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Description of two novel and as-yet uncultured  
endosymbionts of *Acanthamoeba* spp.

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# 1. Introduction

## 1.1 Endosymbiosis and other definitions

Symbiosis is ubiquitous in nature and is becoming increasingly recognized as a significant field of study, because of the fundamental and unifying role across ecological interactions, physiological and developmental processes, as well as evolutionary diversification, even the impacts on humans. The term symbiosis originates from the ancient Greek and in the literal sense it describes a “way of life in company”. It is defined as an intimate and long-term association between different dissimilar organisms, whether the outcome of the interactions is beneficial, neutral or harmful for one of the parties involved. In such a symbiosis, the host organism is defined as the provider of resources, while the symbionts are the consumers of those resources and may or may not offer something in return (Ferrière et al., 2007; Leung & Poulin, 2008). According to Campbell’s biological textbook (Campbell & Reece, 1984), symbiotic associations are divided in three distinctive categories: mutualism, commensalism and parasitism. Mutualism is characterized as an interspecies relationship, where host and symbiont directly benefit from each other. In this case both partners have elevated fitness. If the symbiont makes use of the host’s services without affecting it in a neither harmful nor beneficial way, it is considered commensal. A symbiont qualifies as parasitic if it utilizes the host as a resource reducing its fitness. A newer perception of symbiosis points out a continuous gradation and fine lines between mutualism, commensalism and parasitism. It has frequently been argued how this continuum can be highly variable and circumstantial, and how easily such symbiotic associations can switch from one to another in response to the slightest environmental change (Leung & Poulin, 2008).

In contrast to ectosymbiosis, where partners live in close proximity to each other, but still maintain their cellular integrity, endosymbiosis involves the internalization of bacterial organisms by a variety of eukaryotes. Often symbionts directly benefit from nurturing, protection from hostile environments for stable replication and transport and the host in return receives access to unique metabolites, assistance in defense against natural enemies or sometimes enhanced reproduction (Hétériet et al., 2008). Interspecies interactions of both ecto- and endosymbiosis involve a broad variation of obligate and non-obligate or facultative associations. Facultative intracellular symbionts or in terms of microbial ecology secondary symbionts are still capable of growing and replicating outside a host cell; a characteristic feature well known for *Legionella pneumophila*, the causative agent of Legionnaire’s Disease (Horwitz & Silverstein, 1980) or *Listeria monocytogenes*. However, such a facultative association still increases fitness and survival of *Legionella pneumophila*. In contrast to facultative endosymbiosis, obligate associations between two or more species, such as *Coxiella burnetii* are disadvantageous in many ways. They are unable to survive and replicate alone outside of the host and in tight and long-term relationships they lose genetic material (Nikoh et al, 2011; Ochman & Moran, 2001).

## 1.2 Diversity of bacterial endosymbionts in free-living amoebae

Free-living amoebae, such as *Acanthamoeba*, *Hartmannella* and *Naegleria* are ubiquitous unicellular eukaryotes (protozoans), living mostly in the soil, water and the air as well as several anthropogenic ecosystems. Protozoans play a key role in maintaining the variety and amount of microbial species and the diversity of microbial communities (Brown & Barker, 1999; Rodríguez-Zaragoza, 1994). Inside such biofilms they have key functions in the regulation of the microbial biomass and maintenance of a high bacterial mineralization rate of organic matter through predation (Molmeret, Horn, Wagner, & Santic, 2005). It is well known that free-living amoebae are predators of prokaryotic and eukaryotic microorganisms, performed through phagocytosis and pinocytosis (Greub & Raoult, 2004; Weekers et al., 1993). *Acanthamoeba* preferentially graze on gram-negative bacteria; a fact widely used in isolation assays (Khan, 2006). Some are opportunistic pathogens of animals and humans, such as *Acanthamoeba* spp., a free-living and amphizoic protozoa causing amoebic keratitis (Province, 2012) and Granulomatous Amebic Encephalitis (Khan, 2006). However, most importantly amoebae represent frequent hosts for diverse bacterial endosymbionts.

Basically, one can distinguish between two different developmental stages. Trophozoites resemble the metabolically active and vegetative form (Bowers & Korn, 1968). Cell migration is mainly achieved by pseudopodia, temporary projections of the cell. By constantly changing the cell shape through reversible extension and contraction of their cytoskeleton, amoebae move forward, induced by a nutrient gradient like free-living bacteria. These pseudopods facilitate attachment to a variety of surfaces and are responsible for the typical amoebal cell morphology and irregular shape. Cysts are metabolically inactive and provide increased protection from hazardous environmental conditions like temperature and pH fluctuations as well as UV irradiation and desiccation (Bowers & Korn, 1969). Cysts of *Acanthamoeba* exhibit double walls unlike *Hartmannella* cysts, which are characterized by a round, smooth-walled morphology (Khan, 2006). Additionally, cysts are capable of facilitating the recurrence of infection and thereby enabling amoebae to withstand periods of unfavorable environmental conditions (Khan, 2006). The developmental cycle is closed upon the emergence of trophozoites from cysts, if favorable conditions have reoccurred.

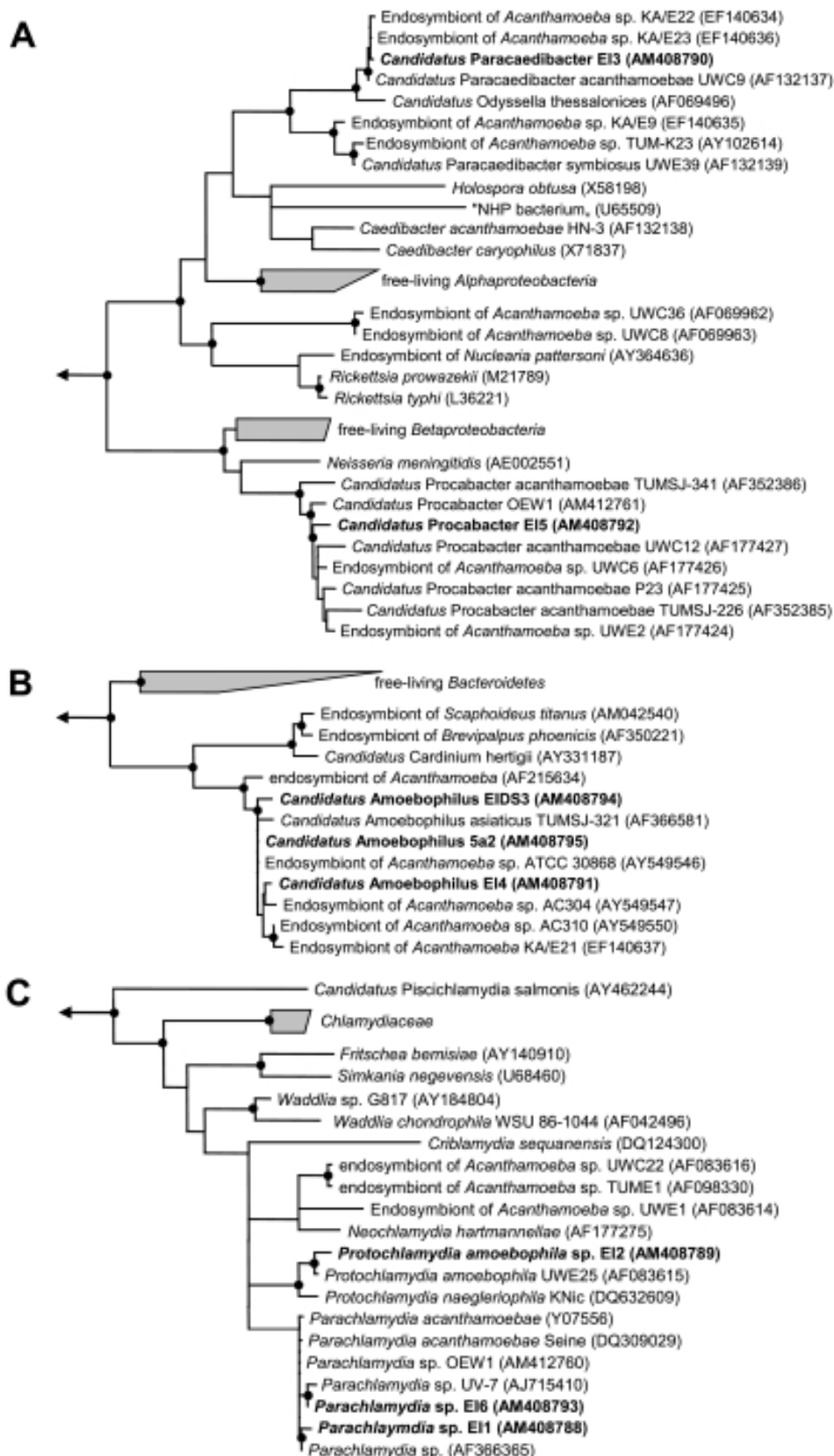
Establishing an intracellular interspecies relationship is often initialized through bacteria taken up by phagocytosis but somehow evading host cell defense mechanisms and becoming resistant to internalization and digestion by the host's phago-lysosomes. This has first been shown in 1956 (Drozanski, 1956). Resistance to digestion can be managed through inhibition of the formation of phago-lysosomes, modulation of endosomes through host manipulation (Isberg & Heidtman, 2009), simple escape from phagosomes or capability to grow at acidic pH inside the phago-lysosome (Khan, 2006) using defense mechanisms such as toxins, toxic pigments or outer membrane structures (Weekers et al., 1993).

Once the adaption to an intracellular life style is complete, bacteria may begin to exploit the host cell for massive replication and spread utilizing metabolic pathways and substrates. Induction of host cell lysis through host cell apoptosis is frequent among obligate intracellular pathogens (Ojcius et al., 1998).

Indeed, the constant pressure of adapting to hostile intracellular environment of protozoa, may well prime pathogenic bacteria for virulence, extension of their host range (Horn & Wagner, 2004; Molmeret et al., 2005) and guarantee the transport into humans (Barker & Brown, 1993). A variety of reports have confirmed, that *Acanthamoeba* and *Hartmannella* provide a general resort for intracellular bacteria resistant to free-living amoebae (Greub & Raoult, 2004a), which are established pathogens of humans. These include the facultative pathogenic mycobacteria like *Mycobacterium avium*, the causative agent of respiratory disease (Krishna-Prasad & Gupta, 1978; Taylor et al., 2003) or *Legionella pneumophila* the causative agent of Legionnaire's Disease, (Holden et al. , 1984; Kwaik, 1996; Rowbotham, 1980) or *Chlamydophila pneumonia*. Examples of emerging pathogens are *Simkania negevensis* and *Parachlamydia acanthamoebae*. However, various bacteria will in return confer access to its own metabolic capabilities, thereby the utilization of primary or secondary metabolites that had been inaccessible before or reprogramming of host gene expression patterns. Eventually, host cells may be able to conquer and subdue unique ecological niches (Oldroyd, 2009; Wernegreen et al., 2004).

Most of these bacteria have developed intimate and long-term associations with their hosts and include members of the phyla *Alphaproteobacteria* (Birtles et al., 2000; Fritsche, 1999; Horn et al., 1999; Xuan et al., 2007), *Betaproteobacteria* (Heinz et al., 2007a; Horn et al., 2002), *Bacteroidetes* (Horn, Harzenetter, Linner, Michel, et al., 2001), *Chlamydiae* (Collingro, 2005a; Collingro, 2005b; Fritsche et al., 2000; Heinz et al., 2007a; Horn et al., 2000) and very recently also *Gammaproteobacteria* (unpublished). Diversity of obligate intracellular symbionts of amoebae, their host range and global distribution in the environment have been highly underestimated (Horn & Wagner, 2004; Schmitz-Esser et al., 2008). In the last decade, an increased diversity has been recognized within the phylum of environmental chlamydiae in particular. Molecular evidence indicates that the actual diversity is even larger. According to former studies (Fritsche, 1993) about 5 % of all *Acanthamoeba* isolates carry chlamydia-like endosymbionts. For example, sequences related to chlamydiae were recovered from various aquatic, terrestrial and even putative environments, such as activated sludge, marine sediment, hydrothermal vents, permafrost soil, lava cave or hot spring microbial mats (Northup et al., 2011; Siegl et al., 2012). The sample analyzed in this study was a green colored microbial biofilm of a littoral cave wall from the Hawaiian Islands.

Such an intimate symbiotic interaction is the key feature that totally undermines traditional culture-dependent identification assays. Deeper insights into this complexity could only be gained through the development of culture-independent techniques, for example the 16S rRNA full-cycle approach in combination with various phylogenetic tools.



**Figure 1.1 16S rRNA based phylogenetic tree of *Acanthamoeba* endosymbionts. (A) Proteobacterial symbionts, (B) the *Bacteroidetes* symbionts and (C) the chlamydial symbionts (Schmitz-Esser et al., 2008)**

## 1.3 General physiology of bacterial endosymbiosis

### 1.3.1 The intracellular life-style

Some microorganisms have evolved to resist the internalization by the protists or become able to survive, grow, and exit free-living amoebae after engulfment. Reports suggest that the fate of internalized bacteria falls into three main groups; those which multiply and cause lysis of amoebal host such as *Legionella* and *Listeria*; those which multiply without causing cell lysis, thus considered endosymbionts with maintenance of a stable host-parasite ratio; and those which survive without multiplication (Barker & Brown, 1993; Fritsche et al., 1993; Hall & Voelz, 1985; Preer et al., 1974). We are particularly interested in the obligate intracellular bacteria that again show a variety of different life-styles. Either they live directly in the cytoplasm or enclosed in host-derived vacuoles suggesting further differences in the mechanisms of host-cell interactions.

The discovery that *Legionella pneumophila* infects and multiplies within some species of free-living amoebae (Rowbotham, 1980) has been the first that confirmed the ability of bacteria to exploit a normally hostile intracellular environment to ensure survival. Indeed, survival and intracellular growth of bacterial species in protozoa may well prime pathogenic bacteria for virulence (Swanson & Hammer, 2000). Following phagocytosis by acanthamoebae, legionellae multiply within the cytoplasm, evading the host lysosomal attack by blocking the phagosome-lysosome fusion and modulating host processes to use it for replication. The final effect of infection is lysis of the cell and liberation of many motile bacteria into the environment (Barker & Brown, 1993; Isberg & Heidtman, 2009). Given the fact that *Rickettsia*-like endosymbionts apparently have a narrow host range and that *Rickettsia* spp. may be considered commensal endosymbionts of ticks, this deep branching may correspond to the time of divergence of the protozoan and arthropods or to the time of their acquisition by ancestral ticks. The human pathogenicity of this *Rickettsia*-like lineage remains to be defined, as do its host range, prevalence, distribution, and interactions with free-living amoebae. *Ralstonia pickettii* of the *Burkholderiaceae* family and *Procabacter acanthamoeba* of the *Procabacteriaceae* are as yet the only two species of *Betaproteobacteria* shown to naturally infect free-living amoebae. *R. pickettii* may act as an opportunistic pathogen. The pathogenic role of *Procabacter acanthamoeba* is largely unknown, but given its obligate intracellular lifestyle, infection may remain undiagnosed if axenic cultures alone are used.

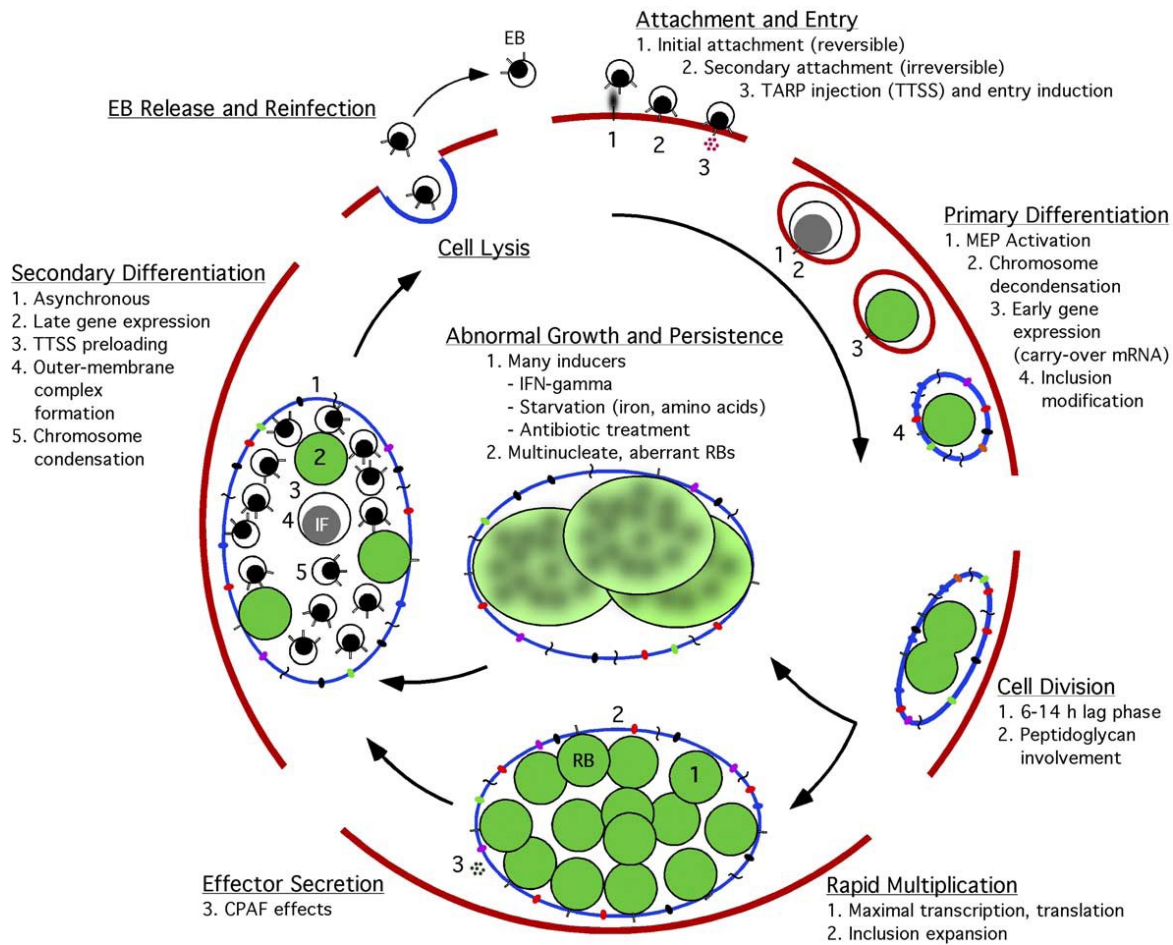
During the past decade, the *Chlamydia*-like organisms have been defined as obligate intracellular bacteria that naturally infect a large variety of organisms, such as insects, mammals, fish and unicellular eukaryotic species. Additionally, several have been considered as emerging pathogens, responsible for respiratory tract infections (G Greub, 2009; Haider et al., 2008; Lamothe & Greub, 2010), abortion and premature birth in humans as well as animals (Baud et al., 2008; Lamothe & Greub, 2010; Lamothe et al., 2011; Ruhl et al., 2009). The *Parachlamydiaceae* family in particular, comprising the obligate intracellular bacterial species *Parachlamydia acanthamoebae* and *Protochlamydia amoebophila*, is associated with free-living amoebae. The infectious bacterial form is endocytosed by

eukaryotic cells and resides within a cytoplasmic inclusion, where it transforms into a vegetative form and replicates by binary fission (Mouldert, 1991), described more in detail in the next paragraph. The endosymbiont of free-living amoebae *Protochlamydia amoebophila* modify the inclusion membrane through insertion of unique proteins, which are involved in interaction with and manipulation of the host cell (Heinz et al., 2010). *Parachlamydia acanthamoebae* have even been shown to be able to resist the microbial effectors of human macrophages, lung fibroblasts as well as pneumocytes and the first member of the order *Chlamydiales* to be shown to traffic through the endocytic pathway within monocyte derived macrophages (Greub, 2009; Greub et al., 2005). Apparently the bacteria replicate in vacuoles by modifying the vacuole biogenesis, preventing the fusion of the PCV with the hydrolases-rich lysosomal compartment and partially inhibiting the acquisition of the v-ATPase, a multisubunit complex that is involved in the acidification of the endocytic pathway. The strategy of *P. acanthamoebae* to subvert innate immune cells is very similar to that used by *Salmonella* (Meresse et al., 1999; Steele-mortimer et al., 1999). In contrast the *Chlamydiaceae* appear to completely bypass the early endocytic pathway and replicate within an inclusion that is trafficked to the peri-Golgi region where it fuses with exocytic vesicles (Greub et al., 2005; Hackstadt et al., 1995; Scidmore-Carlson & Hackstadt, 2000).

### 1.3.2 Chlamydial metabolism and physiologic activity

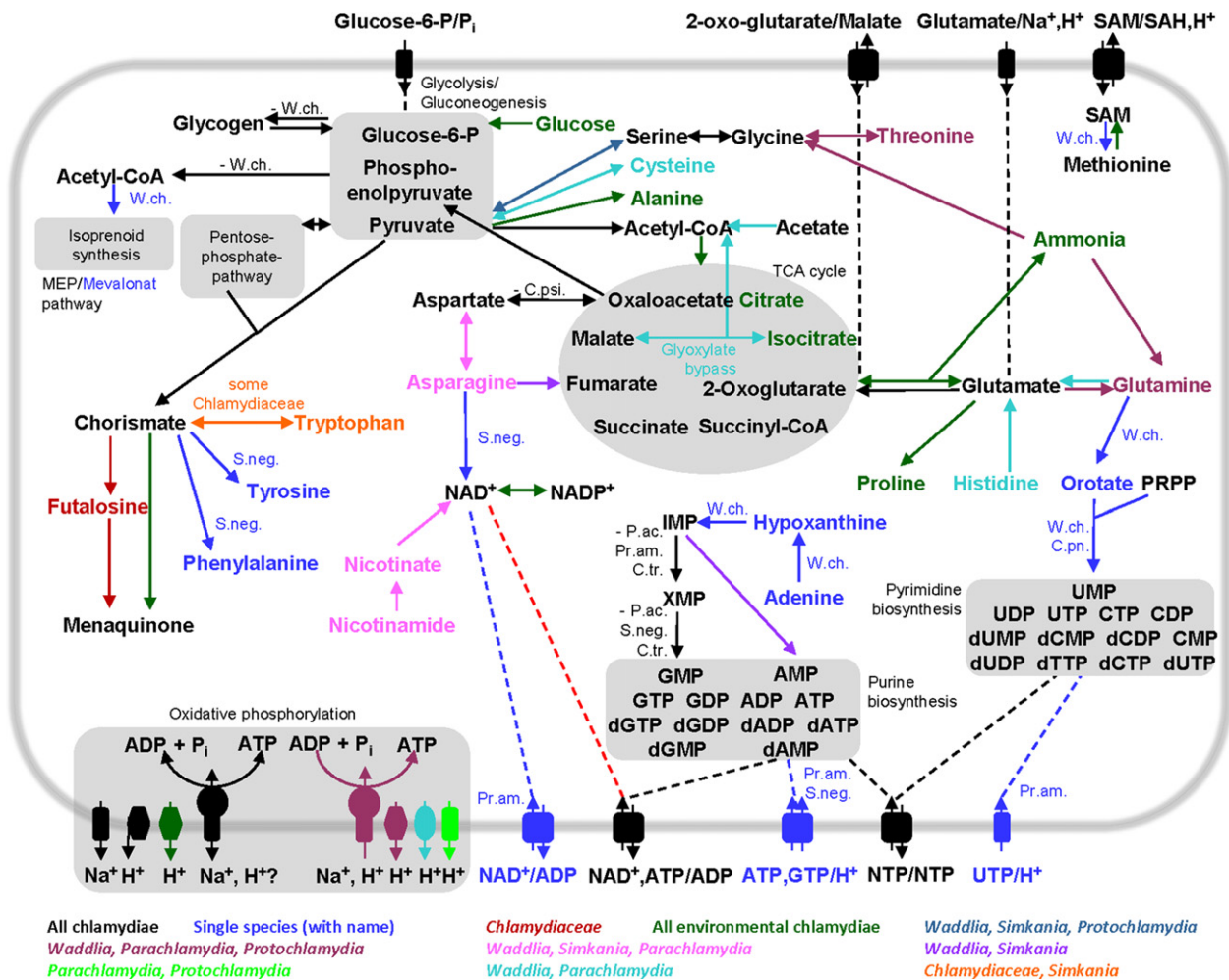
The *Chlamydiaeae* are a group of obligate intracellular bacteria including some of the most important human and animal pathogens such as *Chlamydia trachomatis* as well as endosymbionts of protozoa. Intracellular bacteria often have deep impact on their hosts, which either might have lytic effects or on which the hosts depend for survival and reproduction. As a consequence of this specialized life-style in the interior of eukaryotic cells, these bacteria have highly reduced metabolic capabilities and rely on a large variety of host-derived metabolites (Omsland et al., 2014; Zientz et al., 2004).

All members of the *Chlamydiales* share similar features in their biphasic developmental cycle, which consists of the alternation between two main morphological forms. An infection is thus initiated by the attachment of the extracellular elementary body (EB) known as the infectious form followed by the internalization into a membrane-bound compartment termed inclusion. EBs differentiate into the intracellular replicative form called reticulate body (RB) which then start dividing by binary fission. Finally, the largely accumulated RBs differentiate back to infectious EBs and are released into the environment by extrusion or host cell lysis. One of the essential characteristics of the small, round, electron dense form of *Chlamydia* spp. EBs is their high resistance to harsh extracellular conditions including osmotic and physical stress due to their rigid and highly cross-linked outer membrane (Abdelrahman & Belland, 2005; Hatch, 1996; Hybiske & Stephens, 2007; Mouldert, 1991; Omsland et al., 2014; Sixt et al., 2013). The ultrastructure of EBs is characterized by highly condensed chromatin, which may lead to inactivation of transcription and a reduced RNA to DNA ratio. Thus, EBs have been thought to be totally metabolically inert particles for a long time (Pedersen, 1996).



**Figure 1.2 The biphasic chlamydial developmental cycle.** The host cell cytoplasmic membrane is indicated as a red line and illustrates the interactions with Chlamydial EBs. The formation of the inclusion membrane, as well as the major events in the developmental cycle are described in the text (Abdelrahman & Belland, 2005).

In a more recent study, a profound characterization of the metabolic activities of EBs of the amoeba endosymbiont *Protochlamydia amoebophila* was performed (Sixt et al., 2013). Especially, the effect of nutrient availability or deprivation on chlamydial infectivity was closely investigated. The study revealed that extracellular *Protochlamydia amoebophila* EBs maintain their respiratory activity as well as the uptake and utilization of D-glucose. By replacing the substrate with the nonmetabolizable stereoisomere L-glucose, a rapid decline in the amount of infectious particles was measured. The major route of D-glucose catabolism was identified as the pentose phosphate pathway, but to some extent host-independent activity of the tricarboxylic acid (TCA) cycle was also observed. Genome sequence analysis also revealed that *Protochlamydia amoebophila* and many other environmental chlamydiae encode a glucokinase, which enables them to phosphorylate and activate D-glucose. Furthermore D-glucose availability was even demonstrated to be essential for the survival and maintenance of *Protochlamydia amoebophila* infectivity.



**Figure 1.3** The comparative metabolic repertoire of environmental chlamydiae and the *Chlamydiaceae*. (Omsland et al., 2014)

In contrast to this, genomic studies reveal that the *Chlamydiaceae* lack a glucokinase gene as well as substrate-specific components of the phosphotransferase system. Thus, these bacteria depend on the import of the phosphorylated D-glucose-6-phosphate from the host by a sugar-phosphate/inorganic phosphate antiporter UhpC (Iiffe-Lee & McClarty, 1999) instead of phosphorylating D-glucose on their own. Comparative genome analyses, including sequences of four environmental chlamydiae, *Protochlamydia amoebophila*, *Parachlamydia acanthamoebae*, *Simkania nevegensis* and *Waddlia chondrophila* indicate a significant divergence and little synteny to each other as well as to genomes of the *Chlamydiaceae* (Bertelli et al., 2010; Collingro et al., 2011; Horn et al., 2004; Omsland et al., 2014). A number of the genes that were only found in environmental chlamydiae comprised such encoding proteins involved in metabolic processes. This suggests that these environmental chlamydiae still retain a higher metabolic potential than the *Chlamydiaceae*, but that there are significant species- and strain-specific differences among the environmental chlamydia concerning their metabolic activity. We are far

from understanding the interaction of chlamydiae with their intracellular and extracellular environment, nutrient requirement and the physiological changes during the course of the developmental cycle.

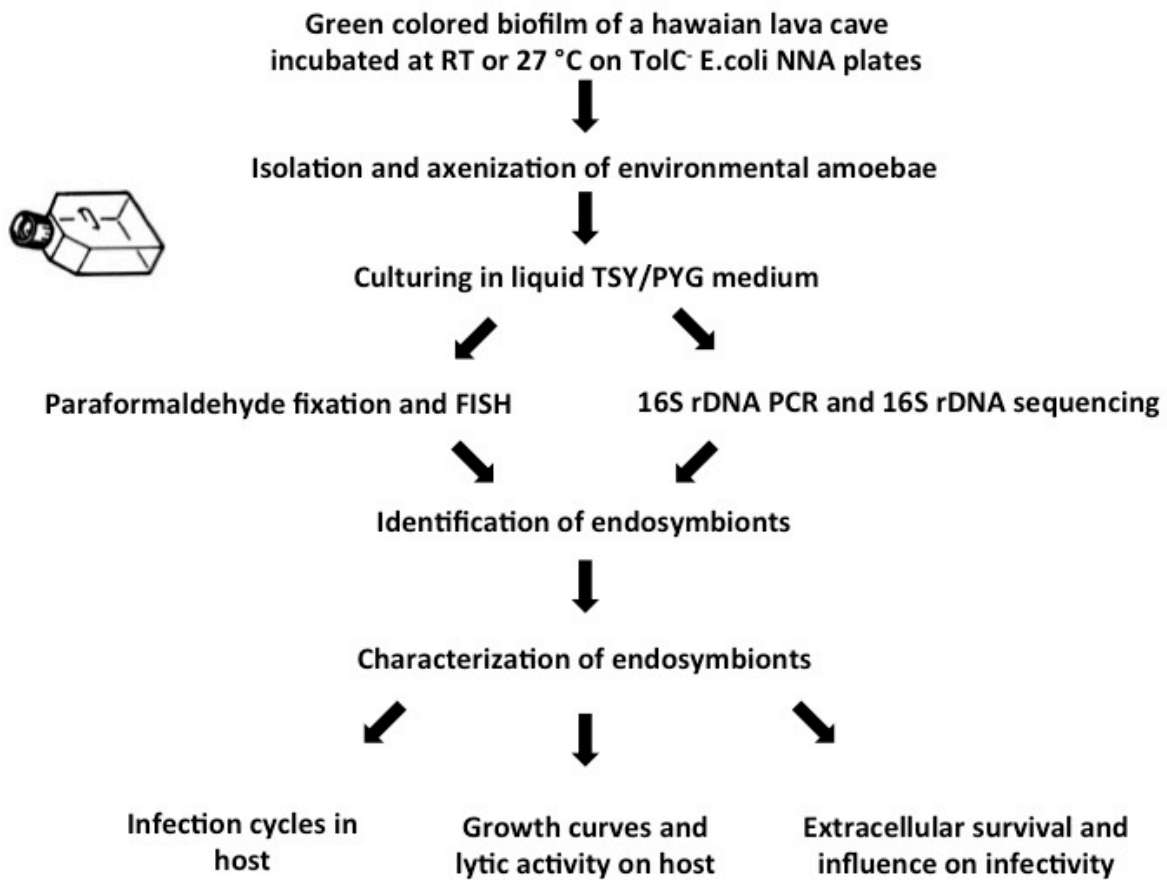
## 1.4 Aim of the study

Until now, the diversity of amoeba endosymbionts has been underestimated and the bacteria have rarely been identified or poorly described. (Horn, 2008) Therefore, the aim of this project was the isolation of amoebae from a biofilm sample, their axenization, as well as the identification and characterization of their endosymbionts. In this work a combination of Fluorescent in-situ Hybridization assays and molecular methods was applied.

First, amoebae had to be isolated from the environmental sample. As was previously shown, amoebae are able to move by means of cytoplasmic flow, thus isolation of environmental amoebae is based on migration on agar surfaces covered with a live *E. coli* layer. (Neff, 1958) The amoebae spread out from the inoculation site and can be transferred to new agar plates. In order to obtain axenic amoebae cultures, amoebae are eventually grown in *E. coli*-free growth media (TSY, PYG), supplemented with ampicillin.

As illustrated in fig. 3 an axenic amoebae culture can be used for the molecular approach of fluorescence in-situ hybridization to detect intracellular bacterial endosymbionts. Identification and phylogenetic association was possible through 16S rRNA gene sequencing.

Here, the detection of two novel species of the *Chlamydiales* and the *Betaproteobacteria*, encourages a further in-depth characterization of the endosymbionts. These enclose the description of infection cycles in hosts, analysis of host range, their impact on amoebae growth and fitness and finally their ability of extracellular survival and maintenance of infectivity. To lead on the study, whole genome sequencing of the strains HSC3 and HSC8 will provide an efficient means of acquiring data relevant to the more detailed molecular characterization of the endosymbionts.



**Figure 1.4 Flow chart** illustrating the steps of amoebae isolation and axenization, followed by the identification and characterization of amoebae endosymbionts.

## 2. Material and Methods

### 2.1 Technical supply and computer software

Most of the work described in this thesis was done at the Division of Microbial Ecology (University of Vienna), with one exception. The Tecan Infinite M200 microplate reader needed for the quantification of total amount of DAPI stained DNA as well as propidium iodide stained cells was located at the Division of Terrestrial Ecosystem Research (University of Vienna).

**Table 2.1** Technical equipment with indication of the corresponding software.

| Machine                                                  | Software         | Manufacturer          |
|----------------------------------------------------------|------------------|-----------------------|
| PowerPac Basic (electrophoresis power supply)            |                  | BioRad                |
| SmartSpec™ 3000 (spectrometer)                           |                  | BioRad                |
| Sub-Cell GT (agarose gel electrophoresis system)         |                  | BioRad                |
| Sub-cell GT gel tray                                     |                  | BioRad                |
| UST-C30M-8R (UV transilluminator)                        | Argus X1 (4.1)   | Biostep               |
| Centrifuge 5804 R                                        |                  | Eppendorf             |
| Eppendorf Research® pipettes                             |                  | Eppendorf             |
| MiniSpin® plus                                           |                  | Eppendorf             |
| Incubation water bath 1004                               |                  | GFL                   |
| Mikro 20 (centrifuge)                                    |                  | Hettich               |
| LaminAir Model 1.2 (laminar flow hood)                   | ND-1000 (3.2)    | JOUAN Nordic A/S      |
| CLSM (confocal laser scanning microscope)                |                  | Leica                 |
| Neubauer haemocytometer (counting chamber)               |                  | Marienfeld            |
| Hybridization oven                                       | AxioVision (4.7) | Memmert               |
| Milli-Q Water Purification System                        |                  | Millipore             |
| C-5050 Zoom (digital camera, transilluminator)           |                  | Olympus               |
| BL3100 (balance)                                         |                  | Sartorius             |
| BL6100 (balance)                                         | i-control™(1.6)  | Sartorius             |
| Vortex Genie 2                                           |                  | Scientific Industries |
| Infinite® M200 (microplate reader)                       |                  | Tecan                 |
| Haake® DC 10-P5/U (heating circulator)                   |                  | Thermo Scientific     |
| NanoDrop ND-1000 (UV-visible spectrometer)               |                  | Thermo Scientific     |
| Galaxy Mini C12XX (microcentrifuge)                      |                  | VWR                   |
| Ino Lab® pH Level 1 (pH meter)                           |                  | WTW                   |
| Axio Imager M1 (epifluorescence microscope)              |                  | Zeiss                 |
| Axioplan 2 imaging (epifluorescence microscope)          |                  | Zeiss                 |
| AxioCam HRc (digital camera, epifluorescence microscope) |                  | Zeiss                 |

## 2.2 Consumables

Consumables used for the cultivation and infection assays were only opened and used in a laminar flow hood to avoid contamination. The outer surfaces were additionally cleaned with 70 % (v/v) ethanol prior to moving them into the laminar flow hood. PCR consumables were only opened at designated PCR hoods and radiated with UV light for 15 min before use. The work benches designated for DNA isolations, including consumables such as pipettes and tip boxes were cleaned with DNA AWAY before starting to work.

**Table 2.2** Consumables.

| Consumable                                                                              | Manufacturer    |
|-----------------------------------------------------------------------------------------|-----------------|
| 0.6 ml reaction tubes                                                                   | Biozym          |
| 0.2 ml PCR tubes                                                                        | Biozym          |
| Premium Tips (with filters)                                                             | Biozym          |
| Omnifix® single-use syringes, 30 and 50 ml                                              | Braun           |
| Microscope slides                                                                       | Carl Roth       |
| Pipette tips                                                                            | Carl Roth       |
| 1.5 ml reaction tubes                                                                   | Greiner Bio-One |
| 2 ml reaction tubes                                                                     | Greiner Bio-One |
| 15 ml reaction tubes                                                                    | Greiner Bio-One |
| 50 ml reaction tubes                                                                    | Greiner Bio-One |
| Microplates, 96-well, flat bottom, chimney, black                                       | Greiner Bio-One |
| Microscope slides, polyethylene coated, black mask with 10 wells                        | Marienfeld      |
| Glass coverslips, 24 x 50 mm                                                            | Marienfeld      |
| Nalgene syringe filters (0.2 µm)                                                        | Nalgene         |
| Standard cell culture flasks, Nuclon™Δ surface, vent/close cap, 25 cm <sup>2</sup>      | Nunc            |
| TripleFlask™ cell culture flasks, Nuclon™Δ surface, vent/close cap, 500 cm <sup>2</sup> | Nunc            |
| Minisart® NML syringe filters, 1.2 and 5.0 µm                                           | Sartorius       |
| Glass bottles                                                                           | Schott          |
| Petri dishes, 90 mm                                                                     | Sterilin        |
| Cell culture flasks, standard cap, 182 cm <sup>2</sup>                                  | VWR             |
| Serological pipettes, 2, 10 and 25 ml                                                   | VWR             |

## 2.3 Molecular biology kits

**Table 2.3** Molecular biology kits.

| Kit                            | Manufacturer |
|--------------------------------|--------------|
| TOPO® TA Cloning Kit           | Invitrogen   |
| DNeasy® Blood & Tissue Kit     | QIAGEN       |
| QIAquick® Gel Extraction Kit   | QIAGEN       |
| QIAquick® PCR Purification Kit | QIAGEN       |

## 2.4 Probes and primers

Oligonucleotide probes were generally single-labelled with 5' (6)-Carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS), Cy3 or Cy5 and sometimes double-labeled and are listed in Table 2.4. All primers and probes were produced by Thermo Fisher Scientific.

**Table 2.4** Oligonucleotide probes for rRNA targeted fluorescence *in situ* hybridization.

| Probe             | Target organism                                 | 5' - 3' sequence        | % FA | Author                 |
|-------------------|-------------------------------------------------|-------------------------|------|------------------------|
| <b>AcRic90</b>    | <i>Rickettsia</i> -like                         | TGC CAC TAG CAG AAC TCC | 20   | Fritsche <i>et al.</i> |
| <b>AcRic1196</b>  | <i>Rickettsia</i> -like                         | CCT ATT GCG TCC AAT TGT | 10   | Fritsche <i>et al.</i> |
| <b>Alf968</b>     | $\alpha$ - <i>Proteobacteria</i>                | GGT AAG GTT CTG CGC GTT | 20   | Neef <i>et al.</i>     |
| <b>Aph1180</b>    | <i>Amoebophilus asiaticus</i>                   | CTG ACC TCA TCC CCT CCT | 20   | Horn <i>et al.</i>     |
| <b>CC23a</b>      | <i>Caedibacter</i> -related                     | TTC CAC TTT CCT CTC TCG | 20   | Springer <i>et al.</i> |
| <b>Chls-0523</b>  | <i>Chlamydiales</i>                             | CCT CCG TAT TAC CGC AGC | 25   | Poppert <i>et al.</i>  |
| <b>CFB286</b>     | <i>Bacteroidetes</i>                            | TCC TCT CAG AAC CCC TAC | 20   | Weller <i>et al.</i>   |
| <b>Gam42a</b>     | $\gamma$ - <i>Proteobacteria</i>                | GCC TTC CCA CAT CGT TT  | 35   | Manz <i>et al.</i>     |
| <b>Bet42a</b>     | $\beta$ - <i>Proteobacteria</i>                 | GCC TTC CCA CTT CGT TT  | 35   | Manz <i>et al.</i>     |
| <b>EUB338-I</b>   | Most bacteria                                   | GCT GCC TCC CGT AGG AGT | 0-60 | Amann <i>et al.</i>    |
| <b>EUB338-II</b>  | <i>Planctomycetales</i>                         | GCA GCC ACC CGT AGG TGT | 0-60 | Daims <i>et al.</i>    |
| <b>EUB338-III</b> | <i>Verrucomicrobiales</i>                       | GCT GCC ACC CGT AGG TGT | 0-60 | Daims <i>et al.</i>    |
| <b>HSC8</b>       | <i>Betaproteobacteria</i> sp. endosymbiont HSC8 |                         | 20   | Unpublished            |
| <b>Proca438</b>   | <i>Procabacter</i> sp.                          | CGA TTT CCT CCC RGA CAA | 20   | Horn <i>et al.</i>     |
| <b>Ric1395</b>    | <i>Rickettsia</i> -like                         | GGC TTG ACG GGC AGT GTG | 20   |                        |

**Table 2.5** Primers used in standard 16S and 18S PCR.

| Primer       | Target organism     | Target molecule | 5' - 3' sequence                    | Author                   |
|--------------|---------------------|-----------------|-------------------------------------|--------------------------|
| <b>616V</b>  | Bacteria            | 16S rRNA        | AGA GTT TGA TYM TGG CTC AG          | Kim <i>et al.</i>        |
| <b>1492R</b> | Bacteria            | 16S rRNA        | RGY TAC CTT GTT ACG ACT T           | McAllister <i>et al.</i> |
| <b>PanF</b>  | <i>Chlamydiales</i> | 16S rRNA        | CGT GGA TGA GGC ATG CRA GTC G       | Corsaro <i>et al.</i>    |
| <b>PanR</b>  | <i>Chlamydiales</i> | 16S rRNA        | GTC ATC RGC CYT ACC TTV SRC RYY TCT | Corsaro <i>et al.</i>    |
| <b>18SF</b>  | Eukaryota           | 18S rRNA        | GTA GTC ATA TGC TTG TCT C           | White <i>et al.</i>      |
| <b>18SR</b>  | Eukaryota           | 18S rRNA        | CGR ARA CCT TGT TAC GAC             | White <i>et al.</i>      |

## 2.5 Growth media, buffers and other chemical solutions

The listed growth media, buffers and solutions were autoclaved at 121°C and 1013 x 10<sup>5</sup> Pa for 20 min and were stored at room temperature, if not stated otherwise. Solidification of the culture media was assured by adding 15 g/l agar prior to autoclaving, supplemented with antibiotics (if required) and poured into 90 mm petri dishes (Sterilin).

**Table 2.6** Growth media for *Acanthamoeba* cultivation and *E. coli* cultivation.

| Ingredients                                                                           | Amount for 1 L |
|---------------------------------------------------------------------------------------|----------------|
| <b>TSY Broth (Trypticase Soy broth with Yeast extract)</b>                            |                |
| Trypticase Soy Broth (DIFCO, Detroit, USA)                                            | 30 g           |
| Yeast extract                                                                         | 10 g           |
| H <sub>2</sub> O <sub>dest.</sub>                                                     | add 1000 ml    |
| pH 7.3                                                                                |                |
| <b>PYG (Peptone-Yeast-Glucose-Medium)</b>                                             |                |
| Pepton                                                                                | 20 g           |
| Glukose                                                                               | 18 g           |
| Hefeextrakt                                                                           | 2 g            |
| Natriumcitrat                                                                         | 1 g            |
| MgSO <sub>4</sub> *7 H <sub>2</sub> O                                                 | 980 mg         |
| Na <sub>2</sub> HPO <sub>4</sub> *7 H <sub>2</sub> O                                  | 355 mg         |
| KH <sub>2</sub> PO <sub>4</sub>                                                       | 340 mg         |
| Fe(NH <sub>4</sub> ) <sub>2</sub> (SO <sub>4</sub> ) <sub>2</sub> * 6H <sub>2</sub> O | 20 mg          |
| H <sub>2</sub> O <sub>dest.</sub>                                                     | add 1000 ml    |
| pH 6.5                                                                                |                |
| Autoclaved at 110 °C                                                                  |                |
| <b>NNA (Non Nutrient Agar)</b>                                                        |                |
| Page's Amoebic Saline (10x)                                                           | 100 ml         |
| H <sub>2</sub> O <sub>dest.</sub>                                                     | 900 ml         |
| Agar                                                                                  | 15 g           |
| <b>LB-medium (Luria-Bertani-medium)</b>                                               |                |
| Trypton                                                                               | 10.0 g         |
| NaCl                                                                                  | 5.0 g          |
| Yeast extract                                                                         | 5.0 g          |
| H <sub>2</sub> O <sub>dest.</sub>                                                     | add 1000 ml    |
| pH 7.0-7.5                                                                            |                |
| <b>DMEM (Dulbecco's Modified Eagle Medium)</b>                                        |                |

**Table 2.7** Buffers.

| Ingredients                                             | Amount for 50 ml | Amount for 1 L |
|---------------------------------------------------------|------------------|----------------|
| <b>Page's Amoebic Saline (10 x PAS)</b>                 |                  |                |
| NaCl                                                    |                  | 1.2 g          |
| MgSO <sub>4</sub> *7 H <sub>2</sub> O                   |                  | 0.04 g         |
| CaCl <sub>2</sub> *2 H <sub>2</sub> O                   |                  | 0.04 g         |
| Na <sub>2</sub> HPO <sub>4</sub>                        |                  | 1.42 g         |
| KH <sub>2</sub> PO <sub>4</sub>                         |                  | 1.36 g         |
| H <sub>2</sub> O <sub>dest.</sub>                       |                  | 1000 ml        |
| pH 6.9                                                  |                  |                |
| <b>Phosphate Buffered Saline (10 x PBS)</b>             |                  |                |
| NaCl                                                    |                  | 80 g           |
| KCl                                                     |                  | 2.0 g          |
| Na <sub>2</sub> HPO <sub>4</sub>                        |                  | 14.4 g         |
| KH <sub>2</sub> PO <sub>4</sub>                         |                  | 2.4 g          |
| H <sub>2</sub> O <sub>dest.</sub>                       |                  | 1000 ml        |
| pH 7.4                                                  |                  |                |
| <b>Dulbecco's Phosphate Buffered Saline (10 x DPBS)</b> |                  |                |
| pH 7.2-7.6                                              |                  |                |
| <b>Tris/HCl buffer</b>                                  |                  |                |
| Tris                                                    |                  | 121.1 g        |
| H <sub>2</sub> O <sub>reinst</sub>                      |                  | Add 1000 ml    |
| pH 8.0                                                  |                  |                |
| <b>10 x TBE (Tris/Borate/EDTA) buffer</b>               |                  |                |
| Tris                                                    |                  | 107.8 g        |
| Boric acid                                              |                  | 55.0 g         |
| EDTA disodium salt dehydrate                            |                  | 7.4 g          |
| H <sub>2</sub> O <sub>reinst</sub>                      |                  | Add 1000 ml    |
| pH 8.3                                                  |                  |                |
| <b>50 x TAE (Tris/ Acetate/EDTA) buffer</b>             |                  |                |
| Tris                                                    |                  | 242 g          |
| Acetic acid                                             |                  | 57.1 ml        |
| EDTA disodium salt dehydrate                            |                  | 18.6 g         |
| H <sub>2</sub> O <sub>reinst</sub>                      |                  | Add 1000 ml    |
| pH 8.3-8.7                                              |                  |                |
| <b>1 x TE</b>                                           |                  |                |
| <b>Buffer A Without EDTA</b>                            |                  |                |
| 35 mM Tris-HCl                                          | 22 g             | 4,4 g          |
| 250 mM Sucrose                                          | 4,28 g           | 85,6 g         |

|                                    |            |                                                            |
|------------------------------------|------------|------------------------------------------------------------|
| 25 mM KCl                          | 0,09 g     | 1,8 g                                                      |
| 10 mM MgCl <sub>2</sub>            | 0,1 g      | 2 g                                                        |
| H <sub>2</sub> O <sub>dest</sub>   | add 50 ml  | add 1000 ml                                                |
| pH 7.5                             |            |                                                            |
| <b>Buffer A With 250 mM EDTA</b>   |            |                                                            |
| 35 mM Tris-HCl                     | 0,44 g     | 4,4 g                                                      |
| 250 mM Sucrose                     | 8,56 g     | 85,6 g                                                     |
| 250 mM EDTA                        | 9,3 g      | 93,1 g                                                     |
| 25 mM KCl                          | 0,18 g     | 1,8 g                                                      |
| 10 mM MgCl <sub>2</sub>            | 0,2 g      | 2 g                                                        |
| H <sub>2</sub> O <sub>dest</sub>   | add 100 ml | add 1000 ml                                                |
| pH 7,5                             |            |                                                            |
| <b>Hybridization buffer</b>        |            |                                                            |
| 5 M NaCl                           |            | 180 µl                                                     |
| 1 M Tris/HCl pH 8.0                |            | 20 µl                                                      |
| Formamid                           |            | x µl *                                                     |
| H <sub>2</sub> O <sub>reinst</sub> |            | add 1000 µl                                                |
| <b>Washing buffer</b>              |            |                                                            |
| 1 M Tris/HCl pH 8,0                |            | 1 ml                                                       |
| 5 M NaCl                           |            | y µl *                                                     |
| 0.5 M EDTA                         |            | 500 µl (above 20 %<br>formamid in<br>hybridization buffer) |
| H <sub>2</sub> O <sub>reinst</sub> |            | add 50 ml                                                  |

**Table 2.8** Antibiotics. All antibiotics listed were filter sterilized (0.2 µm filter) and stored at -20 °C.

| Antibiotic         | Mechanism                                                             | Antibiotic Class | Stock concentration | Dissolvent                         |
|--------------------|-----------------------------------------------------------------------|------------------|---------------------|------------------------------------|
| <b>Ampicillin</b>  | Inhibition of transpeptidase needed for cell wall synthesis           | Aminopenicillin  | 100 mg/ml           | 50% (v/v) 96% EtOH                 |
| <b>Kanamycin</b>   | Interaction with bacterial 30S RSU and inhibition of translocation    | Kanamycin        | 100 mg/ml           | H <sub>2</sub> O <sub>reinst</sub> |
| <b>Rifampicin</b>  | Inhibition of bacterial DNA-dependent RNA- polymerase (RNA synthesis) | Rifamycin        | 10 mg/ml            | 50% (v/v) DMSO                     |
| <b>Tetracyclin</b> | Binding of bacterial 30S RSU and blocking of charged aminoacyl-tRNA   | Tetracyclin      | 10 mg/ml            | H <sub>2</sub> O <sub>reinst</sub> |
| <b>Doxycyclin</b>  | Binding of bacterial 30S RSU and Aminoacyl-tRNA                       | Tetracyclin      | 10 mg/ml            | H <sub>2</sub> O <sub>reinst</sub> |

For the proper preparation of these solutions, only distilled, UV-light treated, filtered, deionized water from the MILLI-Q Water Purification System was used. Only PCR reagents were prepared with double distilled water. PH was adjusted using NaOH platelets or HCl solution.

**Table 2.9** Chemical solutions.

| Ingredients                                            | Amount for 1 L           |
|--------------------------------------------------------|--------------------------|
| <b>Sodium chloride solution</b>                        |                          |
| NaCl                                                   | 292.2 g                  |
| H <sub>2</sub> O <sub>reinst</sub>                     | Add 1000 ml              |
| <b>Paraformaldehyde (PFA) solution</b>                 | <b>Amount for 50 ml</b>  |
| Formaldehyde (37 % solution)                           | 5.4 g                    |
| H <sub>2</sub> O <sub>reinst</sub>                     | Add 50 ml                |
| Filter sterilized, not autoclaved                      |                          |
| <b>EDTA (Ethylenediaminetetraacetic acid) solution</b> | <b>Amount for 250 ml</b> |
| EDTA disodium salt dihydrate                           | 46.5 g                   |
| H <sub>2</sub> O <sub>reinst</sub>                     | Add 250 ml               |
| pH 8.0                                                 |                          |

**Table 2.10** Hybridization buffer (46 °C). Amounts are indicated in µl.

| FA % | 0 % | 5 % | 10 % | 20 % | 25 % | 30 % | 35 % |
|------|-----|-----|------|------|------|------|------|
| NaCl | 180 | 180 | 180  | 180  | 180  | 180  | 180  |
| Tris | 20  | 20  | 20   | 20   | 20   | 20   | 20   |
| MQ   | 800 | 750 | 700  | 600  | 550  | 500  | 450  |
| FA   | 0   | 50  | 100  | 200  | 250  | 300  | 350  |
| SDS  | 1   | 1   | 1    | 1    | 1    | 1    | 1    |

**Table 2.11** Washing buffer (48 °C). Amounts are indicated in µl.

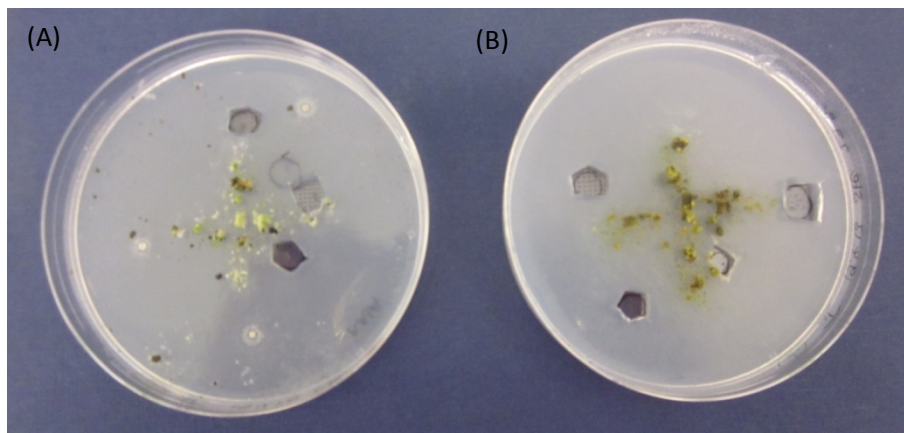
| FA % | 0 %      | 5 %      | 10 %     | 20 %     | 25 %     | 30 %     | 35 %     |
|------|----------|----------|----------|----------|----------|----------|----------|
| NaCl | 9 ml     | 6.3 ml   | 4.5 ml   | 2150     | 1490     | 1020     | 700      |
| Tris | 1 ml     | 1 ml     | 1 ml     | 1 ml     | 1 ml     | 1 ml     | 1 ml     |
| EDTA | 0        | 0        | 0        | 500      | 500      | 500      | 500      |
| MQ   | Ad 50 ml | Ad 50 ml | Ad 50 ml | Ad 50 ml | Ad 50 ml | Ad 50 ml | Ad 50 ml |

## 2.6 Methods

### 2.6.1 Isolation of amoebae of free-living amoebae using non-nutrient agar plates seeded with food bacteria

In nature, prokaryotic and eukaryotic microorganisms are an important food source for free-living amoebae (Khan, 2006). Therefore, in this study amoebae were isolated by luring them away from the inoculation site as the agar plate is covered with a bacterial layer, precisely *Escherichia coli* (NEFF, 1958).

According to a modified “walk out” method described by Lagkouvardos and colleagues (Lagkouvardos et al., 2014), two Non-nutrient agar plates (NNA) were covered with 100 µl live TolC<sup>-</sup> *E. coli* mutants and environmental sample was added to the center. One Petri dish was incubated at 27 °C, the other one at RT, and the amoebae were let to migrate away from the inoculum. This method is used extensively in the isolation of amoebae from both environmental and clinical samples worldwide.



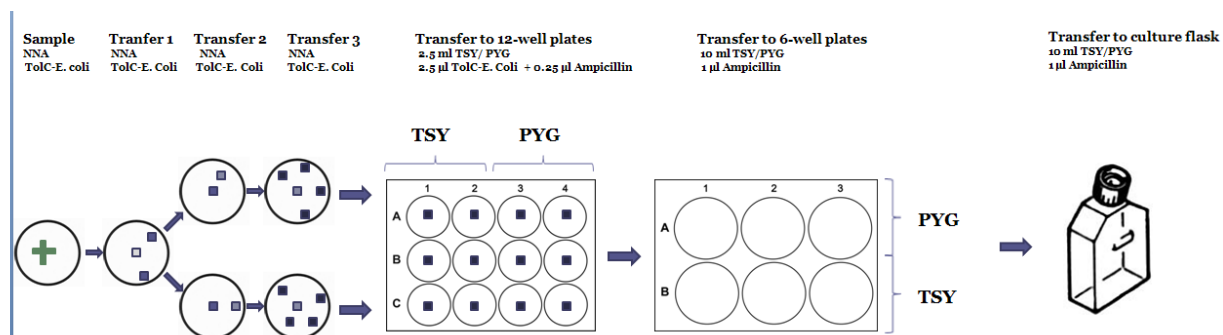
**Figure 2.1** Biofilm plated in the middle of a Petri dish with NNA covered with TolC<sup>-</sup> *E. coli*. (A) Sample incubated at RT, (B) sample incubated at 27 °C.

Each plate was observed daily for the presence of the typical amoebae trophozoites by light microscopy, and then 6 and 9 locations respectively were chosen and excised as agar cubes with a spatula. These were transferred upside down onto a new *E. coli* NNA plate and incubated at the respective temperatures until the amoebae reached the edge of the plate. In order to keep the plates free of contaminants, agar cubes with amoebae were transferred another two times, until 4 pieces of each isolate were dedicated to be grown in a 12-well plate with 2.5 ml liquid media (TSY and PYG) supplemented with 2.5 µl TolC<sup>-</sup> *E. coli* and 0.25 µl ampicillin (100 mg/ml stock solution to a final concentration of 10 µg/ml).

### 2.6.2 Axenization of free-living amoebae

An axenic culture is typically grown in the absence of external live food bacteria. In order to eliminate the necessity of amoebae for the food source over time a single-gene knockout *E. coli* mutant was used, whose gene coding for the outer membrane transporter TolC is replaced by a kanamycin resistant coding gene. Thus, the TolC<sup>-</sup> *E. coli* are hypersensitive to ampicillin and gentamycin and can selectively be eliminated by sublethal amounts of these antibiotics (Baba et al., 2006; Lagkouvardos et al., 2014; Tamae et al., 2008).

As soon as an increase in the numbers of amoebae could be observed by light microscopy, 1 ml amoeba culture was transferred to a 6-well plate, filled with 9 ml of growth medium, supplemented with 1 µl ampicillin. No TolC<sup>-</sup> *E. coli* were added in order to get rid of the necessity of amoebae to feed on them. Further enhanced amoebal growth indicated the passage to axenization and the culture is transferred into 25 cm<sup>2</sup> polystyrene culture flasks, containing 8-10 ml TSY or PYG medium. These were emptied every week, refilled with fresh growth medium and supplemented with 1 µl ampicillin. The culture could be considered axenic, when amoebae continued growing without any further addition of antibiotics and no extracellular growth of bacteria could be observed.



**Figure 2.2** Working scheme for isolation and axenization of amoebae. The transfer steps of agar cubes containing amoebae are indicated.

### 2.6.3 Fluorescence in-situ hybridization (FISH)

In order to detect and identify endosymbiotic bacteria of free-living amoebae, a hybridization using specific oligonucleotide probes can be applied. The best way to get the probes to enter the cells is to fix the amoebae with 4 % formaldehyde, acting as denaturing agent for membrane proteins.

The stringency of the hybridization of oligonucleotide probes to any target 16S rRNA depends on the concentration of formamid added to the Hybridization buffer and the NaCl concentration in the washing

buffer. These conditions differ with every single probe, thus have to be adapted most efficiently. Formamid has a stronger dipole moment than water and therefore destabilizes hydrogen bonds, while NaCl stabilizes nucleic acid hybrids.

An oligonucleotide probe contains a sequence complementary to the target sequence on one hand, on the other hand a fluorescent dye (typically Cy-3 with the red emission, or FLUOS in green) which visualizes the organism of interest under a fluorescence microscope.

#### **Fixation of amoebae cells on FISH slide**

For the *in situ* hybridization preferentially axenized amoebae cultures were used. The culture flasks were vigorously shaken, so that the amoebae attached to the surface were loosened, 1-2 ml of the culture were pipetted into a 2 ml Eppendorf tube, centrifuged for 5 min at 5000 rpm. After removing the supernatant, the pellet was resuspended in a small amount of 1 x PAS (50-120 µl). 20 µl of the cell suspension was left to attach on a Teflon-slide well during 1-2 hours at RT, until the supernatant drop was almost dry. After adding 20 µl of 4 % formaldehyde, the sample was incubated for 10 min, before carefully removing the supernatant drop by pipetting. To wash the wells and get rid of any remaining 4% formaldehyde 20 µl MilliQ were added and removed again immediately. The slide was then air-dried.

#### **Fluorescence in-situ hybridization**

For the *in situ* hybridization 10 µl hybridization buffer and 1 µl fluorescently labeled oligonucleotide probe was dropped onto each well and carefully mixed. The slide was put into a 50 ml screw-top tube, together with a wetted tissue paper. The tube was placed horizontally into a hybridization oven and incubated for 1.5 h at 46°. After that the slide was washed during 10 min in 50 ml washing buffer that has been preheated to 48 °C in a water bath, dipped for 2 s into ice-cold ddH<sub>2</sub>O and dried with compressed air. Prior to analysis under an epifluorescence microscope (Zeiss Axioplan 2) the samples were mounted with Citifluor and covered with a glass coverslip.

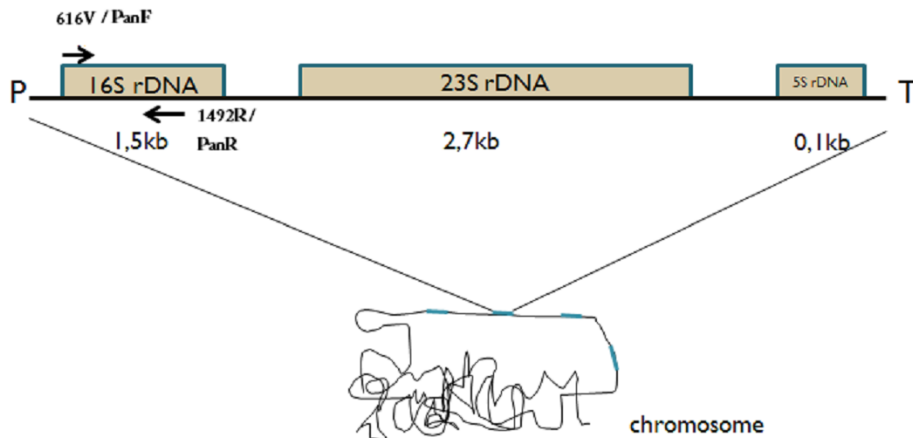
All samples were labeled with the probe EUB338-mix that targets most bacteria, labeled in green by FLUOS and another specific probe targeting single classes of bacteria in red by Cy3. The amoeba hosts were marked by a probe EUK516 targeting eukaryotic cells in general and a probe specific for *Acanthamoeba* sp. both labelled in blue by Cy5.

### **2.6.4 16S rRNA gene sequencing of axenized amoeba cultures**

The 16S rRNA is part of the 30S subunit of the bacterial ribosome and memorizes evolutionary information about a prokaryotic organism. The ribosome itself is the protein machinery of a cell and has one of the most essential functions in an organism. The fact that these functions are responsible for the viability of the bacteria applies a strong selective pressure on the organisms and eliminates the mutated ones immediately. The rRNA genes are functionally homologous between all known bacterial species and contain several highly conserved regions, which can be targeted by “universal primers” like the forward primer 616V and the reverse primer 1492R used in this study. Primers specific for chlamydiae,

such as the forward primer PanF and the reverse primer PanR target the conserved regions of chlamydial 16S rRNA genes.

Along with the conserved regions there are hyper variable sequences that differ from one genus or species to another due to mutations. Therefore the ribosomal 16S rRNA genes act as a molecular clock and allow a classification of unidentified bacteria and a phylogenetic analysis.



**Figure 2.3** Bacterial rRNA operon with indication of primer binding regions used for the 16S rRNA gene amplification.

### DNA extraction

Bacterial DNA was extracted according to the Blood and Tissue Kit (Qiagen). 1 ml of an axenic amoeba culture in TSY/PYG media was centrifuged for 10 min at 7'500 rpm and the pellet was resuspended in 180 µl Buffer ATL, 20 µl proteinase K was added and thoroughly vortexed, before the samples were incubated for another 2 h at 55 °C. After the lysis was complete, the samples were vortexed for 15 s, 200 µl Buffer AL were added and mixed immediately and thoroughly by vortexing. After another incubation at 70 °C for 10 min, 200 µl ethanol absolute were added and vortexed. The mixture was then pipetted onto a DNeasy spin column placed in a 2 ml collection tube. After a first centrifugation step at 8'000 rpm for 1 min, the flow through was discarded and in order to get rid of the remaining ethanol the samples were washed twice: 500 µl Buffer AW1 was added, followed by a second centrifugation step at 8'000 rpm for 1 min, then another 500 µl Buffer AW2 was pipetted onto the column and centrifuged for 3 min at full speed, to dry the DNeasy membrane. The extracted DNA could afterwards be eluted into a new 2 ml tube by addition of 100 µl Buffer AE, incubation during 1 min at RT and then centrifugation during 1 min at 8'000 rpm. This was repeated twice, so that in total an amount of 200 µl of dissolved DNA could be used for further analysis. The concentration of extracted DNA was measured by a NanoDrop Spectrophotometer using Buffer AE as a blank.

## Ribosomal RNA-gene amplification by conventional PCR and clean-up of the PCR product

Conventional PCR was done to amplify bacterial 16S rRNA genes or specific chlamydial 16S rRNA genes on one hand and amoebal 18S rRNA genes on the other hand. The Master Mix contained the following reagents respectively and was run with PCR programs that perform 35 cycles.

### Bacterial 16S PCR

|                      |                |
|----------------------|----------------|
| DNA                  | 1 µl           |
| MgCl <sub>2</sub>    | 4 µl           |
| PCR Buffer           | 5 µl           |
| Nucleotide Mix       | 5 µl           |
| Taq polymerase       | 0.2 µl         |
| Forward Primer 616V  | 1 µl           |
| Reverse Primer 1492R | 1 µl           |
| MilliQ               | <u>32.8 µl</u> |
|                      | 50 µl          |

### Chlamydial 16S PCR

|      |                |
|------|----------------|
|      | 1 µl           |
|      | 4 µl           |
|      | 5 µl           |
|      | 5 µl           |
|      | 0.2 µl         |
| PanF | 1 µl           |
| PanR | 1 µl           |
|      | <u>32.8 µl</u> |
|      | 50 µl          |

### Amoebal 18S PCR

|      |                |
|------|----------------|
|      | 1 µl           |
|      | 4 µl           |
|      | 5 µl           |
|      | 5 µl           |
|      | 0.2 µl         |
| 18SF | 1 µl           |
| 18SR | 1 µl           |
|      | <u>32.8 µl</u> |
|      | 50 µl          |

|                  | °C | Sec    |      |
|------------------|----|--------|------|
| Denaturation     | 95 | 5 min  |      |
| Denaturation     | 95 | 30 sec | 35 x |
| Annealing        | 55 | 40 sec |      |
| Elongation       | 72 | 1 min  |      |
| Final Elongation | 72 | 10 min |      |

|                  | °C | Sec     |      |
|------------------|----|---------|------|
| Denaturation     | 95 | 2 min   |      |
| Denaturation     | 95 | 30 sec  | 35 x |
| Annealing        | 65 | 30 sec  |      |
| Elongation       | 72 | 1.5 min |      |
| Final Elongation | 72 | 7 min   |      |

|                  | °C | Sec    |      |
|------------------|----|--------|------|
| Denaturation     | 95 | 2 min  |      |
| Denaturation     | 95 | 45 sec | 35 x |
| Annealing        | 61 | 45 sec |      |
| Elongation       | 72 | 3 min  |      |
| Final Elongation | 72 | 10 min |      |

For the control of the PCR product formation, a gel electrophoresis on a 1.5 % agarose gel was made. A 1:10 KBL (5 µl) was used for PCR product verification and 8 µl PCR product was added to a drop of loading dye and filled into the gel pockets.

To clean up the 16S PCR products, the Qia Quick PCR Purification Kit was applied. A column was filled with a mixture of the 40 µl PCR sample complemented with 200 µl of PB (5 x the volume). After a 1 min 13'000 rpm centrifugation step, the sample was washed with 750 µl PE and centrifuged again for 1 min at 13'000 rpm into a new 2 ml Eppendorf Tube. The DNA was eluted then by addition of 35 µl Elution buffer, 1 min incubation time at RT, before a spin down at 13'000 rpm for 1 min. The obtained DNA concentration could be measured by a NanoDrop Spectrophotometer using Elution buffer as a blank.

A second method was used to clean up the 18S PCR products, the Qia Quick Gel Extraction Kit. 42 µl PCR sample were loaded onto another 1.5 % agarose gel complemented with 7 µl loading dye. The bands were shortly checked under the UV light. As more than one band could be detected the fragment with the appropriate length was excised from the gel using a clean, sharp scalpel, then put into Eppendorf tubes that were previously weighted. 3 volumes of Buffer QC was added to 1 volume gel (100 mg ~ 100 ml) and incubated at 50 °C for 10 min until the gel slice had completely dissolved. 1 gel volume of isopropanol was added to the sample and mixed. To bind the DNA the sample was applied to a Qiaquick spin column and centrifuged for 1 min at 13'000 rpm. The flow-through was discarded and 500 µl of Buffer QC was added. After centrifugation for 1 min at 13'000 rpm and discarding of the flow-through 750 µl Buffer PE was added and centrifuged for 1 min at 13'000 rpm. Again the flow-through was discarded and another centrifugation step followed for 1 min at 13'000 rpm. To elute DNA 50 µl of MilliQ was added to the center of the column and centrifuged for 1 min at 13'000 rpm into a new 1.5 ml Eppendorf tube. This last step was repeated using 30 µl MilliQ.

### **Cloning of the amplified 16S rRNA gene sequence**

As the samples analyzed in this study were no pure cultures, but possibly a more complex composition of diverse bacterial endosymbionts, as is very common among environmental samples, a mere amplification and sequencing of 16S rRNA genes would not be efficient. The diverse PCR products amplified by the universal primers had to be integrated into single cloning vectors. Each recombinant DNA molecule was transferred into a living host organism (typically *E. coli*), and then replicated along with the host DNA, before 16S rRNA gene sequencing.

The Taq DNA polymerase used for the PCR adds an overlapping adenine (dATP) to the 3' end of every amplicate during the last elongation step. Because of the lacking 3'-5' proofreading activity of the Taq DNA polymerase the mistake is not being adjusted. Fortunately, this fact can be used for TA cloning where a vector in its linearized form is used, carrying a complementary 3' thymine overhang (ddTTP) on each blunt end. Hybridization is catalyzed by a topoisomerase. Not every cloning vector incorporates the 16S rRNA insert of interest, thus selection processes are being needed. A newer method, also applied in this study the vector pCR 4 contains two antibiotic resistance genes (kanamycin und ampicillin) and a suicide gene, which kills its host cell. This gene is thought to serve as insertion site, which means that if the plasmid takes up an insert the suicide gene is destroyed and keeps the host organism alive.

The cloning of the PCR products was done according to the TOPO TA Cloning Kit. 4 µl of each amplicate was mixed with 1 µl salt solution and 1 µl suicide vector (pCR 4), before short centrifugation and 15 min incubation at RT.

After a successful ligation, the transformation step was prepared. Chemically competent *E. coli* TOP10 were thawed on ice, SOC-medium was preheated to 37 °C and LB agar plates were supplemented with a final ampicillin concentration of 100 µg/ml. 3 µl of the ligation product was added to the tube with the chemically competent *E. coli* TOP10 cells and stirred carefully in order not to damage the cells. After 30 min incubation on ice, the cells were heat-shocked at 42 °C in a water bath, followed by 2 min incubation on ice. 250 µl SOC-medium was added to the reaction and the tubes were fixed on a shaker for 1 h at 37 °C and 200 rpm. The reaction mix was then plated on the previously prepared LB agar plates, 100 µl and 150 µl each, which were then stored over-night at 37 °C. Because of the suicide gene of the vector pCR 4, only *E. coli* TOP10 that have taken up the vector with an insert can build colonies on the LB-ampicillin agar plates.

***E. coli* transformants:** A 96-well plate was filled with 150 µl LB medium and ampicillin, and inoculated with one clone. On one hand a forward primer T3, on the other hand a reverse primer T7 was applied for each clone in order to obtain the whole sequence of about 1000 bp. The plate was incubated for 4 h with gentle shaking at 37 °C and labeled with the respective barcode, prior to sending it to the company Microsynth for sequencing.

**Purified PCR Products:** In cases where a conventional PCR reaction was sufficient, using the chlamydia-specific forward primer PanF and the reverse primer PanR or the universal forward primer 616V and reverse primer 1492R, a gel was run to ensure, that the insert of interest was present, before purifying the PCR product using the QIA Quick PCR Purification Kit, as described on page 12 and 13. Microsynth requires 22.5 ng per 100 bp in 15 µl total volume, which was aliquoted into Sarstedt screw cap tubes labeled with the respective barcode and send for sequencing.

### 2.6.5 Cultivation of identified *Acanthamoeba* spp. HSC

Continuous axenic cultures of *Acanthamoeba* spp. HSC were transferred from 25 cm<sup>2</sup> polystyrene culture flasks containing 8-10 ml TSY to 500 cm<sup>2</sup> polystyrene culture flasks and fed with 150 ml of the same medium. This is above all necessary to attain a higher biomass that will be needed for the eventual isolation of extracellular endosymbionts. For a good maintenance of the amoeba cultures medium was exchanged every 2 weeks and in general kept at the room-temperature or if needed for experiments cultivated at 20 °C or 27 °C respectively. Regular observations of morphological characteristics by light microscopy guaranteed the usage of a well-grown culture indicated by densely packed amoebae attached to the surface of the culture flask.

### 2.6.6 Curing of *Acanthamoeba* spp. HSC

The identified *Acanthamoeba* sp. cultures infected with the endosymbionts HSC3 and HSC8 were harvested in 12-well plates filled with 2.5 ml TSY. The cultures were treated with different concentrations of 5 classes of antibiotics, in order to eliminate the bacteria while keeping the amoeba culture alive. All antibiotics used have specified mechanisms of action, mentioned in table 2.8. Several antibiotic treatments were applied according to the following table 2.12.

**Table 2.12** Type of antibiotics used for the curing of the *Acanthamoeba* spp. Hosts with concentrations indicated.

| Isolate | Antibiotic   | Final concentration in media |
|---------|--------------|------------------------------|
| HSC3    | Doxycycline  | 50, 70, 100 ng/ml            |
|         | Tetracycline | 50, 70, 100 ng/ml            |
| HSC8    | Rifampicin   | 100 ng/ml                    |
|         | Kanamycin    | 50, 70, 100 ng/ml            |
|         | Ampicillin   | 50, 70, 100 ng/ml            |

The plates were kept at 20 °C and the same concentration of antibiotics was added every 3 days. General condition of the amoebae was checked regularly by light microscopy and infection rates were observed by repeated screening using specific probes for FISH until in at least one well only uninfected *Acanthamoeba* spp. were detected. After 8 weeks of several dilution steps an *Acanthamoeba* spp. culture free of endosymbionts was grown.

### 2.6.7 Defining the host range of endosymbionts

The endosymbionts HSC3 and HSC8 were added at an MOI of 500 to four different *Acanthamoeba* sp. strains as well as to an S2 insect cell line listed in table 2.13. The infection was then incubated for one week and detected by FISH and confocal laser scanning microscopy as described in 2.6.2.

**Table 2.13** List of hosts that were subject to infection with endosymbionts HSC3 and HSC8.

| Host                                 | MOI | Temperature |
|--------------------------------------|-----|-------------|
| <i>Acanthamoeba</i> sp. C1           | 500 | 20 °C       |
| <i>Acanthamoeba</i> sp. 5a2          | 500 | 20 °C       |
| <i>Acanthamoeba polyphaga</i>        | 500 | 20 °C       |
| <i>Acanthamoeba castellanii</i> NEFF | 500 | 20 °C       |
| S2 cells                             | 500 | 27 °C       |

### 2.6.8 Isolation of genomic DNA

Whole-genome sequencing is a useful tool for understanding the evolution, diversity, physiology and biology of symbiosis of the endosymbionts described in this study. Many different methods are available for isolating the total genomic DNA of bacteria. Choosing the appropriate method needs consideration of the organism that the DNA comes from, as well as the yield, purity and integrity of the DNA that is needed in the end for subsequent steps.

In these cases, the bacteria are obligate intracellular endosymbionts of *Acanthamoeba* spp., which means the cultures frequently contain low levels of endosymbiont DNA, yet large amounts of amoebal DNA. Thus the procedure acquires a lot of technical adaptations and precautions.

First of all several 500 cm<sup>2</sup> polystyrene culture flasks of well-grown infected *Acanthamoeba* spp. HSC were gathered into 50 ml Greiner tubes and centrifuged at 6'600 rpm for 5 min at 4°C. The pellets were resuspended in cold 10 ml Buffer A complemented with EDTA and homogenized 15 times with a tight Dounce Homogenizer. The lysate was then spun down at 300 x g for 2 min at 4°C. The supernatant was stored at 4°C, whereas the pellet was resuspended once more in 10 ml Buffer A complemented by EDTA to increase the yield and homogenized 15 times with the tight douncer. The lysate was centrifuged at 300 x g for 2 min at 4°C. All supernatants were then pooled, filtered with 5 µm and centrifuged again at 11'000 rpm for 5 min at 4 °C. The pellet was washed with 5 ml cold Buffer A, centrifuged again at 11'000 rpm for 5 min at 4 °C and resuspended in 1 ml Buffer A. The suspension was then transferred into a new 2 ml Eppendorf tube, where 10 µl DNase 1 (Thermo Scientific) were added. After an incubation of 1h at 4°C or on ice the digestion was stopped by adding 1/10 Vol. 0.5 M EDTA (100 µl) and centrifuged at 11'000 rpm for 5 min at 4 °C. The pellet was washed once with 1 ml cold Buffer A complemented with EDTA, again centrifuged at 11'000 rpm for 5 min at 4 °C and resuspended in 250 µl

TE buffer. The suspension was then mixed with 675 µl of DNA extraction buffer and 20 µl Proteinase K, before incubating it for 30 min at 37°C by inverting once after 15 min. Then, 75 µl of 20% SDS were added and incubated in a 65°C waterbath for 1 h with gentle inversions every 15-20 min. After mixing with an equal volume of room temperature Phenol/Chloroform/Isoamylalcohol (25:24:1, v/v; pH > 7.8), the Eppendorf tube was carefully inverted a few times then centrifuged at 14'000 rpm for 5 min at RT. The aqueous phase was recovered and transferred into a new Eppendorf tube, avoiding any materials from the interphase or phenol. This was mixed with an equal volume of room temperature Chloroform/Isoamylalcohol (24:1, v/v) and again carefully inverted a few times. The following centrifugation step at 14'000 rpm for 5 min at RT produced another aqueous phase that was transferred once more into new Eppendorf tube. A 1/10<sup>th</sup> volume of 3M Sodium acetate or 2M Sodium chloride and 1µl of glycogen was added to the sample. The nucleic acids were then precipitated by adding 0.7 volume of isopropanol at room temperature for at least 1h until they were pelleted by centrifugation at 16'000 x g for 30 min at 4°C. The pellet was washed with 500 µl ice-cold 70% EtOH for 5 min and spun down at 14'000 rpm for 10 min at RT. After that, the pellet was air-dried for 15 min, resuspended in 30 µl Tris/HCl complemented with RNase [1µl RNase (Qiagen, 100 mg/ml) + 1 ml Tris/HCl] and incubated for 20 min at 37°C.

### 2.6.9 HeLa cell infection assay

The *Chlamydiae* comprise the well-known family *Chlamydiaceae* that includes a number of important pathogens causing human diseases, such as *Chlamydia trachomatis* and *Chlamydia pneumonia*. Furthermore, there is evidence for a potential pathogenicity of some environmental chlamydiae, such as *Simkania negevensis* and *Waddlia chondrophila* which have been shown to infect both amoebae and mammalian cells and associated with respiratory and miscarriage in humans. Even some members of the family of *Parachlamydiaceae* seem too have a limited capacity to grow in a number of nonprotozoan host cells (Omsland, 2014). Within the *Betaproteobacteria*, some important agents of a number of human diseases include the pathogenic *Neisseria gonorrhoeae* (causing gonorrhea), *Neisseria meningitides* (the cause of meningococcal meningitis), and the genus *Burkholderia* whose pathogenic members include *Burkholderia mallei* (responsible for glanders, mostly in horses), *Burkholderia pseudomalleia* (causative agent of melioidosis) and *Burkholderia cepacia* (causing pulmonary infections in people with cystic fibrosis). *Ralstonia pickettii* is one of the only two known species of *Betaproteobacteria* shown to naturally infect free-living amoebae, but also acts as an opportunistic pathogen. The pathogenic role of the second obligate endosymbiont of amoebae *Procabacter acanthamoeba* is largely unknown (Gilbert Greub & Raoult, 2004b). Because of the close relation of the endosymbionts of *Acanthamoeba spp.* HSC3 and HSC8 according to the phylogenetic trees shown in 4.2, to the listed pathogens it seemed important to test their ability to not only infect amoebal strains, but also human cell lines, such as HeLa 229. The HeLa cell is a cell type in an immortal cell line, derived from cervical cancer cells and most commonly used in scientific research.

The first step consisted in harvesting and seeding of HeLa 229 cells. The DMEM HeLa cell medium was carefully removed from the culture flasks, then 3 ml DPBS was added to wash cells but was removed instantly. 0.5 ml 1 x TE was added and the cells were incubated for 10 min at 37 °C and 5% CO<sub>2</sub>. Afterwards, 5 ml DMEM were added, the suspension was transferred to 50 ml Greiner and mixed by pipetting. A small aliquot was used for cell counting with a Neubauer haemocytometer, then a concentration of  $7 \times 10^4$  cells per well were transferred onto glass coverslips in a 24-well plate and incubated for several hours at 37 °C until the cells were fully attached. Then the medium was removed and fresh DMEM was added.

For the infection of HeLa 229 cells, extracellular HSC3 and HSC8 respectively were harvested from well grown cultures, filtered with 1.2 µm or 5 µm, resuspended in 5-10 ml DMEM then added to the HeLa cells. The 24-well plate was spun down for 15 min at 37 °C followed by an incubation of 24 h at 37 °C, 5% CO<sub>2</sub>.

A fixation of the infected HeLa 229 cells followed. After 24 h incubation at 37 °C, 5% CO<sub>2</sub> the medium was removed and 1 ml 4% PFA was added to fix the cells during 1 hour at RT. Then 4% PFA was removed again and the wells were washed with 1 ml DPBS. The glass coverslips were transferred to a new 24-well plate containing 1 ml DPBS/well and could be stored at 4 °C.

The DPBS was removed and an EtOH series was applied, starting with a 0.5 ml 50% EtOH solution incubated for 3 min at RT and removed. Then 0.5 ml 80% EtOH was added incubated for 3 min at RT, removed and finally 0.5 ml 96% EtOH was added, incubated for 3 min at RT, removed again before the coverslips were let to dry out completely. For fluorescent in-situ hybridization 200 µl hybridization buffer and 20 µl probe were added to each well and the plate was sealed with PCR film, green tape and aluminum foil. Hybridization takes place in an oven for 3 h at 46 °C. 1.5 ml preheated washing buffer (48 °C) was added to each well of a new 24-well plate, the coverslips were transferred to the wells and incubated for 10 min at 48 °C. Then the coverslips were tipped in ice-cold MilliQ water and dried under compressed air, before they were embedded upside down on glass slides in mowiol. The slides were let to dry overnight.

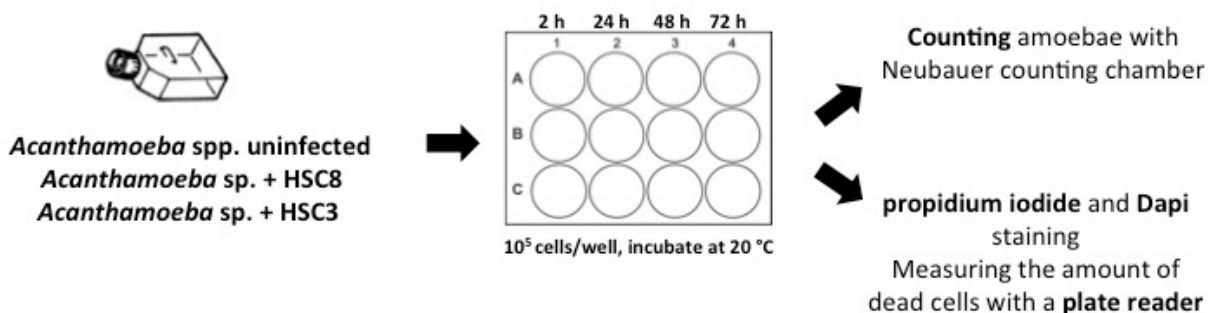
#### **2.6.10 Growth comparison**

A growth comparison between endosymbiont-free *Acanthamoeba* spp. HSC and infected *Acanthamoeba* spp. HSC was used to study the influence of the endosymbionts on amoebal growth. In parallel a live/dead staining with propidium iodide was applied to analyze a possible host cell lysis caused by the endosymbionts.

Propidium iodide (PI) binds to DNA by intercalating between the bases with little or no sequence preference and is membrane impermeant, which means it is generally excluded from viable cells. PI is commonly used for identifying dead cells in a population, in this case as a parameter for host cell lysis

(Riccardi & Nicoletti, 2006). Due to its sufficiently large shift, it allows simultaneous detection of nuclear DNA, for example by DAPI staining targeting the total amount of DNA.

First of all, endosymbiont-free *Acanthamoeba* spp. HSC as well as infected *Acanthamoeba* spp. HSC were collected for a growth comparison, starting with a centrifugation step at 6'000 rpm for 6 min, taken up in 10 ml TSY and an amount of  $10^5$  cells per well were distributed into a 12-well plate (three wells per time point).



**Figure 2.4** An experimental setup for the comparison of HSC8 or HSC3 infected *Acanthamoeba* spp. and their uninfected original *Acanthamoeba* spp. hosts by making growth curves and measuring the lytic activity of the endosymbionts.

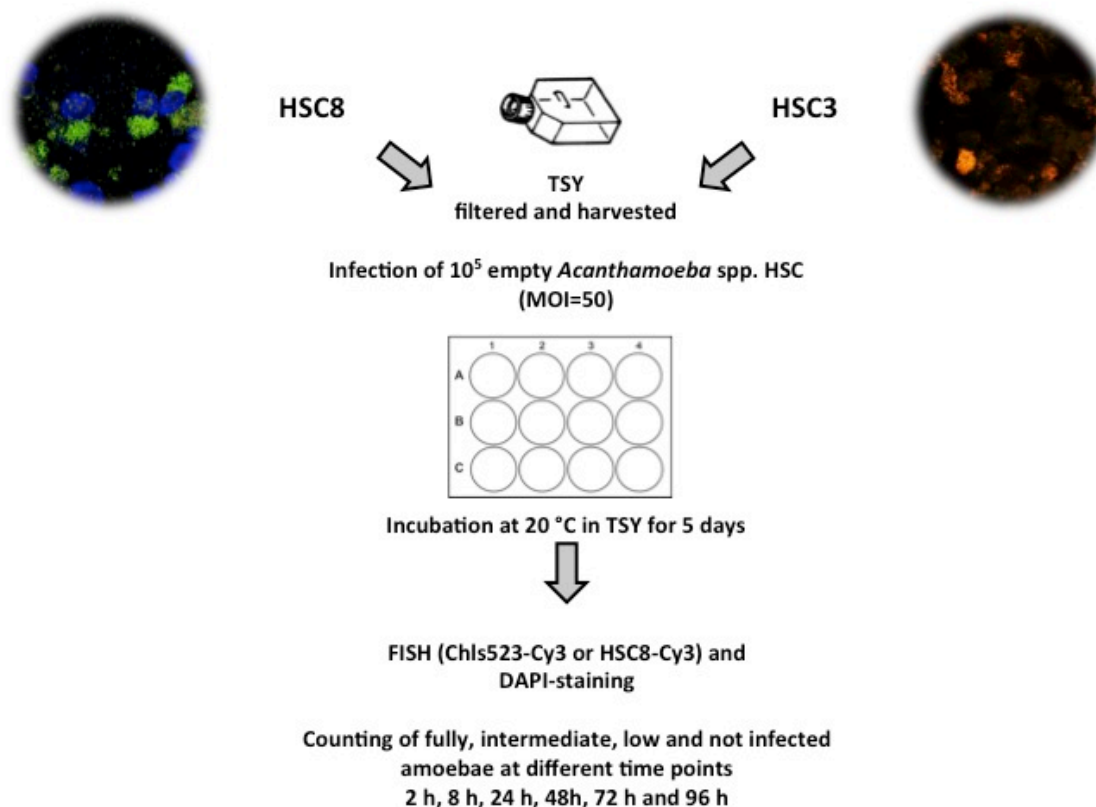
After 2 h, 24 h, 48 h and 72 h incubation at 20 °C in 2 ml TSY, amoebae were harvested in 2 ml Eppendorf tubes. A small amount was used for quantification with a Neubauer haemocytometer, while the remaining cells were stained with propidium iodide (PI) for live/dead differentiation. First, the samples were spun down using an Eppendorf 5804R microcentrifuge at 6'000 rpm for 6 min at RT. The supernatant was decanted and the pellet resuspended in 100 µl 1 x PAS with 1 µM PI solution prior to incubation for 20 min in the dark at RT. For additional quantification of the total DNA, 10 µl of a 1:10'000 dilution of DAPI solution was added together with the PI solution. After another centrifugation step at 6'000 rpm for 6 min at RT the pellet was resuspended in 150 µl 1 x PAS and transferred to a black 96-well plate reader dish. The PI and DAPI fluorescence intensity was measured with a Tecan Infinite M200 microplate reader according to the following protocol.

**Table 2.14** Protocol for the measuring of PI and DAPI fluorescence intensity.

|                                 | <b>Propidium iodide</b>   | <b>DAPI</b>               |
|---------------------------------|---------------------------|---------------------------|
| <b>Shaking</b>                  | 5 s                       | 5 s                       |
| <b>Fluorescence top reading</b> | without lid               | without lid               |
| <b>Gain</b>                     | 100                       | 125                       |
| <b>Reads</b>                    | 4 reads per well          | 4 reads per well          |
| <b>Excitation wavelength</b>    | A <sub>exc</sub> = 535 nm | A <sub>exc</sub> = 350 nm |
| <b>Emission wavelength</b>      | A <sub>em</sub> = 617 nm  | A <sub>em</sub> = 470 nm  |

### 2.6.11 Infection cycles

To analyze the endosymbiont's infection cycle, empty *Acanthamoeba castellanii* NEFF as well as the original *Acanthamoeba* spp. HSC host were collected by centrifugation at 6'000 rpm for 6 min, taken up in 10 ml TSY and an amount of  $10^5$  cells/well were filled into a 12-well plate (three wells per time point). Extracellular HSC8 were filtered from the supernatant of an *Acanthamoeba* spp. culture with 2 x 5 µm syringe filters, and then centrifuged at 7'500 rpm for 6 min. The pellet was resuspended in a small volume of fresh culture medium TSY of which 10 µl were dissolved in PAS and stained with 500 µl DAPI (dilution 1: 5'000) for quantification and determination of MOI (average cell count x  $15'126$  x Dilution = cells/ml). The same procedure was done with extracellular HSC3, except they were isolated with 1 x 5 µm and 1 x 1.2 µm syringe filters, centrifugation steps were done at  $12'800 \times g$  for 10 min. Extracellular HSC8 or HSC3 respectively were added to endosymbionts-free *Acanthamoeba* spp. at a multiplicity of infection of 50, incubated for 15 min at RT followed by centrifugation at  $130 \times g$  during 15 min. The infection was then incubated at 21 °C for 2 h, washed with PAS and fresh culture medium was added. Cells were fixed onto microscope slides after 2 h, 8 h, 24 h, 48 h, 72 h and 96 h incubation using 4 % formaldehyde, followed by a washing step with MilliQ. Cells were visualized by FISH using either the HSC8-specific probe or the *Chlamydia*-specific probe. Amoebae were counted by distinguishing fully infected cells (> 30 bacteria), intermediate infected cells (6-30 bacteria), low infected cells (1-6 bacteria) and uninfected cells.



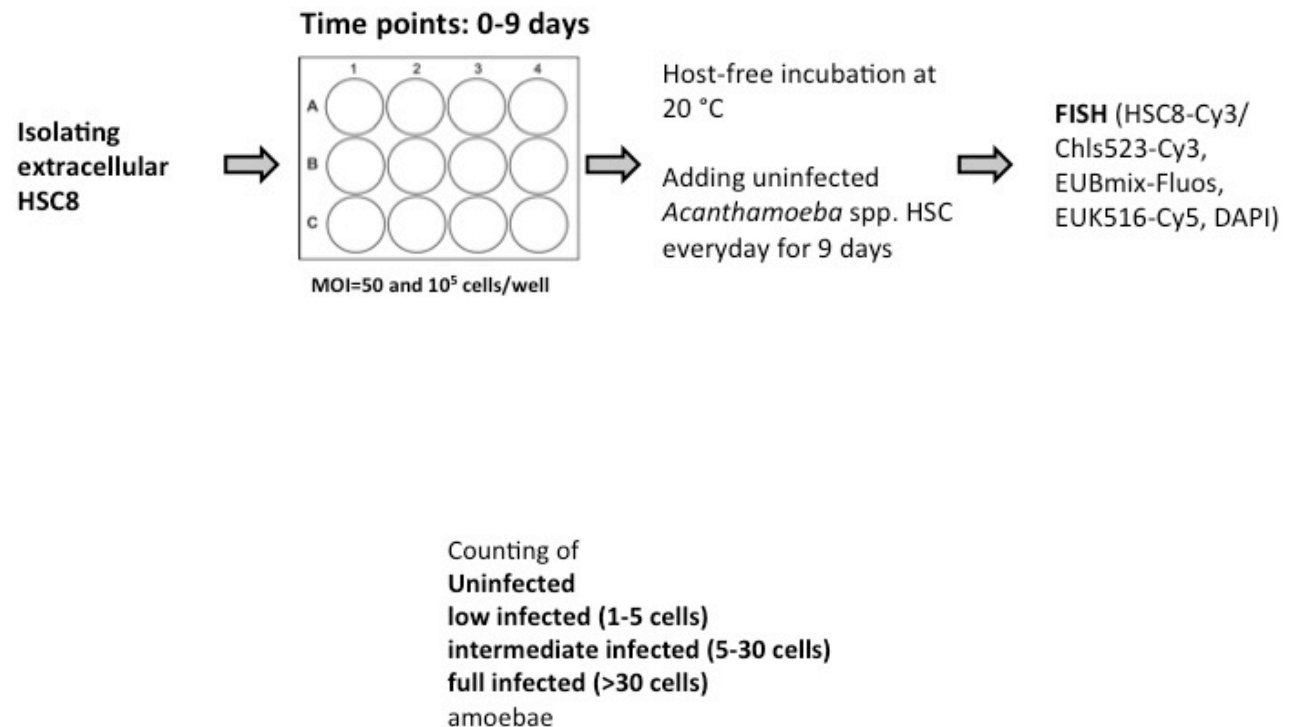
**Figure 2.5** Analysis of the infection cycles of the endosymbionts of *Acanthamoeba* spp. HSC8 and HSC3 in uninfected *Acanthamoeba* spp. hosts.

### 2.6.12 Extracellular infectivity Assay

The endosymbiont of *Acanthamoeba* sp. HSC8 from simple observation seems to be a novel obligate intracellular bacterium of free-living amoebae. In order to demonstrate a somehow limited extracellular survival and maintenance of infectivity in the rich media TSY, the following assay was planned.

Extracellular HSC8 were harvested from a host cell culture and filtered twice with 5  $\mu$ m syringe filters, then collect by centrifugation at 7'500 rpm for 6 min. The pellet was resuspended in a small volume of fresh TSY of which 10  $\mu$ l of the suspension was diluted in 5 ml PBS for quantification with DAPI (500  $\mu$ l of a 1:5000 dilution in PBS). The bacterial suspension was then transferred into 12-well plates for extracellular incubation at 20 °C, at a final multiplicity of infection of 50. In order to investigate the survival and maintenance of infectivity, empty *Acanthamoeba* sp. HSC were added in an amount of  $10^5$  cells per well after 2 h, 24 h, 48 h, 72 h and 96 h. The amoebae were fixed then after 48 h onto microscope slides using 4 % formaldehyde, followed by a washing step with MilliQ. Cells were visualized by FISH using a HSC8-specific probe. Amoebae were counted by distinguishing fully infected cells (> 30

bacteria), intermediate infected cells (6-30 bacteria), low infected cells (1-6 bacteria) and uninfected cells.



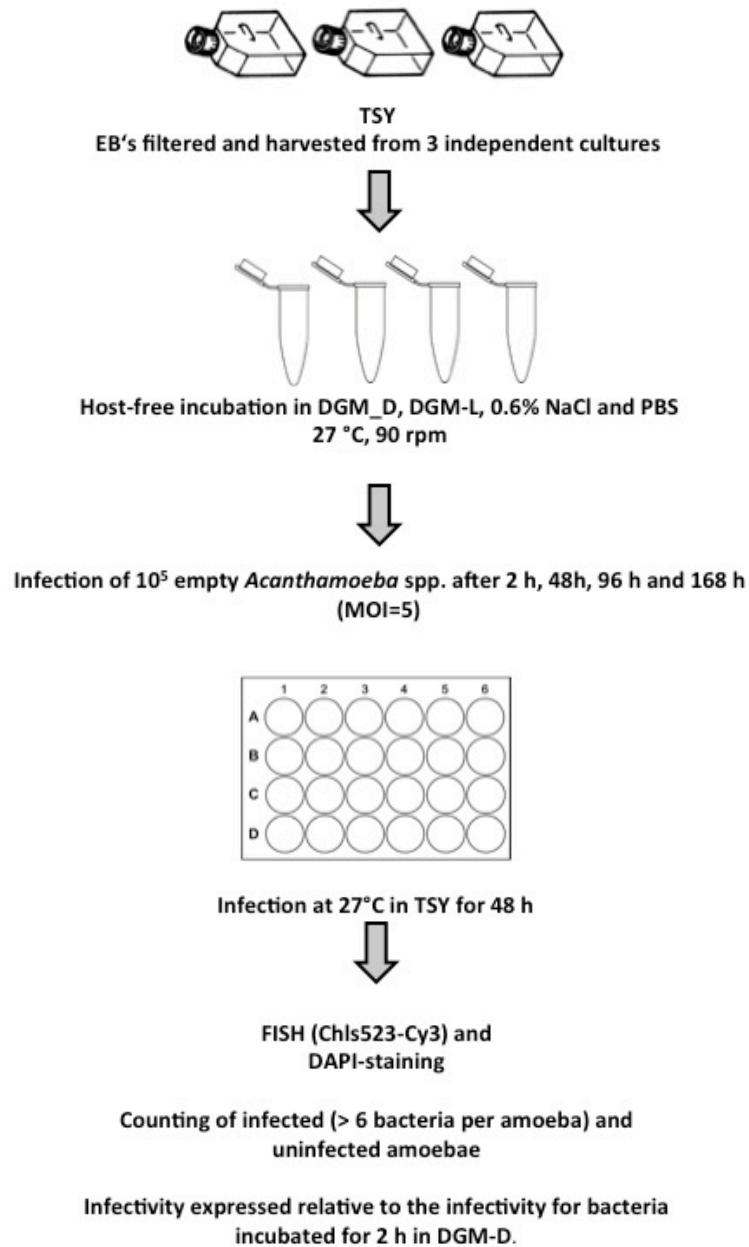
**Figure 2.6** Experimental set-ups for the analysis of host-free survival and maintenance of infectivity of the endosymbiont of *Acanthamoeba* sp. HSC8, by calculating the infectivity rates of different incubation times.

### 2.6.13 Glucose Assay

The endosymbiont of *Acanthamoeba* sp. HSC3 as is shown later in the phylogenetic tree under 4.2 clusters together with *Protochlamydia acanthamoebae* UWE25 within the family *Parachlamydiaceae*. *Protochlamydia acanthamoebae* UWE25 is one of the better studied environmental chlamydiae and former findings indicate that *Protochlamydia acanthamoebae* UWE25 EBs depend on the availability of D-glucose in a host-free environment that is converted to D-glucose-6-phosphate by a self-encoded glucokinase (Sixt et al., 2013). To investigate whether the metabolic activities of the closely related endosymbiont of *Acanthamoeba* sp. HSC3 EBs act in a similar manner an assay for substrate availability was applied.

The endosymbiont HSC3 was harvested from host cell culture and filtered with 1.2 µm syringe filters, then collected by centrifugation at 12'800 x g for 10 min. The pellet was washed with 20 ml PBS and resuspended in a small volume of PBS. 10 µl of the suspension was diluted in 5 ml PBS for quantification with DAPI (500 µl of a 1:5000 dilution in PBS). The bacterial suspension was then transferred into 1.5 ml Eppendorf tubes for parallel host-free incubation in triplicates of different media. To investigate the importance of nutrient availability for HSC3's host-free survival, these media included the nutrient-rich DGM-D containing D-glucose as a supplement, a modified medium DGM-L, in which D-glucose is replaced by L-glucose as well as a nutrient-free salt solution 0.6 % NaCl (0.1 M or 100 mM) and a buffer PBS of same pH and osmolarity. All of them were incubated at 27 °C and 90 rpm for 2 h, 48 h, 94 h, and 168 h respectively, then added to 10<sup>5</sup> amoebae in 24-well plate at MOI=5 containing 1 ml TSY medium.

The plate was incubated for 15 min at 27 °C followed by spinning down at 130 x g for 15 min at 23 °C. Fixation happened after 48 h of incubation at 27 °C using 4% formaldehyde, followed by a washing step with MilliQ. Cells were visualized by FISH using the *Chlamydiae*-specific probe ChIs-0523. Infected amoebae containing at least six intracellular bacteria were counted (~600 cells per sample) and infectivity was expressed relative to the infectivity of bacteria incubated for 2 h in DGM-D.



**Figure 2.7** Experimental set-ups for the analysis of host-free survival and the maintenance of infectivity of the endosymbiont of *Acanthamoeba* sp. HSC3 in the presence or absence of different nutrients.

### 3. Results

#### 3.1 Isolation of free-living amoebae using non-nutrient agar plates seeded with Gram-negative food bacteria and axenic cultivation

The two environmental samples analyzed in this study were a green colored biofilm of a Hawaiian sea cave wall and a sample from a Hawaiian coral sand beach. In total 15 amoebae cultures were isolated from the biofilm of the littoral cave. In order to include possible growth preferences of amoebae into the study, we chose two distinct incubation temperatures. Six amoebae cultures were incubated at RT and nine cultures were grown at 27 °C. Three of the RT cultures were axenized successfully the other 3 were no well-grown cultures. Two of the three axenized cultures harbored endosymbionts. Simultaneously, seven out of nine amoebae cultures grown at 27 °C were axenized and two of them carried intracellular bacteria. No amoebae were isolated from the sample of coral sand.

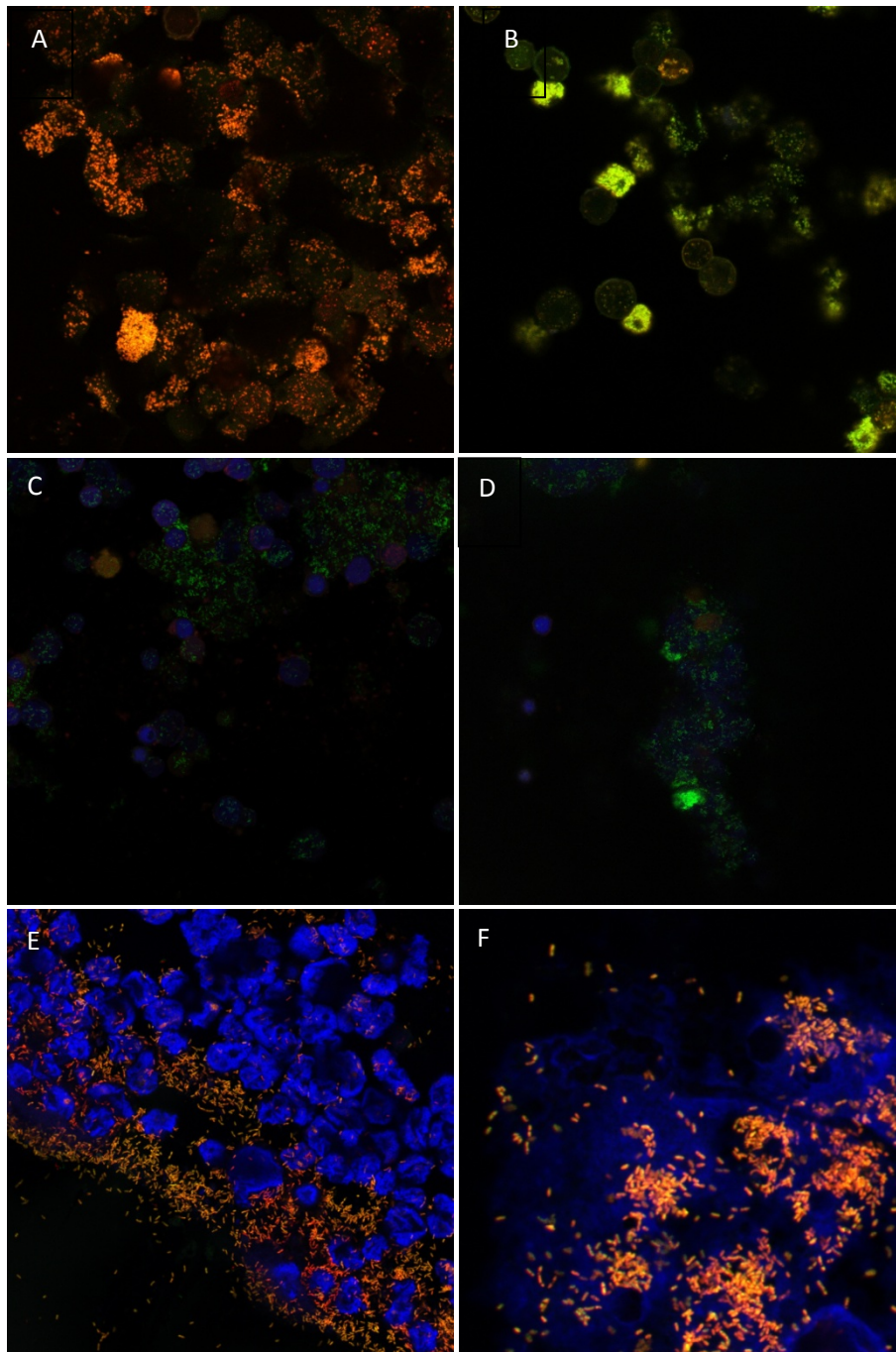
**Table 3.1 The isolation of amoebae from two environmental samples, axenization and the following detection of endosymbionts.** In total 15 amoebae cultures were isolated from the biofilm of the littoral cave of which four were shown to carry intracellular bacteria.

| Environmental Sample  | Incubation T (°C) | Amoebae cultures              | Axenization | Infection |
|-----------------------|-------------------|-------------------------------|-------------|-----------|
| I – Littoral cave     | RT                | 3 unknown                     | -           | -         |
|                       |                   | 1 uninfected amoebae culture  | +           | -         |
|                       |                   | 2 cultures with endosymbionts | +           | +         |
|                       | 27 °C             | 2 unknown                     | -           | -         |
|                       |                   | 5 uninfected amoebae cultures | +           | -         |
|                       |                   | 2 cultures with endosymbionts | +           | +         |
| II – Coral sand beach | RT                | No culture                    | -           | -         |
|                       | 27 °C             | No culture                    | -           | -         |

## 3.2 Identification of novel endosymbionts of *Acanthamoeba* spp.

### 3.2.1 Fluorescence in-situ hybridization results

For a first insight into the presence of endosymbionts all amoebae cultures were subject to a screening by Fluorescence *in-situ* hybridization. This method gives then additional information about the morphology, size and a hint of intracellular location of bacteria as well as the amoeba hosts.



**Figure 3.1** (A-B) Isolates HSC3 hybridized with *Chlamydiales* specific probes in Cy3, Chls-0523. (C-D) Isolates HSC6, hybridized with probes that are specific for *Rickettsiales*-related microorganisms in Cy3, AcRic90 and Rick1395. (E-F) Isolates HSC8 and HSC9 hybridized with EUB338-mix in FLUOS, targeting most bacteria.

In Figure 3.1, the different endosymbionts are shown inside their natural *Acanthamoeba* sp. HSC host. Confocal laser scanning microscopic analysis revealed that *Acanthamoeba* sp. HSC trophozoites are fully infected with coccoid, chlamydia-like endosymbionts that reside in single-cell inclusion vacuoles (figure 3.1 A and B). Two morphological forms could be differentiated; an extracellular stage which is slightly smaller than the intracellular one of about 0.5-0.7  $\mu\text{m}$ . Figure 3.1 C and D show the identity and intracellular location of a second isolate using FISH in combination with laser scanning microscopy. The endosymbionts were more or less rod-shaped, cytoplasmic and varied in size. A third amoebae culture labelled with FISH and subsequently analyzed with confocal laser scanning microscopy was shown to harbor rod-shaped bacteria spread evenly throughout the cytoplasm of its *Acanthamoeba* sp. HSC host. The trophozoites were fully infected and additionally plenty of extracellular bacteria could be detected in the medium. (figure 3.1 E and F)

### 3.2.2 16S/18S rRNA gene sequencing results of amoeba cultures harboring endosymbionts

There were several amoebae isolated from the Non-nutrient agar plates, harboring different intracellular bacteria. First of all, the endosymbiont of *Acanthamoeba* sp. HSC3 was identified as a close relative of the endosymbiont of *Acanthamoeba* sp. UWE1 with a sequence similarity of 99% and belongs to the order *Chlamydiales*. It was isolated from the *Acanthamoeba* sp. UWE1 from a soil sample in western Washington State. Details are indicated in Table 3.2. The best blast hit according to 16S rRNA gene sequencing of the endosymbiont of *Acanthamoeba* sp. HSC6 was shown to be the endosymbiont of *Acanthamoeba* sp. UWE8 as well as the *Acanthamoeba* sp. UWC36 with a maximum identity of 99%. Both isolates were recovered from the *Acanthamoeba* sp. UWC8 and *Acanthamoeba* sp. UWC36 respectively found in infected human corneal tissues and assigned to the order *Rickettsiales*. The two rod-shaped endosymbionts of *Acanthamoeba* sp. HSC8 and HSC9 were found to be identical with 100 % sequence similarity and as-yet unknown and uncultured bacteria. According to the 16S rRNA gene sequencing both cluster in the suborder *Betaproteobacteria* and the best blast hit was given with 94 % sequence similarity as uncultured proteobacterium clones sf-42 and sf-25. Table 3.2 gives the detailed information of all best blast hits for the endosymbionts of *Acanthamoeba* sp. HSC3, HSC6, HSC8 and HSC9, such as the maximum identity, accession number, habitat and reference.

**Table 3.2 Isolated, identified endosymbionts by 16S or 18S rRNA gene sequencing.**

| Isolate      | Best blast hit based on 16S rRNA gene sequencing | Max. Identity | Accession number | Habitat                                                        | Reference              |
|--------------|--------------------------------------------------|---------------|------------------|----------------------------------------------------------------|------------------------|
| <b>HSC3</b>  | Endosymbiont of <i>Acanthamoeba</i> sp. UWE1     | 99 %          | AF083614         | <i>Acanthamoeba</i> sp. UWE1<br>soil, western Washington State | Fritsche et al. (2000) |
| <b>HSC6</b>  | Endosymbiont of <i>Acanthamoeba</i> sp. UWC8     | 99 %          | AF069963         | <i>Acanthamoeba</i> sp. UWC8<br>human corneal tissue           | Fritsche et al. (1999) |
|              | Endosymbiont of <i>Acanthamoeba</i> sp. UWC36    | 99 %          | AF069962         | <i>Acanthamoeba</i> sp. UWC36<br>human corneal tissue          | Fritsche et al. (1999) |
| <b>HSC8</b>  | Uncultured beta proteobacterium clone sf-42      | 94 %          | JQ278953         | groundwater                                                    | Unpublished            |
|              | Uncultured beta proteobacterium clone sf-25      | 94 %          | JQ278943         | groundwater                                                    | Unpublished            |
|              | Uncultured betaproteobacterium clone 26-2_44     | 92 %          | FJ517704         | epithelium of hydra                                            | Fraune et al. (2009)   |
|              | Uncultured betaproteobacterium clone 14-1_27     | 92 %          | FJ517688         | epithelium of hydra                                            | Fraune et al. (2009)   |
| <b>HSC9*</b> | Uncultured beta proteobacterium clone sf-42      | 94 %          | JQ278953         | groundwater                                                    | Unpublished            |
|              | Uncultured beta proteobacterium clone sf-25      | 94 %          | JQ278943         | groundwater                                                    | Unpublished            |
|              | Uncultured betaproteobacterium clone 26-2_44     | 92 %          | FJ517704         | epithelium of hydra                                            | Fraune et al. (2009)   |
|              | Uncultured betaproteobacterium clone 14-1_27     | 92 %          | FJ517688         | epithelium of hydra                                            | Fraune et al. (2009)   |

\*Sequenced with only forward primer

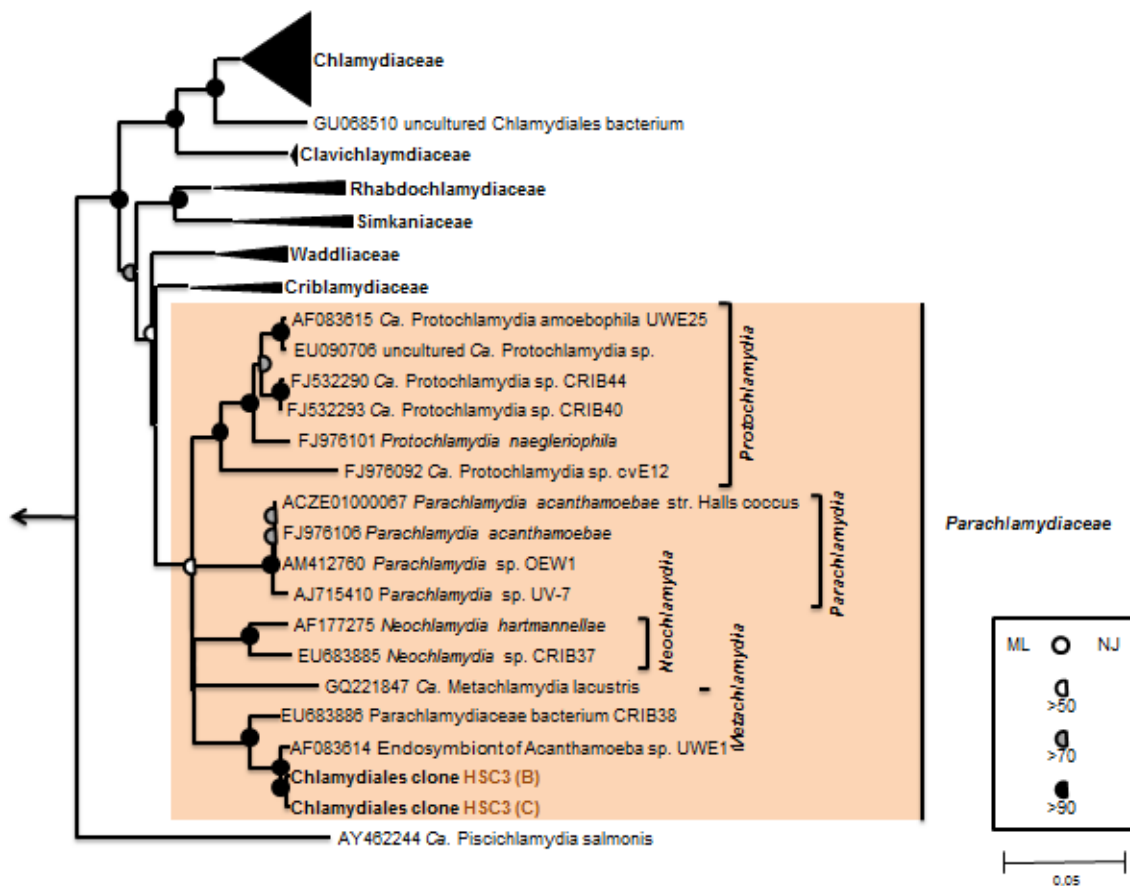
The native amoebae hosts of the endosymbionts HSC3, HSC6, HSC8 and HSC9 were found to be identical with a sequence similarity of 100 %. Based on 18S rRNA gene sequencing the best blast hit was given with 100 % maximum identity as *Acanthamoeba* sp. KA/MSG18 and KA/MSG10.

**Table 3.3 Isolated, identified hosts based on 18S rRNA gene sequencing.**

| Isolate    | Best blast hit based on 18S rRNA gene sequencing | Max. Identity | Accession number | Habitat         | Reference   |
|------------|--------------------------------------------------|---------------|------------------|-----------------|-------------|
| <b>HSC</b> | <i>Acanthamoeba</i> sp. KA/MSG18                 | 100 %         | AY173009         | Marine Sediment | Unpublished |
|            | <i>Acanthamoeba</i> sp. KA/MSG10                 | 100%          | AY173005         | Marine Sediment | Unpublished |

### 3.3 Phylogenetic analysis

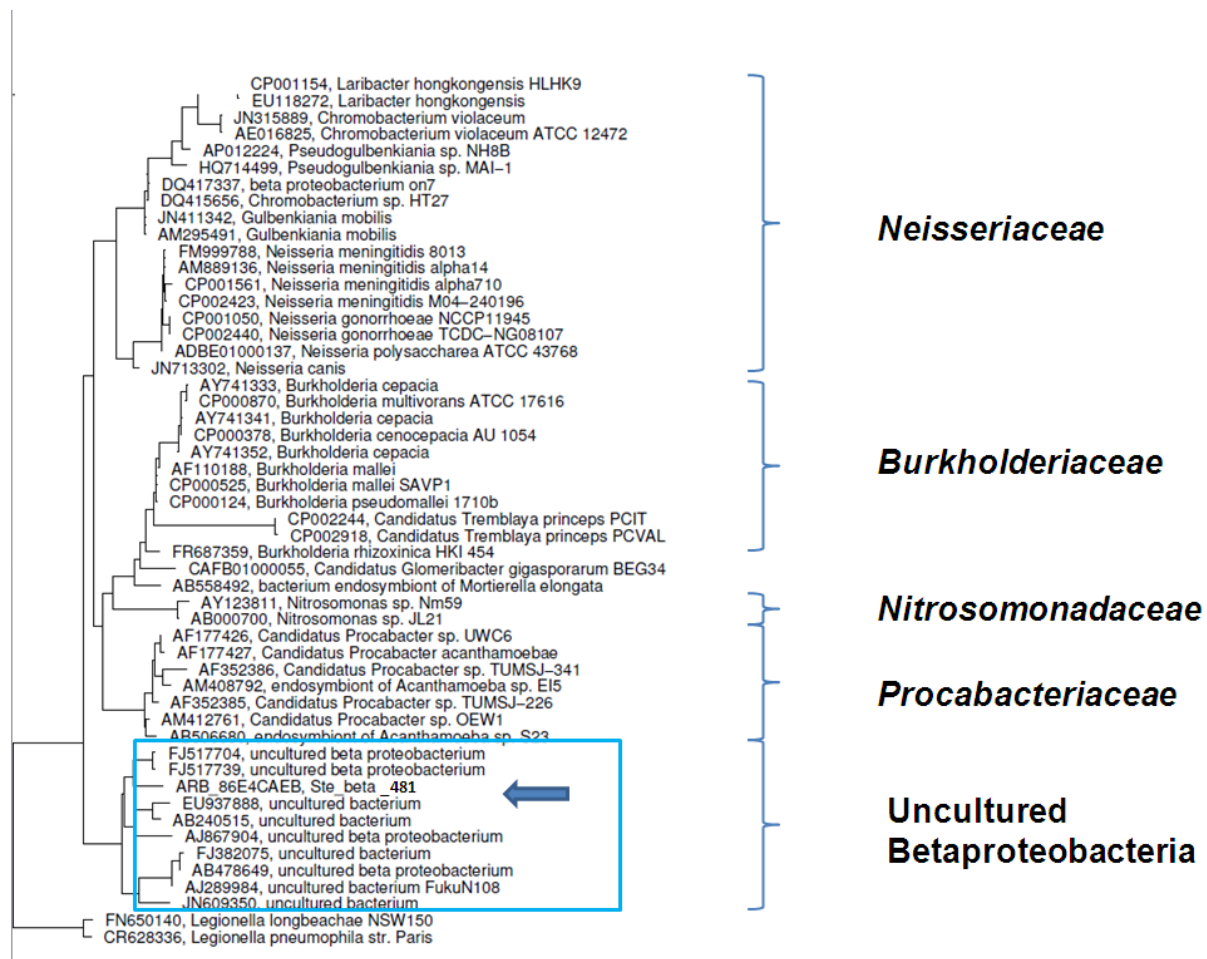
Comparative sequence analysis using maximum-likelihood and neighbor joining revealed the phylogenetic relationships of the endosymbiont isolates to other bacteria. The endosymbiont of *Acanthamoeba* sp. HSC3 showed 99 % sequence identity with each other and were classified as members of the *Chlamydiales* order. Blast search revealed a 99 % sequence similarity with the endosymbiont of *Acanthamoeba* sp. UWE1 isolated from a soil sample from western Washington State (Fritsche et al., 2000). Phylogenetic analysis confirmed that the strains clustered with other members of the *Chlamydiales* and moreover formed a lineage with the endosymbiont of *Acanthamoeba* sp. UWE1, *Parachlamydia* bacterium CRIB38 and more distantly *Ca. Metachlamydia lacustris*, *Neochlamydia hartmannellae* and *Neochlamydia* sp. CRIB37.



**Figure 3.2** 16S rRNA based phylogenetic tree of the endosymbiont of *Acanthamoeba* sp. HSC3. A maximum-likelihood and neighbor-joining dendrogram shows phylogenetic relationships of the sequenced endosymbiont of *Acanthamoeba* sp. HSC3 to other members of the *Chlamydiales*.

The endosymbiont of *Acanthamoeba* sp. HSC6 appears to be related to members of the alpha subclass of *Proteobacteria*, among which the closest neighbors are the endosymbiont of *Acanthamoeba* sp. UWC8 and the endosymbiont of *Acanthamoeba* sp. UWC36 with a sequence identity of 99 %, as well as *Rickettsia australis*, *Rickettsia sibirica* and *Rickettsia typhi* (Fritsche et al., 1999). Further analysis reveals that the endosymbiont of *Acanthamoeba* sp. HSC6 form an independent, distinct lineage within the *Rickettsiales*, together with the UWC8 and UWC36 endosymbionts.

Comparative sequence analysis reveals that the two endosymbiont of *Acanthamoeba* sp. HSC8 and HSC9 were almost identical with a sequence identity of 99% and assembled with members of the *Betaproteobacteria*. When taking a closer look the isolate does not only show close relationship with members of *Procabacteriaceae*, *Nitrosomonadaceae* and *Neisseriaceae* but formed a distinct lineage with a number of uncultured *Betaproteobacteria* among which the closest relative has a sequence similarity of 94 %. Therefore this isolate might represent a novel species and most likely even a novel family within the *Betaproteobacteria*.



**Figure 3.3 'Endosymbiont of *Acanthamoeba* sp. HSC8'.** 16S rRNA based phylogenetic relationships of the 'endosymbiont of *Acanthamoeba* sp. HSC8' to other members of the *Betaproteobacteria*.

### 3.4 Host range of the endosymbionts HSC3 and HSC8

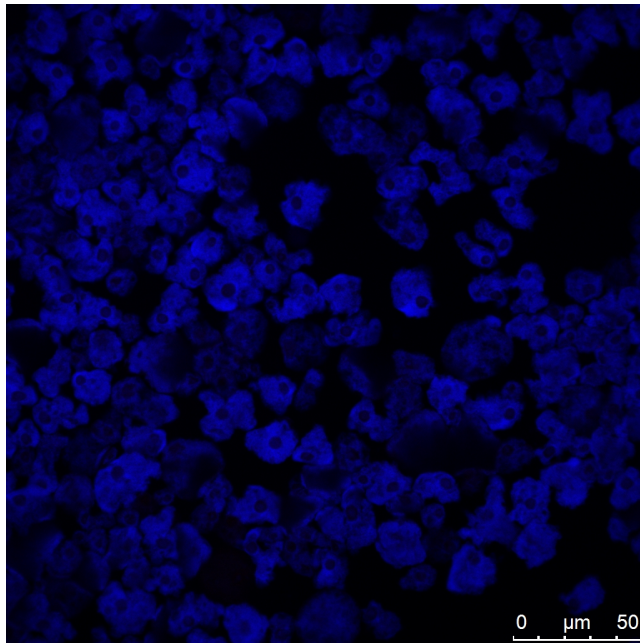
#### 3.4.1 Curing of the natural *Acanthamoeba* sp. HSC host

The original *Acanthamoeba* sp. HSC host was successfully cured from its endosymbionts HSC3 and HSC8 respectively. Cultures were cured from HSC8 with Rifampicin and Kanamycin, whereas HSC3 was eliminated by Doxycyclin without harming the amoeba hosts.

**Table 3.4.** List of Antibiotics that successfully freed acanthamoeba cultures from endosymbionts HSC3 and HSC8 respectively.

| Target organism | Antibiotic  | Mechanism                                                            | Final concentration | Successful curing | Observations                                                |
|-----------------|-------------|----------------------------------------------------------------------|---------------------|-------------------|-------------------------------------------------------------|
| <b>HSC8</b>     | Ampicillin  | Inhibition of transpeptidase needed for cell wall synthesis          | 50, 70, 100 ng/ml   | No                | No curing                                                   |
|                 | Kanamycin   | Interaction with bacterial 30S RSU and inhibition of translocation   | 50, 70, 100 ng/ml   | Yes               | Amoebae in very good condition                              |
|                 | Rifampicin  | Inhibition of bacterial DNA-dependent RNA-polymerase (RNA synthesis) | 100 ng/ml           | Yes               | Amoebae in good condition but several reinfections observed |
| <b>HSC3</b>     | Tetracyclin | Binding of bacterial 30S RSU and blocking of charged aminoacyl-tRNA  | 50, 70, 100 ng/ml   | No                | No curing                                                   |
|                 | Doxycyclin  | Binding of bacterial 30S RSU and Aminoacyl-tRNA                      | 50, 70, 100 ng/ml   | Yes               | Amoebae in good condition                                   |

The uninfected culture is represented in the image made with confocal laser scanning microscope (figure 3.4). The blue Cy5 signal was the only one detected, showing the typical acanthamoeba trophozoite morphology. The absences of FLUOS or Cy3 signals indicate that all endosymbionts were eliminated from the amoebae culture.



**Figure 3.4 Cured natural *Acanthamoeba* sp. HSC host of endosymbiont HSC8.** The bacteria were eliminated by the antibiotic Rifampicin and the amoebae were visualized with FISH using the probes HSC8-Cy3, EUB338-Fluos and Acanth412a-Cy5.

### 3.4.2 Infection of different *Acanthamoeba* spp., human and insect cell lines

In order to evaluate the potential of the endosymbiont of *Acanthamoeba* sp. HSC8 and the endosymbiont of *Acanthamoeba* sp. HSC3 to infect cells other than the original *Acanthamoeba* spp. HSC host, purified bacteria were added to various *Acanthamoeba* strains and cell lines. All four *Acanthamoeba* spp. listed in table 3.5 were susceptible to both symbionts, and reached an infection rate of 100 % after seven days. In contrast, the human HeLa 229 cell line and the *Drosophila* Schneider 2 (S2) cells could not be permanently infected by either of them.

**Table 3.5 Infection of various *Acanthamoeba* spp., human and insect cell lines.**

| Host                                 | Endosymbiont of<br><i>Acanthamoeba</i> sp. HSC3 | Endosymbiont of<br><i>Acanthamoeba</i> sp. HSC8 |
|--------------------------------------|-------------------------------------------------|-------------------------------------------------|
| <i>Acanthamoeba</i> sp. C1           | +                                               | +                                               |
| <i>Acanthamoeba</i> sp. 5a2          | +                                               | +                                               |
| <i>Acanthamoeba polyphaga</i>        | +                                               | +                                               |
| <i>Acanthamoeba castellanii</i> NEFF | +                                               | +                                               |
| HeLa 229 cells                       | -                                               | -                                               |
| S2 cells                             | -                                               | -                                               |

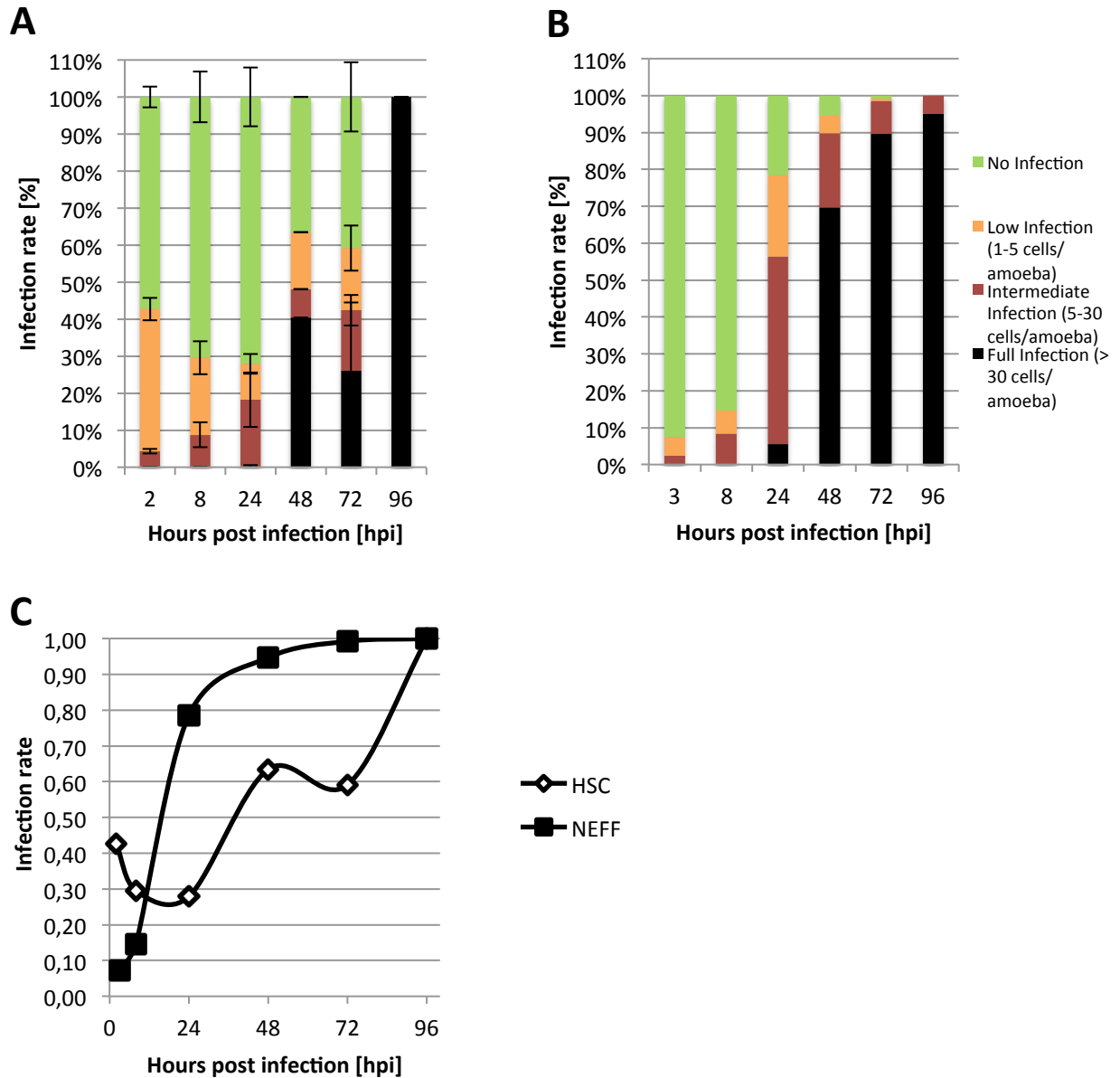
## 3.5 Characterization of Endosymbiont of *Acanthamoeba* sp. HSC3

### 3.5.1 The endosymbiont HSC3 has a biphasic infection cycle

Fluorescence *in situ* hybridization and phase contrast microscopy gave insights into the infection cycle of the chlamydia endosymbiont HSC3 was in its natural *Acanthamoeba* sp. HSC host as well as in *Acanthamoeba castellanii* NEFF. Cell morphologies, infection levels and metabolic activity of the endosymbiont HSC3 were illuminated by these synchronized infection assays. In a second part, the infection rates of HSC3 in its original host was compared to the infection levels of HSC3 in the laboratory strain *A. castellanii* in a second experimental set up. At different time points before and after infection, the overall cell condition was determined visually. Most cells were attached to the surface and in trophozoite cell stage, indicating well grown amoeba cultures.

As we distinguished low infected, intermediate infected and fully infected *Acanthamoeba* spp., these numbers were expressed in relation to each other as well as to the number of uninfected *Acanthamoeba* spp. The infection rate was calculated for both amoebae cultures and at several time points after infection by counting all infected amoebae and dividing it by the total number of cells including the uninfected amoebae. The average number of counted uninfected, low infected, intermediate infected and fully infected amoebae as well as the infection rate throughout one full infection cycle is illustrated in figure. The infection rate of HSC3 in *Acanthamoeba castellanii* NEFF was then compared to the infection levels in the original *Acanthamoeba* sp. HSC host.

The infection of HSC3 in *Acanthamoebae* sp. HSC started with a percentage of 42.7 % of which mostly all amoebae were only low infected at 2 hpi. Only 4.3 % of the amoebae contained more than 5 particles. At this early time point the particles were hardly detectable by FISH, but the DAPI signals showed small coccoid bacteria very close to the hosts outer membrane. After 24 h most FISH signals correlated with the DAPI signals and the morphology of the particles switched to slightly bigger coccoid-shaped cells. At later time points after 48 hpi a larger number of amoebae were fully infected with chlamydiae. Up to 40.4 % of the hosts harbored over 30 particles seemingly distributed throughout the cell in single cell inclusions. Additionally the proportion of infected amoebae slowly increased up to 63.5 %. The infection cycle seemed to be complete after 96 hpi, indicated by 100 % full infection and a larger number of particles in the amoebae surrounding medium.



**Figure 3.5 The course of infection of the endosymbiont HSC3 in different *Acanthamoeba* spp. strains.** Percentage of amoebae containing HSC3 over 96 hours post infection. Green: No infection meaning that no bacteria had infected a host cell. Orange: Low infection, 1-5 symbionts have entered a cell. Red: Intermediate infection, 5-30 cells per amoebae were observed. Black: Full infection for > 30 cells per host. **(A)** The infection cycle of HSC3 in the original host *Acanthamoeba* sp. HSC. The number of infected cells increased over time and 96 hours post infection (hpi) all acanthamoebae were fully infected with the endosymbiont HSC3. **(B)** The infection cycle of HSC3 in *Acanthamoeba castellanii* NEFF. The infection rate in *A. castellanii* NEFF started low, only 10 % of acanthamoebae were infected after 3 to 8 hours post infection, but increased over time until the cycle ends between 72 hpi and 96 hpi with a 100 % infection rate. **(C)** The infection rates of endosymbiont HSC3 in *A. castellanii* NEFF compared to its original host, *Acanthamoeba* sp. HSC. throughout one round of the infection cycle. White: HSC for the

infection in *Acanthamoeba* sp. HSC; black: NEFF for the infection in *A. castellanii* NEFF. At 2-3 hpi HSC3 was present in 40% of all *Acanthamoeba* sp. HSC whereas only 10% of *Acanthamoeba castellanii* NEFF were infected when the same multiplicity of infection was applied. After 24 hpi the infection levels were higher *A. castellanii* NEFF compared to *Acanthamoeba* sp. HSC. Only 60% of *Acanthamoeba* sp. HSC was fully populated at 48 hpi in comparison to the 95% infection rate in *A. castellanii* NEFF. Nevertheless both strains reach an infection rate of 100% after 96 hpi.

In contrast to the infection of HSC3 in the natural *Acanthamoeba* sp. HSC, the starting percentage of infected *Acanthamoeba castellanii* NEFF at 3 hpi seemed much lower with 7.3 %. Most of all amoebae contained single particles or rarely more than five chlamydiae. At the early time points after 2-8 hpi, it seemed even harder to detect the infectious particles by FISH, than it was in the study of *Acanthamoebae* sp. HSC. After 24 h most FISH signals correlated with the DAPI signals and showed slightly bigger coccoid cell morphology. Now the proportion of infected amoebae rapidly increased up to 78.5 % of which 50.7 % of the amoebae contained up to 30 particles. The percentage was still slowly increasing after 48 hpi with now 94.7 % infected amoebae, most harboring over 30 particles (69.7 %). The chlamydia seem to be widely distributed throughout the amoeba host and organized in single cell inclusions. The 100 % full infection was reached between 72 and 96 hpi, indicating completion of the infection cycle in *A. castellanii* NEFF. The bacteria were released into the surrounding medium.

The evaluation of the endosymbiont of *Acanthamoeba* sp. HSC3 infection cycle in *Acanthamoeba* sp. HSC as well as in *Acanthamoeba castellanii* NEFF using FISH and DAPI staining, suggests that the bacteria are taken up after 2-3 hours post infection. As is known for EBs, their detection by FISH is weak, due to low metabolic activity and therefore less rRNA target molecules. As the amount of EBs that enter the hosts at the first time points is low, especially in the infection cycle in *A. castellanii* NEFF, this indicates that most bacterial particles were still EBs. After 24 h most chlamydiae seemed to have switched from EBs to the more active RBs, as the FISH signals mainly correlated with the DAPI signals. A longer replicative phase followed that lasted until 72 hpi with an increase in numbers from four to 30 bacteria per amoeba. *Acanthamoeba* spp. were fully infected with HSC3 after 96 hpi, which were then released into the environment for new infections.

### **3.5.2 Uninfected *Acanthamoeba* sp. HSC show faster growth in comparison to *acanthamoebae* harbouring the endosymbiont HSC3**

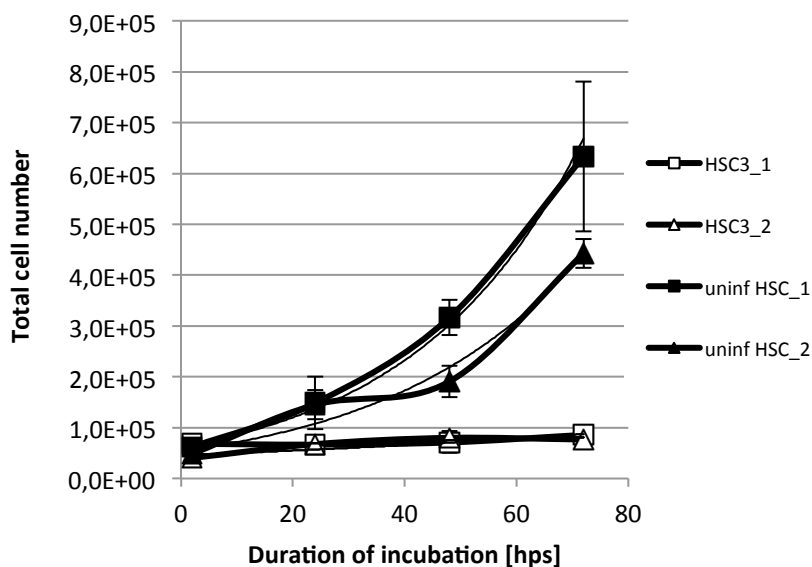
The influence of an endosymbiont on amoebal growth was analyzed by phase contrast microscopy and by calculating the total cell number in each well. The overall cell condition was determined visually after each time point, in order to be sure of well grown cultures where most amoebae are attached to the surface and in trophozoite cell stage. To avoid bias in cell counting we detached all amoebae, mixed them carefully and only used a small amount of the substrate for counting. As is commonly recognized

amoebae have a mobile lifestyle, resulting in an unequal distribution throughout the wells. A higher density can often be observed in the center compared to the areas near the border.

The average number of counted uninfected and infected amoebae are represented in figure and compared to each other. The uninfected acanthamoebae grew much faster than the ones infected with the chlamydia endosymbiont HSC3. A significant difference in cell numbers could first be observed at 48 hps with uninfected amoebae displaying higher numbers. *Acanthamoeba* sp. HSC cultures infected with HSC3 started growing from 54'167 cells up to 81'300 cells in average, which indicates that they keep the growth rate relatively constant over time. In comparison, the uninfected *Acanthamoeba* sp. HSC cultures grow exponentially and reach the ten-fold amount of cells three days after seeding.

To further define the difference in acanthamoebae replication rate, the duplication time for uninfected as well as for infected amoebae were calculated and represented in table. Uninfected acanthamoebae doubled their numbers after only 9 hours post seeding, which is very fast compared to the amoebae cultures harbouring HSC3. In average, their duplication time was later than 72 hours, not represented in this study. Table 3.7 shows the average duplication time of uninfected and infected acanthamoebae. Moreover, empty *Acanthamoeba* sp. HSC of the first run displayed the highest number of cells in total.

**A**



**Figure 3.6 Growth of uninfected and infected acanthamoebae.** The curves illustrate average values for two separate runs. White: first and second run of amoeba cultures infected with HSC3. Black: first run and second run of empty controls. The growth of uninfected amoebae was faster than the growth of amoebae infected with HSC3.

**Table 3.6** The equation and  $R^2$  value of the trend line of each culture in figure.

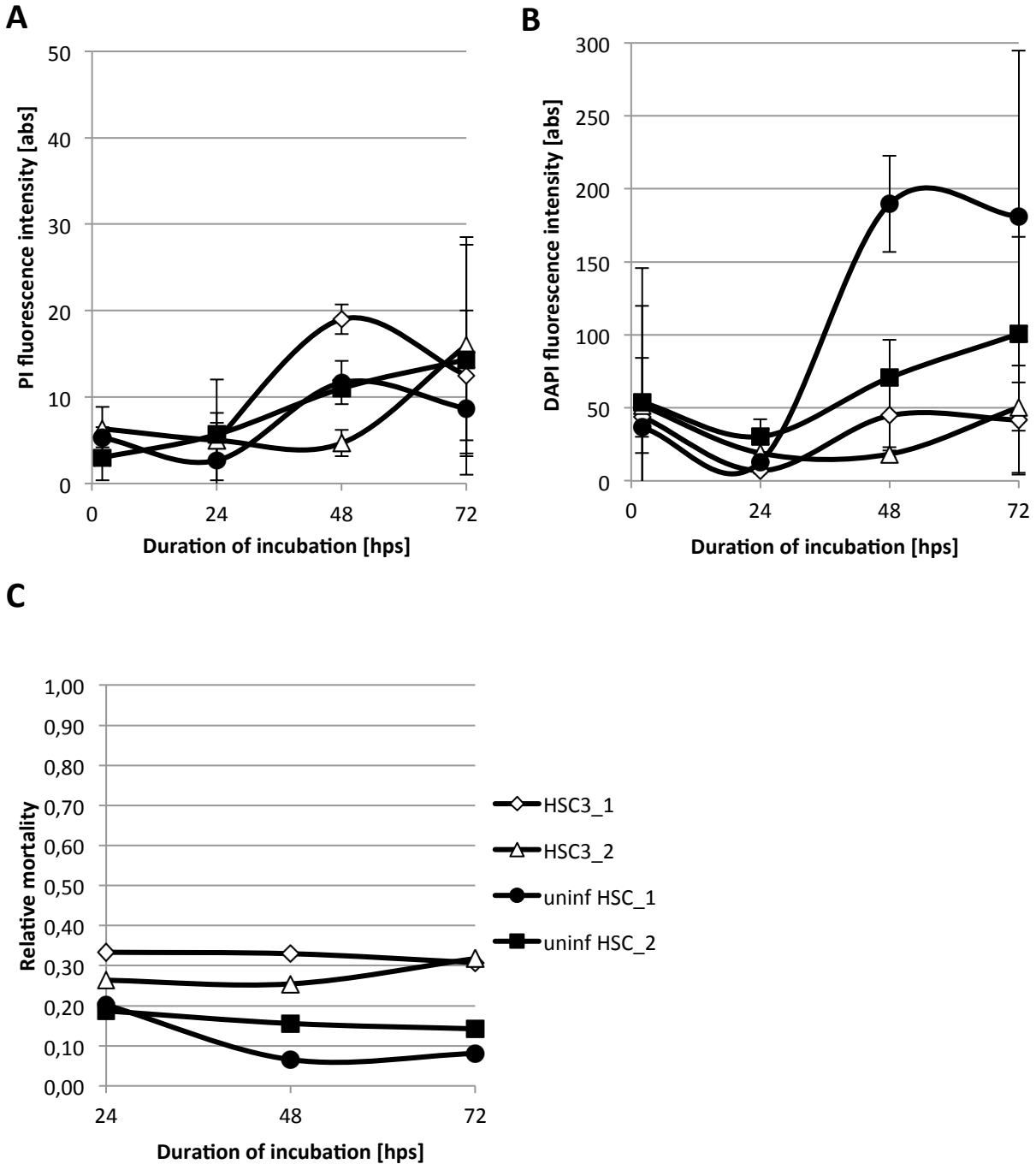
|                     | Equation               | $R^2$ value    |
|---------------------|------------------------|----------------|
| Uninfected HSC_Run1 | $y = 61994e^{0.033x}$  | $R^2 = 0.9946$ |
| Uninfected HSC_Run2 | $y = 53287e^{0.0294x}$ | $R^2 = 0.948$  |
| Infected HSC_Run1   | $y = 64534e^{0.0032x}$ | $R^2 = 0.7217$ |
| Infected HSC_Run2   | $y = 46026e^{0.009x}$  | $R^2 = 0.7171$ |

**Table 3.7** Duplication time of uninfected and infected amoebae in the first and second run respectively. The duplication time of uninfected acanthamoebae ranged around 20 h post seeding. In contrast, acanthamoebae that harbor the endosymbiont HSC3 need at least 61.4 h post seeding to duplicate their number.

|                | Run_1 | Run_2 |
|----------------|-------|-------|
| Uninfected HSC | 20.9  | 20.3  |
| Infected HSC   | 234.5 | 61.4  |

### 3.5.3 Influence of the endosymbiont HSC3 on acanthamoeba viability

The presence of host cell death within HSC3 infected *Acanthamoeba* sp. HSC cultures was compared to uninfected *Acanthamoeba* sp. HSC using propidium iodide for visualization of dead cells. No significant PI fluorescence intensity peak happens throughout the course of the experiment, which indicates that no cell lysis was caused by the endosymbiont of *Acanthamoeba* sp. HSC3 (Figure). Additionally, amoebal growth in continuous cultures did not seem to be influenced by the presence of HSC3 at temperatures below 27 °C. The amoebal fitness is constant over time in both uninfected and infected acanthamoebae cultures. Nevertheless, a slow increase in PI values was observed in both cultures until 48 hps. The differences between infected amobae and uninfected controls were only significantly divergent at that very time point. The trends were rather contradictory after 48 hps, decreasing in the first run and further increasing in the rerun observed in both cultures. For better understanding, a DAPI staining was applied in parallel, which visualizes the total amount of DNA in the sample. The DAPI fluorescence intensity measured could then be put in relation to the PI fluorescence intensity, in order to represent the relative mortality in the culture. The total amount of DNA is constant over time in cultures infected with HSC3, whereas an increase of DAPI fluorescence intensity is observed in uninfected amoebae cultures. Both cultures show a drop after 24 h. nevertheless, the values are very low, indicating an insignificant difference between all cultures, independent on infection with endosymbiont HSC3.



**Figure 3.7** *Acanthamoeba* sp. HSC fitness depending on the presence or absence of the chlamydial endosymbiont HSC3. The data represent average values for two different runs. White: first and second run of amoeba cultures infected with HSC3. Black: first run and second run of empty controls **(A)** Propidium iodide fluorescence intensity measured for infected amoebae cultures and uninfected controls. The amoebal viability is constant over the measured time in all cultures, independent on HSC3

infection. **(B)** DAPI fluorescence intensity in uninfected and infected amoeba cultures. The total amount of DNA is increasing over time in all amoeba cultures tested, with higher values for uninfected cultures compared to cultures containing the endosymbiont HSC3. **(C)** The mortality rate in uninfected and infected amoebae cultures. The relative mortality of a culture results from the relation between the propidium iodide fluorescence intensity and the DAPI fluorescence intensity different cultures over time. We observe increased mortality rates in infected amoebae cultures, but an overall constant relative mortality over time in all cultures.

The relative mortality was calculated for each uninfected and infected amoebae culture and for each incubation period as the total number of dead cells divided by the initial amount of DNA. No significant increase in relative mortality was observed over time in all cultures. Although the values were slightly higher in the infected cultures, the relative mortality rates based on these measurements of PI and DAPI fluorescence intensities may be biased and the difference cannot be described as significant.

#### **3.5.4 Influence of nutrient availability on infectivity**

The chlamydial endosymbiont of *Acanthamoeba* sp. HSC3 were incubated in the absence of hosts for several days in different nutrient-rich and nutrient-free media (2.6.11). After infection of empty *Acanthamoeba* sp. HSC with host-free HSC3 at defined time points, the infectivity, relative to that observed for 2 h incubation in DGM-D, was calculated. The average values for relative infectivity of every growth condition are illustrated in figure and compared to each other.

In general, the amount of infectious chlamydia appeared to decline over time in all four media that were tested. In fact, under the applied incubation and infection conditions the infection rate was reduced to an average of 30 % after a host-free period of 7 days. The initial infectivity, observed after 2 h incubation ranged around the 100 % infection, except for the chlamydiae incubated in salt solution only infected 80 % of the amoebae. Then a rapid increase followed after 2 days and the trend of the infectivity curve was similar to all other tested media, but the values stayed higher. These findings suggest that despite the lack of essential substrates chlamydial survival and infectivity was maintained over a long period of time.

To further determine the differences between the courses of infectivity in the four media, the exact time point where the relative infectivity of the chlamydiae declined to 50 %, 25 % and 0 %, was calculated using the equations from table. The relative infectivity of chlamydiae incubated in DGM-L began to decline between 48 h and 96 h, reached an infectivity of 50 % after 142 h. Although no measurements were done after 168 h, the equation gave an approximate time point of 180.5 h, where no infectivity would be observed. The infectivity of chlamydiae incubated in DGM-D seemed to slightly increase at the start, but then sank down to 50 % after 155.4 h until after 186 h no amoebae would be left infected. The nutrient-free buffer PBS showed a rather constant infectivity during the first four days. But then decreased and 50 % infected amoebae remained after 157 h and no infectivity would be

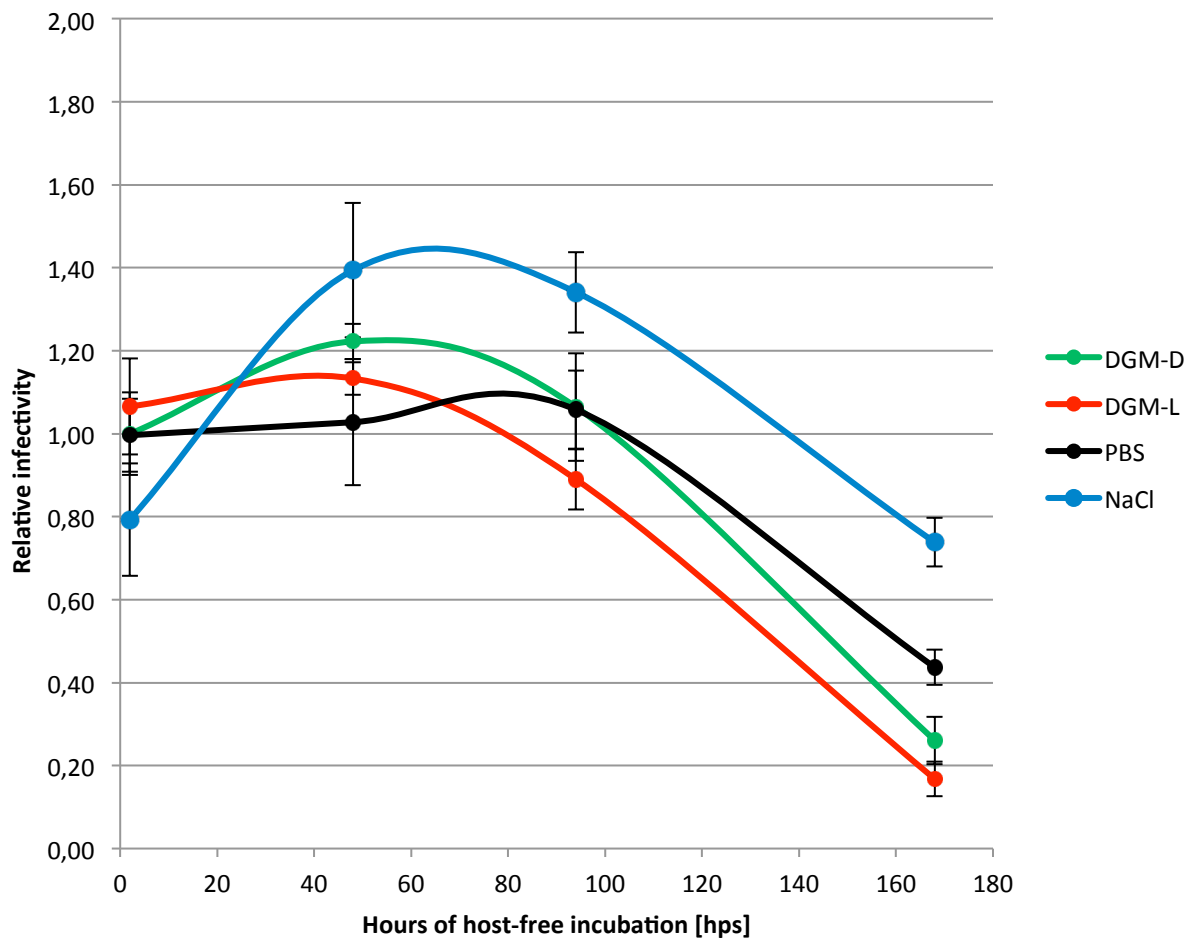
detected after 196 h. The highest values were observed for chlamydiae incubated in nutrient-free salt solution. Starting with lower values compared to all other chlamydiae, a rapid and striking increase reached a relative infectivity of 140 %, which declined only after 96 h. Seven days of host-free incubation did not sink lower than the initial infectivity. According to the equation of the trend line the relative infectivity would be zero after a host-free incubation of 212.5 h, which means the chlamydiae approximately survive for 9 days in nutrient-free salt solution.

**Table 3.8 The equation and R<sup>2</sup> value of the trend line of each growth media in figure.**

|              | Equation                           | R <sup>2</sup> value    |
|--------------|------------------------------------|-------------------------|
| <b>DGM-D</b> | $y = -7E-05x^2 + 0.0077x + 0.9934$ | R <sup>2</sup> = 0.9981 |
| <b>DGM-L</b> | $y = -5E-05x^2 + 0.0031x + 1.0703$ | R <sup>2</sup> = 0.9971 |
| <b>PBS</b>   | $y = -5E-05x^2 + 0.0049x + 0.9638$ | R <sup>2</sup> = 0.9724 |
| <b>NaCl</b>  | $y = -9E-05x^2 + 0.0154x + 0.7919$ | R <sup>2</sup> = 0.9674 |

**Table 3.9 Hours of host-free incubation needed until a fraction of 50 %. 25 % and 0 % infectivity remained in the culture.** The relative infectivity of chlamydiae incubated in DGM-L was the first to decline, followed by chlamydiae in DGM-D and PBS. The highest values were observed for chlamydiae incubated in nutrient-free salt solution.

| Relative infectivity | DGM-D   | DGM-L   | PBS   | NaCl    |
|----------------------|---------|---------|-------|---------|
| <b>50 %</b>          | 155.4 h | 142 h   | 157 h | 188.3 h |
| <b>25 %</b>          | 172 h   | 163 h   | 178 h | 201 h   |
| <b>0 %</b>           | 186 h   | 180.5 h | 196 h | 212.5 h |



**Figure 3.8 Influence of substrate availability on maintenance of infectivity after host-free incubation.**

Relative infectivity of HSC3 previously incubated over a period of 7 days in the absence of host cells. Green: HSC3 incubated in the in nutrient-rich medium DGM-D, containing D-glucose as a supplement. Red: chlamydiae incubated in a modified medium, containing L-glucose. Black: chlamydiae incubated in a nutrient-free PBS buffer Blue: HSC3 incubated in nutrient-free salt solution. The figure indicates average relative infectivity values from three biological replicates. The endosymbionts incubated in DGM-D and in salt solution experienced an initial increase of infectivity, whereas the chlamydiae in DGM-L and PBS keep a constant infectivity rate for 4 days. After 96 h the infectivity declined in all tested media. The infectivity of the endosymbionts incubated in DGM-L was the first to drop. The ones incubated in NaCl were still highly infective after 7 days of host-free incubation.

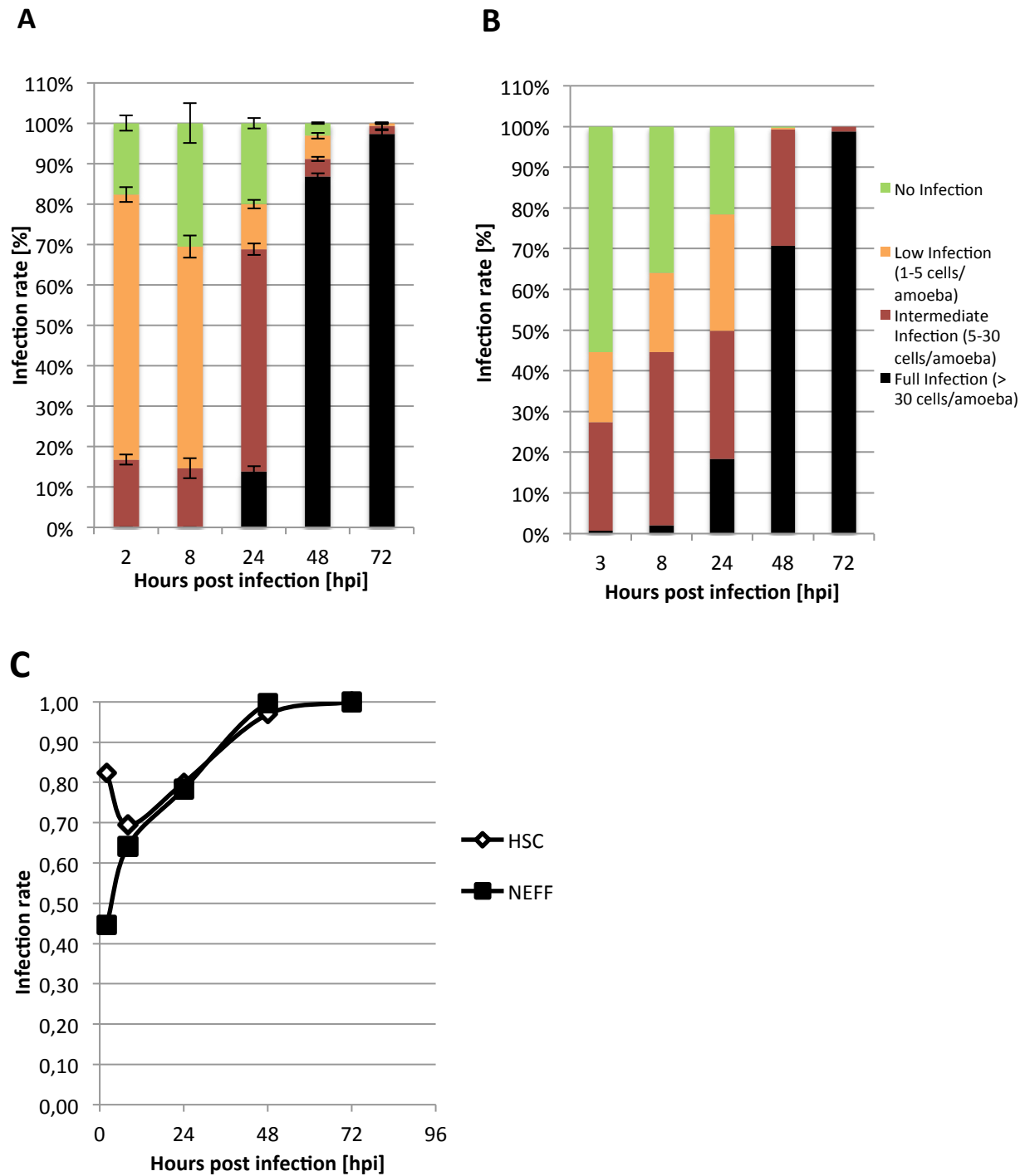
### 3.6 Endosymbiont of *Acanthamoeba* sp. HSC8

#### 3.6.1 The infection cycle of endosymbiont HSC8

The infection cycle of the endosymbiont HSC8 in its natural *Acanthamoeba* sp. HSC host as well as in *Acanthamoeba castellanii* NEFF was analyzed by fluorescence *in situ* hybridization and phase contrast microscopy. These synchronized infection assays give insight into cell morphologies, infection levels and metabolic activity of the endosymbiont HSC8. Furthermore the infection rate of endosymbiont HSC8 in its original host was compared to the infection levels of HSC8 in the laboratory strain *A. castellanii* and differences were pointed out.

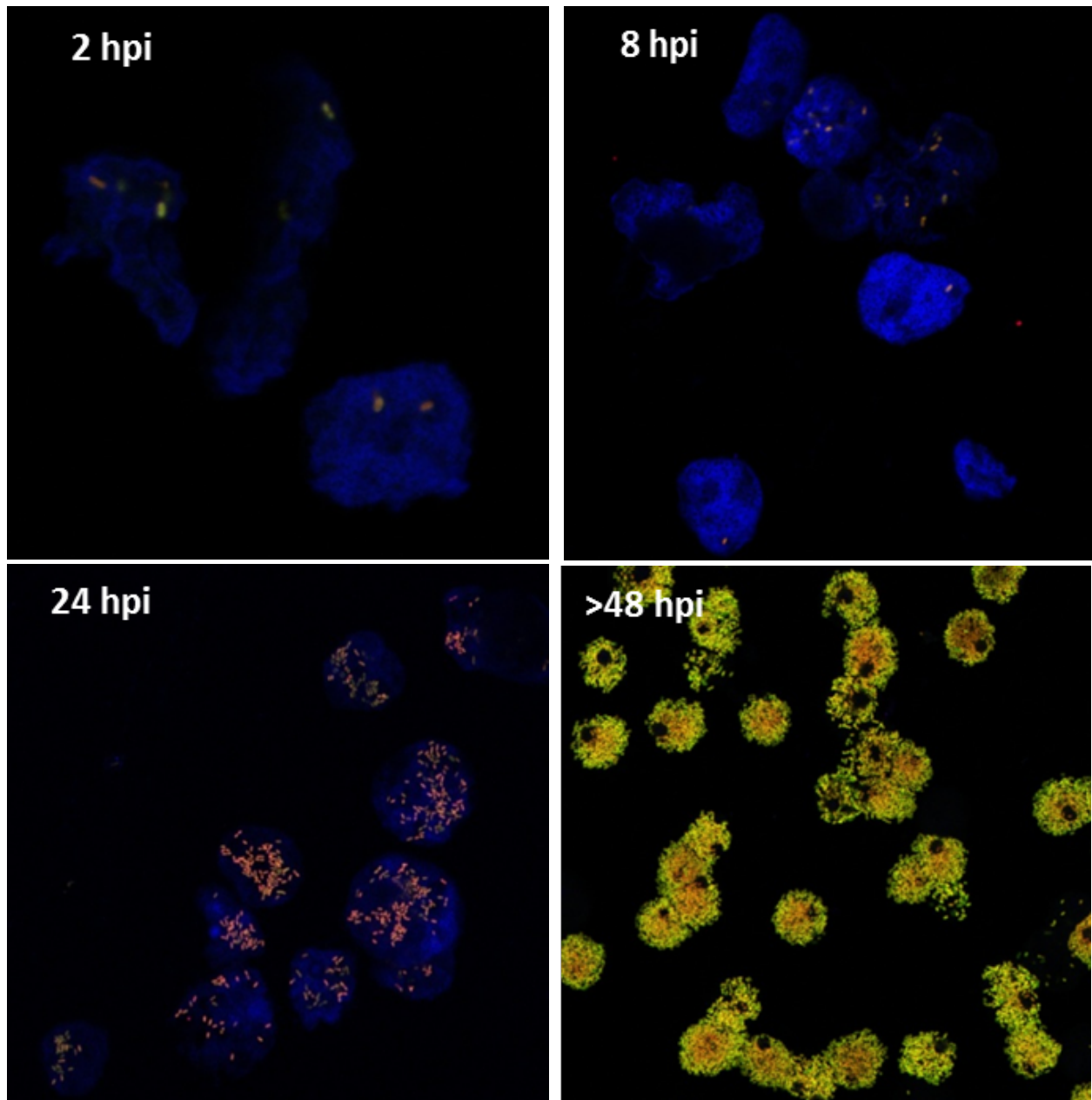
We distinguished low infected, intermediate infected and fully infected *Acanthamoeba* spp. over time and expressed the numbers relative to each other as well as to the number of uninfected *Acanthamoeba* spp. At different time points before and after infection, the overall cell condition was determined visually. Most cells were attached to the surface and in trophozoite cell stage, indicating well grown amoeba cultures. The infection rate was calculated for two amoebae cultures and at several time points after infection by summing up the low infected, intermediate infected and fully infected *Acanthamoeba* spp. divided by the total number of counted cells. The infection rate of HSC8 in *Acanthamoeba castellanii* NEFF was then compared to the infection levels in the original *Acanthamoeba* sp. HSC host.

The infection of HSC8 in *Acanthamoebae* sp. HSC started with a very high percentage of 82.3 % at 2 hpi of which most amoebae were infected with one to five particles. Only 16.7 % of the amoebae contained more than five intracellular bacteria. FISH and DAPI signals showed small rod-shaped bacteria inside the cytoplasm. After 24 hpi most amoebae harbored between five and 30 endosymbionts with a relative percentage of 55 % and some were already fully infected (13.8 %). It took them only until 48 hpi for replication inside the cells, so after 48 hpi with 96.9 % almost every cell was fully infected with HSC8. The bacteria were very evenly spread throughout the cytosol. The infection cycle seemed to be completed between 48 and 72 hpi, indicated by 100 % infection and a large number of extracellular particles in the surrounding medium. For more detail, the infection rate of HSC8 in *Acanthamoeba castellanii* NEFF was then compared to the infection levels in the original *Acanthamoeba* sp. HSC host. The starting percentage of infected *Acanthamoeba castellanii* NEFF was significantly lower than in *Acanthamoeba* sp. HSC with 44.6 %. Between 3 hpi and 48 hpi, the numbers of infected cells increases to 64.1 % then 78.4 % and reaches almost a full infection after only 48 hpi with 99.7 %. At the early time points 26.5 % were already intermediately infected whereas only 17.2 % of the amoebae harbored less than five cells. The number of fully infected cells increased up to 70.6 % after 48 hpi and at 72 hpi all cells were infected, the majority of them containing over 30 endosymbionts. This indicates that the infection cycle completes at 72 hpi.



**Figure 3.9 The course of infection of HSC8 within different *Acanthamoeba* spp. strains. (A)** The infection cycle of HSC8 in the original *Acanthamoeba* sp. HSC host. The number of of infected cells increased over time and after 48 hours post infection (hpi) the endosymbiont HSC8 reached an infection rate of 100 %. **(B)** The infection cycle of endosymbiont HSC8 in *A. castellanii* NEFF. The infection rate

increased over time until 48 hours post infection (hpi) all acanthamoebae were fully infected and the cycle ended. **(C)** The infection rate of endosymbiont HSC8 in *A. castellanii* NEFF compared to the infection rate of HSC8 in its original *Acanthamoeba* sp. HSC host throughout one round of the infection cycle. The infection levels in both amoeba cultures were similar for the periods between 8 hpi and 72 hpi. At 2-3 hpi HSC8 was present in in 80% of all *Acanthamoeba* sp. HSC whereas only 40% of *Acanthamoeba castellanii* NEFF were infected when the same multiplicity of infection was applied. Nevertheless both strains reached an infection rate of 100% after 48 hpi.



**Figure 3.10 Visualization of the infection cycle of the endosymbiont HSC8 in *Acanthamoeba* sp. HSC.** The course of infection was analyzed over a period of 96 h and visualized with FISH using the probes EUK516 (light blue), a combination of EUBmix and HSC (orange) and the DNA dye DAPI (blue). Single bacteria start entering the cell after 2-3 hours post infection, then replicate between 8 hpi and 24 hpi. After 48 hpi, amoebae were fully infected and bacteria were finally released between 48 and 72 hpi.

In summary, the analysis of the infection cycle of the endosymbiont of *Acanthamoeba* sp. HSC8 in its natural *Acanthamoeba* sp. HSC host as well as in *Acanthamoeba castellanii* NEFF using FISH revealed a quite similar process. Bacteria enter the cell after 2-3 hours post infection, followed by a replicative phase after 8 hpi. Numbers of bacteria per amoeba increase from four up to 30 observed at 24 hpi. *Acanthamoeba* spp. were fully infected with HSC8 after 48-72 hpi, which were then released into the environment for new infection. Differences were only observed as early as 2-3 hpi. Starting with infection rates of 40 % and 80 % after 2-3 hpi, a fully completed cycle was observed after 48 hpi infection in both cultures.

### **3.6.2 Uninfected *Acanthamoeba* sp. HSC show faster growth in comparison to acanthamoebae harbouring the endosymbiont HSC8**

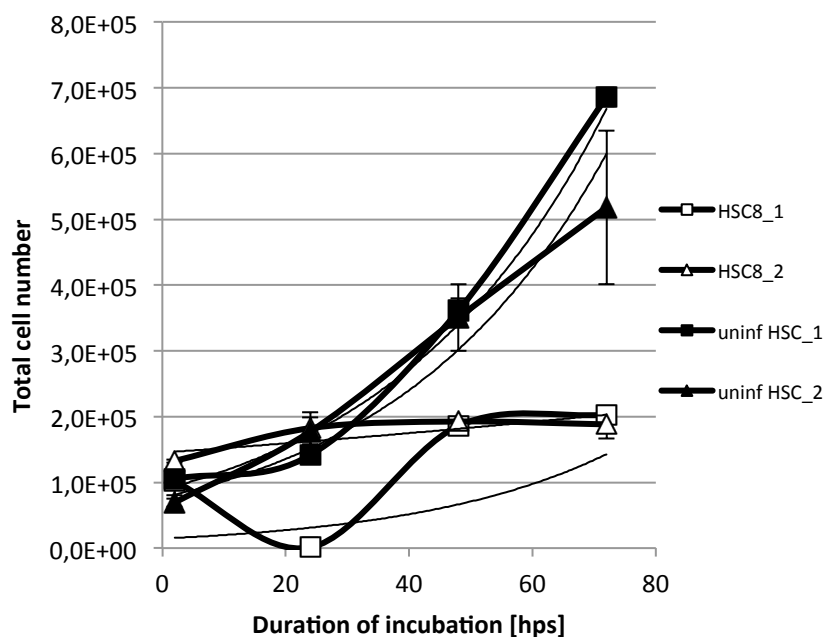
The influence of an endosymbiont HSC8 on amoebal growth was determined by phase contrast microscopy and the total cell number was calculated over time. At different time points after seeding, the overall cell condition was determined visually. Indeed after 2 hours, most cells were attached to the surface and in trophozoite cell stage, which suggests good growth conditions. As the amoebae are not equally distributed throughout the wells, but higher density can often be observed in the center compared to the area near the border, we detach the amoeba and use a small amount of the substrate to avoid bias in cell counting.

The average number of counted uninfected and infected amoebae are represented in figure and compared to each other. The growth rate of uninfected acanthamoebae was much faster than the replication time of infected acanthamoebae. A significant difference in cell numbers could first be observed at 48 hps with uninfected amoebae displaying higher numbers. The results indicate that the *Acanthamoeba* sp. HSC cultures infected with HSC8 keep a low, even relatively constant growth rate resulting in an average of 195'600 cells after 72 h incubation. The total cell number doubled around 72 h post seeding, whereas the uninfected culture grows much faster. The numbers were doubled around 24 h post seeding and reach a six fold maximum of about 602'000 cells in average after three days (Figure 3.11). Indeed, the uninfected amoeba culture of the first run reached the highest cell numbers.

**Table 3.10** The equation and  $R^2$  value of the trend line of each culture in figure.

|                     | Equation                | $R^2$ value |
|---------------------|-------------------------|-------------|
| Uninfected HSC_Run1 | $y = 88716e^{0.028x}$   | 0.9726      |
| Uninfected HSC_Run2 | $y = 76488e^{0.0286x}$  | 0.9603      |
| Infected HSC_Run1   | $y = 98470e^{0.0108x}$  | 0.9346      |
| Infected HSC_Run2   | $y = 145196e^{0.0047x}$ | 0.6423      |

To further define the difference in acanthamoebae replication rate, the duplication time for uninfected as well as for infected amoebae were calculated with the equations of the trend lines from table 3.10. Table 3.11 indicates the calculated duplication time.



**Figure 3.11** Amoeba growth in the absence and presence of endosymbiont HSC8. Total cell counts were calculated for an incubation period of 72 hours. Every infection type is illustrated as average values for two different runs. White: first and second run of amoeba cultures infected with HSC8. Black: first run and second run of empty controls. The growth of uninfected amoebae was faster than the growth of amoebae infected with HSC8.

**Table 3.11 Duplication time of uninfected and infected amoebae in the first and second run respectively.** Uninfected *acanthamoebae* reach double of the starting number between 20.7 h and 31.1 h post seeding, which means it takes them about one day. *Acanthamoebae* that harbor the endosymbiont HSC8 need 67.2 h or more to duplicate their number, in average at least three days.

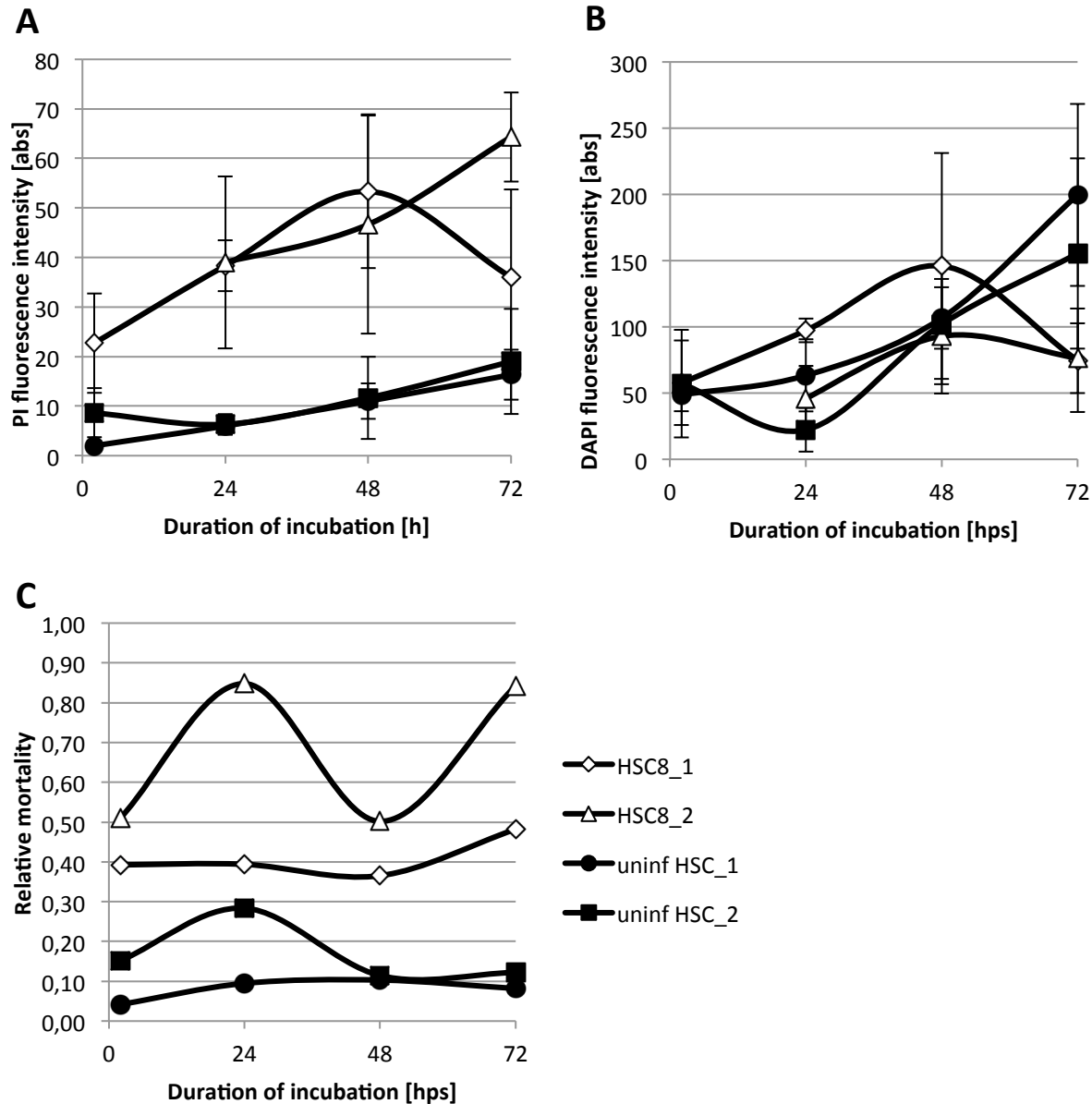
|                | Run_1 | Run_2 |
|----------------|-------|-------|
| Uninfected HSC | 31.1  | 20.7  |
| Infected HSC   | 67.2  | 128.1 |

### 3.6.3 Influence of the endosymbiont HSC8 on *acanthamoeba* viability

Host cell lysis of infected *Acanthamoeba* sp. HSC cultures was analyzed and quantified using a live/dead staining and was then compared to uninfected *Acanthamoeba* sp. HSC as a negative control. The propidium iodide visualizes dead cells with leaking cell membranes. No significant PI peak indicating host cell death could be observed throughout the course of the experiment (Figure 3.12A). Additionally, amoebal fitness in continuous cultures did not seem to be influenced by the presence of HSC8 at temperatures below 27 °C and stays constant over time in both uninfected and infected *acanthamoebae* cultures.

The PI fluorescence intensity is slightly increasing over time in both uninfected and infected *acanthamoebae*. Amoebae harboring the endosymbiont HSC8 have consistently higher PI fluorescence intensity compared to the uninfected control. The differences in amoebal fitness observed between infected amoebae and uninfected controls during the 72 h of incubation were significant at all the time points, but in general so low that the effect of HSC8 on host cell growth is negligible. Moreover, at the later time point of 72 hps the PI values of the infected culture during the first run decreased, in contrast to all other amoeba cultures. In conclusion, since the values are very low the results indicate that the presence of HSC8 did not seem to have an influence on amoeba growth in continuous cultures and that in general no cell lysis takes place in the original *Acanthamoeba* sp. HSC host at 27 °C.

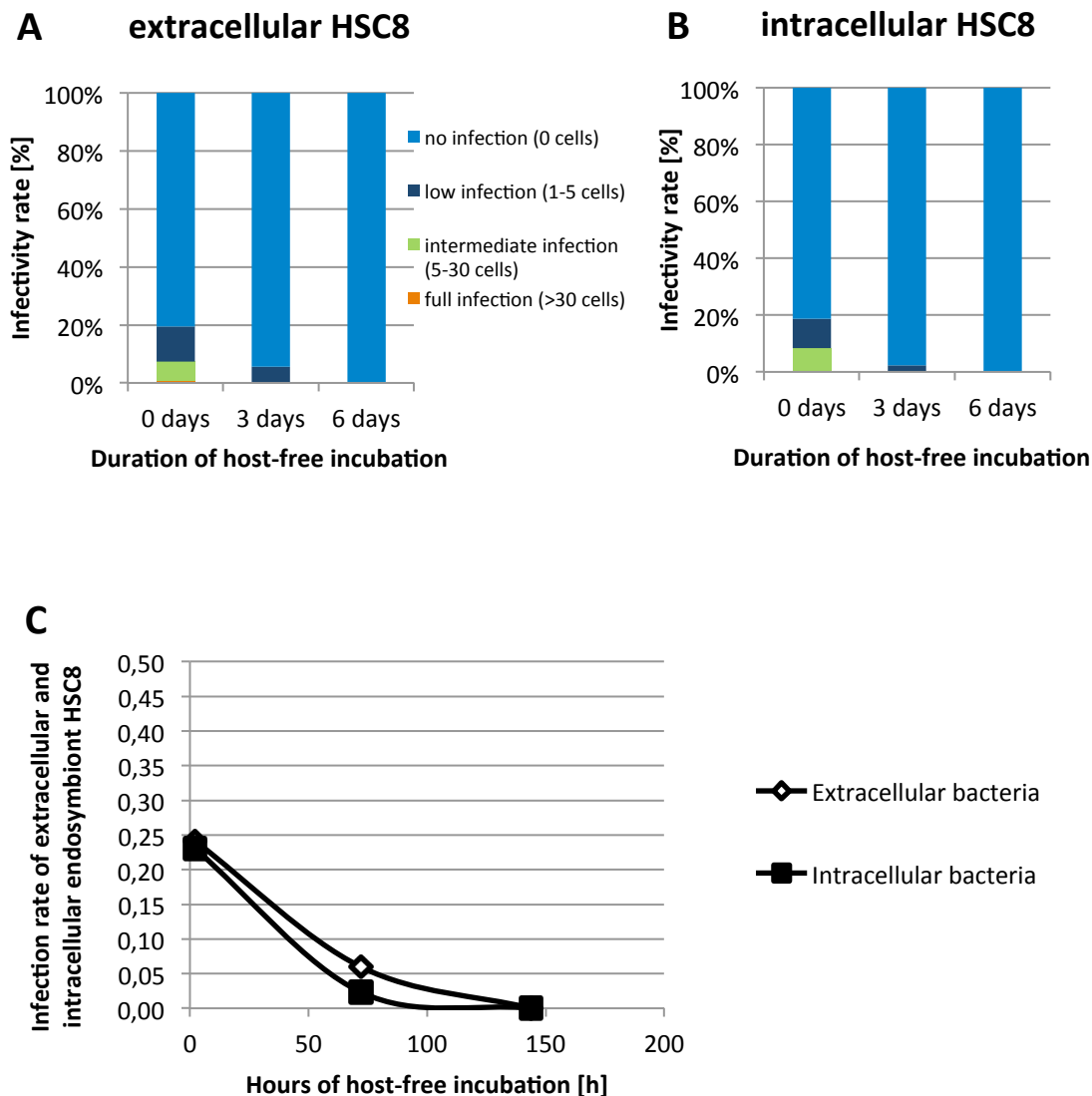
For better comparison, a simultaneous DAPI staining detected the total amount of DNA in the sample. The DAPI fluorescence intensities are illustrated in figure 3.12B. The total amount of DNA increased constantly over time in all cultures, independent on infection with the endosymbiont HSC8, but experienced a drop at the later time points in the infected cultures. DAPI fluorescence intensity was then depicted in relation to the PI fluorescence intensity. The resulting relative mortality was calculated for each culture and for each time point as the total number of dead cells divided by the initial amount of DNA. Although the values were slightly higher in the infected cultures, the relative mortality rates were constant over time. We observe elevated mortality rates for amoeba cultures containing endosymbionts, but an overall constant relative mortality over time in all cultures.



**Figure 3.12 *Acanthamoeba* sp. HSC fitness dependent on the presence or absence of endosymbiont HSC8.** The data represent average values for two different runs. White: first and second run of amoeba cultures infected with HSC3. Black: first run and second run of empty controls **(A)** PI fluorescence intensity over an incubation period of 72 h. The amoebal viability is constant in empty amoebae. Host cell death is elevated in cultures containing endosymbionts and increasing over time. **(B)** DAPI fluorescence signaling represents the total amount of DNA in uninfected and infected amoeba cultures. The DAPI fluorescence intensities increased in all cultures for two days, followed by a drop in the infected cultures and a continuous increase in uninfected cultures. An important difference can be observed after 72 h. **(C)** The relative mortality rate of uninfected and infected *acanthamoebae* for different cultures over a period of 72 h. We observe elevated mortality rates in infected amoeba cultures, but an overall constant relative mortality over time in all cultures.

### 3.6.4 Maintenance of infectivity during host-free incubation

The first step in analyzing the infectivity of the HSC8 consisted in the comparison of extracellular to intracellular HSC8 both recovered from a well-grown amoeba culture. An infectivity assay was performed by incubating extracellular and intracellular symbionts host-free during six days, while adding uninfected *Acanthamoeba castellanii* NEFF every third day. No significant difference in infectivity could be observed in both stages. The infectivity curves were similar, starting with an infectivity rate of 20% followed by a rapid decrease after 3 days. No infected amoebae were detected after 6 days of extracellular incubation.



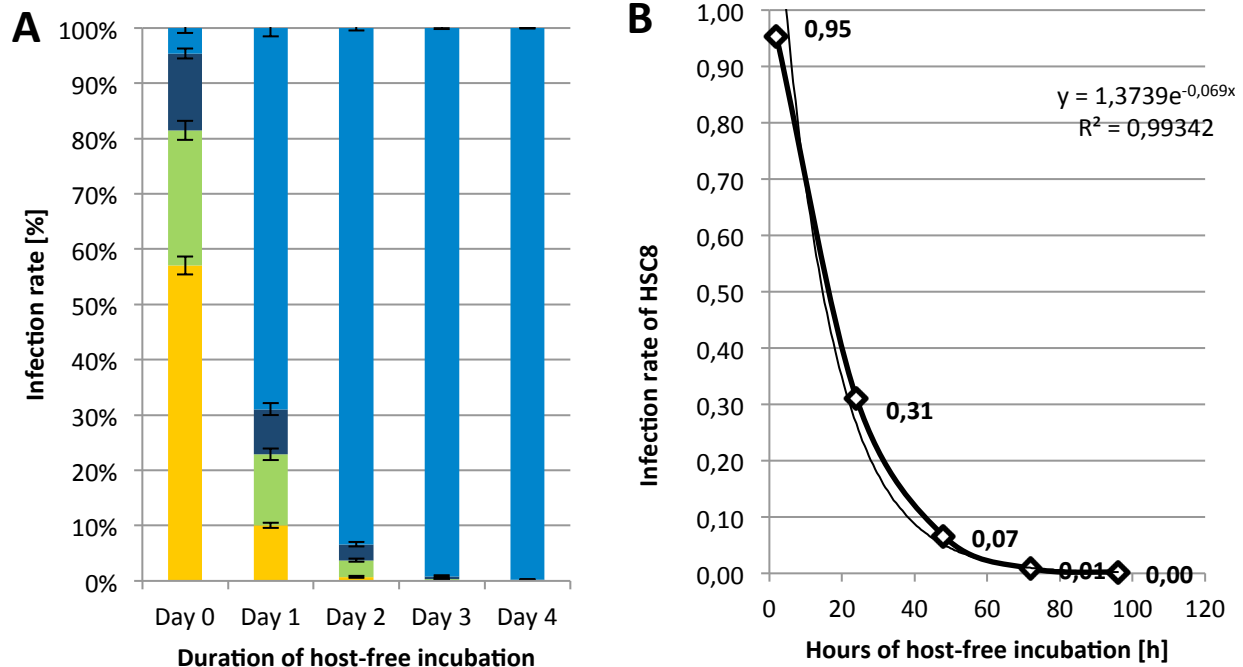
**Figure 3.13 The maintenance of infectivity of the extracellular cell stage compared to the intracellular cell stage of HSC8 after host-free incubation in TSY (MOI 50).** **(A)** Percentage of host cells infected with extracellular HSC8 previously incubated over a period of 6 days in host-free medium. **(B)** Percentage of host cells infected with HSC8 previously isolated from the cytoplasm of amoebae (intracellular stage) followed by an incubation period of 6 days in host-free medium. Light blue: No infection, meaning no bacteria had infected a host cell after 24 hours post infection (hpi). Dark blue: Low infection, meaning that 1-5 symbionts have entered a cell after 24 hpi. Green: Intermediate infection, when 5-30 bacteria per amoebae were observed after 24 hpi. Orange: Full infection, for > 30 cells per host after 24 hpi. **(C)** Infection rate of both stages over the course of 6 days. White: extracellular HSC8 isolated from the medium. Black: intracellular HSC8 isolated from the cytosol of host cells by lysis. An obvious decrease of infected amoebae hosts was detected in both extracellular and intracellular bacteria, resulting in a loss of infectivity after six days of host-free incubation. No difference was observed between both stages.

Noticing that in this study, only 20 % of amoebae were infected right from the beginning a second infectivity assay was applied. To further analyze the infectious behaviour of extracellular HSC8 in the original *Acanthamoeba* sp. HSC host, amoebae were fixed after 48 hours post infection. A decrease of infected cells is observed over time, with an especially dramatic drop of fully infected amoebae. The number of fully infected cells dropped down to 10 % after one day of host-free incubation. After 5 days host-free incubation, all infectivity of HSC8 was lost.

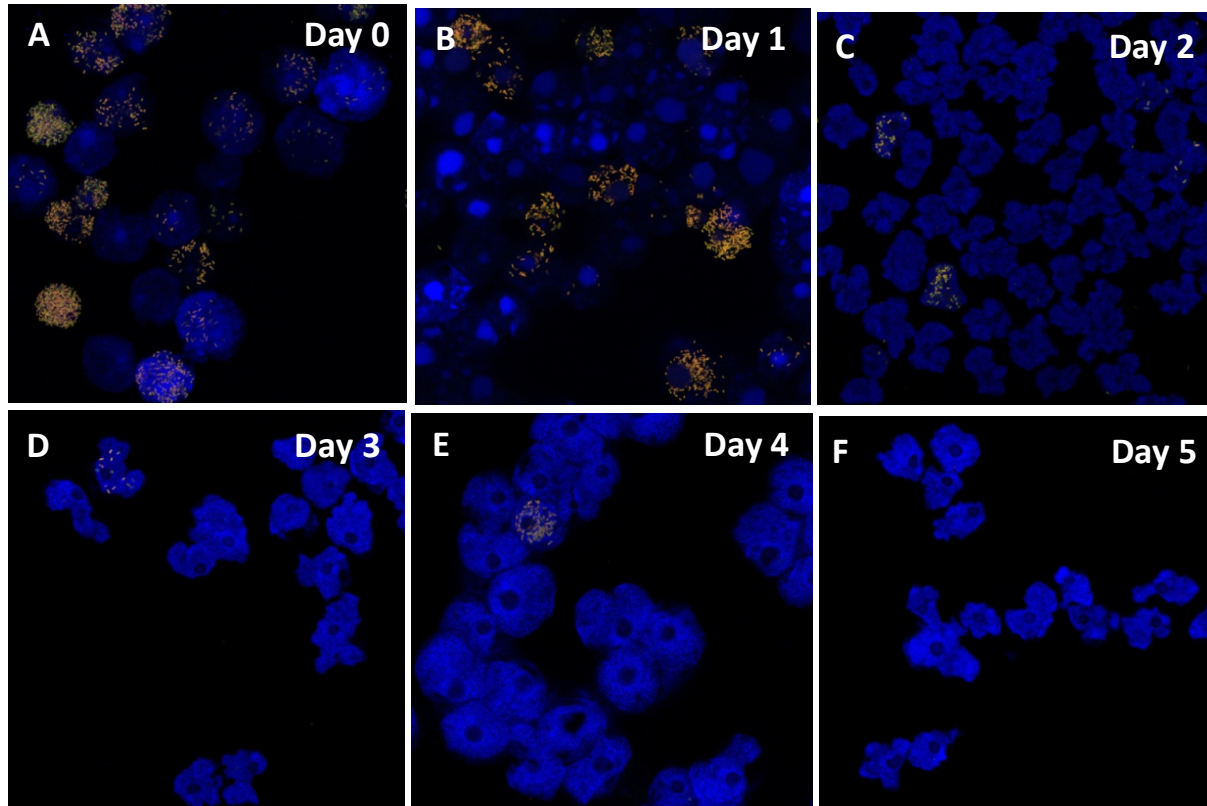
In addition, we calculated the period of time necessary for the infectivity of HSC8 to sink down to  $\frac{1}{2}$  and  $\frac{1}{4}$  in relation to the infection rate measured after 2 h of host-free incubation. We used the equation shown in figure 3.14B. Half of the infectivity was lost as early as 14.6 h and after one day of host-free incubation only 25 % of the endosymbionts were observed within infected amoebae.

**Table 3.12 Half-lives of the endosymbiont's ability to infect the acanthamoebae hosts.** The amount of time required for the infectivity to fall to  $\frac{1}{2}$  and  $\frac{1}{4}$  its value as measured at the beginning of the time period was calculated with the equation shown in figure, After 14.6 h the infectivity decreased down to 50 % and after one day only 25 % of the amoebae have been infected.

| Fraction remaining | Hours of host-free incubation |
|--------------------|-------------------------------|
| $\frac{1}{2}$      | 14.6                          |
| $\frac{1}{4}$      | 24.7                          |



**Figure 3.14 Maintenance of infectivity of HSC8 after host-free incubation in TSY (MOI 50). (A-B)** Infectivity rates indicating the ability of the obligate endosymbiont HSC8 to maintain infectivity after a period of 5 days host-free incubation. **(A)** Percentage of amoebae infected with HSC8, starting with 95 % after 2 h host-free survival, followed by a quick decline over the following 4 days. Light blue: No infection, meaning no bacteria had infected a host cell after 48 hours post infection (hpi). Dark blue: Low infection, meaning that 1-5 symbionts have entered a cell after 48 hpi. Green: Intermediate infection, when 5-30 bacteria per amoebae were observed after 48 hpi. Yellow: Full infection, for > 30 cells per host after 48 hpi. Error bars indicate standard deviation **(B)** The exponential decrease in infectivity over time, after 4 days only few survivors of extracellular HSC8 was observed. The equation underlines exponential decline.



**Figure 3.15 Visualization of maintenance of infectivity after host-free incubation in TSY.** The host-free incubation and successive infection was monitored over a period of 5 days with FISH using EUK516 (light blue), a combination of EUBmix and HSC8 (yellow) and by DAPI stain (blue). **(A)** Nearly every amoeba intermediate to fully infected with HSC8 after 2 hours host-free incubation **(B)** Less than half of the total number of amoebae on the panel contain endosymbionts, most are intermediate to fully infected **(C)** Only few infected cells are observed on the second day of host-free incubation. **(D-E)** One or few surviving and infective particles left among empty amoebae hosts **(F)** No bacterial infection observed within amoebae, only endosymbiont-free trophozoites distributed throughout the plate.

## 4. Discussion

### 4.1 Detection of endosymbionts of free-living amoebae

#### 4.1.1 Isolation and axenization of free-living amoebae

The isolation of free-living amoebae from both environmental and clinical samples using non-nutrient agar plates seeded with Gram-negative bacteria (*E. coli*) is used extensively and worldwide (Lagkouvardos et al., 2014). The method was successfully applied in this study. We isolated 15 amoebae cultures in total, of which 10 were successfully grown in TSY or PYG media in the absence of supplementary food organisms. Within several days, amoebae switch from grazing to pinocytosis of TSY or PYG medium as food source. As soon as multiplication of amoebae can be observed in the absence of external live food microorganisms, the culture is typically referred to as axenic. The screening by fluorescence *in situ* hybridization resulted in the detection of three distinct strains of intracellular bacteria among which one strain may most likely represent a novel species and can thus be recognized as a valuable tool. However, the approach has its limitations simply due to the restricted ability of cultivation. The media used may not be suitable for many amoebae that remained unrecognized, together with all possible endosymbionts. Furthermore not only the cultivation is critical, but some amoebae might not be able to adapt to axenic culture conditions and will be dependent on food bacteria, mostly *Escherichia coli*, that may mask the detection of endosymbionts. If a prospective investigation would concentrate on the diversity of amoebal endosymbionts in a defined environmental sample, this method will have to be complemented by other tools, media and supplements. One possibility might be to use alternative food sources, another one would be an alternative isolation procedure. Cocultivation of environmental samples containing symbiont-free axenic amoebae has already been successfully used in the past, but has its own limitations (Collingro et al., 2005b).

#### 4.1.2 Detection of endosymbionts within free-living amoebae using FISH

Fluorescence *in situ* hybridization using rRNA-targeted oligonucleotide probes has been a valuable tool for investigations of uncultured bacteria in complex microbial communities. For the detection of endosymbionts within environmental amoebae, all 15 isolated cultures were treated with a universal probe (EUBmix) in FLUOS together with a specific probe. The specific probe mostly labeled in Cy3 was first chosen among a list of common bacterial orders, families and species, known to be frequent endosymbionts of other amoebae. Among these probes were oligonucleotides targeting *Alpha*-, *Beta*-, and *Gammaproteobacteria* as well as *Chlamydiales* and *Bacteroidetes* in general, more specifically we used probes labeling *Rickettsia*-like and *Caedibacter*-related bacteria, as well as *Amoebophilus asiaticus*

and *Procabacter* sp. The isolate HSC3 gave a strong signal when labeled with the probe specific for the order *Chlamydiales*.

We detected another endosymbiont using a unpublished probe targeting *Rickettsia*-like bacteria (Rick1395). To take a deeper look into the *Alphaproteobacteria*, we applied two other oligonucleotide AcRic90 and AcRic1196 originally designed by Fritsche (Fritsche et al., 1999) to target the closely related endosymbionts UWC36 and UWC8. Interestingly, our isolate HSC6 gave a fluorescent signal only with Rick1395 and AcRic90, and not AcRic1196, which suggests a mismatch in the target region of HSC6 compared to the endosymbionts UWC36 and UWC8.

A successful detection of the endosymbiont HSC8 and HSC9 was achieved by the probe that usually targets *Gammaproteobacteria*, Gam42a. The fact that according to the phylogenetic classification (under 3.3) the strain HSC8 was assigned to the order *Betaproteobacteria* is contradictory to these findings. Gam42a (5'- GCC TTC CCA CAT CGT TT -3') targets the 23S rRNA of *Gammaproteobacteria* and differs from the probe targeting 23S rRNA of *Betaproteobacteria* Bet42a (5'- GCC TTC CCA CTT CGT TT -3'), only by one single base. The simple inability to detect HSC8 with the *Betaproteobacteria*-specific probe Bet42a has also been reported for the distantly related "*Candidatus* *Procabacter acanthamoeba*". The 23S rRNA gene sequence analysis of these *Betaproteobacteria* revealed the presence of one polymorphism at the target site of the oligonucleotide probe Bet42a that is otherwise conserved in all as yet sequenced *Betaproteobacteria*. The presence of this single T-U mismatch in the center of the target sequence destabilizes the probe-rRNA interaction. Consequently, the application of the oligonucleotide Bet42 results in a nearly undetectably weak fluorescent signal (Horn et al., 2002). To further investigate if a similar polymorphism is displayed in the 23S rRNA gene of the endosymbiont of *Acanthamoeba* sp. HSC8, resulting in a target site with stronger affinity for the oligonucleotide probe Gam42a, a 23S PCR can be done followed by a target sequence analysis. In the meantime, a specific probe targeting the endosymbiont HSC8 was designed.

## **4.2 Identification and phylogenetic analysis of endosymbionts of *Acanthamoeba* sp.**

The sample analyzed in this study was a green colored microbial biofilm of a littoral cave wall from the Hawaiian Islands. Such a sea cave is typically formed along a fault or dike by the wave action of the sea. Hawaii's warm tropical climate, ocean shore and volcano landscape make it an interesting natural scenery with great biological diversity. Former studies compared different cave types by analyzing their microflora, and found a high overlap. Especially *Actinobacteria*, *Proteobacteria*, *Acidobacteria*, *Verrucomicrobia*, *Planctomycetes*, *Nitrospirae* and *Bacteroidetes* have been recovered from all cave types. The only phyla found in lava tubes but not yet documented as part of other cave microflora, were *Chlamydiae* and *Ktedonobacteria* (Northup et al., 2011).

In this study, the isolated bacteria were identified as obligate intracellular endosymbionts, which means that they are in need of an amoebal host to replicate. This has been reported for many other bacterial endosymbionts isolated from free-living protozoa, such as *Parachlamydia* sp., *Protochlamydia amoebophila*, "*Candidatus* Paracaedibacter acanthamoebae", "*Candidatus* Amoebophilus asiaticus" and "*Candidatus* Procabacter acanthamoebae" (Beier et al., 2002; Collingro et al., 2005; Everett et al., 1999; Horn et al., 2002; Horn et al., 2001; Schmitz-Esser et al., 2008). The effects that these intracellular organisms may have on their hosts are varied. Previous studies tell us that not only the endosymbiont depends on the host's intracellular environment, but there exist some free-living protozoa whose endosymbionts are just as necessary for their survival (T R Fritsche et al., 1993). We suggest that the study of such relationships in combination with polymerase chain reaction targeting the 16S rRNA gene and sequencing of the 16S rRNA gene are to be important sources of phylogenetic and evolutionary analysis. Unfortunately the further characterization is still very limited due to the inability to cultivate most bacterial endosymbionts free of their protozoal host and this fact nothing but emphasizes the high degree of adaptation to an intracellular life-style.

Phylogenetic analysis was done using MEGA and ARB software and indicates the relationships of the new isolates with their closest relatives and the assignment to orders and families. Comparative sequence analysis revealed that the isolate HSC3 showed highest 16S rRNA sequence similarity (97-99 %) to previously described members of the *Parachlamydiaceae*, including the endosymbiont of *Acanthamoeba* sp. UWE1 (sequence similarity of 99 %) and *Parachlamydiaceae* bacterium CRIB38 (sequence similarity of 97 %) recovered from a water treatment plant. Thus the isolate HSC3 undoubtedly clusters together with the two strains, forming a distinct lineage within the family *Parachlamydiaceae*. Further obligate endosymbionts of free-living amoebae were reported within this family. While *Protochlamydia amoebophila* UWE25 and *Parachlamydia* spp. count as the better studied intracellular chlamydiae, the more recently identified bacteria such as "*Candidatus* Mesochlamydia elodaea", "*Candidatus* Metachlamydia lacustris" and *Neochlamydia hartmanellae* seem to be the closer relatives to this isolate HSC3 according to Figure 8 (Corsaro et al., 2010; Corsaro et al. , 2013; Horn et al., 2000).

We applied the techniques to characterize another isolate HSC6 and it was revealed that these endosymbionts of *Acanthamoeba* sp. were clustered unequivocally within the *Rickettsiales*. In fact most members of this order are associated with arthropods; however the finding is consistent with previous identifications of endosymbionts of *Acanthamoeba* sp. that demonstrate their reliance upon intracellular growth. The two closest relatives were found to be the previously described endosymbiont of *Acanthamoeba* sp. UWC8 and the endosymbiont of *Acanthamoeba* sp. UWC36 (sequence similarity 99 %). More specifically, according to former studies the endosymbiont of *Acanthamoeba* sp. UWC8 and UWC36 show sequence identities of 99.6 % and thus the three organisms may form a well-separated lineage within the *Rickettsiales*. The oligonucleotide probes AcRic90 (5'- TGC CAC TAG CAG AAC TCC -3') and AcRic1196 (5'- CCT ATT GCG TCC AAT TGT -3') were designed complementary to shared target regions on 16S rRNA of both strains UWC8 and UWC36 (Fritsche et al., 1999). Although in this study, only AcRic90 was able to properly detect HSC6 16S rRNA. Because this organism has previously been described by Fritsche et al., no further investigations have been done in this study.

It is commonly recognized that the majority of the described bacteria with an intracellular life-style are members of the *Alpha*- or *Gammaproteobacteria* (Schmitz-Esser et al., 2008). Currently, only a few endosymbiotic organisms have been identified as *Betaproteobacteria*, including the obligate intracellular “*Candidatus Procabacter acanthamoebae*”, recovered from *Acanthamoeba* spp. (Heinz et al., 2007a; Horn et al., 2002). So rather surprisingly, we found that two of the strains HSC8 and HSC9 could be assigned to the *Betaproteobacteria*, with a sequence identity of 100 % to one another, but less than 94 % sequence similarity to the best blast hits in the Genbank. Phylogenetic analysis revealed that the isolated endosymbionts of *Acanthamoeba* sp. HSC8 and HSC9 might represent a distinct novel species within the *Betaproteobacteria* and very likely a new lineage. Most phylogenetic trees show the sequence within a clade of uncultured *Betaproteobacteria* and more distantly related to a few previously described members of the *Neisseriaceae* family. The uncultured *Betaproteobacteria* were isolated from a number of different locations, such as the epithelium of basal metazoan hydra, iron oxidizing biofilms or lake and seawater samples, listed in Table S1, whereas the isolate HSC8 was recovered from a littoral cave in Hawaii. As protozoa hosts are ubiquitous in aquatic and terrestrial habitats such as freshwater, soil and air, these findings are not especially surprising. However the presence of similar bacterial sequences in samples from aquatic environments worldwide might indicate a preference for these conditions.

### **4.3 Curing of natural *Acanthamoeba* spp. HSC host**

Kanamycin is an aminoglycoside antibiotic, used for treatment of infections caused by Gram-negative bacteria. Kanamycin interacts with the bacterial 30S subunit of ribosomes, induces mistranslation and inhibits translocation during protein synthesis (Misumi et al., 1978; Misumi & Tanaka, 1980). After the regular treatment of the amoebae culture with Kanamycin, the endosymbiont HSC8 was successfully eliminated and as a result we obtained a well-grown endosymbiont-free amoebae culture.

Rifampicin is a bactericidal drug of the Rifamycin group, and is typically used to treat *Mycobacterium* infections, but also *Neisseria*, *Listeria* or *Legionella* infections. Rifampicin inhibits bacterial DNA-dependent RNA-polymerase by binding to the active center, thus inhibits RNA synthesis by physically blocking the formation of the phosphodiester bond in the RNA backbone. This “steric occlusion” mechanism prevents an extension of RNA products longer than 2-3 nucleotides (E. A. Campbell et al., 2001). Rifampicin resistance develops quickly during treatment due to mutations that alter residues of the Rifampicin binding site on RNA polymerase. The affinity for Rifampicin decreases, and as a consequence monotherapy bears great risks. The amoebae culture was successfully cured with Rifampicin, but the final culture free of the endosymbiont HSC8 was achieved only after two attempts due to reinfection. Alternatively Rifampicin should be used in combination with other antibiotics, such as kanamycin, Doxycyclin or Erythromycin, which would in addition increase the selective pressure on bacteria and accelerate the process.

Ampicillin is part of the aminopenicillin family and is active against a number of Gram-positive and some Gram-negative bacteria. Ampicillin acts in the inhibition of an enzyme called transpeptidase needed for cell wall synthesis. As most intracellular bacteria are Gram-negative, Ampicillin might not be able to penetrate the outer membrane of the endosymbiont HSC8. The curing of the amoebae culture infected with HSC8 was not successful using Ampicillin as the only antibiotic. Nevertheless, it may be interesting to apply it in combination with other drugs, such as Rifampicin.

Tetracyclin is a broad-spectrum antibiotic and the name refers to the four-ring system of the compound. Although it can be used against a variety of bacterial infections, we point out that it remains especially useful in the treatment of infections by certain obligate intracellular pathogens such as *Chlamydia*, *Mycoplasma* and *Rickettsia*. Tetracyclin inhibits protein synthesis by binding the bacterial 30S ribosomal subunit and by blocking the A site for attachment of the charged aminoacyl-tRNA. Bacteria acquire resistance to Tetracyclin from horizontal gene transfer of a gene that encodes an efflux pump that actively eliminate tetracycline from the cell cytoplasm (Chopra & Roberts, 2001), or a ribosomal protection protein that dislodges tetracycline from the ribosome. Tetracyclin also binds eukaryotic 40S ribosomal subunit, but as eukaryotic cells do not actively pump the antibiotic into their cytoplasm even against a concentration gradient as bacteria do, they remain less vulnerable (Connell et al., 2003). As the amoebae culture treated with Tetracyclin was not successfully cured, we suggest that either the bacteria might have developed resistance very quickly or the concentration of antibiotics was too low. Doxycycline is part of the Tetracycline antibiotic class and has a similar mechanism of action as the other Tetracyclines do. It has been used successfully to treat sexually transmitted, respiratory and ophthalmic infections including the genera *Chlamydia*, *Streptococcus*, *Ureaplasma* and *Mycoplasma*. Interestingly doxycycline is used as antiprotozoal drug in the prophylaxis against malaria, but not much is known about the effects on amoebae. In this study the amoebae treated with Doxycycline did not show any impairment due to the antibiotics and the chlamydial endosymbiont HSC3 was successfully eliminated from the culture.

Other antibiotics such as Erythromycin, Gentamycin, Phosphomycin or Ofloxacin were not tested, but in combination with Rifampicin worth a trial if any further studies in this area were planned. In general, the curing was successful, but to study the drug resistances of these two intracellular bacteria in detail, new combinations would be needed.

## **4.4 Host range of endosymbionts of *Acanthamoeba* spp. HSC3 and HSC8**

### **4.4.1 HSC3 and HSC8 are able to infect a variety of *Acanthamoeba* spp.**

By extending the host range, endosymbionts might be able to further assure their survival in the harsh environments, their continuous distribution as well as limit possible bottlenecks due to transmission. It is also commonly recognized that various amoebae species in distinct geographic regions can harbor closely related strains of endosymbionts (Molmeret et al., 2005; Schmitz-Esser et al., 2008). Such a

global distribution indicates that free-living amoebae such as *Acanthamoeba* spp. function as a unique ecological niche for intracellular bacteria, more than the surrounding habitat does. Within the nutrient-rich intracellular of amoebal trophozoites these bacteria are protected against unaccustomed environmental conditions. Considering the extend of how these bacteria evolved the ability to adapt to the intracellular life-style within early eukaryotic cells it seems possible that many virulence strategies were developed during these interactions, long before plant or animal cells existed. Examples are the ATP/ADP translocase importing ATP from the host in exchange for ADP, the nucleoside triphosphate transporters, taking up nucleotides or the type III secretion system, injecting effector proteins into eukaryotic cells used by several bacterial pathogens. Nowadays pathogenic chlamydiae use these strategies for the infection of humans (Horn & Wagner, 2004). HSC3 as well as HSC8 were shown to permanently infect various *Acanthamoeba* spp. strains, which are members of the same family as the native hosts of HSC3 and HSC8, *Acanthamoeba* sp. HSC. However, they remained unable to infect any eukaryotes of higher developmental order, neither mammalian nor insect cells. Other amoebae such as *Hartmanella*, *Naegleria* or *Dictostelium* were not tested, but should be studied in the near future. In particular, it is known that the closely related *Acanthamoeba* sp. Symbiont UWE25 is able to multiply not only within various *Acanthamoebae* hosts but also in the distantly related amoeba *Dictostelium discoideum* (Fritsche et al., 1998; Skriwan et al., 2002).

#### **4.4.2 HSC3 and HSC8 are incapable to infect mammalian and insect cells**

The study of intracellular bacteria or environmental chlamydiae in particular, represents a unique opportunity to further define characteristic features of host adaption as well as to identify factors that contribute to pathogenicity. HSC3 and HSC8 both are distantly related to established pathogens, such as *C. trachomatis*, *Nesseiria gonorrhoe* and *Burkholderia cepacia*. There already is evidence for a potential pathogenicity of environmental chlamydiae, such as *Simkania negevensis* and *Waddlia chondrophila*, which are able to infect both amoebae and mammalian cells (Corsaro & Greub, 2006; Horn, 2008). However, members of the family *Parachlamydiaceae*, including *Parachlamydia acanthamoebae* and *Protochlamydia amoebophila* appear to have limited ability to thrive in nonprotozoan host cells (Collingro et al., 2003; Maurin et al., 2002; Omsland et al., 2014). No permanent infection of neither HSC3 (*Parachlamydiaceae*) nor HSC8 in HeLa 229 and *Drosophila melanogaster* macrophage-like cell line S2 could be observed. In contrast to *Acanthamoeba* spp. and other amoebae, where uptake of bacteria is achieved through the classical phagocytosis, HeLa 229 cells are non-phagocytic cells. Receptor-mediated endocytosis is a mechanism common among eukaryotic cells for the uptake of macromolecules or small particles such as viruses. This pathway involves plasma membrane domains that later on are transformed into clathrin-coated vesicles (Clerc & Sansonetti, 1987). Several invasive bacteria such as *Shigella flexneri* induce a mechanism similar to phagocytosis, involving condensations of filamentous actin beneath the plasma membrane of HeLa cells as well as myosin accumulations at the entry site. *Shigellae*, *yersiniae*, *salmonellae*, *rickettsiae* and *chlamydiae* are all capable of entering

nonphagocytic cells (Moulder, 1985). S2 cells derived from a macrophage-like cell line of a late embryonic stage of *Drosophila melanogaster* and might as well take up macromolecules by clathrin-dependent endocytosis (Schneider, 1972).

Receptor-mediated endocytosis has also been suggested for the internalization of chlamydiae. The obligate intracellular bacterium *Chlamydia trachomatis* induces its own entry into host cells, a process mediated by a tyrosine-phosphorylated protein at the site of attachment of surface-associated chlamydiae. This chlamydial protein, termed Tarp (Translocated actin-recruiting phosphoprotein) is rapidly translocated across the membrane into the host cell by type III secretion and recruits actin (Clifton et al., 2004). Furthermore, the analysis of total chlamydial genomes has given us valuable information about protein groups, including two important ones, at present, unique to all Chlamydiae. Inclusion membrane (Inc) proteins and the polymorphic membrane proteins (Pmp proteins) are both involved in adhesion and formation of inclusion membrane (Rockey et al., 2000). However, genomic sequencing of the HSC3 related strain *Protochlamydia amoebophila* UAE25 indicated that the outer-membrane of this organism lacks a number of these proteins thought characteristic for all *Chlamydiaceae*. Among these proteins the Pmp proteins, which might be involved in adhesion to mammalian cells might be a reason for the low infectivity of mammalian cells (Collingro et al., 2004). Little information is available on the internalization mechanisms of *Betaproteobacteria*. As we observed no actual infection of either HeLa229 cells or insect S2 cells by the endosymbiont strain HSC8 in this study, we suggest a similar lack of outer membrane proteins. The analysis of the whole genome sequence of the strains HSC3 and HSC8 would add depth to our understanding of this inability to infect mammalian cells and of course further information of chlamydia-specific processes not covered here. Professional phagocytes, such as macrophages, i.e. the mouse macrophages THP-1 were not tested in this study, but would generally be a good attempt for further studies.

Another aspect of non-phagocytic cells is their critical role in the intrinsic immune defense mechanism in response to microbial infection: triggering apoptotic, necrotic or pyroptotic host cell death. On one hand, apoptosis kills pathogens at a very early stage; on the other hand it induces dendritic cells to eliminate infected apoptotic bodies and inducing further protective immune responses. Some pathogenic bacteria have highly evolved strategies to manipulate cell death pathways in order to enhance their own survival and replication. Examples are various gastrointestinal pathogens such as *Salmonella* Typhimurium, *Yersinia*, *Shigella flexneri*, *Helicobacter pylori* and enteropathogenic *E. coli* (EPEC) as well as *Chlamydia trachomatis* and *Legionella pneumophila* (Ashida et al., 2011). If HSC3 and HSC8 do not possess the ability to inhibit host cell death, it might also be a reason why they do not succeed in infecting non-phagocytic cells.

## 4.5 Characterization of the endosymbiont of *Acanthamoeba* sp. HSC3

### 4.5.1 The developmental cycle of the chlamydia HSC3

Fluorescence *in situ* hybridization with rRNA-targeted oligonucleotide probes is commonly used for investigations of uncultured complex microbial communities. In this study it has been a successful tool to track the developmental cycle of the endosymbiont HSC3 in both the native *Acanthamoeba* sp. HSC and the well characterized *A. castellanii* NEFF.

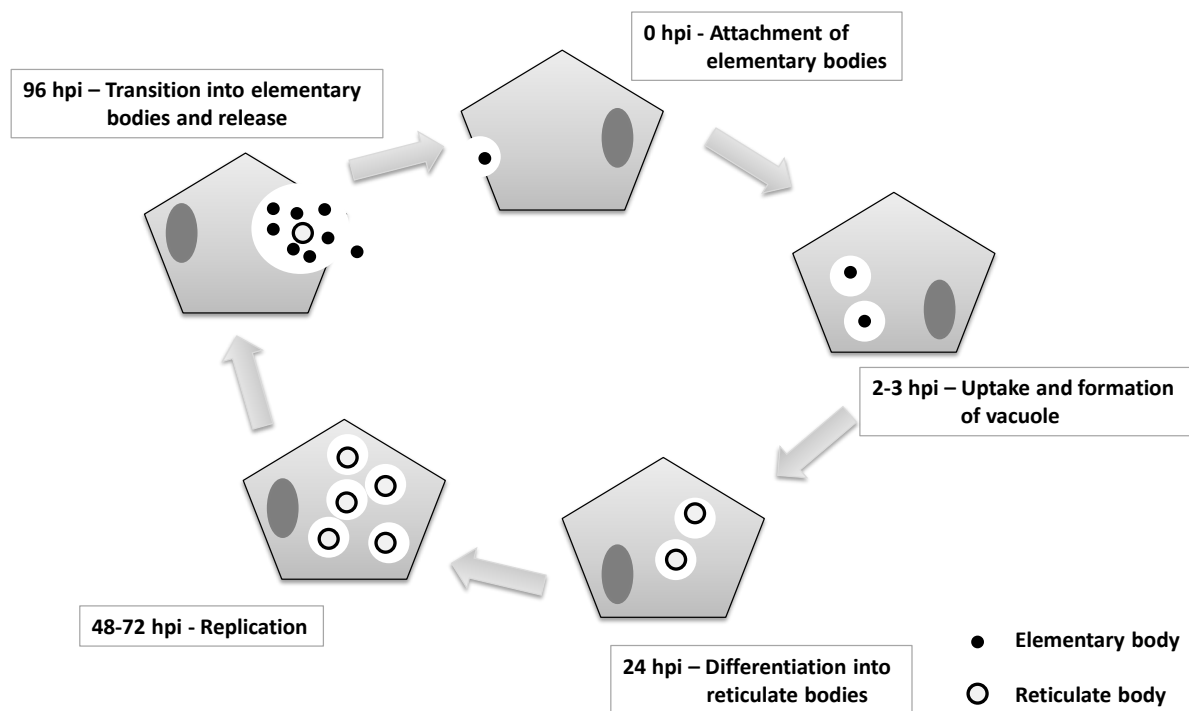
Known chlamydial symbionts display a number of remarkable characteristics shared by all members of the *Chlamydiales*, especially concerning the unique biphasic developmental cycle. It consists of the transition between two main stages, the infectious extracellular elementary bodies (EBs) and the intracellular replicative reticulate bodies (RBs). The endosymbiont of *Acanthamoeba* sp. HSC3 makes no exception, as the combination of fluorescence in situ hybridization and the DNA stain DAPI clearly differentiates between the EBs exhibiting a lower ribosomal content, and the metabolically active RBs. It is well established that FISH does not result in strong fluorescent signals earlier than 12 hpi (Poppert et al., 2002). The only known exception so far is *Simkania negevensis* whose developmental stages both have been described as infectious suggesting a higher extracellular stability of replicative forms (Kahane et al., 2002). However, further insight into the infection cycle of this novel member of the family *Parachlamydiaceae* could reveal yet unknown characteristic features.

The uptake of EBs into the *Acanthamoeba* spp. host occurred after 2-3 hours post infection. As bacterial FISH signals were very rare and at this time point we suggest that the EBs might still have a low metabolic activity but that the number of ribosomes is already increasing. Presumably, the bacteria begin the transition from infectious stage to replicative cell form until the first appearance of mature RBs at 24 hours post infection. The more extensive multiplication continues until up to 72 hours post infection, when at last all *Acanthamoeba* spp. hosts were fully infected with HSC3 after 96 hours post infection. At times, it seems that the intracellular life form of HSC3 has a slightly larger and more elongated shape but to reveal the detailed morphological differences the ultrastructure can be analyzed by transmission electron microscopy. This could as well be a useful tool to look for possible infectious crescent bodies, as have been observed by Greub and coworkers in *Parachlamydia acanthamoebae* (Greub & Raoult, 2002a). According to the study, crescent bodies are defined as a third developmental stage found extracellularly as well as inside the vacuoles of the host. To further investigate the replicative course of HSC3 an additional quantitative real-time PCR can be done.

It is commonly recognized, that all *Chlamydiae* replicate within a host plasma membrane derived vesicular compartment, termed inclusion that is formed during the entry process (Heinz, 2010). We distinguish single cell inclusions from multiple cell inclusions, but unfortunately little is known about neither the intracellular trafficking of inclusions nor how it is involved in the resistance against the host defense mechanisms. HSC3 seems to form single cell inclusions, which would similar to *Protochlamydia amoebophila*, which form small inclusions containing one or few particles (Collingro et al., 2005), but

differ from other members of the *Parachlamydiaceae*. *Parachlamydia acanthamoeba* contains huge fully packed replicative vacuoles (G. Greub & Raoult, 2002; Gilbert Greub et al., 2005). Still it cannot be excluded that the observed features correspond to multiple cell inclusions due to the densely packed amoeba hosts. It remains uncertain as long as it is not further investigated by repeating the infection cycles using a lower multiplicity of infection. Transmission electron microscopy has always been a useful tool for the evaluation of such modalities and has been used frequently to elucidate the life cycle of a variety of obligate intracellular bacteria (Greub & Raoult, 2002b; Kahane et al., 2002)

We conclude, that the endosymbiont of *Acanthamoeba* spp. HSC3 completed its developmental cycle after 96 hours post infection, which is consistent with the information we have about related organisms. *Protochlamydia amoebophila* releases its progeny after 96 hours post infection. In contrast other *Chlamydia*-like bacteria can show huge differences concerning the time of cycle completion. *Parachlamydia acanthamoebae* and *Waddlia chondrophila* both exhibit release of first infectious particles after 24 hpi and 36 hpi followed by lysis of the macrophages (Goy et al., 2008; Gilbert Greub et al., 2003). Undoubtedly, the experimental setup is crucial when comparing two or more organisms, including the temperature, media composition, multiplicity of infection at the beginning of the infection assay and most importantly the host.



**Figure 4.1 The infection cycle of chlamydial endosymbiont HSC3.** Note the two distinct cell types indicating the typical biphasic developmental cycle of the *Chlamydiales* and the single cell inclusions.

#### 4.5.2 Influence of the endosymbiont on growth and fitness of amoebae hosts

Enumeration of amoebae and additional PI staining followed by PI fluorescence measurement give insights into amoebal growth and fitness over the course of an experiment. We analyzed the influence of an endosymbiont on amoebal growth by phase contrast microscopy and calculating the total cell number for all cultures during replication. First of all, we observed that the uninfected *acanthamoebae* grew much faster than the ones infected with the chlamydia endosymbiont HSC3. These findings suggest that HSC3 definitely slows down the exponential growth observed in the empty controls.

A first difference in cell numbers could be observed as early as 24 hps with uninfected amoebae displaying higher numbers, but a remarkable difference occurred at 48 hps. It seems as if the 24 hps are crucial within the course of amoebal replication. This is consistent with the fact that in general *Acanthamoeba* display replication rates between 8–24 hours depending on the species/genotype under optimal growth conditions. (Khan, 2006) However, it is delicate to claim that hosting bacterial endosymbionts is no optimal growth condition, as it is the symbiosis occurs naturally, and the continuous amoeba cultures infected with HSC3 kept at 20 °C are stable. We therefore propose a parasitic life style of HSC3 within *Acanthamoeba* sp. HSC, exploiting the host cell for replication utilizing metabolic pathways and substrates, and thereby slowing down the amoebal growth. Reports have shown that endosymbionts can affect host cell growth of diverse eukaryotic hosts in many ways (Collingro et al., 2004).

Another explanation for the striking difference between infected and uninfected amoebae cultures might involve a bias resulting from density, followed by enhanced nutrient utilization that ultimately leads to a faster depletion of substrates. Our results indicate that such a nutrient depletion might be delayed in the absence of endosymbiont HSC3. Additional density-dependent quorum sensing systems present in many Gram-negative bacteria comprise can influence amoebal growth. Quorum sensing is a system of cell–cell communication mediated by diffusible effector *N*-acyl homoserine lactone (AHL) molecules, also known as autoinducers. Once a certain threshold of autoinducer signaling molecules is achieved, the regulation of virulence genes among others occurs. High cell density has typically been synonymous with cessation of exponential growth (Brown & Barker, 1999), which leads us to the second possible explanation for the constant growth rate of infected *Acanthamoeba* sp. HSC compared to the uninfected ones. We speculate that the presence of bacterial endosymbionts in the amoebae culture leads to a faster depletion of nutriment in the media and as intracellular growth results in early high cell density this could give rise to an early expression of such density-dependent phenomena. Consequently an earlier entry into stationary phase will be achieved in contrast to endosymbiont-free amoebae cultures. However, there is little evidence for such phenomena, either in chlamydiae nor amoebae let alone in a symbiosis between the two organisms and will have to be further analyzed. For example, such an approach could incorporate lower starting numbers to prevent any bias resulting from density. Considering that a period of 3 days is not a long period of time we could speculate that indeed the endosymbionts have an inhibiting effect on amoeba growth, but that the culture might recover after a few more days. This would indicate that infected amoebae cultures just show a delayed starting phase.

Further experiments may prove such a phenomenon if measurements were done over a longer time period.

Based on the results we found regarding the inhibiting effect of HSC3 on its host, it seemed interesting to investigate the degree of damage caused by the endosymbiont. Induction of host cell lysis is frequent among obligate intracellular bacteria; therefore we used a propidium iodide staining for visualization of dead cells. The presence of host cell death within HSC3 infected *Acanthamoeba* sp. HSC cultures was compared to uninfected *Acanthamoeba* sp. HSC. As is commonly known, the cellular exit of *Chlamydiae* can be mediated by two distinct mechanisms. The first pathway is calcium-dependent permeabilization within the cell, starting with rupture of the inclusion membrane followed by other intracellular compartments, resulting in the ultimate lysis of the plasma membrane. The second mechanism is referred to as extrusion pathway, a packaged release process that leaves the host cell intact. (Hybiske & Stephens, 2007) In this study, no significant PI fluorescence intensity peak happens throughout the course of the experiment, indicating that no cell lysis was caused by the endosymbiont of *Acanthamoeba* sp. HSC3 (Figure). Nevertheless, we did not observe packaged release, which is consistent with the findings that HSC3 forms single cell inclusions. The closely related *Protochlamydia amoebophila* UAE25 resides within small inclusions containing only one or few particles, dispersed through the cytoplasm. Both differ from other members of the *Parachlamydiaceae* forming large inclusions with higher number of cells inside the amoebae hosts (Fritsche et al., 2000; Horn et al., 2000; Ludwig et al., 1997). We can only speculate that every chlamydial particle might be packaged within a single cell extrusion and exits the amoebae host by leaving it intact. Further experiments, would give insight into the HSC3 specific mechanism of host cell exit. We could investigate whether extruded chlamydial inclusions were released out of the amoebae entirely or whether they remained surrounded by plasma membrane. In former studies, the cytoplasm and plasma membrane of HeLa cells were labelled with cytosolic-red fluorescent protein and palmitoyl-GFP respectively. These cells were infected with *Chlamydia* and observed with live fluorescence videomicroscopy after 72 h. (Hybiske & Stephens, 2007)

Additionally, amoebal growth in continuous cultures did not seem to be influenced by the presence of HSC3 at temperatures below 27 °C. The amoebal fitness is constant over time in both uninfected and infected acanthamoebae cultures. Nevertheless, we have no information about how HSC3 can affect amoebal fitness at higher temperatures or different growth conditions (e.g. media). *Chlamydia* might grow a lytic effect on its host cells at temperatures around 30 °C. Such investigations would give additional insight into adaption of endosymbiont HSC3 to temperatures higher than in its natural habitat.

The slow increase in PI values in both cultures until 48 hps, the greater divergence between infected amobae and uninfected controls at 48 hps and the contradictory trends after 48 hps can be explained by the extremely low PI values in general. Regarding the threshold of 300, differences in the values ranging between 3 and 19 are quite negligible. The small fluctuations are likely underestimated, due to the greater chance of losing parts of the pellet during washing steps and measuring errors of the Tecan Infinite M200 microplate reader.

A DAPI staining was applied in parallel to visualize the total amount of DNA in the sample, including amoebal as well as bacterial DNA. Results are relatively consistent with the findings of cell counts. Indeed an increase of DAPI fluorescence intensity is observed in uninfected amoebae cultures, whereas the total amount of DNA is constant over time in cultures infected with HSC3. It seems logic that the DAPI values are not as strikingly divergent as the cell counts can be explained by the simple fact that infected cultures contain more DNA due to the endosymbionts, but still less amoebal DNA. Interestingly both cultures show a drop after 24 h, most likely due to a continuous measurement error at the first time point. Nevertheless, the values are very low, indicating that the variations might as well be random and therefore insignificant between all cultures, independent on infection with endosymbiont HSC3. At 48 hps a significant divergence between uninfected and infected cultures occurred, which is kept constant until 72 hps. The first two days seem to be a remarkable time point as the findings collide with the divergence in PI fluorescence intensities.

DAPI fluorescence intensity put in relation to the PI fluorescence intensity represents the relative mortality in an amoeba culture. The relative mortality was constant over time in all cultures. The values were slightly higher in the infected cultures. We might assume that the endosymbiont HSC3 has a very slight effect on the amoebal growth and fitness. However, as the relative mortality rates are based on measurements of PI and DAPI fluorescence intensities, the results of the uninfected amoebae cultures may be biased and underestimated. In that case the difference would be more or less negligible. Further investigations should be done with an improved experimental set-up to avoid measurement errors.

In conclusion, considering the more or less continuously increasing amoebal numbers and no significantly elevated PI fluorescence intensities, we assume an overall non-detrimental effect on amoebal fitness. Bacteria-induced host cell lysis most likely played no major role in the transmission of infective particles. We suggest a non-lytic mode of exit of HSC3 from the host, as has been reported to occur in *Protochlamydia amoebophila* UWE25 (Schulz, 2011) in contrast to the lytic one proposed for *Parachlamydia* sp. (Gilbert Greub & Raoult, 2002c) and for *C. trachomatis* (Hybiske & Stephens, 2007). However in contrast to other free-living protozoa who rely on the endosymbionts for survival, *Acanthamoeba* sp. HSC grow perfectly well and show improved replication rates in absence of endosymbionts HSC3.

#### **4.5.3 Host-free survival capability and maintenance of infectivity in relation to nutrient availability**

Living inside eukaryotic host cells, chlamydiae inhabit an ecological niche in which energy rich metabolic intermediates are readily available. Consequently, many metabolic reactions are dispensable for the *Chlamydiaceae* and therefore these organisms often lack key enzymes of several biosynthetic pathways and are auxotrophic for most amino acids, nucleotides and cofactors (Ilfie-Lee & McClarty, 1999). A similar dependency is also reflected in the genome of the environmental *Chlamydia*. The chlamydial endosymbiont of an *Acanthamoeba* sp., for which a complete genome sequence is available, and a close

relative of HSC3, has been termed *Protochlamydia amoebophila* UWE25 (Collingro et al., 2005; Fritsche et al., 2000). Glycolysis as well as the oxidative pentose phosphate pathway need metabolically active, phosphorylated hexoses as a starting point. In contrast to the *Chlamydiaceae*, *Protochlamydia amoebophila* UWE25 is not only able to import host-derived phosphorylated glucose using a glucose-6-phosphate transporter *uhpC*; (Schwöppe et al., 2002), but encodes a glucokinase (*glk*) and is thus additionally able to generate phosphorylated hexoses independently from its amoeba host cell.

Our findings of this study suggest sustained metabolic activity in HSC3 EBs as their infectivity was hypothetically maintained for 8-9 days in different nutrient-rich and nutrient-free growth media in the absence of host cells. As our tested time period did not involve measurements beyond 7 days, we can only guess a survival of 9 days, further experiments with later time points need to be assessed in this regard. After 7 days of host-free incubation the infectivity rate was reduced to 30 % in average. Nevertheless, they support previous studies about the respiratory activity in *Protochlamydia amoebophila* EBs. Interestingly, the EBs survived in host-free environments apparently independent of the nutrient availability tested in this study, with particular reference to presence or absence of D-glucose.

Indeed, not all host-free metabolic activities detected in *Protochlamydia amoebophila* EBs are expected to occur in exactly the same manner in other chlamydial species due to the differences in their genome repertoire. Thus, the obviously indifference in infectivity for the tested growth conditions, we could suggest that HSC3 might also lack essential proteins for phosphorylation of glucose and therefore rely on import of D-glucose-6-phosphate from their host cell similar to the *Chlamydiaceae*. Hopefully, the analysis of the HSC3 genome sequence will give a deeper insight into the metabolic pathways of this new member of the environmental chlamydiae, especially to enlighten if, for example, it generally encodes the complete pathway for D-glucose catabolism, like the *Protochlamydia amoebophila* genome does or if their metabolism is totally independent from the uptake and/or use of D-glucose in the environment. For example, host-free HSC3 may utilize other internal or external carbon compounds or substrates that enter downstream of the central carbon metabolism. A similar co-occurrence of alternative carbon substrates compensating D-glucose shortage has been suggested in *Protochlamydia amoebophila* (Sixt et al., 2013). As HSC3 EBs survive despite the lack of allegedly essential substrates in buffer and salt solution, we could propose a complete independency from the environmental conditions, explained by glycogen storages or similar storage compounds. Even more likely HSC3 may metabolize products from imported amino acids (in case of the nutrient rich media), protein or lipid degradation.

Nevertheless, most of these speculations do not explain the constantly higher infection rates of EBs grown in nutrient free PBS and salt solution. Even after 7 days of host-free incubation in NaCl the infectivity rate did not sink below 80 %, which corresponds to the initial infection rate. Compared to the other media this was the lowest of the initial infectivity rates. All others ranged round 100 % relative infectivity. Interestingly, we observed a rapid increase immediately afterwards, reaching a peak of 140 % after 2 days. There is a possibility that the adaption of EBs to the salt solution was delayed. As the NaCl solution alone should not vary in osmolarity or pH, we speculate that the EBs are not inhibited directly, but maybe self-induced stress factors or the absence of signaling that trigger survival and infective

activity delayed the process. The mechanisms by which HSC3 can survive the host-free nutrient deprived conditions and the source of energy or substrates that are crucial for the maintenance of infectivity, remains to be elucidated.

Altogether, the HSC3 EBs seem to be very effective in the adaptation for survival in host-free environments. As EBs are indeed the infective stage such abilities can be crucial in their biological role as dispersal stage depending on the density of protozoa hosts in their natural habitat. Despite the evidence of quick adaption to adverse host-free environments provided in this study, the exact requirements for host-free survival and a metabolic potential that has been suggested for *Protochlamydia amoebophila* EBs (Sixt et al., 2013) remain to be enlightened. Further investigations using different growth conditions, changing the nutrient availability, testing other incubation media supplemented with a diversity of substrates need to be assessed.

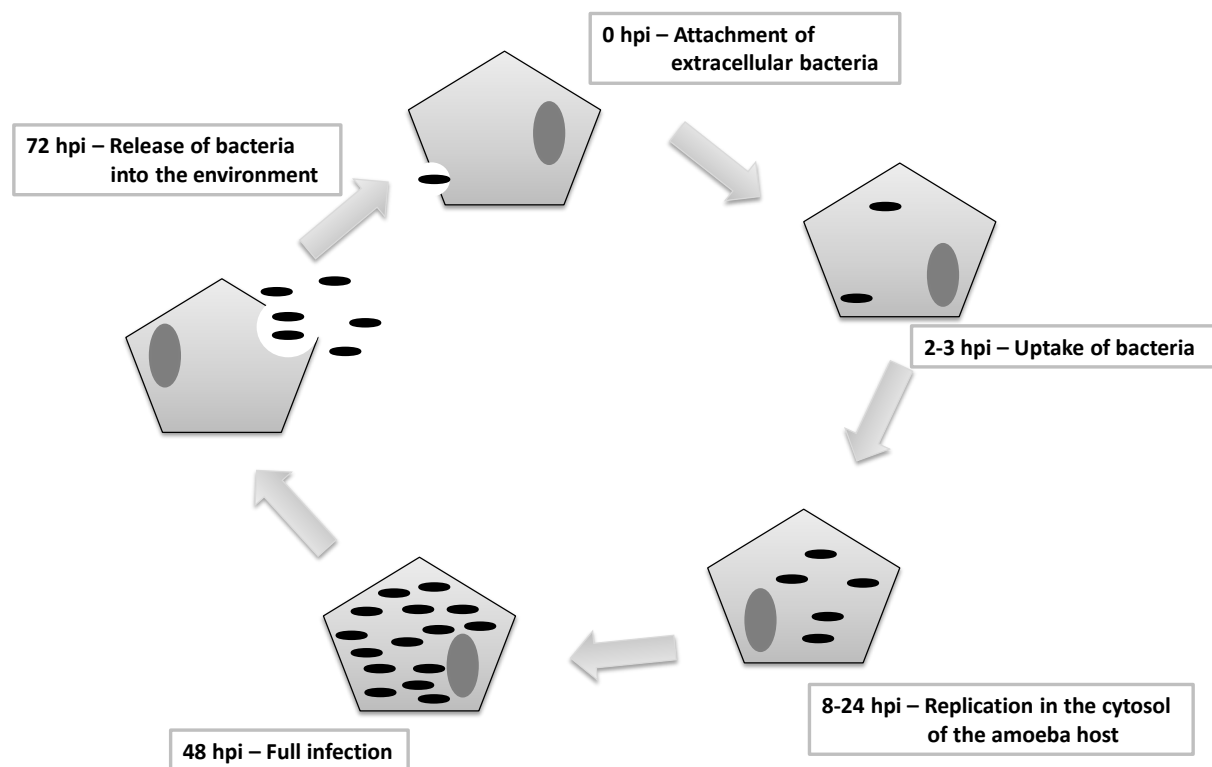
## **4.6 Characterization of the endosymbiont of *Acanthamoeba* sp. HSC8**

### **4.6.1 The infection cycle of the endosymbiont HSC8**

In this study, Fluorescence *in situ* hybridization with rRNA-targeted oligonucleotide probes has been a successful tool to track the developmental cycle of the endosymbiont HSC3 in both the native *Acanthamoeba* sp. HSC and the well characterized *A. castellanii* NEFF (4.5.1). In the same manner, we analyzed the characteristic features of the life cycle of the endosymbiont HSC8 in both *Acanthamoeba* sp. HSC and *A. castellanii* NEFF. As we explored the host range of HSC8, we noticed that they were well able to infect a variety of *Acanthamoeba* spp., but small differences in the developmental cycles are probable (4.4.1).

The uptake of HSC8 into the *Acanthamoeba* spp. host occurred as early as 2-3 hours post infection. Most cells containing one or few particles and begin their massive replication between 8 and 24 hpi. It is commonly recognized, that the mode of entry of endosymbionts into amoeba hosts is by phagocytosis (Khan, 2006). However, we have no information the mechanism of leaving the endocytic pathway and intracellular trafficking for this novel organism. More extensive multiplication continues until up to 48 hpi, when at last all *Acanthamoeba* spp. hosts were densely packed with HSC8. *Chlamydiae* replicate within a host plasma membrane derived vesicular compartment, termed inclusion which is formed during the entry process (Heinz et al., 2010). Other obligate intracellular bacteria, members of the *Alphaproteobacteria* form vacuoles. *Procabacter* sp., a closer relative of HSC8, were observed equally distributed in the cytoplasm (Horn et al., 2002) or enclosed by a host-derived membrane in a multiple-partner association (Heinz et al., 2007b). As was already proposed for the chlamydial endosymbiont HSC3, transmission electron microscopy is a suitable tool for the evaluation of intracellular localization and trafficking in combination with the visualization of vacuoles. FISH revealed a continuous rod-shaped form of the bacteria within, but in the process of infection the detailed morphologies were lost because

of excessive density. To further analyze the ultrastructure, we could use transmission electron microscopy. In the past, it has been used frequently to elucidate the life cycle of a variety of obligate intracellular bacteria (Gilbert Greub & Raoult, 2002a; Kahane, 2002). The replicative course of other obligate intracellular bacteria has been elucidated using an additional quantitative real-time PCR, which would give insight into further characteristics of the HSC8 life cycle. In the end, bacteria were detected in massive amounts in the extracellular environment after 72 hpi, indicating that the developmental cycle is complete. Depending on how the bacteria are enclosed or freely trafficking inside the amoeba host cell, the mode of exit remains to be investigated.



**Figure 4.2 The infection cycle of the bacterial endosymbiont HSC8.** Rod-shaped betaproteobacteria spread evenly throughout the amoeba host cell, no apparent single cell inclusions or clustering.

In conclusion, the endosymbiont of *Acanthamoeba* spp. HSC8 completed its developmental cycle after 72 hours post infection, which is a little earlier than observed with the endosymbiont of *Acanthamoeba* sp. HSC3. The chlamydia-related organism ended its life cycle after 96 hours post infection. Other more closely related Betaproteobacteria, such as *Candidatus Procabacter acanthamoebae* are as-yet the only known obligate intracellular symbiont of amoebae in this phylum. *Ralstonia picketti* is frequently isolated from patient and environmental samples, but has as well been detected inside free-living

*Acanthamoeba* sp., where they form large parasitophorous vacuoles and leave the amoeba hosts by rupture or lysis. They have been shown capable of infecting a variety of *Acanthamoebae* strains. *Burkholderia pseudomallei* and *Burkholderia thailandensis* are related pathogens of the *Betaproteobacteria* invading a variety of cell types, replicate in the cytoplasm, and use a cryptic flagellar system for intracellular movement and cell-cell spread (French et al., 2011; Inglis et al., 2000; Michel & Hauröder, 1997; Ralston et al., 1973).

The direct comparison of the infection rate of endosymbiont HSC8 in its original *Acanthamoeba* sp. HSC host to *A. castellanii* NEFF pointed out differences between the two amoebae strains at the beginning of the developmental cycle. Indeed, especially around 2-3 hpi, when HSC8 started to enter their amoebal hosts, a discrimination between symbionts attached to the outer part of the cell membrane and symbionts that just penetrated the cell membrane to the intracellular. These visual assessments may have led to erroneous results during the first time point and the number of infected *A. castellanii* NEFF may be underestimated. In previous studies FISH slides were therefore manually screened through different focal planes of the amoebae. FISH signals that are distributed throughout the cell in more than one focal plane, the symbiont is most likely intracellular, whereas if FISH signals are found in one single focal plane, the symbionts might still be attached to the outside of the amoebae cell membrane (Diplomarbeit Harreither, 2013). As the course of infection *A. castellanii* NEFF is very consistent with the one in *Acanthamoeba* sp. HSC later in the developmental cycle, the variation at the time of invasion may indicate a so called delayed infection. *A. castellanii* NEFF is not the original host of the endosymbiont HSC8 and may need time to adapt to the absence of a native host.

#### **4.6.2 Influence of the endosymbiont on growth and fitness of amoebae hosts**

Obligate intracellular bacteria can have a variety of effects on the growth and fitness of eukaryotic hosts. As we already observed during the investigations of the chlamydial endosymbiont HSC3, the bacteria slowed down the exponential growth of infected amoebae. Similar results were found during the counting and calculating of uninfected and HSC8 infected amoebae cell numbers. The growth rate of uninfected *acanthamoebae* was much faster than the *acanthamoebae* infected with the endosymbiont HSC8. The endosymbiont somehow reduces amoebae host cell replication, indicated by the relatively constant, non-exponential growth rate observed for uninfected *Acanthamoeba* sp. HSC. We suppose this is due to HSC8 utilizing nutrients from the host as energy source.

A significant difference in cell numbers could first be observed at 48 hps, when uninfected amoebae doubled their numbers compared to infected ones. In 4.5.1, we spoke of density-dependent cell-cell communication systems that can influence amoebal growth by diffusible AHL molecules. Once a certain threshold of autoinducer signaling molecules is achieved, the regulation of various genes occurs, such as virulence genes. High cell density has typically been correlated with cessation of exponential growth (Brown & Barker, 1999). Additionally, the presence of bacterial endosymbionts in the amoebae culture leads to a faster depletion of substrates and as intracellular growth results in early high cell density this

could explain the constant growth rate of infected amoebae. In our case, regarding the starting numbers, such termination of exponential growth in the presence of HSC8 may indeed have happened as early as 24 hps.

The membrane impermeable DNA dye propidium iodide was used to verify any lytic effect of the endosymbiont HSC8 on *Acanthamoeba* sp. HSC. Indeed, PI fluorescence intensity was slightly increasing over time in both uninfected and infected acanthamoebae. Amoebae harboring the endosymbiont HSC8 have consistently elevated PI fluorescence intensity compared to the uninfected control. Despite the few differences in amoebal fitness between infected amoebae and uninfected controls, in general such a small proportion was stained with propidium iodide, that we suppose the host cell membranes stayed intact in both cases. Consequently no host cell lysis was observed and the influence of HSC8 on amoebae fitness was negligible at temperatures below 27 °C. The threshold of 300 was not reached throughout the course of the experiment and even the slightly elevated values of infected cells increasing between 23 and 64 are negligible. Moreover, at the later time point of 72 hps the PI values of the infected culture during the first run decreased, in contrast to all other amoeba cultures. The variations are most likely due to technical issues, such as loss of parts of the pellet during washing steps or measuring errors of the Tecan Infinite M200 microplate reader. However, the PI fluorescence intensities were higher for HSC8 infected than HSC3 infected amoebae. We could assume that HSC8 might have a more prominent effect on host cell fitness at these temperatures than HSC3 has. On the other hand, the chlamydial endosymbiont HSC3 was incubated with a lower starting density. As we already explained, cell density can have an inhibiting effect on amoeba growth due to enhanced substrate turnover which ultimately leads to a faster depletion of nutrients in the media. Host cell death comes along with density-dependent substrate depletion and may consequently be increased in such a way. Additionally, as indicated in our results nutrient depletion might as well occur rather early in the presence of intracellular bacteria, due to parasitic behavior and exploitation of resources. Amoebal exponential growth is inhibited accompanied by host cell death due to starvation. This would not imply any direct symbiont-induced host cell lysis, but neither can we deny such events. Further analysis is necessary to make conclusions about the proportion of cells that die of density-dependent nutrient depletion and the proportion that is lysed due to evasion of intracellular parasites.

For more precise comparison between two intracellular organisms concerning the influence on host amoeba replication and lytic behavior, further experiments must involve lower as well as the same starting densities. Moreover, recent studies reported that temperature does play an essential role in the development of host-symbiont interactions (Fels & Kaltz, 2006; La Scola et al., 2002, 2004; Ohno et al., 2008), the mode of transmission and even modulation of virulence factors of parasites (Restif & Kaltz, 2006). *Parachlamydia acanthamoeba* UV7 grows lytic effects on its *Acanthamoeba* spp. host at temperatures above 30°C, while lower temperatures keep it endosymbiotic for the host cell (Greub et al., 2003). Varying the incubation temperatures for both organisms would give insight into their lytic behavior, thus their influence on host cell fitness in any given environment.

The second DNA dye DAPI is a marker for the total amount of DNA present in the culture. DAPI fluorescence intensity increased constantly over time in all cultures, independent on infection with the endosymbiont HSC8, until 48 hps. A first diversification was observed at 72 hps, where the values for

infected amoebae experienced a drop or cessation of increase, whereas uninfected cultures show continuously rising DAPI signals. In general, these results are consistent with the findings of amoeba cell growth in presence or absence of intracellular bacteria. However, the difference between infected and uninfected cultures was significantly more outstanding by using the enumeration technique than DAPI fluorescence measurements. DAPI is indeed a universal DNA staining dye that binds not only amoebal but also bacterial DNA. Consequently, the values for infected cultures may be overestimated.

The relative mortality rate was calculated by putting the two DNA binding dyes in a relation and was thus used to reveal differences between the cultures. The slightly elevated values in the infected cultures underlined our idea of a growth inhibiting, parasitic life style of HSC8 within *Acanthamoeba* sp. HSC, exploiting the host cell for replication, utilizing metabolic pathways as well as substrates and thereby slowing down the amoebal fitness all the while keeping them alive.

#### **4.6.3 Host-free survival capacity and maintenance of infectivity**

Host-free survival capacity and maintenance of infectivity of HSC8 in nutrient-rich incubation medium was our first attempt to provide a link to their biological role as extracellular survival forms. Simultaneously, host-free survival is likely a critical factor for cell-cell spreading. The extracellular phase of the developmental cycle demands quick adaption to a host-free environment to ensure survival and dispersal. Indeed, a long-term extracellular metabolic activity has been assessed for chlamydiae (Haider et al., 2010; Matsuo et al., 2010), such as *Protochlamydia amoebophila* EBs (Sixt et al., 2013) and *Parachlamydia acanthamoebae* surviving for several weeks in liquid medium (Fukumoto et al., 2010). More recent studies reported that chlamydia-like organisms remain infective for weeks in a number of different conditions, even in sterile tap water. *Parachlamydia acanthamoebae* lose infectivity after 2 weeks, but a few survivors remain displayed even after 10 weeks of host-free incubation in PYG (Coulon et al., 2012). Extracellular survival of endosymbiont HSC3 for more than a week has been observed in this study (4.5.1). Consequently, we suppose that *Chlamydiales* might be environmentally persistent. In contrast, our results indicate that the infectivity rate of endosymbiont HSC8 encounters a quick decrease over time, with no survivors displayed after 5 days of host-free incubation in TSY. The infectivity rate has sunken down to 25 % after only 24 hours. Extracellular dispersal forms of HSC8 do not seem to be as environmentally persistent as *Chlamydiales* are, and might not display such specialized features for facing adverse extracellular conditions. Considering the different growth media used, the dramatic decline of infected amoebae after 24 h may as well be due to the differences in media composition. DGM is a chemically defined nutrient-rich incubation medium, supplemented with various amino acids among other components, whereas TSY is a standard growth medium used for amoeba culturing. Furthermore, as the quick drop happened during the first 24 h, a dilution effect might play an additional role. Generally, *Acanthamoebae* display replication rates between 8–24 hours depending on the species/genotype under optimal growth conditions (Khan, 2006). Uninfected amoebae may at one time replicate faster than infected amoebae and this dilution effect may lead to a bias of the actual infectivity of HSC8.

Studies also revealed that *Chlamydia*-like organisms present better survival capacities than the pathogen *C. trachomatis*. In fact, pathogens infecting mammalian and human cell tissue do not need to remain in host-free conditions for longer time periods, because the cell density is much higher than the amoebal density in environmental sources. When we refer to the native environment of *Acanthamoeba* HSC and its endosymbiont HSC8 in the littoral cave, we could speculate that the density of amoebae is high enough for HSC8 to survive after evading one amoeba host until encountering another one.

A number of studies now revealed a extracellular metabolic activity in chlamydiae (Haider et al., 2010; Matsuo et al., 2010). *Protochlamydia amoebophila* EBs for example, are able to utilize D-glucose even in the absence of amoebae (Sixt et al., 2013). This is most likely a critical factor for long-term survival under host-free environmental conditions. Extracellular HSC8 has not yet been tested for metabolic activity. In our preliminary infectivity assay on the difference between extracellular and intracellular bacteria, we observed none. Both stages were similarly infective, no infective and replicative forms could be distinguished as is typical for chlamydiae. The infectivity curves were similar, starting with an infectivity rate of no more than 20%. The low values are most likely due to technical problems and issues in the experimental set-up as it was a very preliminary experiment. Nevertheless, this underlines the fact that extracellular HSC8 might not be as perfectly adapted to host-free environment and need a healthy environment with relatively high amoebal density to assure survival and dispersal. Indeed the exact requirements for host-free survival, a metabolic potential as has been assessed for chlamydial EBs or the process of adaption to the extracellular conditions remain to be enlightened. We need to pursue possible changes triggered during extracellular stages, on a genomic, transcriptomic and proteomic level to get deeper insight into the issue.



## 5. Abstract

Endosymbiosis involves the internalization of bacterial organisms by a variety of eukaryotes, a field of study gaining more and more recognition. Most of these bacteria have developed intimate and long-term associations with their amoeba hosts and include members of the phyla *Alphaproteobacteria*, *Betaproteobacteria*, *Bacteroidetes*, *Chlamydiae* very recently also *Gammaproteobacteria*. Diversity of obligate intracellular symbionts of amoebae, their host range and global distribution in the environment have been highly underestimated and the bacteria have rarely been identified or poorly described.

In this study, the sample analyzed was a green colored microbial biofilm of a littoral cave wall from the Hawaiian Islands. Free-living amoebae were isolated from the biofilm using a “walk out” method, then axenized to eliminate their necessity of food bacteria. A Fluorescence *in situ* hybridization and 16S rRNA full-cycle approach allowed identification of two novel endosymbiotic bacteria. According to various phylogenetic trees, HSC3 clusters within the *Parachlamydiaceae* family and HSC8 is very likely a member of a novel family within the *Betaproteobacteria*. Such an intimate and complex symbiotic interaction between host amoeba and endosymbiotic bacteria is the key feature that encourages a further in-depth characterization of the novel endosymbionts. These enclose first of all the analysis of host range, as by extending the host range, endosymbionts might be able to further assure their survival in harsh environments and global distribution.

Both HSC3 and HSC8 were able to permanently infect various *Acanthamoeba* spp. strains, but remained incapable of invading non-phagocytic eukaryotic cells neither human HeLa229 nor insect S2 cells. In contrast to their pathogenic relatives, these endosymbionts might not be able to trigger receptor-mediated endocytosis or lack important membrane proteins unique to *Chlamydiales*. By a further description of infection cycles in host amoebae, their impact on amoebae growth and fitness and finally their ability of extracellular survival and maintenance of infectivity, we characterized HSC3 and HSC8 as endosymbiotic bacteria, rather than parasites. Indeed, they seemed to slow down the amoebal replication rate, but overall no direct detrimental effect on amoebal fitness was observed. Bacteria-induced host cell lysis most likely played no major role in the transmission of infective particles. For both endosymbionts, we suggest a non-lytic mode of exit from the host. Our findings of host-free incubation of chlamydial HSC3 showed sustained infectivity over more than 7 days in different nutrient-rich and nutrient-free growth media in the absence of host cells. As HSC3 EBs survive despite the lack of allegedly essential substrates, we suppose they might be environmentally persistent. Indeed host-free survival capacity and maintenance of infectivity of HSC3 and HSC8 very likely provide a link to their biological role as extracellular survival and dispersal forms as well as to quick adaption to a host-free environment. In contrast to HSC3, few survivors of Betaproteobacteria HSC8 were observed after 4 days of host-free incubation, suggesting a less persistent extracellular form.

To lead on the study, whole genome sequencing of the strains HSC3 and HSC8 will provide an efficient means of acquiring data relevant to the more detailed molecular characterization of the endosymbionts.



## 6. Zusammenfassung

Eine Endosymbiose beschreibt die völlige Internalisierung eines Bakteriums durch eine Reihe unterschiedlicher Eukaryoten und gewinnt als neueres Studienfeld mehr und mehr an Bedeutung. Die meisten dieser Bakterien haben eine enge und andauernde Verbindung zu ihren Wirtszellen entwickelt und schließen viele Vertreter der Phyla *Alphaproteobakterien*, *Betaproteobakterien*, *Bakteroidetes*, *Chlamydien* und seit kurzem auch *Gammaproteobakterien* ein. Die Diversität der obligaten intrazellulären Symbionten von Amöben, ihre Bandbreite an Wirten und globale Verbreitung in der Umwelt wurde bisher stark unterschätzt und die Bakterien sind selten identifiziert oder mangelhaft beschrieben worden.

In dieser Studie galt es einen grünfarbenen, mikrobiellen Biofilm zu analysieren, der von der Wand einer Meereshöhle der hawaiianischen Inseln stammte. Wir isolierten freilebende Amöben mithilfe einer „Auswanderungs“-Methode um nach erfolgreicher Axenisierung mögliche intrazelluläre Bakterien zu detektieren. Fluoreszenz *in situ* Hybridisierung und 16S rRNA Gensequenzierung waren Mittel zur Identifizierung von zwei neuartigen Endosymbionten. Diverse phylogenetische Bäume zeigten, dass sich HSC3 innerhalb der *Parachlamydiaceae* Familie einreicht und HSC8 sehr wahrscheinlich eine neue Familie innerhalb der *Betaproteobakterien* bildet. Solch intime und komplexe Interaktionen zwischen Amöben und intrazellulären Bakterien machen eine tiefgehendere Charakterisierung dieser neuartigen Endosymbionten sehr interessant. Deswegen sollte zu allererst eine Analyse der Ausbreitungskapazität durchgeführt werden, da diese ausschlaggebend für das Überleben unter rauen Umweltbedingungen und die globale Verbreitung der Bakterien ist.

Sowohl HSC3 als auch HSC8 gelang eine permanente und volle Infektion diverser *Acanthamoeba* spp., konnten aber keine nicht-phagozytischen Zellen, wie menschliche HeLa229 Zellen oder S2 Insektenzellen infizieren. Im Gegensatz zu ihren nächsten pathogenen Verwandten, scheinen diese Endosymbionten keine rezeptorabhängige Endozytose hervorzurufen oder es fehlen ihnen die notwendigen Membranproteine, wie sie zum Beispiel bei den Vertretern der *Chlamydiales* vorkommen. Die Beschreibung der Lebenszyklen innerhalb der Wirtszellen, der Einfluss der Bakterien auf Überleben und Wachstum der Amöben, sowie die Fähigkeit ohne Wirtszelle infektiös zu bleiben, belegten, dass beide Organismen HSC3 und HSC8 als Endosymbionten statt Parasiten eingestuft werden können. Obwohl wir beobachtet haben, dass die Bakterien die Vermehrung der Wirtszellen verlangsamen, konnte kein direkt zerstörerischer Effekt (Lyse) bei der Übertragung infektiöser Partikel festgestellt werden. Höchstwahrscheinlich liegt bei beiden Endosymbionten ein nicht-lytischer Ausscheidungsmechanismus vor. Weitere Befunde zeigten, dass die Chlamydien HSC3 ihre Infektivität über 7 Tage in verschiedensten nährstoffreichen und -armen Wachstumsmedien beibehalten, obwohl keine Wirtszellen vorhanden waren. Dass HSC3 Ebs ebenso lange überleben, wenn gar keine Nährstoffe zugegen sind, kann ein Hinweis auf ihre extreme Persistenz sein. Sowohl das reine Überleben in Abwesenheit einer Wirtszelle, als auch die Fähigkeit über einen längeren Zeitraum trotzdem infektiös zu bleiben, sind wahrscheinlich wichtige Kriterien für ihre biologische Rolle als extrazelluläre Überlebens- und Verbreitungsformen. So ist es ihnen möglich, sich sehr schnell und effektiv an ungewohnte

Lebensräume anzupassen. Im Gegensatz zu den Chlamydien, scheinen die Betaproteobakterien HSC8 weit weniger persistent zu sein. Für eine weiterführende und detailliertere, molekulare Charakterisierung, wäre die Sequenzierung und Analyse des gesamten Genoms der beiden Organismen HSC3 und HSC8 sehr effizient.

## Appendix

### 16S/18S rRNA gene sequence of the endosymbionts and *Acanthamoeba* hosts

#### HSC3

CGGTTGGAAACGATCGCTAATACCAAATATGGTGCAAGAAGTATCTTCTTGTTATTAAAGTGGGGGATCGCAAGA  
CCTCGCGGTTAAAGAGGGGCCCATGAGATATCAGCTAGTTGGTGAGGTAAGAGCTCACCAAGGCTAAGACGTMT  
AGGCGGATTGAGAGATTGACCGCYAACACTGGGACTGCAGCAACTGCCCTAGACTCCTACGGGAGGCTGCAGTC  
GAGAATCATTGCAATGGGCGAAAGCCTGACGATGCGACGCTGTGTGAGTGATGAAGGCCTTCGGGTCGTAAAG  
CTCTTTCGCTGGGAAAAAGAGAGGTAAGCTAATATCTTACCGATTGAGAGTATCAGGTAAAGAAGCACCGGCT  
AACTCCGTGCCAGCAGCTGCGGTAATACGGAGGGTGCAAGCATTAAATCGGATTTATTGGGCGTAAAGGGCGCGT  
AGGCGGAAAAATAAGTCGGATGTGAAATCCCGGGGCTCAACCCCGGAACAGCATTTGAACTATTTTCCTTGAGG  
GTAGGCGGAGAAAACGGAATTCCACAAGTAGCGGTGAAATGCGTAGATATGTGGAAGAACACCCGTGGCGAAG  
GCGGTTTTCTAGCTTACTCTGACGCTGAGGCGCGAAAGCAAGGGGATCAAACAGGATTAGATACCCTGGTAGTC  
CTTGCCGTAACTATGTATACTTGGTGTAAGTGGACTCAACCTAGTTGTGCCGTAGCTAACGCGATAAGTATACC  
GCCTGAGGAGTACGCTCGCAAGGGTGAACTCAAAGAATTGACGGGGACCCGCACAAGCAGTGGAGCATGTGG  
TTTAATTCGATGCAACGCGAAGAACCTTACCCAGGTTTGACATGCAAAGGACAATACTAGAGATAGTATCTCCCTT  
CGGGGCTTTGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTGGGTTAAGTCCCGCAACGAG  
CGCAACCTTATCATTAGTTGCCAACATTTAAGGTGGGAACTCTAATGAGACTGCCTGGGTTAACCAGGAGGAAG  
GTGAGGATGACGTCAAGTCCGCATGGCCCTTATGTCTGGGGCTACACACGTGCTACAATGGTCGGTACAGAAGGC  
AGCGAAGCCGCGAGGTGAAGCAAATCCCGAAAACCGATCTCAGTTCAGATTGTAGTCTGCAACTCGACTACAAGA  
AGACGGAATTGCTAGTAATGGCGAGTCAGCAACATCGCCGTGAATACGTTCCCGGGTCTTGTAACA

#### HSC6

TTATGGCTCAGAGTGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGAGTAAGAGCCGTAGCAATACGG  
AGTTCTGCTAGTGGCAGACGGGTGAGTAATACATGGGAATCTACCTTAAAGTCTGGGATAACTGTTGGAAACGAC  
AGCTAATACCGGATATTGCCGAGAGGTGAAAGATTTATTGCTTTAAGATGAGCCCATGCAAGATTAGCTTGTGGT  
GGGGTAATGGCCTACCAAGGCTACGATCTTTAGCTGGTTTGAGAGGATGATCAGCCACACTGGAAGTGAACGACG  
GTCCAGACTCCTACGGGAGGACAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCGACGCCGCT  
GAGTGATGAAGGCCTTCGGGTTGTAAAGCTCTTTTAGTAGGGGAAGATAATGACGGTACCCACAGAAAAAGCCCCG  
GCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTG  
CGTAGGCGGCTTGGAAGTTGGAAGTGAAAGCCTAGGGCTCAACCTTAGAATTGCTTTCAAAGTGCCTGGCTAG  
AGTACTAGAGAGGATAGCGGAATTCCTAGTGTAGAGGTGAAATTCGTAGATATTAGGAGGAACACCGGAAGCGA  
AAGCGGCTATCTGGCTAGACACTGACGCTGTTGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA  
GTCCACGCAGTAAACGAAGAGTGCTAGATATTGGAATTTAATTTTCAGTGTCAAAGCTAACCGGTTAAGCACTCCG  
CCTGGGGAGTACGGTCGCAAGATTAAGCTCAAAAGGAATTGACGGGGACTCGCACAAAGCGGTGGAACATGTGGT  
TTAATTCGATGCTACGCGAAAAACCTTACCAGGCCTTGACATGTTGGTCATATCATGAAGAGATTCATGAGTCAGC  
TCGGCTGGACCATCACAGGTGTTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG  
AGCGCAACCTCATCTTAGTTACCAACAGGTTATGCTGGGCACTCTAAGGAAACTGCCGGTGATAAGCCGGAGG

AAGGTGGGGATGACGTCAAGTCAGCATGGCCCTTACGGCCTGGGCTACACACGTGTTACAATGGTGGTGACAATT  
GGACGCAATAGGGCGACCTGGAGCAAATCCCTAAAAGCCACCTCAGTTCGGATTGTACCCTGCAACTCGGGTACA  
TGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATGCTGTGGTGAATACGTTCTCGGGTCTTGTACACACCGCCC

### HSC8

TTCGCCCTTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTACCCGCAGCATGCTGATCTGCGATTACTAGCG  
ATTCCGACTTCATGCACTCGAGTTGCAGAGTGCAATCCGGACTACGATAGGCTTTCTCAGATTAGCTCCCCCTCGCG  
GGTTGGCAACCGTTTGTACCTACCATTGTATGACGTGTGAAGCCCTGCTCATAAGGGCCATGAGGACTTGACGTCA  
TCCCCACCTTCTCCGGCTTAGCACCGGCAGTCCCACTAGAGTTCAAACTTAATTAGGGCAACTAGTAGTAAGGG  
TTGCGCTCGTTGCGTCACTTAAGACAACATTTACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCCAGGT  
TCCCGAAGGCACAATCACATCTCTGCGATCTTCTGGCATGTCAAGAGCAGGTAAGGTTCTTCGCGTTGCATCGAA  
TTAATCCACATCATCCACCGCTTGTGCGGGTCCCCGTCATTCTTTGAGTTTAACTTTGCGACCGTACTCCCCAGG  
CGGTCTACTTCTCGCGTTAGCTTCGCTACTAAGATTACTCCCAACAGCAAGTAGACATCGTTTAGGGCGTGGACTA  
CCAGGGTATCTAATCCTGTTTGCTACCCACGCTTTCGTGCCTGAGCGTCAGTTCTATCCAGGGGGCTGCCTTCGCC  
ATCGGTATTCTCCAAATCTCTACGCATTTCACTGCTACACTTGGAATTCTACCCCCCTCTGACAAACTCTAGACATA  
CAGTCTTAAATGCCGTTCCAGGTTAAGCCCGGGGATTTACATCTAACTTATATATCCGCTGCGCACGCTTTACG  
CCCAGTAATTCCGATTAACGCTCGCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTATTCTT  
CTGGTACTCTCAGTCGCTCATGCTGTTAACACCAGCGGTTTGCTCCAGATAAAAAGAACTTTACAACCCGAAGGCC  
TTCTTATTACGCGGCGTTGCTGGATCAGGGTCCCCCATTGTCCAAAATTCCCCACTGCTGCCTCCCGTAGGAG  
TCTGGGCCGTGTCTCAGTCCAGTGTGGCTGATCTTCTCTCAGAACAGCTACCGATCATCGCCTTGTAAGCCGTT  
ACCTCACCAACTAGCTAATCGGCCATCGGCCGCTTTGTAACGCCAGGCCCGAAGGTCCCCAGCTTTCTCCTCAG  
AGATTATGCGGTATTAGCCAATCTTTCGATTGGTTATCCCCCTCTACAAGGCACGTTCCGATGGTTTACTCACCCGT  
TCGCCACTTGCCATCAACCCGAAGGTCATGCTGCCGTTGACTTGTCATGTGTAAAGCACGCCGCCAGCGTTCAATC  
TGAGCCATGA

### HSC

ATACGGCGAGACTGCGGATGGCTCATTAATCAGTTATAGTTTATTTGATGGTCTCTTTTGTCTTTTTTTTACCTACT  
TGGATAACCGTGGTAATTCTAGAGCTAATACATGCGCAAGGTCCCGAGCGCGGGGGACGGGGCTTCACGGCTCT  
GTTCTCGCATGCGCAGAGGGATGTATTTATTAGGTTAAAAACCAGCGTAGCCAGCAATGGCTACTCAATCTCCTGG  
TGATTCATAGTAACTCTTTCGGATCGCATTATGTCTCCTTGTGGGGACGGCGACGATTATTCAAATTTCTGCCC  
TATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGTCGTAACGGGTAACGGAGAATTAGGGTTCGATTCCGGA  
GAGGGAGCCTGAGAAATGGCTACCACTTCTAAGGAAGGCAGCAGGCGCGCAAATTACCAATCCCGACACGGGG  
AGGTAGTGACAATAAATAACAATACAGGCGCTCGATAAGAGTCTTGTAATTGGAATGAGTACAATTTAAACCCCTT  
AACGAGTAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGT  
TGTTGCAGTTAAAAAGCTCGTAGTTGGATCTAGGGACGCGCATTTCAAGCGCCCGTGCCATCGGGTCAAACCGGT  
GGCTGCGTT

## Uncultured *Betaproteobacteria* that are best blast hits of endosymbionts HSC8

**Table S1.** List of uncultured *Betaproteobacteria* that were isolated from various locations, complemented with the accession numbers of the sequences.

| Acc. Nr         | Name                                                   | Source                                                                                             | Title                                                                                                                 | Author                |
|-----------------|--------------------------------------------------------|----------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------|-----------------------|
| <b>FJ517739</b> | Uncultured beta proteobacterium clone AEP-eGFP-peri_16 | embryonic stages of the basal metazoan Hydra (spike and cuticle) epithelium                        | In an early branching metazoan, bacterial colonization of the embryo is controlled by maternal antimicrobial peptides | Fraune et al.         |
| <b>FJ517688</b> | Uncultured beta proteobacterium clone 14-1_27          | <i>H. magnipapillata</i> , 4 days after temperature treatment, epithelium                          | Disturbing epithelial homeostasis in the metazoan Hydra leads to drastic changes in associated microbiota             | Fraune et al.         |
| <b>EU937888</b> | Uncultured bacterium clone 3BR-5CC                     | riparian iron oxidizing biofilm                                                                    | Biogeochemistry of Iron Oxidation in a circumneutral Freshwater Habitat                                               | O.W. Duckworth et al. |
| <b>AB240515</b> | Uncultured bacterium clone SRRB42                      | PCR-derived sequence from root- base (80 to 160 mm) of Phragmites at Sosei River in Sapporo, Japan | Analysis of microbial community structure in rhizosphere biofilm of Phragmites at Sosei River in Sapporo, Japan       | Y. Nakamura et al.    |
| <b>JN609350</b> | Uncultured bacterium clone 15-4-27                     | activated sludge from lab-simulated sequencing batch reactors                                      | Comparative impacts of nanoscale Ag, ZnO and TiO <sub>2</sub> on wastewater treatment process                         | J. Chen et al.        |
| <b>AJ867904</b> | uncultured betaproteobacterium clone A11-B10           | lake water                                                                                         | Environmental Fluctuations Significantly Influence the Microbial Community Composition Present in Nival Lakes         | M. Yuhana             |
| <b>AJ289984</b> | Uncultured bacterium FukuN108                          | three different lakes (Lake Gossenköllesee, Lake Fuchskuhle, and Lake Baikal)                      | Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters             | F.O. Glöckner et al.  |

|                 |                                                 |                                                          |                                                                                                                                             |                             |
|-----------------|-------------------------------------------------|----------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------|
|                 |                                                 |                                                          | including an abundant group of Actinobacteria                                                                                               |                             |
| <b>AB478649</b> | Uncultured beta proteobacterium clone 004-Cadma | submerged biofilm mats of a montane wetland              | SSU rRNA sequences of bacteria from submerged biofilm mats of a montane wetland in the Alps close to Cadagno di Fuori                       | T.D. Horath and R. Bachofen |
| <b>FJ382075</b> | Uncultured bacterium clone 11W_04c09            | winter hospital shower water from a BMT unit             | Potentially pathogenic bacteria in shower water and air of a stem cell transplant unit                                                      | S.D. Perkins et al.         |
| <b>FJ825812</b> | Uncultured marine bacterium clone BM1-1-44      | filtered surface sea water in the period of diatom bloom | Succession of bacterial community during spring diatom bloom in the Yellow Sea                                                              | L. Min                      |
| <b>JF178540</b> | Uncultured bacterium clone ncd2077d12c1         | skin, popliteal fossa                                    | Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis                      | H.H. Kong                   |
| <b>AB199579</b> | Uncultured bacterium clone: RVW-12              | PCR-derived sequence from river water in Japan           | Selective phylogenetic analysis targeted at 16S rRNA genes of thermophiles and hyperthermophiles in deep-subsurface geothermal environments | H. Kimura                   |
| <b>JF737894</b> | Uncultured bacterium clone RT2-ant07-a11-S      | Rio Tinto                                                | Comparative microbial ecology study of the sediments and the water column of the Río Tinto, an extreme acidic environment                   | A. Garcia-Moyano            |
| <b>HM445124</b> | Uncultured bacterium clone GBL17O32             | yellow microbial mat from lava tube walls, Portugal      | Investigation of Novel Microbial Diversity in Azorean and Hawaiian Lava Tubes                                                               | J.J. Hathaway               |
| <b>AY963481</b> | Uncultured bacterium clone BS43                 | soil                                                     | 16S rRNA gene analyses of bacterial community structures in the soils of                                                                    | O.C. Chan                   |

|                                                                          |                                                                                                                                            |                                                                               |                                                                                                                                         |               |
|--------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------|---------------|
|                                                                          |                                                                                                                                            |                                                                               | evergreen broad-leaved forests in south-west China                                                                                      |               |
| <b>FJ946566</b>                                                          | Uncultured beta proteobacterium clone MWR-B12v                                                                                             | arctic meltwater                                                              | Microbial sequences retrieved from environmental samples from seasonal arctic snow and meltwater                                        | C. Larose     |
| <b>EU104028</b>                                                          | Uncultured bacterium clone M0111_24                                                                                                        | activated sludge                                                              | Evidence for the Comamonadaceae as determinants of activated sludge settling performance                                                | C.E. Brown    |
| <b>FJ236051</b>                                                          | Uncultured beta proteobacterium clone Joinville2                                                                                           | drinking water                                                                | Assessment of phylogenetic diversity of bacterial microflora in drinking water using serial analysis of ribosomal sequence tags         | J.B. Poitelon |
| <b>EU156142</b>                                                          | Uncultured beta proteobacterium clone pCOF_65.7_B2                                                                                         | Coffee Pots Hot Spring                                                        | Molecular characterization of the diversity and distribution of a thermal spring microbial community by using rRNA and metabolic genes. | J.R. Hall     |
| <b>HM481366</b><br><b>HM481365</b><br><b>HM481313</b><br><b>HM481335</b> | Uncultured bacterium clone FL185<br>Uncultured bacterium clone FL176<br>Uncultured bacterium clone FL29<br>Uncultured bacterium clone FL89 | TCE-contaminated field site                                                   | Phylogenetic microarray analysis of a microbial community performing reductive dechlorination at a TCE-contaminated site                | P.K. Lee      |
| <b>DQ129259</b>                                                          | Uncultured bacterium clone AKIW598                                                                                                         | urban aerosol                                                                 | Urban aerosols harbor diverse and dynamic bacterial populations                                                                         | E.L. Brodie   |
| <b>AJ583179</b>                                                          | uncultured beta-proteobacterium clone S15D-MN120                                                                                           | ground water from a monitoring deep-well at a radioactive waste disposal site | Molecular analysis of bacterial communities in ground waters of the deep-well injection site Tomsk-7, Siberia, Russia                   | M. Nedelkova  |

## Glossary

|          |                                                                |
|----------|----------------------------------------------------------------|
| % (v/v)  | volume/volume percentage                                       |
| % (w/v)  | weight/volume (or, more accurately, mass/volume) percentage    |
| % (w/w)  | weight/weight (or, more accurately, mass/mass) percentage      |
| 16S rRNA | prokaryotic rRNA from the small ribosomal subunit              |
| 18S rRNA | eukaryotic rRNA from the small ribosomal subunit               |
| 23S rRNA | prokaryotic rRNA from the large ribosomal subunit              |
| bp       | base pairs (length unit for doublestranded nucleotide chains)  |
| DAPI     | 4',6-diamidino-2-phenylindole                                  |
| DNase    | Deoxyribonuclease                                              |
| dNTP     | Deoxyribonucleotide                                            |
| EDTA     | ethylenediaminetetraacetic acid                                |
| em       | emission wavelength                                            |
| exc      | excitation wavelength                                          |
| FA       | formamide                                                      |
| FISH     | fluorescence in situ hybridization                             |
| FLUOS    | 5(6)-Carboxyfluorescein-N-hydroxysuccinimide ester             |
| hpi      | hours post infection                                           |
| hps      | hours post seeding                                             |
| kb       | kilobases (1 kb = 1000 bp)                                     |
| LSM      | laser scanning microscope                                      |
| MOI      | multiplicity of infection                                      |
| mRNA     | messenger RNA                                                  |
| nt       | nucleotides (length unit for singlestranded nucleotide chains) |
| NTC      | notemplate control (e.g. in PCR or qPCR)                       |
| o/n      | overnight                                                      |
| PCR      | polymerase chain reaction                                      |
| PFA      | paraformaldehyde                                               |
| PI       | propidium iodide                                               |
| qPCR     | quantitative realtime PCR                                      |
| RNase    | ribonuclease                                                   |
| rpm      | revolutions per minute                                         |
| rRNA     | ribosomal RNA                                                  |
| RT       | room temperature                                               |
| SDS      | sodium dodecyl sulfate                                         |
|          | T3SS type three secretion system                               |
|          | T6SS type six secretion system                                 |
| TEM      | transmission electron microscopy                               |
| UV       | ultraviolet                                                    |

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### AUSBILDUNG

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**Masterarbeit** an der Division of microbial ecology, Universität Wien  
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**Universität Zürich**  
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Spezialisierung im 5. und 6. Semester in den Gebieten:  
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Bachelor of Science in Biologie am 7. März 2011

1999 – 2006

Athénée de Luxembourg, Luxemburg  
Sektion C (Schwerpunkt in Naturwissenschaften)  
Matura am 4. Juli 2006

## BERUFSERFAHRUNG

|                              |                                                                                                                                                                                                                                                                |
|------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Januar 2015                  | <b>Greenpeace CEE, Wien</b><br>Direkt Dialog Fundraiserin, Präsentation der Kampagnen und Projekte an die Öffentlichkeit, Menschen inspirieren und überzeugen die Organisation zu unterstützen, Betreuung der Förderer, Ausfüllen von Bankformularen.          |
| August 2011 – September 2011 | <b>Dupont de Nemours, Luxemburg</b><br>Praktikantin, technisches Projekt, <i>"Impact of natural and accelerated ageing on Proshield®, Tyvek® and Tychem® products properties"</i> , Ausführung und Auswertung physikalischer Labortests von Fließprodukten.    |
| März 2011 – Juni 2011        | <b>Departement für Lebensmittelmikrobiologie, ETHZ, Zürich</b><br>Praktikantin: eigenes Forschungsprojekt, Isolierung, Kultivierung und Sequenzierung von komplexer Mikroflora auf Oberfläche von Hartkäse, mikrobiologische und molekularbiologische Methoden |
| Januar 2011 – Februar 2011   | <b>Wildlife rescue center Paradero, Costa Rica</b><br>Freiwilligendienst: Betreuung und medizinische Versorgung von diversen Wildtieren                                                                                                                        |
| Juli 2010 – August 2010      | <b>Dupont de Nemours, Luxemburg</b><br>Praktikantin, Auswertung diverser Laborberichte über Schutz vor biologischen Gefahren durch Spezialkleidung, Internetrecherche, Normen                                                                                  |

## AUßERBERUFLICHE TÄTIGKEITEN

|             |                                                                                                                                                                                                                                                                                                                  |
|-------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 2015        | <b>Aktivistin bei Greenpeace CEE</b> in Wien, NVDA-Training im Februar 2015.                                                                                                                                                                                                                                     |
| 2011        | <b>Mitglied im Animationsteam bei der viertägigen Challenge 2011</b> in Anzere, ein jährlich stattfindender Wettbewerb zwischen den beiden renommiertesten Schweizer Hochschulen, der ETH Zürich und der EPF Lausanne auf und abseits der Skipiste. Vorbereitung des Abendprogramms und Animation währenddessen. |
| 2009 – 2010 | <b>Mitglied im Organisationsteam für die viertägige REEL</b> , réunion européenne des étudiant(e)s luxembourgeois(es), in Zürich im Oktober 2010. Organisation der Events während der vier Tage, Besichtigungen, Veranstaltungen zur Diskussion rund um das Studium, Restaurants und Abendprogramm.              |

2006 – 2007

**Vorstandsmitglied im Verein der Luxemburger Studenten in Zürich** (LSZ) Organisation diverser Events und Ausflüge, Züricher Bal.

2004 – 2007

**Wöchentliche Leitung einer Pfadfindergruppe**, Betreuung von Kindern im Alter zwischen 5 und 8 Jahren, Organisation von Versammlungen, Ausflügen und Pfadfinderlagern. Teilnahme an internationalen Pfadfinderlagern in Moldavien, Polen, Österreich, Spanien und Italien. Formation mit Diplom als ACC (Assistant Chef de Colonie) und CC (Chef de Colonie)

## SPRACHLICHE UND SONSTIGE KENNTNISSE

Luxemburgisch

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MS Office (fortgeschritten), *R Studio* (Grundkenntnisse)

Techniken

Mikrobiologisches Arbeiten; Steriles Arbeiten; DNA-Isolierung & Sequenzierung aus Umweltproben; Kultivierung von Amöben und Bakterien; PCR; Klonieren von Plasmiden;

Arbeitserfahrung mit

*Escherichia coli*, *Protochlamydia acanthamoeba*, *Acanthamoeba castellanii*

Auslandsaufenthalte

Volontariat Costa Rica, Wildlife rescue center, 2011, sechs Wochen  
Studienreise Frankreich, Station biologique Roscoff, 2010, zwei Wochen

## HOBBYS UND INTERESSEN

Hobbys

Individualreisen, Pfadfinder, Sport, Lesen, Musik

Sportliche Aktivitäten

Schwimmen, Indoor- und Beachvolleyball, Inline Skaten, wandern, joggen, Yoga, Snowboarden, Wellen-, Wind- und Kitesurfen, Segeln

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Umwelt, Naturschutz, Ernährungswissenschaften, Medizin, Zoologie



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### EDUCATION

|                                 |                                                                                                                                                                                                            |
|---------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| April 2013 –<br>now             | <b>Master thesis</b> at the Division of microbial ecology,<br>University of Vienna<br>Topic: <i>„Description of two novel endosymbionts of Acanthamoeba<br/>spp.’, under Univ. Prof. Dr. Matthias Horn</i> |
| October 2011 –<br>June 2014     | <b>University of Vienna</b><br>Master study in Molecular Microbiology and Immunology                                                                                                                       |
| October 2006 –<br>December 2010 | <b>University of Zurich</b><br>Bachelor study in biology<br>Areas of specialization:<br>- Microbiology<br>- Molecular biology<br>Bachelor of Science in Biology on 7 <sup>th</sup> March 2011              |
| 1999 – 2006                     | Athénée de Luxembourg, Luxembourg<br>Section C (Scientific)<br>Graduation on 4 <sup>th</sup> July 2006                                                                                                     |

# EMPLOYMENT HISTORY

|                              |                                                                                                                                                                                                                                        |
|------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| January 2015                 | <b>Greenpeace CEE, Vienna</b><br>Direct dialoguer, face-to-face fundraising, presenting information about the campaigns to the public, inspiring people to want to be part of the organization, supporter care, completing bank forms. |
| August 2011 – September 2011 | <b>Dupont de Nemours, Luxembourg</b><br>Intern, technical project, studying the impact of natural and accelerated ageing on products properties, Execution and analysis of physical laboratory tests, internet research.               |
| March 2011 – June 2011       | <b>Departement of food microbiology, ETHZ, Zurich</b><br>Intern, research project, isolation, cultivation and sequencing of a complex microflora on the surface of hard cheese, microbiological and molecular biological methods       |
| January 2011 – February 2011 | <b>Wildlife rescue center Paradero, Costa Rica</b><br>Volunteer: Sitting and medical care of various wild animals                                                                                                                      |
| July 2010 – August 2010      | <b>Dupont de Nemours, Luxembourg</b><br>Intern, analysis of various test reports about biological hazards and protective wear, internet research.                                                                                      |

# EXTRA-PROFESSIONAL ACTIVITIES

|             |                                                                                                                                                                                                                                                                                            |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 2015        | <b>Greenpeace CEE activist, NVDA training, February 2015</b>                                                                                                                                                                                                                               |
| 2011        | <b>Member of the animation team at the Challenge 2011</b> in Anzere, a yearly contest between the two renomated Swiss universities, the ETH Zurich and the EPF Lausanne on and off the ski slope. Preparation and animation of the evening's entertainment during four days.               |
| 2009 – 2010 | <b>Member of the organisation commitee for the REEL 2010</b> , réunion européenne des étudiant(e)s luxembourgeois(es), in Zurich in October 2010. Organisation of events during four days, visitations, discussions around and about the studies, restaurants and evening's entertainment. |
| 2006 – 2007 | <b>Member of the organisation commitee of the Luxemburgish Students in Zurich (LSZ)</b> Organisation of various events and excursions, „Züricher Bal“                                                                                                                                      |

2004 – 2007

**Weekly leadership of a scouts group**, care of children aged between 5 and 8 years, organisation of gatherings, excursions and scouts camps. Participation at international scouts camps in Moldova, Poland, Austria, Spain and Italy.  
Formation with diploma as ACC (Assistant Chef de Colonie) and CC (Chef de Colonie)

## SPOKEN LANGUAGES AND OTHER SKILLS

|                    |                                                                                                                                                                                                             |
|--------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Luxembourgish      | Mother language                                                                                                                                                                                             |
| German             | Mother language                                                                                                                                                                                             |
| English            | Fluent                                                                                                                                                                                                      |
| French             | Fluent                                                                                                                                                                                                      |
| Spanish            | Basic knowledge                                                                                                                                                                                             |
| PC                 | MS Office (advanced), <i>R Studio</i> (Basic knowledge)                                                                                                                                                     |
| Working techniques | Microbiological work; sterile work; DNA isolation & sequencing from environmental samples; cultivation of amoebae and bacteria; cell culturing; PCR; cloning of plasmids; Fluorescent in-situ hybridization |
| Working experience | <i>Escherichia coli</i> , <i>Protochlamydia acanthamoeba</i> , <i>Acanthamoeba castellanii</i>                                                                                                              |
| Stays abroad       | Volunteering in Costa Rica, Wildlife rescue center, 2011, six weeks<br>Study trip to France, station biologique Roscoff, 2010, two weeks                                                                    |

## HOBBIES AND INTERESTS

|                      |                                                                                                                                             |
|----------------------|---------------------------------------------------------------------------------------------------------------------------------------------|
| Hobbies              | Travelling, scouting, sports, reading, music                                                                                                |
| Sports               | Swimming, indoor and beach volleyball, inline skating, running, hiking, Yoga, snowboarding, surfing, windsurfing and kite boarding, sailing |
| Scientific interests | Environment, nature, nutritional sciences, zoology, medicine                                                                                |

