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Characterization of the infection cycle of the intracellular *Acanthamoeba* symbiont *Amoebophilus asiaticus*

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“A mind is like a parachute. It doesn’t work if it is not open.“

Frank Zappa

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1 ABSTRACT

Free-living amoebae (FLA), which can be found in various habitats, are able to serve as host to harbor bacterial human pathogens such as *Legionella pneumophila, Mycobacteirum spp.* and *Francisella tularensis* and are also thus considered as the „Trojan horses“ in the microbial world (Barker and Brown, 1994). In addition, long-term symbiosis between host and FLA was described, our knowledge about the intracellular life of endosymbionts belonging to the *Bacteriodetes,* which is one of the four major groups of obligate intracellular endosymbionts associated with *Acanthamoba*, is still very limited. The aim of this project was to characterize the symbiosis between of *Acanthamoeba* with the bacteria symbiont *Amoebophilus asiaticus* 5a2. The progress of infection as well as morphological changes of *A. asiaticus* 5a2 were monitored by utilizing Fluorescence-In-Situ-Hybridisation (FISH) with a specific probe designed for targeting *A. asiaticus* 5a2, and key events such as uptake, replication of the endosymbiont and spread of the infection were determined. Interestingly newly infected *Acanthamoeba* culture with a high rate of infection seemed initially impossible to achieve. However, the infection of *Acanthamoeba* with *A. asiaticus* 5a2 appeared to be dependent on the amoeba growth medium since the infection efficiency was pronouncedly higher when incubated in the medium TSY instead of PYG. The extracellular *A. asiaticus* 5a2 not only exhibited a much greater infectivity but also showed a different morphology compared to intracellular *A. asiaticus* 5a2. The same feature was also observed for other strains of *A. asiaticus*, namely *A. asiaticus* US1 and *A. asiaticus* EIDS3. This suggested an bi-phasic life style of *A. asiaticus*. The initial uptake of *A. asiaticus* 5a2 in form of small and roundish rods by the *Acanthamoeba* host occured within six hours post infection (hpi). The morphology of *A. asiaticus* 5a2 changed during the replication around 72 hpi becoming more elongated and rod-like. The infection cycle was completed 6 dpi, when lysed amoebae, extracellular *A. asiaticus* 5a2 as well as newly infected acanthamoebae could be observed. Ultrastructure analysis of extracellular *A. asiaticus* 5a2 by TEM showed structures within the cell, which could represent the afp-like prophage. Taken together the analysis of the infection cycle of the bacterial symbiont *A. Asiaticus* in *Acanthamoeba* revealed a bi-phasic life style of *A. asiaticus*. I suggest

the existence of a fundamentally different life stages required for the successful establishment of symbiosis with amoeba hosts.

2 ZUSAMMENFASSUNG

Frei lebende Amöben (FLA), die in verschiedenen Lebensräumen vorkommen, sind potentielle Träger von humanpathogene Erreger wie *Legionella pneumophila*, *Mycobacterium* spp. und *Francisella tularensis* und werden deshalb auch als "Trojanische Pferde" der Mikroben bezeichnet. Unser Wissen über die intrazelluläre Lebensweise der Endosymbionten gehörig zu den *Bacteriodeten*, eine der vier Hauptgruppen der obligat intrazelluläre Endosymbionten der Acanthamöben, ist noch sehr begrenzt. Das Ziel dieses Projektes war es, die Symbiose zwischen Acanthamöben und dem bakteriellen Symbiont *Amoebophilus asiaticus* 5a2 zu charakterisieren. Der Verlauf der Infektion und die morphologischen Veränderungen von *A. asiaticus* 5a2 wurden mit der Verwendung von Fluoreszenz-In-Situ-Hybridisierung (FISH) mit *A. asiaticus* spezifische Sonde erfasst. Unter anderem wurden wichtige Schlüssel-Ereignisse wie die Aufnahme und Replikation der Endosymbionten sowie der Ausbreitung der Infektion bestimmt. Interessanterweise schien es anfänglich nicht möglich zu sein eine neue Kultur mit einer hohen Infektionsrate zu infizieren. Die Infektion von *Acanthamöben* mit *A. asiaticus* 5a2 schien von der verwendeten Wachstumsmedium abhängig zu sein, da bei der Verwendung vom Medium TSY eine deutlich höhere Infektions-Effizienz zu beobachten war als beim Medium PYG. Der extrazelluläre *A. asiaticus* 5a2 zeigte nicht nur eine viel größere Infektiosität an, sondern wies auch eine andere Morphologie im Vergleich zur intrazelluläre *A. asiaticus* 5a2 auf. Das gleiche Phänomen wurde auch bei anderen Stämmen von *A. asiaticus* nämlich *A. asiaticus* US1 und *A. asiaticus* EIDS3 beobachtet. Es deutete einen bi-phasischen Lebensstil von *A. asiaticus* an. Die Aufnahme von *A. asiaticus* 5a2 als kleine und rundliche Stäbchen erfolgte innerhalb der ersten sechs Stunden nach der Infektion des Acanthamöben Wirts. Die Morphologie des Endosymbionts veränderte sich während der Replikation und wurde 72 Stunden nach der Infektion länglicher und stabförmiger. Der Infektions-Zyklus war nach 6 Tagen vollendet und lysierte Amöben, extrazelluläre *A. asiaticus* 5a2 sowie neu infizierte Amöben konnten dann beobachtet werden. Ultrastruktur Analyse der extrazellulären *A. asiaticus* 5a2 mit TEM zeigte intrazelluläre Strukturen, die den afp-like Prophagen darstellen könnten. Die Analyse des Infektions-Zyklus des

bakteriellen Symbionten *A. asiaticus* mit Acanthamöben zusammengefasst, offenbarte einen bi-phasischen Lebensstil von *A. asiaticus*. Ich schlage vor, dass eine fundamental unterschiedliche Lebensphase existiert, die für die erfolgreiche Etablierung einer Symbiose mit Amöben als Wirt notwendig ist.

3 INTRODUCTION

3.1 Forms of symbiosis

The first person who conceptually postulated what we nowadays call "symbiosis" was Simon Schwenderer from swizerland in 1868 (Sapp, 1994). But in general, Heinrich Anton de Bary, who was a german surgeon and scientist, was acknowledged in coining the term symbiosis, which he described as "the phenomena of unlike organisms living together" (de Bary, 1879). Although this description does highlight the importance of physicial proximity of two different species, it neglects the different types of interaction which can occur between host and symbiont and the effect on their fitness. Three distinctive categories of symbiosis can be distinguished: mutualism, parasitism and commensalism (Sapp, 1994). Mutualism is a relationship, which is beneficial for both the host and the symbiont, as in case of *Buchnera aphidicola* and aphids. *Buchnera aphidicola* is a member of the Gamma proteobacteria and an obligate intracellular symbiont of the aphid. *Buchnera aphidicola* is dependent on the aphid host to provide nutrients and protection from outer environment. In return, *Buchnera aphidicola* provides essential nutrients that the host lacks, because of its diet in form of plant sap, which is rich in carbohydrates, but poor in nitrogenous or other essential compounds (Munson et al., 1991). Parasitism describes the situation where the symbiont increases its own fitness at the expense of the host. For example parasitic flatworms belonging to the Cestoda also commonly known as tapeworms live in the digestive tract of vertebrate for nutrients uptake with no benefits given to the host. Commensalism is the relationship between two organisms where one organism benefits but the other is neutral, meaning there is no harm or benefit. *Staphylococcus epidermis* is a Gram-positive, cocci-shaped bacterium and belongs to the normal microflora on healthy human skin (Schönfelder et al., 2010). It is hypothesized that a peptide produced by *Staphylococcus epidermis* might act as an antimicrobial shield and contribute to normal defense at the epidermal skin (Cogen et al., 2010).

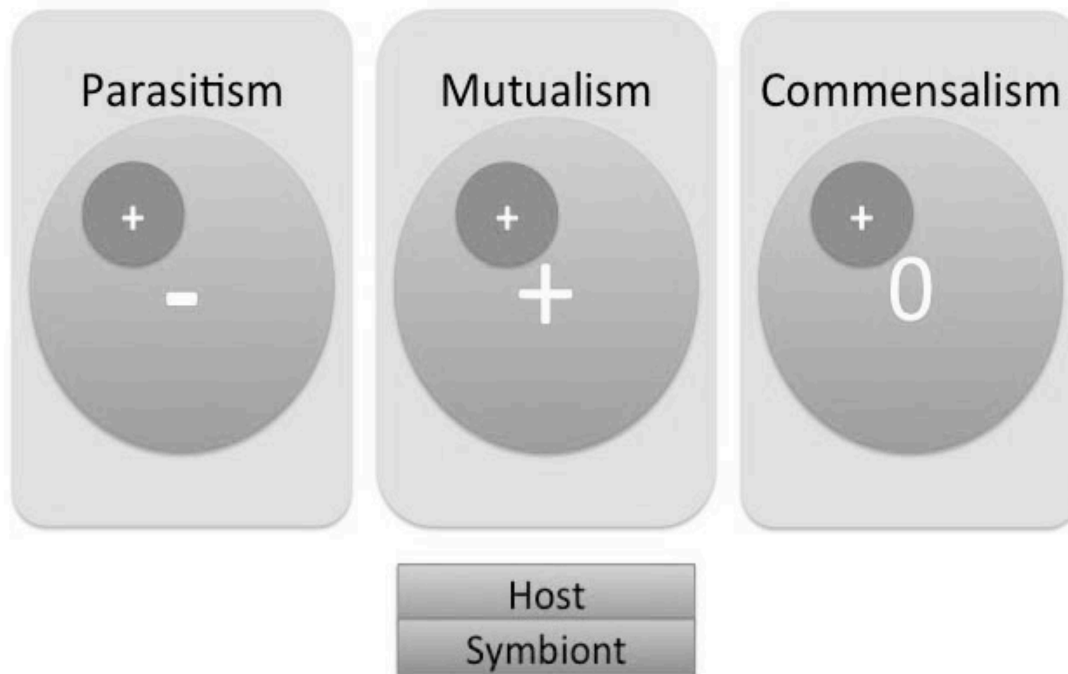


Figure 1. Three different types of symbiosis between a host cell and intracellular symbiont. The bigger circle represents the host and the smaller circle inside represents the endosymbiont. + symbolizes improved fitness; - symbolizes decreased fitness; 0 symbolizes no evident change in fitness

While the aforementioned three types of symbiosis are based on the fitness of the host and symbiont, symbiosis can also be differentiated in endosymbiosis / ectosymbiosis and obligate / facultative. In case of endosymbiosis, one organism (usually referred as the endosymbiont) lives intracellularly inside the body or cells of another organism. The subject of this study for example, the amoeba endosymbiont *Amoebophilus asiaticus* 5a2, lives intracellularly within the amoeba host (Figure 1). Although endosymbionts can be either eu- or prokaryotic, most host cells are predominantly eukaryotes due to their larger cell size, complexity and phagocytosis. The eukaryotic cells are an ideal space for their prokaryotic counterpart to inhabit since they offer a stable and nutrient rich environment. In some cases the biochemical versatility of the endosymbiont allows the host to survive in previously inhabitable environments. For example the symbionts of beetles and haematophagous insects may provide B vitamins where as cockroach symbiont and symbionts of Homoptera provide essential amino acids (Douglas, 1989; Hoffmeister &

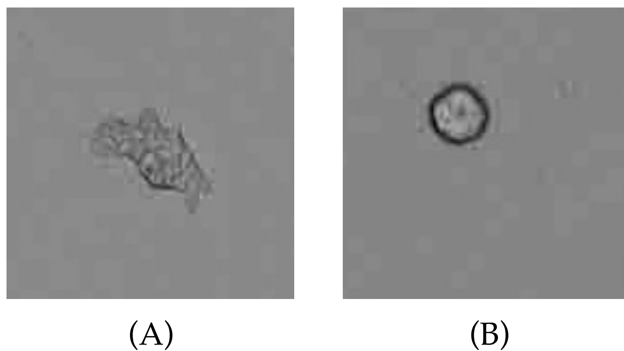
Martin, 2003). *Buchnera aphidicola* also provides the aphid essential nutrients (Munson et al., 1991). Ectosymbiosis refers to the situation when the symbiont lives on the body surface of the host, also including internal surfaces like the digestive tracks. One of the examples is the hemi-parasitic plant belonging to the order of Santalales, also known as the mistletoe. It attaches to other trees for exploitation of water and mineral nutrients (Hawksworth, 1996).

In case of an obligate symbiotic relationship between an intracellular endosymbiont and host, the symbiont can only survive within the host. The symbiont resides within the host and is protected against outer influences. In some examples, the relationship is a necessity for the survival of both the host and the endosymbiont (Baumann P. et al., 1995). The aforementioned *Buchnera aphidicola* lives in a tight relationship with the aphid host, which has also lead to a drastic reduction of the symbiont genome, retaining only essential genes for its specialized lifestyle (Gil et al., 2002). A facultative symbiotic relationship is the case, when the survival of the symbiont isn't dependent on the symbiotic lifestyle, but merely an option. An example for the facultative intracellular symbiont is *Legionella pneumophila*, which is also able to survive extracellularly.

3.2 Free-living amoeba belonging to the genus *Acanthamoeba*

The acanthamoebae belongs to the free-living amoebae (FLA), ubiquitous widespread protozoa comprising also different genera like e.g. *Hartmanella, Naegleria* and *Balamuthia*. FLA are important bacterial predators as well as regulators of the microbial community. Additionaly they also feed themselves on funghi and even other protozoa. The presence of FLA can be found in various habitats ranging from natural territories like fresh water, salt water, soil and air to artificial environment like the sewage system and air-conditioning units. Some FLA have even been isolated from the human body (Rodriguez-Zaragoza, 1994; Khan, 2006) and are recognized as opportunistic human pathogens causing severe diseases in humans. *Naegleria fowleri* e.g. can cause primary amoebic encephalitis (Marshall et al., 1997). *Acanthamoeba* on the other hand is responsible for granulomatous amebic encephalitis in immuno-comprimised persons and amebic keratitis associated with the use of soft

contact lenses, which can even lead to blindness (Armstrong, 2000).
The wide distribution of the FLA is partly due to the two different life stages of the amoebae. One is the motile and metabolically active trophozoite stage allowing the amoeba to graze the surrounding environment (Figure 2A). In this stage, the replication is achieved through cell division and the amoebae are able to change their shape. Their irregular shape is mostly due to the pseudopods, used by the amoebae for surface attachment (Khan, 2001). The other stage is the cyst, transforming the amoeba into a metabolically inactive stage in order to cope with hazardous environment like elevated temperature, shifted pH-value, desiccation, toxicities, UV-radiation and starvation (Khan, 2006) (Figure 2B). The cyst form thus allows the amoeba to survive and outlive rapidly changing environmental conditions over a long period of time.



(A) (B)

Figure 2. The two stages of the acanthamoebae (A) An amoeba in trophozoite stage, the motile and metabolically active stage. (B) An *Acanthamoeba* in resistant stage. It is protected against unfavourable environmental conditions.

3.2.1 Endosymbionts of *Acanthamoeba spp.*

The first observation of long-term symbiotic relationship between bacteria and acanthamoebae was in the 70's (Proca-Ciobanu et al., 1975). Acanthamoebae can act as "Trojan horses" for bacterial pathogens of humans, serving as vectors for transmitting pathogens to humans (Weissenberger et al., 2007; Barker & Brown, 1994). Acanthamoebae might represent an "evolutionary training ground" enabling an adaptation of bacterial cells to eukaryotes, effectively broadening their host range (Albert-Weissenberger et al., 2007; Barker et al., 1994; Darby et

al., 2007; Harb et al., 2000; Horn et al., 2004; Horn et al., 2005). This hypothesis is supported by studies of *Legionella pneumophila*, the causative agent of Legionnaire's disease. Studies showed that *L. pneumophila* grown within amoebae are several fold more invasive for macrophages and mice cells compared to agar-plate grown bacteria (Brieland et al., 2007; Cirillo et at., 1994). *L. pneumophilae* that have undergone a prior passage in amoebae also showed an increase in virulence (Cirillo et al., 1999). Other well-known human pathogens, which are also able to thrive within amoebae, are e.g. *Legionella pneumophila* (Greub & Raoult, 2004; Rowbotham, 1986), *Francisella tularensis* (Abd et al., 2003), *Helicobacter pylori* (Winiecka-Krusnell et al., 2002), *Escherichia coli* (Barker et al., 1999), *Listeria monoctogenes* (Ly and Müller, 1990), *Mycobacterium avium* (Greub & Raoult, 2004; Steinert et al., 1998) and *Shigella boydii* (Jeong et al, 2007). Understanding the basic mechanisms that underlie the interaction of bacteria infecting amoebae may provide insights into the evolution of infection mechanisms and the related intracellular life style.

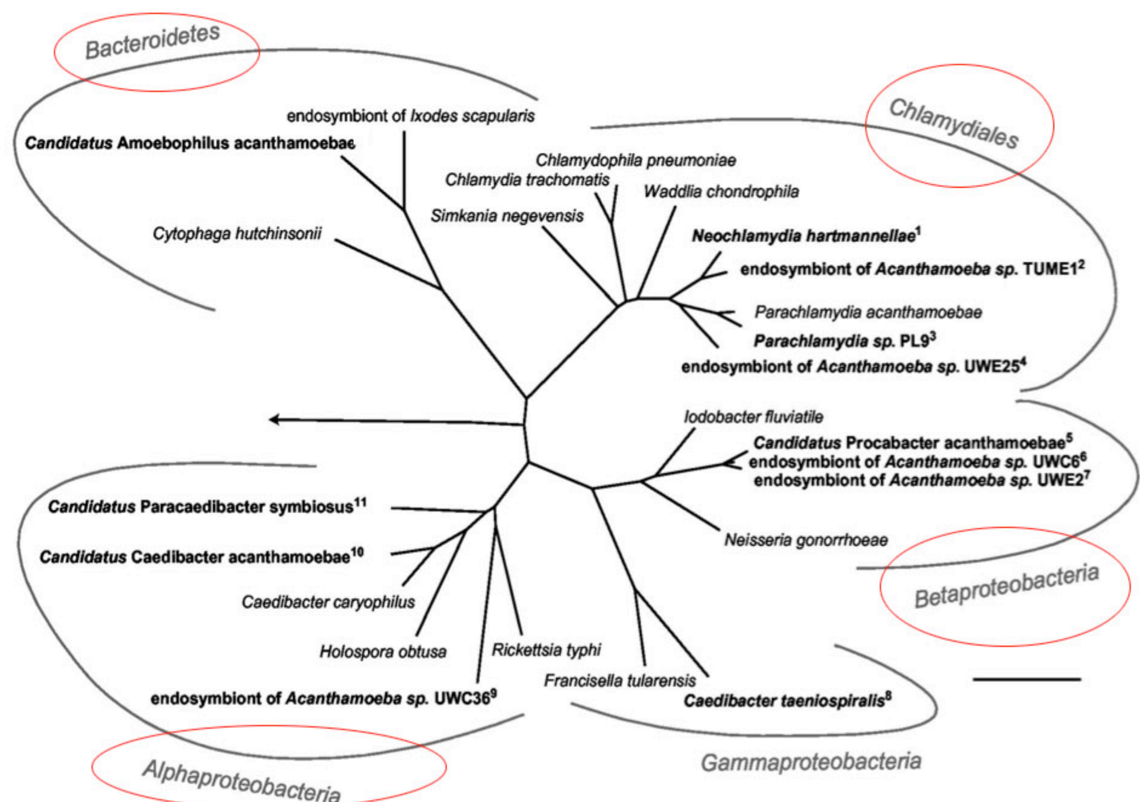


Figure 3. 16S rRNA –based neighbour joining phylogenetic tree showing the relationship of amoeba endosymbionts belonging to the bacteria. The Bacteriodetes, Chlamydiales, Alphaproteobacteria and Betaproteobacteria represent the four groups of obligate intracellular symbiont found in amoebae. (modified from Schmitz-Esser et al., 2004)

Although bacteria are the main food source of FLA, some bacteria developed various strategies to escape the phagocytosis and can exploit the amoebae for their own fitness. These microorganisms manage to prevent being digested by the amoeba and are able to multiply within FLA (Casadevall, 2008). Once escaped the phagocytosis, the symbionts can reside within an *Acanthamoeba*, being protected against unfavorable outer environmental conditions (Fields, 1996; Essig et al., 1997; Ly and Müller, 1990). Especially after encystation, the amoeba can provide protection against extreme extracellular conditions, such as oxygen free conditions in case of anaerobic bacteria (Tomov et al., 1999), dissection (Steinert et al., 1998), high temperatures and chlorination (Kilvington and Price, 1990). 24% of axenically grown *Acanthamoeba* isolates, intracellular bacteria were identified (Fritsche et al., 1993). Most of these symbiont, undergoing a long-term symbiotic relationship with amoebae, are members affiliated with the following four bacterial lineages: *Alphaproteobacteria* (Birtles et al., 2000; Fritsche et al., 1999; Horn et al., 1999; Xuan et al., 2007) and *Betaproteobacteria* (Heinz et al., 2007; Horn et al., 2002), *Bacteriodetes* (Horn et al., 2001; Xuan et al., 2007) and the *Chlamydiae* (Greub et al., 2003; Horn et al., 2004; Horn, 2008) (Figure 3). This taxonomic distribution suggests an independent rise of amoebae-symbiont interaction at several time points during the evolution. Members of the four aforementioned groups are also globally distributed, since similar strains were found in amoebae isolates in geographically distinct regions (Horn & Wagner, 2004; Schmitz-Esser et al., 2008).

3.3 The genomic features of the amoeba symbiont *Amoebophilus asiaticus* 5a2

Amoebophilus asiaticus 5a2, first discovered within an amoeba isolated from alkaline lake sediment (Schmitz-Esser et al., 2008), is a Gram-negative, rod shaped, obligate intracellular amoeba symbiont belonging to the *Bacteriodetes*; a phylogenetically highly diverse pylum (Gherna & Woese, 1992; Schmitz-Esser et al., 2010). *A. asiaticus* 5a2 is a super group of *Cardinium hertigii*, an obligate intracellular parasite of the *Encarsia* wasps also belonging to the *Bacteriodetes*

(Zchori-Fein et al., 2004). The genome of *A. asiaticus* 5a2 consists of 1.89 Mbp and has a G+C content of 35%. It encodes 1.557 proteins with a coding density of 81.8% (Schmitz-Esser et al., 2010). Compared to the genome size of obligate mutualistic symbiont of insects, which usually varies from 0.14 to 0.8 Mbp (McCutcheon et al., 2009; Moran et al., 2008), the genome of *A. asiaticus* 5a2 is only moderately reduced (Merhej et al., 2009; Wernegreen, 2005).

3.3.1 A. asiaticus 5a2 has a moderately reduced genome with limited biosynthetic capabilities

Surprisingly, despite its only moderately reduced genome, *A. asiaticus* 5a2 has an extremely limited biosynthetic capability. Although the genome encodes most enzymes affiliated with glycolysis pathway, one crucial glycolytic enzyme, phosphofructokinase (PfkA), is absent (Schmitz-Esser et al., 2010). It is suspected that the enzymes are used for gluconeogenesis instead of glycolysis, since the key enzyme of gluconeogenesis, fructose biphosphatase, is present. In addition, the tricarboxylic acid cycle as well as known sugar uptake systems are missing (Schmitz-Esser et al., 2010). *A. asiaticus* 5a2 also lacks an electron transport chain and pathways needed for biosynthesis of cofactors, amino acids and vitamins (Schmitz-Esser et al., 2010). Recent study suggests that *A. asiaticus* 5a2 utilizes amino acids, oligopeptides or other substrates of the host as the main nutrient source (Schmitz-Esser et al., 2010). In addition to a variety of different transport proteins (n = 82) for oligopeptides and amino acids uptake, *A. asiaticus* 5a2 uses an ATP / ADP translocase as well. The limited metabolic capability of *A. asiaticus* 5a2 also suggests it to be a parasite, exploiting the host of essential nutrients, since no essential substrates are provided to the amoeba host (Schmitz-Esser et al., 2010). This is supported by an experiment, which demonstrated that the original host also survived without its *A. asiaticus* symbiont. However acanthamoeba infected with *A. asiaticus* have shown a reduction in growth rate. Similar results of *Acanthamoeba* infected with *Protochlamydia amoebophila* have been published, where amoebae infected with the endosymbiont multiplied more slowly than those without endosymbiont. But not all obligate intracellular endosymbionts have a negative impact on their

amoeba host, since the FLA *Hartmannella vermiformis* infected with *Neochlamydia hartmannellae* grow more rapidly than those without the symbiont (Collingro, 2004).

3.3.2 Significant enrichment of proteins with eukaryotic domains in *A. asiaticus*

Another interesting feature of *A. asiaticus* 5a2 genome is the high abundance (8% of all CDSs, n = 129) of proteins with eukaryotic domains (Schmitz-Esser et al., 2010). These proteins with eukaryotic domains consist of ankyrin repeats, leucine-rich repeats, TPR/SEL1-repeats and domains from eukaryotic ubiquitin system, which seems highly likely to be involved in host-symbiont interaction (Schmitz-Esser et al., 2010). Leucine-rich repeat are involved in protein-protein interaction (Bella et al., 2008), where as proteins containing ankyrin repeat motif are important for cytoskeleton formation as well as cell cycle and transcriptional regulation (Li et al., 2006; Mosavi et al., 2004). Studies have also shown the importance of ANK protein for intracellular replication of *L. pneumophila* (Al-Khodor et al., 2008). Proteins needed for gliding motility, a common feature in *Bacteriodetes*, has also been found in the genome (McBride, 2001). Furthermore, five patatin-like proteins, two phospholipases D, a eukaryotic serine/threonine protein kinase and two proteins similar to a toxin complex found in Photorhabdus spp. and Gammaproteobacteria have also been identified. The two putative phospholipase D share 40% similarity to the one of *Rickettsia* spp. for vacuole exit (Hybiske & Stephens, 2008; Renesto et al., 2003). The number and percentage of proteins able to interfere with the host ubiquitination system is with 15 proteins containing F-box domains and nine proteins containing U-box domain unchallenged among bacteria (Rytkonen et al., 2007; Schmitz-Esser et al., 2010). The ubiquitation system is important for controlling protein degradation, cell cycle progression, signal transduction, transcriptional regulation or even resistance to pathogens (Welchmann et al., 2005). *A. asiaticus* 5a2 might be able to transfer and remove ubiquitin to/from target host's proteins to interfere with the hosts ubiquitin system (Schmitz-Esser et al., 2010).

3.3.3 Interaction between *A. asiaticus* 5a2 and its amoeba host cell

Interestingly the endosymbiont lacks the means to transport from periplasm across the outer membrane in form of type II secretion system (Schmitz-Esser et al., 2010). *A. asiaticus* 5a2 encodes a newly discovered putative type VI secretion system, a distant relative of the described type VI secretion system, commonly used by proteobacterial pathogens and symbionts. A recent study has shown a high similarity between proteins of a gene cluster in *A. asiaticus* 5a2 and the proteins of the antifeeding prophage (afp) found in the insect pathogen *Serratia entomophila* (Hurst et al., 2004). The afp prophage of *Serratia entomophila* functions as a protein secretion apparatus, that delivers toxins into the eukaryotic host of *S. entomophila*. In various *Photorhabdus* spp. and other marine bacteria including many *Bacteriodetes*, similar eukaryotic toxin-encoding prophages have also been found (Hurst et al., 2004; Sen et al., 2010; Yang et al., 2006). It is proposed, that the afp-prophage in *A. asiaticus* 5a2 encodes also for a protein secretion apparatus for delivering effector proteins into the amoeba host cell. The secretion system might either be utilized for the transport of specific effectors or as a more generally used secretion system for the proteins containing eukaryotic domains (Penz et al., 2010).

3.3.4 *A. asiaticus* 5a2 shows high abundance of IS-elements

A. asiaticus 5a2 has a high abundance of mobile genetic elements compared to other bacteria, where 24% (n = 354) of all open-reading frames (ORFs) are genes encoding transposases (Schmitz-Esser et al., 2010). Usually the percentage of IS elements in bacterial chromosomes is below 3% (Siguier et al., 2006). The transposase genes of *A. asiaticus* 5a2 are IS-elements, which are widespread and abundant within bacterial and archaeal genomes (Aziz et al., 2010; Newton et al., 2010; Siguier et al., 2006; Touchon et al., 2007; Wagner et al., 2007). *A. asiaticus* 5a2 has 16 different IS elements, which are represented by two to 24 full-length copies (Schmitz-Esser et al., 2010). The IS elements may have played an important role during the adaptation of *A. asiaticus* to the intracellular life style. Interestingly despite the high amount of IS elements, no major

rearrangements seem to have occurred recently. The high sequence divergence, high number of truncated IS element copies and the absence of direct repeats in most IS elements indicate, that the IS elements might actually be inactive. It is suspected that the IS elements in the *A. asiaticus* 5a2 genome are evolutionary remnants, being inactive and currently undergoing the process of degradation.

3.4 Amoeba symbionts show different lifestyles

All hitherto described obligate intracellular symbionts of amoeba belong to the *Alphaproteobacteria* (Birtles et al., 2000; Fritsche et al., 1999; Horn et al., 1999; Xuan et al., 2007), *Betaproteobacteria* (Heinz et al., 2007; Horn et al., 2002), the *Bacteriodetes* (Horn et al., 2001; Xuan et al., 2007) or the *Chlamydiae* (Greub et al., 2003; Horn et al., 2004; Horn, 2008) (Figure 3). These bacteria all show different lifestyles with the developmental cycle of chlamydia-related amoeba symbionts being best described (Greub et al., 2003; Greub et al., 2005; Greub and Raoult, 2002; Horn, 2008; Horn et al., 2004), suggesting fundamentally different mechanisms of host-cell interactions among bacterial symbiont of amoebae.

Chlamydiae are obligate intracellular bacteria with a broad spectrum of eukaryotic hosts. Several members are human pathogens, like *Chlamydia trachomatis,* which is the most prevalent bacterial sexually transmitted disease (WHO, 2001) and the leading cause of preventable blindness (WHO, 2008). *Chlamydia pneumoniae* can cause community acquired pneumonia and also chronic diseases like arteriosclerosis and multiple sclerosis (Mahony et al., 2003; Mussa et al., 2006). The symbiont *Protochlamydia amoebaphila* UWE25 (referred after as *P. amoebophila*), a Gram-negative coccoid bacterium, was originally found intracellularly within an *Acanthamoeba* sp. isolated from a soil sample (Fritsche et al., 1993; Fritsche et al., 2000). *P. amoebophila* is distributed throughout the cytoplasm of the amoeba. All chlamydia-like bacteria show a biphasic developmental cycle with morphologically and physiologically distinct stages, the infectious elementary body (EB) and the other is the non-infectious but metabolically active reticulate body (RB) (Moulder, 1991; Abdelrahman & Belland, 2005). The *Acanthamoeba* takes up the chlamydial EB by phagocytosis.

The EBs will differentiate within the host to RBs, which then start to replicate by binary fission within inclusions. Unlike other members of the *Chlamydiaceae* who form larger inclusions, each *P. amoebophila* cell seems to be surrounded by an inclusion membrane of its own (Collingro et al., 2005; Fritsche et al., 2000). Eventually the RBs will undergo a re-differentiation and turn into EBs again. This event is often accompanied by the lysis of the host, where infectious EBs are released and a new infection cycle can start again. Alternatively the exit from host cell can also be mediated by extrusion of membrane-engulfed bacterial packages, where no harm is done to the host (Hybiske & Stephens, 2007).

Legionella pneumophilae (referred after as *L. pneumophilae*) are Gram-negative, rod-shaped bacteria belonging to the *Gammaproteobacteria*. Differently than members of the aforementioned four main phyla of obligate intracellular endosymbiont, *L. pneumophilae* are facultative intracellular bacteria, able to survive extracellularly without host cells (Fields, 1996; Lau & Ashbold, 2009). They are parasites able to escape amoeba phagocytosis and eventually harm their host. *L. pneumophilae* are known to have a broad host range not limited only to protozoa but also mammalian cells, causing e.g. Legionnaires disease in humans (Winn, 1988). The developmental cycle of *L. pneumophila* consists of two different stages, the transmissive phase and the replicative phase (Molofsky et al., 2004; Weissenmayer et al., 2011). After host cell entry and inhibition of phagosome-lysosome fusion, *L. pneumophila* start to replicate inside the amoebae within a vacuole protected by a layer derived from the endoplasmatic reticulum. After the host cell nutrient pool is depleted, *L. pneumophilae* change from the replicative form into the transmissive form, lysing the host cell and infecting other potential hosts in the surroundings (Bryne & Swanson, 1998).

A. asiaticus 5a2 in comparison is not able to survive extracellularly for extended periods of time, since it is an oblige intracellular symbiont. After exiting phagocytosis, *A. asiaticus* 5a2 does not form a protective layer around itself and will replicate within the cytoplasm of the amoeba host. Host cell lysis take place and *A. asiaticus* is released able to infect other amoebae. It is unclear whether *A. asiaticus* possesses different cell stages like in case of the *L. pneumophila*.

Compared to *L. pneumophila*, the infection of *A. asiaticus* 5a2 does not seem as aggressive since acanthamoebae infected with *A. asiaticus* can be incubated for an extended period of time and without amoeba cell lysis.

3.5 Aims of this study

Within the last years, a lot of research has been done in the field of intracellular bacteria and their *Acanthamoeba* host. Both *P. amoebophila* and *L. pneumophila* show a biphasic lifestyle with distinct stages. But a development cycle of the obligate intracellular endosymbiont belonging to the *Bacteriodetes,* such as *A. asiaticus* 5a2 has not been described yet. The genome of *A. asiaticus* has been already been sequenced and its analysis indicated a parasitic lifestyle. The aim of this project was to characterize the infection of *Acanthamoeba* host with the endosymbiont *A. asiaticus* 5a2. Issues of interest were the time course of infection with emphasis on the replication of the symbiont within the host as well as the further spread to surrounding host cells. Therefore, infection experiments were carried out to establish a stable host-symbiont system of *Acanthamoeba* infected with *A. asiaticus* 5a2. Progress of infection was visualized by utilizing Fluorescence-In-Situ-Hybridisation (FISH) with specific probe designed for targeting the *A. asiaticus* 5a2 and morphological changes of *A. asiaticus* 5a2 were monitored. Interestingly newly infected *Acanthamoeba* culture with a high rate of infection seemed initially impossible to achieve. The infection rate was usually less then 5%. Thus, another goal was to optimize infection efficiency to achieve a highly infected *Acanthamoeba* culture. Further, the effects of an *A. asiaticus* infection on host growth were studied by duplication time comparison between uninfected acanthamoebae and acanthamoebae carrying *A. asiaticus* 5a2 at different incubation temperature and media.

4 METHOD AND MATERIALS

All chemicals used in this study were purchased in pro analysi quality, if not stated otherwise. Double distilled and filtered (5 μm) water (hereafter named ddH_2O) were used for the production of all media, buffers and solutions. The ddH_2O used were fabricated by the water purification system "MILLI-Q® biocel" (Millipore GmbH, Vienna, Austria). Hydrochloric acid solution (HCl) and sodium hydroxide solution (NaOH) were used for pH adjustment. All buffers and general media were sterilized for 20 min at 121°C and 1.013 x 105 Pa pressure using a water-vapour high pressure autoclave and were stored at RT prior to use if not stated otherwise.

4.1 Technical equipment

Table 1. Technical equipment used

Instrument	Manufacturer
Accu-jet® pro pipette aid	Brand GmbH+Co KG, Wertheim, Germany
CCD camera AxioCam HRc	Eppendorf AG, Hamburg, Germany
Dounce tissue grinder 15 ml, 7 ml	Wheaton Science Product, Milville, USA
Centrifuges	
Centrifuge 5804 R	Beckman Coulter, Inc., Palo Alto, CA, USA
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
Filter devices	
Vacuum pump 220 V/50 Hz	
Fritted glass base (2.5 cm diameter) + silicone stopper	Millipore GmbH, Vienna, Austria
Glass funnel 15 ml, glass	
spring clamp, aluminium	
Filtering flask 100 ml, glass	Schott Austria GmbH, Vienna, Austria

Gasprofi 1 SCS micro	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Gelelectrophoresis devices Electrophoresis cell (Sub-Cell GT) Electrophoresis power supply (PowerPac Basic) Sub-Cell GT UV-Transparent Gel Tray (15 x 15) Standard combs UV transilluminator	Bio-Rad Laboratories GmbH, Munich, Germany
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
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, Göttingen, Germany
Spectral photometer SmartSpecTM 3000	Bio-Rad Laboratories GmbH, Munich, Germany
Vortex-Genie 2	Scientific Industries Inc., Bohemia,NY, USA
UV sterilizing PCR workstation	PeqLab Biotechnologie GmbH, Erlangen, Germany
Water baths Haake DC10-P5/U Heating circulator bath	Thermo Fisher Scientific Inc., Waltham, MA, USA
Incubation bath GFL 1004	Gesellschaft für Labortechnik GmbH, Burgwedel, Germany
Water purification system MILLI-Q® biocel	Millipore GmbH, Vienna, Austria
Watervapour high pressure autoclaves Varioclav® 135 S H+P Varioclav® 25 T H+P	H+P Labortechnik GmbH, Oberschleißheim, Germany

4.2 Software

Table 2. Software

Software	Manufacturer
AxioVision 4.8	AxioVision 4.8 Carl Zeiss MicroImaging GmbH, Jena, Germany
GraphPad Prism 5.0 for Mac	GraphPad Software Inc., La Jolla, CA, USA
LSM Image Browser 4.2	Carl Zeiss MicroImaging GmbH, Jena, Germany
Microsoft Office 2011 for Mac	Microsoft Corporation, Redmond, WA, USA

4.3 Disposable items, kits and ready-to-use solutions

Table 3. Disposable items used

Disposable items	Manufacturer
25 cm2 Tissue culture flasks	Asahi Techno Glass Corporation, Iwaki Glass Co., Ltd., Funabashi-City, Japan
500 cm2 Tissue culture flasks	Nunc, Roskilde, Denmark
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
Greiner tubes (15 ml, 50 ml)	Greiner Bio-One GmbH, Frickenhausen, Germany
Isopore™ polycarbonate membrane filters (0.22 µm pore size, 25 mm diameter, black)	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
Plastic pipettes (2 ml, 10 ml)	Barloworld Scientific Ltd., Staffordshire, UK

Plastic tips (various sizes)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Reaction tube 1.5 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Reaction tube 2 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
SafeSeal-Tips® Premium (various sizes)	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Syringe filter, cellulose acetate (0.2 μm)	Asahi Techno Glass Corporation, Iwaki Glass Co., Ltd., Funabashi-City, Japan
Syringe filter, cellulose acetate (5 μm)	Asahi Techno Glass Corporation, Iwaki Glass Co., Ltd., Funabashi-City, Japan
Syringe Injekt®-F 1 ml, single use, sterile	B. Braun Melsungen AG, Melsungen, Germany
Syringe Omnifix® 50 ml, single use, sterile	B. Braun Melsungen AG, Melsungen, Germany

Table 4. Kits used.

Kits	Manufacturer
DNeasy Blood & Tissue Kit	QIAgen, Hilden, Germany
Qiaquick PCR purification Kit	QIAgen, Hilden, Germany

Table 5. Ready-to-use-solutions used

Solutions	Manufacturer
Gene RulerTM 1kb DNA ladder	Fermentas Inc., Hanover, MD, USA

4.4 Chemicals and enzymes

Table 6. Chemicals used.

Chemicals	Manufacturer
4′,6-Diamidino-2-phenylindole (DAPI)	Lactan Chemikalien und Laborgeräte GmbH, Graz, Austria
Boric acid (H_3BO_3)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Citifluor AF1	Agar Scientific Ltd., Stansted, UK
di-Sodiumhydrogen phosphate	Carl Roth GmbH & Co KG, Karlsruhe, Germany

dihydrate (Na_2HPO_4*2 H_2O)	
Dimethyl sulfoxid (DMSO)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Ethanol absolute ($EtOH_{abs.}$)	AustrAlco Österreichische Alkoholhandels GmbH, Spillern, Austria
Ferrous ammonium sulfate hexahydrate ($Fe(NH_4)_2(SO_4)_2$*6 H_2O)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Formaldehyde 37% (w/w) Rotipuran®	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Formamide deionized	Carl Roth GmbH & Co KG, Karlsruhe, Germany
α-D(+)-Glucose monohydrate	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Hydrochloric acid 37% (w/w) (HCl)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
L-Glutamic acid	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
Potassium dihydrogen phosphate (KH_2PO_4)	Mallinckrodt Baker B.V., Deventer, Holland
Proteose peptone	Oxoid Ltd., Hampshire, England
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_2 PO_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
Sucrose	Merck GmbH, Vienna, Austria
Trypticase Soy Broth	Oxoid Ltd., Hampshire, England
Tris Pufferan®	Carl Roth GmbH & Co KG, Karlsruhe, Germany
tri-Sodium citrate-dihydrate	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Yeast Extract (for amoebae cultivation)	Oxoid Ltd., Hampshire, England

Table 7. Enzymes used.

Enzymes	Manufacturer
Taq DNA Polymerase (5U/μl)	Fermentas Inc. Hanover, MD, USA

Table 8. Antibiotics used.

Antibiotic	Manufacturer
Cycloheximide	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Geneticindisulfat	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Rifampicin	Carl Roth GmbH & Co KG, Karlsruhe, Germany

4.5 Primers and probes

Table 9. Primers used. All primers were manufactured by Thermo Fisher Scientific GmbH, Ulm, Germany.

Name	Sequence (5'-3')	Target molecule	Specificity	Comment	Reference
PanR	GTCATCRGCCYYACCTTVSRCRYYTCT	16S rRNA gene	Chlamydiales	Ta: 65°C	Corsaro et al ., 2002
PanF	CGTGGATGAGGCATGCRAGTCG	16S rRNA gene	Chlamydiales	Ta: 65°C	Corsaro et al ., 2002
PcR	TTNNGGATTTGCTTCVCC	16S rRNA gene	Para-chlamydiaceae, Waddliaceae	Ta: 58°C	Horn and Wagner, 2001
PcF	TCAGATTGAATGCTGAC	16S rRNA gene	Para-chlamydiaceae, Waddliaceae	Ta: 58°C	Horn and Wagner, 2001
Aas 79F	ACA CTT CGG TGT TGC TGG	16S rRNA gene	*A. asiaticus*	Ta: 50°C	
Aas 1281R	ATT GGC CGC TTG TTA CAA	16S rRNA gene	*A. asiaticus*	Ta: 50 – 50.9°C	
Aas 1467R	GTC GCT GAT CTA ACC CTA	16S rRNA gene	*A. asiaticus*	Ta: 50.9°C	
ISCaa2F	TTG GGT GTA AAA AGA GGC AAT	IS element	*A. asiaticus*	Ta: 63,7°C	Schmitz-Esser et al. in prep.
ISCaa2R	GCA TGG CGA TAA CAT TTT CA	IS element	*A. asiaticus*	Ta: 63,7°C	Schmitz-Esser et al. in prep.
ISCaa4F	AGA GGT AGA CAG CGC TAC CAG	IS element	*A. asiaticus*	Ta: 63,7°C	Schmitz-Esser et al. in prep.

ISCaa4R	TAG CTT GGC CAT TAG CAG GT	IS element	*A. asiaticus*	Ta: 63,7°C	Schmitz-Esser et al. in prep.
ISCaa7F	AAA GCT CGC CAA TTC TCA AC	IS element	*A. asiaticus*	Ta: 63,7°C	Schmitz-Esser et al. in prep.
ISCaa7R	TTG GCC ATC GGA TGT ACT TT	IS element	*A. asiaticus*	Ta: 63,7°C	Schmitz-Esser et al. in prep.

Table 10. List of used probes. All probes were manufactured by Thermo Fisher Scientific GmbH, Ulm, Germany.

Name	Sequence (5'-3')	Target molecule	Specificity	Comment	Reference
APH-1180	CTGACCTCATCCCCTCCT	16S rRNA	*A. asiaticus*	Cy3-labelled	Horn et al., 2001
Chls-0523	CCTCCGTATTACCGCAGC	16S rRNA	Chlamydiales	Cy3-labelled	Poppert et al., 200
Acanth-412a	ACTCTTATCGAGCGCCTG	18S rRNA	*Acanthamoeba* sp.	Cy5-labelled	In prep.
EUK516	ACCAGACTTGCCCTCC	18S rRNA	Eukarya	Cy5-labelled	Amann et al., 1990
EUB338	GCTGCCTCCCGTAGGAGT	16S rRNA	most Bacteria	FLUOS-labelled	Amann et al., 1990
EUB338	GCTGCCTCCCGTAGGAGT	16S rRNA	most Bacteria	FLUOS-double-labelled	Amann et al., 1990

4.6 Organisms used in this thesis

All flask and vessels containing *Acanthamoeba* sp. 5a2 and *A. asiaticus 5a2* or other symbionts of the *Acanthamoeba* were only opened in a laminar flow in order to reduce the risk of contamination and due to safety precautions for the workers.

Table 11. Organism used.

Organism	Symbiont	Source	Reference
Acanthamoeba sp. 5a2	none	Salt lake sediment	Horn et al., 2001
Acanthamoeba sp. 5a2	*A. asiaticus* 5a2	Salt lake sediment	Horn et al., 2001
Acanthamoeba sp. 5a2	*A. asiaticus* US1	Salt lake sediment	Horn et al., 2001
Acanthamoeba sp. 5a2	*A. asiaticus* EIDS3	Salt lake sediment	Horn et al., 2001

4.7 General media, buffers and solutions

4.7.1 Media for cultivation of amoebae

Trypticase Soy Broth with Yeast Extract (TSY)	
Trypticase Soy Broth	30 g
Yeast extract	10 g
ddH_2O	ad 1000 ml
pH 7.3	

PYG	
Peptone	20 g
Glucose	18 g
Yeast extract	2 g
MgSO4 x 7 H_2O	980 mg
Sodiumcitrate	1 g
Na_2HPO_4 x 7 H_2O	355 mg
KH_2PO_4	340 mg
$Fe(NH_4)_2(SO_4)_2$ x 6 H_2O	20 mg
ddH_2O	ad 1000 ml
pH 6.5	

PYNFH (modified)	
Bacteriological peptone	10 g
Yeast extract	10 g
Yeast nucleic acid	1 g
Folic acid	15 mg
Hemin	1 g
KH_2PO_4	2.6 mM
Na_2HPO_4*$2H_2O$	2.8 mM
ddH_2O	ad 1000 ml

4.7.2 General buffers

10 x Page's Amoebic Saline (PAS)	
NaCl	1.20 g
$MgSO_4$ x $7H_20$	0.04 g
$CaCl_2$ x H_20	0.04 g
NaH_2PO_4 x $2H_20$	1.78 g
KH_2PO_4	1.36 g
ddH_2O	ad 1000 ml

1 x Page's Amoebic Saline (PAS)	
10 x PAS	100 ml
ddH_2O	ad 1000 ml

SPG-Buffer	
Sucrose	75 g
KH_2PO_4	0.52 g
Na_2HPO_4 x 2 H_2O	2.30 g
Glutamic acid	0.75 g
ddH_2O	ad 1000 ml

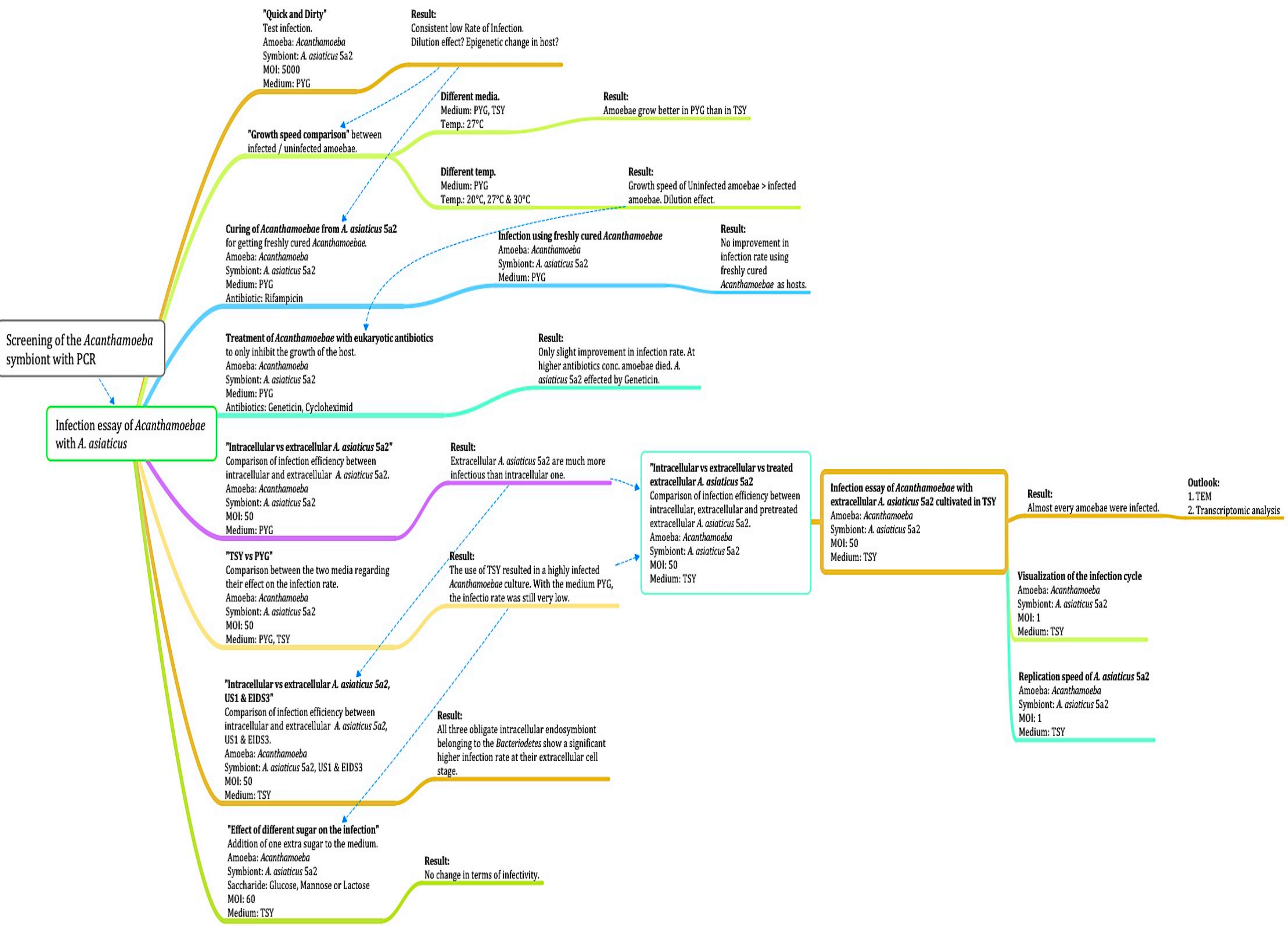
4.7.3 Gel electrophoresis: Buffers, solutions and standards

10 x TBE buffer	
Tris-HCl	162.0 g
Boric Acid	27.5 g
EDTA	9.3 g
ddH_2O	ad 1000 ml

1 x TBE buffer	
10 x TBE	100 ml
ddH_2O	ad 1000 ml

Loading buffer	
Ficoll	25 % (w/v)
Bromphenol blue	0.5 % (w/v)
Xylencyanol	0.5 % (w/v)
EDTA	50 mM

Ethidium bromide solution	
Ethidium bromide stock solution:	10 mg/ml Ethidium bromide (EtBr) in ddH_2O
Ethidium bromide staining solution:	Ethidium bromide stock solution diluted 1: 10.000 in ddH_2O



Scheme 1. The scheme of the diploma thesis.

4.8 General Methods

Scheme 1 shows the chain of thought of this diploma thesis. Those thick lines resemble the performed experiments and their results. The blue arrows indicate that the experiment they point to is subsequently influenced by the result of former experiments.

4.8.1 **Cultivation of acanthamoebae and obligate intracellular endosymbionts of the *Acanthamoeba***

Cultures of *Acanthamoeba* sp. 5a2 harboring *A. asiaticus* 5a2, *Acanthamoeba* sp. harboring *A. asiaticus* EIDS3 as well as non-infected *Acanthamoeba* sp. 5a2 were maintained as adherent culture in 25 cm^2 polystyrene culture flasks (Nunc) containing 10 ml PYG medium (4.7.1) at 27°C in the dark if not stated otherwise. *Acanthamoeba* sp. harboring *A. asiaticus* US1 were incubated under the same condition by using 10 ml PYNFH medium (4.7.1) instead of PYG medium. A backup 500 cm^2 polystyrene culture flask (Nunc) containing 150 ml PYG medium or 150 ml PYNFH medium for acanthamoebae harboring *A. asiaticus* US1 respectively was cultivated for each culture. Cultures were passaged at confluence by 1:10 dilution of the culture every five to ten days, depending on the condition of the amoebae. A 1:10 dilution couldn't be applied to a culture consisting mainly of cysts due to the resulting high loss rate of acanthamoebae. In this case the culture will be diluted 1:3. Two to three days prior to an experiment, the cultures in need were freshly fed by exchanging half of the media. The condition of *Acanthamoeba* cultures was monitored on a regular basis by light microscopy. For determination of the growth condition, the total cell number and the morphology of the amoebae were taken into account. A culture was considered as well-grown when the culture flask's bottom surface is densely covered with attached amoebal cells in trophozoite stage and only a low amount of detached amoebal cells floating in the surrounding medium were present. A high percentage of cyst-forming acanthamoebae in a culture often indicated a lack of nutrients or other unbeneficial environmental factors like changes in pH value and temperature.

4.8.2 **Harvesting of amoebae**

Culture flasks containing well-grown *Acanthamoeba* with or without endosymbionts were shaken vigorously to detach amoebae from the surface. The resulting cell suspension was poured into 50 ml Greiner tubes. Amoebae were obtained by centrifugation with 6600 rpm for six minutes at RT. The supernatant was decanted and the pellet resuspended in a desired amount of media of choice. A larger amount of cells were harvested by pooling the pellet of several Greiner tubes together.

4.8.3 **Quantification of amoebal cells**

The concentration of harvested acanthamoebae was determined by using a Neubauer haemocytometer. The culture flask was shaken vigorously in order to detach all amoebae. 12 µl of the suspension was pipetted at each side of the counting chamber covered by a cover glass. The amount of amoebae residing in four big squares at each side of the counting chamber was counted by using an inverse phase contrast microscope (10x objective, no immersion, Zeiss). The total number of counted cells was divided by 8 (the size of the counting area was 8 x 1 mm^2) and the depth of the chamber (0.1 mm).

$$\text{Conc. of cells in 1 ml} = \left(\frac{\text{Number of cells counted}}{\left(\text{Number of squares counted x 1 mm}^2\right)\left(0.1\ \text{mm}\right)} \right) x\ 1000$$

Highly concentrated acanthamoebae suspension were diluted 1:10 (10 μl of acanthamoebae suspension + 90 μl PYG medium) for an accurate counting.

4.8.4 **Harvesting of *A. asiaticus***

4.8.4.1 **Harvesting intracellular *A. asiaticus* 5a2, EIDS3 or US1**

Acanthamoebae containing *A. asiaticus* endosymbionts of choice were collected from a culture flask in a 50 ml Greiner tube and pelleted by centrifugation with 6600 rpm for six minutes at RT. The pellet was resuspended in five ml PYG buffer and transferred into the dounce homogenizer. The tight glass pestle was used to disrupt the amoeba for 15 times for breaking up the acanthamoebae and releasing the intracellular symbionts. This homogenized suspension was filtered (five µm pore) to separate cell debris and unlysed acanthamoebae from the intracellular *A. asiaticus* 5a2. The harvested intracellular *A. asiaticus* could now be used for infection.

4.8.4.2 **Harvesting extracellular *A. asiaticus* 5a2, EIDS3 or US1**

The media of a well grown acanthamoebae culture harboring *A. asiaticus* of choice was taken from a 500 cm^2 polystyrene culture flask, filtered (5 µm pore) and then poured into a 50 ml Greiner tube without detaching the *Acanthamoeba* first. After centrifugation with 9000 rpm for five minutes at RT, the pellet was resuspended in five ml PYG medium. The harvested extracellular *A. asiaticus* could now be used for infection.

4.8.5 **Quantification of isolated *A. asiaticus***

The concentration of *A. asiaticus* suspension was determined as followed.
A 1:5000 dilution was done for 1 µl of *A. asiaticus* suspension by addition of five ml sterile 1x PAS. The filter device was flame sterilized and assembled by using two different filters. The first filter was the 0.45 µm cellulose acetate membrane support filter (Sartorius), which came underneath the main filter, a black 0.22 µm polycarbonate membrane filter (Millipore). A vacuum pump (Millipore)

running at 250 mmHg was attached onto the filter device. The funnel was first rinsed with 5 ml sterile 1x PAS followed by the filtration of the symbiont suspension. Another rinsing step was needed to clean the funnel from attached bacteria. Subsequently, the vacuum pump was switched off enabling the membrane being incubated with 400 µl of 1:1000 DAPI solution for five minutes in the dark. Afterwards the vacuum pump is turned on again and the DAPI solution pumped off. The last rinsing step with five ml sterile 1x PAS will follow. The membrane was then transferred to an uncoated glass microscope slide. A drop of the anti-bleaching mounting medium Citifluor was added to the middle of the membrane and serves as an adhesive for attaching the cover glass. The cover slide should be stored in the dark in order to prevent the DAPI from bleaching before subjected to epifluorescence microscopy.
At least 10 random spots on the membrane were chosen for counting. The 100x oil immersion objective was used for counting the DAPI signals within the 10 x 10 grid.

Concentration of cells was calculated as follows:

$$M_1\,(\text{Microscope factor 1}) = \frac{\text{membrane area exposed to filtration (j=15 mm)}}{\text{grid area (j= 12.5 mm/100)}} = \frac{\pi\ x\ r^2}{a^2}$$

$$= \frac{\pi \text{ x } 7.5\,(\text{mm}^2)}{0.125\,(\text{mm}^2)} = \frac{176\ \text{mm}^2}{0.015625\ \text{mm}^2} = 11264$$

$$\text{Cells/ml} = \left(\frac{\text{Total number of counted cells}}{\text{Number of randomly counted grids}}\right) \text{x } 11264 \text{ x } 1000$$

4.8.6 Curing of *Acanthamoeba* from intracellular endosymbionts.

This assay was performed to remove all intracellular endosymbionts and successfully cure an *Acanthamoeba* culture. Acanthamoebae containing *A. asiaticus* 5a2 cultivated in PYG medium at 27ºC were treated with 100 µg/ml Rifampicin for 4 weeks. Fresh media containing Rifampicin was changed on

weekly basis. Progress of curing was examined by FISH with *A. asiaticus* 5a2 specific probes and PCR with specific 16S rRNA primer pairs for *A. asiaticus*.

4.8.7 Isolation of acanthamoebae and *A. asiaticus* 5a2 DNA

The DNeasy Blood and Tissue Kit from QIAgen was used for the total DNA isolation from acanthamoebae harbouring *A. asiaticus* 5a2. $EtOH_{abs}$ was added to the AW1 and AW2 buffer as indicated on the bottle. All reagents needed were supplied in the kit except for $EtOH_{abs}$. All steps were performed according to the instruction manual of the manufacturer. 50 μl DEPC-treated ddH_2O were used for elution instead of the supplied AE elution buffer. The Nano Drop® device was used for the spectrophotometrical determination of the DNA concentration.

4.8.8 Polymerase Chain Reaction

Distinction between *A. asiaticus* 5a2, EIDS3 and US1 could only be achieved by using IS-element specific primers, since they have IS-elements.

Table 12. Different symbionts with their distinctive IS element pattern.

	A. asiaticus 5a2	*EIDS3*	*US1*
ISCaa4	+	-	-
ISCaa5	+	+	+
ISCaa7	+	-	-
ISCaa2	+	+	-

All isolated DNA were diluted to 100 ng/μl. In the negative control, ddH_2O was used instead of the template DNA.

Table 13. Components and concentration used for PCR.

Component	Manufacturer	Final conc.	Volume/50 μl reaction

$MgCl_2$ (25 mM)	Fermentas Inc., Hanover, MD, USA	2 mM	4 μl
10 x Taq buffer (+ KCL)	Fermentas Inc., Hanover, MD, USA	1 x	5μl
dNTP mix (2mM each)	Fermentas Inc., Hanover, MD, USA	0,2 mM of each	5μl
Taq DNA polymerase (5 u/μl)	Fermentas Inc., Hanover, MD, USA	1 u	0.2 μl
Forward primer (50 pmol/μl)	Thermo Fisher Scientific GmbH, Ulm, Germany	25 pmol	0.5 μl
Reverse primer (50 pmol/μl)	Thermo Fisher Scientific GmbH, Ulm, Germany	25 pmol	0.5 μl
ddH_2O	Mayrhofer Pharmazeutika GmbH & Co KG, Leonding, Austria	-	32.8 μl
DNA template		100 ng	1 μl

Table 14. Parameters of PCR

Step	Temperature (°C)	Time	Nr. of cycles
Denaturation	95	5 min	
Denaturation	95	30 sec	
Annealing	63.7	30 sec	35 x
Elongation	72	45 sec	
Final Elongation	72	10 min	

Table 15. Expected fragments sizes.

Primer used	Primer sequence (5'-3')	Length of amplificate (bp)	Annealing temp (°C)
ISCaa4 F	AGA GGT AGA CAG CGC TAC CAG	602 bp	63.7
ISCaa4 R	TAG CTT GGC CAT TAG CAG GT		
ISCaa5 F	GCC TTA TAC TTT GGC GGA CA	423 bp	63.7
ISCaa5 R	TGC ACT ACC AGC AGG CAT AG		
ISCaa7 F	AAA GCT CGC CAA TTC TCA AC	443 bp	63.7
ISCaa7 R	TTG GCC ATC GGA TGT ACT TT		
ISCaa2 F	TTG GGT GTA AAA AGA GGC AAT	745 bp	63.7
ISCaa2 R	GCA TGG CGA TAA CAT TTT CA		

4.8.9 Gel electrophoresis

5 μl of the 50 μl PCR reaction product was mixed with 5 μl loading buffer. PCR products and 6 μl Gene Ruler^{TM} 1kb DNA ladder (Fermentas) were loaded on 1,5 % TBE gel (3 g agarose, 200 ml 1x TBE buffer). Parameter for gel electrophoresis was 125 mV for 1 hour. The staining of the DNA bands took place in an ethidium bromide bath (100 μl ethidium bromide stock solution in 1l ddH_2O) for 30 - 45 min.

4.8.10 Visualization of infection by Fluorescence in situ hybridization (FISH) utilization

4.8.10.1 Preparation of Sample

Two ml acanthamoebae with and without symbionts were pelleted in a 2 ml microcentrifuge tube by centrifugation with 6600 rpm, six minutes at RT. The supernatant was decanted and the pellet resuspended in 60 – 200 μl 1x PAS depending on the size of the acanthamoebae pellet on the bottom of the microcentrifuge tube.

4.8.10.2 Fixation of Sample

20 μl of the prepared sample was pipetted onto a well of a Teflon-coated glass slide with 30 min waiting time for the amoebae to attach to the surface. Afterwards the liquid was removed carefully by pipetting and 20 μl 4% PFA solution were applied onto the sample for 12 min at RT. The PFA was removed after fixation, followed by an immediate washing step with 20 μl ddH_2O. The ddH_2O should be taken off immediately after two sec of washing in order to

prevent detaching of the amoebal cells. The slide was put into an incubation oven (46ºC) to let the samples dry. Slides prepared in this way were used directly for FISH or stored at -20ºC.

4.8.10.3 Hybridisation

Hybridisation was performed using 10 µl hybridisation buffer together with 1 µl of each probe for one well. If not stated otherwise, the formamide concentration of the hybridisation buffer was 20%. The slides were incubated in a closed and hybridisation buffer saturated environment at 46ºC in the dark for 90 – 120 min. Afterwards the slides were washed in prewarmed (48ºC) washing buffer for 13 min. The last step consists of a hyperstringent 2 sec washing step in ice-cold ddH_2O followed by immediate air-compressed drying.

Table 16. Composition for FISH Hybridisation buffer (20% Formamide)

Hybridisation Buffer	
5 M NaCl	180 µl
1 M Tris/HCl (pH8)	20 µl
ddH_2O	600 µl
Formamide	200 µl
10% (w/v) SDS	1 µl
Total Volume	1000 µl

Table 17. Composition for FISH Washing buffer (20% Formamide)

Washing Buffer	
5 M NaCl	2150 µl
1 M Tris/HCl (pH8)	1000 µl
0.5 M EDTA	500 µl
ddH_2O	Ad 50 ml
10% (w/v) SDS	50 µl
Total Volume	50 ml

4.8.10.4 Image Analysis

Slides needed for microscopy analysis were pre-treated prior to the analysis. The surface of the side with the samples was mounted evenly in Citifluor to prevent bleaching and enclosed with a cover glass. Visualisation of the cells was utilized by epifluorescence microscopy (Zeiss Axioplan 2) or by using a confocal laser-scanning microscope (CLSM, LSM 510 by Zeiss). The latter one is equipped with two helium-neon lasers (633 nm, 543 nm), an argon-krypton laser (488 nm) and a UV laser (351-364 nm). Image analysis processing was performed with the standard software suite delivered with the instrument.

4.9 Infection of *Acanthamoeba* sp. 5a2 with *A. asiaticus* 5a2

Two days prior to the experiments well-grown uninfected *Acanthamoeba* sp. were harvested by detaching the amoebae from a 500 cm^2 culture flask, transfer five ml of media into each 25 cm^2 culture flask and filled it up with fresh PYG medium ad 10 ml. Prior to the infection assay, the amoebae cultures were examined under the light microscope for their growth. The amoebae should form a monolayer on the culture flask surface for this experiment. Non-attached amoebae were removed by decanting the media without shaken it first and filled up with fresh PYG medium ad 10 ml again. After the addition of *A. asiaticus* 5a2 to the *Acanthamoeba* host, the infection was stimulated by centrifugation of 25 cm^2 culture flasks with 1000 rpm for 15 minutes at RT. Progress of the infection was carefully examined by utilizing fluorescence in situ hybridisation (4.8.10) with specific probes for *A. asiaticus* 5a2 16S rRNA genes (Table 10. List of used probes). All infection assays were performed as specified above if not stated otherwise.

4.9.1 Infection of acanthamoebae with PYG-cultivated *A. asiaticus* 5a2

The intracellular *A. asiaticus* 5a2 was harvested (4.8.4.1) and 2 ml of the symbiont suspension were added to the pre-cultivated uninfected acanthamoebae for a MOI of 5000. The infected acanthamoebae were incubated at 27°C. Six biological replicates with three technical replicates each had been

done. Time range of observation was from 1 hour post infection (hpi) to 3 weeks post infection.

4.9.2 **Treatment of acanthamoebae with eukaryotic antibiotics**

Uninfected *Acanthamoeba* sp. 5a2 and intracellular *A. asiaticus* 5a2 both cultivated in PYG were harvested and quantified as described in (4.8.2, 4.8.4.1). The *A. asiaticus* 5a2 pellet was resuspended in SPG medium instead of PYG. 1 ml of the *A. asiaticus* 5a2 suspension was used for the infection. 10 μl/ml and 100 μg/ml of the antibiotics geneticin and cycloheximide were used for the treatment. Each cell culture was treated with just one concentration of an antibiotic. All other steps were performed as described in 4.9.1. Two biological replicates with two technical replicates had been done.

4.9.3 **Comparison of infection rate between intracellular and extracellular *A. asiaticus* 5a2**

The uninfected host *Acanthamoeba* sp. were harvested, quantified and seeded in 25 cm^2 culture flasks. The extracellular *A. asiaticus* 5a2 were harvested (4.8.4.2) followed by a brief washing step to remove all extracellular *A. asiaticus* 5a2 from the attached acanthamoebae inside the culture flask. Subsequently the intracellular *A. asiaticus* 5a2 cells were harvested (4.8.4.1). The host amoebae were then either infected with the extracellular *A. asiaticus* 5a2 or the intracellular *A. asiaticus* 5a2 using a MOI of 50. Two biological replicates with three technical replicates had been done. The infection was observed from 4 hpi to 120 hpi.

4.9.4 **Comparison of infection rate between PYG and TSY grown acanthamoebae and *A. asiaticus* 5a2**

The uninfected acanthamoebae culture as well as the acanthamoebae

harbouring *A. asiaticus* 5a2 pregrown in TSY medium for two weeks had only been filled up with fresh TSY medium instead of PYG medium. PYG-grown cultures were treated as stated above. Harvested TSY-grown *A. asiaticus* 5a2 was only used to infect acanthamoebae cultivated in TSY; PYG-grown *A. asiaticus* 5a2 was only used to infect acanthamoebae cultivated in PYG. All other steps were performed as stated in 4.9.1. Three biological replicates with three technical replicates had been done. The infection was observed up to 168 hpi.

4.9.5 Comparison of infection rate between extra- and intracellular cell-stage of *A. asiaticus* 5a2, *A. asiaticus* US1 and *A. asiaticus* EIDS3

All cultures used in this assay were only cultivated in TSY medium except acanthamoebae infected with *A. asiaticus* US1, which were cultivated in the medium PYNFH (4.7.1). All other steps were performed as stated in 4.9.3. Two biological replicates with three technical replicates had been done. The infection was observed up to 72 hpi.

4.9.6 The effect of different sugars on the infection of acanthamoebae with *A. asiaticus* 5a2.

TSY-media with addition of one saccharide (mannose, lactose or glucose) for an end-concentration of 100 mM and 200 mM were mixed. In total, seven 500 cm^2 culture flask were prepared, two concentration for each saccharide plus an untreated culture as control. Extracellular *A. asiaticus* 5a2 was used to infect acanthamoebae, all grown in TSY. All other steps were performed as stated in 4.9.1 except the use of MOI 60 instead of 5000. The infection was observed up to 48 hpi.

4.9.7 Comparison of infection rate between intracellular, extracellular and pre-treated extracellular *A. asiaticus* 5a2.

All organisms were incubated in TSY-medium for this assay. Intracellular and extracellular *A. asiaticus* 5a2 were harvested and quantified. The extracellular *A. asiaticus* 5a2 was splitted and one part was pre-treated prior to the infection. The pre-treated part was homogenized by a dounce homogenizer and resuspended in amoeba cell lysate of uninfected acanthamoebae to simulate the treatment of intracellular *A. asiaticus* 5a2. All other steps were performed as in 4.9.3.

4.9.8 **Infection assay using TSY medium and extracellular *A. asiaticus* 5a2 to visualize the cycle**

All steps were performed as in 4.9.4. Extracellular *A. asiaticus* 5a2 was used instead of intracellular *A. asiaticus* 5a2 for this assay. All cultures used in this assay were cultivated in TSY-medium. Extracellular *A. asiaticus* 5a2 was used to infect $2x10^6$ acanthamoebae in one culture flask. A MOI of 1 was used. Three biological replicates with three technical replicates had been done. The infection was observed up to 144 hpi. The time points of host cell entry, replication and lysis of the host amoebae were determined.

4.9.9 **Determination of *A. asiaticus* 5a2s duplication time**

An infection assay was performed as described in 4.9.8. Of each time point, the quantity of intracellular *A. asiaticus* 5a2 was counted and analysed. Based on the number, the duplication rate of *A. asiaticus* 5a2 was calculated. Three biological replicates with three technical replicates had been done.

4.9.10 **Sample preparation for transmission electron microscopy (TEM)**

An infection assay with TSY-grown acanthamoebae as well as acanthamoebae infected with *A. asiaticus* 5a2 at the MOI 50 was started. At the time point 5 hpi, 72 hpi and 144 hpi, 1,5 ml acanthamoebae suspension was transferred from a 25

cm² culture flask into a 1,5 ml microcentrifuge tube with screw-cap. In addition, a sample of the extracellular *A. asiaticus* 5a2 was also taken according to 4.8.4.2. The samples were then centrifuged (for amoebae: 6600 rpm, 6 min, RT; for *A. asiaticus* 5a2: 9000 rpm, 5 min, RT). After centrifugation, the supernatant was decanted and the pellet carefully resuspended in 1 ml fixation buffer (3,5% glutaraldehyde in 0,1M phosphate buffer). The sample was stored at 4˚C until further use.

4.10 Difference in growth rate between infected / uninfected amoebae

4.10.1 **Growth in PYG medium under three different temperatures**

Uninfected amoebae and amoebae infected with *A. asiaticus* 5a2 were harvested (4.8.2) and resuspended in 5 ml fresh sterile PYG. Three 6-Well multiwell plates were prepared, one plate for each temperature point (20ºC, 27ºC and 30ºC). 3 ml of fresh sterile PYG medium was put into each well. In the upper row 10 μl of the uninfected amoebal cell suspension were seeded in each well on all three plates, where as in the lower row 10 μl of the infected amoebal cell suspension were added. The multiwell plates were cultivated at 20˚C, 27˚C and 30˚C. Every 24 h (including 1 h post seeding) 10 pictures of each well were randomly taken with the inverse microscope. The average number of amoeba inside a field of vision was calculated for each condition (infected, uninfected, 3 different temperatures). The growth was observed till 120 h post seeding. Two biological replicates with three technical replicates had been done.

4.10.2 **Growth under 27˚C using PYG and TSY.**

Uninfected amoebae and amoebae infected with *A. asiaticus* 5a2 were harvested (4.8.2) and resuspended in 5 ml PYG. Two 6-Well multiwell plates were prepared, one plate for each medium. All two plates were incubated at 27˚C. On one plate, 3 ml of fresh sterile PYG medium was put into every well where as

the plate used TSY medium. In the upper row of both plates 10 μl of the uninfected amoebal cell suspension were seeded. In contrast 10 μl of the infected amoebal cell suspension were added in the lower row of both plates. Every 24 h (including 1 h post seeding) 15 pictures of each well were randomly taken with the inverse microscope. The average number of amoeba inside a field of vision was calculated. The growth was observed till 96 h post seeding. Two biological replicates with three technical replicates had been done.

5 RESULTS

5.1 IS-elements specific PCR for examination of *A. asiaticus* strains

The cultures acanthamoebae carrying *A. asiaticus* 5a2, acanthamoebae infected with *A. asiaticus* EIDS3 and acanthamoebae harbouring *A. asiaticus* US1 were checked for the endosymbiont by means of PCR prior to the infection experiments to verify the strain of the intracellular endosymbiont. The differentiation between *A. asiaticus* 5a2, EIDS3 and US1 with PCR using *A. asiaticus* specific 16S gene primers was not possible. To verify the identity of the endosymbionts in the acanthamoebae harboring them, they needed to be distinguished by the IS-elements they possess (Table 18). A PCR was done with the isolated genomic DNA of acanthamoebae harboring *A. asiaticus* different strains. All cultures were harbouring the expected endosymbiont.

Table 18. A list of present IS-elements in *A. asiaticus* 5a2, *A. asiaticus* EIDS3 and *A. asiaticus* US1.

	A. asiaticus 5a2	*A. asiaticus* EIDS3	*A. asiaticus* US1
ISCaa4	+	-	-
ISCaa5	+	+	+
ISCaa7	+	-	-
ISCaa2	+	+	-

5.2 Utilization of a grading system for approximation of infection efficiency

For the analysis of infection efficiency, a grading system was developed, where different efficiencies of infection were given a score. The scale was based on the amount of infected cells in the total culture as well as the amount of intracellular endosymbionts per cell.

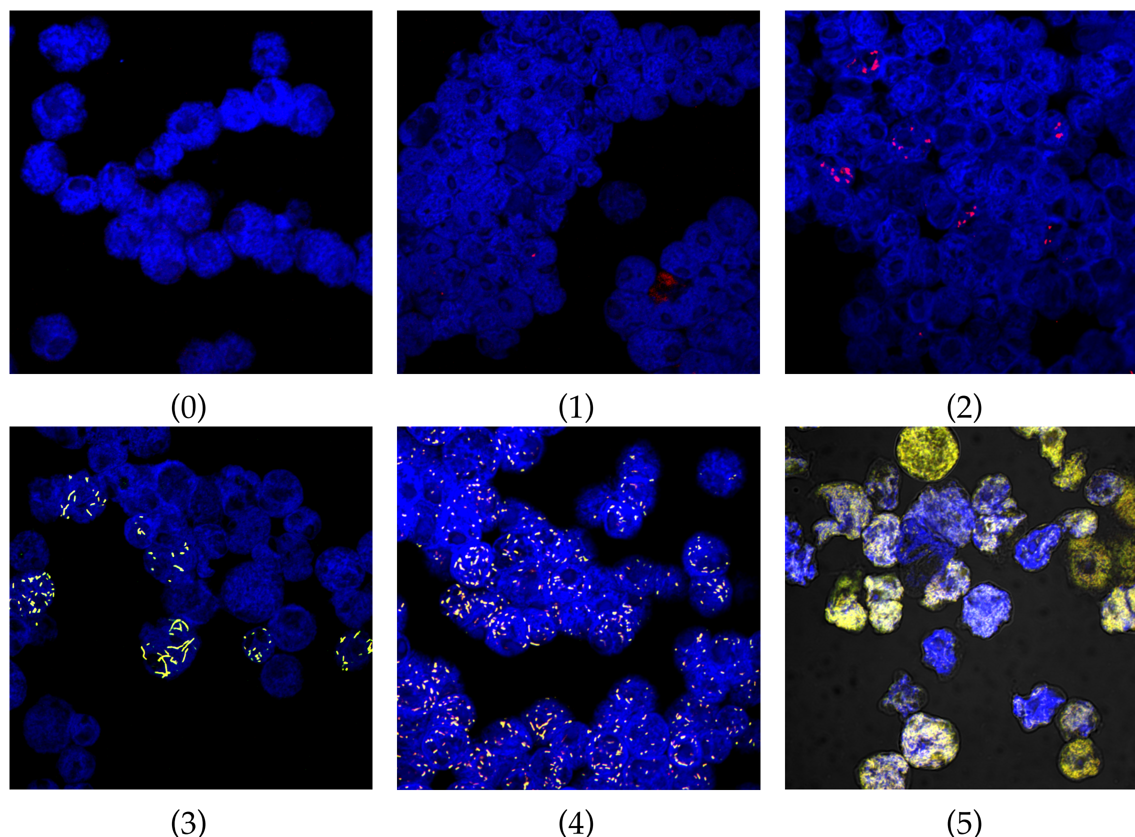


Figure 4. The grading system for approximation of infection efficiency. 0 is the lowest score resembling no infected cells where as 5 resembles the continuous culture with more than 90% of the cells being infected.

5.3 Infection of *Acanthamoeba* sp. with *A. asiaticus* 5a2

Initially an infection of *Acanthamoeba* sp. with *A. asiaticus* 5a2 was performed by using a fast and straightforward protocol established in the lab for chlamydial symbionts. (4.9.1)

After 48 hours of infection with *A. asiaticus* 5a2, only few amoebae were infected, resulting in a low efficiency of infection with a grade of 1 based on Figure 4. The intracellular symbionts can be seen in Figure 5 as yellow short rods. The color yellow results from the binding of the *A. asiaticus*-specific APH1180-Cy3 probe and the bacterial-specific probe EUB338-FLUOS. At later time points the symbiont cells started to elongate by a small margin. The infection efficiency remained at 1 throughout the whole observation time and the number of infected amoebae even began to drop slightly after 13 days post

infection. The repetitive use of an excessive quantity of *A. asiaticus* 5a2 up to MOI 5000 did not improve the number of infected cells.

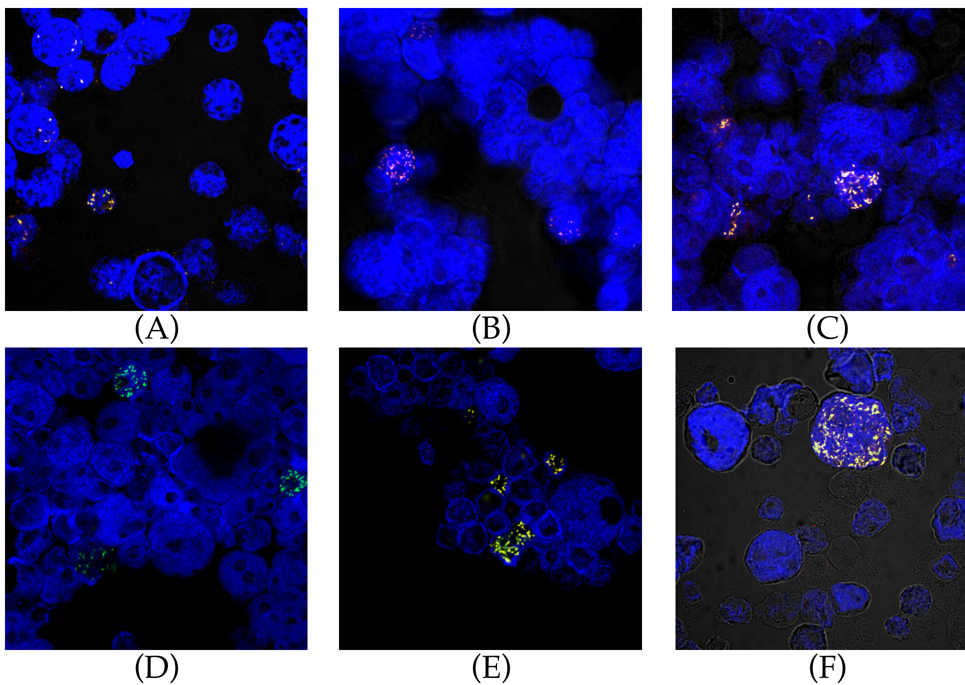


Figure 5. Infection assay with acanthamoebae and *A. asiaticus* 5a2 at MOI 5000. Probes used: APH1180-Cy3 (red), EUB338-FLUOS (green), EUK516-Cy5 (blue). (A) 48 hpi. (B) 128 hpi. (C) 176 hpi. (D) 9 dpi. (E) 11 dpi. (F) 13 dpi

5.4 Infection assay with *A. asiaticus* 5a2 with cured acanthamoebae as host

This assay was performed to rule out the possible resistance to infection due to long term in vitro propagation that could have occurred in the *Acanthamoeba* host. Acanthamoebae harbouring *A. asiaticus* 5a2 were cured and then used as host for this infection assay.

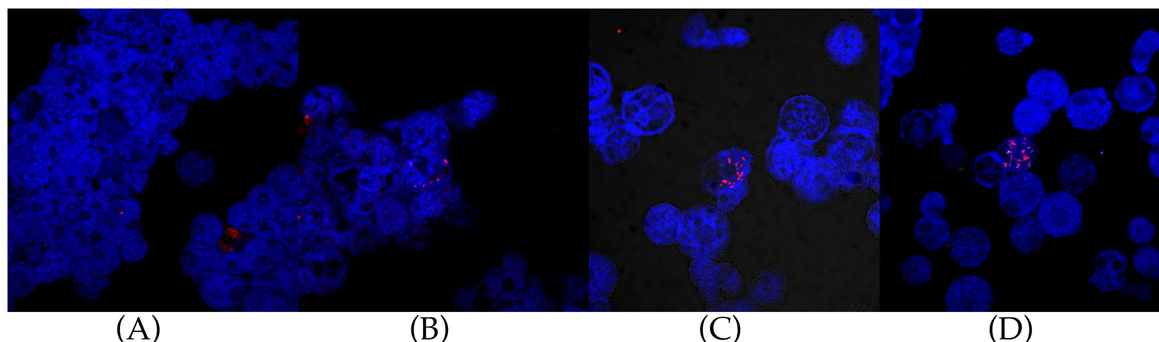


Figure 6. Infection with cured amoebae cells. Used Probes: APH1180-Cy3 (red), EUK516-Cy5 (blue) & Acanth412-Cy5 (blue). Pictures taken at (A) 27 hpi. (B) 48 hpi (C) 72 hpi and (D) 96 hpi

The infection efficiency remained at 1 comparable with the infection using non-cured uninfected acanthamoebae. There were no visible differences regarding the infection efficiency with the use of cured acanthamoebae. *A. asiaticus* 5a2 seemed to replicate normally since the amount of intracellular endosymbiont per infected amoebal cell increased with later time points. (Figure 6)

5.5 Uninfected acanthamoebae show a faster growth compared to acanthamobae harbouring *A. asiaticus* 5a2

The difference in growth rate between *Acanthamoeba* sp. infected with the *A. asiaticus* 5a2 and uninfected *Acanthamoeba* sp. was determined by phase contrast microscopy. Acanthamoebae in division were still counted as one single cell (4.10.1).

5.5.1 Growth of uninfected *Acanthamoeba* sp.

Regardless of which temperature (20°C, 27°C and 30°C), at 1 hour after seeding (hps), most cells were already attached to the surface with only very few cells still floating in the medium. Determination of cell attachment was done by gently shaking the multiwell plate to stir the medium. A large portion of the cells was at the trophozoite cell stage, suggesting a positive cell condition. The centre of the multiwell plate always showed a higher density of amoebae than the area near the rim of the well. Pictures (like Figure 7) of the amoebae were

taken and the amount of attached acanthamoebae counted. In order to avoid bias in cell counting, the spots for counting were chosen randomly.

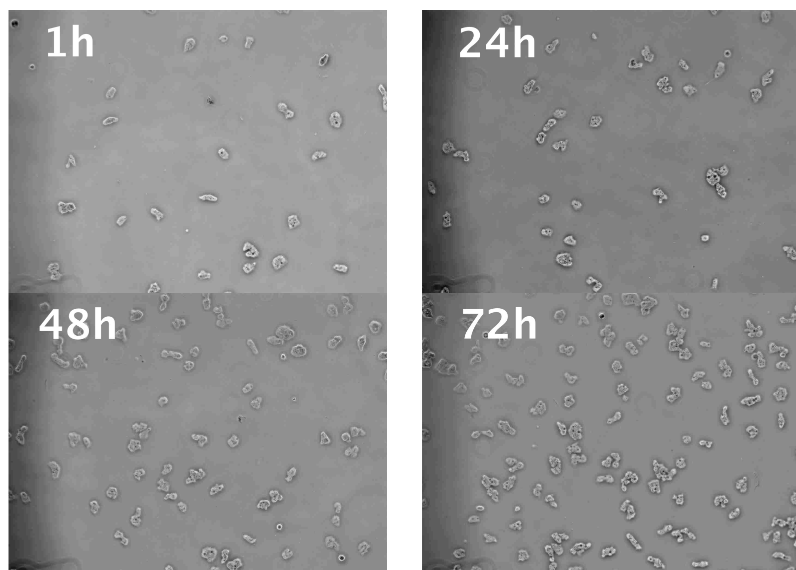


Figure 7. Example of acanthamobae grown in a multiwell plate for determination of growth rate. The amoebae were uninfected acanthamoebae incubated at 20ºC.

The average number of counted amoebae were recorded (Table 19) and compared to each other.

Table 19. The average number of counted uninfected acanthamoebae at different timepoints after seeding at 20˚C, 27˚C and 30˚C. The replication time at 30˚C was much faster than at 27˚C and 20˚C. All numbers were rounded up.

Uninf. amoeabae	1 hps	24 hps	48 hps	72 hps
20˚C	23	37	67	110
27˚C	19	66	190	302
30˚C	19	78	273	804

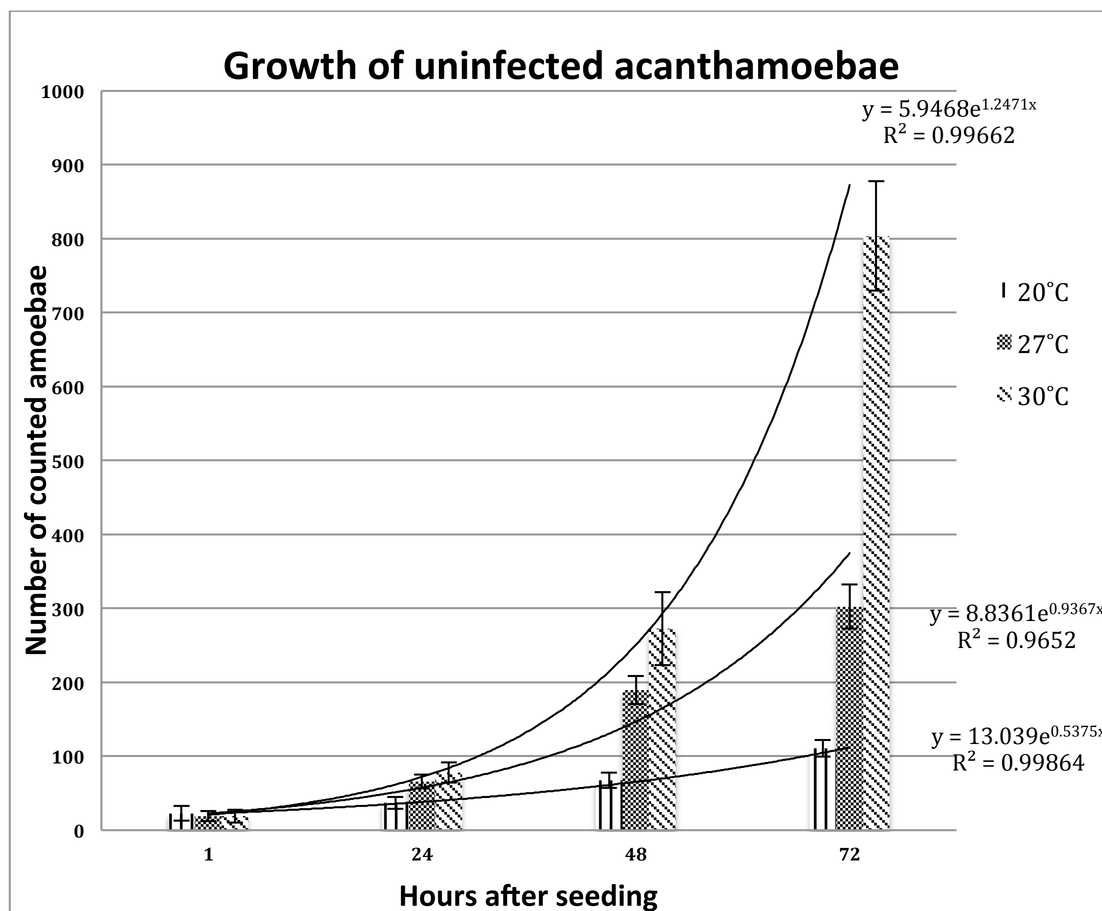


Figure 8. Growth of uninfected acanthamoebae as a column chart. The data were taken from **Table 19**. X- axis resembles the hours after seeding. Y-axis resembles the total number of counted amoebal cells. The growth at 30˚C was visibly faster than at 27˚C and 20˚C.

When comparing the growth of uninfected amoebae at different temperatures, the culture cultivated at 30˚C had a significantly faster replication rate than the culture cultivated at 27˚C and 20˚C. At 20˚C the growth was slow resulting only in five fold more amoebae after 72 hours of incubation where as at 30˚C the growth multiplied more than forty-fold. (Figure 8)

5.5.2 Growth of *Acanthamoeba* sp. infected with *A. asiaticus* 5a2

When compared to the uninfected acanthamoebae, a smaller number of amoebae were seeded in the beginning. It was irrelevant for this experiment since the emphasis was on the replication rate and not the total number of the amoebae. The average number of amoebae inside a field of vision at different

timepoints was also calculated (Table 20). The amoebae cultivated at 30°C showed the highest replication rate where as the amoebae cultivated at 20°C showed a low replication rate, identical to the outcome of 5.5.1. (Figure 9) (2.10.1)

Table 20. The average number of counted acanthamoebae infected with *A. asiaticus* 5a2 at different timepoints after seeding at 20°C, 27°C and 30°C. The amoebae grew at 30°C faster than at 27°C and 20°C. All numbers were rounded up.

Acanthamoebae + *A. asiaticus* 5a2	1 hps	24 hps	48 hps	72 hps
20°C	5	7	77	16
27°C	3	10	22	47
30°C	6	15	35	74

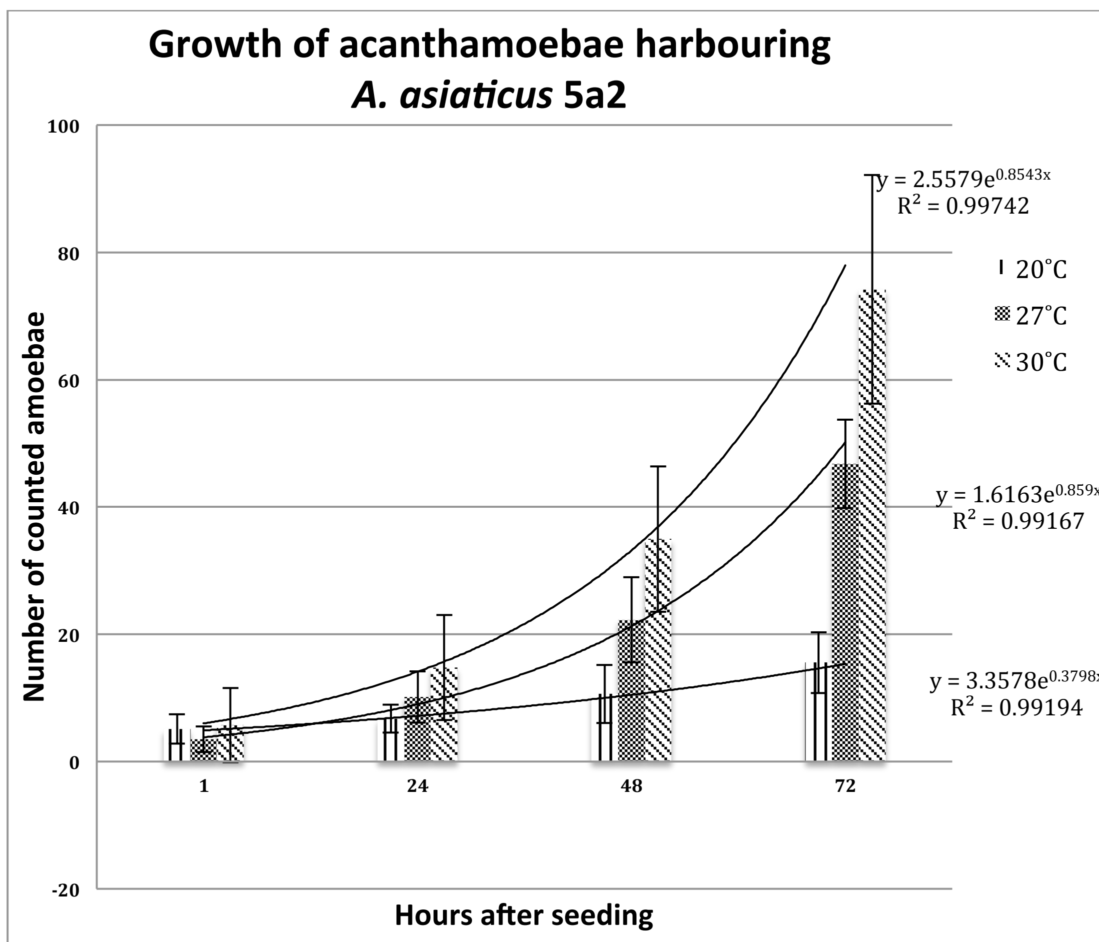


Figure 9. Growth of *Acanthamoeba* sp. infected with *A. asiaticus*. X- axis resembles the hours after seeding. Y-axis resembles the total number of counted amoebae. The amoeba growth at 30°C was faster than at 27°C and 20°C.

5.5.3 Growth rate comparison between infected and uninfected amoebae

Both infected and uninfected amoebae showed a positive correlation regarding their growth rate with the temperature they were incubated at (up to 30˚C). The difference between the amoebae numbers became more pronounced with time. Therefore the uninfected acanthamoebae cultivated at 30˚C had almost eight fold more amoebae than the one cultivated at 20˚C. (Figure 8)

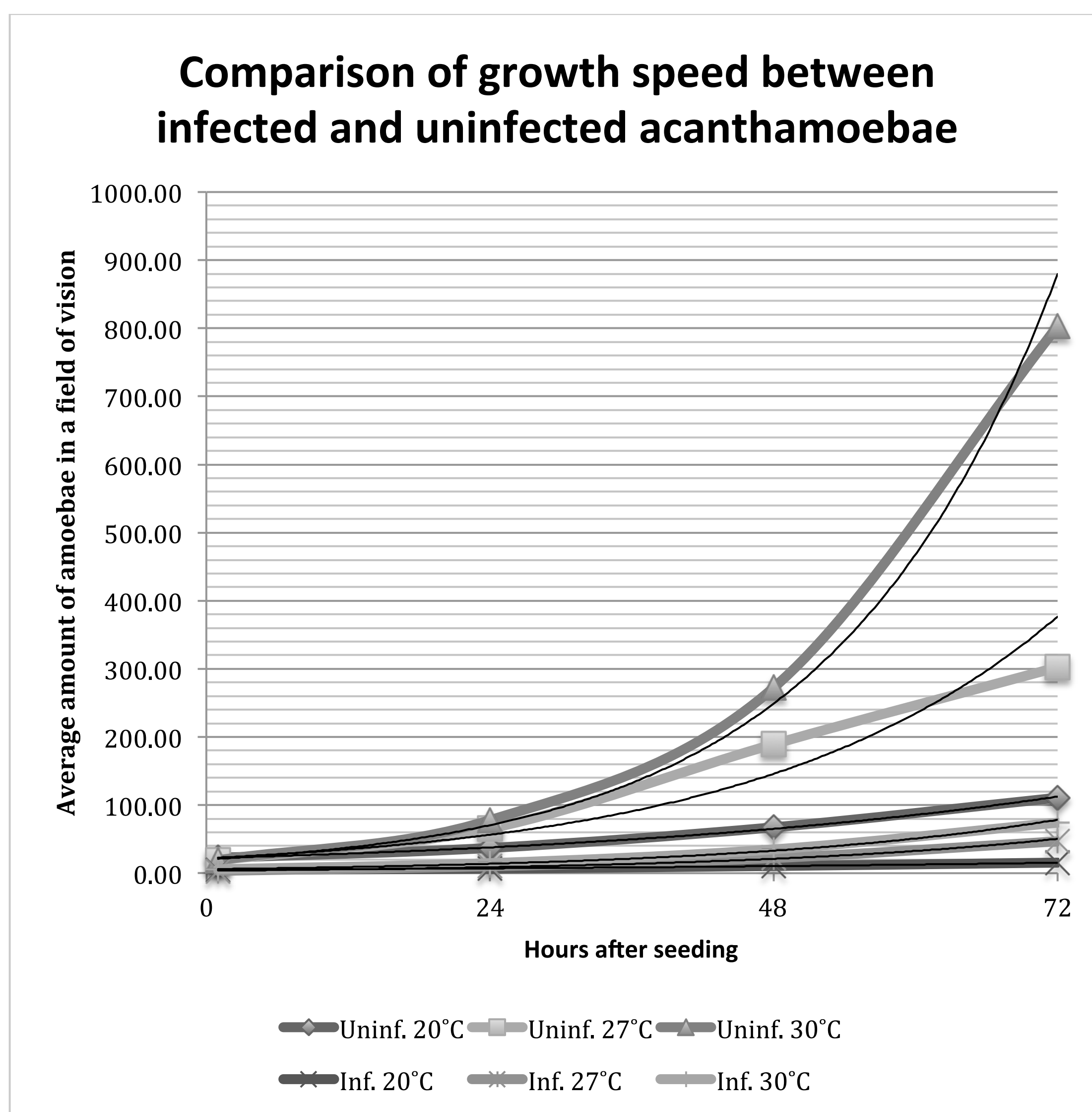


Figure 10. Comparison of growth rate between acanthamoebae with and without *A. asiaticus* 5a2. X- axis resembles the hours after seeding. Y-axis resembles the total number of counted amoebae. "Uninf" stands for uninfected amoebae; "inf" stands for infected amoebae. The temperature specification is the cultivation temperature. The acanthamoebae with no endosymbiont at all temperature grew much faster compared to the ones harbouring *A. asiaticus* 5a2.

Table 21. The equation and R^2 value of the trendline of each culture in Figure 8.

	Equation	R^2 Value
Uninf. 20°C	$y = 21.965e^{0.0227x}$	$R^2 = 0.99883$
Uninf. 27°C	$y = 21.929e^{0.0395x}$	$R^2 = 0.96191$
Uninf. 30°C	$y = 19.975e^{0.0526x}$	$R^2 = 0.99542$
Inf. 20°C	$y = 4.8514e^{0.016x}$	$R^2 = 0.99345$
Inf. 27°C	$y = 3.7284e^{0.0362x}$	$R^2 = 0.98972$
Inf. 30°C	$y = 5.8628e^{0.036x}$	$R^2 = 0.99637$

The acanthamoebae replication rate increased with the temperature they were incubated at. Infected amoebae constantly showed a much lower replication rate compared to the uninfected amoebae cells. Based on Table 21 the duplication time for infected as well as uninfected amoebae were calculated. Table 22 shows that the uninfected amoebae had a faster replication time then the infected amoebae.

Table 22. Duplication time of uninfected and infected amoebae at different temperatures.

	Uninfected amoebae	Infected amoebae
20°C	30,5 hours	43,3 hours
27°C	17,5 hours	19,2 hours
30°C	13,4 hours	19,3 hours

5.6 Growth rate comparison between *acanthamoebae* grown in PYG and TSY

This assay was done to compare the growth of acanthamoebae and acanthamoebae harbouring *A. asiaticus* 5a2 in the media PYG and TSY. Pictures of the amoebae inside a well of multiwell plate were taken and the amount of amoebae determined. (Figure 11) (4.10.2.)

5.6.1 Incubation of acanthamoebae in PYG medium

The counted number of amoebae is stated inside the Table 23. Uninfected acanthamoebae replicated as expected where as acanthamoebae harbouring *A. asiaticus* 5a2 replicated very slow and duplicated only 96 hours after seeding (Figure 12).

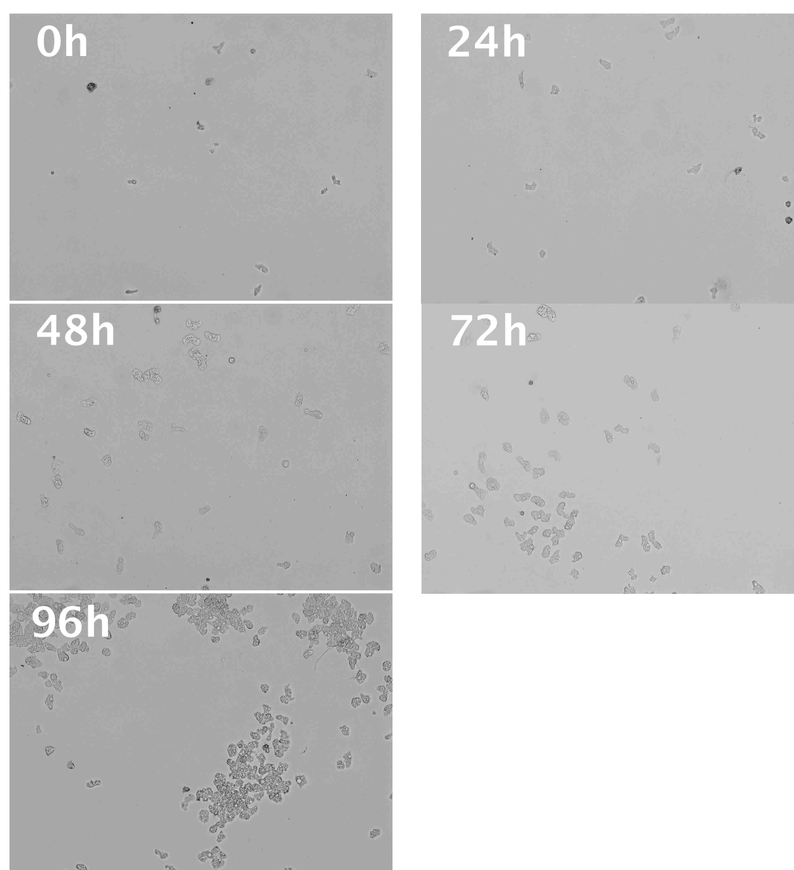


Figure 11. Example of a set of pictures taken for this assay. The amoebae were uninfected and cultivated in PYG at 27°C.

Table 23. Average number of counted uninfected amoebae as well as acanthamoebae infected with *A. asiaticus* 5a2 cultivated in PYG at different timepoints inside a field of vision. The numbers were rounded up. At 24 hps, 48 hps and 72 hps the unrounded average number is stated inside the bracket.

PYG	0 hps	24 hps	48 hps	72 hps	96 hps
Uninfected amoebae	4	11	20	43	115
Acanthamoebae carrying *A. asiaticus* 5a2	2	3 (2.89)	3 (2.79)	3 (3.13)	4

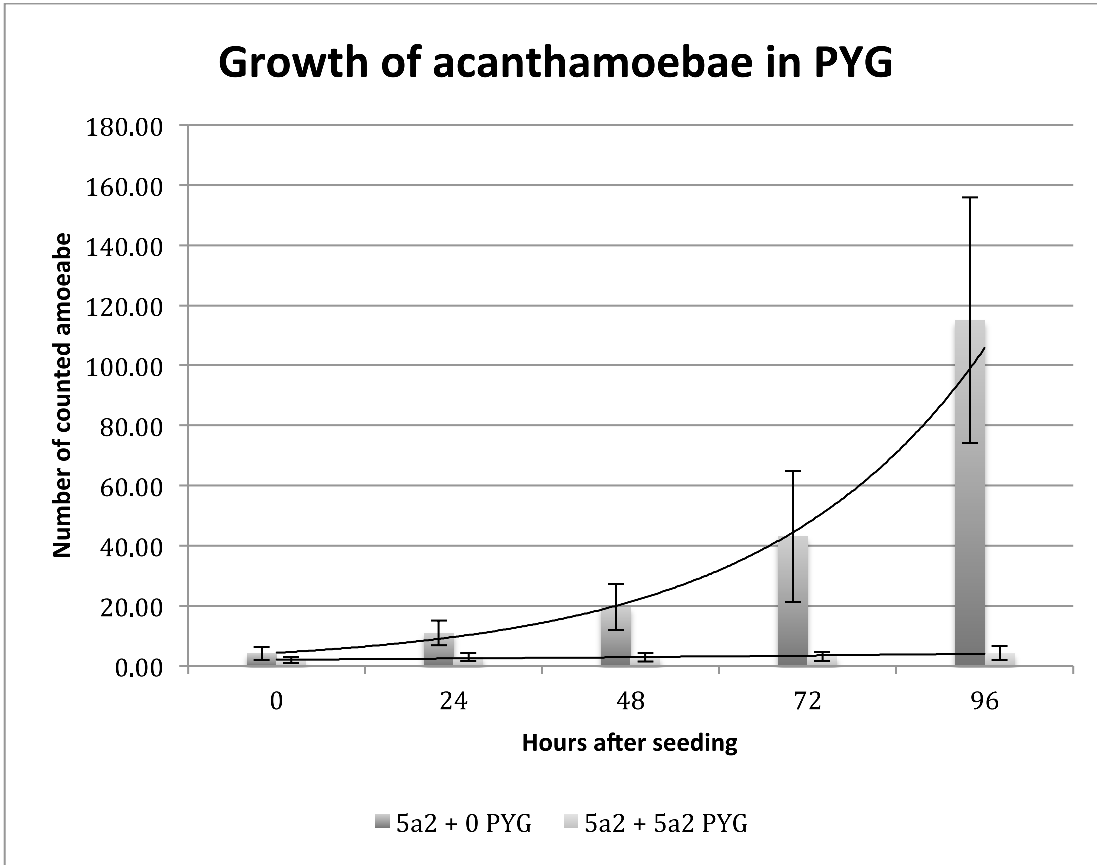


Figure 12. Comparison of growth of uninfected and infected amoebae in PYG. X- axis resembles the hours after seeding. Y-axis resembles the total number of counted amoebal cells. The darker bar "amoebae" represents the uninfected acanthamoebae where as the light gray bar represents the acanthamoebae infected with *A. asiaticus* 5a2. The growth of the uninfected acanthamoebae was much faster than the growth of infected amoebae that almost didn't multiply at all.

5.6.2 Incubation of acanthamoebae in TSY medium

The course of replication was similar to the same assay done in PYG (5.6.1), displaying a very low replication rate of the infected amoebae and vastly higher replication rate of the uninfected amoebae.

Table 24. Average number of counted uninfected amoebae as well as acanthamoebae infected with *A. asiaticus* 5a2cultivated in TSY at different timepoints inside a field of vision. The numbers were rounded up.

TSY	0 hps	24 hps	48 hps	72 hps	96 hps
Counted amoebae	4	5	9	16	46
Acanthamoebae carrying *A. asiaticus* 5a2	3	3	4	5	7

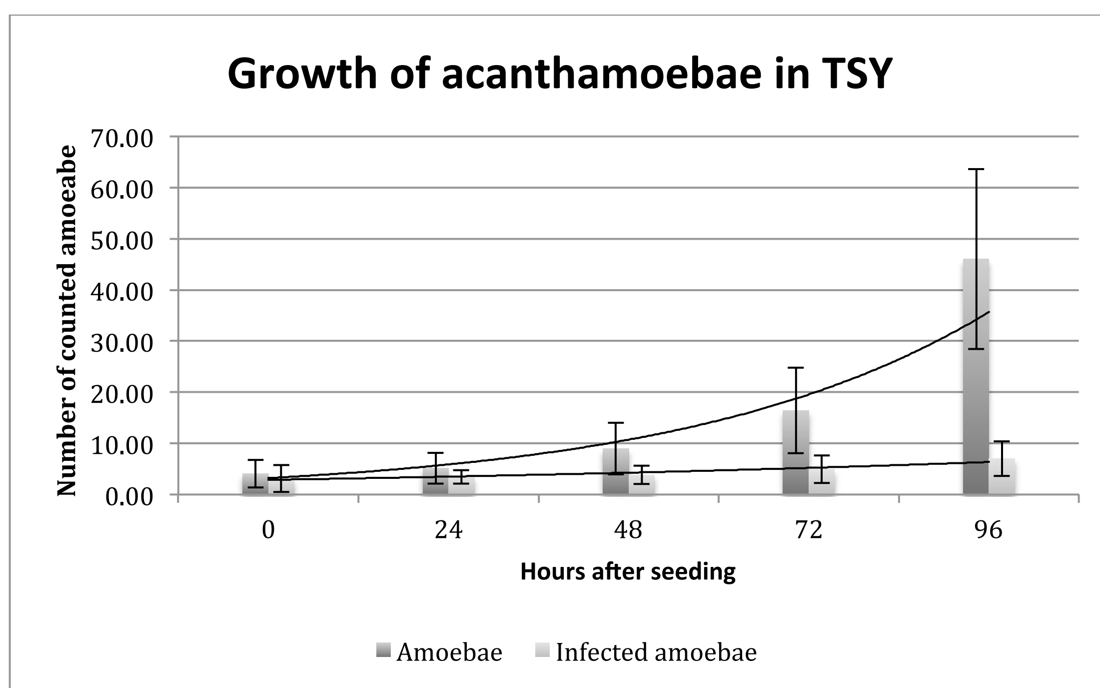


Figure 13. Comparison of growth between infected and uninfected amoeba cultivated in TSY. X- axis resembles the hours after seeding. Y-axis resembles the total number of counted amoebal cells. The darker bar "amoebae" represents the uninfected acanthamoebae where as the light gray bar represents the acanthamoebae infected with *A. asiaticus* 5a2. The uninfected amoebae grew better than the infected amoebae over the course of observation.

5.6.3 Comparison between growth in PYG and TSY medium

While comparing the growth of acanthamoebae in TSY and PYG, the acanthamoebae harbouring no symbionts grew faster in PYG medium than in TSY medium. The replication rate of acanthamoebae infected with *A. asiaticus* 5a2 was low in both TSY and PYG both only doubled their original amount of amoebae after 96 hours. There were no major differences between these two

(Figure 14). Overall, amoebae grew better in PYG than in TSY.

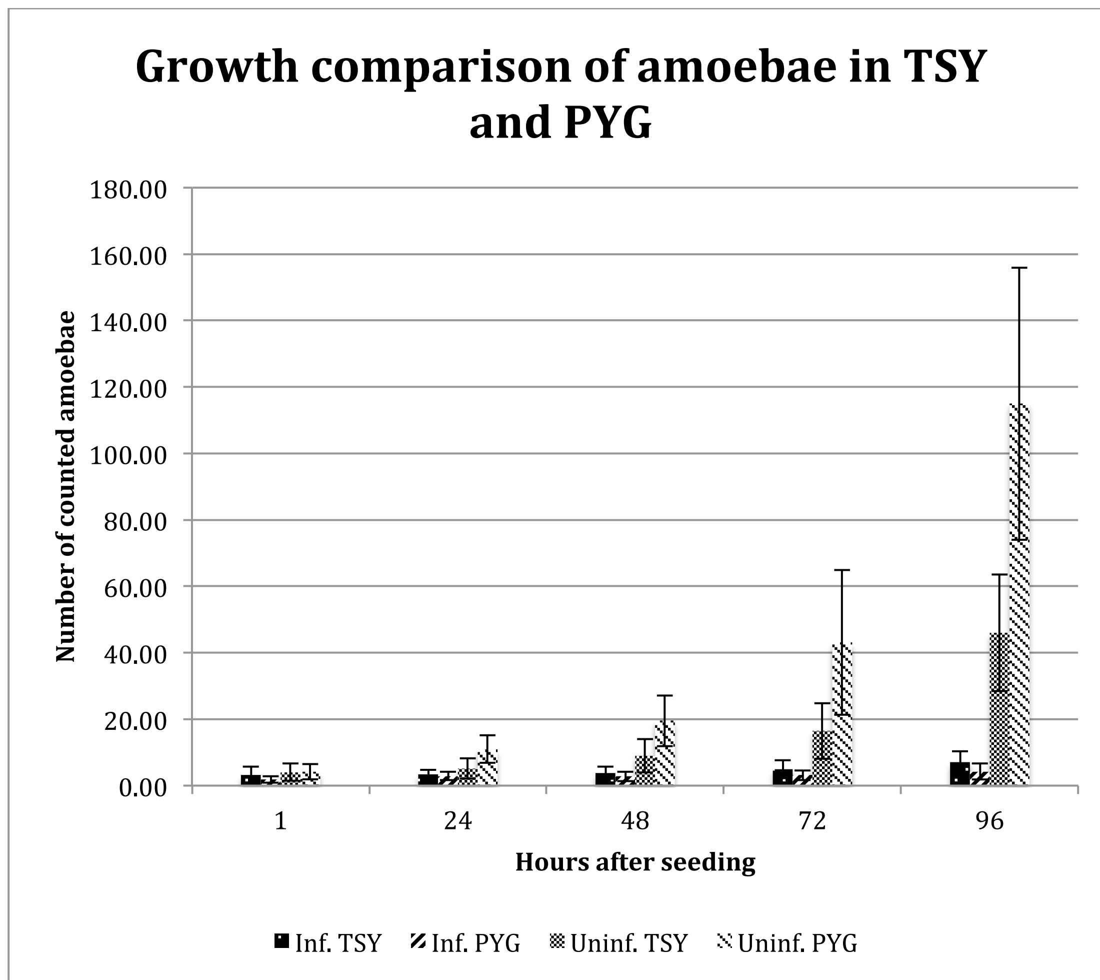
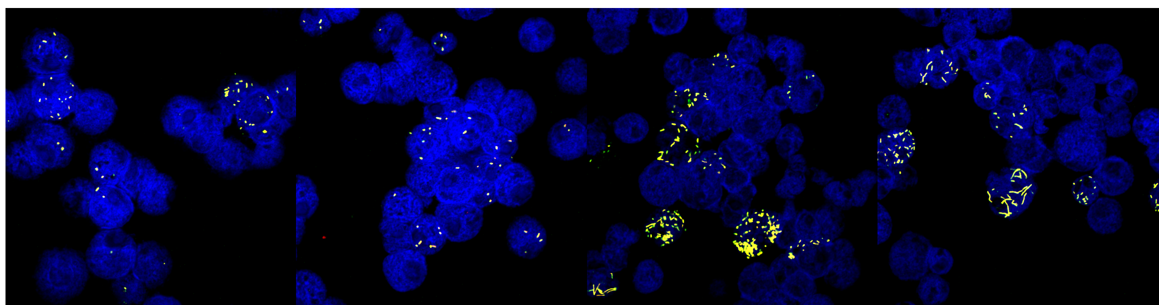


Figure 14. Comparison of growth rate between amoebae cultivated in TSY and PYG. X- axis resembles the hours after seeding. Y-axis resembles the total number of counted amoebal cells. "Inf" stands for infected amoebae; "uninf" stands for uninfected amoebae. TSY = cultivated in TSY medium; PYG = cultivated in PYG medium. While comparing the media TSY and PYG, uninfected acanthamoebae grew better in PYG than in TSY, while there were almost no difference between infected acanthamoebae grown in TSY and PYG.

5.7 Uninfected acanthamoebae and acanthamoebae harbouring *A. asiaticus* 5a2 cultivated in TSY medium exhibit a higher infectivity

To investigate the effect of the two media TSY and PYG regarding the infection rate, two infection assays were performed. One was done with uninfected acanthamoebae and *acanthamoebae* harbouring *A. asiaticus* 5a2 both cultivated only in TSY medium, where as the second assay was performed by utilizing organisms cultivated in PYG medium. For this assay, the intracellular *A. asiaticus* 5a2 were harvested. (4.9.4.)



(A) (B)

Figure 15. Acanthamoebae infected with *A. asiaticus* 5a2, cultivated in TSY. Used Probes: APH1180-Cy3 (red), EUB338-Fluos-DG (green), EUK516-Cy5 (blue) & Acanth412-Cy5 (blue). Pictures were taken at (A) 24 hpi and (B) 72 hpi. Both the amount of infected amoebae as well as the quantity of *A. asiaticus* 5a2 inside each infected amoebae were much higher than in the assay performed with organisms cultivated in PYG.

When comparing the infection rate between PYG-cultivated acanthamoebae containing *A. asiaticus* 5a2 (Figure not shown, similar to Figure 5) with TSY-cultivated acanthamoebae and *A. asiaticus* 5a2 (Figure 15), the latter one exhibits an infection rate of around 3. The infection of PYG-cultivated acanthamoebae were just 1 instead. The quantity of infected amoeba hosts and the number of *A. asiaticus* 5a2 inside the infected amoebae was thus higher in the TSY cultivated group. The *A. asiaticus* 5a2 also replicated inside the infected amoebae as shown in Figure 15B, where not only the amount of intracellular *A. asiaticus* 5a2 was higher, but they also gave a stronger fluorescence signal.

5.8 Treatment of acanthamoebae with eukaryotic inhibitors

As demonstrated, the growth of uninfected acanthamoebae is significantly faster than the growth of infected amoebae. This could resolve in the dilution effect, where uninfected amoebae outgrow infected amoebae and decrease the rate of infection. Therefore in this infection assay, the growth of the host was inhibited by addition of eukaryotic antibiotics, limiting only the growth of the host but not *A. asiaticus* 5a2. (4.9.2.)

Geneticin is an aminoglycoside antibiotic, which can block the polypeptide synthesis by inhibiting the elongation step in treated cells. Cycloheximide on the other hand is an inhibitor of protein biosynthesis in eukaryotic organisms.

5.8.1 Infection in the presence of cycloheximide

At the presence of 10 μg/ml cycloheximide resulted in a infection efficiency of 1 to 2 at the 24 hpi time point. More amoebae had taken up *A. asiaticus* 5a2 resulting in more infected amoebae with just few endosymbiont within. Furthermore, the density of amoebae were also reduced due to cycloheximide. At 72 hpi, the infection rate was 2 while untreated cell culture were just 1. The density of amoebae cells increased, suggesting that the effect of antibiotics weakened.

At 100 μg/ml concentration, more endosymbionts were taken up, as suggested in stronger fluorescence signal inside the amoebae. The fluorescence signal of *A. asiaticus* 5a2 were clumped, overlapped and roundish as opposed to the single cell form found in untreated cell cultures or one treated with a low concentration of cycloheximide. At 72 hpi, there were more endosymbionts per amoeba compared to the 10 μg/ml culture, since the amount of uptake in the beginning was higher. The efficiency of infection was 2 at both timepoints.

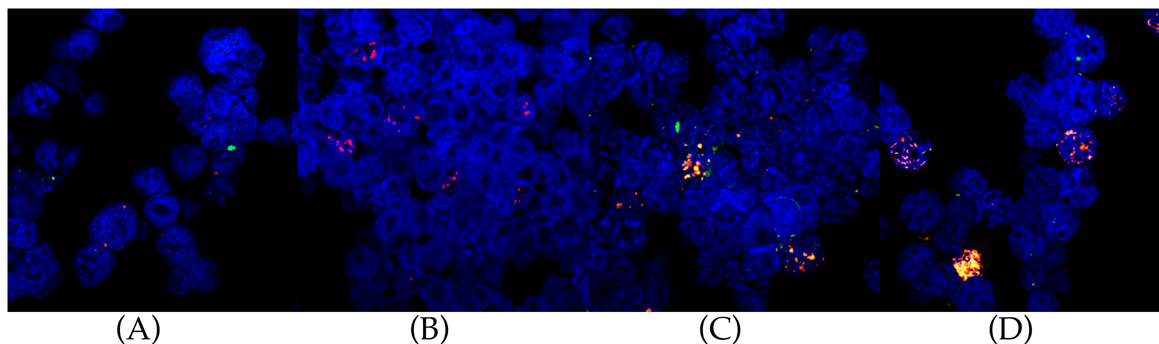


Figure 16. Infection in the presence of cycloheximide. Used Probes: APH1180-Cy3 (red), EUB338-Fluos (green), EUK516-Cy5 (blue) & Acanth412-Cy5 (blue) (A) Cycloheximide concentration: 10 µg/ml. 24 hpi (B) Cycloheximide concentration: 10 µg/ml. 72 hpi (C) Cycloheximide concentration: 100 µg/ml. 24 hpi (D) Cycloheximide concentration: 100 µg/ml. 72 hpi

5.8.2 Infection in the presence of geneticin

At the lower concentration of 10 µg/ml, the growth of acanthamoebae was inhibited and the uptake of of *A. asiaticus* 5a2 was increased. The symbiont signals inside the amoebae were more clustered and vacuole like instead of single cells. At 72 hpi, the morphology of *A. asiaticus* 5a2 changed drastically, forming long and snake like shapes. The infection efficiency was 1 to 2 at both time points.

The uptake of the endosymbiont at 24 hpi was higher when a more concentrated doses of geneticin (100 µg/ml) was used. The endosymbionts gave a strong roundish signal, indicating their location inside vacuoles. The infection efficiency was 2-3. No pictures were taken of the 100 µg/ml geneticin treated cell culture at the 72 hpi time point since a large amount of the cells were lysed. (Figure 17)

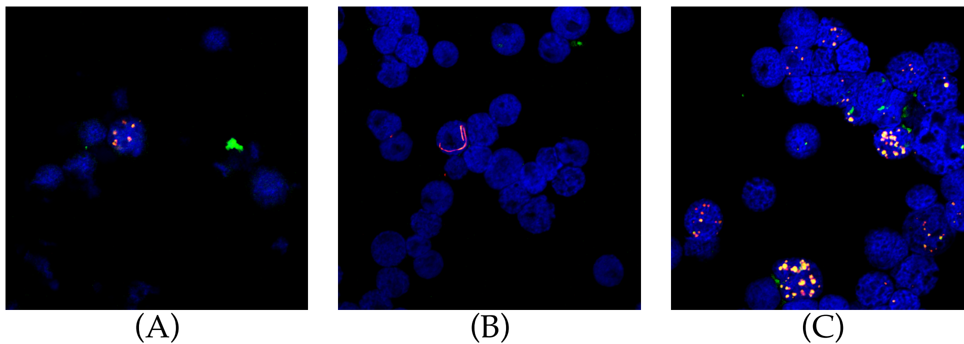


Figure 17. Treatment with geneticin. Used Probes: APH1180-Cy3 (red), EUB338-Fluos (green), EUK516-Cy5 (blue) & Acanth412-Cy5 (blue). (A) Geneticin concentration: 10 µg/ml. 24 hpi. The symbiont signal are clustered compared to non-treated infection cycle. (B) Geneticin concentration: 10 µg/ml. 72 hpi. *A. asiaticus* 5a2 formed long and elongated shapes. (C) Geneticin concentration: 100 µg/ml. 24 hpi. The amount of uptaken *A. asiaticus* 5a2 per amoeba increased.

5.8.3 Effect of geneticin and cycloheximide

Both antibiotics inhibited the growth of acanthamoebae resulting in a higher infection rate. It appeared that the uptake of *A. asiaticus* 5a2 was stimulated in the presence of eukaryotic inhibitors because the FISH-signal of the symbiont were located inside vacuole-like compartments, probably food vacuoles. The inhibitory effect of 10 µg/ml cycloheximide vanished after 72 hpi where as 100 µg/ml still seemed to have an inhibitory effect. The use of 100 µg/ml geneticin was too strong as most of the amoebal cells were lysed after incubation for 72 hours. With the use of 10 µg/ml geneticin, *A. asiaticus* 5a2 had a drastic change in morphology resulting in elongated rod-shape. Both eukaryotic inhibitors increased the efficiency of infection from 1 to up to 3.

5.9 Extracellular *A. asiaticus* 5a2 show a higher infectivity compared to intracellular *A. asiaticus* 5a2

In order to investigate, whether *A. asiaticus* 5a2 exhibits a more complex cell cycle than previously assumed, the infectivity between intracellular and extracellularly collected *A. asiaticus* 5a2 was compared. Both stages were harvested from a single culture flask. (4.9.3.)

There is a clear difference between extracellular and intracellular *A. asiaticus* 5a2 incubated in PYG regarding infectivity. On a FISH-slide well fixated with intracellular infected amoebae, only few infected amoebae could be found, which was almost an infection efficiency of 0. The efficiency was even lower compared to the usual infection cycle, which was 1. However when the extracellular *A. asiaticus* 5a2 was used for the assay, symbiont-containing amoebae could be found regularly on the well, resulting in a higher infection efficiency of 2-3. Both extracellular and intracellular *A. asiaticus* 5a2 were replicating normally, once infected an amoeba. At later time points (Figure 18D), the number of symbionts within the amoebae increased compared to previous time points. This trend was comparable to all infection assays without the presence of additional eukaryotic inhibitors. The main difference between the infection with extracellular *A. asiaticus* 5a2 and intracellular *A.* asiaticus 5a2 was the efficiency of infection with the former one being a 2 – 3 where as the intracellular one was almost 0.

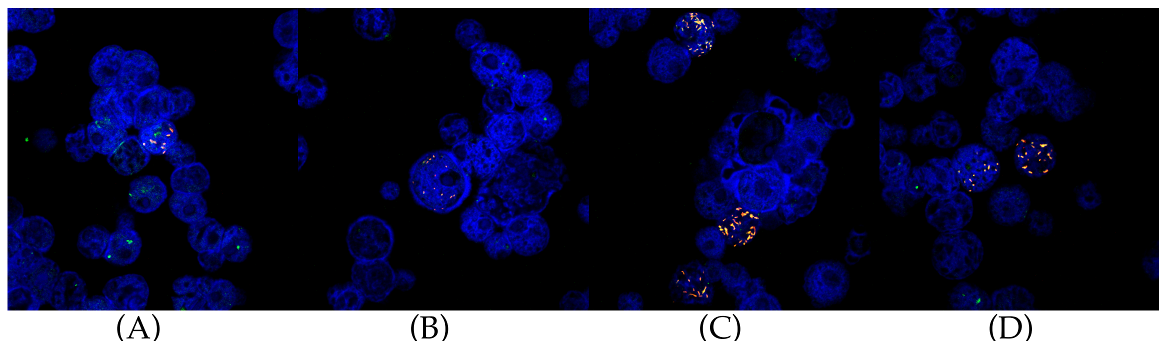


Figure 18. Infection experiment of intracellular and extracellular *A. asiaticus* 5a2. Used Probes: APH1180-Cy3 (red), EUB338-Fluos-DG (green), EUK516-Cy5 (blue) & Acanth412-Cy5 (blue). (A) Intracellular symbiont, 72 hpi. (B) Intracellular symbiont, 96 hpi. (C) Extracellular symbiont, 72 hpi. (D) Extracellular symbiont, 96 hpi. There were almost no infected amoebae to be found in (A) and (B). Very few cells showing (A) were found on the well where as (C) can be found regularly. The infection rate with extracellular *A. asiaticus* 5a2 was much higher than using the intracellular *A. asiaticus* 5a2 for infection. The endosymbionts were replicating inside the amoebae.

The same infection assay was performed again by using TSY-cultivated host and symbiont instead of PYG-cultivated ones. The use of TSY-incubated acanthamoebae and *A. asiaticus* 5a2 resulted in highly infected amoebae culture

(Figure 19) with an infection efficiency of 5. Almost every amoeba was infected with *A. asiaticus* 5a2. In comparison, a much lower infection efficiency of 1 resulted by using PYG incubated organisms (Figure 5, Figure 6). The infection was stable and *A. asiaticus* 5a2 replicated normally inside the amoeba. The number of *A. asiaticus* 5a2 inside infected amoebae was increasing during the whole course of infection. The infection was stable with no evident percentage drop of infected cells. Replicas all showed the same phenomena.

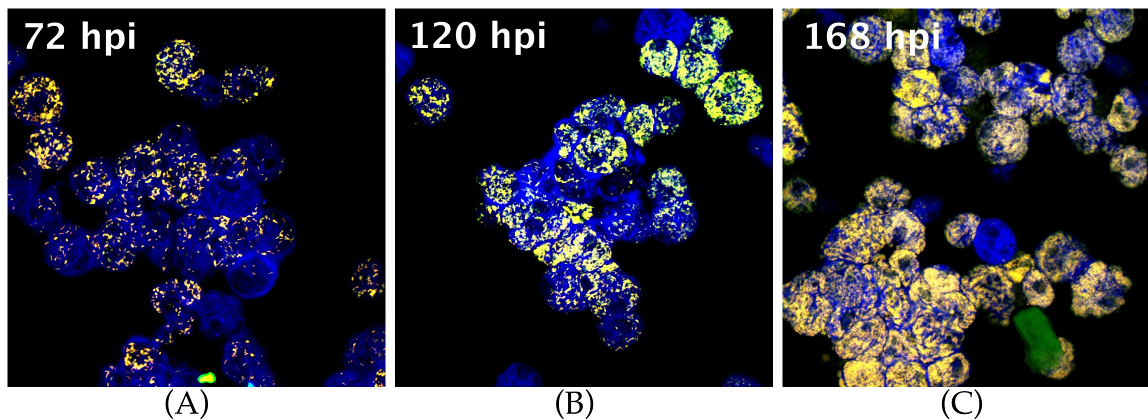


Figure 19. Infection assay with TSY cultivated acanthamoebae and extracellular *A. asiaticus* 5a2. Used Probes: APH1180-Cy3 (red), EUB338-Fluos-DG (green), EUK516-Cy5 (blue) & Acanth412-Cy5 (blue).

5.10 Extracellular *A. asiaticus* 5a2 pre-treated the same way as intracellular *A. asiaticus* 5a2 still show a higher infection rate

To avoid the potential bias, which could be induced through different harvesting method, the extracellular *A. asiaticus* 5a2 were pre-treated the same way as the intracellular *A. asiaticus* 5a2. The pre-treatment consisted of homogenizing with the tissue homogenizer and resuspension in amoebae cell lysate of uninfected acanthamoebae. An infection assay was then performed comparing the infectivity between intracellular, extracellular and pre-treated extracellular *A. asiaticus* 5a2. (4.9.7).

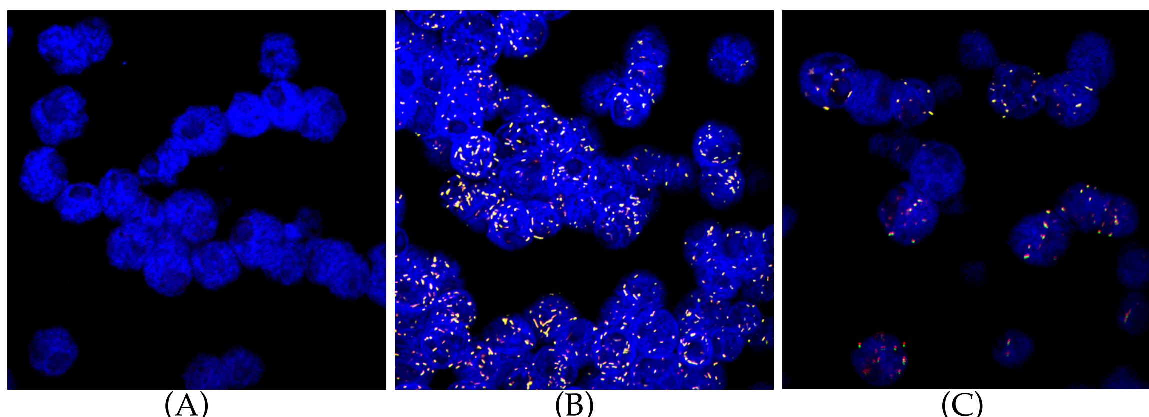


Figure 20. Intracellular, extracellular and pre-treated extracellular *A. asiaticus* 5a2. All pictures were taken at 24 hpi. The used MOI was 50. The cultures used in this assay were incubated in the medium TSY. Used Probes: APH1180-Cy3 (red), EUB338-Fluos-DG (green), EUK516-Cy5 (blue) & Acanth412-Cy5 (blue). (A) Infection with intracellular *A. asiaticus* 5a2. (B) Infection with extracellular *A. asiaticus* 5a2. (C) Infection with pre-treated extracellular *A. asiaticus* 5a2

No infected amoebae were found in the culture infected with intracellular *A. asiaticus* 5a2. The infection efficiency was 0. When untreated extracellular *A. asiaticus* 5a2 was used, the increase in infection efficiency was very evident and resulted in a score of 4 to 5 (Figure 20B). Using pre-treated extracellular *A. asiaticus* 5a2, the infection efficiency (3 to 4) was lower than using untreated extracellular *A. asiaticus* 5a2 (4 to 5) but still higher than using intracellular *A. asiaticus* 5a2 (0).

5.11 Comparison between the intracellular and extracellular cell stage of *A. asiaticus* 5a2, US1 and EIDS3

The result of 5.9 suggested a higher infectivity of the extracellular cell stage of the endosymbiont *A. asiaticus* 5a2. This assay was performed to examine whether this also holds true for other *A. asiaticus* strains. The extracellular stages of *A. asiaticus* 5a2, US1 and EIDS3 were compared to the intracellular form regarding the infectivity. All steps were performed as stated in 4.9.5.

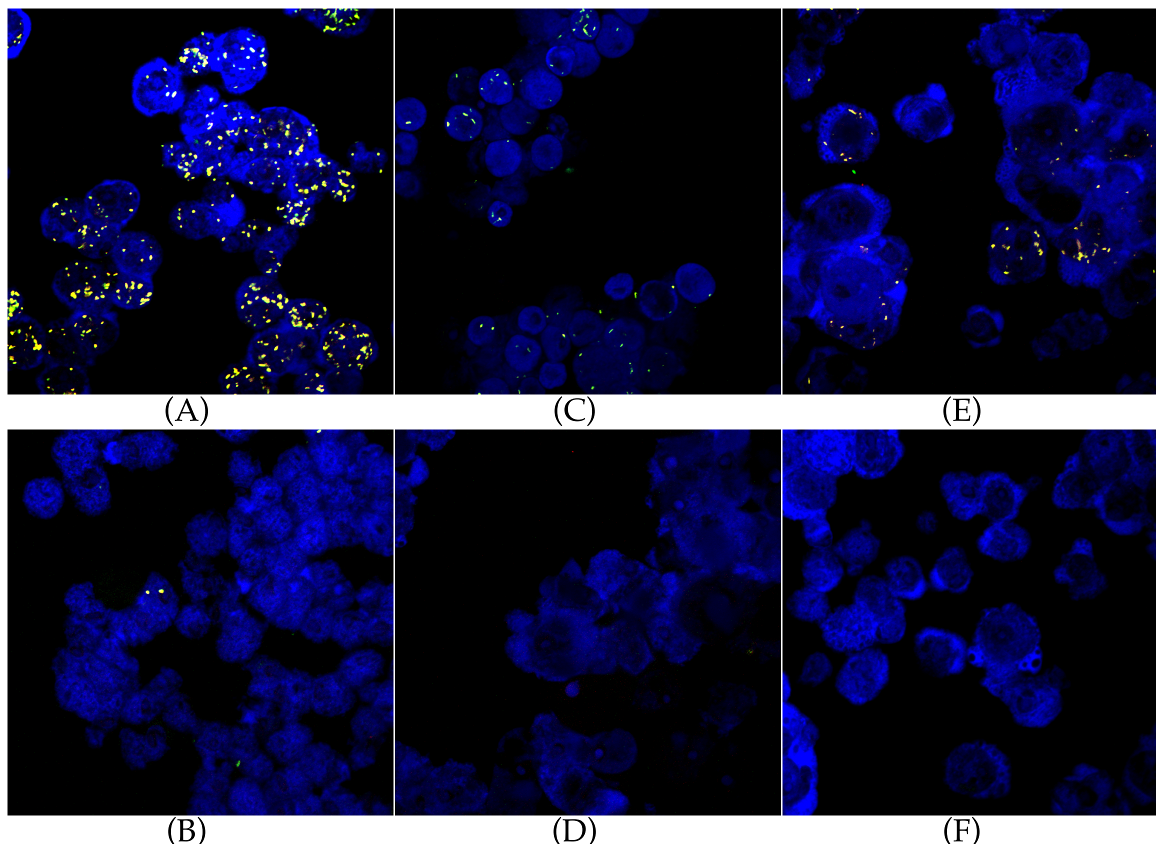


Figure 21. Comparison between extracellular and intracellular form of *A. asiaticus* 5a2, EIDS3 and US1. All pictures were taken 24 hours post infection. Used Probes: APH1180-Cy3 (red), EUB338-Fluos-DG (green), EUK516-Cy5 (blue) & Acanth412-Cy5 (blue). (A) Infection with extracellular *A. asiaticus* 5a2. (B) Infection with intracellular *A. asiaticus* 5a2. (C) Infection with extracellular *A. asiaticus* EIDS3. (D) Infection with intracellular *A. asiaticus* EIDS3. (E) Infection with extracellular *A. asiaticus* US1. (F) Infection with intracellular *A. asiaticus* US1.

In case of *A. asiaticus* 5a2, only few infected cells were found in the intracellular infected culture, which led to an efficiency score of 0 to 1. In comparison, no infected amoebae were found at all while using intracellular *A. asiaticus* EIDS3 and US1. Both had an infection efficiency of 0. Infected amoebae could be found in all three cultures infected with the extracellular form of *A. asiaticus*. While using extracellular *A. asiaticus* 5a2 the infection efficiency was 4. In case of extracellular *A. asiaticus* EIDS3 and US, they had an infection efficiency of around 3. *A. asiaticus* 5a2 had also the highest amount of uptaken symbiont, around ten symbiont within an amoebae. *A. asiaticus* EIDS3 showed around three symbiont cells where as *A. asiaticus* US1 had around five cells (Figure 21).

5.12 Infection in the presence of different sugars

A study (Declerck et al., 2007) has shown the interference of *L. pneumophilae* uptake by addition of certain saccharide. The main difference between the components of PYG and TSY is the addition of glucose in PYG. This assay was performed to investigate whether the low infection rate of experiments performed with PYG-cultivated amoebae were directly influenced by presence of the sugar. This would be in accordance with a recent study on *L. pneumophilae*, which demonstrated the interference *L. pneumophilae* uptake by addition of certain sugars. In this assay three different sugars (mannose, galactose, glucose) were added to the TSY media and their effects on the infection were investigated. (4.9.6.)

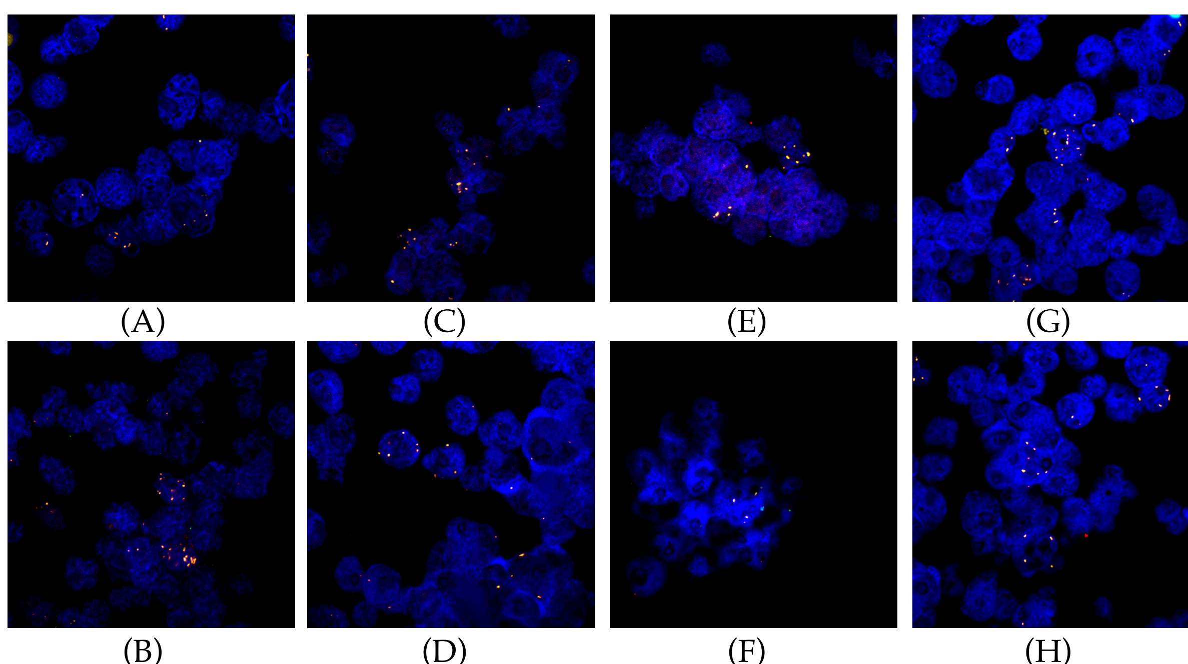


Figure 22 Infection assay with addition of various saccharides. All pictures were taken at 24 hpi: chosen MOI was 50. Used Probes: APH1180-Cy3 (red), EUB338-Fluos-DG (green), EUK516-Cy5 (blue) & Acanth412-Cy5 (blue). (A) Glucose 100 mM. (B) Gluose 200 mM. (C) Lactose 100 mM (D) Lactose 200 mM (E) Mannose 100 mM (F) Mannose 200 mM (G, H) TSY only

There was no visible inhibition or decrease regarding infection efficiency visible with the addition of various saccharides at the concentration used (Figure 22). The infection efficiency was 1 in all setups. The morphology of the symbiont was as expected from a 24 hpi time point, with shapes of small rods. The

amoebae in the culture with addition of 200 mM mannose were more detached compared to the other culture.

5.13 Infection of acanthamoebae with *A. asiaticus* 5a2 using a low MOI to visualize the infection cycle

Important for this assay was the visualization the different key events of the infection cycle like the attachment and entry of *A. asiaticus* 5a2, the replication inside the amoebae and the host cell lysis. Thus high MOIs of 50 or even 5000 weren't suited since the differentiation between single *A. asiaticus* 5a2 cells was difficult. Instead a MOI of 1 was used to visualize the development of the infection cycle. (4.9.8.)

Six hours after the infection, *A. asiaticus* 5a2 FISH signals could already be detected inside of amoebae. The shape of the symbiont was roundish and coccus like (Figure 23A). For the next 48 hours, the average number of *A. asiaticus* 5a2 inside an amoeba was rising, suggesting growth of the endosymbiont within the host. At 72 hpi, the shape of *A. asiaticus* 5a2 began to change. It became more elongated and rod like (Figure 23D). Around 120 hpi, the cytosol of the infected acanthamoebae slowly began to fill up with *A. asiaticus* 5a2. A differentiation between every *A. asiaticus* 5a2 cell was difficult. At this stage most infected acanthamoebae were fill completely with *A. asiaticus* 5a2 144 hours after the initial infection (Figure 23G). First lysis of infected amoebae could also be detected. It resulted in release of the intracellular *A. asiaticus* 5a2. Newly infected amoebae could also be observed. The morphology of released intracellular endosymbiont were short rods, where as the symbionts inside the newly infected cells seemed a little bit more elongated.

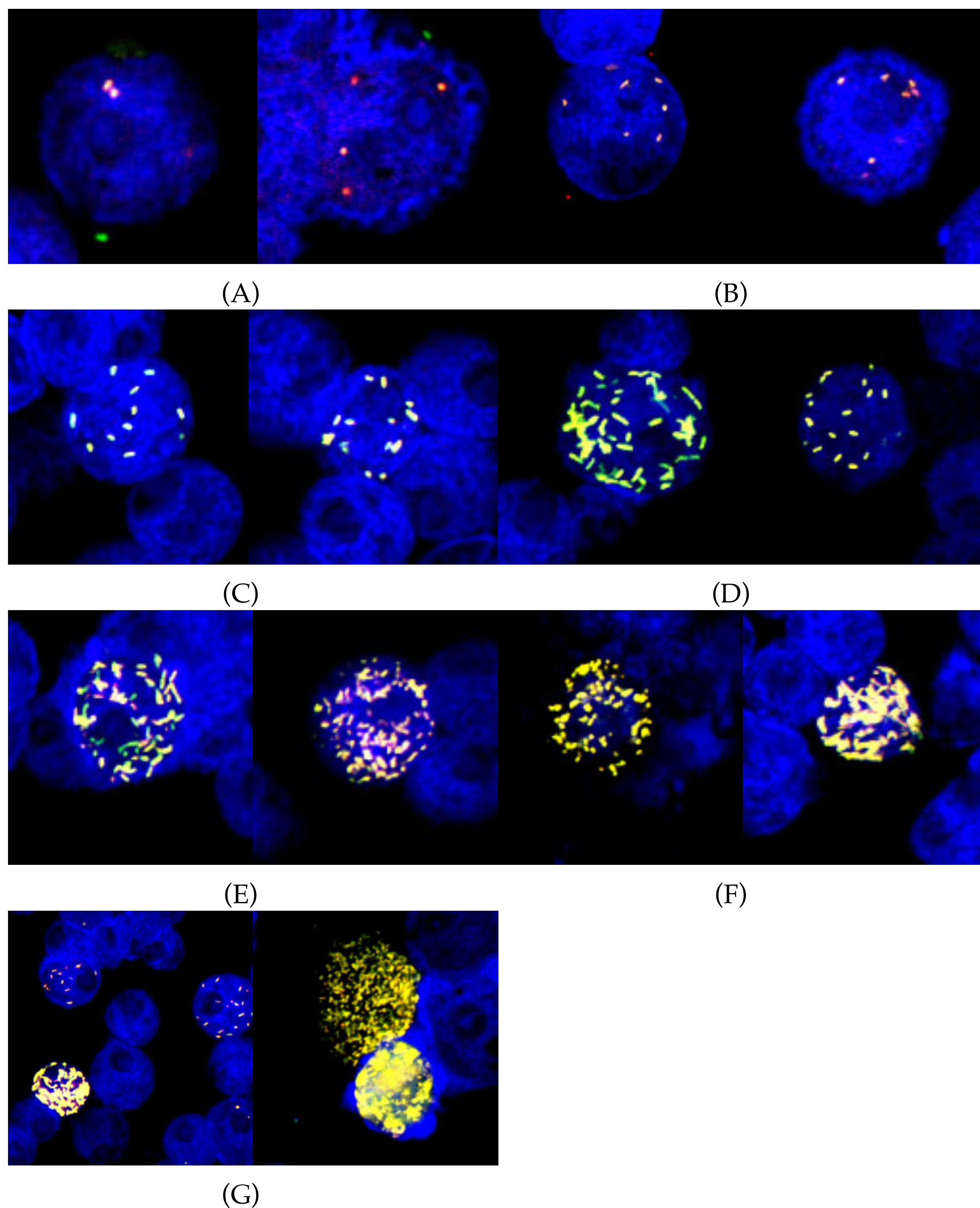


Figure 23. Acanthamoebae infected with *A. asiaticus* 5a2 to visualize the infection cycle. Used Probes: APH1180-Cy3 (red), EUB338-Fluos-DG (green), EUK516-Cy5 (blue) & Acanth412-Cy5 (blue). Pictures taken at (A) 6 hpi (B) 24 hpi (C) 48 hpi (D) 72 hpi (E) 96 hpi (F) 120 hpi (G) 144 hpi

5.14 The determination of duplication time of *A. asiaticus* 5a2

An infection experiment with acanthamoebae and *A. asiaticus* 5a2 using a MOI = 1 was started. In order to estimate the replication time of *A. asiaticus* 5a2, the average number of the symbiont inside an amoeba was determined every 24 hours (Figure 24). All steps were performed as described in 4.9.9.

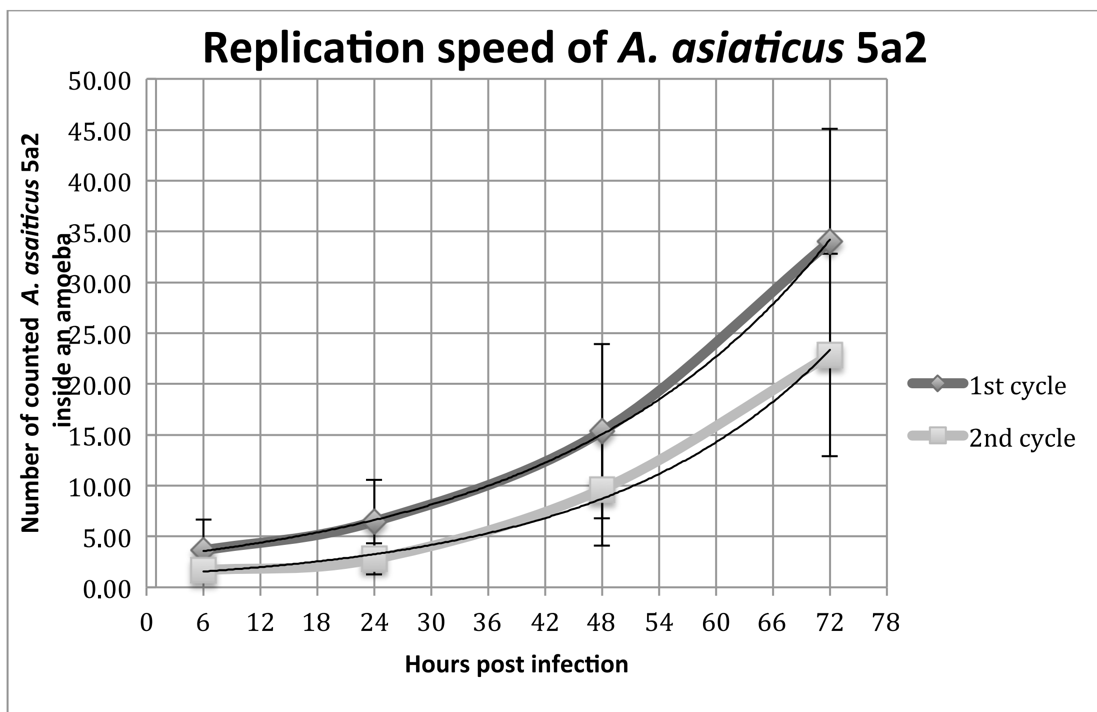


Figure 24. Average number of counted *A. asiaticus* 5a2 inside an infected amoeba. Two independent infection cycles were done and analysed. The amount of *A. asiaticus* 5a2 per amoeba at different time points was counted.

Table 24. The equation and R^2 value of the trendlines for *A. asiaticus* 5a2 growth.

	Equation	R^2 Value
1st cycle	$y = 2.9125e^{0.0342x}$	$R^2 = 0.99966$
2nd cycle	$y = 1.2154e^{0.041x}$	$R^2 = 0.99034$

Based on the equations in Table 24, the duplication time for intracellular *A. asiaticus* 5a2 incubated at 27°C was determined; *A. asiaticus* 5a2 duplicates every 17 h to 20 h.

5.15 Ultrastructure of *A. asiaticus* 5a2 in the extracellular stage

Since a difference regarding the infection efficiency between extracellular and intracellular *A. asiaticus* 5a2 was demonstrated, the ultrastructure of *A. asiaticus* 5a2 in its extracellular stage was investigated by the means of transmission electron microscopy.

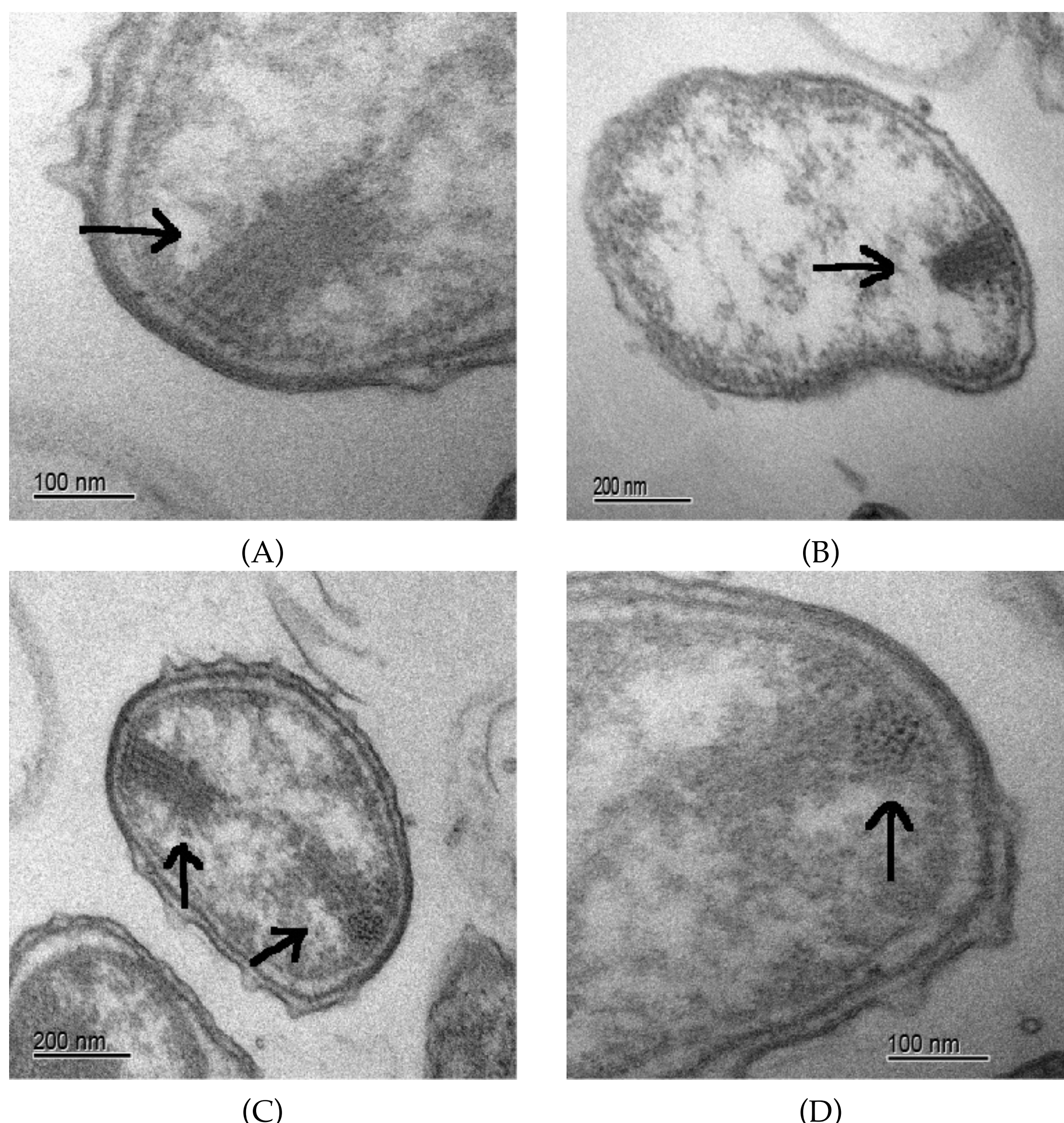


Figure 25. TEM pictures of extracellular *A. asiaticus* 5a2 showing the afp-like-apparatus. (A, B & C) The vertical cut of probabply the afp helical sheath can be seen. (D) A horizontal cut of probably the bell-shaped structure and the inner tube of the afp-like-apparatus. **(pictures made by Rok Konstanjsek)**

The *A. asiaticus* 5a2 cells were roundish and not elongated, which coincide with the observations made in 5.13. In the TEM pictures (Figure 25) of the extracellular *A. asiaticus* 5a2, the cells were comprised of a double membrane and their size various between 0.8 μm and 1 μm in length and around 0.5 μm in width. Structures that could represent the afp-like prophage were also found inside *A. asiaticus* 5a2 cells. Those structures, which could represent the helical sheath, were 200 nm long and 120 nm wide (Figure 25A, C). The other structure visible in Figure 25D, which looks like an assembly of pores could represent the bell like structure of the afp-like prophage or caused by the horizontal cut of the helical sheath during the preparation of the sample.

6 DISCUSSION

6.1 Infection of *Acanthamoeba* sp. 5a2 with *A. asiaticus* 5a2

Free-living amoebae can be found in various environments and are known to harbour human pathogens (Barker & Brown, 1994; Rodriguez-Zaragoza, 1994; Weissenberger et al., 2007). Therefore their importance for the medical health is gaining more attention in the last years. The goal of this experiment was to gain more understanding in the developmental cycle of *A. asiaticus* 5a2 as not much was known about endosymbionts of the acanthamoebae belonging to the *Bacteriodetes*. The different vital key stages as well as the time points during infection were investigated. Stages of interest are for example, the initial entry and uptake of *A. asiaticus* 5a2 inside the amoebae through phagocytosis, the escape of *A. asiaticus* 5a2 from phagocytosis and replication inside the acanthamoebae, the total occupation of the amoeba cytosol with the endosymbiont, lysis of the amoeba host and finally the infection of other acanthamoebae.

6.1.1 Dependency of infection by *A. asiaticus* on the amoeba growth media

Before the assay of comparing the infection rate of *A. asiaticus* 5a2 in different media was done, the acanthamoebae harbouring *A. asiaticus* 5a2 were cultivated in PYG. Therefore all acanthamoebae used for the assays were also cultivated in PYG medium. For the first fast and straightforward infection assay (5.3), freshly harvested intracellular *A. asiaticus* 5a2 has to be used, since it is an obligate intracellular symbiont and is not able to survive under extracellular conditions for an extended period of time. When the infection rate of this condition was compared to the continuous culture of acanthamoebae harbouring *A. asiaticus* 5a2, it was vastly lower. Even though the incubation condition was the same for both amoebae culture. The number of infected cells was dropping over the course of infection but the amount of intracellular *A. asiaticus* 5a2 per infected amoeba was steadily rising. This partially ruled out the explanation, that the

symbiont weren't able to replicate and therefore led to a low infection rate. Nine days after the initial infection, some weakly infected amoebae occurred, suggesting that cell lysis of infected amoebae took place, resulting in newly infected amoebae.

The infection rate of the first infection assays was very low. Not only were just few infected amoebae to be seen, but the percentage of infected amoebae was also dropping over time. It was suspected that the finding was caused by the dilution effect. The dilution effect is caused by the different growth rate between infected amoebae and uninfected amoebae. In this case, the supposed faster replication rate of the uninfected amoebae might out-dilute the infected amoebae. To verify the impact of possible "dilution effect" on the infection rate the growth rates of both infected and uninfected amoebae were tested out (5.5). Regardless of incubation temperature all acanthamoebae infected with the endosymbiont *A. asiaticus* 5a2 showed a slower duplication time than uninfected acanthamoebae. This answered the question about why the amount of infected amoebae seems to decrease overtime but not the initial amount of few infected cells. It is suggested that *A. asiaticus* is an energy parasite of the amoebae, exploiting amino acids, oligopeptides and other factors from the host. An infected amoeba would require more time than an uninfected amoeba to grow since it needs to compensate for the loss of energy caused by the symbionts. Because the number of uninfected amoebae was vastly higher than the number of infected amoebae, the uninfected ones outgrew the others, leading to a decrease in infection rate. New amoeba couldn't get infected because it could only be attained by lysis of infected amoebae, not occurring before days after infection. When both events happened at the same point, infected amoebae got out-diluted resulting in the "dilution effect". Only a high number of initially infected cell, subsequently leading to more cells being newly infected than the replication of uninfected amoebae, can minimize the dilution effect.

Since the initial number of infected cells was constantly low, the growth of the amoebae host must be inhibited for *A. asiaticus* 5a2 to catch up. Two different eukaryotic cell growth-inhibiting antibiotics were utilized to reduce the growth

of the amoebae (4.9.2), geneticin and cycloheximide.

Geneticin also known as G418 is an aminoglycoside antibiotic produced by *Micromonospora rhodorangea*. It blocks the polypeptide synthesis by inhibiting the elongation in not only eukaryotic cells but also prokaryotic cells. Cycloheximide is an antibiotic produced by the bacterium *Streptomyces griseus.* Its antibiotic effect comes from blocking the translational elongation.

After 24 hours of treatment with higher concentrated antibiotics, the impact on the host cells growth was clearly visualized by FISH. Not only were the amoebae more roundish and less trophozoite like, but also the uptake of symbiont per cell increased. The amoebae seem to have increased the uptake of potential food in order to counter the toxic effect of the antibiotics. The endosymbionts weren't visible as single cells residing in the cytosol but as accumulated signals inside vacuoles. Geneticin seems to be unsuitable because the amoebae died when a higher concentration was used. Since geneticin also affects prokaryotic cells, the morphology of *A. asiaticus* 5a2 changed while treated with Geneticin. The elongated form of the symbiont could be a cell undergoing binary fission but unable to segregate as a side effect of the antibiotic. Cultures treated with cycloheximide on the other hand did not show this elongated form of *A. asiaticus* 5a2, but some unknown effect couldn't be eliminated. Cycloheximide appeared to be less toxic compared to geneticin. The increase in infected cells compared to none treated culture were subtle.

Further experiments showed that *A. asiaticus* 5a2 at different life stages displays different grades of infectivity. But a highly infected amoebae culture like the continuous culture was still unachievable. Therefore the infection efficiency between TSY–grown and PYG-grown acanthamoebae and acanthamoebae harbouring *A. asiaticus* 5a2 was compared (5.7). Interestingly the use of TSY medium instead of PYG medium improved the infection rate visibly even by using intracellular *A. asiaticus* 5a2. A highly infected amoebae culture was finally achieved by utilizing the findings of both experiments (Figure 19), when both host and symbiont were cultivated in TSY-medium and the extracellular form of *A. asiaticus* 5a2 were used. Since all parameter were identical while

comparing the infection with PYG and TSY cultivated organisms, it suggest that either TSY contains factors beneficial for the infection or the medium PYG contains factors acting inhibitory for the infection.

Proteins of the outer membrane are important for host-cell-interaction (Caldwell et al., 1981; Swanson et al., 1994; Wyllie et al 1998). For bacterial pathogens both attachment and uptake by the host are important events as it is the first step, which will lead to the survival and replication inside the host (Abu Kwaik et al., 1994; Samrakandi et al., 2002). As in case of *L. pneumophila* studies show that the uptake is receptor mediated and that the process can be interfered by presence of saccharide structures (Harb et al., 1998). Dose dependent addition of mannose e.g. can be used to inhibit the uptake of *L. pneumophila* by the *Acanthamoeba castellani* (Allen and Dawidowicz, 1990; Alsam et al., 2005). A new study has also shown that the addition of glucose inhibits the uptake of *Legionella pneumophila* by acanthamoebae (Declerck et al., 2007).

To test out whether this also holds true for *A. asiaticus 5a2*, sugars in form of lactose, glucose and mannose were each added in different concentrations to a newly infected amoebae culture. An inhibitory effect of the *A. asiaticus* 5a2 uptake mediated by the addition of saccharides was expected. But regardless of the added saccharide, there was no visible difference between treated and untreated culture.

6.1.2 Comparison of *A. asiaticus* 5a2s developmental cycle with other endosymbionts of *acanthamoebae*

When compared to other obligate intracellular symbionts of the acanthamoebae, for example the *Chlamydiae,* the chlamydial developmental cycle is actually quite different. Bacteria belonging to the *Chlamydiae* possess the chlamydial developmental cycle, which consists of two distinct morphological as well as functional stages. One is the metabolically active stage, where the symbionts can thrive in inclusions called reticulate bodies (RBs) and multiply within their eukaryotic host cells. Furthermore, they can switch to an inactive

stage and reside as inactive elementary bodies (EBs), which can be taken up by the *Acanthamoeba* for further infection (Moulder, 1991; Abdelrahman & Belland, 2005). But not all RBs are the same. Members of *Chlamydiacea* form larger inclusions with many symbionts, where in case of *Protochlamydia amoebophila*, there are just one single cell inside the inclusion.

L. pneumophila, a human pathogen known to be able to thrive in acanthamoebae, also shows a biphasic life style. It consists of two distinctive stages, the transmissive phase and the replicative phase (Molofsky et al., 2004; Weissenmayer et al., 2011). Host cell lysis will only occur when *L. pneumophila* change into the transmissive form where it can infect other potential hosts.

A. asiaticus 5a2 does not form inclusions like in case of *P. amoebophila* after the uptake by an *Acanthamoeba.* Each *A. asiaticus* 5a2 resides in the cytosol of the amoeba as a single cell and is not clustered together. *A. asiaticus* 5a2 replicates in the cytosol until the host amoeba is filled up with the endosymbiont, followed by the lysis of the *Acanthamoeba*. Unlike *Chlamydiae*, where exit from host cell can also be mediated by extrusion of membrane-engulfed bacterial packages (Hybiske & Stephens, 2007) and leaving an intact host behind, the exit of *A. asiaticus* 5a2 from the host amoeba is hitherto only observed with the amoeba cell lysis. As demonstrated here, it seems that *A. asiaticus* 5a2 also has a cell cycle with functionally different cell stages, just like *P. amoebophila* and *L. pneumophila.* While comparing the infection assay of extracellular and intracellular *A. asiaticus* 5a2 (5.9), the infection rate was much higher when extracellular *A. asiaticus* 5a2 was used for the infection (Figure 15). Although infected cells could be detected in both assays (Figure 15), the amount of infected amoebae in the assay with extracellular endosymbiont was more abundant. On the other hand, in the assay with intracellular symbionts, only few infected amoebae could be found on the FISH-slide well. These few *A. asiaticus* 5a2 fluorescence signal could be extracellular *A. asiaticus* 5a2 still attached on the outer membrane of the acanthamoebae while the intracellular *A. asiaticus* 5a2 were harvested (4.8.4.1). Since when a refined version of this assay was performed (5.10), where the extracellular *A. asiaticus* 5a2 was compared to the intracellular and the pre-treated extracellular ones, there were

no infected cells found when intracellular *A. asiaticus* 5a2 was used (Figure 20A). Both extracellular and pre-treated extracellular *A. asiaticus* 5a2 showed many infected amoebae instead (Figure 20B, C). Based on those findings it appears that *A. asiaticus* 5a2 has at least two different cell stages, with the extracellular one being more infectious than the intracellular one, similar to two stages of *L. pneumophila.* This would also explain, why the infection efficiency of the initial fast and straightforward infection (5.3) was so low. Usually the media of the culture were replaced two days prior to the experiments. That led to the removal of all extracellular *A. asiaticus* 5a2, which was more infectious than the intracellular *A. asiaticus* 5a2. So even when a high MOI of 5000 was used, it still resulted in a low infection efficiency.

Since *A. asiaticus* 5a2 showed a biphasic lifestyle, it was interesting to check, whether other *A. asiaticus* strains were showing the same traits of a bi or even multiphasic lifestyle like *A. asiaticus* 5a2 (5.11). Indeed did both *A. asiaticus* US1 and *A. asiaticus* EIDS3 show the same feature as in *A. asiaticus* 5a2 with the extracellular form being more infectious than the intracellular one. Having multiple life stages seems to be an adaptation of the endosymbiont to their specific lifestyle. The various strategies are used to cope with different environment. Having several cell stages for dealing with different situation is one of them. For example *Theileria annulata* is a protozoa able to infect the B-lymphocytes and macrophages of cows, causing uncontrolled proliferation of infected blood cells as well as phenotypes of typical tumor cells (Dessauge et al., 2005; Heussler et al., 2006). It usually resides in the salivary gland of the tick and is transferred to the cattle when it gets bitten by the infected tick. *T. annulata* will infest the leucocyte of the new host as sporozoite. But for further infection, it needs to differentiate into merozoites able to infect erythrozoites. Infecting the red blood cells, which are much more numerous than the leucocytes, it will wander into the gut and salivary gland of the tick, that is feasting on the blood of the cattle (Shiels et al, 2006). Without the different cell stage allowing *T. annulata* to infect merozoites, the infection cycle cannot be completed. But not only protozoan are can show a multiphasic life style, but also prokaryotes e.g. the aforementioned *P. amoebophila* und *L. pneumophila*. The two different stages of *A. asiaticus,* an energy parasite of the amoeba (Schmitz-

Esser et al., 2010), might allow it to only expresses certain factors for invading new hosts when it is necessary and therefore preserving the energy for other vital metabolic interactions. *A. asiaticus* 5a2 is also the first described bacterium belonging to the *Bacteriodetes* with a bi-phasic life style.

For example, important for the chlamydial life cycle are the proteins in the outer membrane of chlamydiae, as they are involved in attachment to host cells, uptake of nutrients and waste removal. They are also responsible for the disparity between EBs and RBs. In case of *P. amoebophila* E25, no gene similar to the most abundant outer membrane protein MOMP (major outer membrane protein) was found. Instead it is suggested that the putative porin pc1489, which was found in *P. amoebophila* E25, might act as a functional replacement of MOMP in *P. amoebophila* (Heinz et al., 2010).

L. pneumophila needs to express macrophage infectivity factor (mip) in order to establish a successful infection (Cianciotto & Fields, 1992). *L. pneumophila* utilizes an Icm/Dot type IV secretion system to interact with the host through injections of effector proteins or factors for remodeling endosome membrane (Swanson & Isberg, 1995; Vogel, 1998). The release from the host is achieved by the production of pore forming toxins, which induces holes into cytoplasma membrane of the amoeba (Byrne and Swanson, 1998).

Not much was known about *A.* asiaticus until recently, when the genome of it was sequenced. (Schmitz-Esser et al, 2010). The most revealing thing was the high abundance of proteins with eukaryotic domains such as ankyrin-repeats, TPR/SEL1 repeats, leucine-rich repeats, as well as F- and U box domains. It is suggested that these domains are important for the host cell interaction, in order to facilitate infection or to maintain the intracellular survival as well as replication (Schmitz-Esser et al, 2010). But it wasn't clear how the proteins were presented to the host cell due to lack of known secretion and transport system except an incomplete type six secretion system. Recently a new study shows that *A. asiaticus* 5a2s genome encodes proteins similar to proteins from the antifeeding prophage (afp) (Penz et al., 2010). The afp was found on the plasmid pADAP (amber disease associated plasmid) in the insect pathogen

Serratia entomophila (Hurst et al., 2007) where it acts as a toxin delivery system into the host. Although different variants of afp have been identified (Hurst et al, 2007), it is known that are two different configuration, the extended configuration and the retracted configuration, where the core region is exposed (Hurst et al., 2007; Sen et al., 2010). The afp particle consists of a helical sheath, a bell shaped structure and an inner tube (Sen et al., 2010). It is suggested, that afp-like proteins of *A. asiaticus* 5a2 represents some sort of novel protein secretion apparatus (Penz et al., 2010).

Interestingly when TEM pictures of *A. asiaticus* 5a2 at different time points (5 hpi, 72 hpi, 144 hpi and extracellular *A. asiaticus* 5a2) of the infection was taken, the afp-like- apparatus structure can clearly be seen inside the extracellular *A. asiaticus* 5a2 (Figure 25). It is possible, that *A. asiaticus* 5a2 utilizes the afp-like-apparatus for escape from the amoeba host by injecting cell lysis inducing factors or *A. asiaticus* 5a2 uses it for infection or entry of the *Acanthamoeba* where the symbiont can attach itself on the outer membrane of the host. What factors are involved in these processes remain to be revealed.

6.1.3 The infection cycle of A. asiaticus 5a2 in Acanthamoeba sp. 5a2

Characterization of the infection cycle is of great interest, since not much is hitherto known about amoebal symbionts belonging to the *Bacteriodetes.* Much insight can be gained when the infection of *A. asiaticus* 5a2 is described.
The entry or uptake of the endosymbiont occurs within 4-6 hours post infection, since intracellular *A. asiaticus* 5a2 could still be detected with FISH after the 4 hpi washing steps. Between the time points 4 – 72 hpi, the morphology of *A. asiaticus* 5a2 can be considered as small clinched rods, almost coccus-like. This forms a contrast to the rod-shaped nature of *A. asiaticus* 5a2, which can be observed intracellularly. After the 72 hpi mark, the shape of the endosymbiont began to change, becoming more elongated and rod-like. This could be the result of extensive cell division through binary fission, indicating a replicative stage of the symbiont. At 144 hpi, most infected amoebae were full with *A. asiaticus* 5a2 and the first lysis of infected amoebal cells occurred. Not only

extracellular *A. asiaticus* 5a2 but also newly infected cells could also be detected. Comparing the shape of extracellular and newly infecting *A. asiaticus 5a2* with the shape around 72 hpi time point, it was visible that the symbiont weren't as elongated as before but shorter, suggesting either a stop in replication due to possible depletion of nutrients inside the amoebae or a change into some transmissive form, able to infect other amoebae. This also coincides with the observation, that the extracellular *A. asiaticus* 5a2 is more infectious than the intracellular one. (Figure 26)

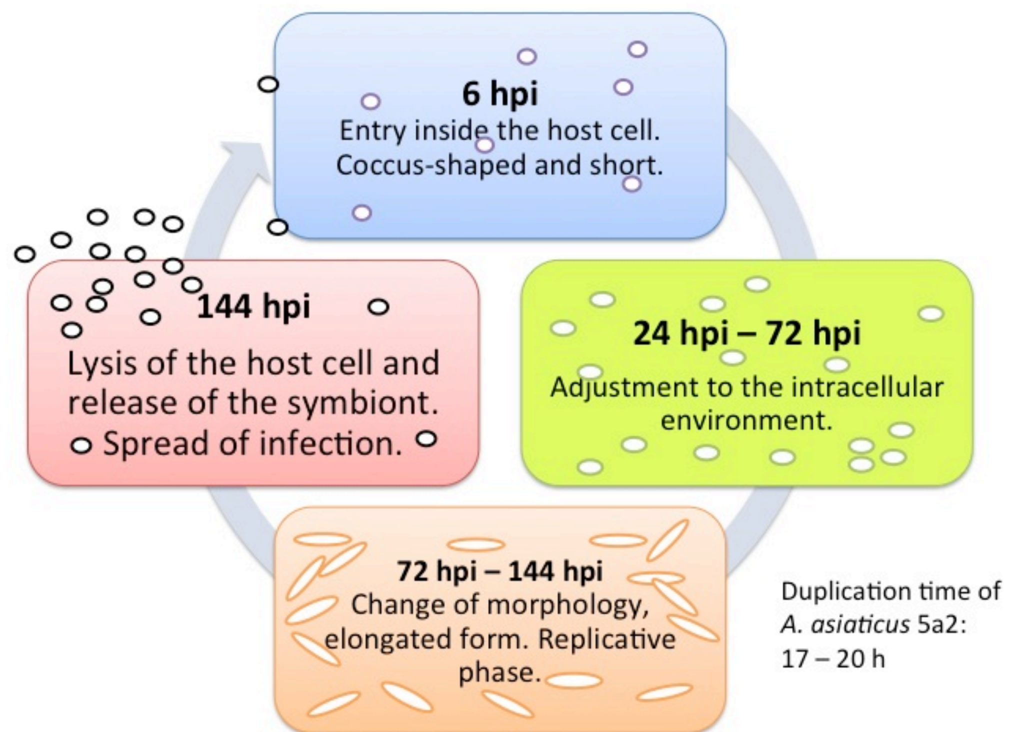


Figure 26. The infection cycle of *A. asiaticus* 5a2. Note the existence of two distinct morphotypes suggesting a biphasic life-style of *A. asiaticus* 5a2.

6.1.4 **Outlook**

Further analysis of the ultrastructure of *A. asiaticus* 5a2 at different life stages should be performed to better understand the morphological differences. The results of this study serve as in-depth analysis of the infection cycle of *A.*

asiaticus using transcriptomics. The expression pattern will help to better understand the role of genes and mechanisms of *A. asiaticus* 5a2 for the infection of eukaryotic cells.

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8 LIST OF ABBREVIATIONS

16S rDNA	small subunit ribosomal RNA-encoding gene of prokaryotes
%	percentage
°C	degree celcius
µ	micro (10^{-6})
16S rRNA	small subunit ribosomal RNA of prokaryotes
A. asiaticus	*Amoebophilus asiaticus*
bp	basepairs
L. pneumophila	*Legionella pneumophila*
Cy3	indocarbocyanine
d	days
DAPI	4',6-diamidino-2-phenylindole
$_{dd}H_2O$	double distilled and filtered water
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
$EtOH_{abs}$	ethanol absolute
extrac.	extracellular
FISH	fluorescence in situ hybridization
FLUOS	fluorescein N-hydroxysuccinimidester
g	gram
h	hours
hpi	hours post infection
hps	hours post seeding
intrac.	intracellular
kb	Kilobases
l	liter
m	milli (10^{-3})
M	Molar
Mb	Megabases
Mg	magnesium
min	minutes

MOI	multiplicity of infection
n	nano (10^{-9})
P. amoebophila	*Protochlamydiae amoebophila*
PAS	Page's amoebic saline
PBS	phosphate buffered saline
PFA	paraformaldehyde
PYG	peptone-yeast extract-glucose
RNA	ribonucleic acid
rpm	rounds per minutes
rRNA	ribosomal RNA
RT	room temperature
s	seconds
sp.	species
SPG	sucrose-phosphate-glutamate
v/v	volume to volume

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