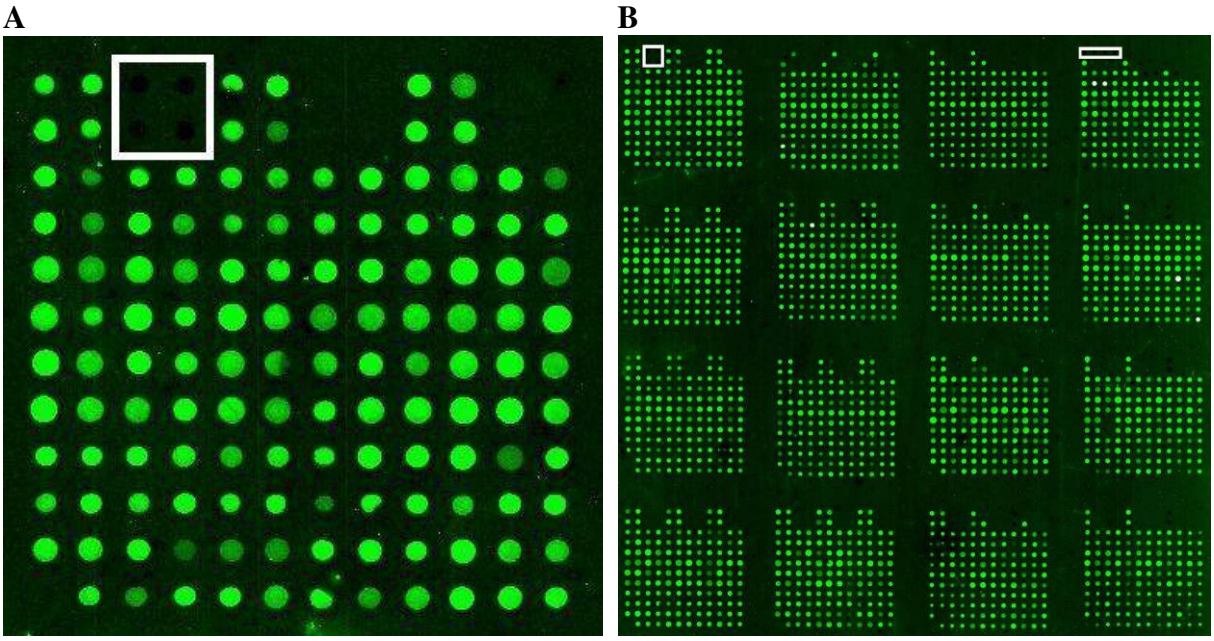

Figure 11. Hybridization with genomic DNA. White squares indicate negative controls. (A) Detail of subgrid 1 shows high intensities for all the spots except the negative controls with low background fluorescence. (B) Detail of block 1.

D.7. Hybridization with cDNA

Hybridization was evaluated by calculating the mean SNR of the triplicates for every spot. When the mean SNR was >3 the spot was considered to give a signal. A mean of 61% of predicted genes in the *P. amoebophila* genome were found to be transcribed during infection (Haider, unpublished). For this reason, detection of around 60% of the genes was set as a target for the optimization process. Figure 13 shows an overview over the optimization process.

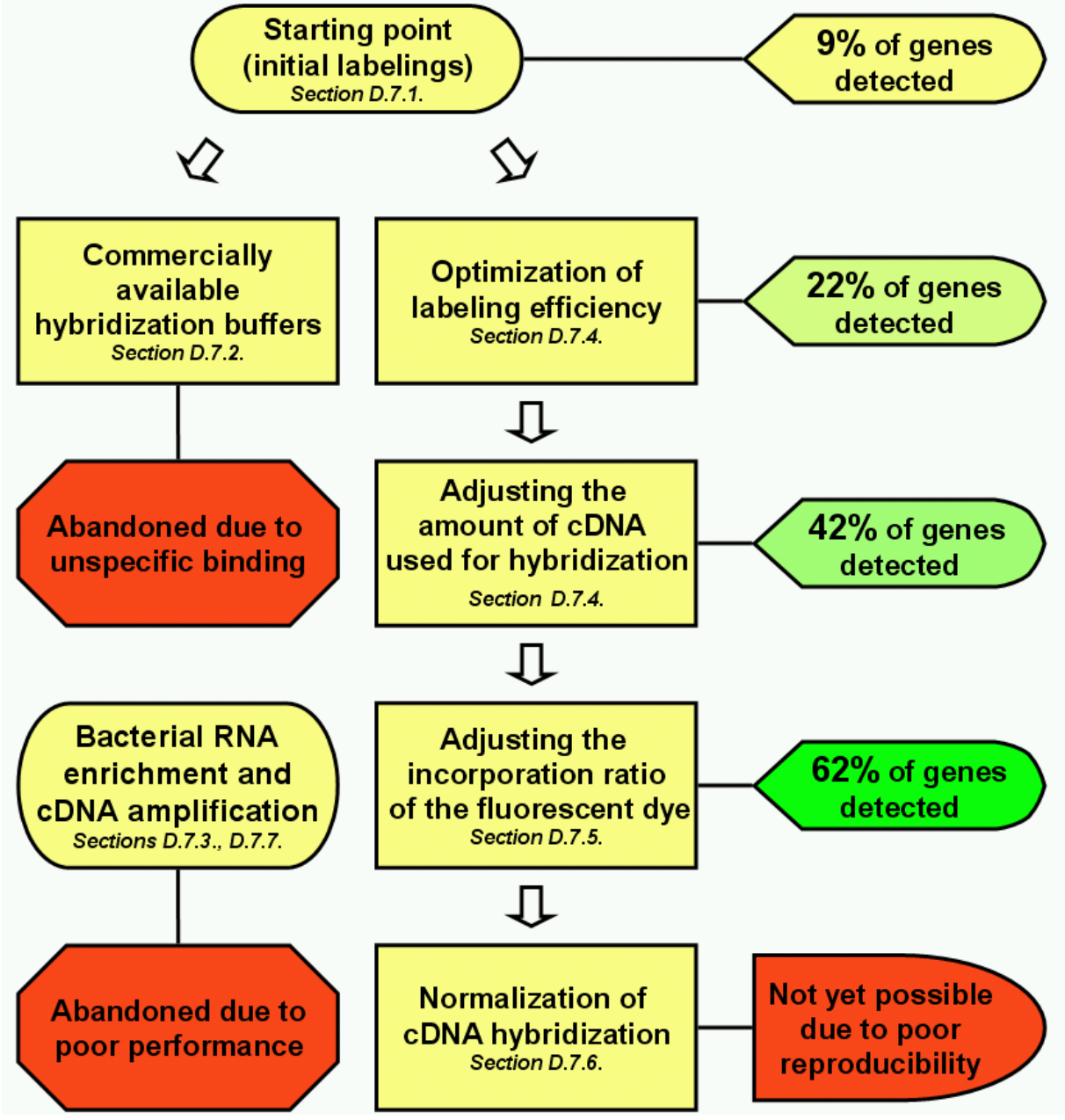

Figure 13. Overview of the hybridization optimization process.

D.7.1. Initial hybridizations

Initially, one labeling reaction with 20 µg of template RNA was used for one hybridization. Hybridization signals were generally very weak and the background intensities were relatively high so that only between 5% and 11% ($\bar{x}$ = 9%) of the spots could be detected (Table 15). Following image (Figure 13) shows representative details of a hybridization of the first series. In that hybridization 11% of the spots had an SNR greater than 3 and were thus qualified as signals.

Table 15. Hybridizations with initial labelings

Slide	Initial amount of RNA	CyDye	Labeling parameters			Signals detected
			cDNA (µg)	CyDye (pmol)	FOI	
#2503-12	20 µg	Cy5	1.2	194	55	5%
#2503-13	20 µg	Cy5	1.7	290	56	11%
#2503-14	20 µg	Cy5	1.6	188	58	9%
#2503-15	20 µg	Cy5	1.4	249	57	11%

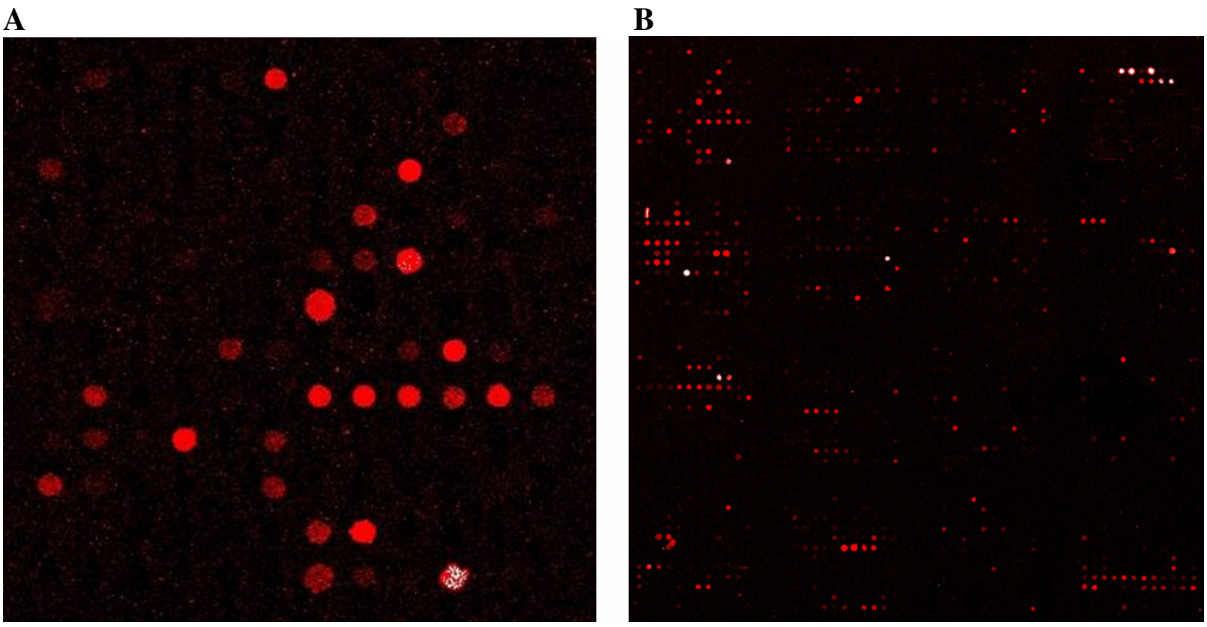

Figure 13. Hybridization of Cy5-labeled cDNA on slide #2503-13 (A) Detail of subgrid 1 illustrates the background fluorescence that is relatively high compared to the fluorescence intensity of the signals. Under such circumstances only the spots with the highest intensities are able to cross the detection threshold and many genes go undetected. (B) Detail of block 1 shows the hybridization pattern of the chip.

D.7.2. Commercially available hybridization buffers

Two commercially available hybridization buffers were tested: Sigma's PerfectHyb Hybridization Buffer and Ambion's SlideHyb Glass Array Hybridization Buffer #1. Both

buffers produced very high signal intensities at the expense of specificity, since most of the negative controls showed signals. Attempts at increasing the specificity by increasing the stringency were not successful, but lead only to an overall decrease in signal intensity without restoring specificity. In the case of PerfectHyb Hybridization Buffer an additional problem was that the hybridization pattern of the microarray chip was completely different from the pattern observed in all the previous hybridizations with RNA from asynchronous cultures and was thought to not accurately represent the gene expression. Details of hybridizations using PerfectHyb and SlideHyb buffers are shown in Figure 14 and Figure 15, respectively.

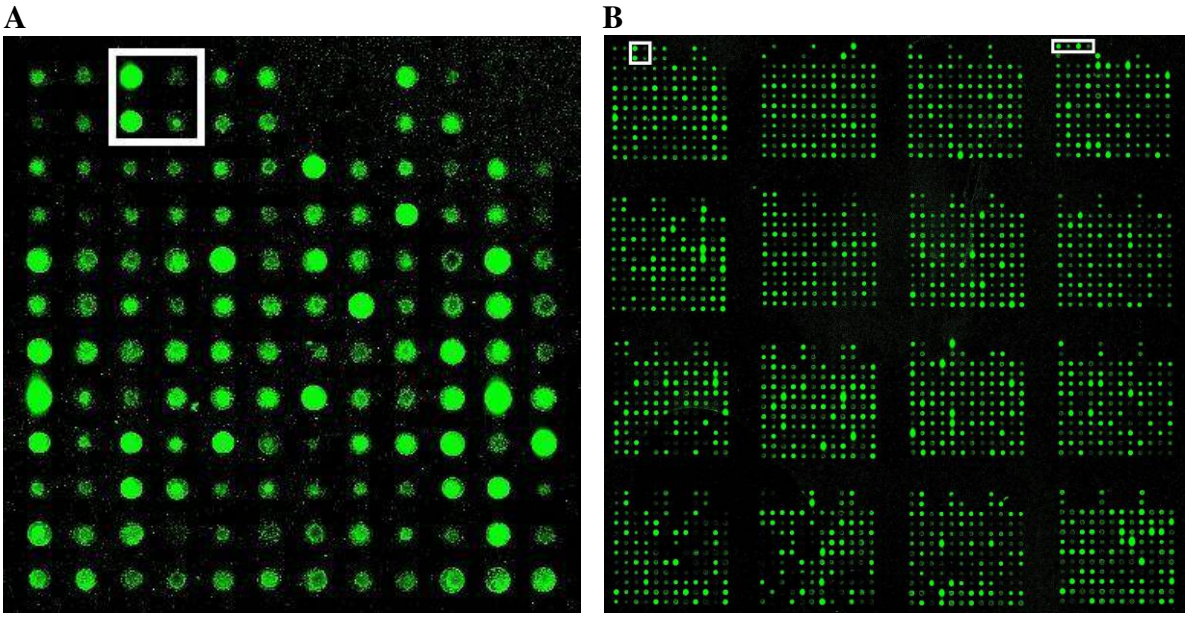

Figure 14: Hybridization using PerfectHyb Hybridization Buffer on slide #2304-02. Negative controls are indicated by white squares. (A) Detail of subgrid 1. The signal intensity is very high and so is the signal to noise ratio, as the intensity of the background is relatively low. 70% of the spots produced signals. The hybridization, however, is completely unspecific and seems to occur on almost every spot, even on the negative controls that are curiously among the most intense signals observed. (B) Detail of block 1 shows the completely altered hybridization pattern of hybridizations using PerfectHyb Hybridization Buffer.

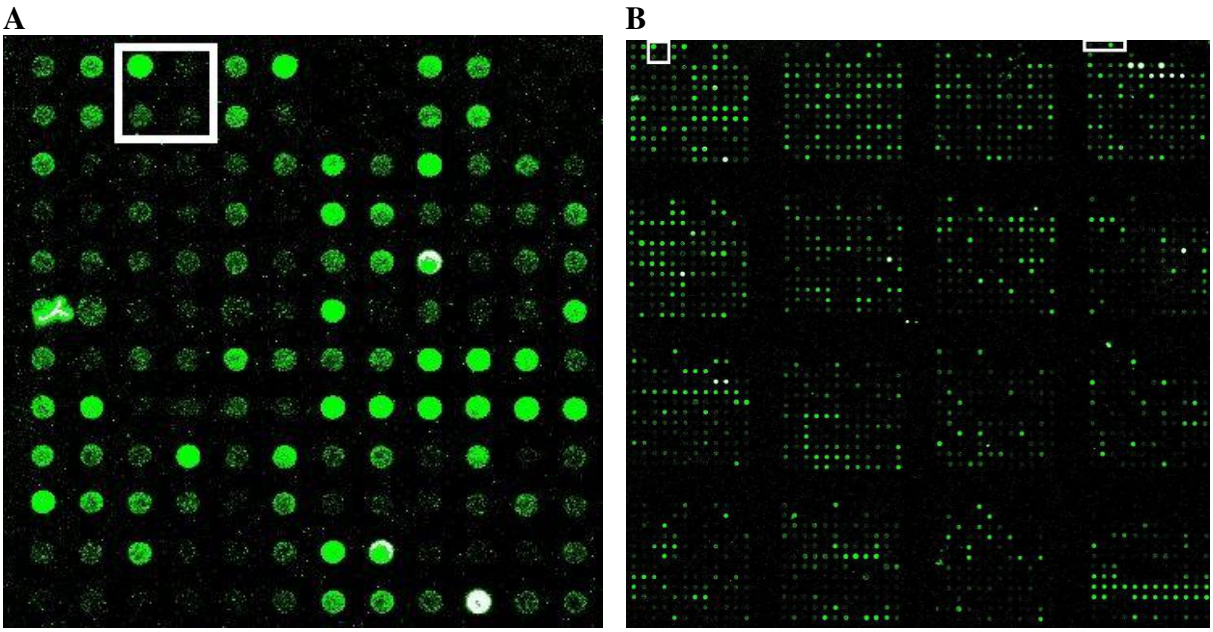

Figure 15. Hybridization using SlideHyb Glass Array Hybridization Buffer #1 on slide #2304-16. Negative controls are indicated by white squares. (A) Detail of subgrid 1. Signal intensities are comparably high but signal-to-noise ratios are lower when using SlideHyb than when using PerfectHyb due to higher background. (B) Detail of block 1. Using SlideHyb the microarray once again shows its familiar hybridization pattern.

D.7.3. Hybridization after bacterial RNA enrichment

Hybridization after bacterial RNA enrichment could not improve the signal intensities (Table 16). Hybridizations with labelings of 5 µg of enriched RNA as template produced results similar to hybridizations with labelings of 20 µg of total RNA as template: slides #2503-16 and #2503-17 were hybridized with initial labelings using the preliminary nucleotide mix and can be directly compared to the respective hybridizations from total RNA (section 7.1., Table 15). When more RNA was used as a template for bacterial RNA enrichment and therefore more cDNA was used for hybridization, the percentage of detected signals increased (slide #0807-12). Curiously, when two labelings from 5 µg of enriched cDNA were pooled and used, the hybridization showed positive negative controls (slide #0807-11).

Table 16. Hybridizations after bacterial RNA enrichment

Slide	Initial amount of RNA	CyDye	Labeling parameters			Signals detected
			cDNA (µg)	CyDye (pmol)	FOI	
#2503-16	5 µg	Cy5	0.9	180	67	9%
#2503-17	5 µg	Cy5	1.8	245	41	11%
#0807-11	10 µg	Cy5	3.9	746	62	65%*
#0807-12	16 µg	Cy5	2.7	593	72	22%

* unspecific binding

D.7.4. Optimization of cDNA amount used in hybridization

Since neither the application of the commercially available hybridization buffers nor the bacterial RNA enrichment could improve the hybridization, it was tried to optimize the standard labeling procedure (section D.3.). Avoiding the overloading of the purification columns lead to an increased labeling efficiency that was also reflected in the performance of the hybridizations, the number of signals detected could be raised to 22% (Table 17).

Table 17. Hybridization with labeling reaction that was split for purification

Slide	Initial amount of RNA	CyDye	Labeling parameters			Signals detected
			cDNA (µg)	CyDye (pmol)	FOI	
#0807-03	20 µg	Cy3	3.0	758	79	22%

After that, experiments were done to determine the optimal amount of cDNA to be hybridized (Table 18). The percentage of detected signals could be improved from 24% to 41% by increasing the amount of cDNA used for hybridization from 2.8 µg to 6.4 µg. If the amount of DNA was increased further to 8 µg of labeled cDNA, the percentage of detected signals dropped to 15%, possibly due to quenching effects or steric hindrance of DNA molecules.

Table 18. Optimization of cDNA amount used in hybridization

Slide	Initial amount of RNA	CyDye	Labeling parameters			Signals detected
			cDNA (µg)	CyDye (pmol)	FOI	
#0807-08	20 µg	Cy5	2.8	461	53	24%
#0807-09	30 µg	Cy5	4.6	850	60	33%
#0807-10	40 µg	Cy5	6.4	1200	61	41%
#0807-11	60 µg	Cy3	8.0	1640	67	15%

D.7.5. Optimization of cDNA hybridization by adjusting the incorporation rates

Another major step forward was the optimization of the nucleotide mix used for reverse transcription as described before (section D.3.). Up to that time a nucleotide mix was used that employed dTTP and aa-dUTP in a ratio of 1:6.25, which lead to an average FOI of 63. Adjusting this ratio to 1:1, an optimal average FOI of 33 was obtained. Table 19 shows hybridizations that were conducted during and after the optimization of the dTTP:aa-dUTP ratio.

In the beginning, a labeling using the 1:2-nucleotide mix (FOI 34) was compared to a labeling that used the preliminary nucleotide mix with a 1:6.25 ratio (FOI 62) and the labelings were hybridized to slide #1908-02 and #1908-04, respectively, so that in both hybridizations approximately the same amount of CyDye was present. Although the amount of cDNA was therefore considerably lower for the hybridization with the 1:2 ratio, the percentage of detected signals was considerably higher (47% versus 21%). The next labelings with the 1:2-nucleotide mix produced higher FOIs that performed accordingly in hybridization, the labeling with an FOI of 74 detected only 8% of the genes (slide #1908-05), the labeling with an FOI of 58 detected at least 34% (slide #1908-06). Consequently the amount of aa-dUTP in the nucleotide mix was further decreased to a dTTP:aa-dUTP ratio of 1:1 and even 2:1, that were used to produce labeled cDNA with FOIs of 34 and 28, respectively. When hybridized, the respective samples could only detect 26% (slide #1908-07) and 21% (slide #1908-08), but at least the FOI achieved with the 1:1-nucleotide mix seemed to be suitable. The following Cy3 labelings (slides #1908-09 to 13) further supported the superiority of the 1:1-nucleotide mix concerning the FOI, but satisfying hybridization results were not obtained until the first hybridization with Cy5 labeled cDNA (#1908-14). On this slide, to which the pooled products of two labeling reactions, containing 5.9 µg of cDNA and 635 pmol of Cy5, with an optimal FOI of 35 were hybridized, 62% of the genes could be detected (Figure 16). Reproducing this result was not easy, because labelings with similar amounts of cDNA and incorporated Cy5 produced varying hybridization results by detecting 48% (slide #1908-17), 59% (slide #0807-15), 25% (slide #0807-16), 26% (slide #2110-02), 44% (slide #2110-03), 27% (slide #2110-04), 60% (slide #0711-01), and 55% (slide #0711-03). The reasons for the lacking stability of the hybridization results are not known, although it is suspected that the varying quality of different spotting series has a major impact on the hybridizations, as for example the hybridizations using slides from the #2110 series were considerably worse and this series was also shown to perform inferiorly in test hybridizations using poly-dTTP spacer probes

(section D.5.). Further labelings with Cy3 produced considerably lower signals, even after further optimization of the nucleotide mix it was not possible to obtain more than 43% signals. The four hybridizations that were considered successful and detected a sufficient number of genes ($\bar{x}$ =59%) are shown in bold letters in Table 19.

Table 19. Optimization of cDNA hybridization

Slide	Initial amount of RNA	CyDye	dTTP:aa-dUTP ratio	Labeling parameters			Signals detected
				cDNA (µg)	CyDye (pmol)	FOI	
#1908-02	20 µg	Cy3	1:2	4.0	416	34	47%
#1908-04	17 µg	Cy5	1:6.26	2.2	410	62	21%
#1908-05	20 µg	Cy3	1:2	4.1	932	74	8%
#1908-06	20 µg	Cy3	1:2	2.5	448	58	34%
#1908-07	20 µg	Cy3	1:1	2.4	252	34	26%
#1908-08	20 µg	Cy3	2:1	1.9	168	28	21%
#1908-09	15 µg	Cy3	1:2	3.2	624	61	8%
#1908-10	20 µg	Cy3	1:1	3.2	420	43	9%
#1908-11	40 µg	Cy3	1:1	5.2	632	40	43%
#1908-12	30 µg	Cy3	1:2	6.5	1308	65	35%
#1908-13	40 µg	Cy3	1:1	6.5	864	64	36%
#1908-14	**40 µg**	**Cy5**	**1:1**	**5.9**	**636**	**35**	**62%**
#1908-15	15 µg	Cy3	1:1	3.4	518	50	24%
#1908-16	25 µg	Cy3	1:1	6.4	966	49	34%
#1908-17	40 µg	Cy5	1:1	7.6	804	34	48%
#1908-19	33 µg	Cy5	1:1	4.7	354	24	32%
#0807-15	**53 µg**	**Cy5**	1:1	**6.0**	**641**	**35**	**59%**
#0807-16	37 µg	Cy5	1:1	5.9	658	37	25%
#0807-17	40 µg	Cy3	1:1	5.9	832	47	22%
#0807-18	40 µg	Cy3	1:1	5.6	820	47	23%
#2110-02	40 µg	Cy5	1:1	6.2	669	35	26%
#2110-03	40 µg	Cy5	1:1	6.8	770	37	44%
#2110-04	40 µg	Cy5	1:1	5.0	499	33	27%
#0711-01	**20 µg**	**Cy5**	**1:1**	**6.4**	**648**	**33**	**60%**
#0711-03	**30 µg**	**Cy5**	**1:1**	**6.4**	**751**	**38**	**55%**
#0711-18	40 µg	Cy3	3:2	7.5	656	28	23%
#0711-19	40 µg	Cy3	4:3	6.2	704	37	20%

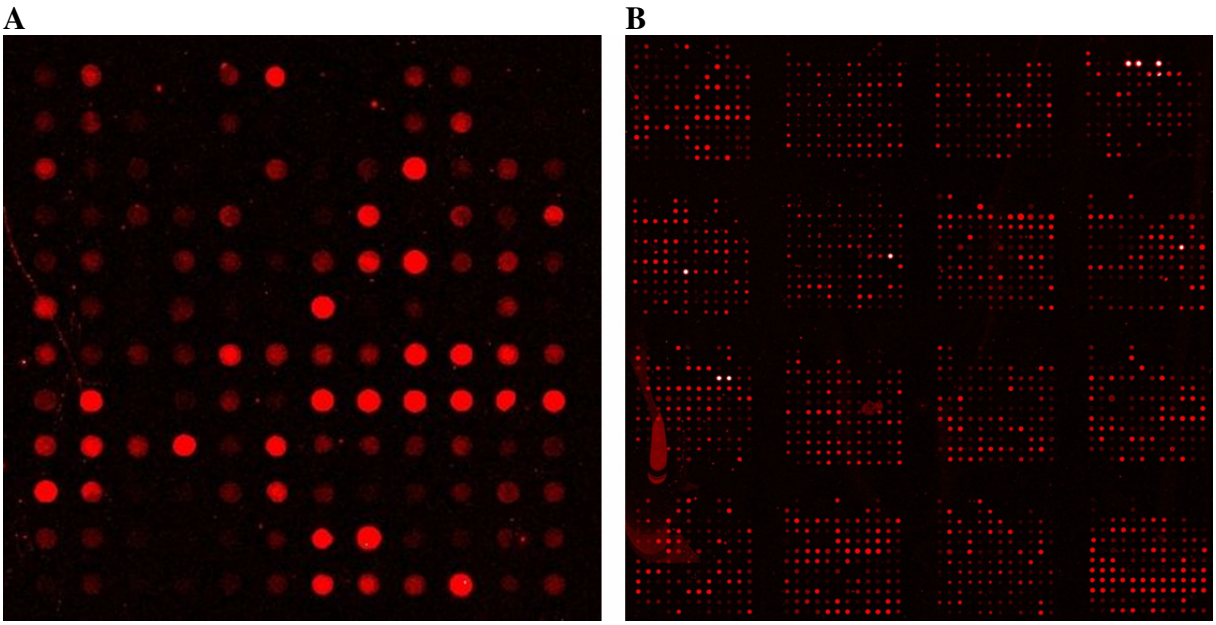

Figure 16. Hybridization of Cy5-labeled cDNA on slide #1908-14. (A) Detail of subgrid 1 illustrates the high signal-to-noise ratio generated by high signal intensities and low background fluorescence. Most spots visible on the picture are above the signal threshold. (B) Detail of block 1. Altogether, 62% of the genes are detected.

D.7.6. Normalization with genomic DNA

The next step was to normalize the cDNA hybridization by co-hybridization with genomic DNA. Unfortunately, the good performance of the cDNA hybridization could not be reproduced when hybridized in combination with genomic DNA (Table 20). While genomic DNA hybridization always detected ≥99% of the genes, the percentage of signals detected from cDNA was never above 26% (Figure 17). In the first two attempts to normalize the cDNA hybridization of an asynchronous culture with genomic DNA only 25% and 24% of the genes could be detected. To determine whether the co-hybridization of gDNA was responsible for the low hybridization signals or it was an independent problem, two duplicate hybridizations were performed, one of them with addition of 1 or 2 µg of gDNA and one without addition of gDNA. The normalized hybridization did always detect less genes than the hybridization without gDNA addition (21% versus 23%, 13% versus 18%), an effect that was even more pronounced when the CyDyes were switched (12% versus 25%), but this reduction was not responsible for the comparably poor performance of these hybridizations.

Table 20. Normalization with genomic DNA

Slide	Initial amount of RNA	Labeling parameters			Cy3-gDNA	Signals detected
		cDNA (µg)	Cy5 (pmol)	FOI		
#0711-12	25 µg	5.5	644	38	1 µg	25%
#0711-13	27 µg	6.1	614	31	1 µg	24%
#0711-14	40 µg	4.8	422	29	-	23%
#0711-15	40 µg	4.8	422	29	1 µg	21%
#0711-16	20 µg	3.9	512 (Cy3)	43	-	25%
#0711-17	20 µg	3.7	476 (Cy3)	42	1 µg (Cy5)	12%
#2411-02	40 µg	9.2	758	27	-	-
#2411-03	40 µg	9.2	758	27	1 µg	26%
#0212-02	27 µg	6.6	659	32	-	18%
#0212-03	27 µg	6.6	659	32	2 µg	13%

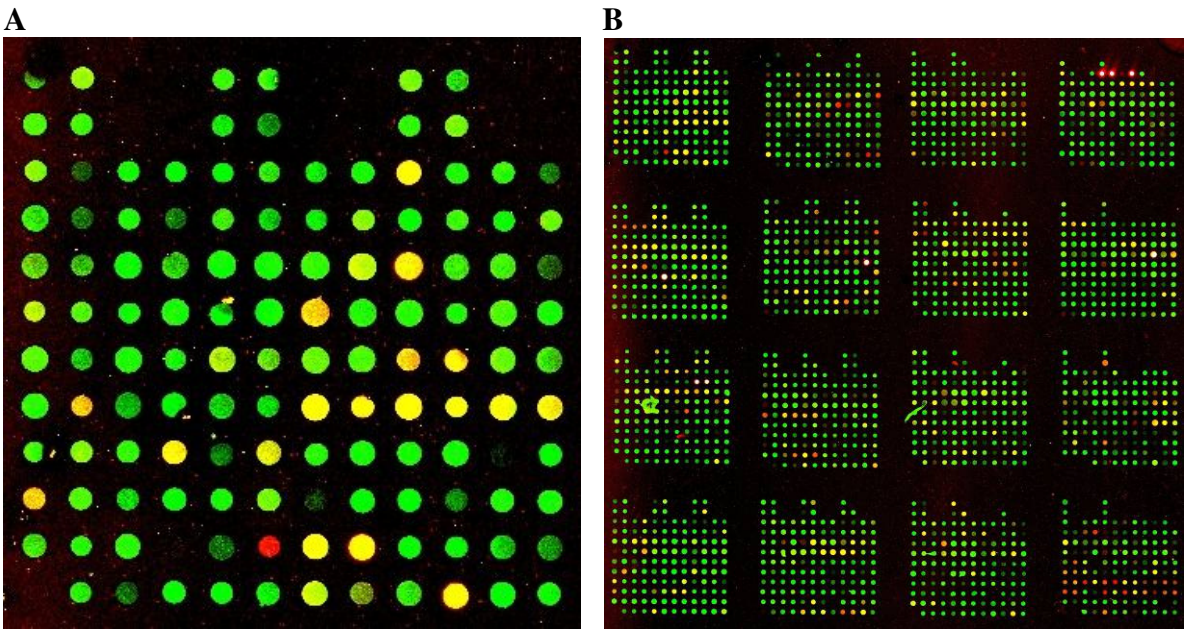

Figure 17. Co-hybridization of cDNA (Cy5, red) and genomic DNA (Cy3, green) on slide #2411-03. Only about a quarter of the spots (26%) are detected by both samples and therefore appear yellow. (A) Detail of subgrid 1. (B) Detail of block 1.

D.7.7. Hybridization of amplified cDNA

In order to enhance signal intensity, the DNA amplification method GenomiPhi was tested. But hybridizations of cDNA that was amplified with the GenomiPhi kit and directly labeled using a Klenow fragment based labeling protocol did not fulfill the expectations. 7 out of 8 hybridizations with labeled amplified cDNA did not produce any hybridization signals at all (Table 21). The single hybridization signal that did show hybridization (slide #2811-15) obtained 24% signals, no unspecific binding to negative controls, but a hybridization pattern

that was different from the conventional cDNA hybridizations. Besides, hybridization of genomic DNA after amplification showed unspecific binding in one of out of two runs.

Table 21. Hybridization with amplified and directly labeled cDNA

Slide	Template	RNA enrichment	Reverse transcription	cDNA amplification	Restriction digestion	Purification	Signals detected
#2811-04	DD18	✓	✓	✓		✓	0%
#2811-05	DD18	✓	✓	✓			0%
#2811-12	DD18	✓	✓	✓		✓	0%
#2811-14	DD18	✓	✓	✓		✓	0%
#2811-15	RNA-TRI	✓	✓	✓	✓	✓	24%
#2811-16	RNA-TRI	✓	✓	✓	✓	✓	0%
#2811-17	RNA-easy	✓	✓	✓	✓	✓	0%
#2811-18	RNA-easy	✓	✓	✓	✓	✓	0%
#2811-19	gDNA			✓	✓	✓	99%
#2811-20	gDNA			✓	✓	✓	99%

A **B**

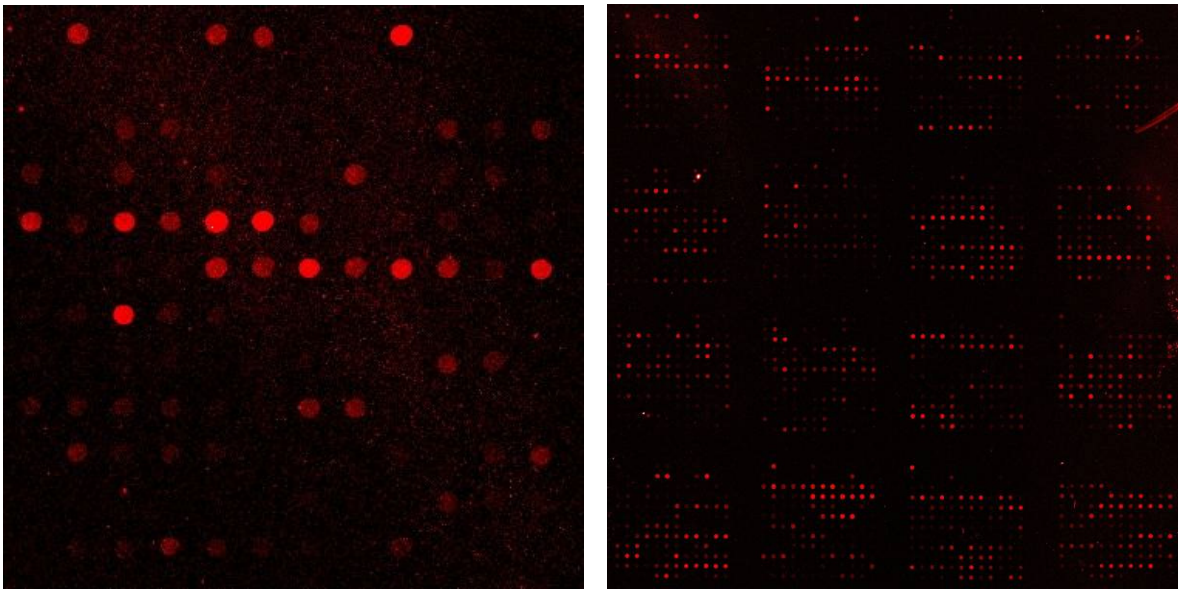

Figure 18. Hybridization of amplified cDNA on slide #2811-15. Most signals are below the threshold and the hybridization pattern is altered (compare to Figures 12 and 15). (A) Detail of subgrid 1. (B) Detail of block 1.

D.8. Amoeba isolation

D.8.1. Extraction of amoebae on non-nutrient agar

The objective of the experiment was the isolation of amoebae from environmental samples and to culture them under axenic conditions. For this purpose, two different samples were taken in Feldkirch (Vorarlberg), soil from a molehill in a garden (sample A) and sediment from the shores of a quarry lake (sample B). Three replicate extractions were performed for each sample (samples AAI-1 to 3 and BAI-1 to 3). Table 22 shows the six amoeba isolates and the respective outcome of the extraction.

Table 22. Extraction of amoebae on non-nutrient agar

Sample	Amoeba isolate	Result of extraction
Sample A	AAI-1	No axenic growth in liquid media
	AAI-2	No growth on NNA plates with dead *E. coli*
	AAI-3	No growth on NNA plates with dead *E. coli*
Sample B	**BAI-1**	**Axenic culture could be established**
	BAI-2	No growth on NNA plates with dead *E. coli*
	BAI-3	No attachment to the surface of the culture flasks

After the samples were placed on NNA plates seeded with living *E. coli*, amoebae were feeding on the bacteria and multiplying, thereby moving out of the sample. Amoebae could be transferred to new NNA plates seeded with living *E. coli* by cutting out small pieces of agar from the edges of the plates. The transfer of fast growing contaminations, especially fungi, could be successfully avoided. After five rounds of transfer, the living *E. coli* cells were replaced with heat-inactivated *E. coli* cells to cautiously prepare them for axenization. The dead cells, however, were not readily accepted as a food source, and amoebal growth was either substantially slowing down or stopped altogether. Three of the six amoeba isolates did not grow at all on heat-inactivated *E. coli*, namely isolates AAI-2, AAI-3 and BAI-2. From the other three extractions, three different types of amoebae could be separated on non-nutrient agar plates and were subsequently transferred to cell culture flasks.

When examined under the light microscope, two of them phenotypically resembled *Acanthamoeba* species (AAI-1 and BAI-1) while the third (BAI-3) was phenotypically similar to *Naegleria* species. All three survived the hydrochloric acid treatment and excysted when incubated in PYG. Isolate AAI-1, however, did not grow at all neither in PYG nor in Chang medium and the culture was eventually discarded. The *Naegleria*-like BAI-3 cells did grow well at first, but only when supplied with heat-inactivated bacterial cells as a food source.

However, after a few days the cultures were lost as the medium was replaced since the amoebae failed to attach to the surface of the culture flasks. BAI-1 cells grew very slowly in PYG and Chang medium, but showed fast growth in PYNFH medium and could be established as axenic culture.

D.8.2. Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) is a method of cultivation independent identification of bacteria using fluorescently labeled oligonucleotide probes and therefore it is particularly suited for detecting the presence of uncultivable intracellular endosymbionts within acanthamoebae.

DAPI-staining (section C.20.3.) of the amoeba isolate BAI-1 could show the presence of intracellular coccoid bacteria (data not shown). For identification, the isolated amoebae were subjected to FISH analysis. A *Chlamydiales*-specific probe (Chls-0523) was used simultaneously with a universal probe targeting most bacteria (EUB338). The bacterial probe generated multiple signals in most amoebal cells (Figure 19), confirming the presence of intracellular bacteria within the isolated amoebae already detected by DAPI-staining. All of these signals matched with the signals obtained with the *Chlamydiales*-specific probe (Figure 19), indicating that all the symbionts belong to the order *Chlamydiales*. No signals were obtained using the nonsense probe NONEUB, the *Betaproteobacteria*-specific probe BET42a, and the *Alphaproteobacteria*-specific probe ALF968.

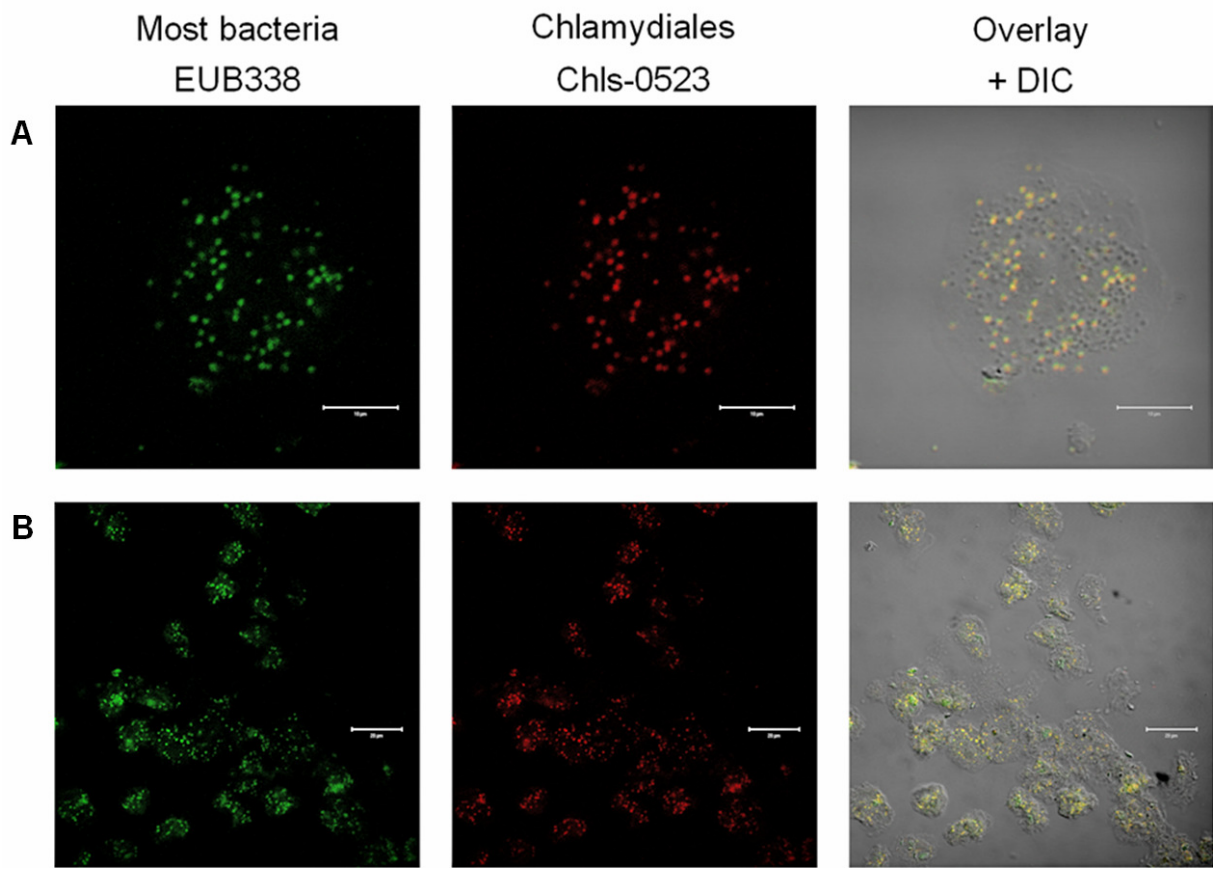

Figure 19. Fluorescence *in situ* hybridization using universal bacterial probe EUB338 (FLUOS, green) and *Chlamydiales*-specific probe Chls-0523 (Cy3, red). Signals of both probes match in an overlay. Differential interference contrast (DIC) is shown for better overview. (A) Detail showing one amoeba cell. Bar indicates 10 µm. (B) Larger detail showing several amoebae. Bar indicates 20 µm. CLSM pictures by K. Aistleitner.

D.8.3. Identification of isolated amoebae and their symbionts

For further characterization of the isolate BAI-1, DNA was isolated from the amoebae and their intracellular symbionts. Using primers suitable for amplification of the 18S rRNA gene of acanthamoebae, a 2197 nucleotide long sequence of the 18S rRNA gene was obtained and BLAST analysis (Altschul et al. 1990) confirmed their affiliation to the genus *Acanthamoeba* as assumed from phenotypical appearance. The 18S rRNA sequence displayed 100% identity (100% query coverage) to *Acanthamoeba castellanii* Neff ATCC 50373 18S rRNA gene sequence. Figure 20 shows the position of amoeba isolate BAI-1 in a phylogenetic tree and its affiliation to sequence type 4 (T4).

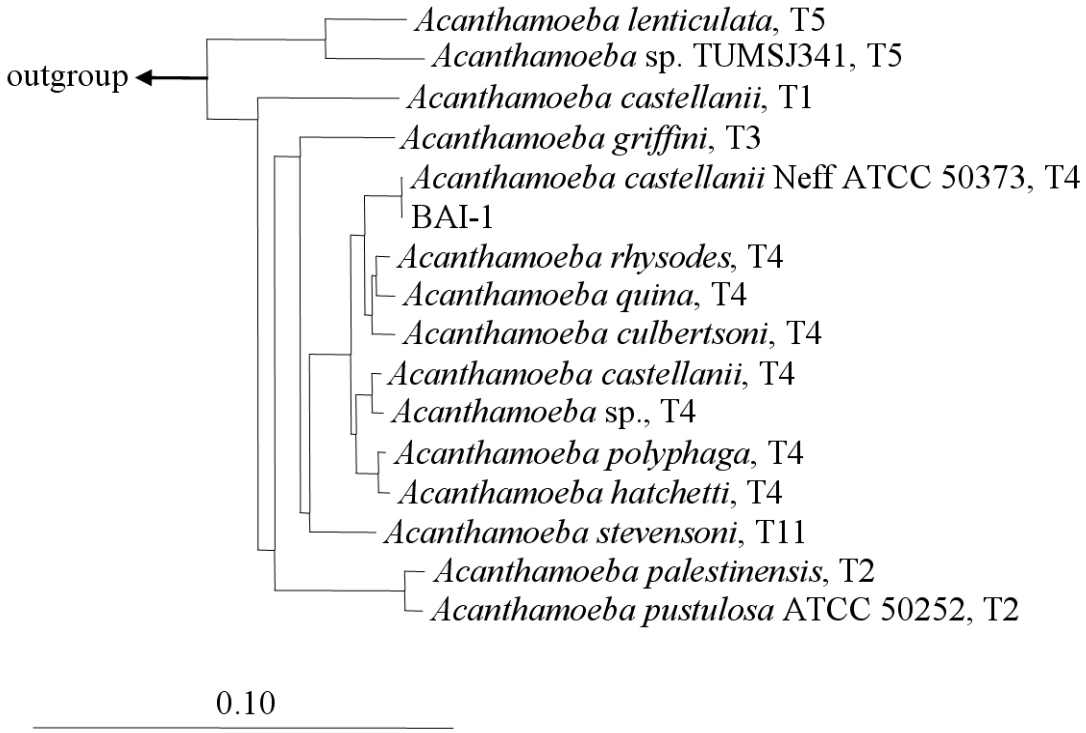

Figure 20. Phylogenetic affiliation of amoeba isolate BAI-1 based on 18S rRNA sequence analysis. Tree calculation was performed using the neighbour joining algorithm (jukes cantor distance correction) in ARB (Ludwig et al. 2004), scale bar represents 10% estimated evolutionary distance.

The 16S rRNA gene sequence of the intracellular symbiont BAI-1 obtained with primers specific for the order *Chlamydiales* was 1378 nucleotides in length and showed highest similarities to following 16S rRNA sequences in the database (Table 23):

Table 23. Most similar 16S rRNA sequences found by BLAST analysis

16S rRNA gene sequence	Mismatches	Max identity
Endosymbiont of *Acanthamoeba* sp. UWC22	4	99%
Endosymbiont of *Acanthamoeba* sp. TUME1	8	99%
Neochlamydia sp. CRIB37	13	99%
Neochlamydia hartmannellae	46	96%

Phylogenetic analysis confirmed the similarity inferred affiliation of the BAI-1 symbiont (Figure 21). It belongs to the family *Parachlamydiaceae* and is closely affiliated with the genus *Neochlamydia*.

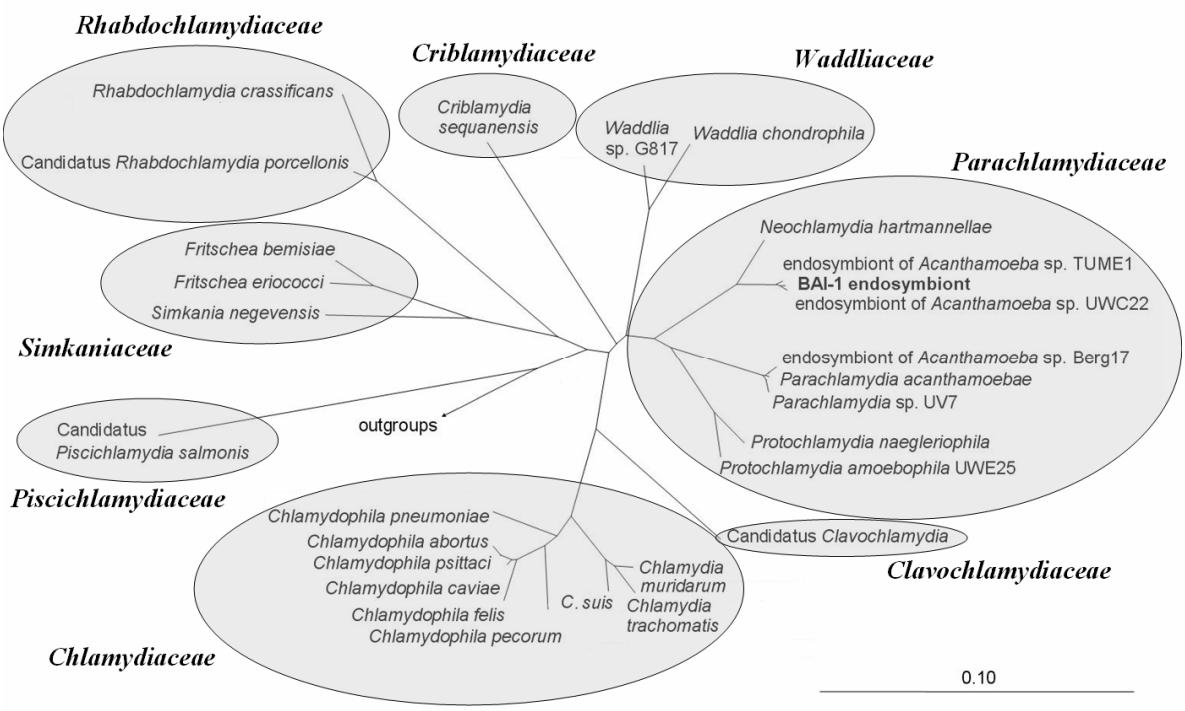

Figure 21. Phylogenetic affiliation of the BAI-1 endosymbiont based on 16S rRNA analysis. Tree calculation was performed using the neighbour joining algorithm (jukes cantor distance correction) in ARB (Ludwig et al. 2004), scale bar represents 10% estimated evolutionary distance.

E. Discussion

E.1. Optimization of the *P. amoebophila* whole genome microarray

E.1.1. Preconditions

The availability of the complete genome sequence of the obligate intracellular symbiont *P. amoebophila* (Horn et al. 2004), a member of the environmental chlamydiae, provided the opportunity to design a whole genome microarray to explore its gene expression during intracellular growth and reveal new insights into the interaction with its host (Haider, unpublished). However, microarray analysis of obligate intracellular bacteria is technically challenging and the procedure was experiencing problems the precise nature of which was not entirely clear. One particular issue being the application of the commercially available two-step random labeling kit CyScribe (Amersham Biosciences) that had to be replaced by another labeling method (section C.9.) due to a sudden breakdown in performance (Haider, unpublished). However, it was still not possible to detect a sufficient number of genes with the microarray. An estimated 61% of genes in the *P. amoebophila* genome were found to be transcribed during infection (Haider, unpublished), a number that is comparable to the proportion of transcribed genes reported for other bacterial species that range between 40% and 76% expressed genes (Talaat et al. 2002; Corbin et al. 2003; Guina et al. 2003; Wei et al. 2006). For this reason, detection of around 60% of the genes was set as a target for the optimization process. Initially, however, the number of genes that could be detected was only 9% (section D.7.1.).

E.1.2. Application of commercially available hybridization buffers

To solve this problem, two different commercially available hybridization buffers were obtained and tested, but without success (section D.7.2.). They both produced very high hybridization signals, but did so at the expense of specificity and it was not possible to obtain strong and specific hybridization signals by increasing the stringency during hybridization and washing. PerfectHyb Hybridization Buffer (Sigma) generated very high signal intensities, but some of the negative controls were among the strongest signals and the hybridization pattern was completely changed in comparison to all previous hybridizations. SlideHyb Glass Array Hybridization Buffer #1 (Ambion) preserved the usual hybridization pattern, but the signal intensities were lower compared to PerfectHyb hybridizations and unspecific binding occurred nevertheless.

E.1.3. Optimizing hybridization with aminoallyl labeled cDNA

A major focus of this study was the optimization of the labeling method used to prepare the samples for hybridization. An indirect two-step labeling strategy (section C.9.) was employed to produce cDNA with incorporated aminoallyl-dUTPs to which a fluorescent dye was coupled in a second step. During optimization of the labeling procedure (section D.3.) it was found that the labeling efficiency was increased by using two purification columns for one labeling reaction in order not to exceed the maximum capacity. Progress in hybridization optimization could be made by increasing the amount of labeled cDNA that was used for hybridization, raising the number of genes that could be detected to 41% (section D.7.4.). More headway was made by adjusting the frequency of incorporation (FOI) of the fluorescent dye into the cDNA (D.7.5.). It is generally recommended to use FOI values between 20 and 50 (incorporation of 20 to 50 labeled nucleotides per 1000 nucleotides of cDNA) for successful hybridizations, since lower or higher FOIs might generate weak signals upon hybridization due to poor incorporation and quenching effects between fluorophores, respectively (GE Healthcare 2008). The FOI can be adjusted by altering the composition of the nucleotide mix used for reverse transcription. The amount of aa-dUTP, to which the fluorescent dye is coupled after reverse transcription, in proportion to the amount of dTTP determines the dye incorporation. Using the preliminary nucleotide mix that contained dTTP and aa-dUTP in a ratio of 1:6.25, the FOIs were much higher than the recommended values. After a few experiments with different nucleotide mixes, the nucleotide mix containing equal concentrations of dTTP and aa-dUTP was found to give the best results with an average FOI of 33. With this nucleotide mix, good hybridization results with up to 62% detected signals could be obtained. However, the results were not easily reproducible and sometimes the hybridizations did not work for no obvious reason. One of the technical issues that seemed to be responsible for low signal-to-noise ratios in a number of hybridizations was the varying quality of the different slide spotting series that for some reason were not equally well suitable for hybridization (Tables 13 and 14, Figure 9). An influence of different reduction times in sodium borohydride was assumed, but could not be confirmed using poly-dTTP hybridization (Figure 10).

E.1.4. Normalization using genomic DNA

Nevertheless, it was attempted to normalize the cDNA hybridizations in order to obtain quantitative transcriptional data about an asynchronous culture of *P. amoebophila*. The normalization procedure to be used was most similar to the genomic normalization procedure described in Talaat et al. 2002 and is based on the comparison of gene expression levels to signals generated from genomic DNA. Genomic DNA was found to be a reliable universal standard for gene expression measurements, since all genes are represented with constant copy number (Pollack et al. 1999; Talaat et al. 2002; Weil et al. 2002; Williams et al. 2004; Gadgil et al. 2005). Genomic DNA hybridization performed well, all the spots with the exception of the negative controls and occasional spotting failures gave signals above the threshold. The number of detected genes was always ≥99%. When hybridized simultaneously with Cy3-labeled genomic DNA, however, the Cy5-labeled cDNA never generated more than 26% signals. Generally, the signals obtained with cDNA were approximately 2.5 times lower than signals obtained from genomic DNA hybridization, contradictory to a previous report stating that most RNA samples generate higher signals than genomic DNA samples due to the presence of multiple transcripts per gene (Talaat et al. 2002). The poor performance of the cDNA hybridization during normalization, however, could not be attributed to a detrimental effect of the additional genomic DNA, since only marginally more signals were generated in comparable cDNA only hybridizations.

E.1.5. Bacterial RNA enrichment

The good performance of the microarray when used with genomic DNA sharply contrasts to the problems that were experienced when cDNA samples were analyzed and indicates that the preparation of the RNA samples might be of fundamental importance. Actually, the extraction of adequate quantities of intact bacterial mRNA is considered to be the most important factor for a successful application of microarray technology (Mangan et al. 2002). In this study, asynchronous cultures of amoebae infected with *P. amoebophila* were used to isolate large quantities of a mixture of bacterial and amoebal RNA. The actual fraction of mRNA derived from the chlamydial endosymbiont, however, might still be present in concentrations too low for successful analysis and might be subject to fast degradation processes. Indeed, bacterial mRNA constitutes only a marginal proportion of the total RNA, since generally only 3% of the entire RNA content of a cell is mRNA, the rest being mainly rRNA and tRNAs (Alberts 1994). Furthermore, in case of intracellular symbionts, the amount of eukaryotic host RNA by far exceeds the amount of bacterial RNA, as shown for *Rickettsia prowazekii*, where

rickettsial RNA makes up less than 10% of the total RNA extracted from rickettsia-infected host cells (Audia et al. 2008). By default, only total RNA can be labelled by random priming, because specific labeling of mRNA is not possible since bacterial mRNA lacks polyadenylation. This fact, however, can be taken advantage of by using the poly(A) tail that is characteristic for eukaryotic mRNA to selectively remove these sequences from a complex host-bacteria RNA mixture. The MICROB*Enrich* kit employs magnetic beads, derivatized with oligonucleotides that hybridize not only to the polyadenylated 3' ends of eukaryotic mRNA but also to 18S and 28S rRNAs, and was used to enrich the total RNA samples extracted from infected amoebae in chlamydial RNA. Hybridization with enriched RNA, however, did not lead to an observable improvement of the hybridization. When raising the amount of enriched cDNA used for hybridization for some reason generated unspecific binding to the negative controls, the method was put aside until the application of a linear amplification technique promised a new way to benefit from bacterial RNA enrichment.

E.1.6. cDNA amplification

The GenomiPhi DNA Amplification Kit utilizes the bacteriophage Phi29 DNA polymerase to linearly amplify DNA templates during a strand displacement reaction that is highly accurate due to the polymerase's proofreading activity (Lizardi et al. 1998; Dean et al. 2001). The kit had been tested for use in transcriptional microarray analyses of low abundance prokaryotic RNA sources and was found to provide linear amplification with equivalent results in terms of relative messenger abundance as those obtained by conventional direct reverse-transcription (Francois et al. 2007) and has already been used successfully in transcriptional gene expression analysis of intracellular bacteria (Garzoni et al. 2007; Renesto et al. 2008). In this study, the GenomiPhi DNA amplification Kit was applied without success (D.7.7.). Reasonable hybridization results – although in one case showing unspecific binding – were obtained with genomic DNA control amplifications but not when cDNA was used as a template. Only one out of eight hybridizations with amplified cDNA showed any hybridization signals at all and they were not particularly promising as only 24% of genes were detected and the hybridization pattern was altered to such a degree that the fact that the cDNA originated from a different RNA isolation experiment might be not sufficient to serve as an explanation (Figure 18).

E.1.7. Conclusion

In conclusion, progress could be made in the optimization of the microarray procedure and successful cDNA hybridizations were performed, in which 55% - 62% ($\bar{x}$ =59%) of genes were found to be expressed during growth of *P. amoebophila* in asynchronous infection. Unfortunately, the procedure did not show stable performance, as results were often not readily reproducible and quantitation using genomic DNA for normalization was not successful. It is tempting to speculate that the achievement of quantitative normalized results is just a matter of time and effort and might soon be accomplished, but more energy has to be spent on optimizing the reproducibility and solving technical issues until the microarray can be used for more sophisticated expression profiling studies.

E.2. Amoeba isolation

Another aim of this thesis was the isolation of amoebae from environmental samples and to test them for the presence of endosymbionts. Environmental samples were taken (section C.20.1.) and amoebae isolated from sediment of the shore of a quarry lake could successfully be established as an axenic culture (section D.8.1). Using DAPI-staining (section C.20.3.) a great amount of intracellular bacteria of coccoid shape could be detected within the amoebal cells, equally dispersed in the cytoplasm. Due to their coccoid morphology it was speculated that the symbionts might be chlamydiae, since an estimated 24% of *Acanthamoeba* species harbour bacterial endosymbionts, 20% gram negative rods and 5% gram negative cocci (Fritsche et al. 1993), and the latter were shown to belong to the order *Chlamydiales* (Fritsche et al. 2000). This hypothesis was supported by FISH analysis (section C.20.4.) that identified all intracellular bacteria as members of the order *Chlamydiales* by the use of general bacterial (EUB-338) and *Chlamydiales*-specific (Chls-0523) probes (section D.8.2.). To confirm these results and to phylogenetically classify both host amoebae and symbionts, sequences of the respective small subunit ribosomal RNA genes were obtained by DNA isolation, PCR amplification and direct sequencing (section D.8.3.). BLAST analysis could identify the host amoebae as *Acanthamoeba castellanii* since the 18S rRNA gene showed 100% sequence identity with the *A. castellanii* Neff ATCC 50373 strain. When the 16S rRNA gene sequence of the bacterial symbionts was compared to the sequences in the NCBI database, they showed a similarity of up to 99% to *Neochlamydia*-like symbionts of *Acanthamoebae* species (Table 23). Furthermore, with the obtained sequences it was possible to use the ARB software to align the sequences with a database and calculate phylogenetic trees from these alignments. Unsurprisingly, the 18S rRNA sequence of the isolated amoebae clustered with *A. castellanii* Neff within the T4 sequence type of *Acanthamoebae* species (Figure 20). The T4 sequence type is by far the largest of the 12 *Acanthamoeba* lineages described so far (sequence types T1-T12) and contains six closely related nominal species, including almost all isolates from amoebic keratitis (Gast et al. 1996; Stothard et al. 1998). The 16S rRNA tree showed the affiliation of the BAI-1 symbiont with the family *Parachlamydiaceae*, more specifically with a branch comprising species of the genus *Neochlamydia* (Figure 21). The *Parachlamydiaceae* are the family of environmental chlamydiae represented by the highest number of identified organisms. It contains three genera, *Parachlamydia*, *Neochlamydia*, and *Protochlamydia*, members of which are almost always associated with free-living amoebae (Amann et al. 1997; Horn et al. 2000; Casson et al. 2008; Kuo et al. 2008). The most similar (99% sequence

identity) and most closely related sequences belong to endosymbionts of *Acanthamoeba* sp. UWC22 and TUME1 that were recovered from infected corneal tissue (UWC22) and municipal sewage sludge from Munich, Germany (TUME1) (Fritsche et al. 2000). The closest relative that has been thoroughly described as a species is *Neochlamydia hartmannellae* that was found living intracellularly in the amoeba *Hartmannella vermiformis* (Horn et al. 2000). Affiliation with the genus *Neochlamydia* is supported by the high sequence similarity (99%) to *Neochlamydia* sp. CRIB37, a strain isolated from a sediment sample of a drinking water treatment plant (Corsaro et al. 2009). The successful isolation of a *Neochlamydia*-like endosymbiont once again shows the ubiquitous distribution of environmental chlamydiae.

F. Summaries

F.1. Summary

Chlamydiae are obligate intracellular bacteria that share a unique biphasic developmental cycle (Ward 1988; Moulder 1991; Abdelrahman et al. 2005). They were long viewed as a phylogenetically well-separated group comprising a few closely related pathogenic species (Schachter 1999), until the recent discovery of a huge chlamydial diversity in the environment (reviewed in Horn 2008). The availability of the complete genome sequence of the environmental chlamydia *Protochlamydia amoebophila* UWE25 (Horn et al. 2004) provided the opportunity to design a whole genome microarray to explore its gene expression during intracellular growth and reveal new insights into the interaction with its host (Haider, unpublished). The aim of this study was the optimization of this whole-genome microarray and the establishment of a procedure suitable for studying the gene expression of *P. amoebophila*. It is shown that optimization of the aminoallyl labeling procedure lead to successful hybridization of *P. amoebophila* cDNA and detection of 59% expressed genes during asynchronous infection. Unfortunately, the hybridization results were not easily reproducible and quantitation of gene expression by normalization with genomic DNA was not possible. A recurring problem were low signal intensities upon hybridization. Sophisticated commercially available methods were employed to solve this issue, but without success. The total RNA samples were enriched in bacterial RNA by removal of eukaryotic host RNA using MICROB*Enrich* (Ambion), yet the enriched samples did not show an improved performance. In order to increase signal intensities, the linear amplification method GenomiPhi (GE Healthcare) was used for cDNA amplification, also without satisfactory results. In addition, the two commercially available hybridization solutions PerfectHyb (Sigma) and SlideHyb (Ambion) were unsuccessfully tested. In conclusion, progress could be made in optimization of the microarray procedure and successful cDNA hybridizations were performed, but more energy has to be spent on improving the reproducibility and solving technical issues until the microarray can be used for more sophisticated expression profiling studies.

This study also reports on the successful isolation of the *Neochlamydia*-like symbiont BAI-1 living intracellularly in an *Acanthamoeba castellanii* strain extracted from sediment from the shores of a quarry lake.

F.2. Zusammenfassung

Chlamydien sind obligat intrazelluläre Bakterien, die sich durch einen einzigartigen zweiphasigen Entwicklungszyklus auszeichnen (Ward 1988; Moulder 1991; Abdelrahman et al. 2005). Lange Zeit wurden sie als phylogenetisch gut abgesonderte Gruppe betrachtet, bestehend aus einigen wenigen nah verwandten pathogenen Arten (Schachter 1999), bis zur kürzlichen Entdeckung einer enormen Diversität an Chlamydien in der Umwelt (zusammengefasst in Horn 2008). Die Verfügbarkeit einer kompletten Genomsequenz der Umweltchlamydie *Protochlamydia amoebophila* UWE25 (Horn et al. 2004) ermöglichte die Entwicklung eines globalen Microarrays, um die Genexpression von *P. amoebophila* während des intrazellulären Wachstums zu erforschen und neue Erkenntnisse über die Interaktionen mit ihrem Wirt zu erzielen (Haider, unveröffentlicht). Das Ziel dieser Diplomarbeit war die Optimierung dieses Microarrays und die Etablierung eines Verfahrens, das es erlaubt die Genexpression von *P. amoebophila* zu untersuchen. Es wird gezeigt, dass die Optimierung des Aminoallyl-Labeling-Protokolls zu erfolgreichen Hybridisierungen der *P. amoebophila* cDNA und zur Detektion von 59% exprimierten Genen während einer asynchronen Infektion führte. Unglücklicherweise waren die Hybridisierungsergebnisse nicht leicht zu reproduzieren und die Quantifizierung der Genexpression durch Normalisierung mit genomischer DNA war nicht möglich. Ein wiederkehrendes Problem waren niedrige Signalintensitäten bei der Hybridisierung. Um dieses Problem zu lösen, wurden neben den kommerziell erhältlichen Hybridisierungslösungen PerfectHyb (Sigma) und SlideHyb (Ambion) auch komplexe kommerziell erhältliche Methoden angewandt, allerdings ohne Erfolg. So wurde MICROB*Enrich* (Ambion) verwendet, um den Anteil an bakterieller RNA in den Gesamt-RNA-Proben durch Entfernung der eukaryotischen Wirts-RNA zu erhöhen, doch die Anreicherung hatte keinen positiven Effekt auf die nachfolgende Hybridisierung. Um die Signalintensitäten zu verstärken, wurde auch die lineare Amplifikationsmethode GenomiPhi (GE Healthcare) zur cDNA Amplifizierung getestet, jedoch ebenfalls ohne befriedigende Ergebnisse. Zusammenfassend wurden Fortschritte bei der Optimierung des verwendeten Verfahrens erzielt und erfolgreiche cDNA Hybridisierungen durchgeführt. Bis der Microarray für anspruchsvolle Genexpressionsstudien verwendet werden kann, sind jedoch neben der Lösung einiger technischer Probleme noch weitere Anstrengungen zur Verbesserung der Reproduzierbarkeit notwendig.

Des Weiteren beschreibt diese Studie die erfolgreiche Isolierung des *Neochlamydia*-ähnlichen, intrazellulären Symbionten BAI-1 innerhalb eines *Acanthamoeba castellanii* Stammes, der aus dem Sediment vom Ufer eines Baggersees extrahiert wurde.

G. Appendix

Supplementary tables:

Table S1. Isolated RNA (before DNase digestion)

Sample	Concentration*	Volume	RNA amount	Ratio 260/280	Ratio 260/230
RNA 01	4509.5 ng/µL	50 µL	225 µg	1.60	1.31
RNA 02	3657.4 ng/µL	50 µL	183 µg	1.96	1.08
RNA 03	4579.4 ng/µL	50 µL	229 µg	1.53	1.34
RNA 04	4627.2 ng/µL	50 µL	231 µg	1.40	1.20
RNA 05	4624.4 ng/µL	50 µL	231 µg	1.31	1.24
RNA 06	1955.6 ng/µL	50 µL	98 µg	2.06	0.92
RNA 07	4603.3 ng/µL	50 µL	230 µg	1.41	1.23
RNA 08	4717.6 ng/µL	50 µL	236 µg	1.34	1.24
RNA 09	3880.9 ng/µL	50 µL	194 µg	1.81	1.06
RNA 10	4162.0 ng/µL	50 µL	208 µg	1.92	1.39
RNA 11	4773.0 ng/µL	50 µL	239 µg	1.05	1.17
RNA 12	4706.4 ng/µL	50 µL	235 µg	1.31	1.16

* Most values pass the detection limit of 3,700 ng/µL, the concentration measurements are therefore inaccurate and probably underestimate the actual concentration.

Table S2. Isolated RNA (after DNase digestion)

Sample	RNA*	Concentration	Volume	RNA amount	Ratio 260/280	Ratio 260/230
DD01	22.0 µL RNA 01	2085.2 ng/µL	100 µL	209 µg	2.14	2.33
DD02	22.0 µL RNA 01	1684.5 ng/µL	100 µL	168 µg	2.14	2.42
DD03	4.5 µL RNA 01 21.5 µL RNA 02	2039.5 ng/µL	100 µL	204 µg	2.14	2.40
DD04	27.0 µL RNA 02	1685.1 ng/µL	100 µL	169 µg	2.14	2.40
DD05	22.0 µL RNA 03	1753.6 ng/µL	100 µL	175 µg	2.13	2.37
DD06	22.0 µL RNA 03	1667.2 ng/µL	100 µL	167 µg	2.12	2.35
DD07	22.0 µL RNA 04	1639.4 ng/µL	100 µL	164 µg	2.12	2..36
DD08	22.0 µL RNA 04	1523.9 ng/µL	100 µL	152 µg	2.11	2.37
DD09	22.0 µL RNA 05	1776.7 ng/µL	100 µL	178 µg	2.12.	2.34
DD10	22.0 µL RNA 05	1035.0 ng/µL	100 µL	104 µg	2.09	2.31
DD11	48,5 µL RNA 06	1376.5 ng/µL	100 µL	138 µg	2.11	2.32
DD12	22.0 µL RNA 07	1763.9 ng/µL	100 µL	176 µg	2.13	2.38
DD13	22.0 µL RNA 07	1969.1 ng/µL	100 µL	197 µg	2.13	2.37
DD14	4.5 µL RNA 03 4.5 µL RNA 04 4.5 µL RNA 05 4.5 µL RNA 07	3643.9 ng/µL	100 µL	364 µg	2.06	2.26
DD15	21.0 µL RNA 08	1509.4 ng/µL	100 µL	151 µg	2.11	2.37
DD16	21.0 µL RNA 08	1658.1 ng/µL	100 µL	166 µg	2.12	2.38
DD17	24.0 µL RNA 09	2000.6 ng/µL	100 µL	200 µg	2.13	2.33
DD18	24.5 µL RNA 09	1928.9 ng/µL	100 µL	193 µg	2.13	2.36
DD19	24.0 µL RNA 10	1516.5 ng/µL	100 µL	152 µg	2.13	2.34
DD20	24.5 µL RNA 10	1558.0 ng/µL	100 µL	156 µg	2.12	2.37
DD21	21.0 µL RNA 11	2020.9 ng/µL	100 µL	202 µg	2.11	2.39

DD22	21.0 µL RNA 11	1891.3 ng/µL	100 µL	189 µg	2.11	2.41
DD23	21.0 µL RNA 12	2188.7 ng/µL	100 µL	219 µg	2.13	2.39
DD24	21.0 µL RNA 12	1266.5 ng/µL	100 µL	127 µg	2.08	2.37
DD25	6.5 µL RNA 08 6.5 µL RNA 11 6.5 µL RNA 12	2470.1 ng/µL	100 µL	247 µg	2.12	2.39

*The RNA samples (Table S1) were distributed to in order not to exceed the maximum amount of RNA per digestion reaction.

Table S3. MICROBEnrich results of 10 performances

Name of RNA sample	total RNA	enriched RNA
LK2	55 µg	5.3 µg (9.6 %)
LK2	100 µg	18.7 µg (18.7%)
LK2	100 µg	29.1 µg (29.1%)
K2	50 µg	10.8 µg (21.6%)
DD03	100 µg	26.1 µg (26.1%)
DD18	50 µg	3.7 µg (7.4%)
DD14	50 µg	3.8 µg (7.6%)
RNA-TRI	50 µg	7.7 µg (15.4%)
RNA-easy	50 µg	6.2 µg (12.4%)

Table S4. Initial labelings with preliminary nucleotide mix

RNA template	Template amount	Fluorophore	cDNA (µg)	Cy3/5 (pmol)	FOI
LK2 (total RNA)	20 µg	Cy5	2.8	514	60
LK2 (total RNA)	20 µg	Cy5	2.5	470	62
LK2 (total RNA)	20 µg	Cy5	1.2	194	55
LK2 (total RNA)	20 µg	Cy5	1.5	249	57
LK2 (total RNA)	20 µg	Cy5	1.7	290	55
LK2 (total RNA)	20 µg	Cy5	1.6	288	58
LK2 (total RNA)	20 µg	Cy3	2.8	516	60
LK2 (total RNA)	20 µg	Cy3	2.7	544	66
LK2 (total RNA)	20 µg	Cy3	3.2	692	70
LK2 (total RNA)	20 µg	Cy3	3.0	668	73
LK2 (total RNA)	20 µg	Cy3	2.7	588	70
K1 (total RNA)	20 µg	Cy3	1.8	480	87
K1 (total RNA)	20 µg	Cy3	1.7	412	79
K1 (total RNA)	20 µg	Cy3	1.6	396	78
K1 (total RNA)	20 µg	Cy3	1.9	452	77
K1 (total RNA)	20 µg	Cy3	1.6	372	73
LK2 (enriched RNA)	5 µg	Cy3	0.9	180	67
LK2 (enriched RNA)	5 µg	Cy5	2.2	130	46
LK2 (enriched RNA)	5 µg	Cy5	1.9	115	41
LK2 (enriched RNA)	10 µg	Cy5	3.3	142	25
LK2 (enriched RNA)	5 µg	Cy3	2.0	352	58
LK2 (enriched RNA)	5 µg	Cy3	1.7	312	61
LK2 (enriched RNA)	5 µg	Cy3	1.7	320	62
LK2 (enriched RNA)	5 µg	Cy3	1.8	344	62
LK2 (enriched RNA)	5 µg	Cy3	1.4	272	61

*RNA templates for the initial labeling were isolated by Susanne Haider.

Table S5. Second labeling series with preliminary nucleotide mix

RNA template	Template amount	Fluorophore	cDNA (µg)	Cy3/5 (pmol)	FOI
DD01 (total RNA)	10 µg	Cy5	1.6	310	62
DD01 (total RNA)	10 µg	Cy5	1.5	262	55
DD01 (total RNA)	10 µg	Cy5	1.6	254	52
DD01 (total RNA)	10 µg	Cy5	1.5	235	51
DD01 (total RNA)	10 µg	Cy5	1.4	278	64
DD01 (total RNA)	10 µg	Cy5	1.5	314	67
DD01 (total RNA)	10 µg	Cy3	3.3	812	80
DD01 (total RNA)	10 µg	Cy3	2.7	644	78
DD02 (total RNA)	10 µg	Cy5	1.6	310	63
DD02 (total RNA)	10 µg	Cy5	1.2	228	62
DD02 (total RNA)	10 µg	Cy5	1.4	257	58
DD02 (total RNA)	10 µg	Cy5	1.8	290	54
DD02 (total RNA)	10 µg	Cy5	1.3	242	62
DD02 (total RNA)	10 µg	Cy5	1.2	206	54
DD02 (total RNA)	10 µg	Cy5	1.3	240	62
DD02 (total RNA)	10 µg	Cy5	1.5	288	63
DD02 (total RNA)	10 µg	Cy3	1.6	340	70
DD02 (total RNA)	10 µg	Cy3	1.3	268	65
DD02 (total RNA)	10 µg	Cy3	1.3	224	57
DD02 (total RNA)	10 µg	Cy3	1.3	260	63
DD02 (total RNA)	10 µg	Cy3	1.3	280	72
DD02 (total RNA)	10 µg	Cy3	1.2	268	71
DD03 (enriched RNA)	5 µg	Cy5	1.8	286	53
DD03 (enriched RNA)	5 µg	Cy5	2.2	461	69
DD03 (enriched RNA)	16 µg	Cy5	2.7	593	70

Table S6. Optimization of the nucleotide mix

RNA template	Template amount	Fluorophore	Ratio dTTP:aa-dUTP	cDNA (µg)	Cy3/5 (pmol)	FOI
DD04	10 µg	Cy3	1:2	2.0	208	34
DD04	10 µg	Cy3	1:2	2.0	208	34
DD04	10 µg	Cy3	1:2	2.1	472	72
DD04	10 µg	Cy3	1:2	2.0	460	76
DD04	10 µg	Cy3	1:2	2.2	456	67
DD04	10 µg	Cy3	1:2	2.1	424	67
DD04	7 µg	Cy3	1:2	1.3	228	56
DD04	7 µg	Cy3	1:2	1.3	252	64
DD04	7 µg	Cy3	1:2	1.3	228	58
DD04	5 µg	Cy3	1:2	1.1	220	66
DD04	5 µg	Cy3	1:2	1.1	216	62
DD04	5 µg	Cy3	1:2	1.2	212	60
DD04	5 µg	Cy3	1:2	1.2	204	55
DD04	10 µg	Cy3	1:2	2.0	420	67
DD04	10 µg	Cy3	1:2	2.3	428	61
DD04	10 µg	Cy3	1:2	1.2	224	58
DD04	10 µg	Cy3	1:2	1.3	224	57
DD05	10 µg	Cy3	1:1	1.2	208	34
DD05	10 µg	Cy3	1:1	1.3	208	33
DD05	10 µg	Cy3	2:1	0.8	84	32
DD05	10 µg	Cy3	2:1	1.1	84	26

Table S7: Further optimization of nucleotide mix for Cy3

RNA template	Template amount	Fluorophore	Ratio dTTP:aa-dUTP	cDNA (µg)	Cy3/5 (pmol)	FOI
DD05	10 µg	Cy3	1:1	1.3	164	40
DD05	10 µg	Cy3	1:1	1.2	160	43
DD05	10 µg	Cy3	1:1	1.8	256	45
DD05	10 µg	Cy3	1:1	2.2	284	43
DD05	10 µg	Cy3	1:1	1.6	224	44
DD05	10 µg	Cy3	1:1	1.5	196	42
DD05	10 µg	Cy3	1:1	1.1	144	42
DD05	10 µg	Cy3	1:1	1.2	168	45
DD05	10 µg	Cy3	1:1	1.4	168	39
DD05	10 µg	Cy3	1:1	1.4	152	34
DD06	10 µg	Cy3	1:1	2.6	372	47
DD06	10 µg	Cy3	1:1	2.5	376	50
DD06	10 µg	Cy3	1:1	2.1	332	52
DD06	10 µg	Cy3	1:1	2.6	404	50
DD09	10 µg	Cy3	1:1	2.3	296	42
DD09	10 µg	Cy3	1:1	1.9	272	46
DD09	10 µg	Cy3	1:1	1.8	260	46
DD09	10 µg	Cy3	1:1	1.7	220	41
DD09	10 µg	Cy3	1:1	1.0	164	51
DD09	10 µg	Cy3	1:1	0.9	132	47
DD09	10 µg	Cy3	1:1	0.9	164	57
DD09	10 µg	Cy3	1:1	0.9	144	50
DD17	10 µg	Cy3	2:1	0.7	56	26
DD17	10 µg	Cy3	2:1	0.7	40	18
DD17	10 µg	Cy3	2:1	0.6	52	29
DD17	10 µg	Cy3	2:1	0.6	52	29
DD23	10 µg	Cy3	4:3	1.4	192	45
DD23	10 µg	Cy3	4:3	1.0	132	43
DD23	10 µg	Cy3	4:3	1.2	144	40
DD23	10 µg	Cy3	4:3	1.5	188	41
DD23	10 µg	Cy3	4:3	1.5	192	42
DD23	10 µg	Cy3	4:3	1.0	140	45
DD23	20 µg	Cy3	3:2	7.2	608	27
DD21	20 µg	Cy3	3:2	7.5	656	28
DD21	20 µg	Cy3	4:3	6.2	704	37

Table S8. Aminoallyl labeling in Cy5

RNA template	Template amount	Fluorophore	cDNA (µg)	Cy3/5 (pmol)	FOI
DD06	10 µg	Cy5	1.3	134	34
DD06	10 µg	Cy5	1.0	113	35
DD06	10 µg	Cy5	1.7	180	35
DD06	10 µg	Cy5	2.0	209	35
DD06	10 µg	Cy5	2.1	199	31
DD06	10 µg	Cy5	1.8	192	34
DD06	10 µg	Cy5	1.8	197	36
DD06	10 µg	Cy5	1.9	216	36
DD07	10 µg	Cy5	0.8	67	27
DD07	10 µg	Cy5	0.8	72	28

DD07	10 µg	Cy5	0.7	50	22
DD07	10 µg	Cy5	0.8	74	29
DD07	10 µg	Cy5	0.8	89	34
DD07	10 µg	Cy5	0.9	77	29
DD07	10 µg	Cy5	0.8	70	27
DD07	10 µg	Cy5	0.7	60	27
DD07	10 µg	Cy5	0.4	46	33
DD07	10 µg	Cy5	0.5	48	31
DD07	10 µg	Cy5	0.7	60	28
DD07	10 µg	Cy5	0.5	48	31
DD09	10 µg	Cy5	1.8	194	35
DD09	10 µg	Cy5	1.8	204	37
DD09	10 µg	Cy5	2.1	254	40
DD09	10 µg	Cy5	1.9	199	35
DD09	10 µg	Cy5	1.8	192	35
DD09	10 µg	Cy5	1.8	185	34
DD13	10 µg	Cy5	1.6	185	37
DD13	10 µg	Cy5	1.7	194	38
DD13	10 µg	Cy5	1.7	192	37
DD13	10 µg	Cy5	1.6	190	38
DD13	10 µg	Cy5	1.6	163	34
DD13	10 µg	Cy5	1.8	194	35
DD13	10 µg	Cy5	1.5	163	36
DD13	10 µg	Cy5	1.5	158	34
DD17	10 µg	Cy5	2.1	250	39
DD17	10 µg	Cy5	2.3	286	41
DD17	10 µg	Cy5	2.0	228	37
DD17	10 µg	Cy5	2.4	290	39
DD17	10 µg	Cy5	2.1	252	40
DD17	10 µg	Cy5	2.2	245	37
DD17	10 µg	Cy5	1.9	230	39
DD17	10 µg	Cy5	2.3	276	39
DD17	10 µg	Cy5	3.4	341	33
DD17	10 µg	Cy5	3.0	307	33
DD23	10 µg	Cy5	1.8	173	31
DD23	10 µg	Cy5	1.6	154	31
DD23	10 µg	Cy5	1.4	137	31
DD23	10 µg	Cy5	1.6	151	31
DD23	10 µg	Cy5	1.8	175	32
DD23	10 µg	Cy5	1.4	139	31
DD23	10 µg	Cy5	1.6	156	33
DD23	10 µg	Cy5	1.3	115	29
DD23	10 µg	Cy5	1.5	132	29
DD23	10 µg	Cy5	1.5	137	30
DD23	10 µg	Cy5	1.3	139	34
DD23	10 µg	Cy5	1.4	122	29
DD21	80 µg	Cy5	18.5	1517	27
DD18	80 µg	Cy5	19.9	1978	32

Table S9. Direct labelings of amplified cDNA and gDNA controls

Template	mRNA enrichment	Reverse transcription	cDNA amplification	Restriction digestion	Purification	Fluoro-phore	labelled DNA (µg)*	Cy3 (pmol)*	FOI*
DD18	✓	✓	✓		✓	Cy3	10.4	207	6
DD18	✓	✓	✓		✓	Cy3	9.9	190	6
DD18	✓	✓	✓			Cy3	7.8	147	6
DD18	✓	✓	✓			Cy3	4.8	60	4
DD18	✓	✓	✓		✓	Cy3	13.6	283	7
DD18	✓	✓	✓		✓	Cy3	12.7	173	4
DD18	✓	✓	✓			Cy3	2.0	10	2
RNA-TRI	✓	✓	✓	✓	✓	Cy3	8.1	100	4
RNA-TRI	✓	✓	✓	✓	✓	Cy3	7.8	83	3
RNA-easy	✓	✓	✓	✓	✓	Cy3	4.8	70	5
RNA-easy	✓	✓	✓	✓	✓	Cy3	6.6	77	4
gDNA			✓	✓	✓	Cy3	10.3	143	5
gDNA			✓	✓	✓	Cy3	11.9	223	6

* The labeling parameters were calculated analogous to the indirect labeling (C.9.4.) but with a different extinction coefficient (e = 50 µg cm mL^{-1}) for double stranded DNA since amplification generates DNA that is mostly double stranded.

H. Abbreviations

16S rRNA	small ribosomal RNA subunit of prokaryotes
18S rRNA	small ribosomal RNA subunit of eukaryotes
28S rRNA	large ribosomal RNA subunit of prokaryotes
%	percent
°C	degree celsius
A	adenine
A.	*Acanthamoeba*
aa-cDNA	aminoallyl cDNA
aa-dUTP	aminoallyl dUTP
ATCC	American Type Culture Collection
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumine
c	centi (10^{-2})
C	cytosine
C.	*Chlamydia; Chlamydophila*
cDNA	complementary DNA
CLSM	confocal laser scanning microscope
CTAB	cetyl trimethylammonium bromide
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
ddH_2O	double distilled water
DAPI	4'-6-di-amidino-2-phenylindol
DEPC	diethylpyrocarbonate
dGTP	deoxyguanine triphosphate
DIC	differential interference contrast
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphat
dUTP	deoxyuracil triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease

DTT	dithiothreitol
E.	*Escherichia*
EB	elementary body
EDTA	ethylenediaminetetraacetic acid
et al.	et alteri (latine, “and others”)
FISH	fluorescence *in situ* hybridization
FOI	frequency of incorporation
g	gram(s); gravitational constant
gDNA	genomic DNA
h	hour(s)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
L	litre(s)
m	meter(s); milli (10^{-3})
M	molar
min	minute(s)
mol	mole
mRNA	messenger RNA
n	nano (10^{-9})
NNA	non-nutrient agar
nt	nucleotide(s)
ORF	open reading frame
P.	*Protochlamydia*
PAS	Page’s amoebic saline
PCR	polymerase chain reaction
p	pico (10^{-12})
PYG	peptone-yeast-glucose medium
PYNFH	peptone-yeast-nucleic acid-folic acid-hemin medium
RB	reticulate body
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	room temperature; reverse transcriptase
S	Siemens

SDS	sodium dodecyl sulfate
sec	seconds
SNR	signal-to-noise ratio
sp.	species
SPG	sucrose-phosphate-glutamate buffer
SSC	sodium carbonate-sodium citrate buffer
T	thymine
T_a	annealing temperature
TAE	Tris-acetate-EDTA buffer
Taq	thermostable DNA polymerase from *Thermus aquaticus*
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
TSY	trypticase soy broth with yeast extract
U	unit
UV	ultraviolet
vol	volume
$\bar{x}$	arithmetic mean
ε	molar extinction coefficient
μ	micro (10^{-6})

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J. Acknowledgements

This diploma thesis was performed from June 2008 to May 2009 at the Department of Microbial Ecology (DOME) at the University of Vienna under the leadership of Professor Dr. Michael Wagner.

I want to thank all DOME members for the nice working atmosphere and for the great time I had during this year.

My special thanks go to …

… Prof. Michael Wagner for raising my interest in microbial ecology by almost making me believe that waste water treatment is the coolest thing in the world and for providing excellent research conditions.

… Prof. Matthias Horn for giving me the opportunity to do my diploma thesis in his group and for being the most pleasant boss imaginable.

… my supervisor Susi for introducing me to the lab routine, teaching me lots of thing, always having time for me, and for being a good example in never giving up despite all the setbacks.

… Christian, for being the one I could bother with all my little lab-related problems and for always taking time to solve them.

… Karin for paving me the way through university by doing everything just one step ahead of me and for being a great friend too.

… my other L2-lab colleagues, Silvia, Barbara, and Lena, who made working there a real pleasure.

… Ilias for instructing me on all things amoeba isolation and for his help with keeping them alive and well.

J. Acknowledgements

… Andrea for the critical discussions and helpful skype conversations about my work and for her ongoing friendship.

… my flat mate Lukas for all the scientific discussions that make me look beyond the rim of my microbiological teacup and for generally being a nice guy to be around.

… all my friends, especially Urs, Claus, and Reini, who are still there for me even though we hardly ever meet.

… my family for always letting me do whatever I saw fit and for supporting me in far-off Vienna.

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