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„*Deciphering the Mysterious*: Interactions between amoebae and their obligate intracellular symbionts“

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„Der erste Trunk aus dem Becher der Naturwissenschaft macht atheistisch, aber auf dem Grund des Bechers wartet Gott.“

Werner Karl Heisenberg

INTRODUCTION

1. INTRODUCTION

1.1 SYMBIOSIS–A DEFINITION OF CONCEPT

The term symbiosis originates from the ancient Greek and consists of two words *σύν* "way of life" and *βίωσις* " in company with, together with" (Henry George Liddell 1940a; Henry George Liddell 1940b). Conceptually, it describes the intimate association between two or more dissimilar biological species living together in long–term interactions. According to the broad public perception, the term symbiosis is often put on a level with a community of two or more species, where all partners obtain and grant severe benefits from each other and may even be incapable of living alone. A conclusion, which does not entirely fit reality for the word symbiosis actually encompasses a continuum of interactions. It was Albert Bernhard Frank, who originally created the term "symbiotismus" during his study of crustose lichens in 1877; a relationship found to be equally mutualistic (Frank AB 1877). Furthermore, the German mycologist Heinrich Anton de Bary came up with a new aspect of symbiosis as he depicted it as "living together of unequally named organisms" (De Bary 1879). According to De Bary's original perception of symbiosis, the outcome of interactions between partners may not contribute to its name thereby including parasitic and pathogenic interactions as well. In modern times, we do differentiate between three distinct ways of interspecies relationships: mutualism, commensalism and parasitism; a classification based on research originating from van Beneden (Sapp 1994). Various efforts in research revealed that besides the physical proximity of participating organisms is of utmost importance, one cannot deny the fact that varying extrinsic and intrinsic parameters heavily contribute to the stability and endurance of such interactions. In agreement with that, symbiosis was described and characterized as long–term relationships that ultimately result in the formation of new structures and gaining of unique metabolic capabilities thereby driving evolution (Douglas 1995). Nowadays, the concept of symbiosis involves various levels of interspecies interactions. In ant–plant symbioses, a severe impact of varying ant behavior on the nature of the interaction, ranging from mutualism to parasitism could be observed (Tillberg 2004). Furthermore, associations between different organisms do also include interactions on a metabolic and genetic level (Moya et al. 2008). Occupying an enormous diversity and variety, the field of ectosymbiosis, where partners maintain their cellular integrity has been studied in detail. Organisms usually live in close proximity to each other, displaying unique behavior and morphological characteristics. Moreover, often partners directly benefit from offered shelter in a controlled environment as well as increased motility and in turn confer access to unique metabolites or

provide additional defense against enemies. Ectosymbiosis is also considered to play a major role in species distribution and species richness thereby enhancing biodiversity (Hétérier et al. 2008). Unlike ecto–symbionts, bacterial organisms that had been internalized by a variety of eukaryotic species subsequently becoming a host may not keep their original morphological, metabolic and genetic integrity. A mutual characteristic of both ecto–and endosymbiosis is a diversification of symbiosis exhibiting obligate and non–obligate associations.

1.2 CLASSES OF SYMBIOSES

In agreement with previous statements, the modern perception of symbioses comprises three distinctive classes of interspecies associations: mutualism, commensalism and parasitism (Sapp 1994). Mutualism resembles interspecies relationships with both partners directly benefitting from each other accompanied by intimate and close proximity. Nowadays, many different examples displaying the modalities of such an association are known for instance the betaproteobacterial endosymbiont *Buchnera aphidicola* residing in aphids (Munson et al. 1991) or *Gingko biloba* of the family of *Ginkgoaceae* containing a green algae inside its host cells (Biloba et al. 2002). Notably, the massive replication and subsequent release of algae cells was confirmed to be directly linked to host cell death. Generally, both partners show signs of elevated fitness and may even accomplish to conquer new ecological niches and gain access to new metabolites. A fine and well–known example for a bilaterally beneficial and therefore mutual interspecies relationship are attine ants (*Formidiacea*), which cultivate fungi in order to fulfill their nutritional requirements through receiving metabolites from the fungi. In return, the fungi is actively feed, propagated and defended by the ants (Mueller et al. 2001). Moreover, ants receive additional protection against a parasitic fungus *Escovopsis* through a mutual relationship with *Pseudonocardia*, a genus of bacteria residing on the ant's integuments and producing antibiotics and secondary metabolites (Currie et al. 2003).

Commensalism is mainly characterized through benefits in terms of fitness accessible for only one of two or more partners, whereas the other(s) are not affected. Examples are *Acinetobacter* strain C6 and *Pseudomonas putida* strain R1 either displaying competition or commensalism in the presence of a single carbon and energy source (Christensen et al. 2002). The definition of parasitism encompasses the increase of fitness for one of the associated partners directly linked to the fitness reduction of the other. Often symbionts are using hosts to exploit their metabolic features and thereby gain access to formerly unusable metabolites. Examples are parasitic wasps belong to the *Hymenoptera* super–families parasitizing on various animals, predominantly other arthropods like larval *Lepidoptera*, a class of caterpillar

hosts. Through oviposition in host bodies or even eggs, adult female wasps ensure safety of own offspring, who benefits from host tissue directly and eventually hatches into larvae. These larvae directly feed on host tissue leading to the ultimate death of the host. Eventually larvae start to pupate and break free, leaving only the shell of the remaining host (Jervis et al. 2001; Sugonyaev 2006).

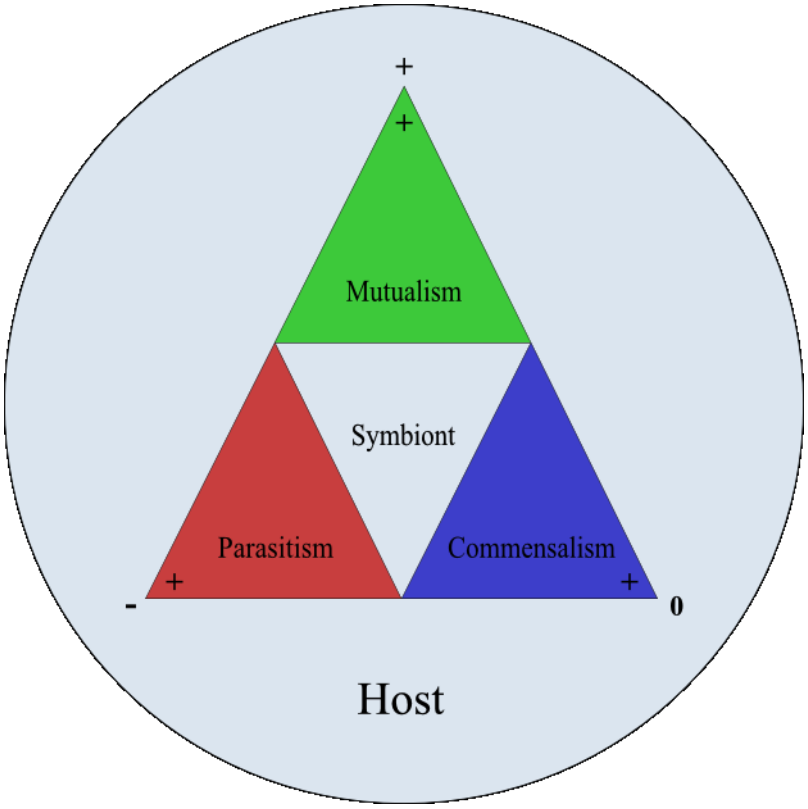

Fig. 1 Concept of host–symbiont relationships in intracellular associations. In symbiosis, host organisms face different impacts on fitness level. + = beneficial, enhanced fitness. **0** = unaltered fitness. – = disadvantageous; decreased fitness.

Further research showed that symbiosis may not solely be reduced to associations like mutualism, commensalism or parasitism (Fig.1) but may as well be greatly influenced by other intrinsic and extrinsic effects. These effects may vary in their importance and impact and can be neutral, positive or even negative for both symbiont and host. Interspecies relationships can vary in strength and impact exhibiting obligate and non–obligate respectively facultative symbiosis. In a facultative association, one or both partners do not solely rely on each other in terms of fitness and survivability and are capable of living alone. In terms of microbial ecology, these symbionts are referred as secondary symbionts, which are often transmitted horizontally thereby mediating the effect of an infection more likely through ecological interactions. However, these interactions still contribute to an enhanced fitness level by offering additional protection against enemies, as it has been shown for *Acyrthosiphon pisum*, a tea aphid and its secondary symbionts (Oliver et al. 2003). Here, *Acyrthosiphon pisum* inhabited by a variety of secondary symbionts (for example members of

the *Rickettsia* and *Spiroplasma*) beside *Buchnera aphidicola*, its primary endosymbiont exhibit a reduced likelihood in supporting the parasitoid development of its natural enemy, the hymenopteran *Aphidius erdi*. Secondary symbionts are still capable of performing replication outside a host cell/tissue; a characteristic feature well known for *Legionella pneumophila*, the causative agent of Legionnaire's Disease (Horwitz & Silverstein 1980).

Furthermore, interspecies associations are often broadly ranged and involve the concept of a defensive mutualism. It has been reported, that fungal endophytes of the ascomycete family *Clavicipitaceae*, once inside a host plant initiate the production of reactive oxygen (ROS) species in order to alter host cell membranes and thereby facilitate leakage of nutrients. Due to increased levels of ROS, the plant in turn starts to produce antioxidant species like flavonoids, which subsequently increases the plant's resistance against a variety of stresses and diseases (drought and metal stress) (White & Torres 2010). The lichen symbiosis is generally considered to display a defensive mutualism, where the symbiosis has ultimately resulted in a combined mutualistic unit possessing greatly enhanced tolerance against various biotic and abiotic stresses. Interestingly, fungal endophytes may further directly contribute to plant stress resistance by production of a variety of carbohydrates and phenolic compounds and thereby providing a direct benefit (Malinowski & Belesky 2006; White & Torres 2010). Concluding, the exact outcome of symbiotic interactions regarding the impact on host and symbiont fitness may depend on conditions that associated species face as well as on the nature of involved organisms itself.

Endosymbiosis, as already depicted with the example of *Buchnera aphidicola* residing in specialized host cells (bacteriocytes) of aphids (family *Aphididae*) (Munson et al. 1991; Oliver et al. 2003), is generally defined by associations involving organisms of unequal size. One species resides in one other, receiving shelter, protection from hostile environment, nourishment and niche for stable replication. Most hosts in these relationships belong to the domain of eukaryotes providing an environment rich in nutrients. Moreover, these hosts often even predate on bacteria and therefore uptake is mainly facilitated through phagocytosis; a process which most bacterial species do not survive. Establishing an intracellular interspecies relationship is often initialized through bacteria escaping or preventing lyses and evading other host cell defense mechanisms. Once protection from the outside and the host has been ensured, bacterial species begin to exploit host cell related pathways and metabolites for their own replication and spread. However, bacteria do as well eventually enable host cells to conquer and subdue unique ecological niches by conferring access to its own metabolic

capabilities, transfer of primary or secondary metabolites or reprogramming of host gene expression patterns (Parniske 2008; Wernegreen 2004; Oldroyd et al. 2009) Eventually, host cells may be able to utilize nutrients, which had been inaccessible before. In contrast to facultative endosymbiosis, obligate associations between two or more species hold several disadvantages: inability to survive alone, tight symbiont–host relationship and loss of genetic capabilities for the symbiont (Nikoh et al. 2011; Ochman 2001). Intracellular symbionts generally consist of both pro–and eukaryotes.

1.3 AMOEBAE–PREDATORS OF BACTERIA

Unicellular eukaryotes like protozoans play a key role inside these biofilms in terms of regulating the microbial biomass. Comprising various kinds of animal–like protists like amoebae, paramecia and trypanosomes, they assist in maintaining the variety and amount of microbial species (Brown & Barker 1999).

Known to be efficient predators of bacteria, fungi, yeasts and algae, free–living amoebae comprise a variety of different, unicellular and shapeless organisms belonging to the eukaryotic domain of life. Bio–ecological features of free–living amoebae are various and complex. The occurrence of free–living amoebae is widespread and amoebae can be isolated frequently from anthropogenic ecosystems such as tap water, air–conditioning units and cooling towers. Moreover, they contribute to plant growth (Bonkowski 2004) and have a great influence on the diversity of microbial communities (Rodríguez–Zaragoza 1994). Feeding on the microbial biofilm, they assist in maintaining a high bacterial mineralization rate of organic matter through predation (Molmeret et al. 2005).

The uptake of prey and food is performed through phagocytosis and pinocytosis. 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. It has been shown, that random pseudopodia initiations in combination with two distinct sensing models (temporal and spatial sensing) are key factors in locomotion (Allena 2013). Moreover, pseudopods facilitate attachment to a variety of surfaces and are responsible for the typical amoebal cell morphology and irregular shape.

Furthermore, free–living amoebae are well known pathogens of humans. *Acanthamoeba spp.*, a free–living and opportunistic, amphizoic protozoa commonly found in environmental habitats like fresh waters is also the causative agent of amoebic keratitis (Rahdar et at., 2012)

and Granulomatous Amebic Encephalitis (Khan 2003). The genus *Hartmannella* belonging to the kingdom of amoebozoa was first characterized in 1967 (Page 1967) and have also been isolated from human tissue (Wang & Feldman 1967). Both genera achieve replication through cell division. Basically, one can distinguish between two different developmental stages. Trophozoites resemble the metabolically active and vegetative form (Bowers & Korn 1968), whereas cysts 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 2006a). Additionally, cysts are metabolically inactive and capable of facilitating the recurrence of infection and thereby enabling amoebae to withstand periods of unfavorable environmental conditions (Khan 2006a). The developmental cycle is closed upon the emergence of trophozoites from cysts, if favorable conditions have reoccurred. Trophozoites of *Acanthamoeba* are usually observed if amoebae are actively growing and replicating for example if cultivated in commonly used media like TSY (Trypticase Soy Broth with Yeast Extract). Like other protists, *Acanthamoeba* carry a large membrane–bound contractile vacuoles filled with water for osmoregulation.

1.4 Emergence of endosymbiosis through bacteria escaping amoebal grazing

Acanthamoeba have first been shown to inhabit bacteria apparently resistant to digestion by the host in 1956 (Drozanski 1956). In the mid–1970s, it was confirmed that *Acanthamoeba* provide shelter and protection for bacteria referred as endosymbionts (Proca–Ciobanu et al. 1975). Uptake of bacteria, the predominant prey of *Acanthamoeba* (Weekers et al. 1993) is mainly facilitated through phagocytosis; a process unlike pinocytosis (unspecific membrane engulfment) mediated through receptors (Greub & Raoult 2004). *Acanthamoeba* preferentially graze on gram–negative bacteria; a fact widely used in isolation assays (Khan 2006a). While many bacteria are taken up by phagocytosis and converted into food and energy, some of them manage to escape their fate. Upon bacterial uptake into large food vacuoles, bacteria are both rapidly killed and digested in phago–lysosomes. However, some manage to escape host–derived digestion either through inhibition of the formation of phago–lysosomes, modulation of endosomes through host manipulation (Isberg et al. 2009) or prior escape from phagosomes. Induction of host cells lysis trough host cell apoptosis is frequent among obligate intracellular pathogens (Ito et al. 2012; Ojcius et al. 1998).

Moreover, bacterial pathogens may even be capable to grow at acidic pH, once inside a phago–lysosome (Khan 2006a). Bacteria may then either adapt to an intracellular life style or exploit the host cell for massive replication and subsequent outbreak. Indeed, survival and intracellular growth of bacterial species in protozoa may well prime pathogenic bacteria for virulence. *Acanthamoeba* may thereby transport pathogens 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 2004), which are well known pathogens of humans. Examples are the facultative pathogenic mycobacteria like *Mycobacterium avium*, the causative agent of respiratory disease (Prasad & Gupta 1978; Taylor et al. 2003). Through constant selective pressure in a hostile environment, bacteria may well get primed to adapt to intracellular life in eukaryotes and subsequently extend their host range (Molmeret et al. 2005; Horn & Wagner 2004). Famous for being the causative agent of Legionnaire's Disease, *Legionella pneumophila* is an intracellular pathogen of humans and has also been found frequently inside amoebae cells (Holden et al. 1984; Kwaik 1996; Rowbotham 1980). Notably, phagosomes of *Hartmannella vermiformis* containing *L. pneumophila* are surrounded by host–cell rough endoplasmic reticulum; a protective compartment rich in nutrients and energy (Kwaik 1996).

However, *L. pneumophila* can also grow outside of host cells and is therefore considered a facultative intracellular pathogen. Intracellular survival can also confer beneficial traits to pathogenic bacteria in terms of virulence and susceptibility to drugs. It was shown, that *M. avium* exhibits elevated levels of virulence in case of mouse models, if grown within *Acanthamoeba* castellanii compared with free–living *M. avium* (Cirillo et al. 1997). Similar observations were made for *L. pneumophila*, where amoeba–grown bacteria could facilitate entry into macrophages via coiling–phagocytosis at a higher frequency than agar–grown *L. pneumophila* did (Cirillo et al. 1994). Additionally, *L. pneumophila* grown in *Acanthamoeba castellanii* exhibited increased levels of virulence in mice models (Cirillo et al. 1999). *Vibrio cholera* (causative agent of cholera) (Thom et al. 1992), *Simkania nevgensis* (causative agent of pneumonia) (Kahane et al. 2001) and *Pseudomonas aeruginosa* (the causative agent of keratitis) are further prominent human pathogens capable of thriving inside *Acanthamoeba*.

Virulence and the ability of intracellular survival and replication also varies between different isolates of bacterial strains. In agreement with that, the invasive *Escherichia coli* K1 exhibited significantly increased levels of association with and invasion of *Acanthamoeba castellanii* host cells, if compared with non–invasive isolate K12 (Alsam et al. 2006). Furthermore,

association, invasion and intracellular survival also depend on symbiont outer membrane proteins as shown for *E.coli* K1 (Alsam et al. 2006). In conclusion, these findings suggests, that *Acanthamoeba* heavily contribute to the transmission of bacterial pathogens to humans and thereby conferring protection against the human immune system. Through the ability of forming cysts under harsh and unfavorable conditions such as extreme temperatures, osmolality and pH, *Acanthamoeba* provide suitable vectors for pathogenic facultative bacteria or obligate endosymbionts. Reports have also shown, that cysts of *Acanthamoeba* and *Hartmannella* can cope with various drugs and chlorine (Kilvington & Price 1990; Kuchta et al. 1993). In agreement with the numerous findings of bacteria residing within amoebae, it was supposed that about 25% of all *Acanthamoeba* contain bacterial endosymbionts (Fritsche et al. 1993).

Most of these bacteria have developed close and long–term associations with their hosts; a key feature, which greatly undermines traditional culture–dependent identification assays. As a result of the development of culture–independent techniques, for example the 16S rRNA full–cycle approach in combination with various phylogenetic tools, more and deeper insights into these complex relationships have been gained. Four bacterial taxa assembling all these different endosymbionts were identified: The *Alphaproteobacteria*, the *Betaproteobacteria*, the *Bacteriodetes* and the *Chlamydia* (Fritsche et al. 1999; Horn & Wagner 2004; Schmitz–Esser et al. 2008). Notably, regions of geographically distinct character do harbor amoebae containing similar strains of members from all lineages mentioned above. This clearly indicates a global distribution (Schmitz–Esser et al. 2008; Horn & Wagner 2004).

1.5 ENDONUCLEAR ENDOSYMBIONTS–LIFE STYLE AND DEVELOPMENTAL CYCLES

Endosymbionts residing in either the host cytoplasm or host endosomes modulated through host manipulation (Isberg et al. 2009) have been described in great detail during the last 30 years (Fritsche et al. 1993; Fritsche et al. 1999; Horn et al. 2002; Molmeret et al. 2005; Barker & Brown 1993). Most of these endosymbionts either use these manipulated host endosomes or the cytoplasm for exploitation of the host's metabolic capabilities and massive replication as well as protective shell against a hostile environment. Only rarely, other host compartments like mitochondria are targeted by endosymbionts (Epis et al. 2008; Sassera et al. 2006). Intracellular bacteria could also be located in chloroplasts of photo–synthetically active dinoflagellates (Wilcox 1986). Since the early 1980's a number a studies focusing on the emerging field of endocytobiosis or cytobiosis has been published offering new insights into

these complex host–endosymbiont associations. For example, the novel relationship between the ciliate *Paramecium caudatum* and its gram–negative endosymbiont *Holospora* residing inside the nucleus gave rise to various kinds of questions regarding host fitness, survivability and replication as well an endosymbiont propagation and developmental cycle (Görtz 1983). Here, *H. undulata* and *H. elegans* exclusively target the host cell micronucleus, a compartment of reduced transcriptional activity and condensed chromatin, whereas *H. obtusa* inhabits the host macronucleus (Fujishima & Görtz 1983). Commonly, these bacteria specifically target the host cell nucleus, but can also be found in the cytoplasm during early stages of infection. Furthermore, transmission easily occurs through residual food vacuoles of already lysed symbiont–bearing cells to symbiont–free cells (Görtz 1980; Fujishima & Görtz 1983). Additionally, besides host nuclei specificity a host specificity was also observed, for *H. elegans* and *H. undulata* were only found in micronuclei of *P. caudatum*, whereas *H. obtusa* could only invade macronuclei of certain strains of *P. caudatum* (Fujishima & Fujita 1985). Moreover, *H. obtusa*–bearing paramecia found in nature only belonged to strains of *P. caudatum* species. Further research revealed that *H. obtusa* exhibits two distinct morphologies reflecting different developmental stages: a reproductive short form inhabiting macronuclei of propagating host cells and an infectious long form only observed in starved host cells (Görtz et al. 1989). In agreement with that observation, *H. elegans* as well possesses these biphasic life cycle (Görtz 1983). Fujishima and Fujita could also show, that a successful infection and maintenance in host macronuclei as well as *H. obtusa* host specificity may not only rely on its ability to penetrate the nuclear membrane, but as well depend on host–related factors (Fujishima & Fujita 1985).

The evaluation of the impact of host–nuclei specific endosymbionts on host DNA replication and transcription became interesting. The identification and characterization of a novel bacterial parasite *Endonucleobacter bathymodioli* targeting the nucleus of deep–sea bathymodiolin mussels (namely *Bathymodiolus puteoserpentis*) highlights small barrier of mutualism and parasitism (Zielinski et al. 2009). In contrast to *Holospora*, where a stable host–symbiont association has been observed at least for some species (Fujishima & Fujita 1985), these endonuclear symbionts are considered to be parasites, actively exploiting the host nucleus for massive replication based on a single cell to more than 80 000 cells. The disruption of the host cell cytoplasmic membrane through a greatly swollen nucleus ultimately leads to the host cell death and bacteria are released after the nuclear membrane has burst. Analyses of greater depth have shown that the *E. bathymodioli* endonuclear developmental cycle consists of six distinct stages comprising various cell morphologies.

Starting with a solitary rod–shaped cell inside the host nucleus, *E. bathymodioli* develops into one or several un–septated filaments. Replication mainly occurs through longitudinal fission; a rare finding among bacteria. Moreover, consequences for the host involve morphologically irregular shape of the nucleus in combination with ongoing chromatin reduction. Later stages exhibit massively replicating bacteria through a shift to transverse binary fission and the occurrence of shorter rod–shaped cells most probably caused by nutrient limitation (Zielinski et al. 2009). Supposing, *E. bathymodioli* utilizes host chromatin thereby leading to its reduction. Escape of endonuclear bacteria is currently not well understood. Most probably, endonuclear bacteria are passively released through burst of the endonuclear membrane. However, recent studies propose a direct interaction between the symbionts and the host nucleus division apparatus in order to facilitate escape (Görtz 2006; Schweikert et al. 2013; Fokin 2004). Here, the highly infectious form of *H. obtusa* is somehow associated to the host nuclear membrane during *B. puteoserpentis* division. Additionally, *B. puteoserpentis* harbors two types of chemosynthetic bacterial symbionts (sulfur–oxidizers and methane oxidizers) in the cytoplasm of its mussel gill bacteriocytes, which grant access to carbon compounds supporting growth and maintenance of host biomass. In return, these endosymbionts receive reductants and oxidants from the host; a mutually beneficial relationship. Interestingly, *E. bathymodioli* could never be detected in these bacteriocytes indicating additional protection conferred by the symbionts.

Unlike *E. bathymodioli* most other endonuclear symbionts target members of the universal clade of protists (Fokin 2004; Schweikert et al. 2013). Therefore, amoebae, which have already greatly contributed to the discovery of various bacterial endosymbionts, may further provide a suitable model for studies of endosymbionts residing in unusual eukaryotic cell compartments. Only recently, the discovery of a new bacterial endosymbiont inhabiting the nucleus of its *Hartmannella sp*. host could be confirmed (Schulz et al. 2014). Exhibiting only moderate relation to known bacteria (~90% 16S and 23S rRNA sequence similarity) the endosymbiont was named *Nucleicultrix amoebiphila*. Further phylogenetic evaluation showed that this organism belongs to a novel clade affiliated with *Rickettsiales* and *Rhodospirillales*. Host–range assays revealed that *N. amoebiphila* could as well infect various *Acanthamoeba* strains. In agreement with previous results, uptake was mainly facilitated through amoebae grazing on endonuclear bacteria. Evaluation through fluorescence in–situ hybridization could reveal further insights into the developmental cycle of this novel symbiont. Similar to *E. bathymodioli*, infection of host nuclei may start with a solitary cell and host nuclei pronouncedly increase in size after 96hpi due to high amounts of cells. In contrast to *E.*

bathymodioli, a stable and long–term maintenance of the symbiont is established, at least in *Hartmannella sp*. Host cell lysis only partly occurs, but confers horizontal transmission beside frequent vertical transmission. Intriguingly, no impact on host fitness could be observed, as it has been for *P. caudatum* exhibiting *H. obtusa* (Fujishima et al. 2005) or *H. elegans* (Hori et al. 2008).

Recent studies have also suggested, that under altered and unfavorable environmental conditions, endonuclear symbionts facilitate alterations in gene expression profiles and thereby contribute to enhanced host stress resistance (Hori et al. 2008; Fujishima et al. 2005). Heat–shock resistance and maintained motility under unfavorable temperatures of *P. caudatum* conferred by infection with the macronucleus–specific endosymbiont *H. obtusa* was irreversibly enhanced, even in host cells cured from bacterial presence and termed "aposymbiotic" compared with empty controls (Fujishima et al. 2005).

A similar observation had been made for *H. elegans* exclusively infecting the micronucleus of *P. caudatum*. Endonuclear symbiont–harboring ciliates as well as aposymbiotic cells exhibited constantly enhanced expression levels of hsp60 and hsp70 mRNA even at 25°C and could maintain this beneficial trait for over a year. Fluorescence in–situ analysis additionally indicated the transfer of *Holospora* genomic DNA from the micronucleus into the macronucleus of symbiotic paramecia (Hori et al. 2008). Furthermore, a growing number of studies have indicated, that bacteria pathogenic for various animals and plants are modulating host defenses through direct interception with gene expression. Hereby, bacterial secondary metabolites termed "nucleomodulins" are secreted and directly enter the nucleus in order to manipulate transcription, chromatin–remodeling, DNA repair and replication and RNA splicing to fulfill their requirements (Bierne & Cossart 2012). Endonuclear bacteria might as well interfere with various host–related processes in an even more comprehensive way and thereby provide an excellent field of research.

1.6 DNA AS A NUTRIENT

Dissolved DNA (dDNA) is an important source of dissolved organic matter (DOM) in aqueous habitats such as marine environments. Production of dDNA mainly occurs through cell death, lysis and secretion. Utilization of dDNA as the primary source for carbon, nitrogen and phosphorus has a huge effect on the composition of microbial communities (Lennon 2007). Exhibiting rapid turnover rates, dDNA supports bacterial communities to meet their nutritional requirements like carbon and nitrogen demand (Jorgensen et al. 1993). For example, studies have shown that single nucleotides can directly be utilized by bacteria for

RNA and DNA synthesis (Redfield 1993; Paul et al. 1988). Methods of dDNA uptake comprise several strategies, such as the secretion of extracellular nucleases to facilitate dDNA degradation and subsequent consumption of hydrolyzed products. Moreover, some bacteria are capable of directly taking up dDNA and therefore considered to be naturally competent (Lennon 2007). Low molecular weight (LMW) dDNA was preferentially used by bacteria, especially if they had already been grown and incubated on LMW dDNA before. This data indicates a potential association with energy costs producing extracellular nucleases and secreting them. Bacterial symbionts inhabiting the nuclei of their hosts may utilize host genomic DNA or histones in order to acquire energy as well as carbon, nitrogen and phosphorus (Pinchuk et al. 2008). Several competition experiments have shown, that uptake of HMW DNA and its hydrolysis products is effectively competed with the uptake of single nucleotides and nucleosides (Paul et al. 1987; Paul et al. 1988). Currently, microbial communities in marine habitats are thought to rapidly bind dissolved DNA to particulate matter by a combination of specific DNA–binding mechanisms and abiotic absorption. Simultaneously, membrane–associated and extracellular DNA exonucleases might facilitate the hydrolysis of bound DNA. Hydrolysis products such as single nucleotides and nucleosides may then be rapidly transported into the cell and already re–synthesized into nucleic acids (Pinchuk et al. 2008; Paul et al. 1987; Paul et al. 1988).

In our study, we were primarily interested in the uptake of exogenous DNA purified from *Acanthamoeba castellanii* Neff by *Nucleicultrix amoebiphila*; an ability thought to be of useful for an endonuclear symbiont of amoebae in order to ensure carbon and energy demands. Respiratory activity indicating continued metabolism can be easily and roughly estimated by the use of 5–cyano–2, 3–ditolyl–tetrazoliumchloride (CTC). Chemically, CTC is a soluble crystal forming a nearly colorless non–fluorescent solution. Once taken up, CTC becomes subsequently reduced by cellular metabolism thereby producing a fluorescent formazan and is especially used to track P450 activity. Upon reduction, insoluble formazans crystals become accumulated by the cell. The emission of red fluorescent light (peak at 630nm), if excited with blue light (480nm) can easily be detected by an epifluorescence microscope. However, it should be noted that it interferes with the electron transfer system thereby replacing oxygen as terminal electron acceptor. Therefore, it is considered to be potentially toxic for bacteria or other unicellular organisms, once the reduction processes are completed. Furthermore, it represents the redox activity of cells at the time observations are made. CTC has been widely been applied to assess the redox activity of microbial communities in environmental samples such as activated sludge (Gruden et al. 2003), stream

water (Araya et al. 2003), soil (Winding et al. 1994) or drinking water (Schaule et al. 1993). Common protozoan hosts of various intracellular endosymbionts such as *Acanthamoeba* spp. have as well been evaluated in terms of metabolic activity (Kobayashi et al. 2012). CTC provides a number of advantages in order to measure cellular respiratory activity for it can easily be distinguished from inactive or dead cells exhibiting no fluorescence. Moreover, reports have also suggested, that bacterial respiratory activity well corresponds with viability assays, if assessed with a CTC staining (Créach et al. 2003). In addition, a CTC staining can be coupled with other methods addressing questions of metabolic activities of bacterial communities for example fluorescence in–situ hybridization or micro–autoradiography, even if it apparently targets only the most active cells (Nielsen et al. 2003).

1.7 THE ORDER RICKETTSIALES

The order of *Rickettsiales* comprises a variety of diverse obligate intracellular bacteria within the subgroup of Alphaproteobacteria. These gram–negative bacteria mostly exhibit a rod–shaped morphology and are known symbionts of arthropods capable of infecting mammalian cells for example *Acanthamoeba* spp. (Fritsche et al. 1999), ubiquitous soil and water bactivores, *Paramecium* spp. (Beier et al. 2002) or even mitochondria of *Ixodes ricinus*, a sheep tick and well–known vector for Lyme's Disease in Europe (Epis et al. 2008; Sassera et al. 2006). Associations with host organisms vary between parasitism and mutualism. In general, the order of *Rickettsiales* is proposed to have originally been associated with aquatic and environmental protista, that have served as ecological and evolutionary reservoir for the *Rickettsiales* infecting animals (Montagna et al. 2013). Based on 16S rRNA phylogeny, the order of *Rickettsiales* consists of four major families: the *Anaplasmataceae*, *Holosporaceae*, *Midichloriaceae* (Montagna et al. 2013) and the *Rickettsiaceae* (Dumler J.; Walker D. 2005,). The family *Holosporaceae* are famous of inhabiting endonuclear symbionts of *Paramecium caudatum* infecting either the host's macro–or micronucleus (Fujishima & Görtz 1983; Hori et al. 2008; Fujishima & Fujita 1985; Fujishima 2009). Common genera of the order *Rickettsiales* are *Rickettsia*, *Cowdria*, *Neorickettsia*, *Bartonella Orientia*, *Ehrlichia* and *Wolbachia*, famous bacteria inheriting the cytoplasm of host cell reproductive tissue thereby inducing alterations to host reproduction program such as cytoplasmic incompatibility (Werren 1997). The life cycles of the majority of the members of *Rickettsiales* typically depend on replication inside cells of eukaryotic hosts in order to ensure growth and survival. Generally, *Rickettsiales* exhibit small and obviously degraded genomes due to long periods of obligate intracellular life style. Despite extensive gene loss, members of the *Rickettsiales* show elevated levels of pathogenicity. Recent reports have shown that high levels of genetic

exchange between different species inhabiting the same host as well as between host and symbiont are responsible for that (Renvoisé et al. 2011). Here, a Type IV secretion system appears to be the main operator in order to facilitate transfer of proteins containing eukaryotic–like repeat motifs and manipulation of host replication (Renvoisé et al. 2011).

Amoeba symbionts affiliated to the order *Rickettsiales* are *Odyssella thessalonicensis* (Birtles et al. 2000), *Paracaedibacter sp.* EI3 (Schmitz–Esser et al. 2008) and *Endosymbiont of Acanthamoeba* UWC36 (Fritsche et al. 1999). *Paracaedibacter sp.* EI3 (highest similarity to *P. acanthamoeba* UWC9 with 99.7%) shows affiliations with the order of *Rickettsiales* and *Rhodospirillales* and belongs to a novel clade of symbionts of protists together with *N. amoebiphila* besides the four already mentioned families (Schulz et al. 2014; Schmitz–Esser et al. 2008). Endosymbiont of *Acanthamoeba* UWC36 has not been characterized in depth and apparently belongs to the order of *Rickettsiales* forming an independent and well–separated lineage together with *Midichloria mitochondrii*. Both organisms show obligate intracellular association to *Acanthamoeba spp*.; unable to replicate outside a suitable host cell. *Paracaedibacter* EI3 was isolated from a rainforest soil sample from Dominica (Schmitz–Esser et al. 2008), whereas Endosymbiont of *Acanthamoeba* UWC36 (Host *Acanthamoeba* sp. belongs to sequence type T4) was retrieved from human corneal tissue (Fritsche et al. 1999). Both *Paracaedibacter* EI3 and Endosymbiont of *Acanthamoeba* UWC36 exhibit cytoplasmic presence and a clear adjacent zone surrounded by a capsule or slime layer. No additional information about the life cycle and impact on host fitness and survivability has been given yet.

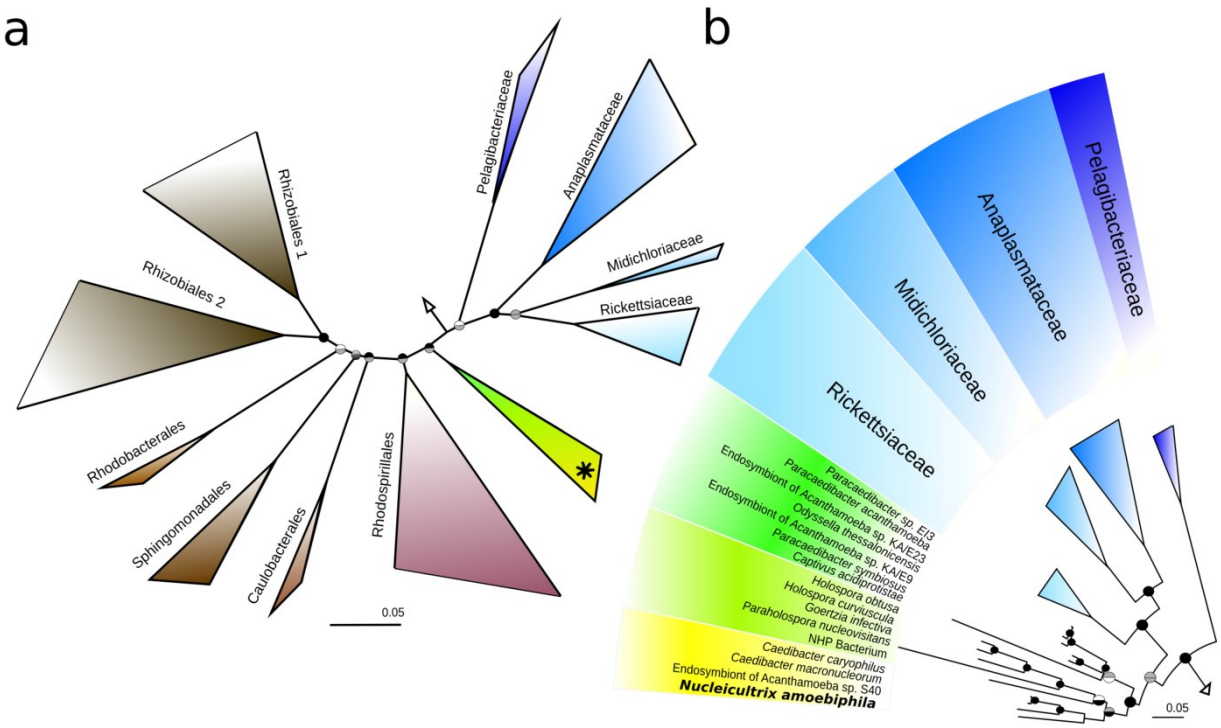

Fig. 2 Diversity of the order of Rickettsiales. (a) Maximum likelihood tree based on a concatenated 16S–23S rRNA alignment. 'Nucleicultrix amoebiphila' (indicated by an asterisk) together with other protist symbionts forms a deeply branching lineage within the Alphaproteobacteria. **(b)** 16S rRNA–based maximum likelihood tree showing members of the protist symbiont clade containing 'Nucleicultrix amoebiphila'. Circles displayed at the nodes represent bootstrap values (upper half) and Bayesian posterior probability values (lower half), respectively. Black indicates support values >80% or probability values >0.8; gray indicates support values of 50–80% or probability values of 0.5–0.8, white indicates no support. Image kindly provided by (Schulz et al. 2014).

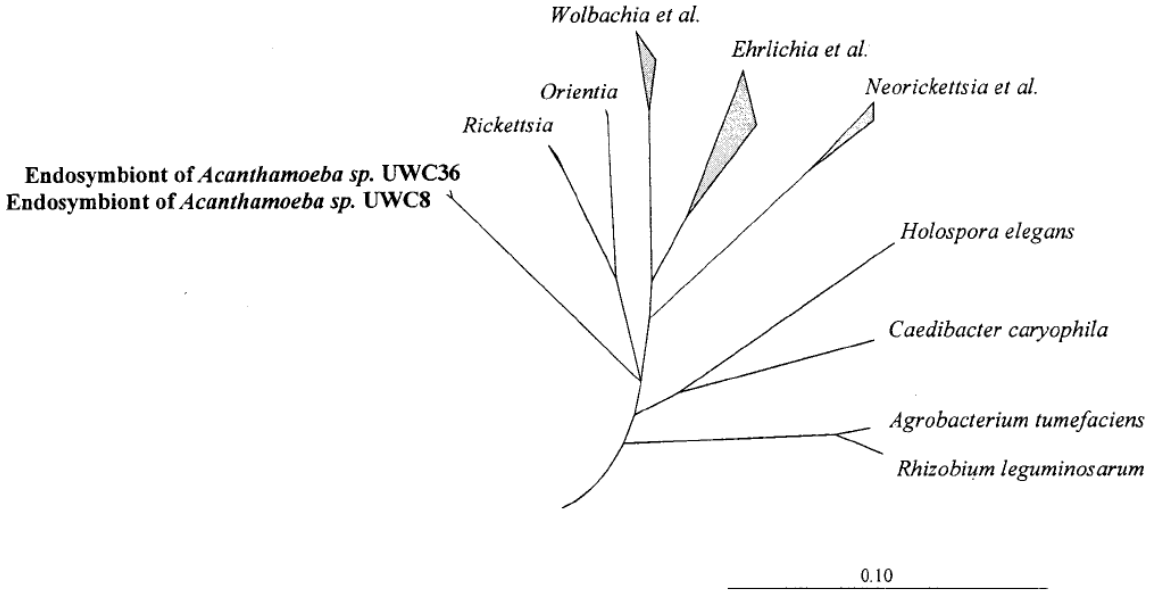

Fig. 3 Neighbor–joining dendrogram showing relationships of endosymbionts of *Acanthamoeba* strains UWC8 and UWC36. Depicted are relations to members of the order of Rickettsiales. All tree–generating methods support deep branching of the retrieved 16S rDNA sequences. Tree provided by (Fritsche et al. 1999).

1.8 AIM OF THIS STUDY

The aim of this study was to describe key elements of the interaction between obligate intracellular bacteria and their *Acanthamoeba* respectively *Hartmannella* hosts. The first part of this thesis focused on *Nucleicultrix amoebiphila* and its amoeba host *Hartmannella sp.* FS5. We were predominantly interested in assessing its capability of utilizing host DNA; an ability that provide a suitable way to ensure its carbon and energy demands once inside the host nucleus. Therefore, purified fractions of *N. amoebiphila* were incubated host–free together with purified DNA from *Acanthamoeba castellanii* Neff and bacterial respiratory activity was measured using of 5–cyano–2,3–ditolyl–tetrazoliumchloride (CTC). Different amounts of DNA as well as single dNTPs were also tested. Additionally, in order to evaluate bacterial viability in face of a host–free environment, Propidium iodide was used as selective marker for cell death. The second part of the thesis focused on the study of the developmental cycles of two obligate intra–cytoplasmic bacterial symbionts of *Acanthamoeba* spp.; Endosymbiont of *Acanthamoeba* UWC36 and *Paracaedibacter Acanthamoeba* EI3. Both are Alphaproteobacteria and thus related to *N. amoebiphila*. Experiments to evaluate key characteristics during an infection cycle were carried out at two distinct temperatures. Fluorescence in–situ hybridization was performed to monitor progress of infection at distinct time points. Growth rates using plate counts and host cell viability using Propidium iodide were obtained to evaluate impact on host organisms. Furthermore, we tried to assess the host range of both bacterial endosymbionts using a variety of *Acanthamoeba* spp. host as well as mouse macrophages and HeLa–cells.

Material & Methods

2. MATERIAL & METHODS

All chemicals used during the entire study were purchased in pro analysis quality. Media, buffer and solutions were prepared utilizing double–distilled and filtered water ($H_2O_{bidest.}$). The water purification system used for this was MILLI–Q®biocel from Millipore GmbH (Vienna, Austria). In order to adjust the final pH of various media, buffers and solutions either Sodium hydroxide (NaOH, dry) or hydrochloric acid (HCl) were used.

2.1 MATERIALS

2.1.1 SOFTWARE

Tab. 1 Software applications and manufacturers

Software	Manufacturer
Axiovision Rel. 4.8	Carl Zeiss MicroImaging GmbH, Jena, Germany
Inkscape	Hinerangi Courtenay and Martin Owens
ImageJ	http://rsbweb.nih.gov/ij/index.html
Leica LAS AF Lite	Leica Microsystems, Wetzlar, Germany
Graphpad Prism 5.0	Graphpad Software Inc., La Jolla, CA, USA
Microsoft Office Word 2007 for Windows	Microsoft Corporation, Redmond, WA, USA

2.1.2 INSTRUMENTS AND TECHNICAL EQUIPMENT

Tab. 2 Laboratory instruments and technical equipment

Instrument	Manufacturer
Accu–jet® pro pipette aid	Brand GmbH+Co KG, Wertheim, Germany
CCD camera AxioCam HRc	Carl Zeiss MicroImaging GmbH, Jena, Germany
Centrifuges	
Centrifuge 5804 R	Eppendorf AG, Hamburg, Germany
Mikro 20 benchtop centrifuge	Andreas Hettich GmbH & Co KG, Tuttlingen, Germany
Incubators	
Microbiological incubator KB 115	Binder GmbH, Tuttlingen, Germany
Hybridization oven UE 500	Memmert GmbH & Co KG, Schwabach, Germany
Laminar flow hood, model 1.8	Holten, Jouan Nordic, Allerød, Denmark
Magnetic stirrer RCT basic	IKA® Werke GmbH & Co KG, Staufen, Germany
Dounce tissue grinder 15 ml, 7 ml	Wheaton Science Product, Milville, USA
NanoDrop® ND1000 UV/Vis spectrophotometer	NanoDrop Technologies Inc., Wilmington, DE, USA
Microscopes	
Epifluorescence microscope Axioplan 2 imaging	Carl Zeiss MicroImaging GmbH, Jena, Germany
Inverse microscope Axiovert 25	Carl Zeiss MicroImaging GmbH, Jena, Germany
Confocal Laser Scanning Microscope LSM 510 Meta	Carl Zeiss MicroImaging GmbH, Jena, Germany
Filter devices	
Vacuum pump 220 V/50 Hz	Millipore GmbH, Vienna, Austria
Fritted glass base (2.5 cm diameter)	Millipore GmbH, Vienna, Austria
Silicone stopper	Millipore GmbH, Vienna, Austria
Glass funnel 15 ml, glass	Millipore GmbH, Vienna, Austria
Spring clamp, aluminium	Millipore GmbH, Vienna, Austria
Filtering flask 100 ml, glass	Schott Austria GmbH, Vienna, Austria
Neubauer counting chamber	Paul Marienfeld GmbH & Co KG, Lauda–Königshofen, Germany
pH meter inoLab pH Level 1	Wissenschaftlich–Technische Werkstätten GmbH, Weilheim, Germany
Scales	
OHAUS® Analytical Plus balance	Ohaus Corporation, Pine Brook, NJ, USA
Sartorius BL 3100	Sartorius AG, Gottingen, Germany
Spectral photometer SmartSpecTM 3000	Bio–Rad Laboratories GmbH, Munich, Germany

Vortex–Genie 2	Scientific Industries Inc., Bohemia,NY, USA
Water baths	
Haake DC10–P5/U Heating circulator	Thermo Fisher Scientific Inc., Waltham, MA, USA
Incubation bath GFL 1004	Gesellschaft für Labortechnik GmbH, Burgwedel,
Water purification system MILLI–Q® biocel	Millipore GmbH, Vienna, Austria
Watervapour high pressure autoclaves	
Varioclav® 135 S H+P	H+P Labortechnik GmbH, Oberschleißheim, Germany
Varioclav® 25 T H+P	H+P Labortechnik GmbH, Oberschleißheim, Germany

2.1.3 DISPOSABLE ITEMS

Tab. 3 Disposable single–use materials and items

Disposable Item	Manufacturer
25cm2 Tissue culture flasks	Asahi Techno Glass Corporation, Iwaki Glass Co., Ltd., Funabashi–City, Japan
500cm2 Tissue culture	Nunc, Roskilde, Denmark
Cellulose acetate membrane filters (0.45µm, 1.2µm, 5µm)	Sartorius Stedim Biotech GmbH, Göttingen, Germany
Cell Culture Dish, 100 x 20 mm, PS, Advanced TC™	Bartelt Labor–und Messtechnik, Graz, Austria
Cover glasses (24 x 50 mm, 24 x 60 mm)	Paul Marienfeld GmbH & Co KG, Lauda–Königshofen, Germany
Coverslips (12 mm)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Glass beads (0.75–1.0 mm)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Greiner tubes (15 ml, 50 ml)	Greiner Bio–One GmbH, Frickenhausen, Germany
IsoporeTM polycarbonate membrane filters (0.22 µm pore size, 25 mm	Millipore GmbH, Vienna, Austria
Microscope slides (76 x 26 mm)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Microscope slides, 10 wells	Paul Marienfeld GmbH & Co KG, Lauda–Königshofen, Germany
Multiwell dishes, polystyrene (6 wells, 12 wells)	Nunc, Roskilde, Denmark
Needles Sterican® (Ø 0.45 x 25 mm, Ø 0.90 x 40 mm)	B. Braun Melsungen AG, Melsungen, Germany
Parafilm® M laboratory film	American National Can Company, Chicago, IL, USA
Plastic cuvettes	Greiner Bio–One GmbH, Frickenhausen, Germany
PCR tubes (0.2 ml)	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Plastic pipettes (2 ml, 10 ml)	Barloworld Scientific Ltd., Staffordshire, UK
Plastic tips (various sizes)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Plastic inoculation loop	Nunc Roskilde, Denmark
Reaction tube 1.5 ml	Greiner Bio–One GmbH, Frickenhausen, Germany
Reaction tube 2 ml	Greiner Bio–One GmbH, Frickenhausen, Germany
SafeSeal–Tips® Premium	Biozym Scientific GmbH, Hessisch–Oldendorf, Germany
Syringe filter, cellulose acetate (0.2 µm)	Asahi Techno Glass Corporation, Iwaki Glass Co., Ltd., Funabashi–City, Japan

2.1.4 CHEMICALS, ENZYMES AND ANTIBIOTICS

Tab. 4 Chemicals and enzymes used

Chemicals	Manufacturer
4',6–Diamidino–2–phenylindole (DAPI)	Lactan Chemikalien und Laborgeräte GmbH, Graz, Austria
5–cyano–2,3–ditolyl–tetrazolium chloride (CTC)	Sigma–Aldrich Chemie GmbH, Steinheim, Germany
Agarose	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Ammonium chloride (NH_4Cl)	Carl Roth GmbH & Co KG, Karlsruhe, Germany

Ammonium formate (NH_4 formate)	Fluka Chemie AG, Buchs, Switzerland
Ammonium iron(II) sulfat hexahydrat ($(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Ammonium molybdate tetrahydrate	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Boric acid (H_3BO_3)	Lactan Chemikalien und Laborgeräte GmbH, Graz,
Bovine serum albumin (BSA)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Calcium chloride dihydrate ($CaCl_2$*2 H_2O)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Citifluor AF1	Agar Scientific Ltd., Stansted, UK
Cobalt(II) chloride hexahydrate ($CoCl_2$)	Fluka Chemie AG, Buchs, Switzerland
Copper(II) sulphate pentahydrate ($CuSO_4$)	Fluka Chemie AG, Buchs, Switzerland
di–Sodium hydrogen phosphate dihydrate (Na_2HPO_4*2 H_2O)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
DL–Phenylalanine	Merck GmbH, Vienna, Austria
Dulbecco's Phosphate–Buffered Saline (DPBS), 10x	Invitrogen Molecular Probes Inc., Eugene, OR, USA
DMEM, High glucose, HEPES, L–glutamine	Invitrogen Molecular Probes Inc., Eugene, OR, USA
RPMI 1640, HEPES, L–glutamine	Invitrogen Molecular Probes Inc., Eugene, OR, USA
0.5% Trypsin–EDTA (10X), w/o phenol red ("TE")	Invitrogen Molecular Probes Inc., Eugene, OR, USA
Ethanol absolute (EtOHabs.)	AustrAlco Österreichische Alkoholhandels GmbH, Spillern, Austria
Ethylenediamine–tetraaceticacid (EDTA)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Foetal Bovine Serum Gold ("FBS")	GE Healthcare Life Sciences, Chalfont St Giles, UK
Formaldehyde 37% (w/w) Rotipuran®	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Formamide deionized	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Glycerol Rotipuran®	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Glutaraldehyde, 25%	Sigma–Aldrich Chemie GmbH, Steinheim, Germany
Glycine	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Hydrochloric acid 37% (w/w) (HCl)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Nicotinic acid (Niacin)	Sigma–Aldrich Chemie GmbH, Steinheim, Germany
Magnesium chloride hexahydrate ($MgCl_2$)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Magnesium sulfate heptahydrate ($MgSO_4$*7 H_2O)	Merck GmbH, Vienna, Austria
Methanol	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Proteose Peptone	Fisher Scientific GmbH, Vienna, Austria
PIPES buffer	Lactan Chemikalien und Laborgeräte GmbH, Graz, Austria
Potassium chloride (KCl)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Mallinckrodt Baker B.V., Deventer, Holland
Propidium iodide (PI)	Invitrogen Molecular Probes Inc., Eugene, OR, USA
Sodium acetate (Na acetate)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Sodium chloride (NaCl)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Sodium dihydrogen phosphate (NaH_2PO_4)	Mallinckrodt Baker B.V., Deventer, Holland
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Sodium hydrogen carbonate ($NaHCO_3$)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Sodium hydroxide (NaOH)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Sodium lactate, 60% (w/v)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Tris Pufferan®	Carl Roth GmbH & Co KG, Karlsruhe, Germany
tri–Sodium citrate–dihydrate	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Yeast Extract	Oxoid Ltd., Hampshire, England
Zinc sulphate heptahydrate ($ZnSO_4$*7 H_2O)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
α–D(+)–Glucose monohydrate	Carl Roth GmbH & Co KG, Karlsruhe, Germany

Tab. 5 Antibiotics used

Antibiotic	Manufacturer
ampicillin sodium salt	Sigma–Aldrich Chemie GmbH, Steinheim, Germany
Gentamycin	Sigma–Aldrich Chemie GmbH, Steinheim, Germany
Phosphomycin disodium salt	Sigma–Aldrich Chemie GmbH, Steinheim, Germany
Ofloxacin	Sigma–Aldrich Chemie GmbH, Steinheim, Germany
Doxycyclin	Sigma–Aldrich Chemie GmbH, Steinheim, Germany
Erythromycin	Sigma–Aldrich Chemie GmbH, Steinheim, Germany
Rifampicin	Sigma–Aldrich Chemie GmbH, Steinheim, Germany
Penicillin–Streptomycin (100 x) ("PS")	Invitrogen Molecular Probes Inc., Eugene, OR, USA

Tab. 6 Ready–to–use kits

Kits and ready–to–use solutions	Manufacturer
DNeasy blood & tissue kit	Qiagen Vertriebs GmbH, Vienna, Austria
VenorGeM, 25 Tests (mycoplasma test kit)	BioProducts, Stockerau, Austria

2.1.5 CHEMICAL BUFFERS, MEDIA AND SOLUTIONS

All media, buffers and solutions were prepared utilizing double–distilled and filtered water (MilliQ). In order to perform adjustment of the pH, either Sodium hydroxide (NaOH, dry) or hydrochloric acid (HCl) was used, if not stated otherwise. All buffers, media and solutions (if required) were generally sterilized for 20 minutes at 121°C and 1.013 x 105 Pa pressure using water – vapor high pressure autoclave. Storage before use was performed at room temperature, if not stated otherwise.

Tab. 7 Chemical buffers and solutions used

Buffers & solutions	Components
DAPI working solution	
DAPI stock solution 10mg/ml	
Sterile 1xPAS	1:5000, 1:1000 dilution
Sterile 1xPBS	1:10000, 1:5000 dilution
Sterile 1x$H_2O_{bidest.}$	1:10000 dilution
Propidium iodide (PI) solution	
PI stock solution 1mg/ml	
Sterile 1xPAS	1:1000 dilution
4% Paraformaldehyde (PFA) solution	
PFA, 37%	1ml
Sterile $H_2O_{bidest.}$	9.25ml
5–cyano–2,3–ditolyl–tetrazoliumchloride (CTC)	
CTC stock (20mM)	6.24mg/ml
Sterile 1xPAS	5ml Filtered through 0.2µm cellulose–acetate filter, storage at 4°C in the dark
CTC working solution (5mM)	
CTC stock solution (20mM)	250µl
Sterile 1xPAS	750µl Filtered through 0.2µm cellulose–acetate filter, storage at 4°C in the dark
10xPage's amoebic saline (PAS)	
NaCl	1.2g

$MgSO_4$ x 7 H_2O	0.04g
$CaCl_2$ x H_2O	0.04g
NaH_2PO_4 x 2 H_2O	1.78g
KH_2PO_4	1.36g
$H_2O_{bidest.}$	Ad 1000ml
FISH Washing buffer (20% FA)	
NaCl 5M	2.15ml
Tris/HCl 1M	1ml
EDTA 0.5M	0.5ml
$H_2O_{bidest.}$	Ad 50ml
FISH Hybridization buffer (20%)	
NaCl 5M	180µl
Tris/HCl 1M	20µl
$H_2O_{bidest.}$	600µl
Formamid deionized	200µl, under a fume hood
10% (w/v) SDS	1µl
20xPBS	
NaH_2PO_4	35.6g (200mM)
Na_2HPO_4	27.6g (200mM)
$H_2O_{bidest.}$	Ad 1000ml
1xPBS	
NaCl	7.6g
20x PBS	50ml
$H_2O_{bidest.}$	Ad 1000ml
Sucrose–phosphate–glutamate buffer (SPG)	
Sucrose	75g
Na_2HPO_4 x	1.53g
KH_2PO_4	520mg
L–Glutamic acid	720mg
$H_2O_{bidest.}$	Ad 1000ml Adjust pH to 7.2
7% Glutaraldehyde	
Glutaraldehyde 25%	1.4ml
$H_2O_{bidest.}$	3.6ml
0.2M Phosphate buffer	
NaH_2PO_4 x 2 H_2O	0.44g
Na_2HPO_4 x 2 H_2O	1.83g
$H_2O_{bidest.}$	50ml
DPBS, 1x	
10x DPBS	100ml
$H_2O_{bidest.}$	850ml Adjust pH to 7–7.2, filter through 0.2µm filter, store at 4°C
1x 0.5% Trypsin–EDTA , w/o phenol red	
10x 0.5% Trypsin–EDTA	5ml
DPBS, 1x	45ml filter through 0.2µm filter, store at 4°C

Tab. 8 General media used

General media	Components
Trypticase Soy Broth with Yeast Extract (TSY)	
Trypticase Soy Broth	30g
Yeast Extract	10g
$H_2O_{bidest.}$	Ad 1000ml
Peptone–Yeast Extract–Glucose (PYG)	
Proteose Peptone	20g
Yeast Extract	2g
α–D(+)–Glucose monohydrate	18g
tri–Sodium citrate–dihydrate	1g

$MgSO_4$ x 7 H_2O	920mg
KH_2PO_4	340mg
Na_2HPO_4 x 7 H_2O	355mg
$Fe(NH_4)_2(SO_4)_2$ x 6 H_2O	20mg
$H_2O_{bidest.}$	Ad 1000ml
	Adjust pH to 6.5
DMEM, High glucose, HEPES, L–glutamine + 10% FBS (v/v)	
DMEM	45ml
FBS	5ml
	filter through 0.2µm filter, store at 4°C
RPMI 1640, HEPES, L–glutamine + 10% FBS (v/v)	
RPMI 1640	45ml
FBS (heat–inactivated for 5 minutes at 95°C)	5ml
	filter through 0.2µm filter, store at 4°C

2.1.6 FISH–PROBES

All probes were generally stored at –20°C. Use required thawing and temporary storage on ice (4°). Preparation of working solutions was carried using small volumes of $H_2O_{bidest.}$ (100–150µl).

Tab. 9 FISH–probes used
All FISH–probes were manufactured by Thermo Fischer Scientific GmbH, Ulm, Germany

Name	Sequence (5'–3')	Target molecule	Specificity	Comments	Reference
EUK516	5'–ACCAGA CTTGCCCT CC–3'	18S rRNA	*Eukarya*	Cy5 – labelled	(Amann et al. 1990)
EUB338	5'–GCTGC CTCCCGTAG GAGT–3'	16S rRNA	Most bacteria	FLUOS–labeled	(Amann et al. 1990)
EUB338 II	5'–GCAGCC ACCCGTAGG TGT–3'	16S rRNA	*Planctomycetales*	FLUOS–labeled	(Daims et al, 1999)
EUB338 III	5'–GCTGCCA CCCGTAGG TGT–3'	16S rRNA	*Verrucomicrobiales*	FLUOS–labeled	(Daims et al. 1999)
CBR125	5'– ACCATTCGGCATGTTCC C–3'	16S rRNA	*Nucleicultrix amoebiphila* FS5	Cy3 – labeled	(Schulz et al. 2014)
AcRic–90	5'– TGC CAC TAG CAG AAC TCC –3'	16S rRNA	*Rickettsia*–related endosymbionts of *Acanthamoeba* strains UWC8 and UWC36	Cy3 – labeled	(Fritsche et al. 1999)
CC23a	5'– TTC CAC TTT CCT CTC TCG –3'	16S rRNA	*Paracaedibacter Acanthamoeba* EI3	Cy3 – labeled	(Springer et al. 1993)
Alf968	5'– GGT AAG GTT CTG CGC GTT –3'	16S rRNA	*Alphaproteobacteria*	Cy3 – labeled	(Neef 1997)
AcRic–1196	5'– CCT ATT GCG TCC AAT TGT –3'	16S rRNA	*Rickettsia*–related endosymbionts of *Acanthamoeba* strains UWC8 and UWC36	Cy3 – labeled	(Fritsche et al. 1999)

All FISH–probes were stored at –20°C until further use. If used, probes required temporary storage on ice and were kept in the dark to prevent bleaching. Preparation of new stock and working solutions was carried out with sterile double–distilled water. Working solutions were

generally prepared in small volumes of 100–200µl. At all stages of an experiment, probes had to be protected from light.

2.1.7 ORGANISMS

All organisms used during the course of the thesis were only handled under a L2–classified laminar flow in order to provide protection for all workers as well as avoid contamination of flasks and vessels containing organisms of choice. Used multi–well plates were sealed with Parafilm® before removing them from the L2 laminar flow. Liquid waste containing organisms of choice were disposed according to standardized safety guidelines for L2–classified laboratories. Both *Acanthamoeba* sp. and *Hartmannella* sp. are classified as L2 organisms.

Tab. 10 Organisms used

Host	Endosymbiont	Source	Reference
Acanthamoeba castellanii Neff	Endosymbiont – free	ATCC, USA	(Neff 1957)
Acanthamoeba sp.	Endosymbiont of *Acanthamoeba* UWC36	Our lab	(Fritsche et al. 1999)
Acanthamoeba sp.	*Paracaedibacter* EI3	Our lab	(Schmitz–Esser et al. 2008)
Acanthamoeba sp. UWC12	Endosymbiont – free	Our lab	In preparation
Acanthamoeba sp. C1	Endosymbiont – free	Our lab	In preparation
Acanthamoeba sp. 5A2	Endosymbiont – free	Our lab	(Horn et al. 2001)
***Acanthamoeba sp. Polyphaga* DOME**	Endosymbiont – free	Our lab	(Khan 2006a)
Hartmannella vermiformis	Endosymbiont – free	Our lab	(Michel et al. 1995)
***Hartmannella* sp. FS5**	*N. amoebiphila* FS5	Our lab	(Schulz et al. 2014)

2.2 METHODS

2.2.1 CULTIVATION OF EMPTY AND INFECTED AMOEBAE

Empty *A. castellanii* Neff, *A. castellanii* C1, *A. castellanii* UWC12, *A. polyphaga*, *A. castellanii* 5A2 and *Hartmannella* sp. as well native UWC36 respectively EI3 *Acanthamoeba* spp. hosts were routinely and periodically cultivated at 20°C and room temperature (24°C). Generally, all cultures were incubated in sterile polystyrene 500cm² culture flasks (Nunc) and 150ml TSY media. Additionally, amoebae were kept in sterile polystyrene 25cm² culture flasks and 10ml TSY media. *Hartmannella* sp. was incubated in sterile polystyrene 500cm² culture flasks filled with 150ml 1xPAS supplemented with 1–5ml *E. coli* tolC– and 30µl ampicillin (100mg/ml). The exchange of the media was carried out once in two weeks, depending on the morphological status of host cells. Examinations were performed by light microscopy. Morphological criteria resembled the encystations and de–attachment of host amoebae as well as the amount of extracellular bacteria in the surrounding media. In order to

maintain properly grown cultures, all flasks were routinely shaken off and old media replaced by freshly prepared one. Cultures exhibiting a lawn of moderately dense distributed amoebal trophozoites and low numbers of floating cells were considered as well–grown and further subjected to experimental assays. The media exchange was carried out by deposing old media in a glass bottle and fresh media was transferred using either disposable 10ml plastic pipettes or transferred directly. All culture flasks and media bottles were sterilized using the Bunsen burner. Poorly grown cultures were inoculated with cells originating from well–grown cultures. All steps were carried out under the L2 laminar flow.

2.2.2 AMOEBAL DNA EXTRACTION AND PURIFICATION

The extraction of *A. castellanii* Neff DNA was performed by use of the DNeasy Blood & Tissue Kit. Amoebal cultures were harvested by shaking off cells covering the flask's surface and 6–10ml of the cell suspension were then spun down at 6600g for 8 minutes at room temperature. The pellet was washed once in sterile 1xPAS and subsequently re–suspended in 180µl buffer ATL (lyses buffer). After the addition of 20µl proteinase K, the cell suspension was incubated at 56°C for 2–4h in order to fully degrade all proteins especially DNases. All following steps were performed along with the manufacturer's instructions. The final concentration and purity of the dissolved DNA was subsequently determined spectro–photometrically by using the NanoDrop device.

2.2.3 SPECTRO–PHOTOMETRICAL DETERMINATION OF DNA

Concentrations (ng/µl) and purity (A260/A280) of DNA were determined using the NanoDrop device. Capable of measuring very small volumes (0.5–2µl) and highly concentrated samples (up to 3700 ng/µl dsDNA) it provides all means necessary for DNA measurements. Each measurement started with using 1.5µl double–distilled water or DNA elution buffer AE (depending on the DNA elution) followed by application of 1.5µl dissolved DNA sample on a fiber optic cable. All data was provided by the NanoDrop software.

2.2.4 QUANTIFICATION OF EXTRACTED AND PURIFIED ENDOSYMBIONT CELLS

All cell suspensions containing different kinds of endosymbionts were quantified by filtration of particles using a filter membrane system. In order to achieve an equal distribution of bacterial cells on the filter, 1–2µl of freshly extracted and purified endosymbionts were diluted with 10ml of sterile 1xPAS. To avoid contamination of purified fractions, which would disturb the quantification process the 1xPAS had been sterilized by filtration through a 0.2µm filter under the L2 laminar flow using a disposable 50ml syringe. All parts of the filter funnel system had been rinsed with double–distilled water and sterilized with 70% EtOH and

a Bunsen burner prior to the quantification process. A 200ml Erlenmeyer forcer had been fixed with clamps and placed under a fume hood. The assembly of the filter device was performed placing a 0.45µm underneath a black 0.22µm polycarbonate filter (Millipore) onto the forcer. Subsequently, the funnel was placed on top of both filters and fixed with additional clamps. In order to rinse the funnel, 10ml filter–sterilized 1xPAS was filtered by application a vacuum pump running at 250 mmHg (millimeter of mercury). After the funnel had been rinsed, the bacterial suspension was quickly stirred and applied slowly onto the black 0.22µm polycarbonate membrane filter. To avoid residual bacterial cells sticking to the funnel or the Greiner tube, an additional rinsing step using sterile 1xPAS was carried out. 500µl of a 1:5000 DAPI solution were transferred on top of the membrane and incubated for 5 minutes in the dark. The DAPI solution was removed using the vacuum pump and the membrane thoroughly washed with 2x 3ml sterile 1xPAS. The quantification of bacterial cells sticking to the black 0.22µm polystyrene membrane was conducted with an epifluorescence microscope. Therefore, the membrane was placed onto a microscope slide and covered with a drop of Citifluor®, an anti –bleaching mounting media. A cover slip was added and slides were either evaluated directly or stored at 4°C in the fridge. All DAPI–positive bacterial cells within the 10 x 10 standardized counting grids were counted using the 100x oil–immersion objective. In order to obtain the exact number of bacteria, the membrane was evaluated starting from the very middle and a quarter was counted in total. Depending on the density of the stained cells, 20–30 different fields were evaluated comprising cell numbers of 100–400 cells per field. Only clearly coccid–or rod–shaped cells (depending on the organism extracted and purified) were considered to be of bacterial origin. The concentration of the cells was calculated with the formula described below:

2.2.5 UWC36 Test infection

The capability of UWC36 to invade and successfully multiply within *A. castellanii* Neff was determined via a Test infection assay. In order to reach an average infection rate of ~30% after 24h past infection, UWC36 was purified from running cultures as described before, filtered with a 0.22µm IsoporeTM polycarbonate membrane filter and the number of cells/µl was determined by a DAPI staining. *A. castellanii* Neff was also purified from running cultures and $3x10^5$ cells/well were seeded into a 12–well multi–well dish. Used MOIs were 10, 30, 50 and 100. To achieve higher infection rates, infectious agents were spun down onto host cells at 370 rcf for 15 minutes and the cultures were incubated at room temperature for 2h. To avoid multiple infection events occurring at single amoebae, the media was removed after 2h; the cultures were washed twice with fresh TSY and finally incubated again in 2ml

TSY/well. The culture was incubated at room temperature for 24h, before amoebae were harvested and prepared for FISH as described earlier. The number of infected host cells was determined using AcRic90–Cy (specific probe), EUB338mix–FLUOS (most bacteria) and EUK516–Cy5 (eukaryotes). DAPI was applied to visualize host nuclei and counter–stain bacterial cells. Evaluation and image obtaining was conducted with the Axioplan 2 imaging epifluorescence microscope.

2.2.6 UWC36 INFECTION CYCLE WITH *A. CASTELLANII* NEFF AS HOST ORGANISM

A. castellanii Neff cells were harvested by shaking off 2 distinct 150ml culture flasks considered to contain well–grown and healthy trophozoites. The cell suspension was subsequently spun down at 6600 rcf for 8 minutes at room temperature. To fully remove any cell media components, the excess supernatant was decanted and the pellet washed once with 50ml sterile 1xPAS. The sterilization of 1xPAS had been carried out using a 0.22µm sterile filter and a disposable 50ml syringe. After removal of the supernatant again, the pellet was finally collected in 1–2ml sterile 1xPAS. 1:10 and 1:100 dilutions were prepared and the cell number was determined by use of the Neubauer's haemocytometer. 2ml of freshly prepared TSY were transferred into 12 x 12 well plates. For each time point and incubation temperature, three technical replicates comprising 3 x 10^5 cells per well designed for infection were inoculated. The same number of cells was used for empty controls incubated at both temperatures. All cultures were then pre–incubated at 20°C and 30°C over night for at least 12h in order to allow amoebae hosts to acclimatize to the temperature. UWC36 was extracted and purified as described before and a theoretical multiplicity of infection of 100 was added to all cultures designed for infection. Amoebal cells designed for TEM were infected with a multiplicity of infection of 500 to ensure a proper number of cells for TEM analysis. All plates were then subsequently placed into appropriate plate holders and UWC36 was pulled down on top of amoebae host cells by centrifugation at 370 rcf for 15 minutes. After 2 hours of incubation at 20°/30°C, the media was exchanged in order to remove all extracellular bacteria, which had not been taken up by host amoebae. The exchange of the media was carried out using a sterile glass pipette and an automated pumping device. All cultures were washed twice with 2ml fresh TSY after removal of the media. To equalize conditions for both empty and infected amoebae, empty controls were treated the same. Subsequently, the first time point 2hpi was harvested by re–suspension and the cell suspension was transferred into 1.5ml Eppendorf tubes. The cells were spun down at 6600rcf for 8 minutes at room temperature and after removing the excess supernatant again washed with 1ml sterile 1xPAS.

The supernatant was again decanted and the pellets were collect in 50µl sterile 1xPAS. 15µl of the cell suspension were incubated on 10–well microscope slides for 30–40 minutes at room temperature to preserve amoebal trophozoite structure. Residual liquid was removed and cells were fixed with 15µl 4% para–formaldehyde for 15 minutes at room temperature. The slides were once washed with 5µl sterile 1xPAS, air–dried and stored at –20 °C. Additional time points 8, 24, 48, 72, 96, 120, 144 and 168hpi were treated equally and as described before. All slides designed for FISH screening were prepared in technical duplicates for each temperature and infection status. At least 250–300 amoebal cells were counted for each technical and biological replicate with the epifluorescence microscope (Axioplan 2 imaging). Additionally, 50µl of each replicate were used to determine the number of cells/µl for each time point and infection status using the Neubauer's haemocytometer. The residual amount of media (1ml) was subjected to a Propidium iodide stain in order to evaluate the amount of dead cells. 10µl of a 1:10 dilution were transferred into 1ml of residual media containing both amoebal and bacterial cells. The cell suspension was then subsequently incubated for 20 minutes in the dark at room temperature. After that, the liquid was spun down at 6600 rcf for 8 minutes and the supernatant removed carefully. The pellet was washed once with 1ml sterile 1xPAS for 8 minutes at 6600 rcf and the liquid was removed again. Finally, the pellet was collected in 150µl sterile 1xPAS and transferred into sterile black 96–well micro titer plates. The emission of red fluorescence light was subsequently measured through a fluorescence reader.

2.2.7 PREPARATION OF SAMPLES FOR TEM

Amoebal cells considered to be infected with UWC36 were carefully spun down at 5000 rcf for 5 minutes and the supernatant was removed by decantation. The remaining pellet was slowly re–suspended in 1ml 7% glutar–aldehyde dissolved in 1ml 0.2M phosphate buffer to avoid rapid fixation and thereby damaging cell ultra–structure. Fixed samples were stored at 4°C prior to shipment.

2.2.8 CELL RESPIRATORY ACTIVITY ASSAY

FS5 was harvested from 8 x 150ml 500cm² polystyrene culture flasks containing FS5–harboring *Hartmannella* sp.. The presence of residual *E. coli* tolC– was checked beforehand by light microscopy to avoid exceeding contamination and falsification of results. The harvest and purification process consisted of filtration of the supernatant through a 1.2µm sterile filter coupled with a disposable 50ml sterile syringe and the subsequent centrifugation step (10500 rcf, 10 minutes, RT). In order to maximize the output of bacterial cells, the filter was washed once with sterile 1xPAS and all pellets were combined after decantation of the

liquid. Consistently, the final pellet was collected in 1ml sterile 1xPAS and stored at room temperature before further processing. Additionally, the total amount of DNA of empty *A. castellanii* Neff had been purified by use of the DNasey Blood & Tissue Kit the way described previously. Final DNA concentration was ~ 100ng/µl. All experimental assays following were carried out in technical duplicates for each of the following time points: 0, 24, 48, 72, 96, 120, 144, 168h. 69 empty sterile 1.5ml Eppendorf tubes had been prepared beforehand as the following: 16 x tubes for evaluation of the infectivity had been filled with 70µl sterile 1xPAS per cap + 30µl of the FS5 cell suspension. Additionally, 8 of these tubes had been supplied with 500ng of previously purified amoebal DNA. The residual 8 tubes were left unaltered. Furthermore, 40 x tubes were filled with 200µl sterile 1xPAS per cap and 7µl of FS5 cell suspension were added to each tube. These tubes were designated for evaluation of host–free respiratory activity of FS5 by application of CTC (5–cyano–2, 3–ditolyl–tetrazolium chloride). Freshly purified amoebal DNA was added into 16 of these caps and 16 tubes were left unaltered. The remaining 8 caps contained heat–inactivated fractions of FS5 in the presence of DNA and were marked as dead controls (no duplicates). Host–free incubations of FS5 destined for infections of *Hartmannella* sp. were applied 0hpi, 48hpi, 120hpi and 168hpi to freshly harvested and seeded amoebae for 24 hours before FISH screening.

2.2.9 PREPARATION OF CTC STOCKS

5ml of a 20mM stock solution of CTC (6.24mg/ml) was prepared in double–distilled water and subsequently filtered through a 0.2µm sterile disposable filter. In order to keep it functional, it was incubated at 4°C and further protected from direct light. 1ml 5mM staining solution (250µl 20mM CTC stock solution + 750µl sterile 1xPAS) was freshly prepared every day before use. All incubations with CTC were carried using a rotating roller plate to ensure a proper distribution.

2.2.10 HOST–FREE RESPIRATORY ACTIVITY ASSAY

Host–free incubations in the presence / absence of freshly purified *A. castellanii* Neff DNA were prepared and the number of infected host cells as well as the number of apparently alive and respiring FS5 cells was obtained.

Incubated fractions of host–free FS5 in the presence/absence of DNA were collected at designed time points (0, 24, 48, 72, 96, 120, 144, 168h) by centrifugation (10500g, 10 minutes, RT). The supernatant/media was carefully removed to avoid re–suspension of the pellet. 25–35µl of a 20mM CTC stock solution was applied together with 75µl–105µl sterile

1xPAS to gain a final concentration of 5mM CTC per sample/cap. The initial incubation period of 2h was extended to 4h and 24h during the course of the experiment. In order to avoid loss of signal intensity and bleaching effects, all samples were protected from light by the use of adhesive tape. After the end of the incubation period, the bacteria were again collected by centrifugation (10500g, 10 minutes, RT) and the media was carefully removed by pipetting. The pellet was re–suspended in 250µl 4% PFA and incubated for 15 minutes at room temperature in the dark. In order to remove the 4% PFA, the cells were spun down by centrifugation at 10500 rcf for 10 minutes at room temperature. The liquid was decanted, the pellet collected in 1ml sterile 1xPAS and washed once by centrifugation. After the removal of the residual liquid, the pellet was again re–suspended in 50µl sterile 1xPAS and 5µl were transferred onto sterile microscope 10–well slides. To accelerate the drying process, the slides were shortly incubated at 46°C in an oven. 15µl of a 1:1000 DAPI solution were applied for 3 minutes followed by a quick and thorough washing step with 15µl sterile 1xPAS. The slides were subsequently dried again and embedded in CitiFluor®, covered with a microscope cover slip and subjected to fluorescence microscope analysis. The cells were quantified by counting of all DAPI signal and the number of CTC–positive cells was determined. Furthermore, the DAPI: CTC ratio was evaluated and a few representative pictures were taken. Both caps containing already fixed cells and microscope slides were stored at 4°C in the dark to avoid loss of signal intensity. The whole assay was repeated several times endosymbionts purified from differently fed bacteria–harboring amoebae concerning feeding times and amount of added *E. coli* tolC– as well as starved amoebae.

2.2.11 Acquisition of new eukaryotic, non – amoebal hosts

Eukaryotic cells designed to be evaluated for potential uptake of endosymbionts and maintenance of stable infection were harvested either shaking off cells from culture flask or by use of 1xTE in order to detach HeLa–or mouse macrophage cells. HeLa–and mouse macrophage cells were then collected by thorough centrifugation at 4000rcf for 5 minutes at room temperature (24°C). The supernatant was carefully removed through pipetting and the pellets washed once with 1ml sterile 1x DPBS. All steps belong to purification and preparation of eukaryotic hosts required constant working under a designated laminar flow. The residual liquid was again disposed and pellets finally re–suspended in 40–50µl of freshly prepared and filtered 1xDPBS. 1:10 and 1:100 dilutions of the cell suspension were prepared. In order to obtain the number of potential host cells, 20µl of diluted cell suspension were transferred onto a Neubauer's haemocytometer© and counted by light microscopy. $3x10^5$ eukaryotic cells were seeded into freshly prepared 12 –well plates containing 2ml of either

DMEM + 10% (v/v) FBS (in case of HeLa–cells) or RPMI + 10% (v/v) FBS (designated for incubation of mouse macrophages). Cultures were incubated at 37°C and 5% CO_2 for several days before freshly purified (described elsewhere) endosymbionts were added. To ensure proper contact between host cells and endosymbionts and accelerate sinking of bacterial cells, a final centrifugation step at 370 rcf for 15 minutes at room temperature (24°C) was conducted. Infected cultures were subsequently kept at 37°C, 5% CO_2 for 2–3 days prior to removing of media and replacing it with fresh media.

2.2.12 NATIVE *PARACAEDIBACTER* EI3 HOST CURING ASSAY

Acanthamoeba spp. containing *Paracaedibacter* EI3 were harvested and $3x10^5$ host cells were seeded into 2x 6–well polystyrene multi–well dishes filled with 5ml TSY. To remove endosymbionts from both inside host cells and extracellular in the surrounding media, 7 different antibiotics with different target sites and mechanisms had been chosen and prepared beforehand.

Tab. 11 *Paracaedibacter* EI3 curing assay
Used antibiotics with different target mechanisms and final concentration in media.

Antibiotic	Target site/mechanism	Antibiotic class	Stock conc.	Dissolvent	Final conc. in media
Rifampicin	Inhibitor of bacterial DNA–dependant RNA – Polymerase	Rifamycins	10mg/ml	50% (v/v) DMSO	20/14/10µg/ml
Ampicillin	Irreversible inhibitor of the enzyme transpeptidase	Aminopenicillins	100mg/ml	50% (v/v) 96% EtOH	200/140/100µg/ml
Phosphomycin	Inhibitor of bacterial cell wall biogenesis through binding to UDP–N – acteylglucosamine	Polypeptides	10mg/ml	$H_2O_{bidest.}$	20/14/10µg/ml
Gentamycin	t–Peptidyl–site of 30S RSU / interference with proofreading activity	Aminoglykosides	10mg/ml	$H_2O_{bidest.}$	20/14/10µg/ml
Doxycyclin	Binds to bacterial 30S RSU / inhibits binding of Aminoacyl – tRNA	Tetracyclines	10mg/ml	$H_2O_{bidest.}$	20/14/10µg/ml
Erythromycin	Inhibitor of bacterial 50S RSU / prevents tRNA transfer from A–to P–site	Makrolides	10mg/ml	50% (v/v) 96% EtOH	20/14/10µg/ml
Ofloxacin	Inhibitor of bacterial DNA–gyrase / bacterial replication	Quinolones	1mg/ml	50mg in 1M NaOH, + 50ml $H_2O_{bidest.}$	2/1.4/1µg/ml

10µl of rifampicin were added to each well, leading to a final conc. of 20µg/ml in the media (5ml TSY). Additionally, each other antibiotic described in the table above, was added to rifampicin–treated wells resulting in 6 different combinations of two differently acting antibiotics. Each combination was prepared in duplicates and the multi–well dishes were then incubated for 72h at room temperature. The addition of antibiotics was carried out under a L2

laminar flow to protect amoebal cultures from contamination. The viability and morphology of treated cells was regularly checked by light microscopy. Amoebal cells were considered to be healthy and well–grown, if the number of floating amoebae cells was low and trophozoites could be detected. Cultures containing vast numbers of floating cells and exhibiting cystic morphology were rescued by removing the media and replacing it with unaltered TSY. Subsequently, the cultures were incubated at room temperature, allowing the amoebae to recover for 4–7 days. After the recovery of host cells was completed and the incubation period extended to 4 days, 5µl of each antibiotic were again added, resulting in a final concentration of 50% of antibiotic concentration originally used (200/20/2µg/ml). The media was removed after 4 days of incubation and replaced with fresh but unaltered TSY. Furthermore, host cells were harvested and fixed for a FISH screening, using CC23a–Cy3 (specific) and EUBmix–FLUOS (general) probes for bacteria and EUK516–Cy5 to visualize amoebae. Fixed cells were also stained with 15µl DAPI 1:5000 in order to visualize host amoebae nuclei and to counter–stain cells. Images were taken with the confocal microscope using the software Leica LAS AF. The antibiotic concentration was slightly increased to 70% of the originally used concentration and 7µl of each antibiotic were subsequently added. Over a period of 6 weeks, all cultures were alternately treated with media supplemented with 7µl of each antibiotic or unaltered TSY and incubated at room temperature. The progress of curing was regularly evaluated by FISH screenings. After 6 weeks of alternately curing and recovery phases, host cells were considered to be endosymbiont–free. No extracellular *Paracaedibacter spp*. EI3 could be found in the surrounding media.

2.2.13 Fluorescence in–situ hybridization (FISH)

Cells were harvested from either 50–150ml culture flasks by shaking or 6–12 well plates by pipetting 1ml for each sample. The volume of the extracted cell suspension was 1ml for each sample. After transferring the suspension into 1,5ml Eppendorf tubes, the samples were spun down at 6500rpm for 8 minutes at room temperature (RT). The supernatant was removed except for 80µl and the pellet was suspended. 20µl were taken out and applied to a Teflon coated FISH glass slide for each spot. Depending on the experimental approach, the slides were either incubated at RT for 25–30 minutes to preserve trophozoite structure or at 46°C for 15 minutes in the incubator to obtain a higher cell density on the spot. To fix the cells onto the glass slides, 15µl 4% PFA were applied to each spot and slides were incubated for 15 minutes at RT. Excess 4% PFA was removed and spots were washed with 15µl 1xPAS. Slides were then air–dried and kept either frozen at –20°C or directly subjected for FISH.

FISH was performed with 10µl of a 20% hybridization buffer and 1µl of each probe on each spot. FISH–probes were thawed on ice at 4°C and kept in the dark to prevent bleaching. Working concentrations of FISH–probes were 30ng/µl for Cy3–and Cy5–labeled probes and 50ng/µl for FLUOS–labeled probes. A probe–hybridization buffer master mix was prepared, in order to prevent diluting biases (Table 3). After applying the probe hybridization master mix to each spot, the remaining amount of hybridization buffer was subjected to a folded paper inside 50ml Blue Greiner tubes. The slides were then carefully transferred into the tubes and incubated at 46°C for 1, 5–18h. The hybridization was stopped by transferring the slides into pre–heated (48°C) Greiner tubes containing 20% washing buffer and incubated for 10 minutes at 48°C. A hyper–stringent washing step was undertaken in ice–cold MQ for 3s. In order to prevent diluting effects, the slides were immediately dried with compressed air. The final step includes mounting the slides with CitiFluor©, an embedding liquid suitable for oil immersion objectives. The Slides were the covered by putting a cover slip (17µm) onto them.

Visualization of samples was performed with an epifluorescence microscopy (Zeiss, Axioplan 2) or by a confocal laser–scanning microscope (Leica, SP8). Samples were visualized by excitation with a white light laser as well as a 405nm Diode. Used channel were FLUOS (505nm to 550nm), Cy3 (560nm to 615nm) and Cy5 (640nm to 750nm). Additionally, emission of fluorescence from cells stained with DAPI was obtained in a range of 410nm to 485nm. Images of samples analyzed with the epifluorescence microscope were obtained with Axioplan 4.8 software.

2.2.14 DAPI STAIN

Before embedding the slides with Citi Fluor, all spots were incubated with 15µl of a 4',6–Diamidino–2–phenylindole (DAPI)1:5000 working solution for 3 minutes. After that, the remaining DAPI solution was removed and all spots were washed with 15µl 1xPAS each. The final step included a 2–minute air–drying step.

2.2.15 PI STAIN

5µl of a 1:10 PI solution were applied to 500µl cell suspension. After an incubation of 20 minutes in the dark, the solution was spun down at 6600 rcf for 8 minutes at room temperature. The supernatant was decanted except for 50µl. The pellet was washed twice with sterile 1xPAS for 8 minutes at 6600 rcf, before the liquid was disposed again. 100µl sterile 1xPAS were added and the solution was transferred into a black micro–titer plate. Fluorescence was measured with a fluorescent reader.

2.2.16 CELL HARVESTING AND CELL NUMBER DETERMINATION

50–150ml culture flasks were manually shaken to detach amoebae from the surface. 50ml of cell suspension were transferred into 50ml Greiner tubes and spun down at 6500g for 8 minutes at RT (24°C). After that, the remaining supernatant was decanted and the cell pellet was re–suspended in 500µl 1xPAS. To obtain the amount of cells/µl, a Neubauer's haemocytometer© was used. All samples were diluted either 1:10 or 1:100 and applied to the haemocytometer with an amount of 20µl/sample. A dilution step became necessary, if the visible amount of cells exceeded 50 cells/square. Cells/µl was calculated with the formula listed below. Depending on number of cells to be seeded, a proper volume of cell suspension was transferred into new x–well plates. Empty culture flasks were refilled with 50ml or 150ml 1xPAS, depending on its size. Cells were feed with 10µl *Escherichia coli* tolC– per x ml 1xPAS (10µl/ml). Ampicillin (100mg/ml) was added with a v/v ratio of 1µl/5ml. All steps were performed under a laminar flow. All culture flasks were stored at 24°C, if not indicated otherwise.

2.2.17 HARVESTING OF ENDOSYMBIONTS AND INFECTION OF NEW HOSTS

Culture flasks containing endosymbionts were slightly shaken to prevent detachment of amoebae cells. A proper volume of cell suspension (8–50ml) was then transferred into a syringe and subjected to sterile–filtration. 1.2µm filters were utilized to purify FS5. The cell suspension was then spun down at 10500g for 10 minutes at RT. The excess supernatant was removed by pipetting except for 1ml to prevent a loss of biomass. After that, the pellet was re–suspended with 30ml 1xPAS and spun down at 10500g for 10 minutes at RT. Excess supernatant was decanted and the remaining pellet was re–suspended in 500µl–1ml 1xPAS, depending on its size. To start the infection, a correspondent amount of cell suspension was applied to each well. Applied cells were pulled down through a final centrifugation step with 370g for 15 minutes. Empty culture flasks were refilled with a proper amount of 1xPAS (150ml), *E. coli* tolC– and ampicillin (100mg/ml) All culture flasks were stored at 24°C, if not indicated otherwise.

Alternatively, culture flask containing endosymbiont–harboring amoebae were shaken of thoroughly in order to completely detach all host cells. The cell suspension was then spun down at 10500g for 10 min at RT. The excess supernatant was removed by decantation and the cell pellet subsequently re–suspended in 5ml 1xPAS/TSY (depending on the host amoebae). In order to achieve complete lyses of all endosymbiont–harboring amoebae cells, 3ml sterile glass beads (Roth) were added and the cell suspension was then vigorously vortexed for 3 minutes at 24°C. Furthermore, the glass beads were once washed in 1xPAS

and subsequently removed and the cell suspension passed through a 5µm/1.2µm (depending on the endosymbiont) disposable sterile filter. The endosymbionts were then collected by spinning down the cell suspension at 10500g for 10min. Depending on the original media; the pellet was once washed in freshly prepared and sterile 1xPAS and finally collected in 1ml sterile 1xPAS. Bacterial cells were quantified as described above.

2.2.18 ESTABLISHMENT OF CLONAL AMOEBAE CULTURES

200µl 1xPAS containing *E .coli tolC*– (5µl/ml) and ampicillin (100mg/ml, 1µl/5ml) were transferred into 20–48 wells of a 96–well micro–incubation plate. Single amoebae cells were carefully picked with the micromanipulator and transferred into each well by pipetting. After sealing it with Parafilm©, the plate was incubated for 3–7 days at RT.

2.2.19 ESTABLISHMENT AND CULTIVATION OF HELA 229–CELL LINES

HeLa 229–cell lines were routinely incubated in 25cm² sterile tissue culture flasks for 2–3 days at 37°C, 5% CO_2. DMEM supplemented by 10% (v/v) FBS was used as appropriate cultivation media. In order to transfer HeLa 229–cells, the medium was removed carefully by using sterile 10ml disposable syringes. Furthermore, the cells were washed once in 6–10ml sterile 1xDBPS to avoid possible contaminations and cross–reactions with 1xTE originating from residual media components. The liquid was fully removed and 300–500µl of 1xTE, depending on the density of attached cells was added. The culture flasks were enclosed with filter–lids and placed horizontally into the incubator at 37°C, 5% CO_2 for 10–12 minutes. Every 5 minutes the flasks were slightly shaken to fully cover all cells with 1xTE. The progress of de–attaching all HeLa cells was evaluated by light microscopy. If all cells had been dissociated from the bottom of the culture flasks, 5–6ml of sterile DMEM supplemented with 10% (v/v) FBS were transferred into each flask in order to inactivate 1xTE. Simultaneously, 4–5ml fresh DMEM supplemented with 10% (v/v) FBS were transferred into new sterile 25cm² tissue culture flasks and inoculated with 0.5–1ml cell suspension. All flasks were incubated at 37°C, 5% CO_2 for 2–3 days and the fitness and health of cells was routinely analyzed by light microscopy, before the cultures were again harvested and split. Cells were considered to be healthy and well–grown, if the majority of cells were still attached exhibiting characteristic unidirectional cell morphology.

2.2.20 ESTABLISHMENT AND CULTIVATION OF MOUSE MACROPHAGE THP–1 CELL LINES

Similar to human HeLa 229–cell lines, mouse macrophage THP–1 cell lines were kept at 37°C at 5% CO_2. 5ml sterile RPMI supplemented with 10% FBS (v/v) were used as incubation media and cultures were split twice a week on a regular basis. Additionally, sterile

FBS was heat–inactivated for 10–15 minutes at 70–90°C and subsequently filtered through a 0.2µm sterile disposable filter. The media exchange and splitting procedure was carried out based on morphological criteria obtained by regular light microscopy. Cells were considered to be healthy and well–grown, if the majority of macrophages were still floating in the fluid rather than being attached to the bottom of the culture flasks. Moreover, macrophages were incubated moderately longer in order to obtain a higher number of cells than HeLa 229–cells. Cultures were incubated in 25cm^2 sterile tissue culture flasks with filter lids in order to facilitate gas exchange. Depending on the macrophage–cell morphology and the number of attached cells, the fluid was removed and subsequently used to inoculate newly prepared culture flasks or attached cells were detached by the use of 1xTE. If macrophages had to be removed, the culture flasks were additionally shaken to enhance the dissociation of cells. The splitting procedure and maintenance of cultures was performed in the way described for HeLa 229–cells. 2–3ml of cell suspension was used in either case to inoculate new cultures and flasks were incubated at 37°C, 5% CO^2.

RESULTS

3. RESULTS

3.1 THE UWC36 INFECTION CYCLE AND ITS INFLUENCE ON HOST FITNESS

The infection of UWC36 in *A. castellanii* Neff was studied at both 20°C and 30°C. We chose two distinct temperatures to analyze possible effects on host cell susceptibility to a UWC36 infection. Moreover, we also evaluated the impact of higher temperatures on UWC36 infection modalities (UWC36 spread, multiplication inside host cells, host cell lysis).

2.2.21 EVALUATION OF OPTIMAL MOI FOR INFECTION STUDIES

First, we determined the impact of different multiplicities of infection (MOI) on overall host infection with the goal to be able to achieve a synchronous infection (Fig. 4). Only a small number of host cells containing single UWC36 cells could be observed 2 hours post infection (h.p.i.). We therefore let the infection proceed for 24 hours prior to analysis. After 24hpi, an increase of infected cells to 4.3 % was detected when using a higher MOI of 10. Host cells were moderately infected with <10 UWC36 cells in the cytoplasm. MOI 1 was also tested; however, we were unable to detect any infected host cell. At MOI 100, about 32.5% of all observed cells were infected, whereas at MOI 30 and 50 about 10.3% respectively 18.6% of all host cells did contain UWC36 in the cytoplasm (Fig. 4). Differences between MOI 10 and MOI 50 respectively 100 were calculated and determined to be significant ($P < 0.01$; $P < 0.001$).

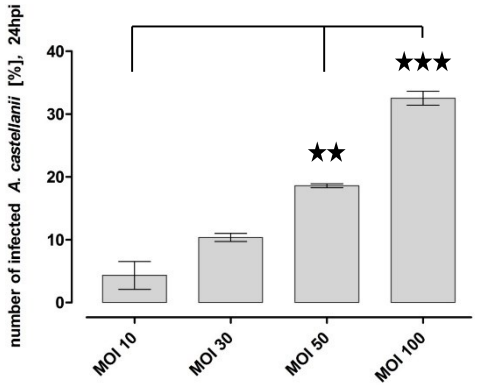

Fig. 4 Proportion of *A. castellanii* Neff cells infected by UWC36 in relation to different multiplicities of infection (MOI). Percentage of infected *A. castellanii* Neff [%] after 24hpi at different MOIs. All data points represent average values of two biological replicates. Experiment performed twice. Error bars show standard deviation.

2.2.22 UWC36 INSIDE ACANTHAMOEBA CASTELLANII NEFF

The UWC36 infection in *A. castellanii* Neff started with an initially low percentage of 12.3% (20°C) respectively 18.4% (30°C) infected host cells 2hpi. Infected amoebae contained mostly single UWC36 particles (Fig. 7 A). We rarely observed amoebae cells already infected

with a higher number of bacterial agents ranging from 2 to 5 cells at 2hpi (Fig. 7 B). Notably, many infected host cells exhibited UWC36 being in close proximity to host cell nuclei at 2hpi and 8hpi (Fig.7 A); a phenomenon that occurred at both incubation temperatures.

Between 2hpi and 8hpi, UWC36 mainly showed small coccid–shaped morphology (Fig. 7 B). After 24hpi, we observed mainly rod–shaped bacterial particles accompanied by a few number of still coccid–shaped UWC36 cells (Fig. 7 C–D). Furthermore, we also detected longer filaments between 24hpi and 72hpi (Fig. 7 G–H). These filaments only occurred in a very small number regardless of the used incubation temperature. At time points after 72hpi, UWC36 was present in higher numbers and distributed over the entire cytoplasm regardless the incubation temperature. By TEM we could detect coccid–shaped rods at later time points. UWC36 showed a typical Gram–negative cell membrane (Fig. 5 B). It replicated by a binary fission, which could be shown at later time points (Fig. 2 A). Moreover, the outer–membrane typically for gram–negative bacteria was clearly visible for both UWC36 inside vacuoles and distributed in the cytoplasm (Fig. 5 B, C, E). Vacuoles containing UWC36 cells were often placed in close proximity to each other showing partly fusion and formation of larger vesicles (Fig. 5 F). Images obtained from late–stage time points suggested lysis of host amoebae and subsequent release of UWC36 into the surrounding media (Fig. 5 F).

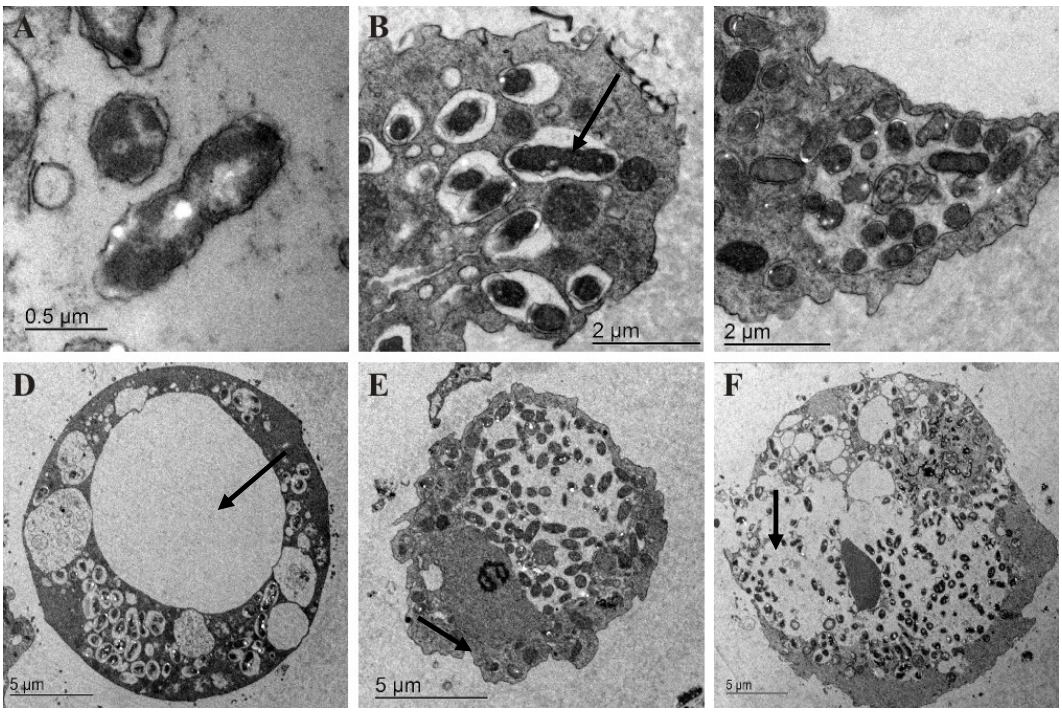

Fig. 5 An UWC36 infection as seen with transmission–electron microscopy (TEM). (A) UWC36 division inside the cytoplasm of *A. castellanii* Neff. **(B)** UWC36 replicating inside vacuoles in a single host cell. Arrow points at dividing bacterial cell inside a vacuole. **(C)** Single vacuole filled with >20 UWC36 cells after 96hpi surrounding by single UWC36 cells inside the host cytoplasm. **(D)** *A. castellanii* Neff containing UWC36 inside vacuoles. A giant vacuole placed in the center of the amoebae (arrow). **(E)** Large vacuole filled with high number of UWC36 at 72hpi, 30°C. Arrow points at the host cell nucleus. **(F)** Single host cell heavily infected, intra–cytoplasmic vacuoles are partially fusing with each other. The host cell membrane appears to be disrupted (arrow).

2.2.23 UWC36 SUCCESSFULLY THRIVES WITHIN *A. CASTELLANII* NEFF AT TWO DISTINCT TEMPERATURES REVEALED BY FLUORESCENCE IN–SITU HYBRIDIZATION

During the first 8hpi, the percentage of infected amoebae incubated at 20°C reached about 18.4% 2hpi and 13.9% 8hpi. At 30°C the initial proportion of UWC36–harboring host cells was slightly lower with about 12.3% after 2hpi, but increased to 15.3% 8hpi (Fig. 8, A–B). The difference in the proportion of bacteria–harboring *A. castellanii* Neff between both incubation temperatures were considered to be significant at 2hpi ($P < 0.05$), but not for 8hpi. After 24 hours, host cells containing >5 UWC36 particles were observed the first time at both incubation temperatures (Fig. 8, A–B). During the first 24h, the total number of infected host cells remained around 14.9 –16.1% at 20°C respectively 30°C. The fraction of amoebae exhibiting >5 UWC36 cells was only slightly higher at 30°C than at 20°C 24hpi. We could also observe this trend at 48hpi with the emergence of *A. castellanii* Neff being already infected with more than 30 bacterial cells at 30°C; an observation we did not make for host cells kept at 20°C.

The percentage of infected host amoebae incubated at 20°C did only slowly increase between 24hpi (15%) and 72hpi (27.9%) (Fig.8 A/6). In comparison with that, the proportion of infected amoebae kept at 30°C increased from 33.4% 48hpi to up to 64.2% 72hpi (Fig. 8 B/6). At 30°C we could detect highly infected *A. castellanii* Neff (>30 UWC36 cells) 48hpi for the first time (Fig. 8 B). In addition, also the percentage of host cells infected with <5 UWC36 cells was significantly higher ($P < 0.05$) after 48 hours (16% vs 9%). We further observed an increasing amount of extracellular particles at 30°C; an observation we did not make for amoebae incubated at 20°C. The total number of infected amoebae/µl remained stable and unvaried between 72hpi and 144hpi.

Elevated proportions of freshly infected *A. castellanii* Neff (<5 UWC36 cells) at 20°C could be seen between 72hpi and 120hpi (Fig. 8 A). Except for 8hpi and 24hpi, the mean percentage of initially infected host cells was generally significantly higher ($P < 0.05$) at 20°C than for amoebae kept at 30°C at all examined time points. Furthermore we also observed a doubling of the total proportion of infected amoebae between 72hpi (27%) and 96hpi (49%) at 20°C; a trend also monitored for host cells incubated at 30°C between 48hpi (33.4%) and 72hpi (61.6%). Between 48hpi (24.8%), 96hpi (49%) and 168hpi (96.5%), the total proportion of infected amoebae incubated at 20°C rose consistently. Elevated proportions of amoebae inhabited by UWC36 were also noticed at 30°C; however in comparison with *A. castellanii* Neff kept at 20°C, the proportion of UWC36–harboring amoebae was significantly higher (P

< 0.05) at 30°C between 72hpi and 120hpi (Fig. 6). Moreover, only a small proportion of host amoebae containing <5 bacterial cells in their cytoplasm were detected between 72hpi and 168hpi at 30°C. Differences in the percentage of *A. castellanii* Neff invaded by <5 UWC36 cells between both incubation temperatures in terms of significance varied between different time points. At 48hpi, host cells held at 30°C displayed a significantly higher ($P < 0.05$) proportion of infected amoebae; whereas between 72hpi and 144hpi this proportion was significantly lower ($P < 0.05$) than at 20°C. However, the number of host amoebae harboring more than 30 UWC36 cells was significantly higher ($P < 0.05$) at 30°C than at 20°C for all time points examined after 48hpi. A similar observation was made for intermediately infected host cells bearing significantly higher ($P < 0.05$) numbers at 30°C than at 20°C except for 120hpi.

Between 120hpi and 144hpi the percentage of infected amoebae further increased from 56.7% to 92.7% at 20°C thereby comprising an increase of ~60% (Fig. 8A/Fig. 6). During the same period of time *A. castellanii* Neff inhabiting UWC36 incubated at 30°C already exhibited almost entirely infected cultures comprising infection proportions of 98.9% (120hpi) and 100% (144hpi). From 144hpi on, infected amoebae maintained a high number of UWC36 cells in their cytoplasm, ranging from 20 to 30 bacterial agents and all replicates exhibited total infections rates around 100% at either temperature. The experiment was stopped 168hpi and all cultures were considered to be entirely infected with UWC36. All replicates exhibited high bacterial load outside of host cells. Furthermore, *A. castellanii* Neff were completely inhabited by UWC36 cells. Bacterial cells were freely distributed in the cytoplasm and seemingly organized within vacuoles.

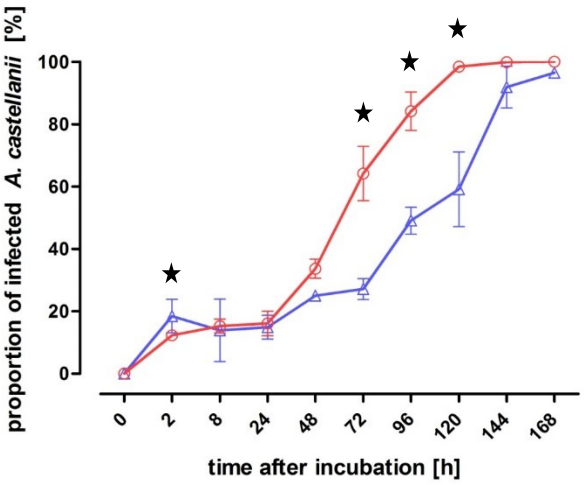

Fig. 6 UWC36 shows significant differences in the proportion of infected amoebae between 20°C and 30°C
Percentage of infected host cells at 20°C (blue line) and 30°C (red line) over the course of 168 hours. Asterisks mark significant differences ($P < 0.05$). Error bars indicate standard deviation. Asterisks mark significant differences.

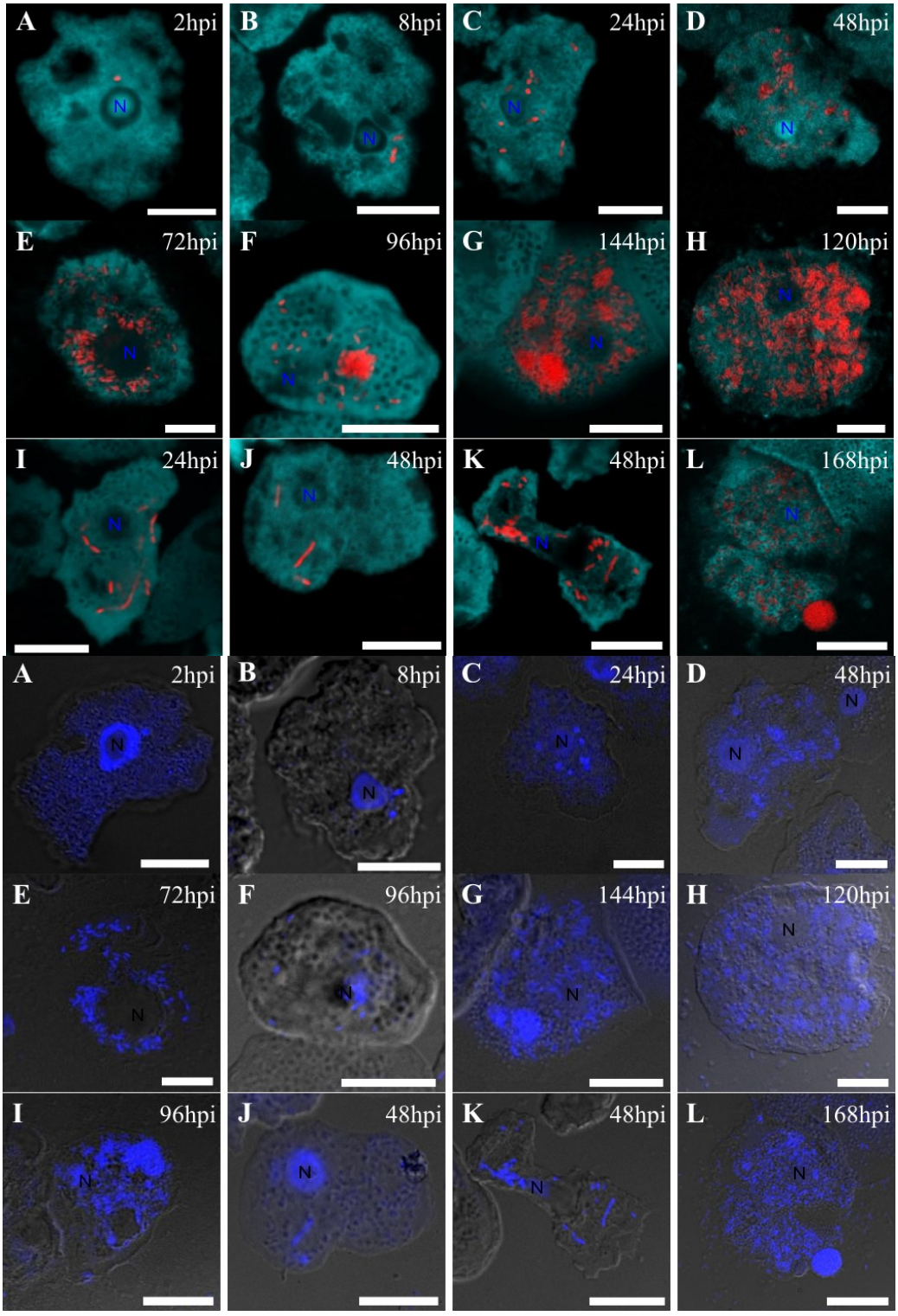

Fig. 7 Visualization of the UWC36 infection cycle in *A. castellanii* Neff. The infection was monitored over 168 hours with FISH using the EUK516 (turquoise) and combination of EUBmix .and AcRic90 (red). (**Panel A):** and by DAPI stain (blue) (Panel B): **(A–F)** Progress of infection, originating from singly infected *A. castellanii* Neff at 2hpi. **(A)** Single UWC36 cell in close proximity to host nucleus 2hpi. **(B)** Three UWC36 cells close to host nucleus 8hpi. Rod–shaped morphology. **(C)** Rod–shaped UWC36 after 24hpi arranged around host cell nucleus. **(D)** UWC36 replication in host cytoplasm. Partially infected host cells 48hpi. **(E)** Occurrence of moderately (<30) infected host cells after 48hpi/72hpi, depending on incubation temperature. **(F–G)** Asynchronous cycle. Multiple infection events at single host cells. Formation of vesicle–like structures containing massive numbers of bacterial cells after 72hpi. **(H–I)** Appearance of almost entirely filled host cells containing both vesicle–like structures and single UWC36 cells 96/120hpi. **(I–J)** Occurrence of longer filaments between 24hpi and 72hpi. **(K)** Vertical transmission of UWC36 during host cell division 48hpi. **(L)** Host amoeba entirely packed with UWC36 after 168hpi. Bacterial agents arranged freely in the cytoplasm and inside vesicle–like structures in host cell tissue 168hpi. Possible secretion of vesicles into surrounding media. N, host cell nucleus. Scale bars represent 10µm.

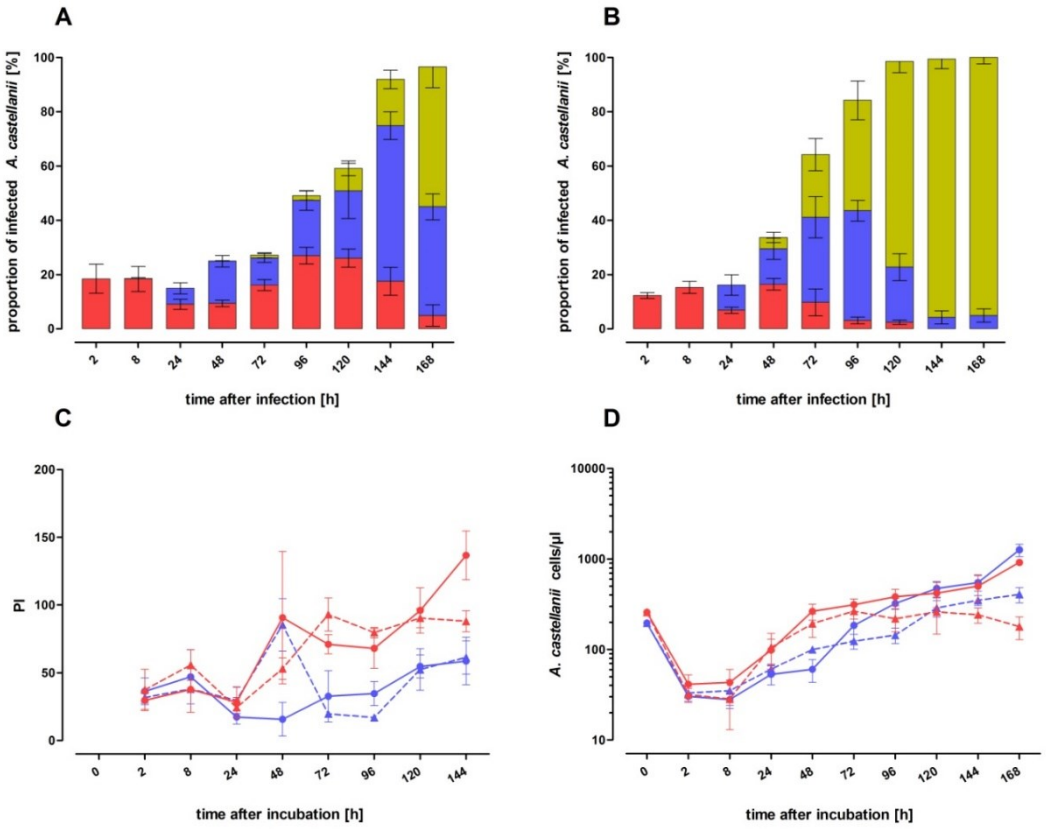

Fig. 8 The course of an UWC36 infection in *A. castellanii* Neff at different temperatures (20°C, 30°C). (A–B) Percentage of infected *A. castellanii* Neff [%] at 20°C (left) and 30°C (right) over time. **Red:** Host cells containing <5 bacterial cells, **Blue:** Host cells containing 5–30 bacterial cells, **Green:** Host cells containing >30 bacterial cells. All bars resemble average values of three biological replicates. Error bars show standard deviation. **(C)** Propidium iodide (PI) fluorescence intensities at 20°C and 30°C over the course of 168 hours. **Red continuous line:** empty controls 30°C. **Red dotted line:** PI values infected host 30°C. **Blue continuous line:** PI values empty control 20°C. **Blue dotted line:** PI values infected host 20°C. **(D)** Amoebae cell numbers over the course of 168 hours. **Red continuous line:** cells/µl empty control 30°C. **Red dotted line:** cells/µl infected host 30°C. **Blue continuous line:** cells/µl empty control 20°C. **Blue dotted line:** cells/µl infected host 20°C. Error bars show standard deviation

The titer of extracellular bacteria increased over time leading to host cells becoming infected multiple times. Notably, we observed vesicle –like structures containing massive numbers of UWC36 cells both inside host cells and inside the extracellular milieu (Fig. 7 E–F). These vacuoles first appeared in cultures incubated at 30°C after 72hpi, whereas amoebae being held at a lower temperature did not display these vesicles at that time point. Some amoeba cells contained vacuoles almost of the size of the entire host cell (Fig. 5 E, D). The number of vacuoles increased with ongoing incubation. Some vacuoles appeared to be in close proximity to the cytoplasmic membrane of already infected amoebae (Fig. 7, L). A release or uptake of vacuoles could not be observed. We were not able to detect vacuoles with the general eukaryotic probe EUK516. However, both EUBmix and AcRic90 led to a FISH signal with high intensity indicating a high bacterial cell activity.

2.2.24 UWC36 DOES NOT INDUCE HOST CELL LYSIS

A PI peak indicating increased host cell death could not be observed throughout the entire course of the experiment. Moreover, differences in observed PI values between infected amoebae and empty controls were only significant at 48hpi (20°C, $P < 0.001$) and 168hpi (30°C, $P < 0.01$). In general, infected amoebae incubated at 30°C did exhibit significantly higher ($P < 0.05$) PI values compared to amoebae incubated at 20°C at all examined time points after 24hpi (Fig. 8 C); an observation we did not make for empty control except at 48hpi (30°C, $P < 0.001$) and 144hpi (30°C, $P < 0.001$).

2.2.25 UWC36 ALTERS GROWTH PATTERNS OF *A. CASTELLANII* NEFF AT LATER TIME POINTS

Comparing the growth rates of amoebae, a difference in cell numbers between infected cultures and uninfected controls could only be identified from 96hpi on, with uninfected cultures displaying significantly ($P < 0.05$) higher numbers of cells/µl (Fig. 8 D) at both incubation temperatures except for 120hpi at 30°C. Between 8hpi and 24hpi as well as 96hpi and 120hpi, infected hosts incubated at 20°C were doubling in cells/µl; an observation as well made for empty controls between 8hpi and 24hpi respectively 48hpi and 72hpi. Moreover, empty *A. castellanii* Neff at 20°C displayed the highest number of cells/µl at 168hpi comprising a doubling after 144hpi. Monitoring uninfected amoebae incubated at 30°C revealed a similar doubling of cells/µl as well between 8hpi and 24hpi respectively 144hpi and 168hpi (Fig. 8 D). When comparing UWC36–harboring amoebae incubated at 20°C and 30°C, significantly ($P < 0.001$) higher values could only be seen for 72hpi (30°C) and 168hpi (20°C). A similar observation ($P < 0.05$) was made for empty controls at 48hpi (30°C) and 168hpi (20°C).

3.2 HOST RANGE OF PARACAEDIBACTER EI3 AND NUCLEICULTRIX AMOEBIPHILA FS5

By extending the host range, endosymbionts might further assure their continuous spread and survival and limit bottlenecks of transmission. In order to analyze the potential of UWC36, *Paracaedibacter* EI3 (hereby referred as EI3) and *Nucleicultrix amoebiphila* FS5 (hereby referred as FS5) to acquire new amoeba and eukaryotic hosts we performed several infection assays.

In order to evaluate the potential of EI3 to infect *A. castellanii* Neff, we performed a test infection experiment. We could not observe any infected host cell 2hpi. A repeat of the experiment did not result in any infected host cell after both 8hpi and 24hpi. Although the

amount of extracellular bacterial cells was considerably high, no EI3 particles were detected inside *A. castellanii* Neff. However, we often observed EI3 in close proximity to the host cell cytoplasmic membrane. FISH was performed with the general probe EUBmix and the specific probe CC23a. Both probes did bind to EI3 and led to considerably high signal intensity. Equal to observations made for EI3 in hybridized native infected *Acanthamoeba* spp. host cells, host–free EI3 exhibited mainly rod–shaped cell morphology.

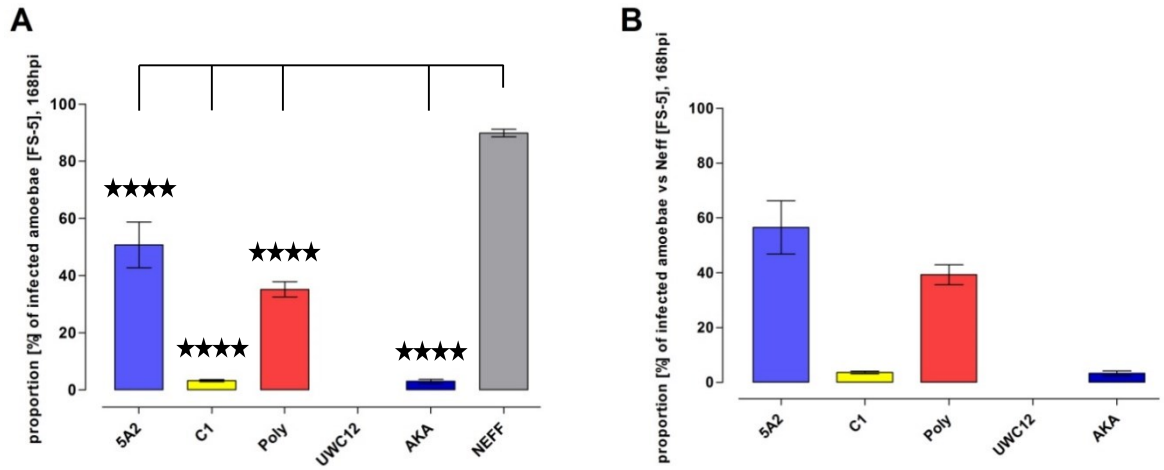

Fig. 9 FS5 infects various *Acanthamoeba* hosts after 168hpi. (A) Number of infected *A. castellanii* cells [%] after 168hpi. **(B)** Proportion of infected *A. castellanii* cells [%] against the control [Neff] after 168hpi. Error bars indicate standard deviation. Asterisks mark significant differences compared to control *A. castellanii* Neff.

The application of FS5 new *Acanthamoeba* hosts resulted in infected host nuclei of *A. castellanii* 5A2, *A. castellanii* C1 and *A. castellanii* Neff after 168hpi (Fig. 9). Additionally, *A. polyphaga* DOME and *Acanthamoeba spp*. AKA did also observe infected host nuclei. *A. castellanii* UWC12 could not be infected by FS5. In general, infection rates varied greatly among the used species. The control *A. castellanii* Neff showed the highest number of infected host cells with an average infection rate of 90% (Fig. 9 A). Compared to *A. castellanii* Neff, the proportion of infected amoebae was significantly lower ($P < 0.0001$) in all other amoeba strains used. *A. castellanii* 5A2 and *A. polyphaga* were also infected and the average number of infected cells was about 50% respectively 35% (Fig. 9 B). The majority of infected cells did not exhibit host nuclei entirely packed with *Nucleicultrix*, but single to few bacterial cells inside host nuclei. Observations made by light microscopy did not indicate extensive host cell lysis.

In contrast to FS5, EI3 was not able to infect any of the used amoebae strains. Moreover, the number of extracellular bacteria in the media was still considerably high after 168hpi. We did not observe cell lysis events by light or fluorescence microscopy.

Endosymbiont of *Acanthamoeba* UWC36 was able to infect *A. castellanii* C1, *A. polyphaga* and *A. castellanii* UWC12 besides *A. castellanii* Neff with considerably high percentage of infected amoebae (data not shown).

3.3 CURING OF THE NATIVE HOST OF PARACAEDIBACTER EI3

To cure the native host of EI3 we combined different antibiotics to increase their impact on bacterial replication, growth and viability. We therefore chose six different antibiotics belonging to six different classes and combined each of them with rifampicin in different concentrations (200µg, 20µg, 2µg/ml; Tab. 12).

After 72h of incubation at 24°C, we observed extensive host cell lysis and formation of cysts in all six cultures. In order to rescue the host cells, the media was completely removed and all cultures were washed once with fresh media. After the addition of new media, we incubated the cells again at 24°C for 72h, after 48 hours of incubation; most cultures had apparently recovered and did contain extensive numbers of host cell trophozoites. A FISH screening revealed that EI3 was still present in–and outside of host cells in considerable numbers. We continuously treated the native host with 50% (100µg, 10µg, 1µg/ml; Tab. 12) reduced concentration of antibiotics for 72h. Recovery of native host amoebae was conducted through replacement of media supplemented with antibiotics with unaltered media for 96h. We continued treating native host amoebae with 70% (Tab. 12) antibiotics (140µg, 14µg, 1.4 µg/ml) for 72h subsequently followed by a 96h recovery phase in unaltered rich media. To avoid host cell over–exposure to unfavorable conditions, the working concentration was further slightly lowered to 50% of the originally applied working concentration. This treatment was conducted for one additional week comprising 72h of exposure to antibiotics subsequently followed by a 96h recovery phase in fresh TSY, before the concentration of added antibiotics was again moderately elevated to 70% (percentage of originally used conc.; Tab. 12).

Cultures treated with rifampicin + ampicillin/doxycyclin/erythromycin did exhibit extensive host cell growth and subsequently a great number of trophozoites. Most of the cells appeared to be in a good shape and only a minor fraction of cysts could be observed. Additionally, the number of extracellular particles had further decreased. A preliminary FISH screening showed that most of the amoebae did not contain any bacterial cells anymore in their cytoplasm after 5 weeks of treatment. In contrast to these observations, a decrease in the proportion of EI3–harboring native host cells as well as the number of extracellular bacteria in samples treated with rifampicin + phosphomycin/gentamycin/ofloxacin could not be detected

after 4 weeks of incubation. To ensure proper replication and complete removal of residual *Paracaedibacter* EI3 cells in symbiont–free samples, the treatment procedure in combination with a subsequent recovery phase was prolonged for one additional week thereby applying 70% of the originally used concentration of antibiotics (Tab. 12). All cured cultures were subsequently split into a recovery cell line and a backup line still treated with 50% and 100% of the originally applied concentration of antibiotics. After one additional week of incubation, EI3 could not be detected by FISH and cultures were considered to be cured. TSY was added thereby replacing antibiotics and native *Acanthamoeba spp*. host cells were further incubated at room temperature (24°C).

Tab. 12 Curing of the native *Acanthamoeba* host of *Paracaedibacter EI3* over period of 6 weeks.
– = negative effect in terms of host cell morphology, replication and viability / number of extra – & intracellular bacterial cells. **0** = no effect. **+** = positive effect

Antibiotic combination		1. week of incubation			2. week of incubation		
1. Antibiotic	**2. Antibiotic**	**Effect on host viability**	**Effect on EI3 intra – & extracellular**	**AB working conc. (µg/ml)**	**Effect on host viability**	**Effect on EI3 intra – & extracellular**	**AB working conc. (µg/ml)**
	Ampicillin	– – –	0	200µg/ml	– – –	0	100µg/ml
	Doxycyclin	– – –	0	20µg/ml	– – –	0	10µg/ml
Rifampicin	**Erythromycin**	– – –	0	20µg/ml	– – –	0	10µg/ml
(20µg/ml)	**Phosphomycin**	– – –	0	20µg/ml	– – –	0	10µg/ml
	Ofloxacin	– – –	0	20µg/ml	– – –	0	10µg/ml
	Gentamycin	– – –	0	2µg/ml	– – –	0	1µg/ml
Antibiotic combination		**3. week of incubation**			**4. week of incubation**		
1. Antibiotic	**2. Antibiotic**	**Effect on host viability**	**Effect on EI3 intra – & extracellular**	**AB working conc. (µg/ml)**	**Effect on host viability**	**Effect on EI3 intra – & extracellular**	**AB working conc. (µg/ml)**
	Ampicillin	–	0	140µg/ml	0	+	100µg/ml
	Doxycyclin	–	0	14µg/ml	0	+	10µg/ml
Rifampicin	**erythromycin**	–	0	14µg/ml	0	+	10µg/ml
(20µg/ml)	**Phosphomycin**	0	0	14µg/ml	– – –	– – –	10µg/ml
	Gentamycin	0	0	14µg/ml	– – –	– – –	10µg/ml
	Ofloxacin	0	0	1.4µg/ml	– – –	– – –	1µg/ml
Antibiotic combination		**5. week of incubation**			**6. week of incubation**		
1. Antibiotic	**2. Antibiotic**	**Effect on host viability**	**Effect on EI3 intra – & extracellular**	**AB working conc. (µg/ml)**	**Effect on host viability**	**Effect on EI3 intra – & extracellular**	**AB working conc. (µg/ml)**
	Ampicillin	+	++	140µg/ml	+++	+++	140µg/ml
	Doxycyclin	+	++	14µg/ml	+++	+++	14µg/ml
Rifampicin	**Erythromycin**	+	++	14µg/ml	+++	+++	14µg/ml
(20µg/ml)	**Phosphomycin**	n.t.	n.t.	14µg/ml	n.t.	n.t.	14µg/ml
	Gentamycin	n.t.	n.t.	14µg/ml	n.t.	n.t.	14µg/ml
	Ofloxacin	n.t.	n.t.	1.4µg/ml	n.t.	n.t.	1.4µg/ml

3.4 PARACAEDIBACTER EI3 REMAINS UNABLE OF RE-INFECTING NATIVE ACANTHAMOEBA HOSTS AFTER CURING

In order to successfully re–infect the cured native host of *Paracaedibacter* EI3 (referred as EI3), we tried to evaluate the impact of different incubation temperatures (24°C, 30°C, 37°C) and media (TSY, 1xPAS +/– *E .coli TolC*) on EI3 infectiousness. The preliminary test infection using EI3 native host incubated at 24°C in TSY did not show any infected host cell after 24hpi. Subsequently, the initial infection time had been prolonged from 2h to 8h and 24h. However, we could not detect any infected host cell after extending the initial infection period.

Incubating amoebae at higher temperatures in combination with different cultivation media did not result in any infected host cell after 24hpi, 72hpi and 168hpi. At 30°C, both empty controls (*A. castellanii* Neff, EI3 native host) did exhibit stable growth and replication in both TSY and 1xPAS supplemented with *E. coli* tolC– indicated by the appearance of trophozoites. Screening the cultures through FISH revealed that only *E. coli* tolC– had been taken up by the host cells. After 168hpi, both infected cultures and empty controls incubated in 1xPAS +/– *E. coli* tolC– had already consumed all *E. coli* tolC– cells. Moreover, both host amoeba strains had become lysed and massive amounts of cell debris could be observed. *A. castellanii* Neff and EI3 native host incubated in TSY +/– *E. coli* tolC– were still growing and we could see many trophozoites. We could observe EI3 in the surrounding media at both 24hpi and 168hpi through light and fluorescence microscopy. Moreover, EI3 bacteria were sometimes detected in close proximity to host cells cytoplasmic membrane. However, we did not discover any host cell taking up EI3 by phagocytosis, not even in 1xPAS lacking additional nutrients. A further increase of the incubation temperature to 37°C did not favor host cell growth, but resulted in extensive host cell death after 7 days of incubation. Both infected cultures and empty controls did not survive this treatment and contained massive amounts of cell debris. Only single amoebae could still be detected. FISH screenings after 24hpi and 168hpi showed that no amoeba host cell had become infected by or did contain EI3 cells in their cytoplasm. Unlike EI3, *E. coli* tolC– had been taken up extensively, at least after 24hpi. The whole approach was repeated twice with equal results.

3.5 DIFFERENTIALLY PURIFIED UWC36 AND EI3 SHOW EXTENDED HOST RANGE AT ROOM TEMPERATURE

Increasing the incubation temperature did not affect the capability of *Paracaedibacter* EI3 to re–infect its native host; we tried to assess the impact of a different purification protocol on

EI3 infectiousness. Moreover, also the host range of both *Paracaedibacter* EI3 and Endosymbiont of *Acanthamoeba* UWC36 (*A. castellanii* C1 & UWC12, *A. polyphaga* DOME, *A. castellanii* 5A2, *Hartmannella* sp., *A. castellanii* Neff, HeLa cell line 229, mouse macrophages THP–1, *Drosophila* Schneider 2 (S2)) was re–evaluated using different media (TSY, PYG, 1xPAS + *E. coli* tolC–). To obtain the entire fraction of intra–and extracellular EI3 and UWC36 cells, all host cells were broke open through bead beating prior to filter purification. HeLa cell line 229, mouse macrophages THP–1 and insect cell line S2 could not be successfully infected by both UWC36 and EI3 24hpi, 72hpi and 96hpi (Tab. 13/Tab. 14).

Tab. 13 *Paracaedibacter* EI3 and Endosymbiont of *Acanthamoeba* UWC36 are able to infect various *Acanthamoeba* hosts under different conditions tested. The table depicts various conditions and parameters tested for potential EI3 and UWC36 infection of different *Acanthamoeba* hosts. – = no infection. + = positive infection. **n. t**. = not tested. All experimental assays were performed using technical duplicates/triplicates. Bacterial removal after 24hpi. Evaluation of proportion of infection after 24/72/96hpi.

Incubation temperature		**24°C**				**30°C**			
Host organism	**Infectious agents**	**TSY**	**TSY + *E. coli***	**1xPAS**	**1xPAS+ *E. coli***	**TSY**	**TSY + *E. coli***	**1xPAS**	**1xPAS+ *E. coli***
Native *Acanthamoeba* host EI3	**UWC36**	+	+	+	+	n.t.	n.t.	n.t.	n.t.
	EI3	–	–	+	+	–	–	–	–
***A. castellanii* Neff**	**UWC36**	+	+	+	+	+	n.t.	n.t.	n.t.
	EI3	–	–	–	–	–	–	–	–
A. castellanii **C1**	**UWC36**	+	n.t.	+	+	n.t.	n.t.	n.t.	n.t.
	EI3	–	n.t.	+	+	n.t.	n.t.	n.t.	n.t.
A. castellanii **UWC12**	**UWC36**	+	n.t.	+	+	n.t.	n.t.	n.t.	n.t.
	EI3	–	n.t.	+	+	n.t.	n.t.	n.t.	n.t.
A. castellanii **5A2**	**UWC36**	+	n.t.	+	+	n.t.	n.t.	n.t.	n.t.
	EI3	–	n.t.	–	–	n.t.	n.t.	n.t.	n.t.
A. polyphaga **DOME**	**UWC36**	+	n.t.	+	+	n.t.	n.t.	n.t.	n.t.
	EI3	–	n.t.	+	+	n.t.	n.t.	n.t.	n.t.
***Hartmannella* sp.**	**UWC36**	–	n.t.	–	–	n.t.	n.t.	n.t.	n.t.
	EI3	–	n.t.	–	–	n.t.	n.t.	n.t.	n.t.

Tab. 14 *Paracaedibacter* EI3 and Endosymbiont of *Acanthamoeba* UWC36 are unable to infect multiple eukaryotic hosts. The table depicts various conditions and parameters tested for potential EI3 and UWC36 infection of different eukaryotic hosts. – = no infection. + = positive infection. **n. t**. = not tested. All experimental assays were performed using technical duplicates/triplicates. Bacterial removal after 24hpi. Evaluation of proportion of infection after 24/72/96hpi.

Incubation temperature 37°C (5%CO_2)	Infectious agents	RPMI + 10%FBS (v/v)	DMEM + 10%FBS (v/v)
HeLa cell line 229	UWC36	–	–
	EI3	–	–
Mouse macrophages THP–1	UWC36	–	–
	EI3	–	–
Insect cell line S2	UWC36	–	–
	EI3	–	–

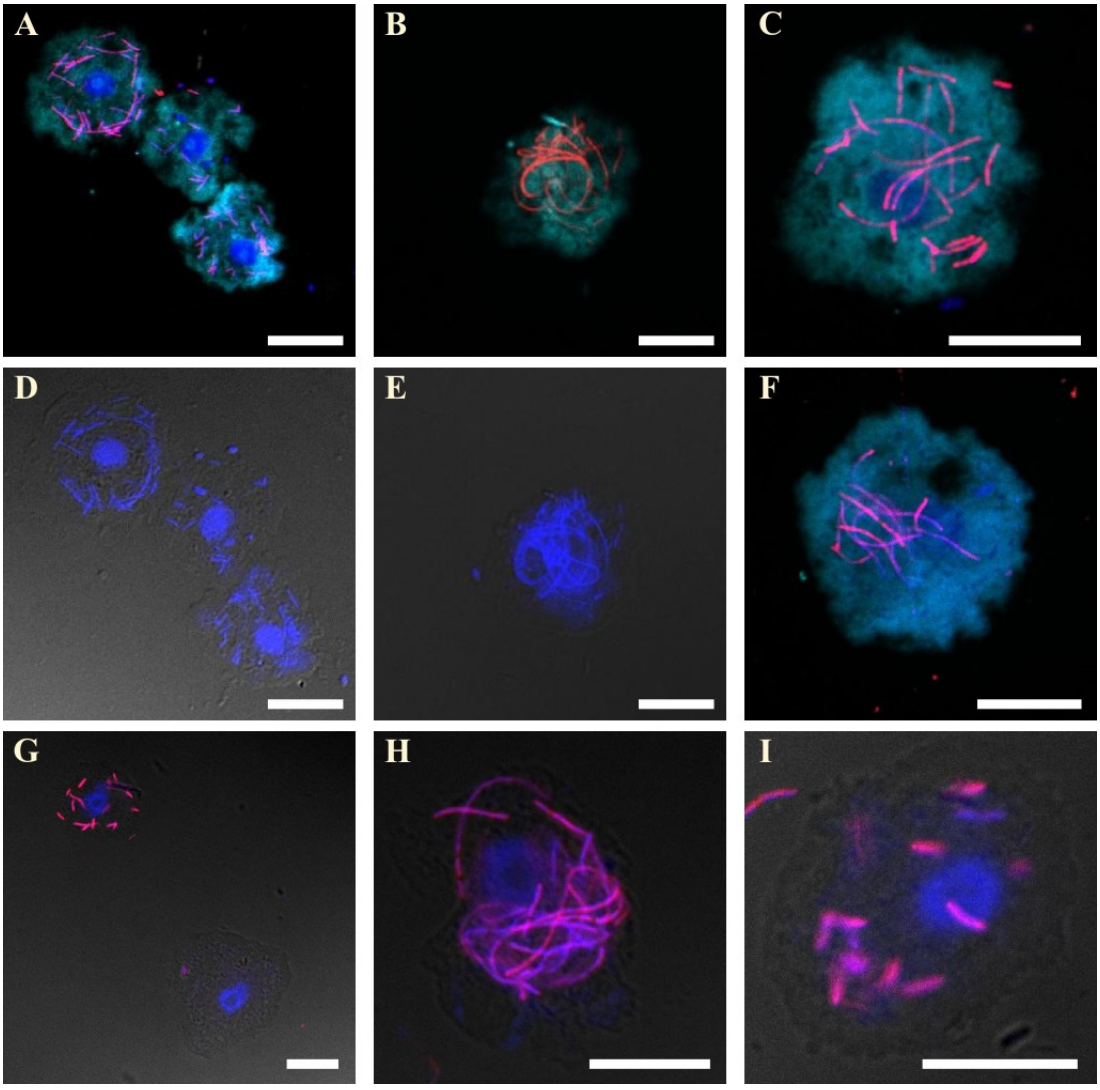

Fig. 10 *Paracaedibacter* EI3 native infection of its cured native host *Acanthamoeba spp.*, 96hpi. (A, D) EI3 native amoebae containing several EI3 cells in the cytoplasm at 96hpi. **(B, E)** Single native host cell containing heavily enlarged and circled EI3 cells. **(C, F)** Single amoeba cell packed with both rod–shaped and enlarged EI3 cells. **(G, I)** Small number of EI3 cells inside native host amoebae. **(H)** Several heavily enlarged EI3 cells circled around host nucleus. Host cells incubated in 1xPAS supplemented with *E. coli* tolC– (10µl/ml) and ampicillin (20µg/ml). Host amoebae were targeted with the general eukaryotic probe EU338mix–FLUOS (not shown) and the specific EI3 probe CC23a–Cy3 (red). Additionally, host cell nuclei, mitochondria and UWC36 were visualized by a DAPI stain (blue). Scale bars represent 10µm.

Furthermore, EI3 was not capable of invading any host amoebae if being incubated in TSY. No infected host cell could be observed at both 24hpi and 96hpi. However, some cultures, which had been cultivated in 1xPAS supplemented with *E. coli* tolC– (10µl/ml) and ampicillin (20µg/ml) turned out to be already infected after 24hpi. *A. castellanii* C1, *A. polyphaga* and *A. castellanii* UWC12 did exhibit EI3 cells inside the host cytoplasm. More importantly, also the cured native host had become infected by EI3 indicated by many freshly infected amoebae containing <5 EI3 cells (Fig. 10). Most EI3 particles inside *A. castellanii* C1 appeared to be enclosed in vacuoles.

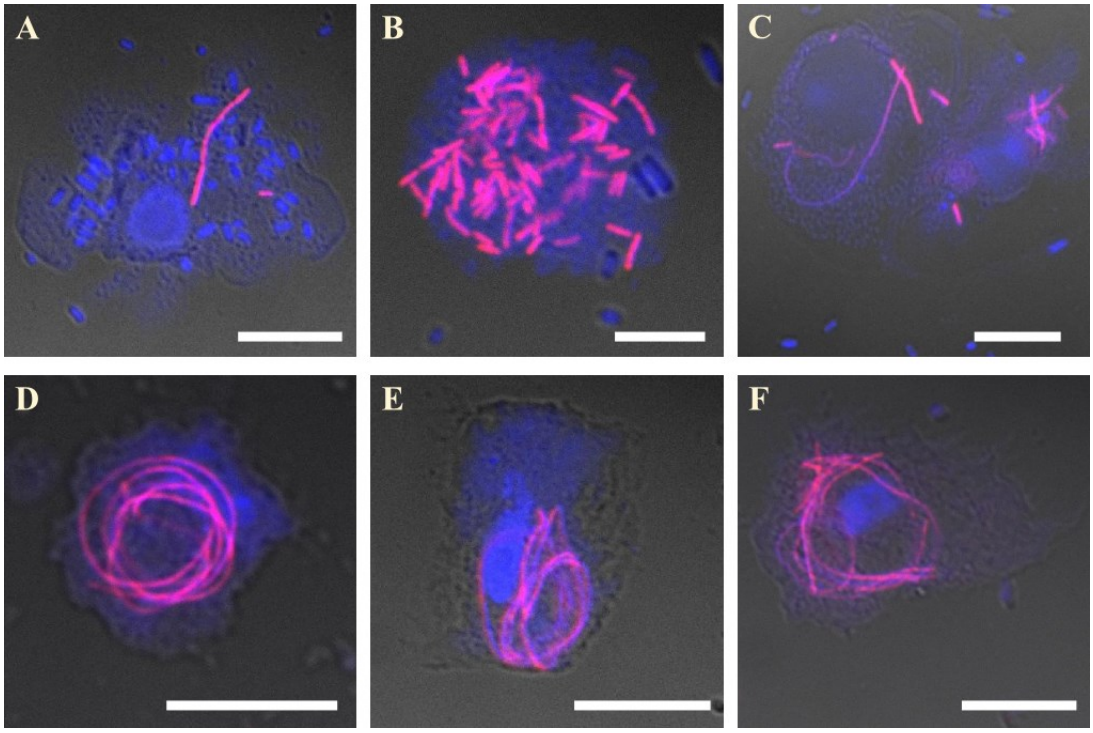

Fig. 11 Morphology of *Paracaedibacter* EI3 varies in different *Acanthamoeba* hosts, 96hpi. (A) Host *A. castellanii* UWC12 containing a single enlarged EI3 cell close to host nucleus at 96hpi. Host amoeba fed with *E. coli* tolC– (Blue cocci–shaped cells). **(B)** Single UWC12 exhibiting many EI3 cells beside single *E. coli* tolC– cells. No DAPI signal from nucleus. **(C)** Two UWC12 cells in close proximity containing a small number of EI3 cells. Single heavily enlarged EI3 cell showing only a partial Cy3 signal. **(D)** *A. castellanii* C1 filled with a single significantly enlarged and circled EI3 cell. **(E)** C1 showing a small number of apparently enlarged and circled EI3 cells in close proximity to the host nucleus. **(F)** *A. polyphaga* DOME containing several significantly enlarged and circled EI3 cells close to the host nucleus. EI3 was detected with a combination of the universal probe EUBmix and the specific probe CC23a. Additionally, host cell nuclei, mitochondria and EI3 were visualized with a DAPI stain. Scale bars represent 10µm.

Morphologically, EI3 mainly appeared to be rod–shaped with an intermediate cell size (Fig. 10 A, G, I). In comparison with the native unaltered host, no obvious difference concerning both host cell and EI3 cell morphology could be observed. Once inside the host cell, EI3 cells appeared to be both clustered together in apparently vacuoles and single cells in the cytoplasm. Interestingly, a small number of infected amoebae contained only a single bacterial cell, which had been heavily enlarged and enrolled (Fig. 10 H). We could detect those morphologically changed EI3 particles in *A. castellanii* C1, *A. polyphaga* DOME, *A. castellanii* UWC12 and in the native host as well as in the surrounding 1xPAS. *E. coli* tolC–,

which had been added as a food source however did not exhibit an altered cell morphology (Fig. 11). *A. castellanii* 5A2, Neff and *Hartmannella* sp. were not infected by EI3, neither in TSY nor in 1xPAS. No controls without added *E. coli* tolC– and ampicillin had been prepared.

Conclusively, EI3 was capable to re–infect its cured native host beside *A. castellanii UWC12*, *A. castellanii* C1 and *A. polyphaga* DOME only in 1xPAS supplemented with *E. coli* tolC– and ampicillin to a currently unknown extend.

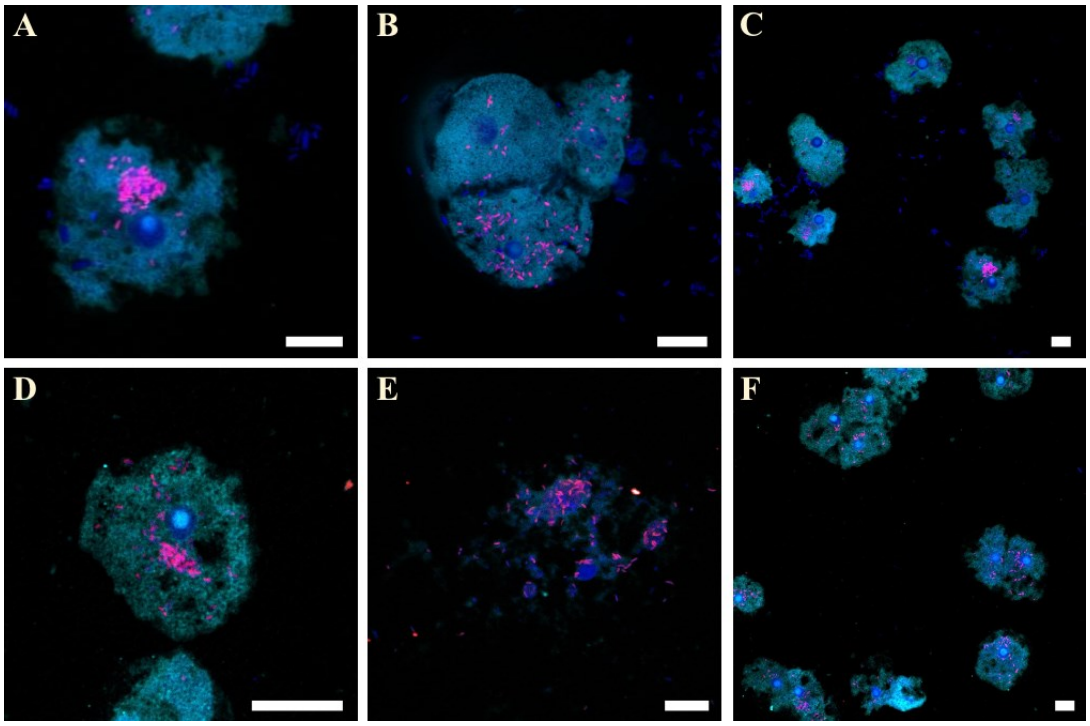

Fig. 12 UWC36 infects *Acanthamoeba castellanii* UWC12 after 24hpi in different media/buffer. (A) Single UWC12 cell (turquoise) showing several single and UWC36 (red) organized in a vacuole–like structure close to the DAPI stained host nucleus (blue). Incubation media 1xPAS. **(B)** Cluster of three UWC12 cells being incubated in 1xPAS infected with different numbers of UWC36. Some UWC36 cells appear to be in close proximity to the host nucleus. **(C)** Progress of infection after 96hpi. Almost every host amoebae filled with differently distributed UWC36 in host cytoplasm. Variable bacterial cell number. **(D)** Single host cell incubated in PYG showing several UWC36 cells clustered together and freely distributed in the cytoplasm. **(E)** Late stage of infection comprising massive host cell lysis events after 96hpi. Host cells kept in TSY at room temperature **(F)** Cluster of host cells with different numbers of UWC36 cells inside. Intermediate stage of infection 72hpi. All host cells incubated in 1xPAS had been supplemented with *E. coli* tolC–. Host amoebae were targeted with the general eukaryotic probe EUK516–Cy5 (turquoise). UWC36 was detected with the universal probe EUBmix–FLUOS (not shown) and the specific probe AcRic90–Cy3 (red). Additionally, host cell nuclei, mitochondria and UWC36 were visualized with a DAPI (blue) stain. Scale bars represent 10µm.

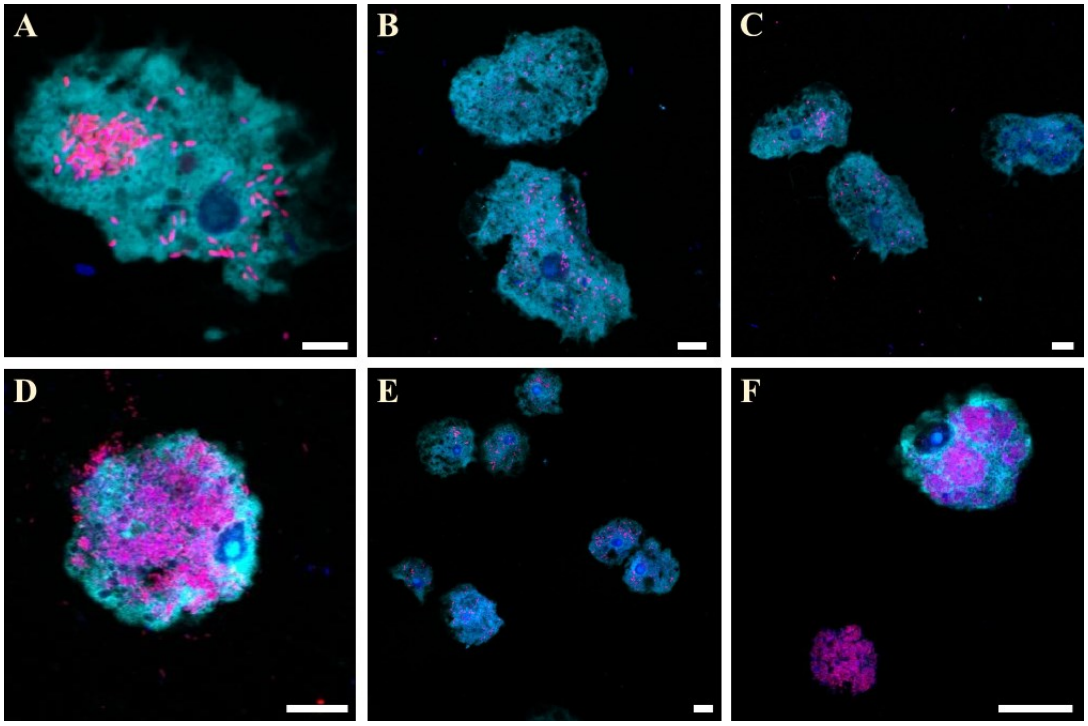

Fig. 13 UWC36 invades *Acanthamoeba castellanii* C1 after 24hpi in different media/buffer. **(A)** Single *A. castellanii* C1 cell incubated in 1xPAS containing UWC36 both freely and accumulated in a vacuole in the cytoplasm 24hpi **(B)** Two host cells exhibiting a moderate number of UWC36 in the cytoplasm. Host cells kept in 1xPAS 24hpi. **(C)** Several *A. castellanii* C1 cells incubated in 1xPAS filled with differently distributed UWC36 cells. The upper left one apparently contains UWC36 cells organized in a vacuole – like structure 24hpi. **(D)** Host cell 96hpi entirely filled with UWC36. Bacterial cells in close proximity to host cell membrane indicate evasion. Host amoebae incubated in TSY. **(E)** Intermediate stage of infection 72hpi. Most host cells display intermediate number of UWC36 cells in the cytoplasm. **(F)** Late stage of infection with host cell lysis and exclusion of bacterial cells. Lower left amoeba have apparently been lysed indicated by the loss of a corresponding FISH signal. No DAPI signal for host nuclei visible. All host cells incubated in 1xPAS had been supplemented with *E. coli* tolC–. Host amoebae were targeted with the general eukaryotic probe EUK516–Cy5 (turquoise). UWC36 was detected with the universal probe EUBmix–FLUOS (not shown) and the specific probe AcRic90–Cy3 (red). Additionally, host cell nuclei, mitochondria and UWC36 were visualized with a DAPI (blue) stain. Scale bars represent 10µm.

UWC36 was able to infect all *Acanthamoeba* strains cultivated in TSY but not *Hartmannella*. Interestingly, only *A. castellanii* Neff, *A. castellanii* UWC12 and *A. castellanii* C1 did contain UWC36 cells in their cytoplasm, when being incubated in 1xPAS supplemented with *E. coli* tolC– (Fig. 12, 13) and ampicillin. Both *A. castellanii* Neff and *A. castellanii* C1 cultures in TSY turned out to be completely infected. Additionally, the amount of extracellular UWC36 was still extremely high after 96hpi. Similar to that, both amoeba strains kept in 1xPAS did as well exhibit high infection rates and moderate amounts of UWC36 in the surrounding media. Once inside the host cell, UWC36 was found to be distributed equally in the host cytoplasm as well as packed in vacuole–like structures (Fig. 12, 13).

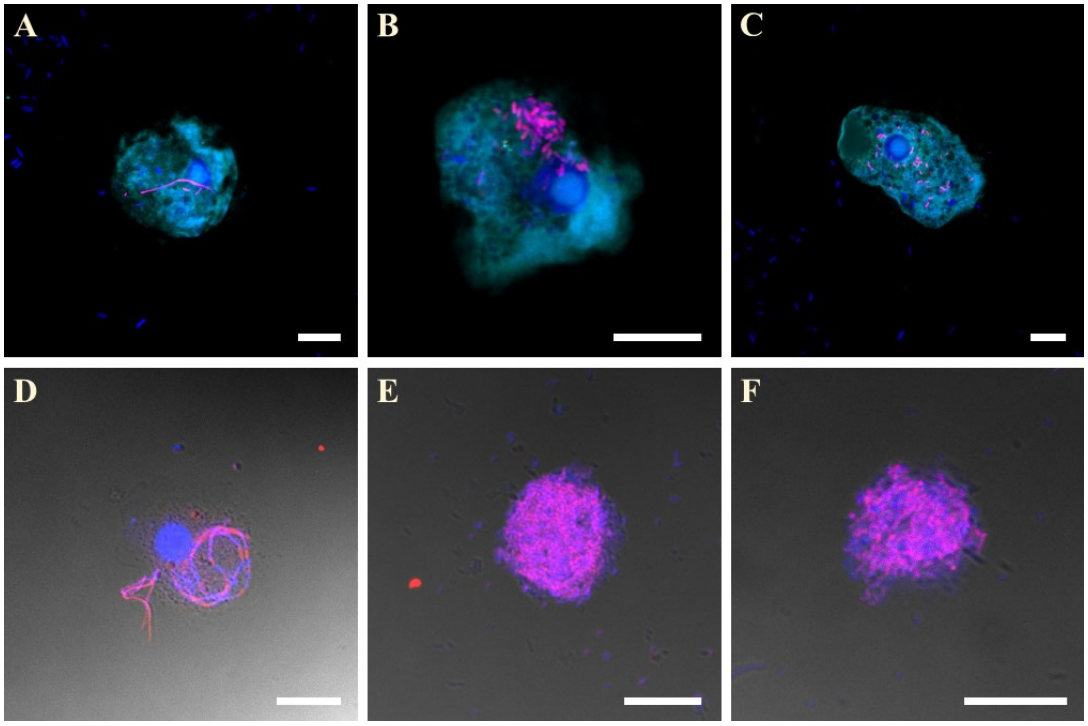

Fig. 14 UWC36 infects other *Acanthamoeba spp.* hosts showing enlarged cell shape in the presence of ampicillin. **(A)** Single enlarged UWC36 cell inside *A. castellanii* Neff incubated in 1xPAS + *E. coli* tolC– and ampicillin 24hpi. Two rod–shaped UWC36 cells in close proximity to host nucleus. **(B)** UWC36 organized in a vesicle–like structure next to *A. castellanii* Neff nucleus 24hpi. **(C)** Few UWC36 cells freely distributed in the cytoplasm of *A. castellanii* Neff. **(D)** Heavily enlarged UWC36 cells inside *A. castellanii* Neff 72hpi. **(E)** Single *A. castellanii* 5A2 incubated in TSY entirely infected by UWC36 72hpi. No DAPI signal from host nucleus **(F)** *A. polyphaga* incubated in TSY exhibiting massive amount of UWC36 distributed in the cytoplasm 72hpi. No DAPI signal from host nucleus. UWC36 was detected with the universal probe EUBmix–FLUOS (not shown) and the specific probe AcRic90–Cy3 (red). Additionally, host cell nuclei, mitochondria and UWC36 were visualized with a DAPI (blue) stain. Scale bars represent 10µm.

A. castellanii UWC12, *A. castellanii* 5A2 and *A. polyphaga* were infected to a moderately lower degree than *A. castellanii* Neff and *A. castellanii* C1, if incubated in TSY. Moreover, we could observe a high number of host cells showing a decrease in cell size and an altered morphology 96hpi. These cells were almost entirely packed with UWC36 and were apparently round–shaped and smaller regarding the cell size than empty controls. Trophozoites could not be detected anymore. Partial cell lysis had already begun, as indicated by both light and fluorescence microscopy. Apparently independent from the surrounding media, these observations were also made for amoebae cultivated in 1xPAS and TSY.

Generally, *A. castellanii* 5A2 and *A. polyphaga* were affected to a higher degree than other hosts (Fig. 14). However, we could not obtain any relevant data concerning the proportion of infected amoebae [%] due to a generally low number of host cells (< 50) attached to the glass surface. Transferring all cultures into freshly prepared media for recovery did not result in any observable host cell growth. After 168h of recovery, almost no host cell could be found suggesting that either the initial cell number had already been to low or cells had already been dead. A repeat of the experiment with only *A. castellanii* C1, Neff and UWC12 in TSY and 1xPAS + *E. coli* tolC– and ampicillin did show similar results.

Morphologically, UWC36 appeared to be both rod–shaped and coccid, resembling the morphology varieties already observed during the infection cycle experiment. However, we could also discover heavily enlarged and rolled up particles both inside host cells and in the surrounding media. Similar to EI3, these particles sometimes appeared to be a single bacterial cell and were only seen in 1xPAS. Interestingly, most infected host cells did also contain moderately sized vacuole – like structures being filled with UWC36 (Fig. 13 A). These vacuoles did not only occur inside host cells, but also in the surrounding media. However, *E. coli* tolC– did not display this altered cell morphology.

3.6 Maintenance of respiratory activity & infectivity of N. amoebiphila FS5 in a host–free environment

Being capable of withstanding various environmental pressures once outside the host cell is critical for the continuous spread and survival of FS5. Preliminary results have already suggested that FS5 remains infectious after 5 days of host–free incubation. By extending this period further, we were interested, if FS5 may further remain infectious. Furthermore, we focused on the evaluation of FS5 cell activity, if being incubated host–free in the presence/absence of purified amoebae DNA.

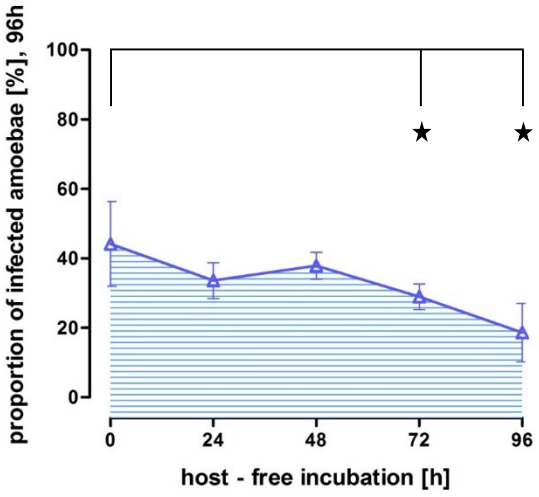

Fig. 15 FS5 remains able to infect *Hartmannella* sp. after 96 hours host–free incubation. The proportion of infected *Hartmannella* sp. nuclei [%] monitored over time. All points resemble the average of four biological replicates. Error bars show standard deviation. Asterisks indicate significant differences compared to control 0hpi.

FS5 remained able to infect *Hartmannella* sp. after 5 days of host–free incubation (Fig. 15). The total infection rate dropped moderately with continuing host–free incubation of FS5 from 44.12 % to 18.62% of infected amoebae. A considerably high number of infected host nuclei did contain only 1–3 FS5 particles at 72 and 96 hours of host–free incubation (data not shown). However, we did not observe decreasing infection rates between 24 and 72 hours of host–free incubation.

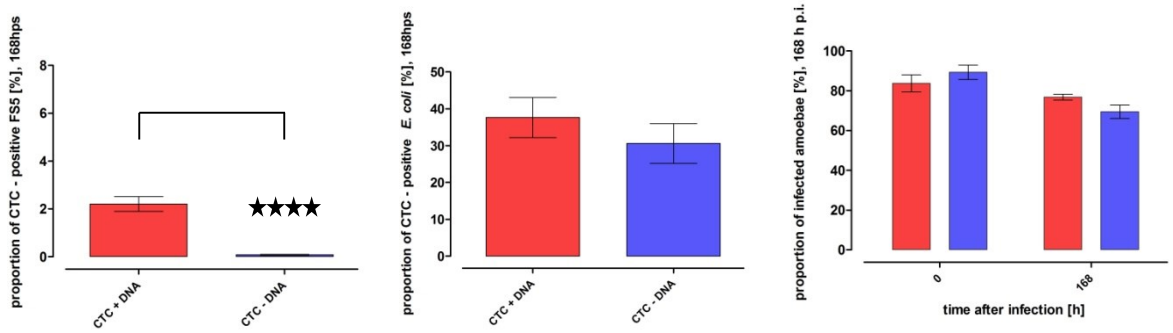

Fig. 16 How the presence or absence of DNA during host–free incubation affects FS5. (A) Percentage of CTC–positive FS5 cells [%] after 168h. **(B)** Number of PI–positive FS5 cells [%] after 168h. All points resemble average value of eight biological replicates. Error bars show standard deviation. **(C)** Number of infected *Hartmannella* sp. cells [%] in the presence / absence of DNA after 0hpi and 168hpi. Red: + DNA. Blue: – DNA. **Red:** + DNA. **Blue:** – DNA. Values resemble the average of four biological replicates. Error bars show standard deviation. Asterisks mark significant differences.

Furthermore, we tried to assess the question, if an endonuclear symbiont might utilize host DNA for its own purpose in order to stay alive and replicate. The total proportion of infected host cells remained stable and did not drop down over time of 168 hours of host–free incubation. The initial proportion of infected amoebae was 83.7% in the presence of DNA 0hpi. Samples incubated without the addition of purified amoebal DNA exhibited 89.3% infected *Hartmannella* sp. 0hpi (Fig.16 C). The difference between both incubations was considered insignificant at the start of the experiment. After 168h of host–free incubation in the presence of DNA, FS5 remained capable of invading *Hartmannella* sp. In comparison to the proportion of amoebae harboring FS5 at 0hpi, 91% of host cells did contained FS5 particles inside their nuclei. In the absence of host DNA as a possible utilizable food source, 69.5% of all host cells were infected 168hpi. Compared with 0hpi as reference, still 77.9% of all host amoebae contained bacterial cells inside their nuclei, if samples had not been supplied with amoebal DNA. The difference in the proportion of *Hartmannella* sp. inhabiting FS5 between 0hpi and 168hpi was found to be insignificant in the presence of DNA. In contrast to that, we could discover a significant ($P < 0.01$) decrease of infected host cells in the absence of DNA between the reference (0hpi) and 168hpi. Moreover, the proportion of FS5–harboring amoebae 168hpi was significantly ($P < 0.05$) higher in the presence of DNA than in the absence thereby exhibiting a total percentage of 76.7% infected host cells (unaltered controls 69.5%).

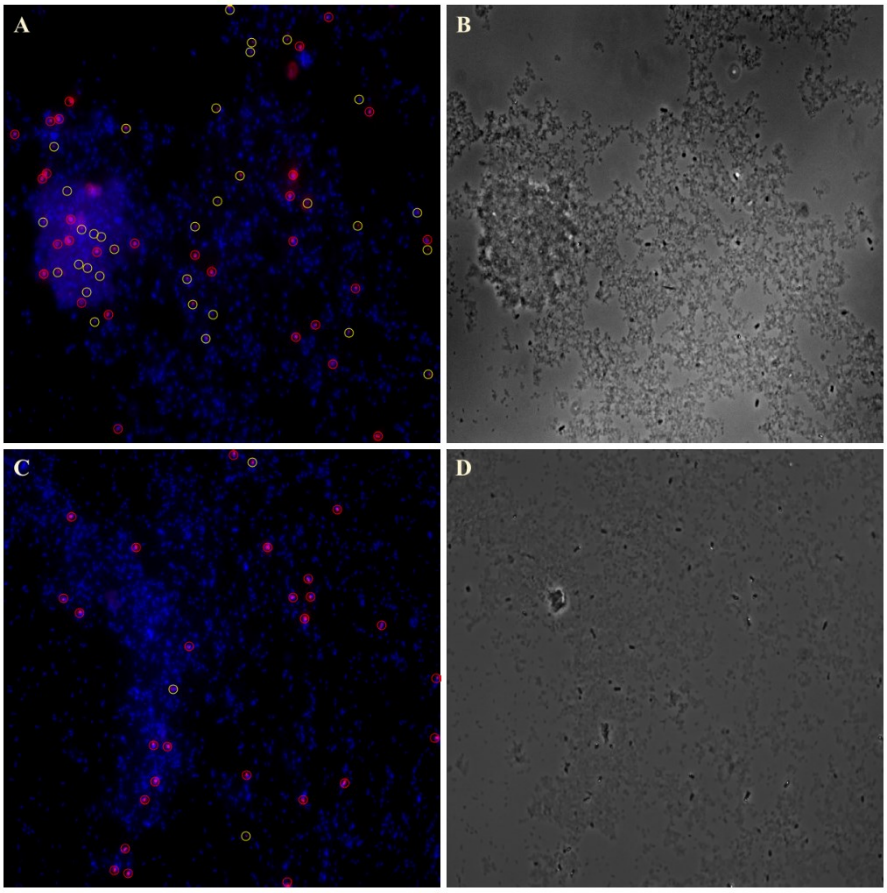

Fig. 17 FS5 displays higher proportion of active cells in the presence of purified amoebal DNA 168hpi (A) DAPI + CTC staining after 168h host–free incubation in the presence of DNA. **Red circles:** CTC–positive *E. coli* tolC–. **Yellow circles:** CTC–positive FS5. **(B)** Phase–contrast image of A. **(C)** DAPI + CTC staining after 168h host–free incubation without DNA. **Red circles:** CTC–positive *E. coli* tolC–. **Yellow circles:** CTC–positive FS5. **(D)** Phase–contrast image of C.

About 2.25% of all FS5 particles did exhibit a CTC–positive signal in the presence of host DNA after 168h of host–free incubation (Fig. 16 A). In contrast to that only 0.1%, active FS5 cells could be detected, if DNA had not been added (Fig. 16 A). The difference between both incubations was significant ($P < 0.0001$) thereby comprising a 28–fold increase of respiratory active FS5 cells in the presence of DNA. *E. coli* tolC–, which was used as positive control did exhibit generally larger sized signals as well as apparently higher signal intensities. Residual *E. coli* tolC– cells inside FS5 incubations originating from the FS5 purification process did also show a distinct signal, which was larger in size and intensity than signals from FS5. At 72 hours of host–free incubation, 3.2% of all monitored FS5 cells were active; a 53.3–fold increase of activity compared with samples lacking DNA. Data obtained at 96h of host–free incubation showed similar results with about 6.2% active FS5 cells in the presence of amoebae DNA and 0.17 % in the absence of DNA. The number of CTC–positive FS5 cells supplied with purified amoebal DNA decreased to about 1% after 240 hours of host–free incubation. In the absence of DNA, about 0.06% of all observed FS5 cells exhibited a CTC–positive signal at this time point; a 16.7 fold difference. The number of CTC–positive FS5

cells varied significantly ($P < 0.0001$) between samples incubated with and without DNA at all examined time points. Moreover, *E. coli* tolC–showed significantly ($P < 0.0001$) higher numbers of respiratory active cells compared with FS5 in both the presence and absence of DNA at all examined time points (Fig. 16) Throughout the entire course of the experiment, at least 24% of all detected *E. coli* tolC– cells were respiratory active at both incubations. After 72 hours of host–free incubation, 40.01% (+ DNA) respectively 24.4% (– DNA) active *E. coli* tolC– cells were monitored. At 96 hours of host–free incubation, about 43.8% (+DNA) respectively 45.2% (–DNA) exhibited a CTC–positive signal. Generally, the number of active *E. coli* tolC– cells in the presence of DNA did not show much variation between different time points. Samples incubated without added DNA, however did exhibit a decrease in the number of CTC–positive cells from 96h to 240h of host–free incubation. However, in contrast to FS5 these differences were never significant. The relative numbers of bacterial cells visualized by application of DAPI did vary moderately between all observed time points, ranging from 100 to 1000 cells/spot (Fig. 17). We could not detect any CTC–positive signals after an incubation time of 2 or 8 hours. The incubation time was therefore increased to 24 hours.

To evaluate the degree of dead cells, PI was added to each replicate separately from CTC. DAPI was applied to visualize the total number of cells. Notably, the PI values obtained after 168h did not indicate a significant increase in the number of dead cells neither in the presence nor in the absence of host DNA (Figure 18 C).

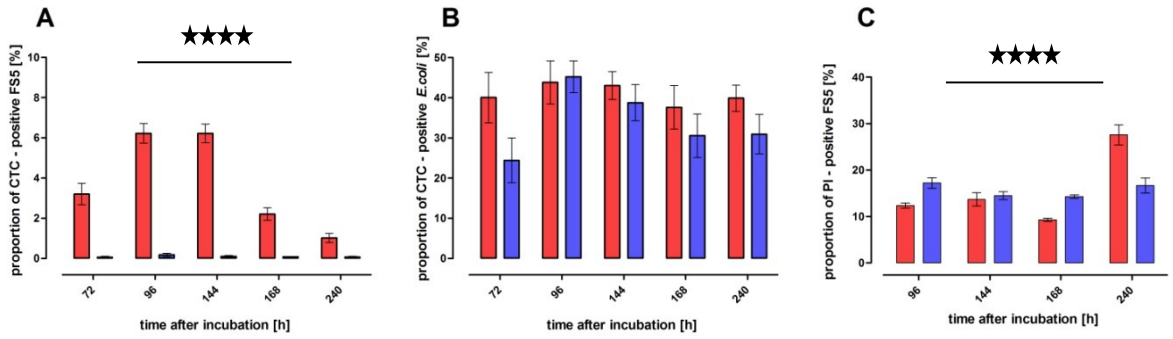

Fig. 18 FS5 & *E. coli* tolC– display respiratory activity in host–free incubation over time. (A) Percentage of CTC–positive FS5 cells [%] at single time points. **(B)** Number of CTC–positive *E. coli* tolC– cells [%] at single time points. **(C)** Number of PI–positive FS5 cells at single time points. **Red:** + DNA. **Blue:** – DNA. All bars indicate average value of eight biological replicates. Error bars show SEM. Asterisks mark significant differences between incubations +/- DNA.

The number of PI–positive FS5 cells in the presence or absence of DNA was significantly different ($P < 0.001$) at all incubations except after an incubation period of 144 hours (Fig. 18 C). At 96h and 168h of host – free incubation, fractions fed with DNA contained less numbers of dead FS5 cells. At 96h, 12.3% of all observed FS5 cells supplied with DNA showed a

positive PI signal, whereas 17.2% of the control samples were dead. Both incubations exhibited less numbers of dead cells after 168h of host–free incubation (9.3% respectively 14.2%). Samples incubated for 144 hours host–free in the presence of amoebal DNA contained about 13.6 % dead FS5 cells. Controls kept host–free in the absence of DNA did show 14.5 % dead cells after 144 hours. In the presence of DNA however, an increase of dead FS5 particles was detected between 168h and 240h of incubation. Apparently, 28% of all observed FS5 cells did as well exhibit a PI–positive signal besides the DAPI signal.

In order to examine the impact of these possible cross–feeding effects originating from residual *E. coli* tolC– cells or other currently unknown factors inside, an additional experiment had been performed. The number of CTC–positive FS5 and *E. coli* tolC– cells after 96 hours of host–free incubation varied among tested conditions (Fig. 19). FS5 had been purified from host amoebae *Hartmannella* supernatant, which had been supplied with live *E. coli* tolC– as a food source. Another fraction of FS5 was purified from host amoebae had been fed with heat–inactivated *E. coli* tolC– instead of live bacteria prior to the experiment. Additionally, the impact of ampicillin on both *E. coli* tolC– and FS5 activity was evaluated.

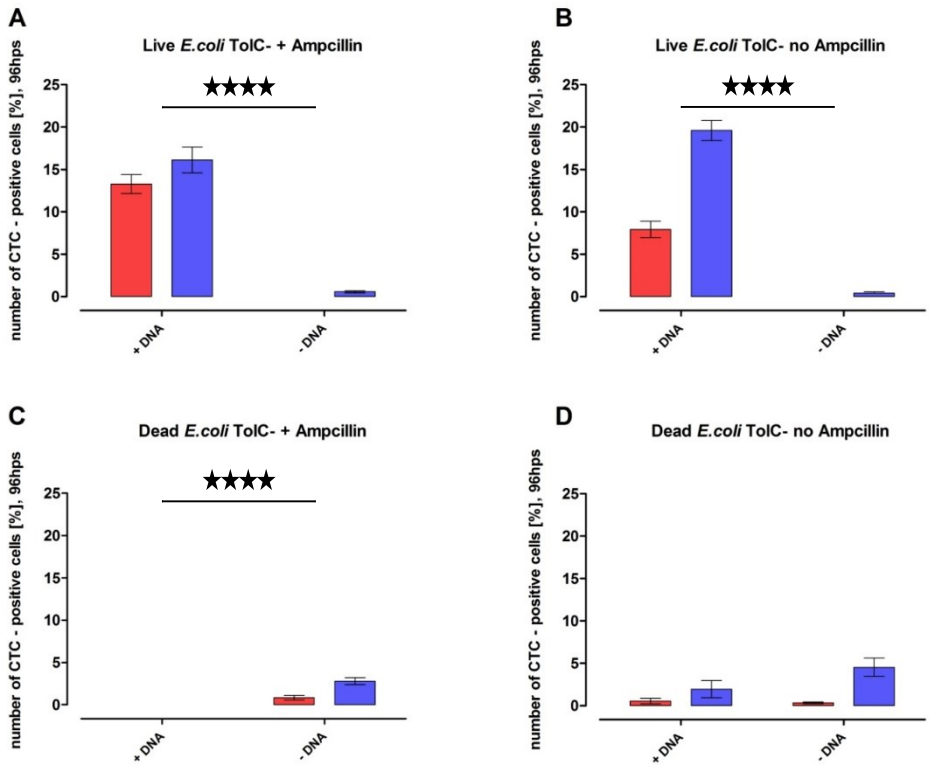

Fig. 19 FS5 & *E. coli* tolC– host–free respiratory activity in the presence of amoebal DNA & ampicillin, 96hps **(A–D)** Number of CTC–positive cells [%] after 96h host–free incubation. **Red:** FS5. **Blue: *E. coli*** tolC–**. (A)** Host–free incubation +/– DNA + ampicillin. **(B)** Host–free incubation +/– DNA. **(C)** Host–free incubation +/– DNA + ampicillin. Host amoebae fed with heat–inactivated *E. coli* tolC–. **(D)** Host–free incubation +/– DNA, heat–inactivated *E. coli* tolC–. All bars resemble average values of 10 replicates. Error bars show standard deviation. Asterisks mark significant differences compared between samples +/- DNA.

After 96h of host–free incubation, a significant difference ($P < 0.0001$) of FS5 cellular activity between fractions supplied with and without DNA in the presence of ampicillin was detected (Fig. 19 A). Differences between samples supplied with or incubated without DNA were as well significant, if ampicillin had not been added ($P < 0.0001$). FS5 fractions originating from amoebae cultures fed with live *E. coli* tolC– contained about 13.3% of active FS5 cells in the presence of host DNA and ampicillin. In the absence of ampicillin, samples supplied with DNA showed 7.9% active cells; a difference found to be significant ($P < 0.01$). No positive signals were found in the absence of DNA for neither samples treated with ampicillin nor unaltered samples. Residual *E. coli* tolC– within FS5 fractions did not emit red fluorescent light in amounts comparable with previous experiments, if incubations had not been supplied with host DNA. However, fresh *E. coli* tolC–, which had been used as a positive control did contain almost 100% of CTC–positive cells. Heat–inactivating *E. coli* tolC– for 30 minutes did result in entirely inactive cells showing no signals. Only double–stained (DAPI + CTC) cells were taken into account. Furthermore, we could also see a significant ($P < 0.05$) difference between fractions incubated with ampicillin and without. About 7.9% of all detected FS5 cells were considered active, if not been treated with ampicillin (Fig. 19 B). No CTC–positive FS5 cell could be found, if ampicillin and amoebal DNA had been absent. Interestingly, we could discover significantly ($P < 0.05$) higher numbers of active FS5 cells in the presence of ampicillin than without, if DNA had been added. Comparing fractions supplemented with ampicillin and without, about 16.1% respectively 19.6% of all discovered *E. coli* tolC– were emitting red fluorescence light, if DNA had been added. In both the presence and absence of ampicillin, the proportion of active *E. coli* tolC– cells was significantly ($P < 0.0001$) lower in samples incubated without DNA than samples supplied with DNA. Differences in terms of presence/absence of ampicillin were not significant.

FS5 fractions originating from host amoebae fed with heat–inactivated *E. coli* tolC– were apparently almost entirely inactive. In the presence of ampicillin, fractions supplied with DNA did not contain any positive bacterial cell (Fig. 19 C). 0.8% of all observed FS5 cells showed bright red signals, if DNA had not been added indicating respiratory activity. The difference between these fractions was approved to be significant ($P < 0.01$). However, we were still able to find *E. coli* tolC– cells emitting red light suggesting, that a partial fraction of bacterial cells had survived the heat–inactivation step. No significant difference between fractions supplemented with host DNA and without in the absence of ampicillin was observed for FS5 (Fig. 19 D). FS5 remained to be active in both fractions incubated with and without

host DNA in only a very small number (0.54% respectively 0.33%, Fig. 20 D). Again, differences, which had occurred between samples incubated with and without ampicillin were not significant regardless the addition or absence of amoebal DNA. The amount of active residual *E. coli* tolC– was slightly higher in the absence of DNA and ampicillin further suggesting an incomplete heat–inactivation procedure. The total number of bacterial cells inside all fractions was considerably low, ranging from 100 to 500 cells showing a DAPI signal. In order to differentiate between FS5 and *E. coli* tolC–, phase–contrast images had also been taken. FS5 remained to be morphologically inherited during the entire experiment showing small rod–shaped cells, similar to *E. coli* tolC–, which was moderately bigger in size. We did not detect any other morphologically different cells indicating, that all fractions were free of contaminants. Additional parameters had also been evaluated like the impact of 5x DNA or single dNTPs.

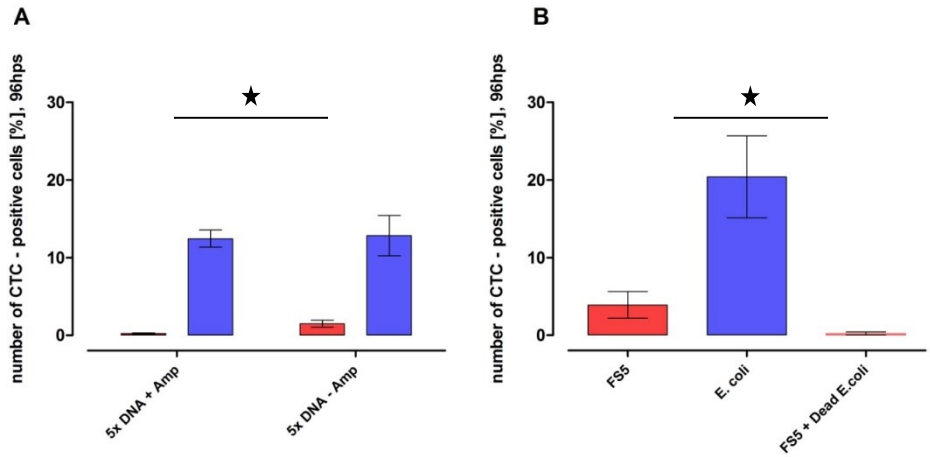

Fig. 20 Respiratory activity of FS5 purified from live *E. coli* tolC– fed amoebae incubated with dNTPs / 5x DNA **(A)** Number of CTC–positive cells after 96h host–free incubation. **Red:** FS5 + 5x DNA. **Blue:** *E. coli* + 5x DNA. **(B)** Number of CTC–positive cells in the presence of single dNTPs. All bars resemble average values of 5 replicates. Error bars show standard deviation. Asterisks indicate significant differences between incubations +/- ampicillin.

Compared with samples incubated with 1x DNA, the addition of 5x DNA to host–free incubations of FS5 did result in a significant decrease in the number of CTC–positive signals ($P < 0.0001$) in the presence of ampicillin. This difference was also observed at samples left unaltered ($P < 0.001$). The total number of active FS5 cells was very low for both fractions kept in the presence of ampicillin and without (0.22% respectively 1.48%, Fig. 20 A). However, statistical analysis revealed a significant difference between those two numbers ($P < 0.05$). Furthermore, the number of actively respiring FS5 cells was also significantly ($P < 0.05$) different between samples containing 1x DNA (Fig. 21 A, B) and samples incubated with 5x DNA or single dNTPs (Fig. 22 A, B). In comparison with control samples lacking DNA, only the addition of single dNTPs led to significantly ($P < 0.05$) elevated numbers of

active cells. 3.9% of all FS5 cells observed did exhibit a red fluorescent signal indicating the reduction of CTC and subsequently cellular respiratory activity (Fig. 20 B). We could not detect any difference, if 5x DNA had been added, compared with unaltered controls lacking amoebal DNA. Moreover, the number of CTC–positive FS5 cells was generally significantly ($P < 0.05$) lower in the presence of single dNTPs than for samples containing 1xDNA regardless the presence/absence of ampicillin. FS5 generally showed little respiratory activity, if incubated with either 5x DNA or single nucleotides.

FS5 purified from *Hartmannella* sp. fed with heat–inactivated *E. coli* tolC– remained entirely inactive, if samples had been incubated with 5xDNA or single dNTPs regardless the presence/absence of ampicillin.

Unlike FS5, *E. coli* tolC– did not exhibit significant differences in the number of positive cells between samples treated with ampicillin and controls (12.43% respectively 12.83%) , if 5x DNA had been added (Fig. 20 A). We also evaluated the differences, which occurred between fractions incubated with different amounts of DNA as well as single dNTPs in the presence and absence of ampicillin. Adding 5x DNA or single dNTPs did not result in significantly elevated numbers of respiratory active *E. coli* tolC– cells compared with samples supplemented with 1x DNA. Moreover, the difference between fractions aided with 5x DNA and samples supplied with single dNTPs were of no significance in terms of respiratory activity. However, we could observe increased numbers of CTC–positive cells comparing control samples with these fractions; a difference, which was considered to be significant ($P < 0.05$).

Furthermore, ampicillin did not inhibit *E. coli* tolC–‘s utilization of amoebae DNA. Moreover, adding ampicillin had no effect on the proportion of active FS5 cells as well. Supplying host–free fractions with single dNTPs however, could not increase the number of CTC–positive *E. coli* tolC– cells (Fig. 20 B). More importantly, FS5 also showed activity if supplied with single dNTPs leading to significantly lower rates ($P < 0.05$) compared with 1x DNA. No controls carried out with heat–inactivated *E. coli* tolC– showed any positive *E. coli* tolC– cell. No further time points were evaluated.

To analyze possible cross–feeding effects, the supernatant of both live and heat–inactivated *E. coli* tolC– supplemented with and without amoeba host DNA for 48h had been added to freshly purified FS5. Additionally, we again used 1xPAS supplemented with and without amoebae DNA and added both live and dead *E. coli* tolC– in order to repeat the previous

experiments. However, we could not detect any CTC–positive cell in the supernatant of pre–incubated dead *E. coli* tolC– in the presence of DNA. Moreover, the fractions incubated in 1xPAS supplemented with DNA and dead *E. coli* tolC– did as well not contain any positive cells. Single randomly distributed signals within some fractions did not appear to be significant due to a relatively high number of plotted cells. FS5 incubated together with living *E. coli* tolC– however, did contain cells showing respiratory activity, if supplemented with DNA. The number of positive cells however, was considerably low compared with previous experiments. In the absence of an additional food source, only single positive cells without any significance could be found. The supernatant of pre–incubated *E. coli s*upplied with amoebae DNA did apparently not affect FS5 host–free activity for that no red fluorescent cells were observed in both fractions with and without DNA.

Discussion

4. DISCUSSION

4.1 THE INFECTION CYCLE OF UWC36 IN A. CASTELLANII NEFF

2.2.26 PREFACE ABOUT THE EXPERIMENTAL SETUP

UWC36 was regularly purified from infected host *Acanthamoeba* spp. ordinarily long–term incubated at 20°C through filtering the supernatant. Consequently, purification of already UWC36–harboring amoebae was prevented and purified UWC36 fractions were considered pure. However, about the general levels of bacterial fitness and infectiousness of host–free symbionts we can only speculate. To avoid possible infection biases regarding a decline of infectiousness of freshly purified UWC36 fractions over time, we immediately applied bacteria on host amoebae. Moreover, to facilitate immediate contact of the symbiont with amoebae upon infection we performed a centrifugation step. This is a commonly used technique and well–established in our lab for analysis of developmental cycles of obligate intracellular bacteria (König 2009; Schulz 2011; Tsao 2011). Alternative approaches include complete lysis of host organisms in order to obtain intra–and extracellular bacteria thereby enabling comparisons between different fractions of differentially purified bacteria regarding bacterial fitness and infectiousness. Various reports have suggested differential impact on the initial proportion of infected host cells, if intra–or extracellular endosymbionts had been applied, most notably the *Chlamydia* spp. with their biphasic life style (Abdelrahman & Belland, 2005; Greub et al, 2003; Horn et al., 2000). However, we did not follow this up further in this study.

Most UWC36 cells found within amoebae exhibited only low FISH signal intensities or did not display any detectable signal at time points earlier than 24hpi. It has been proposed that the general level of cellular activity often correlates well with the amount of ribosomes actively expressed (Wagner et al, 2003). Assuming low bacterial activity and reduced numbers of ribosomes upon uptake through phagocytosis during early stages of the developmental cycle, these time points in the infection cycle remain elusive and inaccessible for FISH analysis. Multiple obligate intracellular bacteria exhibit a metabolically inert infectious form, e.g. members of the order of *Chlamydiales* (Abdelrahman & Belland, 2005; Poppert et al., 2002) and the genus *Legionella* (Garduño et al. 2002). For example, chlamydial elementary bodies are metabolically inert and remain undetectable by conventional FISH applications, as it has widely been shown in our lab (König 2009; Schulz 2011; Tsao 2011). To overcome these technical challenges, we combined FISH with a DAPI stain. DAPI directly interacts with DNA by intercalating into it independent of

cellular/metabolic activity thereby leading to strong and easily detectable signals from both bacterial cells and eukaryotic nuclei/mitochondria. After 24hpi, both the UWC36–specific probe AcRic90 and the general eubacterial probe EUB338mix could efficiently target the symbiont showing high signal intensities and thereby indicating high bacterial metabolic activity. We therefore conclude that all detected bacterial cells were of UWC36 origin and the culture was considered pure. During the entire course of the experiment, FISH combined with DAPI has proven to be efficient in visualization of the UWC36 developmental cycle and thereby delivering substantial information about the infection process (Fig. 7).

2.2.27 The UWC36 developmental cycle in *A. castellanii* Neff

The main aim of this experiment was to determine the specific modalities of host–endosymbiont interaction as well as to decipher key elements of Endosymbiont of *Acanthamoeba* UWC36 (Fritsche et al., 1999) developmental cycle inside *Acanthamoeba castellanii* Neff, a well characterized host (Clarke et al., 2013; Neff, 1957; Neff, 1958). Moreover, we were interested how different incubation patterns like varying temperatures would affect host fitness and viability as well as progress of UWC36 infection. To get deeper insights into host–endosymbiont interactions, it is crucial to understand specific modalities of the infection process. For instance, in the last decade a variety of reports have suggested, that key features of intracellular developmental cycles are influenced by the incubation temperature (Greub et al. 2003). For example *Parachlamydia acanthamoeba* UV7 has been shown to facilitate host cell lysis at incubation temperatures above 30°C, but remains endosymbiotic for its *Acanthamoeba* spp. host at 25–30°C (Greub et al., 2003). Temperature does play an essential role in the development of host–symbiont interactions (Fels & Kaltz, 2006; Ohno et al, 2008; Scola et al, 2004) and greatly influences the process of transmission and even modulates virulence of parasites (Restif & Kaltz 2006). Bacterial endosymbionts living in a mutual/symbiotic relationship might turn into parasites exploiting host resources and ultimately leading to host cell lysis, if incubated under elevated temperatures. During the course of our study, we tested whether a higher temperature of 30°C would induce a switch from symbiotic to parasitic behavior of UWC36 in *A. castellanii* Neff or not.

2.2.28 UWC36 progress of infection over time

Upon entering the host cell, UWC36 was clearly able to thrive and multiply within its host cytoplasm (Fig. 24). Equal to observations made during the preliminary test infection experiment, UWC36 readily established presence in amoebae 2hpi (Fig. 7 A, B). Avoiding multiple infection events at single host cells is critical in order to ensure synchrony of infection and to decipher key elements of the UWC36 developmental cycle. Application of a

MOI of 100 resulted in 20–30% infected amoebae 24hpi; a proportion sufficient for subsequent bacterial intracellular multiplication and spread among host cells (Fig. 4). Consequently, we decided to initialize the UWC36 infection cycle with a MOI of 100. Notably, amoebae incubated at 20°C exhibited a significantly higher proportion of infected host cells than at 30°C ($P < 0.05$). The fact that continuous cultures of UWC36 are maintained at 20°C in our lab suggests adaptation to a lower incubation temperature over time. UWC36 was isolated from human corneal epithelium (Fritsche et al, 1999); a habitat featuring a temperature of usually 37°C. Consequently, UWC36 does seem to possess the capability of withstanding higher temperatures and maintains its infectious potential, but does take some time to acclimatize. Conversely, differential incubation parameters rather affect the host organism influencing host cell survival and fitness than influencing obligate intracellular bacteria.

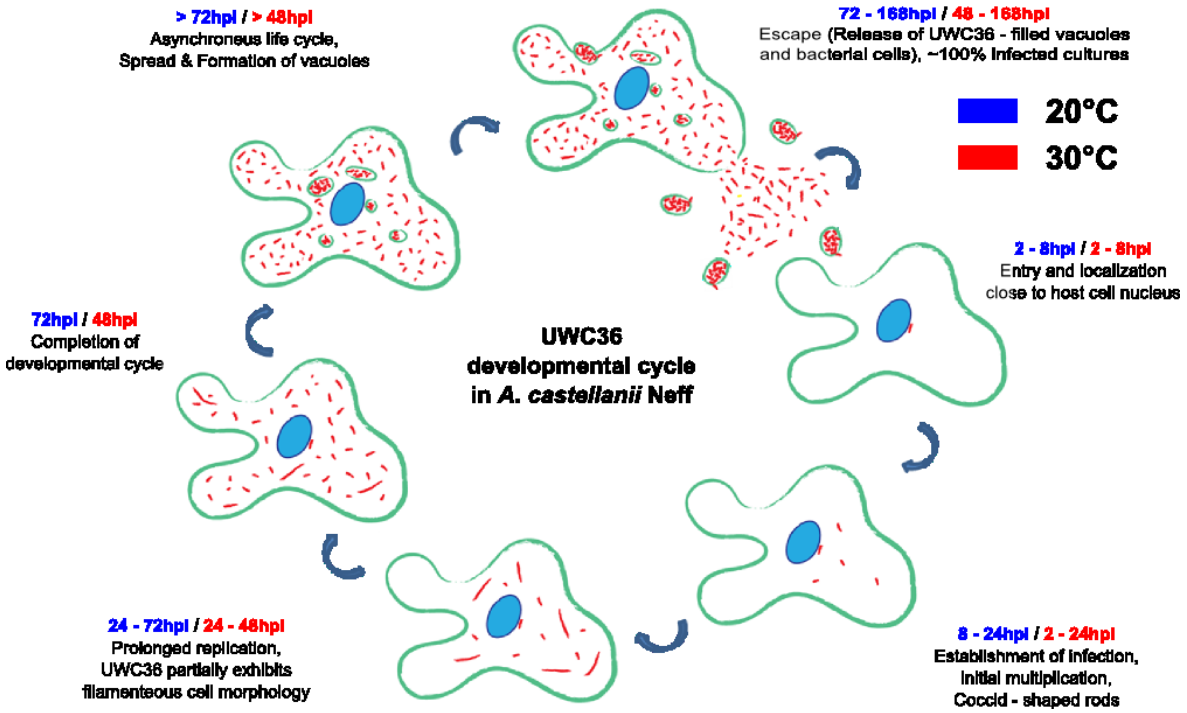

Fig. 21 UWC36 Developmental cycle in *A. castellanii* Neff. UWC36 shows accelerated infection cycle at higher incubation temperatures. Expulsion of vacuoles filled with highly active bacterial cells >48hpi/72hpi. **Red:** Cycle at 20°C. **Blue:** Cycle at 30°C.

Interestingly, host cells were almost exclusively infected by a single bacterial cell located in the cytoplasm in close proximity to the host cell nucleus (Fig. 7 A, B). Between 8hpi and 24hpi, variations in the proportion of UWC36–harboring amoebae remained insignificant between 20°C and 30°C implying equally continuing development and similar growth patterns of UWC36 (Fig. 8 A, B). Once the initial phase of infection and intracellular establishment had been passed, differences between both incubation temperatures concerning

the infection level became prominent. A doubling of infected individuals between 24hpi, 48hpi and 72hpi was only observed for amoebae incubated at 30°C; an observation that directly pointed at accelerated intracellular growth and replication under an elevated temperature (Fig. 8 B). Accompanied by the emergence of highly infected amoebae 48hpi, the bacterial titer in the surrounding media dramatically increased indicating a completion of the developmental cycle. This conclusion was further supported by three–fold increase of freshly infected amoebae between 24hpi and 48hpi at 30°C as well as a general infection level of 64.2% 72hpi (Fig. 5). Within the same period, infection levels of host cells kept under room temperature (20°C) were only slightly rising. Differences between both incubation temperatures were significant between 72hpi and 120hpi ($P < 0.0001$); a doubling of infected host cells at 20°C was only observed after 72hpi leading to a general proportion of 49% infected amoebae 96hpi (Fig. 8A, 5). Evidently, UWC36 does exhibit an accelerated growth pattern at 30°C and transmission seemingly occurs at a higher level than at 20°C. Additionally, host cell division does become accelerated at 30°C thereby leading to a faster spread of UWC36 among amoebae through vertical transmission. The generation time of diverse *Acanthamoeba* species/isolates usually differs from 8h to 24h (Khan 2006); an observation we could also make during the course of our experiments. Quantitative PCR (qPCR) targeting bacterial as well as amoebal housekeeping genes would contribute in deciphering the precise progress of infection and determining bacterial replication rates within an amoebal host over the course of an infection cycle at different incubation temperatures (Brennan et al. 2003).

The developmental cycle was clearly shifted between both temperatures accompanied by a generally higher level of infection and a decrease of freshly infected amoebae after 48hpi at 30°C. The transition between freshly, intermediately and highly infected amoebae exhibited a clear delay at the lower temperature. Temperature often directly influences the process of transmission for example through modulation of bacterial life span or exposure to host (Mitchell et al, 2005). *Holospora undulata* exhibits accelerated production of infectious forms, but limited transmission within a *Paramecium caudatum* culture incubated at 23–30°C (Fels & Kaltz 2006). Moreover, alterations in bacterial virulence over time as well as a biphasic life style consisting of infectious, metabolically inert and reproductive, metabolically active forms are often related to varying environmental conditions especially temperature (Restif & Kaltz 2006). Bacterial cells can be transmitted through vertical–and horizontal transmission within a host cell culture; an observation we also made for UWC36 thriving in *A. castellanii* Neff at both incubation temperatures. Reports have proposed a tradeoff between

horizontal and vertical transmission, which is directly related to appearance of infectious respectively reproductive forms (Restif & Kaltz 2006). Here, the efficacy of vertical transmission is reduced by production of infectious forms actively exploiting the host and presumably preparing for escape and subsequent horizontal transfer.

2.2.29 MORPHOLOGICAL CHARACTERISTICS OF UWC36

Our results showed that UWC36 changes its morphology during the infection cycle from small cocci over coccid–rods to long filamentous rods and back to cocci. Different morphologies would be a hint for variations in cell metabolic activity and different bacterial growth stages throughout the infection cycle. UWC36 thereby showed a typical Gram–negative cell membrane and was performing binary fission; a kind of cell replication commonly found among bacteria. The detection of longer filamentous–shaped bacteria during early stages of the developmental cycle invoked several questions (Fig. 7 I, J / Fig. 23). We only observed these filaments in a subset of UWC3–harboring amoebae. Through TEM, we were not able to detect any of these filaments at all examined time points. A clear distinction between longer filaments and coccid–rods could not be performed unambiguously. Consequently, these filaments are currently considered to originate from UWC36 replicating through ongoing binary fission and may not be distinctive morphological forms.

Upon localization of UWC36 within host–derived vacuoles from 72hpi on, we could observe UWC36–containing vacuoles both in–and outside amoebae (Fig. 7 H, L). Obligate intracellular bacteria such as UWC36 facilitate exit from vacuoles and subsequent release into the surrounding media through diverse ways. For example, for *Chlamydia spp*. two mutually exclusive pathways of cellular escape have been identified (Hybiske & Stephens 2007). Here, *Chlamydia spp*. either facilitate their exclusion through modulation of Ca^{2+} – dependent host cell lysis or host cytoskeleton–mediated extrusion within packages surrounded by two host–derived membranes. While host cell lysis naturally killed the host, exclusion left the host alive. Various reports could show that lysis of host cells is a general phenomenon within *Chlamydia spp*. (Campbell et al, 1989; Neeper et al, 1990; Rockey et al, 1996). However, UWC36 does not induce extensive host cell lysis upon infection as indicated by our FISH analysis. We therefore assume that infected amoebae actively expel vacuoles filled with UWC36 triggered by currently unknown factors.

Application of universal eukaryotic FISH–probe EUK516–Cy5 did not stain these vacuoles (Fig. 7 L). In highly infected amoebae usually multiple vacuoles were present, indicating replication and accumulation of bacteria in these compartments. Additionally, these

compartments were often found in close proximity to already entirely infected *A. castellanii* Neff cells. The detection of these different sized vacuoles filled with different titers of bacteria raised the question, if these vacuoles would subsequently merge and form large vacuoles as partly indicated by transmission–electron microscopy (TEM). However, the exact nature of these vacuoles however has to be elicited through further TEM analysis. A preliminary TEM analysis already gave us insight into the modalities of UWC36 maintenance within host cells. Beside vacuoles roughly packed with various UWC36 cells, we could also monitor vesicles containing only single bacterial cells (Fig. 5). Moreover, UWC36 was also performing binary fission if located inside these vacuoles as shown by TEM. TEM has proven to be a suitable tool for evaluating the specific modalities of life cycles from a variety of obligate intracellular bacteria (Greub & Raoult, 2002; Kahane et al, 2002).

After being taken up by host–mediated phagocytosis, the predominant mode of entry in for bacteria invading amoebae (Barker & Brown, 1993; Khan, 2006; Weekers et al, 1993), UWC36 has to protect itself from host–defense mechanisms. Reports have shown that symbionts either modulate host cell processes to prevent phagosome–lysosome fusion, escape localization in lysosomes or they are capable of surviving acidification and thriving in the phago–lysosome. (Isberg et al, 2009). One way to study the uptake mechanism of the symbiont is visualization of lysosomes by staining them with the acidic dye LysoTracker®, as it has been performed for *P. acanthamoeba* (Greub et al. 2005). Additionally, chemical compounds like calcein or dextran enable differentiation between digestive phago–lysosomes and non–digestive vacuoles (Olofsson et al. 2013). Intriguingly, a dynamic interplay between residence in the host cytoplasm and successful re–entry into the host endocytic pathway has only recently been demonstrated for *Francisella tularensis* (Checroun et al, 2006), which may promote bacterial egress from host cells.

2.2.30 UWC36 INFLUENCE ON HOST FITNESS AND SURVIVAL

Acanthamoeba generally display replication rates between 8–24 hours under optimal growth conditions depending on the species/genotype (Khan 2006). Moreover, bacteria residing in *Acanthamoeba* species are actively transmitted through binary fission from mother to daughter cells (Fig. 7 K). Our data shows that UWC36 does not adversely affect host cell replication regardless the incubation temperature, at least during early and intermediate stages of infection (Fig. 8 C, D). However, reports have shown, that different endosymbionts differentially affect host cell growth of diverse eukaryotic hosts (Collingro et al. 2004). A delay of replication upon infection within an amoebal culture could lead to empty amoebae multiplying faster than UWC36–bearing hosts ultimately resulting in a dilution of the

infection. However, the progress of infection did not alter host cell growth and replication at both incubations temperatures until 96hpi rendering a "dilution of infection" effect obsolete. Statistical analysis revealed significant differences ($P < 0.05$) between empty controls and infected amoebae for both temperatures from 96hpi on. Accelerated replication and growth induced by a higher incubation temperature followed by enhanced nutrient utilization and turnover–rates ultimately leads to a faster depletion of substrates. We observed a slow decline of infected amoebae from 120hpi on, if incubated at 30°C (Fig. 8 D). Conversely, empty controls exhibited continuous growth and replication even at late time points suggesting that nutrient depletion is delayed in the absence of UWC36. Late–stage growth patterns of both UWC36–harboring and *empty A. castellanii* Neff indicated a generally decelerated amoebal propagation at 20°C regardless the presence/absence of UWC36. In full agreement with that, UWC36 thriving within host cells seemingly facilitates a delay in amoebal cell division at both incubation temperatures. UWC36–harboring amoebae incubated at 30°C faced nutrient limitation followed by depletion of substrates earlier than both empty controls (30°C) and infected amoebae held at 20°C; an observation directly associated with enhanced bacterial replication and transmission. In conclusion, temperature does directly affect host cell growth and replication leading to accelerated growth patterns at elevated levels of temperatures. Furthermore, UWC36 infecting amoebae do result in decelerated growth rates and directly contributes to ongoing depletion of substrates. Obligate intracellular bacteria often access the host metabolic capabilities to ensure propagation and therefore directly benefit from enhanced uptake of nutrients (Greub & Raoult, 2004). Subsequent draining of these nutrients e.g. nucleic and amino acids, proteins and lipids by the symbiont can be monitored by using labelled substrates. Furthermore, labelled substrates enable measurement of their depletion in the supernatant and subsequent enrichment in intracellular bacteria residing in the host. Moreover, obligate intracellular bacteria often possess nucleotide–transporters; a common feature which allows exploitation of host cell derived metabolites such as ATP (Schmitz–esser et al. 2004).

Acquiring growth rates represents only one aspect to measure the impact of obligate intracellular bacteria on host fitness. Another approach is to assess the impact of lysis on the host culture. For this purpose, application of propidium iodide, which is an intercalating agent directly interfering with DNA of dead cells, but does not pass the cytoplasmic membrane of living cells, proves to be a suitable tool. While monitoring the course of an UWC36 infection over a period of 168 hours, we did not observe host cell lysis (Fig. 8 C), suggesting that UWC36 does not adversely affect host cell fitness and survivability as it has been shown for

various other obligate intracellular bacteria (Greub et al., 2003; Ojcius et al., 1998; Osaka et al, 2008). This clearly indicates that host cells maintain their cellular activities and functions and are not precluded by UWC36 multiplying within them. Interestingly, a higher incubation temperature resulted in significantly ($P < 0.01$) elevated PI values from 72hpi on (Fig. 8 C). Though obtained PI values were clearly below a threshold of 300, host cell fitness and survivability are clearly influenced by both incubation temperature and presence of UWC36. In fully agreement with previous data obtained through host cell counting, accelerated host cell metabolism, enhanced turnover and UWC36 transmission rates subsequently followed by a faster nutrient depletion are evident. This conclusion is further supported taking observations made for empty controls into account. Although no peak in emitted fluorescence could be measured, empty amoebae were constantly displaying moderately higher PI values at 30°C. Nevertheless, the outcome of UWC36 infecting *A. castellanii* Neff in terms of host cell survivability and fitness can only be estimated during a long–term experiment with time points beyond 168hpi. Often microorganism facilitate modulation of host gene expression through secretion of effector proteins to circumvent host immune defenses, avoid detection, prevent host cell death through apoptosis and successfully establish a niche for replication and life (Greub et al., 2003; Ito et al., 2012; Lamkanfi & Dixit, 2010; Ojcius et al., 1998). Similar observations have been made for obligate intracellular pathogens belonging to the clade of *Alphaproteobacteria* (Batut et al, 2004). Some pathogens have even become vital and essential for their host through continuous host cell manipulation (Pannebakker et al, 2007). A dynamic interplay between mutualistic and parasitic interactions are common among interspecies relationships; a fact which may also be true for UWC36.

4.2 The host range of Endosymbiont of Acanthamoeba UWC36

Extending the range of possible host organisms is a key capability of obligate intracellular bacteria to ensure further spread and survival as well as conquer new ecological niches and limit bottlenecks of transmission (Horn & Wagner, 2004; Molmeret et al, 2005). *Acanthamoeba* spp. and other free–living amoebae provide a unique habitat rich in nutrients and offer protection from various environmental dangers (Greub & Raoult, 2004). Furthermore, through adaptation to intracellular life style and extension of the host range, endosymbionts may even gain access to unique traits and enhance their virulence (Alsam et al., 2006; Cirillo et al., 1997; Cirillo et al., 1999; Cirillo et al., 1994). Moreover, distinct geographic regions harbor amoebae containing similar strains of intracellular bacteria thereby pointing at global distribution (Horn & Wagner, 2004; Schmitz–Esser et al., 2008). Our results clearly indicate that UWC36 easily extends its host range among various

Acanthamoeba species, but remains incapable of invading eukaryotic hosts of higher order (Fig. 12, 13, 14/Tab. 13, 14). A media–dependent effect on UWC36 uptake by *Acanthamoeba* could also be identified. Rich media provides all nutrients essential for *Acanthamoeba* propagation and development and easily induces formation of trophozoites (Khan 2006). This renders active predation of UWC36 in order to fulfill carbon and energy requirements not essential for further survival. *Acanthamoeba* preferentially graze on gram–negative bacteria (Khan 2006). Besides using Trypticase Soy Broth with Yeast Extract (TSY), *Acanthamoeba* species were also incubated in 1xPAS lacking supplemented with *E. coli* tolC–. Adding live *E. coli* tolC– to the buffer system promotes uptake of bacteria through amoebal phago (specific, adsorptive)–and pinocytosis (non–specific, non–adsorptive) and subsequent degradation of bacteria within food vacuoles; however different bacteria induce differential behavior in free–living amoebae (Weekers et al. 1993). Incubating freshly isolated amoebae in a minimal buffer system supplemented only with live *E. coli* tolC– as sole carbon and energy source is widely and commonly used technique in our lab and provides an ideal system for studying amoebal grazing on various bacteria resistant to degradation.

2.2.31 UWC36 DISPLAYS DIFFERENTIAL HOST RANGE IF INCUBATED IN DIVERSE MEDIA

All tested *Acanthamoeba* species were infected with UWC36 24hpi, if incubated in rich media, whereas only *A. castellanii* Neff, *A. castellanii* UWC12 and *A. castellanii* C1 displayed UWC36 cells within their cytoplasm 24hpi in 1xPAS + *E. coli* tolC– (Fig. 12, 13). Various intracellular pathogens facilitate their own uptake by host cells through attaching to their cytoplasmic membranes and binding to secondary receptors, for example *C. trachomatis* (Clifton et al. 2004; Abdelrahman & Belland 2005). Moreover it has been shown that various sugars even block receptor–mediated uptake of bacterial endosymbionts in *Acanthamoeba* (Allen & Dawidowicz 1990). Coiling phagocytosis mediated by receptors is the main mechanism of bacterial uptake by *Acanthamoeba* spp.; an easy way for intracellular bacteria to enter host organisms (Greub & Raoult 2004). Since *Acanthamoeba* might preferentially take up *E. coli* tolC– for maintenance of growth and replication, UWC36 may only partly be recognized as possible food resource by *Acanthamoeba* species. Uptake of *E. coli* tolC– might directly be associated with uptake of UWC36; a process best be addressed by using micro–beads. However, early studies have also shown, that phagocytosis is a highly selective process (Wetzel & Korn 1969; Korn & Weisman 1967). An alternative approach would be the use of specific inhibitors of phagocytosis and pinocytosis such as derivatives of the fungal

Cytochalasins (B, D, F,…) and Methylamine in order to evaluate the potential of UWC36 to invade *Acanthamoeba* spp. in more detail (Cells & Shotts 1991; Bos & De Souza 2000).

In the presence of ampicillin, which had been added to avoid *E. coli* tolC– over growth, UWC36 exhibited an interesting change in morphology comprising single filamentous and enrolled cells (Fig. 14). Ampicillin acts as an irreversible inhibitor of the bacterial enzyme Trans–peptidase, which facilitates building of cell walls. Moreover, it also efficiently blocks bacterial cell division. Though the final concentration in the buffer was considered sub–inhibitory (20µg/ml), we can only speculate about possible impact on UWC36. Antibiotics disturb bacterial cell wall biogenesis and replication, which can result in longitudinal growth without subsequent cell division by binary fission. Interestingly, we observed these filaments both within amoebae and in the surrounding buffer (Fig. 14). No controls lacking ampicillin or *E. coli* tolC– had been prepared rendering a conclusion about the influence of ampicillin impossible. Filamentation of bacteria is often induced by a bacterial stress response to low doses of antibiotics, exposure to ROS or starvation (Imlay & Linn 1987; Davis et al. 1997). Moreover, prominent host cells like macrophages often impair bacterial intracellular replication by production and release of antimicrobial peptides; a defense mechanism which can result in formation of filaments with arrested septation (Rosenberger et al. 2004). Observations of filamentous morphologies have also been made for *Legionella pneumophila* in order to avoid phagocytosis or to quickly grow in biomass and proliferate in great numbers (Piao et al. 2006).

2.2.32 UWC36 DOES NOT INFECT EUKARYOTIC HOSTS OF HIGHER DEVELOPMENTAL ORDER

We did not observe an UWC36 infection in HeLa 229, mouse macrophages THP–1 and *Drosophila melanogaster* macrophage–like cell–line S2 (Tab. 14). Both HeLa 229 cells and mouse macrophages THP–1 are able to perform uptake of bacterial pathogens through receptor– mediated phagocytosis (Clerc & Sansonetti 1987; Aderem & Underhill 1999). S2 cells were derived from a late stage embryonic *Drosophila melanogaster* macrophage–like lineage (Schneider 1972) and are most likely to perform receptor–mediated endocytosis (clathrin–dependent endocytosis). Both macrophages THP–1 and HeLa 229 cells were ordinarily incubated at 37°C (5%CO_2); a temperature difference of 17°C compared with UWC36–harboring *Acanthamoeba* incubations. Similar to TSY/PYG, both DMEM (HeLa) and RPMI (macrophages) are rich media offering various metabolites, which may possibly block receptor–mediated phago– or endocytosis. Conversely, mouse macrophages THP–1 are professional phagocytes destined to remove bacterial/viral pathogens trough uptake and

digestion. S2 cells are regularly kept at 27°C in Schneider's media. One plausible explanation could be the high nutrient content, which may efficiently block receptor–mediated phagocytosis and thereby interfering with bacterial uptake by insect cells. Furthermore, eukaryotic cells of higher developmental order like mouse macrophages or S2 cells are generally characterized by well–defined and complex antimicrobial defense mechanisms, which highly contribute to the successful establishment and maintenance of bacterial infections. Furthermore, higher developed eukaryotic hosts often induce apoptosis in order to prevent continuous bacterial infection and subsequent spread (Ito et al. 2012; Ojcius et al. 1998). The modulation of host cell response through apoptosis is complex and varies with different stages of infection, induced by both the symbiont (Ashida et al. 2011) to facilitate release from host cells or the host to prevent subsequent spread of infection (Mu & Brand 1996). Even more intriguing, bacterial pathogens are capable of inhibiting host cell death through apoptosis, necrosis or pyroptosis during early stages of infection, whereas later on host cell death is actively induced by symbionts (Portillo 2004; Ashida et al. 2011).

Taking together, UWC36 is capable of extending its host range to a certain degree. UWC36 does show a certain preference for *Acanthamoeba* hosts under different conditions. Moreover, UWC36 successfully established presence and maintenance in various *Acanthamoeba* hosts (Fig. 12, 13, 14), performed multiplication inside and became readily spread among the entire cultures through both horizontal and vertical transmission. UWC36 exhibited a preference for incubation in rich media thereby invading all tested *Acanthamoeba* hosts. Conversely, *Hartmannella* sp., HeLa 229, mouse macrophages THP–1 and *Drosophila* S2 cells could not be infected.

4.3 Considerations about Paracaedibacter EI3 and its host range

Paracaedibacter EI3 was isolated from rainforest soil in the Dominican Republic and is most similar to the alphaproteobacterial *Acanthamoeba* endosymbiont *Paracaedibacter* UWC9 (Schmitz–Esser et al. 2008). FISH analysis revealed rod–shaped cells directly located inside the host cell cytoplasm, but no enclosing within capsules or vesicles (Schmitz–Esser et al. 2008); an observation we could extend during the course of our experiment. *Paracaedibacter* EI3 belongs to a novel clade of obligate intracellular bacteria affiliated with the *Rickettsiales* and *Rhodospirillales* (Schulz et al., 2014). We tried to address the question, if EI3 and UWC36, two deep–branching obligate endosymbionts affiliated to the order of the *Rickettsiales,* share common features in their infection cycle. Additionally, we also tried to

evaluate the potential of EI3 to invade host cell nuclei, since members of the proposed new clade are endonuclear bacteria (Schulz et al. 2014).

2.2.33 CURING OF EI3 NATIVE *ACANTHAMOEBA* HOSTS WITH DIFFERENTLY COMBINED ANTIBIOTICS

Experiments with different *Acanthamoeba* and eukaryotic hosts could show that EI3 has a limited *Acanthamoeba* host range compared with UWC36. Consequently, we chose to cure the native *Acanthamoeba* host of EI3; an experiment that resulted in cured amoeba cultures free from extracellular bacteria. Cured native host cells displayed ongoing cell growth and multiplication if incubated at 20°C in TSY indicated by a generally high amount of trophozoites. Using two combined antibiotics with a differential mechanism of action further increases the selective pressure on bacterial pathogens (Tab. 12). Antibiotics have widely been used in order to treat clinical infections with *Acanthamoeba* spp. harboring various intracellular bacteria (Goy & Greub, 2009; Maurin et al , 2002). We could successfully remove both intra–and extracellular bacteria by treating native infected *Acanthamoeba* with combinations of rifampicin + ampicillin/doxycycline/erythromycin for 6 weeks at room temperature (Tab. 12). Detrimental effects on host amoebae were only observed using antibiotic concentrations of 20µg/ml suggesting that amoebae were incapable of removing antibiotics by efflux pumps. Furthermore, rifampicin is regularly dissolved in DMSO; a chemical compound with cytotoxic effects on bacterial and eukaryotic cells. Removal of extracellular bacteria was completed after 4 weeks of treatment and recovery, whereas EI3–harboring *Acanthamoeba* were free of intracellular bacteria after 6 weeks. In contrast to these observations, combinations of rifampicin + phosphomycin/gentamycin/ofloxacin did not result in a reduction of extracellular bacteria or curing of infected native *Acanthamoeba*. Our results suggest that combinations of rifampicin with members of macrolides or tetracycline most efficiently lead to total removal of host–free and intracellular bacteria in an *Acanthamoeba* culture (Tab. 12).

Concluding these observations, *Paracaedibacter* EI3 does exhibit a limited host range in comparison with UWC36. Infected native *Acanthamoeba* were successfully cured and re–infected thereby verifying horizontal transmission. However, a re–infection was only observed in non–nutrient buffer 1xPAS, if EI3 had been purified from already infected native *Acanthamoeba* as well as the surrounding media. Furthermore, EI3 spread within co – cultivations of native cured/native infected *Acanthamoeba* as well as native infected *Acanthamoeba* / *A. castellanii* Neff. However, *A. castellanii* Neff remained inaccessible for

EI3 during the entire course of all experiments; a clear difference to UWC36. No extensive host cell lysis could be monitored during the course of our experiments.

2.2.34 EI3 DISPLAYS LIMITED HOST RANGE UNDER DIFFERENT INCUBATION CONDITIONS

Unlike UWC36, EI3 remained unable of acquiring various new *Acanthamoeba* host species, if incubated in rich media (Tab. 13). Media components like sugars or molecules with high–molecular weight like yeast–extracts or Tryptone effectively interfere with receptor–mediated phagocytosis of bacteria (Allen & Dawidowicz 1990). Moreover, *Acanthamoeba* might not perform phagocytosis if grown in rich media thereby supported with essential nutrients to ensure further proliferation (Khan 2006a). Phago–and pinocytosis are actively promoted by the addition of gram–negative food bacteria, the predominant prey of *Acanthamoeba* (Weekers et al. 1993; Khan 2006a). Moreover, early studies have also shown, that phagocytosis is a highly selective process (Wetzel & Korn 1969; Korn & Weisman 1967). By adding *E. coli* tolC– to *Acanthamoeba* proliferating in TSY, we tried to evaluate the impact of gram– bacteria on amoeba phagocytosis. The presence of *E. coli* tolC– in rich media could not unambiguously be established despite the addition of ampicillin. Cultures most likely became contaminated by *E. coli* tolC– regardless of the incubation temperature, whereas EI3 remained unable of establishing presence within *Acanthamoeba* species. In full agreement with this conclusion, empty controls incubated in TSY and supplemented with *E. coli* tolC– + ampicillin were also affected by extensive contamination. Strikingly, even before *E. coli* tolC– had started to multiply massively, only a very small proportion of host amoebae containing *E. coli* tolC– had been detected indicating that *Acanthamoeba* rather feed on media components than actively predate on bacteria. To further address the question, how phagocytosis might be influenced by the presence of gram– bacteria, the application of inhibitors of phagocytosis (Cells & Shotts 1991; Bos & De Souza 2000) as well as artificial latex beads (Wetzel & Korn 1969; Korn & Weisman 1967) might prove to be useful.

Varying incubation temperatures did not alter EI3's capability to infect tested *Acanthamoeba* species suggesting a rather narrow host range (Tab. 13). EI3 was originally isolated from rainforest soil in the Dominican Republic raising the question if temperatures >30°C confer entry into *Acanthamoeba* hosts (Schmitz–Esser et al. 2008). Furthermore, incubation temperatures above 30°C rather induced accelerated host cell metabolism and rapid turnover of nutrients leading to a faster depletion of substrates within media. *Acanthamoeba* are likely to perform encystment at higher incubation temperatures (Greub & Raoult, 2004). Despite phylogenetic relationship with *Nucleicultrix amoebiphila* FS5 (Schulz et al. 2014), EI3 did

not infect host cell nuclei of its native *Acanthamoeba* host. Additionally, we never observed any bacterial cell located in the nucleus of EI3 native *Acanthamoeba* host. However, a final verification of EI3's exclusive intra–cytoplasmic presence can only be accomplished through transmission–electron microscopy (TEM).

The successful re–infection of its native *Acanthamoeba* host clearly shows that EI3 is capable of performing horizontal transmission beside vertical transfer (Fig. 10). Our results also indicate that host–free and intracellular EI3 populations differ in levels of infectiousness. Our results suggest that EI3 released into the supernatant in our continuous cultures faces a quick and severe loss of infectiousness. Through harvesting both host–free and intracellular EI3, we successfully circumvented this problem and finally obtained EI3 able to infect *Acanthamoeba* species.

Equal to observations made for UWC36, filamentation of bacterial cells was also detected for EI3 residing both in–and outside of host cells (Fig. 10, 11). Moreover, these filamentous bacteria were only observed in cultures incubated in 1xPAS supplemented with *E. coli* tolC– and ampicillin. As already stated above, the formation of filaments can be a stress response to low doses of antibiotics, exposure to ROS or starvation (Imlay & Linn 1987; Davis et al. 1997). Filamentation combined with arrested septation upon exposure to antimicrobial peptides produced and released by macrophages has also recently been demonstrated (Rosenberger et al. 2004). Furthermore, other intracellular bacteria like *L. pneumophila* induce a switch towards filamentous growth to quickly increase their biomass, avoid phagocytosis and to ensure proliferation in great numbers (Piao et al. 2006). FISH signals obtained from both rod – shaped and filamentous bacteria were bright indicating successful binding of both specific and general bacterial FISH–probes.

Overall, EI3 was capable of extending its host range as shown for *A. castellanii* C1, *A. castellanii* UWC12 and *A. polyphaga* DOME, only if host cells had been incubated in 1xPAS supplemented with *E. coli* tolC–. This observation directly correlated with a differentially performed EI3 purification protocol thereby obtaining both host – free and intracellular bacterial cells from infected native *Acanthamoeba* host cultures. Notably, our results indicate a quick and severe loss of infectivity of EI3 after release into the media. Conversely, *A. castellanii* Neff remained empty despite harvesting both in–and extracellular bacteria prior to application on amoebae rendering this host inaccessible for EI3. Higher developed eukaryotic hosts for example mouse macrophages THP–1, Drosophila S2 cells and HeLa–229 cells could not be invaded by EI3 (Tab .14).

4.4 N. AMOEBIPHILA FS5 FEEDS ON PURIFIED A. CASTELLANII NEFF DNA UNDER HOST-FREE CONDITIONS

The specific localization of *N. amoebiphila* FS5 (referred as FS5), an obligate intracellular symbiont residing in the nucleus of its *Hartmannella* host (Schulz et al., 2014) invoked several questions about the utilization of host DNA and chromatin. Moreover, we were interested in evaluating the influence of host–free incubations on FS5 infectivity and extracellular stability and if purified amoebal DNA might confer prolonged maintenance of these important bacterial functions.

2.2.35 FS5 REMAINS INFECTIVE AFTER HOST–FREE INCUBATION PERIODS

Our data clearly shows that FS5 remains capable of invading its native host *Hartmannella* sp. after 96 hours respectively 168 hours of host–free incubation independent of the presence of additional nutrients (Fig. 18). Intriguingly, a significant ($P < 0.001$) decline in the level of infection over time could only be monitored in the absence of amoebal DNA suggesting that utilization of exogenous DNA does confer maintenance of infectivity under host–free conditions. In agreement with that conclusion, FS5 maintained its level of infectivity in the presence of DNA 168hpi over time leading to significantly ($P < 0.05$) higher proportions of infected amoebae compared with samples absent of DNA (Fig. 18).

Direct comparisons between replicate experiments involving FS5 were difficult. Unlike the initial host–free incubation experiment, a higher proportion of infected host cells was monitored in the replicate experiment. Previous experiments in our lab have already indicated that parameters like regular feeding of amoebae with *E. coli* tolC–, the general level of infected host cells, the titer of host–free FS5 in the surrounding media and the incubation temperature directly contribute to FS5's level of infectivity. Moreover, transmission of FS5 occurs both vertically and horizontally and FS5 does not adversely affect fitness and survivability of its native host *Hartmannella* sp. (Schulz et al., 2014). The appearance of freshly infected host cells raised the question, if FS5 may be distributed unequally among daughter cells or rather be a result of ongoing infection. The buffer containing FS5 was not removed after the initial infection events had taken place thereby granting ongoing uptake of bacteria by *Hartmannella* sp. before host cells were harvested 24hpi. However, previous growth experiments could clearly show that FS5 does not induce altered host cell division rates in infected amoebae excluding a possible "dilution of infection" effect. Consequently, differences in the proportion of infected hosts are directly linked to the presence of utilizable nutrients in a host–free environment. Furthermore, FS5 does stay infectious over longer

periods of time after readily being taken up by amoebal phagocytosis (Barker & Brown, 1993; Khan, 2006; Weekers et al, 1993).

2.2.36 FS5 KEEPS UP RESPIRATORY ACTIVITY IN THE PRESENCE OF EXOGENOUS DNA

Obligate intracellular bacteria localized in host cell nuclei such as FS5 might directly utilize host DNA and chromatin. We therefore chose to aid host–free incubations of FS5 with purified amoebal DNA obtained from *A. castellanii* Neff. Dissolved DNA (dDNA) as the sole source for carbon, nitrogen and phosphor can shape microbial communities (Lennon 2007). Moreover, dDNA can rapidly been turned over by microbial communities in order to fulfill their nutritional and energy requirements (Jorgensen et al., 1993). We could clearly identify a direct correlation between presence of additional nutrients such as purified amoebal DNA and prolongation/maintenance of cellular respiratory activity. Strikingly, the FS5 supplied with DNA displayed a 28–fold higher number of active cells than incubations lacking DNA 168 hours post incubation. Observations made for earlier time points revealed a 37–fold respectively 62–fold increase of active bacteria in the presence of DNA verifying a beneficial effect of purified DNA on FS5 metabolic activity in a host–free environment (Fig. 20). Our results indicate that both FS5 and *E. coli* tolC– utilize exogenous DNA in a host–free and non–nutrient habitat. In contrast to FS5, *E. coli* tolC– maintains cellular activity despite the absence of a defined carbon, nitrogen or phosphor source thereby comprising the variable metabolic capabilities of free–living bacteria compared with obligate intracellular microorganisms facing metabolic limitations. Moreover, *E. coli* tolC– might as well necrotrophically feed on dead FS5 cells as well as utilize residual amoebal debris originating from FS5 purification process. Necrotrophic growth of bacteria has also been reported for the facultative intracellular parasite *L. pneumophila* (Temmerman et al. 2006) thereby granting prolonged environmental persistence.

2.2.37 FS5 SHOWS LITTLE RESPIRATORY ACTIVITY IN THE PRESENCE OF 5X DNA / SINGLE DNTPS

According to our results, the addition of 5x DNA or single nucleotides to fractions of FS5 did confer prolonged maintenance of respiratory activity in a host–free environment. However, in comparison with fractions supplemented with 1x DNA, levels of respiratory activity were significantly ($P < 0.001$; $P < 0.05$) lower in samples incubated with 5x DNA or single nucleotides thereby displaying a 28–fold respectively 3.4–fold difference (Fig. 21/22 A, B). Conversely, residual *E. coli* tolC– maintained its cellular activity thereby showing no significant differences between fractions incubated with 1x DNA, 5x DNA and single dNTPs.

Although positive CTC–signals were scarce among FS5 cells supplemented with 5x DNA or single dNTPs, the presence of nutrients does correlate with maintenance of respiratory activity for both FS5 and *E. coli* tolC–. A plausible explanation for this behavior would be that both FS5 and *E. coli* tolC– feed on residual amoebal cell debris originating from the purification protocol; a characteristic which has already been demonstrated for *L. pneumophila* (Temmerman et al. 2006). *E. coli* tolC– may as well possess the ability of growing necrotrophically for instance on dead FS5 cells. However, samples lacking additional nutrients such as purified amoebal DNA or single nucleotides did not contain any CTC–positive bacterial cells. Assuming equal numbers of bacterial cells in all replicate fractions, sustained cellular activity over time may rather derive from inert metabolic capabilities of *E. coli* tolC– and FS5 than being associated with the presence of amoebal cell debris. Previous assays have already shown, that incubation in a non–respectively low nutrient, host–free environment does not adversely affect survivability of FS5 (Fig. 20 C) or *E. coli* tolC– (data not shown) after 168 hours. Consequently, necrotrophic growth on debris of bacteria and amoebae matter might not be the prime cause for sustained cellular activity of both FS5 and *E .coli* tolC–. Notably, FS5 directly benefited from adding single nucleotides as we could measure significantly ($P < 0.05$) higher numbers of active cells than in control samples; an observation we did not make for samples treated with 5x DNA. To this effect, uptake of exogenous DNA is versatile among bacteria. Microbial communities in marine habitats gain access to dissolve DNA trough binding it to particulate matter by a combination of specific DNA–binding mechanisms and biotic adsorption. Moreover, some bacteria are capable of directly taking up dDNA and therefore considered to be naturally competent (Lennon 2007). How and to which degree obligate endonuclear bacteria facilitate uptake and subsequent utilization of DNA is currently not well understood; an issue best be addressed by using DNA labelled with 5–ethynyl–2'–deoxyuridine (EdU), an alkyne–tagged cell proliferation probe (Li et al. 2010). Visualization of uptake of DNA and subsequent conversion conferring prolonged proliferation in a host–free environment can further be monitored by Raman microscopy (Yamakoshi et al. 2011).

Strikingly, maintained respiratory activity could only be monitored in the presence of live *E. coli* tolC–; an observation indicating a beneficial trait enabling maintenance of cellular activity somehow conferred by live *E. coli* tolC–. Secretion of extracellular and cell–associated DNA nucleases facilitates DNA degradation; a process followed by the subsequent uptake and consumption of hydrolyzed products. Re–synthesis of single nucleotides and nucleosides into nucleic acids upon transport into the cell is a common feature of bacteria

(Paul et al., 1987; Paul et al., 1988; Pinchuk et al., 2008). Analyzing the genome of FS5 has revealed the presence of three exclusive nucleotide transporter located in the cytoplasmic membrane (Schulz, unpublished data); an observation, which suggests that FS5 is capable of taking up nutrients from the host. Conclusively, the addition of DNA alone could not ensure maintained cellular activity; yet the presence of *E. coli* tolC– actively growing on these nutrients is seemingly linked to FS5's capability to utilize these substrates (Fig. 21 A, B). Secretory DNA nucleases are one explanation for these observations and their functionality often shows dependency on presence of bivalent cations such as Ca^{2+} (Patil et al., 2005; Rangarajan & Shankar, 1999). Adding different salts like $CaCl_2$ or $MnCl_2$ to fractions of pure *E. coli* tolC–, respectively pure FS5 as well as mixtures of FS5 & *E. coli* tolC– may possibly lead to a boost in metabolic activity of both bacteria. Additionally, chemical agents such as EDTA, Aurintricarboxylic acid (ATA) (Walther et al., 2005), Actin or SDS are well known for their ability to inhibit DNA nucleases. By adding these compounds to our system, DNA hydrolysis and subsequent uptake of hydrolyzed products could effectively be inhibited thereby leading to complete loss of CTC–signals. Moreover, FS5 might not only feed on exogenous DNA, but more likely on various host–derived RNAs, for instance mRNA or rRNA present in the host cell nucleus during transcription. In contrast to DNA, ribonucleic acids are single–stranded molecules containing ribose, which lacks an additional hydroxyl group at the second position. These characteristics are responsible for a lower chemical stability of RNA compared to double–stranded DNA. Since eukaryotic nuclei are rich in ribonucleic acids depending on the host cell's condition, host–free incubations in the presence of various ribonucleic acids and single nucleotides might therefore illuminate the consequences of such an intra–nuclear life style on both the symbionts and host's metabolism.

CTC has widely been applied to various systems (Araya et al., 2003; Gruden et al., 2003; Schaule et al., 1993; Winding et al., 1994), studies have suggested that only the most active bacteria become visualized (Nielsen et al., 2003). Moreover, CTC has efficiently been coupled with FISH or micro–autoradiography in order to gain additional insights about respiratory activity, ribosomal content and utilization/turnover of radioactively labeled substrates (Créach et al., 2003). In contrast to these observations, performing FISH upon CTC staining led to a complete loss of all CTC–signals and low FISH signal intensities for all our samples. Notably, CTC acts as an alternative terminal electron acceptor thereby replacing O_2 and is considered toxic for bacteria over longer periods. Additionally, CTC accumulates within cells in the proximity of the cytoplasmic membrane over time leading to unpredictable side effects on host cell activity and viability. We could not detect any positive signal for both

FS5 and *E. coli* tolC– after 2 and 4 hours of incubation. Controls featuring pure *E. coli* tolC– fractions did not emit red fluorescence suggesting that the incubation was too short for CTC to efficiently trace P450 and become reduced by the cellular machinery. Only after extending the incubation time to 24 hours, active bacterial cells were visualized exhibiting positive CTC–signals. A simultaneous visualization of both active and dead cells through simultaneous application of CTC and PI could not be performed due to emission of red fluorescent light of both compounds.

2.2.38 PRESENCE OF LIVE *E. COLI* TOLC– CORRELATES WITH ENHANCED RESPIRATORY ACTIVITY OF FS5

One explanation for our observations could be the secretion of *E. coli* tolC– derived extracellular DNA, which might enable FS5 to utilize dissolved and hydrolyzed products after DNA degradation. The detection of constantly active *E. coli* regardless the incubation patterns gave rise to the question, if *E. coli* tolC– might contribute to the survival of FS5 under host–free conditions. We could clearly identify a correlation between maintenance of FS5 respiratory activity and the presence of live and active *E. coli* tolC– in a host–free environment. Heat–inactivated *E. coli* tolC– did not confer any beneficial trait on FS5's cellular activity independent from the presence/absence of DNA. Moreover, ampicillin does contribute to a slightly reduced metabolic activity of *E. coli* tolC– in the presence of DNA. Interestingly, FS5 may benefit from that exhibiting significantly ($P < 0.01$) lower numbers of active cells in the absence of ampicillin. Ampicillin adversely affects bacterial cell wall biogenesis and cell division and is considered to act bactericidal on bacteria, but may not preclude metabolic and respiratory activity. Moreover, naturally occurring bacterial communities have been shown to facilitate various processes through secretion of antibiotics in sub–minimal inhibitory concentrations such as mediating host–parasite interactions or acting as signaling molecules in sub–inhibitory concentrations (Sengupta et al., 2013). Reports have also shown a direct impact on bacterial transcription, if applied in sub–minimal inhibitory concentrations (Yim et al., 2006). Interestingly, *E. coli* tolC– did not display any significant alterations of cellular activity in the presence of 5 x DNA, suggesting that sub–inhibitory concentrations of ampicillin do not sufficiently affect bacterial metabolic activities.

2.2.39 NO BENEFICIAL EFFECT OF *E. COLI* TOLC– SUPERNATANT ON FS5 RESPIRATORY ACTIVITY IN THE PRESENCE OF AMOEBAL DNA

Assays performed with the supernatant of *E. coli* tolC– incubated in the presence/absence of DNA to address the question of FS5 feeding on DNA hydrolyzed by *E. coli* tolC– secreted DNA nucleases could not reveal any "cross–feeding effect". However, we did not evaluate the

presence of DNA nucleases experimentally or tested if respiratory activity might be hampered by the use of protein inhibitors like EDTA, SDS or actin. FS5 remained almost exclusively inactive in all fractions suggesting that the secretion of enzymes may not solely be responsible for FS5 prolonged respiratory activity. Consequently, the actual presence of *E. coli* tolC– appears to be the major factor driving survival of FS5 in a host–free environment.

In summary, FS5 does show prolonged respiratory activity in a host–free environment for up to 240 hours in the presence of amoebal DNA. Unaltered controls were primarily inactive. Notably this prolonged cellular activity was somehow linked to the presence and activity of *E. coli* tolC– in a currently unknown fashion. Supernatants of *E. coli* tolC– feeding on amoebal DNA did not exhibit any active FS5 cells suggesting that respiratory activity is linked to the presence of active *E. coli* tolC– cells. Moreover, FS5 remains infectious despite host–free conditions in both the presence and absence of carbon, nitrogen and phosphor sources. Further comprehensive research has to be performed to elucidate the nature of FS5 cellular activity outside a host organism.

5. ABSTRACT

The majority of known obligate and facultative intracellular bacteria reside in the cytoplasm of unicellular / multicellular organisms. However, some symbionts have managed to invade and adapt to a life in specialized eukaryotic compartments such as mitochondria and nuclei. In the first part of the thesis, we addressed the question, if *Nucleicultrix amoebiphila* FS5, an obligate intracellular bacterium thriving in the nucleus of its amoebal host *Hartmannella* sp. possesses the potential to utilize host DNA. Host–free incubations in presence and absence of DNA and bacterial activity and viability were monitored with a combination of CTC, PI and DAPI staining. Our results could clearly show a maintenance of infectivity over time under host–free conditions. Moreover, FS5 exhibits significantly higher numbers of cells displaying respiratory activity in the presence of purified amoebal DNA. Utilization of exogenous DNA was possibly linked to the presence of live *E. coli* conferring currently unknown beneficial traits to FS5, eventually by secreting DNA nucleases. *Nucleicultrix* belongs to a novel class of obligate intracellular bacteria affiliated with the *Rickettsiales*. In the second part of the thesis, we focused on studying the developmental cycle of those relatives of *Nucleicultrix*. Moreover, we were interested if an elevated temperature might induce parasitic behavior. The characterization of infection cycles was carried out using fluorescence in–situ hybridization combined with a DAPI staining. Impact on host cells was evaluated by quantification of amoebae and conduction of a host viability assay using propidium iodide. In contrast to *Nucleicultrix*, Endosymbiont of *Acanthamoeba* UWC36 and *Paracaedibacter* EI3, both thrive in the cytoplasm of their *Acanthamoeba* sp. hosts. We could clearly identify a temperature–dependent acceleration of the infection process at 30°C. The infection cycle was completed 48hpi (30°C) respectively 72hpi (20°C). UWC36 was transmitted both horizontally and vertically leading to entirely infected *amoeba* cultures after 144hpi (20°C) respectively 96hpi (30°C) of incubation. Infected cultures decreased in cell numbers after 120hpi at 30°C, whereas UWC36–harboring amoebae incubated at 20°C continuously proliferated and replicated. Moreover, infected amoebae generally displayed significantly lower cell numbers from 96hpi on independent of the incubation temperature. However, no elevated levels of host cell lysis were observed during the course of the cycle. Conversely, EI3 could not infect *A. castellanii* Neff. Application of distinct antibiotic combinations resulted in completely cured native EI3 *Acanthamoeba* spp. host cells after 6 weeks. EI3 exhibited a limited host range, whereas UWC36 was able to invade other *Acanthamoeba* species. Our results gave first insights into the life style of those fascinating bacteria and can be linked to ongoing genomic,

transcriptomic and proteomic studies to further illuminate the underlying molecular mechanisms of these remarkable symbioses.

6. ZUSAMMENFASSUNG

Die Ausbildung von Lebensgemeinschaften unterschiedlichster Form und Prägung zwischen den verschiedenen Lebensformen innerhalb eines Habitats kennt in ihrer Mannigfaltigkeit und Variation keinerlei Grenzen. Ekto–sowie endosymbiotische Wechselbeziehungen zwischen Organismen nehmen eine essentielle Funktion in der Gestaltung eines Lebensraumes ein und liefern einen wesentlichen Beitrag zur Biodiversität eines Habitats.

Freilebende Amöben, unizelluläre, eukaryotische Organismen mit weitreichender Funktion innerhalb terrestrischer sowie aquatischer Lebensräume spielen eine gewichtige Rolle bei der Regulation bakterieller Biodiversität und Vielfalt. In ihrer Funktion als Räuber nehmen sie über den Prozess der Phagozytose bakterielle Organismen auf und bauen diese anschließend ab. Im Laufe der Zeit haben manche dieser Bakterien Strategien entwickelt, welche es ihnen ermöglichen, dem Abbauprozess zu entgehen und ihrerseits ihren bisherigen Fressfeind effektiv als ökologische Nische zur Reproduktion zu benutzen. Untersuchungen dieses intrazellulären Lebensstils liefern einen wesentlichen Beitrag zum Verständnis interspezifischer Wechselwirkungen auf molekularer und evolutionärer Ebene.

Im ersten Teil unserer Studie beschäftigten wir uns mit dem Einfluss wirtseigener DNA auf die Aufrechterhaltung zellulärer Aktivität und Stabilität von *Nucleicultrix amoebiphila* FS5, einem obligat intrazellulären *Alphaproteobakterium*, welches spezifisch den Zellkern seines Wirtes *Hartmannella sp.* infiziert. In Gegenwart bzw. Abwesenheit aufgereinigter DNA wurden Wirts–freie Fraktionen von *N. amoebiphila* FS5 über den Zeitraum einer Woche inkubiert. Zur Evaluierung und Bestimmung der zellulären Aktivität sowie Stabilität wurden spezifische Marker beziehungsweise Farbstoffe wie DAPI, PI und CTC herangezogen. Der prozentuelle Anteil infizierter Wirtszellen innerhalb einer Amöbenpopulation wurde mittels Fluoreszenz in–situ Hybridisierung ermittelt. Gemäß unseren erhobenen Daten behält *N. amoebiphila* FS5 seine Infektiosität in einer Wirts–freien Umgebung über einen längeren Zeitraum bei. Wir konnten des Weiteren auch eine signifikant erhöhte zelluläre Aktivität mittels CTC in der Gegenwart aufgereinigter DNA feststellen. Die Verwertung exogener DNA steht möglicherweise im Zusammenhang mit der Präsenz lebender *E. coli* Zellen, welche *N. amoebiphila* FS5 einen zum derzeitigen Stand unbekannten Vorteil verleihen. Eine mögliche Erklärung wäre die Sekretion exogener DNA Nukleasen, welche eine effektive Degradierung der DNA herbeiführen und dabei einzelne Nukleotide für *N. amoebiphila* FS5 verfügbar machen.

Nucleicultrix ist Mitglied eines kürzlich beschriebenen, neuartigen Stammes von obligat intrazellulären Bakterien, welcher der Ordnung der *Rickettsiales* zuzuordnen ist.

Während des zweiten Teils unseres Projektes lag der Schwerpunkt auf der Erforschung und Charakterisierung des Entwicklungszyklus zweier verwandter Organismen von *Nucleicultrix*, Endosymbiont of *Acanthamoeba* UWC36 und *Paracaedibacter* EI3. Beides sind Symbionten der Gattung *Acanthamoeba sp*., infizieren jedoch das Zytoplasma ihres jeweiligen Wirtes. Im Mittelpunkt unseres Interesses stand die Evaluierung des Einflusses erhöhter Inkubationstemperatur auf die Ausbildung parasitären Verhaltens beider Organismen. Die Charakterisierung des Lebens–bzw. Infektionszyklus liefert dabei einen wichtigen Einblick in die Art und Ausprägung intrazellulärer Wechselwirkungen und wurde in unserem Fall mittels Fluoreszenz in–situ Hybridisierung in Kombination mit einem DAPI–Färbeansatz zur gleichzeitigen Erfassung der metabolischen Aktivität des Symbionten durchgeführt. Mit Hilfe der Bestimmung der Wachstums–bzw. Teilungsraten infizierter sowie uninfizierter Amöben sowie eines Propidiumiodid (PI)–Färbeansatzes wurde der Einfluss einer bakteriellen Infektion auf den Fitnesslevel und die Überlebensfähigkeit infizierter *Acanthamoeba castellanii* Neff untersucht. Die Infektion von *A. castellanii* Neff durch UWC36 erfolgte rasch, bereits 2 Stunden nach Beginn wurden erste UWC36 Bakterien innerhalb von Wirtszellen beobachtet. Begünstigt durch seine schützende Umgebung innerhalb einer Wirtszelle breitete sich UWC36 rasch innerhalb einer Population von uninfizierter *A. castellanii* Neff aus. Des Weiteren konnten wir eine direkte, Temperatur–abhängige Beschleunigung des Entwicklungszyklus bei 30°C feststellen. Der Abschluss des Entwicklungszyklus erfolgte abhängig von der jeweiligen Inkubationstemperatur 48 (30°C) beziehungsweise 72 (20°C) Stunden nach Beginn der Infektion. Im Laufe der Entwicklungszyklus von UWC36 kam es zu einer kontinuierlichen Infektion der gesamten Wirtspopulation über horizontalem Transfer sowie vertikaler Übertragung mittels Teilung der Wirtszellen. Nach 168 Stunden fortwährender Inkubation waren dementsprechend sämtliche Wirtszellen innerhalb einer Amöbenpopulation vollständig infiziert, unabhängig von der Inkubationstemperatur. Während die Zellzahl infizierter Amöben bei 30°C 120 Stunden nach Beginn der Inkubation beständig abnahm, blieb jene infizierter Wirtszellen bei 20°C über den weiteren Verlauf des Zyklus stabil. Die Zellzahl infizierter Amöben lag generell signifikant niedriger als jene uninfizierter Kontrollen ab 96 Stunden nach Beginn der Infektion unabhängig von der jeweiligen Inkubationstemperatur. UWC36 scheint den gewählten Wirtsorganismus *A. castellanii* Neff in seiner Überlebensfähigkeit und Vitalität nicht

wesentlich zu beeinflussen oder vorzeitige Zelllyse zu induzieren, was durch das Fehlen charakteristischer PI–Peaks während des gesamten Infektionszyklus gekennzeichnet ist.

Paracaedibacter EI3 war im Gegensatz zu UWC36 nicht in der Lage, *A. castellanii* Neff erfolgreich zu infizieren. Mittels eines Antibiotikaansatzes bestehend aus verschiedenen Kombinationen von Antibiotika mit unterschiedlichen Wirkungsspektren gelang es, *Paracaedibacter* EI3 erfolgreich aus seinem nativen Wirt *Acanthamoeba sp*. zu entfernen. Nach 6 Wochen kontinuierlicher Behandlung konnten keinerlei infizierte Amöben sowie EI3 im extrazellulären Milieu mittels FISH und DAPI–Färbung detektiert werden. Im direkten Vergleich mit Endosymbiont of *Acanthamoeba* UWC36 weist *Paracaedibacter* EI3 eine eingeschränkte Reichweite an möglichen Wirten auf, wohingegen UWC36 fähig zur Erweiterung seines Repertoires an möglichen Wirtszellen fähig erscheint.

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„Die Naturwissenschaft beschreibt und erklärt die Natur nicht einfach, so wie sie "an sich" ist. Sie ist vielmehr ein Teil des Wechselspiels zwischen der Natur und uns selbst."

Werner Karl Heisenberg

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PERSONALE, METHODISCHE UND SOZIAL–KOMMUNIKATIVE KOMPETENZEN	**Organisiertes und selbständiges Arbeiten** **Strebsamkeit, Aufmerksamkeit, Verlässlichkeit** **Zielstrebigkeit, Disziplin** (erworben durch meine Ausbildungen und beruflichen Erfahrungen)
EDV	ECDL – Europäischer Computerführerschein Tabellenkalkulation mit Excel Formatierung Einführung in SPSS Autodidakte Grundlagen Adobe Photoshop Ausbildung zum Brandschutzwart und Brandschutzbeauftragten
FÜHRERSCHEINE	B (PKW nicht vorhanden)