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**Unlinking host dependence:
Characterization of the intracellular
Acanthamoeba endosymbiont
*Procabacter acanthamoebae***

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Summary

Free-living amoebae have been described as evolutionary “training grounds” for intracellular bacterial pathogens. They are known to serve as environmental reservoirs for human pathogens such as *Legionella pneumophila*, *Mycobacterium* spp., and *Bacillus anthracis*. In addition they may serve as hosts for obligate intracellular symbionts. *Procabacter acanthamoebae* UWC12 is an obligate intracytoplasmatic *Acanthamoeba* symbiont belonging to the *Betaproteobacteria*. In comparison to the reduced genomes of most endosymbionts, the genome of *Procabacter acanthamoebae* is with 2.45 mega-bases unusually large and encodes less-reduced metabolic pathways. Investigation of the infection cycle shows a monophasic lifestyle with a comparably low infectivity in the natural host *Acanthamoeba* sp. UWC12. *P. acanthamoebae* has a slightly negative impact on host population doubling time and does not lyse the amoeba host at any point during the infection. The host range seems to be limited to phagocytic cells, as it can enter macrophage-like insect (S2) and human (THP-1) cells, but not human HeLa cells. Fast propagation, and a high rate of host lysis was observed in S2 cells. Suprisingly, we noted evidence for limited extracellular growth of *P. acanthamoebae* in Schneider medium. Soft agar based cultivation approaches were used to incubate *P. acanthamoebae* in an oxygen gradient in this medium. Growth was observed primarily in the micro aerobic zone. A 16S rRNA gene-targeted qPCR assay was established for quantification of *P. acanthamoebae*. Under micro aerobic conditions growth was observed in liquid Schneider medium after 16 days. Interestingly, biofilm formation was observed under atmospheric oxygen conditions. Using a crystal violet based photometric assay significantly more biofilm formation was measured under aerobic conditions than under micro aerobic conditions. Taken together, in this study I analysed the infection cycle and host range of the amoeba symbiont *P. acanthamoebae*, and I could show that these bacteria may be grown independently of their hosts in nutrient-rich media under micro-aerobic conditions. This investigation contributes to our understanding of axenic cultivation of the obligate intracellular symbiont *P. acanthamoebae* and paves the way to a novel genetic system to study this symbiosis.

Zusammenfassung

Freilebende Amöben wurden in der Literatur als evolutionäre “Trainingsgelände” für intrazelluläre bakterielle Pathogene beschrieben. Sie sind bekannte Umwelreservoirs für Humanpathogene wie *Legionella pneumophila*, *Mycobacterium* spp. und *Bacillus anthracis*. Zusätzlich können sie auch obligate intrazelluläre Organismen beherbergen. *Procabacter acanthamoebae* UWC12 ist ein obligater intrazytoplasmatischer Endosymbiont in der Gattung *Acanthamoeba* und fällt in die Klasse der *Betaproteobakterien*. Im Vergleich zu den reduzierten Genomen vieler anderer Endosymbionten ist das Genom mit 2,45 Megabasen verhältnismäßig groß und enkodiert kaum reduzierte Stoffwechselwege. Untersuchungen des Infektionszyklus zeigen einen monophasischen Lebenszyklus mit einer niedrigen Infektiosität im natürlichen Wirt *Acanthamoeba* sp. UWC12. *P. acanthamoebae* beeinflusst die Populationsverdopplungszeit des Wirtes negativ, wenn auch in schwachem Ausmaß und Wirtszellen scheinen zu keinem Zeitpunkt der Infektion zu lysieren. Die Bandbreite infizierbarer Wirtsgruppen scheint sich auf phagozytisch aktive Zellen zu beschränken, da der Endosymbiont die makrophagenähnliche insektoide (S2) und humane (THP-1) Zelllinie infizieren kann, jedoch nicht humane HeLa Zellen. Eine schnelle Verbreitung der Endosymbionten und eine hohe Lysierungsrate der Wirtszellen in der S2 Zelllinie wurden beobachtet. Überraschenderweise konnte begrenztes Wachstum von extrazellulären *P. acanthamoebae* in Schneider Medium festgestellt werden. Um in diesem Medium einen Sauerstoffgradienten einzustellen, wurde eine geringe Konzentration an Agar zugesetzt. Mit diesem Ansatz konnte Wachstum vor allem in der mikroaeroben Zone beobachtet werden. Ein qPCR Assay wurde etabliert, der spezifisch für das 16S rRNA Gen von *P. acanthamoebae* ist. Unter mikroaeroben Bedingungen konnte Wachstum in flüssigem SM nachgewiesen werden. Interessanterweise wurde unter atmosphärischen Sauerstoffkonzentrationen ein Biofilm ausgebildet. Mit Hilfe eines photometrischen Kristall-Violett Assays wurde gezeigt, dass signifikant mehr Biofilm unter aeroben, als unter mikroaeroben Bedingungen ausgebildet wird. Zusammenfassend habe ich in dieser Arbeit den Infektionszyklus und die Bandbreite an Wirtszellen des Amöbensymbionten *P. acanthamoebae* analysiert und ich konnte zeigen, dass diese Bakterien unabhängig von ihrem Wirt in nährstoffreichen Medium unter mikroaeroben Bedingungen Wachsen können. Diese Untersuchungen tragen zum Verständnis von axenischer Kultivierung des obligaten, intrazellulären Symbionten *P. acanthamoebae* bei und ebnen den Weg zu einem neuen genetischen System um diese Symbiose zu untersuchen.

Introduction

3.1 An Introduction into symbiosis

The word symbiosis is of greek origin and means “living together”. Our current definition is based on the book “Die Erscheinung der Symbiose” by Anton de Bary (1879) who defines symbiosis as “the living together of unlike named organisms” and was originally used to explain the formation of lichens, consisting of a symbiosis between algae (or cyanobacteria) and fungi. However this term does not determine the properties of these associations. Symbiosis of two phylogenetically different organisms can be beneficial to both (mutualism), beneficial to one without effect on the other (commensalism), or beneficial to one and detrimental to the other (parasitism) (Sapp 1994). A well—known example for a mutualistic relationship is *Buchnera aphidicola*, which is a member of the *Gammaroteobacteria* and an obligate endosymbiont of the aphid. It is dependent on the aphid host to provide nutrients and protection from the outer environment. In return, *Buchnera aphidicola* provides essential nutrients that the host lacks, due to its carbon—rich, but nitrogen—poor diet (Munson et al., 1991). Parasitism is a frequently used strategy in nature. Many human diseases are caused by intracellular pathogens like *Legionella pneumophila* or *Chlamydia trachomatis* (Fraser et al. 1977; Halberstädter and Prowazek 1907). A recent example for commensalism is *Staphylococcus epidermidis*, which can benefit cutaneous immune defense by selectively inhibiting the survival of skin pathogens of humans (Cogen et al. 2010).

3.2 *Acanthamoeba* spp. as a host for intracellular bacteria

Free-living *Acanthamoeba* are ubiquitous and were isolated from a diverse set of environments ranging from soil, salt water, dust, and air to even human cornea. In their natural habitats amoeba are important predators of the microbial community and mineralization of carbon, nitrogen, and phosphorus of soil containing *Acanthamoeba* and bacteria is significantly greater than in soil solely containing bacteria (Rønn et al. 2002). An important feature of *Acanthamoeba* spp. is their resilience to abiotic and biotic stresses. *Acanthamoebae* can be found either in their active form, the trophozoite, displaying their characteristic pseudopods, the spikelike “acanthopodia” (Blair Bowers and Korn 1968) or in cysts that enable them to withstand starvation, desiccation, and temperature stress (B. Bowers and Korn 1969). *Acanthamoeba* have been particularly well—known for facultatively hosting endosymbiotic bacteria for a long time (Proca-Ciobanu et al. 1975; T. R. Fritsche et al. 1993) and are phylogenetically diverse (Fig. 3.1). Additionally several species are implicated in human disease, including *A. astronyxis*, *A. culbertsoni*, *A. polyphaga*, *A. castellanii*, *A. hatchetti*, *A. rhysodes*, *A. divionensis*, *A. lugdunensis*, and *A. lenticulata* (Kong 2009). In Austria, especially *Acanthamoeba keratitis* is well—documented (Walochnik et al. 2001).

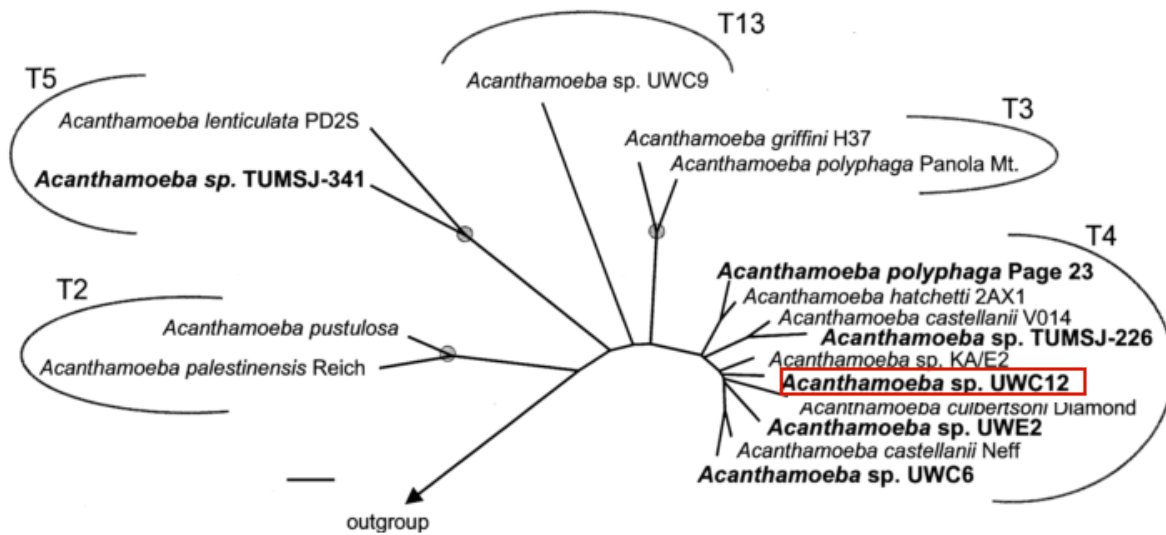


Figure 3.1: Phylogeny of *Acanthamoeba* spp. 18S rDNA based phylogenetic tree using the neighbor—joining algorithm (modified from Horn et al. 2002). Even within the genus *Acanthamoeba* there is still considerable genetic diversity. *Acanthamoeba* sp. UWC12 is highlighted with a red box.

It has been described that 25 % of *Acanthamoeba* spp. are hosting bacterial endosymbionts of diverse taxa, although the roles such endosymbionts play in effective host fitness are unclear (T. R. Fritsche et al. 1993). Already in 2004, 19 bacterial endosymbionts of *Acanthamoeba* sp. and *Hartmannella* sp. belonging to several phyla (eg. *Proteobacteria*, *Chlamydiae*, and *Bacteroidetes*) had been described (Horn and Wagner 2004). The first human pathogen described to thrive intracellularly in *Acanthamoeba* was *Legionella pneumophila* (Rowbotham 1980). These observations led to the hypothesis that amoebae may act as training grounds for intracellular bacterial pathogens (Molmeret et al. 2005). *Acanthamoeba* seem to interact almost exclusively with gram-negative bacteria, with some exceptions such as *Bacillus anthracis* (Dey, Hoffman, and Glomski 2012). Under mesophilic conditions it was even shown that *A. castellanii* can grow on lawns of hyperthermophilic archaea of the genus *Sulfolobus* (Iqbal, Siddiqui, and Khan 2013). Therefore it has been hypothesised that a limited ability of non-spore-forming bacteria to survive hostile conditions may have led to their adaption to associate with a protist host, which then serves as an additional biological reservoir. Under the assumption that amoebae provided a “training ground” to learn to evade phagocytic killing, becoming an intracellular human pathogen was a secondary event (Khan and Siddiqui 2014).

3.3 *Procabacter acanthamoebae* UWC12

Procabacter acanthamoebae UWC12 belongs to the class of β —Proteobacteria (Fig. 3.2), is Gram-negative, has a rod—shaped morphology, and has a size of $0.3\text{--}0.5 \times 1.0\text{--}2.0 \mu\text{m}$ (Horn et al. 2002). It is an obligate intracytoplasmatic endosymbiont of *Acanthamoeba* sp. UWC12, which was isolated from corneal scrapings of *Acanthamoeba* keratitis patients (T. R. Fritsche et al. 1993) and has so far not been cultureable in host—free growth media. Among other amoebal endosymbionts, *P. acanthamoebae* has been reported to increase the rate of the cytopathic effect of *Acanthamoeba* spp. by 19 % in average (T. R. Fritsche, Sobek, and Gautom 1998). An interesting feature of the genus *Procabacter* is the versatility in lifestyle, despite there only being 8 known representatives

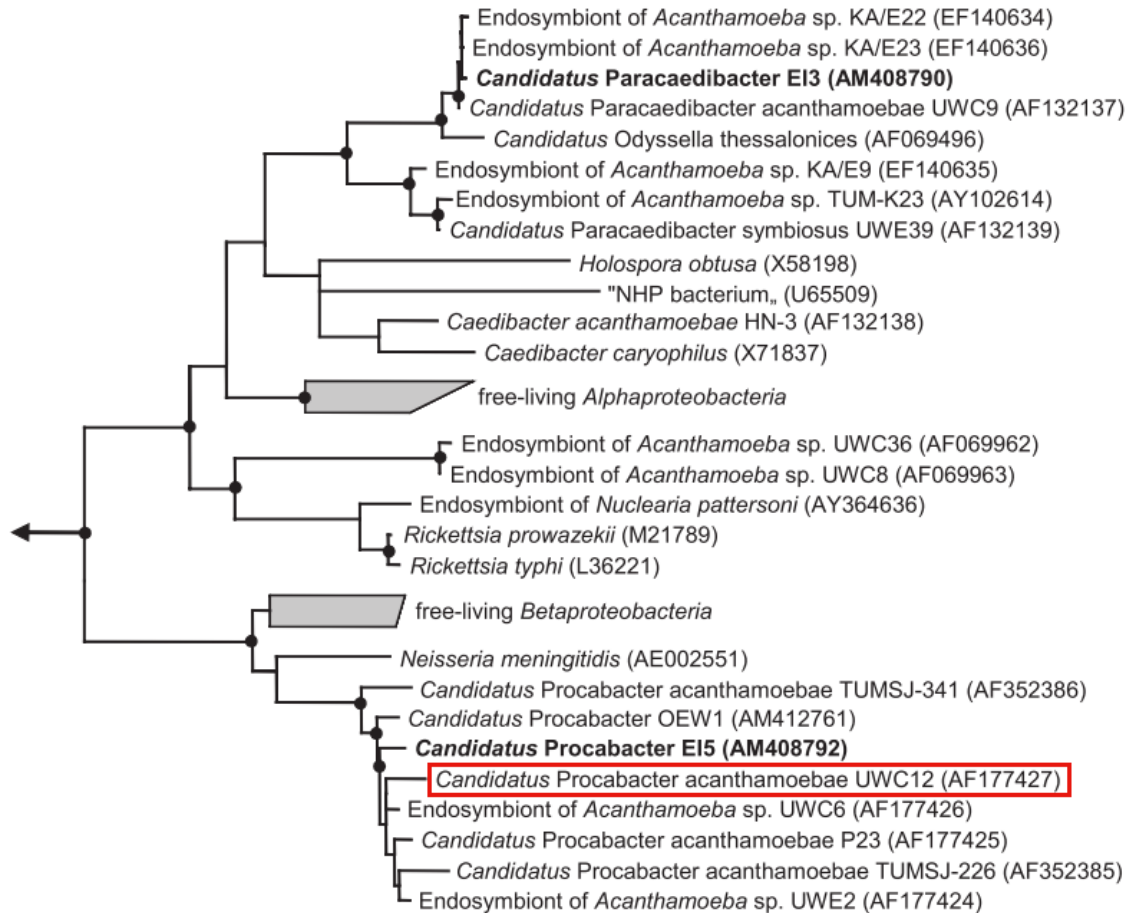


Figure 3.2: Phylogeny of *Proteobacteria* endosymbionts found in *Acanthamoeba* spp. 16S rDNA-based TREE-PUZZLE tree depicting the phylogenetic relationship of *Acanthamoeba* endosymbionts found within the *Proteobacteria* (modified from Schmitz-Esser et al. 2008). *Procabacter acanthamoebae* UWC12 is highlighted with a red box.

(Schmitz-Esser et al. 2008). *P. acanthamoebae* strains Page 23, UWC12, and UWE2 are found directly in the cytoplasm (Horn et al. 2002), while *Procabacter* sp. EI5 and OEW1 are enveloped by a membrane (Schmitz-Esser et al. 2008; Heinz et al. 2007). *Procabacter* sp. OEW1 is an intriguing member of the genus, as it has additionally been isolated in a multi-partner association with *Acanthamoeba* sp. OEW1 and *Parachlamydia* sp. OEW1 (Heinz et al. 2007). Genome sequencing showed that *P. acanthamoebae* UWC12 has a relatively large genome for an obligate endosymbiont with 2.48 megabases (Tsao 2013, unpublished). It has high metabolic capabilities with a complete TCA cycle, glycolysis, pentose phosphate pathway and is auxotrophic only for asparagin, aspartate, cysteine, methionine, glutamate, and glycine (Tsao 2014, unpublished).

3.4 What it takes to thrive in an exclusive niche: the eukaryotic cytosol

The eukaryotic cytoplasm is highly organized system within the eukaryotic cell enclosed by the cell membrane, containing subcellular compartmentation (Weiss and Korge 2001). In this thesis the term cytosol is used for the aqueous cytoplasm as defined by Clegg (1984). To specify the cytosol as a niche for bacterial growth, it is important to understand its nutritional content, osmolarity, and pH. The mammalian cytosol has a high concentration of potassium (140 mM), yet a low concentration of magnesium (0.5 mM), sodium (5–15 mM), and calcium (10^{-4} mM) (Alberts et al. 2014). The cytosolic pH is fairly neutral and has been measured to be between 7.09 and 7.35 in the Swiss 3T3 celline (Bright et al. 1987). The cytosol has further been characterized as a reducing environment by the ratio of reduced to oxidized glutathione (Hwang, Sinskey, and Lodish 1992). Tissue oxygen concentrations were measured to be microoxic in general (Braun et al. 2001). Ray et al. (2009) divide the life cycle of cytosolic bacteria in three stages:

- Escape from the vacuole
- Replication within the cytosol
- Manipulation of the innate immune response

Its noteworthy to mention that, before escaping from a formed vacuole, uptake has to be initialized. Cytosolic endosymbionts usually use either a trigger or zipper mechanism to enter nonphagocytic eukaryotic cells (Pascale Cossart and Sansonetti 2004). The bacteria are taken up into a vacuole, which acidifies within 15 minutes of forming (Yates, Hermetter, and Russell 2005). An additional threat is posed by microbicidal peptides contained in lysosomes that become a threat after lysosome-vacuole fusion (Haas 2007). Therefore it is of utmost importance for a cytosolic endosymbiont to escape from the vacuole before it faces degradation. In *Listeria monocytogenes* escape is mediated by the enzymes listeriolysin O (P. Cossart et al. 1989) and two type C phospholipases (Smith et al. 1995). In *Shigella flexneri* the type III secreted IpaB is used to escape vacuoles and similar to listeriolysin O it shows haemolytic activity (High et al. 1992). *Rickettsia* spp. also use a haemolytic protein (haemolysin C) and phospholipases for the escape from host vacuoles (Whitworth et al. 2005).

3.5 Aims of this study

The general subject of this thesis was the characterization of the intracellular bacterium *Procabacter acanthamoebae* UWC12 and its properties as an endosymbiont in the natural host *Acanthamoeba* sp. UWC12. The aims of this study can be roughly summarized as follows:

1. Establish an aposymbiotic *Acanthamoeba* sp. UWC12 population using antibiotic treatment to investigate effects of a *P. acanthamoebae* UWC12 infection on the natural host.
2. Examine and quantify the infection cycle of *P. acanthamoebae* UWC12 in its natural host *Acanthamoeba* sp. UWC12 and comparison with other known endosymbionts of acanthamoebae using fluorescence in situ hybridization (FISH).
3. Measure the impact of the *P. acanthamoebae* UWC12 infection on *Acanthamoeba* sp. UWC12 growth rates at different temperatures to illuminate possible mutualistic or parasitic properties of the symbiosis.
4. Investigate the host range of *P. acanthamoebae* UWC12 in higher eukaryotic celllines and the persistence in a host-free environment.
5. Set the framework for a genetic model system by establishing a host free cultivation setup for *P. acanthamoebae* UWC12 by finding an axenic medium and cultivation conditions.
6. Develop and evaluate a protocol for quantifying *P. acanthamoebae* in liquid medium.

The study started with an *Acanthamoeba* sp. UWC12 running culture infected with *P. acanthamoebae*. Previous curing attempts did not result in a stable aposymbiotic host population (Köstlbacher 2013, unpublished). It was already known that *P. acanthamoebae* can not infect HeLa cells (Tsao 2013, unpublished). In general cultivation media, axenic endosymbiont growth could not be observed (Horn et al. 2002). Genome assembly and annotation were in parallel conducted by Han-Fei Allen Tsao.

Materials and methods

All laboratory work was done at the Division of Microbial Ecology (University of Vienna). The only exception was the Tecan Infinite M200 microplate reader, which was used for biofilm quantification and is located at the Division of Terrestrial Ecosystem Research (University of Vienna)

4.1 Equipment

Equipment	Company
Agarose gel electrophoresis apparatus Sub-Cell GT	Bio-Rad Laboratories GmbH, Munich, Germany
Beadbeater Fast Prep FP 120	Savant Instruments Inc. Holbrook, NY
Mikro 20 (centrifuge)	Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany
Rotina 35 R (centrifuge)	Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany
Galaxy Mini (centrifuge)	VWR international, West Chester, PA, USA
Sub-Cell GT (agarose gel electrophoresis system)	Bio-Rad Laboratories GmbH, Munich, Germany
PowerPac Basic (Electrophoresis power supply)	Bio-Rad Laboratories GmbH, Munich, Germany
Axioplan 2 imaging (Epifluorescence microscope)	Carl Zeiss MicroImaging GmbH, Jena, Germany
TCS SP8 X (Confocal Laser Scanning)	Leica Camera AG, Wetzlar, Germany
Neubauer counting chamber	Paul Marienfeld GmbH & Co KG, Lauda- Königshofen, Germany
inoLab® pH Level 1 (pH meter)	Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany
OHAUS® Analytical Plus (analytical balance)	Ohaus Corporation, Pine Brook, NJ, USA
Sartorius BL 3100 (balance)	Sartorius AG, Göttingen, Germany

Equipment	Company
SmartSpec™ 3000 (Spectral photometer)	Bio-Rad Laboratories GmbH, Munich, Germany
UV sterilizing PCR workstation	PeqLab Biotechnologie GmbH, Erlangen, Germany
Haake DC10-P5/U (heating circulator bath)	Thermo Fisher Scientific Inc., Waltham, MA, USA
GFL 1004 (Incubation bath)	Gesellschaft für Labortechnik GmbH, Burgwedel, Germany
Varioclav® 135 S H+P (autoclave)	H+P Labortechnik GmbH, Oberschleißheim, Germany
Varioclav® 25 T H+P (autoclave)	H+P Labortechnik GmbH, Oberschleißheim, Germany
Water purification system MILLI-Q® biocel	Millipore GmbH, Vienna, Austria
Infinite® M200 (microplate reader)	Tecan Group Ltd., Männedorf, Switzerland
NanoDrop ND-1000 (UV-visible spectrophotometer)	Thermo Fisher Scientific Inc., Waltham, MA, USA

Table 4.1: Technical equipment used for experimental work.

4.2 Software

Software	Developer
Axiovision Rel. 4.8	Carl Zeiss MicroImaging GmbH, Jena, Germany
ImageJ	http://rsbweb.nih.gov/ij/index.html
Leica LAS AF Lite	Leica Microsystems, Wetzlar, Germany
Microsoft Excel for Mac 2011	Microsoft Corporation, Redmond, WA, USA
Rstudio Version 0.98.953	RStudio, Inc.

Table 4.2: Software used for data collection and analysis.

4.3 Consumables

Consumables	Company
Optical sealing tape (for use with iCycler™ iQ)	Bio-Rad Laboratories GmbH, Munich, Germany

Consumables	Company
0.2 mL PCR tubes	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
0.6 mL reaction tubes	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Pipette tips with filter, various sizes	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Sterican® needles, various sizes	B. Braun Melsungen AG, Melsungen, Germany
Syringe filter, cellulose acetate (0.2 µm and 5 µm)	Asahi Techno Glass Corporation, Iwaki Glass Co., Ltd., Funabashi-City, Japan
PCR plates, 96-well, semi-skirted, colourless (for use in qPCR)	VWR International GmbH, Erlangen, Germany
Reaction tubes (15 ml, 50 ml) Isopore™	Greiner Bio-One GmbH, Frickenhausen, Germany
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
25 cm ² Tissue culture flasks	Asahi Techno Glass Corporation, Iwaki Glass Co., Ltd., Funabashi-City, Japan
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	
Multiwell dishes, polystyrene (6 wells, 12 wells)	Nunc, Roskilde, Denmark
Parafilm® M laboratory film	American National Can Company, Chicago, IL, USA
Syringe Injekt®-F single use, sterile (various sizes)	B. Braun Melsungen AG, Melsungen, Germany

Table 4.3: Consumables used for experimental work.

4.4 Probes and primers

4.4.1 FISH probes

Probe name	Specificity	Probe sequence (5'-3')	Reference
Proca438	Procabacter sp.	CGA TTT CCT CCC RGA CAA	(Horn et al. 2002)
EUB338- DOPE	most Bacteria	GCT GCC TCC CGT AGG AGT	(Amann et al. 1990)
EUK516	Eukarya	ACC AGA CTT GCC CTC C	(Amann et al. 1990)

Table 4.4: Probes used for FISH.

4.4.2 PCR primers

Probe name	Specificity	Primer sequence	Reference
M13 Forward	MCS of many vectors	5'-GTA AAA CGA CGG CCA G-3'	TOPO TA guide, Invitrogen
M13 Reverse	MCS of many vectors	5'-CAG GAA ACA GCT ATG AC-3'	TOPO TA guide, Invitrogen
616V	Most <i>Bacteria</i>	5'-AGA GTT TGA TYM TGG CTC-3'	(Loy et al. 2002)
1389Fmix	Most <i>Bacteria</i> and <i>Archaea</i> (16S rRNA genes)	5'-TGY ACA CAC CGC CCG T-3'	(Loy et al. 2002)
1492R	Most <i>Bacteria</i> and <i>Archaea</i> (16S rRNA genes)	5'-GGY TAC CTT GTT ACG ACT T-3'	(Loy et al. 2002)
117F	<i>Procabacter acanthamoebae</i>	5'-GAA CGT GCC TAG TAG TGG GG-3'	(Tsao 2014, unpublished)
583F	<i>Procabacter acanthamoebae</i>	5'-GGT GGT TTA TCA AGC CAG AT-3'	(Tsao 2014, unpublished)
584F	<i>Procabacter acanthamoebae</i>	5'-GTG GTT TAT CAA GCC AGA TG-3'	(Tsao 2014, unpublished)

Probe name	Specificity	Primer sequence	Reference
744R	<i>Procabacter acanthamoebae</i>	5'-CGT GCT TGA GTG TCA GTA TCA-3'	(Tsao 2014, unpublished)
271R	<i>Procabacter acanthamoebae</i>	5'-ATC GAT CGC AGC CTT GGT AG-3'	(Tsao 2014, unpublished)
Proc-581F	<i>Procabacter acanthamoebae</i>	5-CAG GTG GTT TAT CAA GCC AG-3'	(Tsao 2014, unpublished)
Proc-1212R	<i>Procabacter acanthamoebae</i>	5'-CGT GTG AAG CCC TGC CTA T-3'	(Tsao 2014, unpublished)

Table 4.5: Primers used for PCR.

4.5 Cultivation of *Acanthamoeba* sp. UWC12 and *Procabacter acanthamoebae* UWC12

4.5.1 Endosymbiont cultivation

Acanthamoeba sp. UWC12 containing *Procabacter acanthamoebae* UWC12 endosymbionts were cultivated in Tryptone Soy Yeast Extract Broth (TSY; 30 g l⁻¹ Trypticase Soy Broth, 10 g l⁻¹ Yeast extract) at 20 °C. Cultures were maintained in cell culture flasks (Nunclon delta-surface, Thermoscientific, St Leon Rot, Germany) and the medium was renewed every seven days by removing the supernatant and adding 250 ml of TSY.

4.5.2 Host amoeba cultivation conditions

Acanthamoeba sp. UWC12 were cultivated in TSY at 20 °C. Cultures were maintained separately from infected amoeba in cell culture flasks and the medium was renewed every seven days by removing the supernatant and adding 250 ml of TSY.

4.6 Harvesting of *Acanthamoeba* sp. UWC12 and *Procabacter acanthamoebae* UWC12

4.6.1 Harvesting of amoebae

The culture flasks containing *Acanthamoeba* were shaken vigorously to detach amoebae from the surface. The resulting cell suspension was poured into tubes of varying size depending on the experimental requirements. Amoebae were obtained by centrifugation at 2600 g for six minutes at room temperature (RT). The supernatant was decanted and the pellet re-suspended in a desired amount of medium or fixative of choice. For a higher yield of amoebae, pellets were pooled after resuspension.

4.6.2 Harvesting of intracellular *P. acanthamoebae*

Amoebae containing *P. acanthamoebae* were collected (4.6.1) from a culture flask in a 50 ml Greiner tube and pelleted by centrifugation at 2600 g for six minutes at RT. The pellet was then re-suspended in eight ml of a medium of choice and transferred into the Dounce homogenizer. A tight glass pestle was used to lyse amoeba cells. The pestle was struck for 15 times to release the intracellular bacteria. The homogenized suspension was filtered using filters of 5 μm pore size to separate cell debris and intact amoebae and from bacterial cells.

4.6.3 Harvesting of extracellular *P. acanthamoebae*

Supernatant of an attached *P. acanthamoebae* containing amoebae culture was filtered (five μm pore size) into a 50 ml Greiner tube to obtain an amoeba free endosymbiont suspension. If required the cells density was concentrated by centrifugation at 2600 g for six minutes at RT and suspension in a smaller volume of medium of choice.

4.7 Fixation for fluorescence in situ hybridization (FISH)

4.7.1 Fixation for FISH of amoeba samples

Amoeba containing growth medium was centrifuged at 2600 g at RT for 5 minutes. A washing step with page's amoeba saline solution (PAS; 0.12 g l⁻¹ NaCl, 0.004 g l⁻¹ MgSO₄ × H₂O, 0.004 g l⁻¹ CaCl × 2H₂O, 0.142 g l⁻¹ Na₂HPO₄, 0.136 g l⁻¹ KH₂PO₄) was conducted before the sample was re-suspended in PAS and 20 μl were applied to a well on a FISH-slide. After 20 minutes amoebae were considered to be attached to the slide. The supernatant was removed and the sample was fixed with 20 μl of 4 % (w v⁻¹) paraformaldehyde (PFA) for 15 minutes. The PFA was then removed and after a washing step with Milli-Q water the sample was dried at 46 °C.

4.7.2 Fixation for FISH of endosymbiont samples

P. acanthamoebae containing growth medium was centrifuged at 2600 g. Three volumes of 4 % paraformaldehyde solution were gently mixed with one volume of endosymbiont containing supernatant and incubated at 4°C for 4h. To remove the 4% paraformaldehyde solution the sample was

centrifuged at 18,000 g for 15 min and after disposal of the supernatant the pellet was washed with $1\times$ PBS. Subsequently, the sample was centrifuged again at 13,000 rpm for 15min. The pellet was then re-suspended in one volume of $1\times$ PBS and one volume of ethanol_{abs}.

4.7.3 Fixation for FISH of soft-agar samples

If not stated differently, centrifugation speed was 2600 g and working temperature was RT. Approximately 200 μ l were taken out of the soft agar slants with a 2ml plastic pipette and centrifuged for 8 minutes. To wash the sample, 200 μ l of PBS were added, vigorously mixed, and centrifuged for 5 min. 300 μ l of PFA (4 % w v⁻¹) was added for 10 minutes, vigorously mixed, and removed by centrifugation for 5 minutes. Samples always directly used for FISH analysis.

4.8 FISH procedures and DAPI staining

4.8.1 Hybridization of FISH samples fixed on glass slides

All probes were purchased from Thermo Fisher Scientific (St Leon Rot, Germany). Hybridization of the probes (Table 4.4) to the corresponding samples was achieved in hybridization buffer with 20 % formamide (FA) and washing with the corresponding washing buffer (Daims, Stoecker, and Wagner 2005). The samples then were incubated at 48 °C in washing buffer for 10 minutes, followed by a 2 second washing step in ice-cold water. Afterwards the slides were air—dried until no liquid remained and stored at -20 °C or directly used for further processing or microscope analysis.

4.8.2 Hybridization of soft-agar FISH samples

A highly modified version of FISH in suspension (Sixt 2013, unpublished) was used. For hybridization, 100 μ l of hybridization buffer (20%) and 10 μ l of each respective probe was added the Eppendorf tube containing the fixed sample (4.7.3). Hybridization was performed overnight at 46° C. The hybridization buffer (including the probes) was removed by centrifugation for 5 min at 46°C. It was important to perform the centrifugation at 46 °C to guarantee the stringency of the probe hybridization. 800 μ l of washing buffer were added for 5 min and removed by 5 min of centrifugation at 46 °C. The sample pellet was then re-suspended in 100 μ l of PBS and directly applied to a microscope glass slide. DAPI (dissolved in PBS, 0.5 mg ml⁻¹) was premixed with Citifluor 1:8 (v v⁻¹) and applied to the sample before analysis at a confocal laser scanning microscope.

4.8.3 DAPI staining

Before embedding the samples with Citifluor (Agar-Scientific, Stansted, UK), the slide wells were incubated with 4',6—Diamidino-2—phenylindole (DAPI, 0.5 mg ml⁻¹) for three minutes. The DAPI solution was removed and the wells were washed once with 10 μ l of MQ. The slides were then air dried until no liquid remained.

4.9 Optical determination of cell numbers

4.9.1 Determination of amoeba cell numbers

The host culture was washed with growth medium without detaching amoeba cells attached to the surface of the well bottom. The amoeba were then detached by shaking and knocking the culture flask. 10 μl of the suspension were transferred into a Neubauer haemocytometer. The number of cells per μl was then determined by counting the cell number in the 8 grids. The total count was divided by the size of the counting area 8 grids times the area of 1 mm^2 and the depth of the chamber of 0.1 mm. By reducing the fraction, the following formula can be obtained.

$$\text{amoebae}/\mu\text{l} = \frac{10 \times \text{number of amoebae}}{\text{counted grids}}$$

4.9.2 Determination of bacterial cell numbers

The supernatant of an endosymbiont infected amoeba culture was transferred into a 50 ml centrifugation tube without detaching the amoebae. Extracellular *P. acanthamoebae* cells were separated from the unattached amoebae by filtration (5 μm pore-size, Sartorius stedim biotech, Minisart). The sample was centrifuged for six minutes at 5600 g at room temperature and the supernatant was discarded. The pellet was re-suspended in approximately 10 ml of TSY-medium. Two Falcon tubes with either 2 μl or 5 μl of bacteria containing supernatant diluted with 5 ml of PAS were prepared. The cells were collected and stained with DAPI on a filter using a vacuum-filter-tower. The average bacteria number of 10 random spots was evaluated using an epifluorescence microscope.

$$\text{cells}/\mu\text{l} = \frac{15126 \times \text{number of cells}}{\text{counted spots} \times \text{initial volume}}$$

4.10 Antibiotics treatment to obtain the endosymbiont-free original host *Acanthamoeba* sp. UWC12

1000 amoeba cells were transferred into 2 ml of growth medium in a 12 well multidish. To inhibit the growth of endosymbiotic bacteria and obtain endosymbiont-free Amoebae, a final concentration of 100 $\mu\text{g ml}^{-1}$ Rifampicin (filtered with 0.22 μm pore—size to sterilize) was added to amoebae cultured in TSY. Therefore a Rifampicine stock solution of 100 mg ml^{-1} dissolved in Dimethylsulfoxid (DMSO) was prepared. Rifampicin inhibits the initiation of RNA synthesis by binding to the β -sub-unit of the RNA-polymerase. Upon reaching the stationary growth phase (assumed when a mono-layer was grown at the bottom of a well), 1000 amoebae from each well were transferred into a new well containing 2 ml of TSY (100 $\mu\text{g ml}^{-1}$ Rifampicine). It was assumed that as the amoebae continue growing and endosymbiont growth is inhibited, the endosymbiont cells get out-diluted. After each passage, one ml of each well was used for FISH analyzes to check for the infection rate.

4.11 Growth comparison of infected/non-infected *Acanthamoeba* sp.

4.11.1 Infection of cured *Acanthamoeba* sp. UWC12

Two ml of culture uninfected *Acanthamoeba* UWC12 were put into 4 separate small culture flasks. Cultures meant to stay uninfected were supplemented with 8 ml of sterile TSY medium. 50 ml of the supernatant of an *P. acanthamoebae* UWC12 infected *Acanthamoeba* sp. UWC12 culture were centrifuged at 5600 g for six minutes, re-suspended in 18 ml of sterile TSY, and filtered with a pore—size of five μm twice to purify extracellular endosymbiont cells. The two remaining cultures meant to be infected were supplemented with 8 ml of endosymbiont containing TSY each. Cultures were then incubated at 20°C or 30°C to acclimat to the new temperature before the growth comparison assay. After seven days the cultures were checked for the completeness and absence of infection respectively. The reason for starting a new infection in already cured amoebae was to correct for the bottle—necking the amoeba culture was subject to during the curing procedures. It can not be guaranteed that the cured host cell population is still at the same fitness level as the untreated, infected host population. This could introduce bias concerning retarded growth rates for example. Therefore, using the same starting population (the cured amoebae) stronger statements can be made.

4.11.2 Growth comparison assay

Using the Neubauer counting chamber the cell density of each preincubated culture was determined. 1000 amoebae of the corresponding precultures were transferred into 2 ml of growth medium in a 12 well multidish. The samples were then incubated at 20 °C or 30 °C after a centrifugation step at 300 g for 12 minutes at RT to accelerate cell—attachment to the well. For each condition (infected, uninfected, 20 °C, and 30 °C) duplicates were used. At each time-point ten pictures of random spots in each well were taken with a camera at the inverse light microscope. The number of attached amoeba cells was counted using the imageJ software (C. a Schneider, Rasband, and Eliceiri 2012) and the mean was calculated for each well at each time-point. The values were then averaged to get a mean cell density per well per time-point. After the last time-point, each well was analyzed by FISH to screen for contamination and correct infection rates (0 or 100 percent respectively). To estimate the doubling time, first the intrinsic rate of increase

$$r_m = \frac{\ln \frac{N_t}{N_0}}{t}$$

was estimated (Dixon 1987), where N_t is the cell count at time t and N_0 is the initial cell count of t_0 . Population doubling time was subsequently estimated using the formula $PDT = \ln(2)/r_m$.

4.12 Infection cycle experimental setup

4.12.1 Infection cycle in *Acanthamoeba* sp. UWC12

Endosymbiont free cells were obtained and their cell number was determined as described above (4.9.1). In a 12 well-plate, 3×10^4 cells were transferred into 2 ml of TSY medium per well. Each well

was infected with *P. acanthamoebae* UWC12 and a multiplicity of infection (MOI) of 200 was applied. The samples were then incubated at 20 °C in duplicates for each time-point and a non-infected negative control of amoebae. All wells were washed with one ml of PAS and re-supplemented two ml of growth medium two hours post infection (hpi). At each time-point samples were prewashed with PAS to remove bacteria from the supernatant, which could influence the infection rate quantification. If extracellular endosymbiont cells were in close proximity to an uninfected host cell, they could be classified as infected which would lead to a false positive. Amoebae were then fixed with PFA (4 % w v⁻¹), in situ hybridized, and analyzed using fluorescence microscopy. Host cells containing less than eight *P. acanthamoebae* were deemed lowly infected, while cells containing more than eight endosymbionts were deemed highly infected. For each time-point two wells were in situ hybridized for analysis. For each replicate of each time-point random spots were analyzed until approximately 150 cells were observed and classified.

4.12.2 Infection assay with *Drosophila* Schneider 2 (S2) insect cellline

Drosophila Schneider 2 (S2) cells were maintained at 27°C (ambient atmospheric conditions) in five ml Schneider medium (SM) in 25 cm² sterile tissue culture flasks with filter lids to facilitate gas exchange. The medium was supplemented with 10 % (v v⁻¹) fetal calf serum (FCS, heat inactivated at 56°C for 30 minutes) and sterilized by filtering with 0.2 µm pore-size. Medium was always preheated to 27 °C before passaging cells. To start the infection assay 1.5 × 10⁵ cells were transferred into a 12 well-plate containing 2 ml SM medium per well in duplicates for each time-point and one non-infected negative control. The cell number of S2 cells used for the assay was higher than in 4.12.1 because these cells required a higher density to grow. A MOI of 150 was applied to infect with *P. acanthamoebae* UWC12. After infection the well plate was centrifuged for 10 minutes at 370 g at 27°C to enhance the interaction between bacterial and eukaryotic cells. A washing step with PAS to remove bacteria from the supernatant was performed 24 hpi to exclude extracellular bacteria from starting an infection. New infections should solely be started by *P. acanthamoebae* leaving the host cell. At each time-point S2 cells were fixed with PFA (4 % w v⁻¹), in situ hybridized (duplicates for each well), and analyzed using fluorescence microscopy.

4.12.3 Infection assay with mouse macrophage THP-1 cellline

The running cultures of the THP-1 cell-line were maintained at 37°C (5 % CO₂ atmosphere) in five ml Roswell Park Memorial Institute 1640 medium (RPMI) in 25 cm² sterile tissue culture flasks with filter lids to facilitate gas exchange. The medium was supplemented with 10 % (v v⁻¹) fetal calf serum (heat inactivated at 56°C for 30 minutes) and sterilized by filtering with 0.2 µm pore-size. 50 % of each culture was passaged on a biweekly basis and media used were preheated to 37 °C. Macrophages were considered to viable, if they were not attached to the bottom of the culture flasks. A MOI of 150 was applied to infect with *P. acanthamoebae* UWC12. After infection the well plate was centrifuged for 10 minutes at 370 g at 37°C to enhance the interaction between endosymbiont and eukaryotic host cells. A washing step with PAS (preheated to 37°C) to remove bacteria from the supernatant was performed 24 hpi to exclude extracellular bacteria from starting an infection. Mouse macrophages were fixed (duplicates for each well) with PFA (4 % w v⁻¹), in situ hybridized, and analyzed using fluorescence microscopy.

4.13 Maintenance of infectivity in an extracellular environment assay

P. acanthamoebae cells were transferred into 2 ml of TSY in a 12 well-plate in duplicates for each time-point and incubated at 20 °C. 10^5 uninfected *Acanthamoeba* sp. UWC12 cells were added after zero days, two days, four days, and 6 days. This led to a MOI of 250. 24 h post infection the wells were washed with PAS to stop new infections of cells remaining in the supernatant. After an additional 24 h of incubation (48 h post infection) to increase the endosymbiont number per infected amoeba cell and thereby raise the sensitivity of the assay, the wells were fixed (duplicates for each well) in PFA (4 % w v⁻¹), in situ hybridized, and analyzed using fluorescence microscopy.

4.14 Axenic endosymbiont cultivation in host—free media

4.14.1 Soft—agar (slant) based cultivation

PYG (containing 20 g l⁻¹ proteose peptone, 18 g l⁻¹ glucose, 2 g l⁻¹ yeast extract, 3.4 mM sodium citrate-dihydrate, 4 mM MgSO₄ × 7H₂O, 2 mM Na₂HPO₄ × 2H₂O, 1.7 mM KH₂PO₄, 0.05 mM Fe(NH₄)₂(SO₄)₂ × 6H₂O) and SM were used as a basis. An agar base of 20 ml 1,5 % PAS-Agar was produced and autoclaved. The media and the agar base were warmed to 55 °C in the water bath. The temperature was found to preserve the agar from gelling (gelling temperature: ~35 °C) when mixed with medium. At the same time the complex media were not degraded, as they cannot be boiled or autoclaved due to reactive components (e.g. L-Cysteine in SM). The respective media and the agar supplemented PAS were mixed 3:1 (v v⁻¹) and subsequently cooled to 38°C in the water bath. This temperature was found to be sufficient to enable inoculation of bacteria without killing them. *P. acanthamoeba* was isolated from 40 ml of supernatant of an infected amoeba culture. After centrifugation the pellet was re-suspended in 8ml of PAS and processed as described in 4.6.2. The medium—agar emulsion was inoculated with isolated *P. acanthamoebae* (40 µl ml⁻¹). Sterile glass eprouvettes were supplemented with five ml of the inoculated medium—agar emulsion and closed with metal caps. To enable the formation of an oxygen gradient, the cap was tightly closed with parafilm to prevent the diffusion of oxygen into the eprouvette. Cultures were incubated at 27 °C as this is the optimal operation temperature of SM. For analysis, samples were fixed as described in 4.7.3 and then hybridized as described in 4.8.2.

4.14.2 Liquid medium cultivation of *P. acanthamoebae*

P. acanthamoeba was isolated from the supernatant of an infected, detached amoeba culture. After centrifugation the pellet was re-suspended in 8ml of SM and processed as described in 4.6.2. The inoculate was then diluted 1:20 in SM. Filtered 25 cm² culture flasks were supplemented with 5 ml of inoculate and incubated at 27 °C in microaerobic bags (CampyGen Compact, Oxoid, Thermo Fisher Scientific Inc., USA) as described in the user manual to ensure a microaerobic environment. The provided paper sachet, which is added to the bag, contains ascorbic acid which oxidizes on contact with air to produce the microaerobic conditions.

4.15 DNA—based analyses

4.15.1 Genomic DNA isolation of endosymbiont containing amoebae

The DNeasy Blood and Tissue Kit (QIAGEN) was used for the total DNA isolation from *Acanthamoeba* sp. UWC12. All reagents needed were supplied in the kit except for absolute ethanol (EtOH_{abs}). The extraction was performed as in the company instructions. To measure nucleic acid concentrations, the Nano Drop® device was used for spectrophotometrical quantification.

4.15.2 Genomic DNA isolation of *P. acanthamoebae* UWC12 for the qPCR assay

4.15.2.1 Sampling of *P. acanthamoebae* in liquid SM for DNA extraction

200 µl of *P. acanthamoebae* containing SM medium (4.14.2) were taken out of the culture flask and stored at -80 °C until DNA extraction. A newly opened ascorbic acid bag (CampyGen Compact, Oxoid, Thermo Fisher Scientific Inc., USA) was added to reestablish the microaerobic environment and the bag was resealed.

4.15.2.2 Modified DNA extraction technique of Omsland et al. (2009)

A modified version of the extraction technique by Omsland et al. (2009) was used. The samples (200 µl) were diluted 1:5 (v v⁻¹) with H₂O_{bidest.} (radiated with UV for 30 min) and applied to Lysing matrix E tubes (MP Biomedicals, Germany). 1.4 mm ceramic spheres, 0.1 mm silica spheres, and one 4 mm glass bead are used to mechanically disrupt cells to release bacterial DNA using a FastPrep homogenizer (Savant Instruments Inc. Holbrook, NY) for 30 sec at 6.5 m s⁻¹ speed. Samples were then directly used for qPCR or stored at -80 °C.

4.15.2.3 Modified DNA extraction technique using the DNeasy Blood and Tissue Kit

The DNeasy Blood and Tissue Kit (QIAGEN) was used for the total DNA isolation as described in 4.15.1 with slight modifications. Instead of PBS, Proteinase K digestion was done in SM directly to avoid a loss of bacterial cells, due to the already small cell number for 1 h at 56 °C. For DNA elution, DNA was eluted in 100 µl of buffer AE twice for each spin column to increase total DNA yield.

4.15.3 Agarose gel electrophoresis

Gels were prepared in a microwave with LE Agarose (Biozym) and 1× Tris—Borate—EDTA (TBE) buffer. 6× DNA Loading Dye (Fermentas) was used to load samples. Gels were stained with GelRed Nucleic Acid Gel Stain (Sigma-Aldrich) prerun.

4.15.4 16S rDNA PCR protocols

4.15.4.1 Standard bacterial 16S rRNA gene quantification protocol

Reagent	Amount [μl]
10 $\times$ Taq Buffer with KCl (Fermentas)	5
dNTP Mix (2 mmol L ⁻¹ each, Fermentas)	5
MgCl ₂ (25 mmol L ⁻¹ , Fermentas)	4
Primer Forward (50 pmol μL^{-1})	1
Primer Reverse (50 pmol μL^{-1})	1
Taq DNA polymerase (recombinant, 5 u μl^{-1} , Fermentas)	0.2
Template DNA	1
PCR water	32.8
Total volume	50

Table 4.6: Standard PCR reaction used for 16S rDNA quantification and for M13 PCR.

Step	Sets	Temperature	Time [min:sec]	Note
1	1 $\times$	95 °C	05:00	
2.1	40 $\times$	95 °C	00:40	
2.2		55-62 °C	00:30	detection
2.3		72 °C	01:00-01:30	detection
3	1 $\times$	95 °C	01:00	
4	1 $\times$	55 °C	01:00	
5	80 $\times$	55- 95 °C	00:10	+0.5 °C / repeat, detection

Table 4.7: Standard thermocycler protocol for PCR on 16S rDNA.

4.15.4.2 qPCR assay for 16S rDNA fragments

If not stated otherwise, samples were analyzed in technical triplicates and standard curves were done in technical duplicates. Additionally controls were done in every assay. Duplicates or triplicates of no-template controls (NTC; 10 μl of PCR water) were always included. Optimized by Pester et al. (2010).

Reagent	1 $\times$ reaction [μl]	final concentration
2 $\times$ SuperMix (Bio-Rad, 170-8882)	12	
Primer Forward (50 μM)	0.5	1 μM
Primer Reverse (50 μM)	0.5	1 μM
BSA (1 mg mL ⁻¹)	0.12	0.005 mg mL ⁻¹

Reagent	1 × reaction [μl]	final concentration
Template	10	
PCR water	1.4	
Total volume	25	

Table 4.9: Standard qPCR reaction used for 16S rDNA quantification.

4.15.5 TOPO TA and TOPO TA cloning and M13 PCR protocols

4.15.5.1 M13 PCR

M13 PCR to check for cloning inserts was performed using the TOPO TA or TOPO XL cloning kit (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturers' instructions. A 25 μl reaction size was used for PCR (4.6).

4.15.6 Sequence analyses using BLAST online tool

The Basic Local Alignment Search Tool (BLAST, <http://www.ncbi.nlm.nih.gov/BLAST/>) is an online tool used to compare sequences with the NCBI database. The program compares the sequence of interest to the database by local sequence alignment to identify the most similar sequences in the data-bank and ranks them according to their relevance.

4.16 Quantification of biofilm formation using crystal violet

4.16.1 Incubation settings

Endosymbiont cells were inoculated and as described in 4.14.2, but instead of culture flasks, 24 well multidish plates were used. Samples were split into microaerobic and aerobic incubation conditions. Both were incubated within microaerobic bags, but only the microaerobic group was supplemented with a citric acid bag to establish microaerobic conditions. Incubations, including controls were done in biological quadruplicates. *Pseudomonas aeruginosa* was taken as a positive control and uninoculated SM was used as a negative control.

4.16.2 Crystal violet biofilm staining and quantification

The bio film quantification assay from the technique described by Merritt, Kadouri, and Toole (2011). It relies on Tris(4-(dimethylamino)phenyl)methylum (crystal violet) to stain extracellular matrix produced in biofilms. The fluorophore is used in Gram staining to differentiate between Gram-negative versus Gram-positive bacteria (Gram 1884). Washing and staining procedures were performed in 24 well multidish plates. After 16 days of incubation, wells containing *P. acanthamoebae* in SM were washed twice with H_2O_{bidest} to remove planktonic bacteria using using a 1 ml pipette.

Step	Sets	Temperature	Time [min:sec]	Note
1	1×	95 °C	05:00	
2.1	40×	95 °C	00:40	
2.2		55 °C	00:30	detection
2.3		72 °C	00:40	detection
3	1×	95 °C	01:00	
4	1×	55 °C	01:00	
5	80×	55- 95 °C	00:10	+0.5 °C / repeat, detection

Table 4.8: Standard thermocycler protocol for qPCR with primer pair 1389F/ 1492R.

To stain biofilm, 1 ml of crystal violet ($0.1\% \text{ w v}^{-1}$) was added for 10 minutes. The Crystal violet solution was then taken off and the wells were washed three times with $\text{H}_2\text{O}_{\text{bidest}}$ as described above to remove residual crystal violet. Plates were then allowed to air dry and were either stored at $4\text{ }^{\circ}\text{C}$ or directly further processed. 1.2 ml of 30 % acidic acid were added to each well to dissolve the stained biofilm for 15 minutes. 125 μl of the crystal violet/acetic acid solution from each well were transferred into a clear flat bottom 96 well plate (Greiner Bio-One) in technical duplicates. The optical density (OD) of each of the samples was measured at a wavelength of 600 nm with a with the Infinite M200 microplate reader.

Results

5.1 Establishment of aposymbiotic *Acanthamoeba* sp. UWC12

The natural host was cured of the endosymbiont infection as described in 4.10. The cultures were screened for endosymbionts using FISH (Fig. 5.1). The aposymbiotic cultures were then used for further assays.

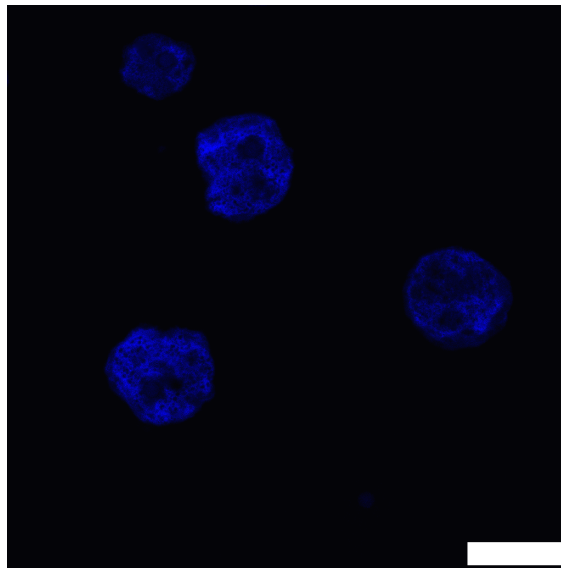


Figure 5.1: Aposymbiotic *Acanthamoeba* sp. UWC12. Bacteria (green), *P. acanthamoebae* UWC12 cells (red) and general eukaryote staining (blue) for *Acanthamoeba* sp. UWC12. After treatment with Rifampicin, amoebae were free of endosymbionts. Scalebar: 25µm.

5.2 Host specific examination of a *P. acanthamoebae* infection

To illuminate host specific characteristics of the endosymbiont *P. acanthamoebae* UWC12, a number of assays was conducted in its natural host *Acanthamoeba* sp. UWC12.

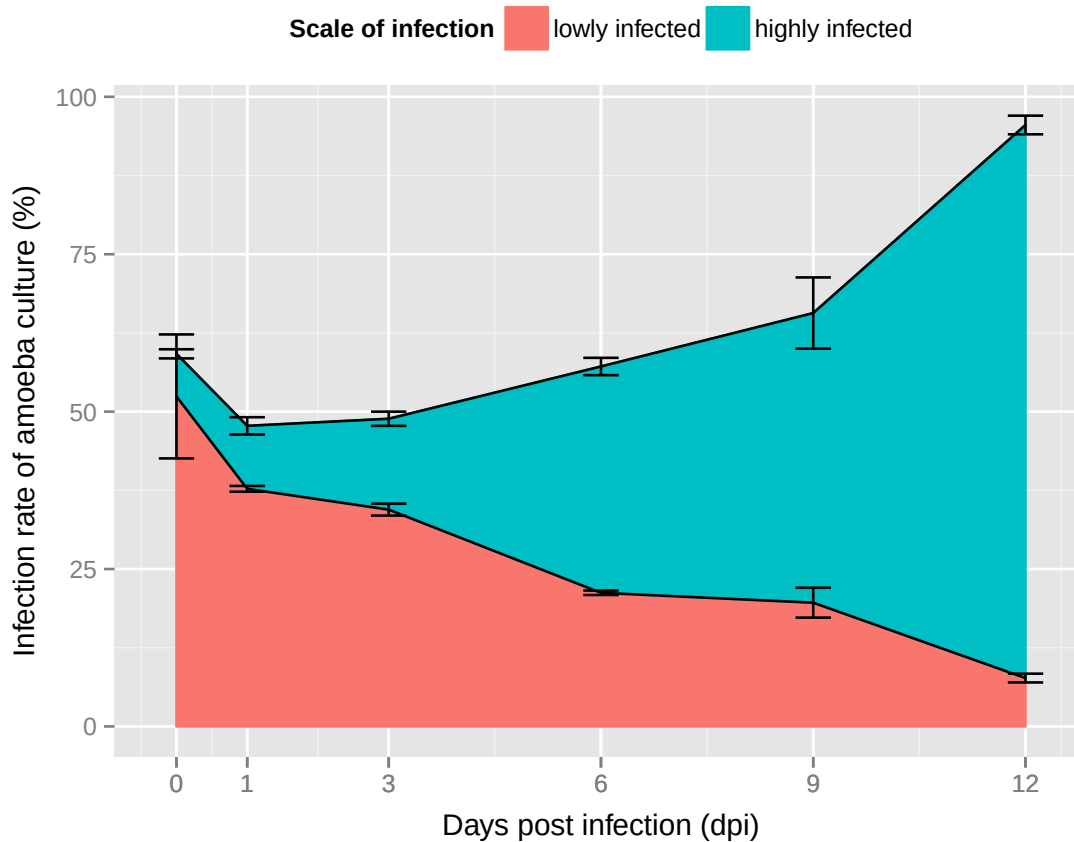


Figure 5.2: Progression of the infection of *Procabacter acanthamoebae* UWC12 in its natural host. Stacked plot depicting the infection cycle of *Procabacter acanthamoebae* UWC12 in *Acanthamoeba* sp. UWC12. Endosymbiont—free host cells were infected with a predefined number of endosymbiont cells. Shown is the average of 2 biological replicates and their standard deviation as error bars. Six timepoints were taken at 2 h, 1 d, 3 d, 6 d, 9 d, and 12 d post infection and host cell infection was determined using specific FISH staining of bacterial endosymbiont cells and general eukaryote staining for host cells. Host cells were divided into 3 groups: uninfected, lowly infected (8 or less bacterial cells per amoeba cell), and highly infected (> 8 bacteria per amoeba).

5.2.1 Infection cycle of *P. acanthamoebae* UWC12 in its natural host

To investigate the infection cycle of *P. acanthamoebae* UWC12 in *Acanthamoeba* sp. UWC12, an infection experiment with a MOI of 200 was conducted as described in 4.12.1. Six timepoints were taken at 2 h, 1 d, 3 d, 6 d, 9 d and 12 d post infection (Fig. 5.2). A classification system based on FISH analysis was used (Tsao 2013, unpublished) to cluster the infection state of amoebae into:

- Uninfected: no endosymbiont cells detected in the host cell
- Lowly infected: eight or less endosymbiont cells detected in a host cell
- Highly infected: more than eight endosymbiont cells detected in a host cell

The initial total infection level 2 hpi was at 59.18 % of the host population (Fig. 5.2). Only 6.76 % of the host population were highly infected at this state of the infection, whereas 52.41 % were lowly infected. At 1 dpi there was a drop in total infection to 47.74 % infection of the host population. This was due to a major drop of the lowly infected fraction by 14.67 %, while the highly infected proportion only increased slightly (by 3.24 %). Until 3 dpi the total infection rate stagnates at approximately 48 % with an increase of the highly infected fraction to 14.43 %. The following timepoints showed a strong increase of the highly infected proportion and in consequence of total infection. At 12 dpi the infection rate of the host population was at 95.52 % and was therefore be declared as a fully infected culture.

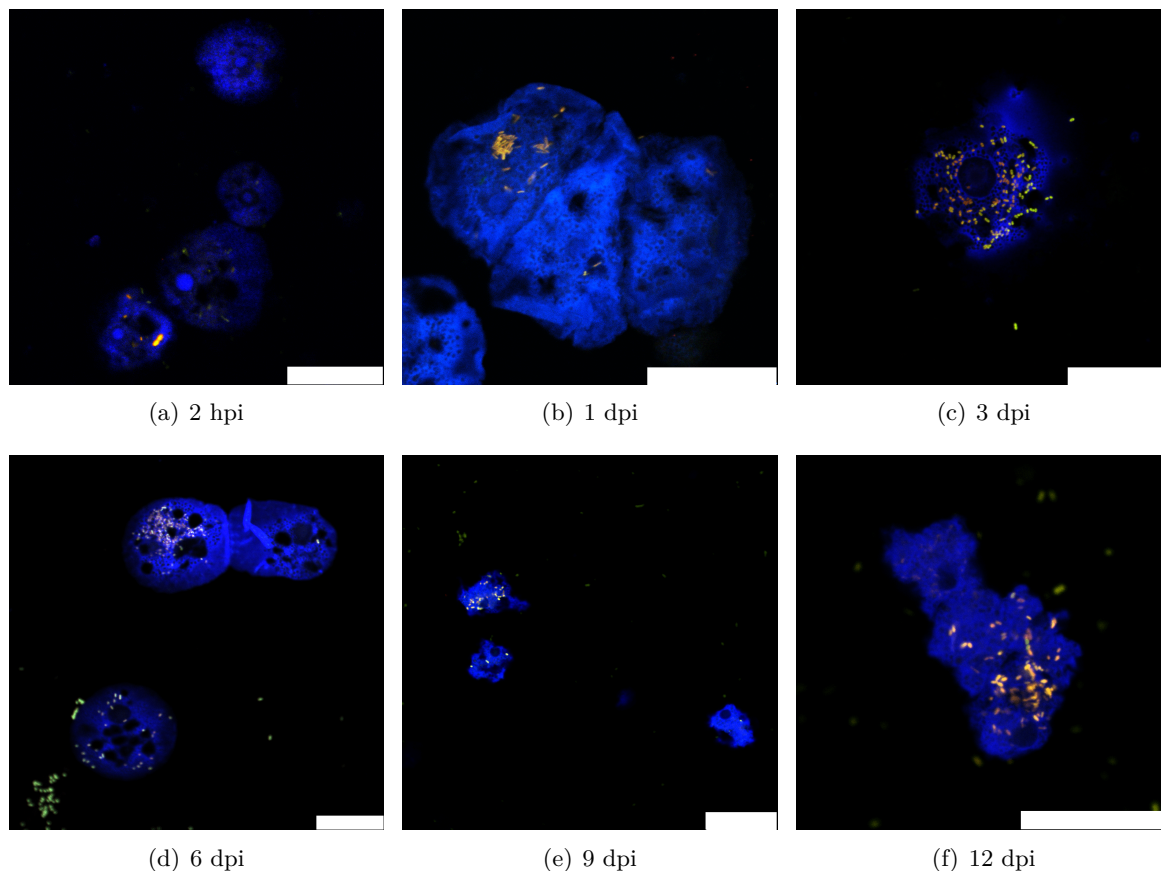


Figure 5.3: Visualization of *Procabacter acanthamoebae* UWC12 in its natural host. Specific FISH staining of *P. acanthamoebae* UWC12 cells (yellow) and general eukaryote staining (blue) for *Acanthamoeba* sp. UWC12. (a) Two hpi a discrepancy in signal intensity of endosymbiont cells can be observed in some amoebae. This could hint to the degradation of endosymbiont cells, which can not establish the infection in a host cell. (b) At one dpi endosymbiont cells are often concentrated within the amoeba cytosol. (c) At three dpi fully infected host cells can be observed and extracellular endosymbiont cells start occurring extracellularly. (d-e) Between six and twelve hpi, besides an increase in total infection rate, the fraction of highly infected cells increases. Scalebar: 25 µm.

At two hpi some host cells were observed to contain *P. acanthamoebae* cells which seem to have weaker FISH signals, compared to those in other infected host cells (Fig. 5.3). Those cells could represent endosymbiont cells, which were not able to establish an infection within the host cell. At one dpi *P. acanthamoebae* cells were often observed in cell-clusters within host cells. Starting from

three dpi, fully infected host cells were observed and extracellular endosymbiont cells emerge. At the later timepoints the fraction of highly infected *Acanthamoeba* sp. UWC12 cells further increased.

5.2.2 Maintenance of infectivity in a host—free environment

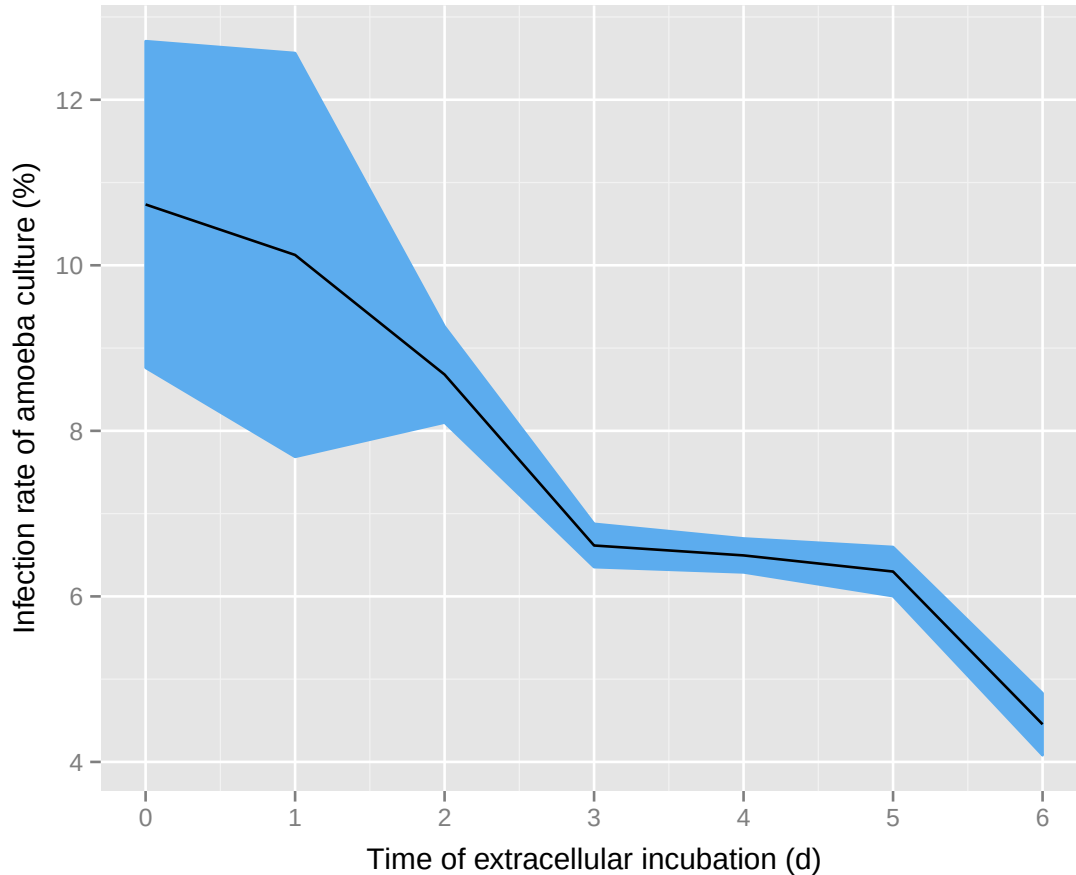


Figure 5.4: Persistence of *Procabacter acanthamoebae* UWC12 in TSY medium. Graph depicting the maintenance of infectivity of *Procabacter acanthamoebae* UWC12 in a host—cell free environment (TSY medium) in 24 hour incubation steps for a total of 6 days. The assay was conducted with the native host, *Acanthamoeba* sp. UWC12. A host cell was counted as infected, if it had contained at least one *Procabacter acanthamoeba* UWC12 cell. Shown is the average of 2 biological replicates and their standard deviation as a blue area stretching the y-axis.

To measure the endosymbiont’s potential to stay infective in an extracellular environment, the infectivity of *P. acanthamoebae* after incubation in a host—free environment (TSY medium) was tested. At the specified time-points (0, 1, 2, 3, 4, 5, 6 days) host cells were added and the endosymbiont’s ability to infect the natural host *Acanthamoeba* sp. UWC12 was measured (Fig. 5.4). The infection rate peaks at the first timepoint, without extracellular incubation at ~11 %. The infection rate then drops constantly until six days of host free incubation. After seven days of host-free endosymbiont incubation none of the added host- cells were observed to be infected by their symbiont. Remarkably even after six days of host—free incubation *P. acanthamoeba* UWC12

is still infective, despite its drop to ~4.5 %. It seems that *Procabacter* can maintain infectivity for at least seven days, which shows strong capabilities of surviving in a host—free environment.

5.2.3 Growth comparison of infected/non-infected *Acanthamoeba* sp. UWC12 under different conditions

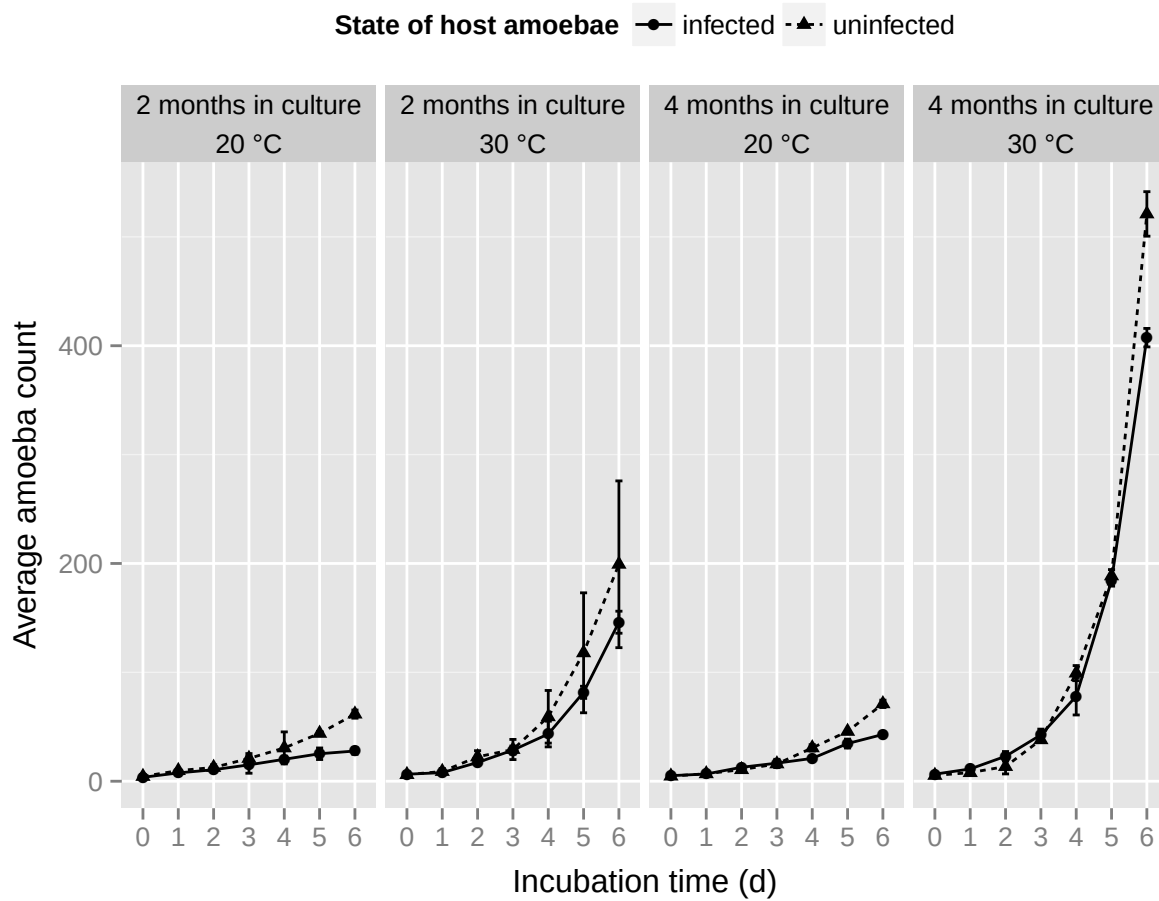


Figure 5.5: Comparison of growth of infected and uninfected host cells. Growth curves of *Acanthamoeba* sp. UWC12 with or without *Procabacter acanthamoebae* UWC12 at incubation temperatures of 20 and 30 °C. The assay was conducted in December and reproduced in February. Shown is the average of 2 biological replicates and their standard deviation as error bars.

To investigate the impact of the endosymbiont on the host fitness a comparison of growth rates between infected and uninfected *Acanthamoeba* sp. UWC12 were compared. To illuminate possible stress dependent effects, the assay was conducted at the usual *Acanthamoeba* sp. UWC12 cultivation temperature of 20 °C and additionally at 30°C (Fig. 5.5). The *Acanthamoeba* sp. UWC12 host was cured of *P. acanthamoebae* UWC12 to capture host-specific effects. Uninfected host amoebae were compared to reinfected host amoebae of the same initial population to exclude bias introduced by the antibiotic curing procedures.

To visualize differences in growth rates the average amoeba counts were scaled logarithmically with

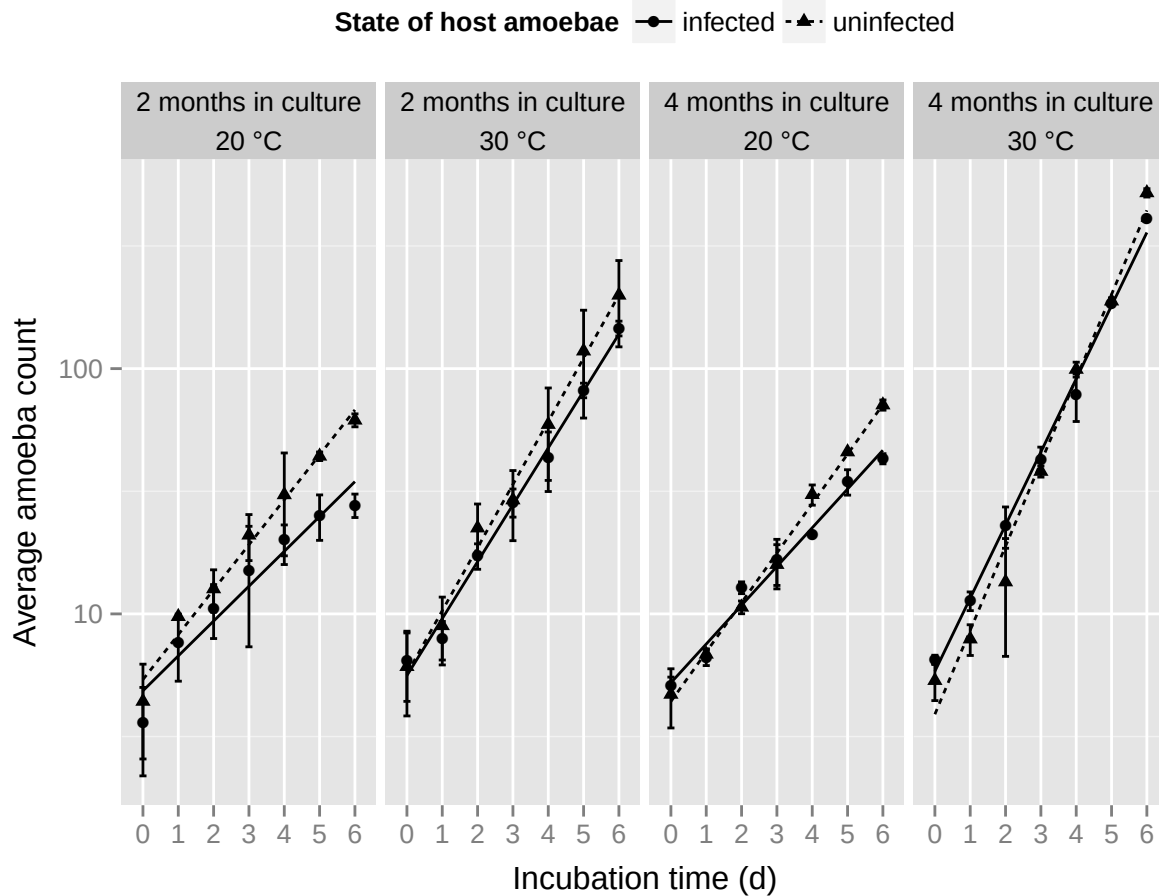


Figure 5.6: Comparison of slope of log transformed cell counts. The slope of *Acanthamoeba* sp. UWC12 growth curves with *Procabacter acanthamoebae* UWC12 at temperatures of 20 and 30 °C conducted in December and february respectively is compared. Shown is the average of 2 biological replicates and their standard deviation as error bars. The y-axis is scaled logarithmically with the power of ten as the base to observe linear growth.

the power of ten as a base (Fig. 5.6). By log-transformation one can observe the slope of the growth function in a linear matter. It can be thereby inferred, that the slope of infected amoebae, which represent the growth rates, is lower than those of uninfected amoebae. Interestingly this trend seems to be weaker at an elevated temperature of 30 °C. Additionally the growth rate of *Acanthamoeba* sp. seems to be higher after a prolonged time in culture. To elucidate these trends further, the intrinsic rate of increase (r_m) and the population doubling time (PDT; see 4.11.2) were estimated by assuming logarithmic growth (Tab. 5.1). It becomes evident that the PDT of infected amoebae populations is consistently higher than that of uninfected ones. Strikingly when looking at the relative change of the PDT, infected cultures at 20 °C had an average increase in PDT of 28.374 % (standard deviation: ± 1.252 %), while at 30 °C the increase is at only 11.198 % (standard deviation: ± 0.773 %).

Further it has to be noted that the PDT decreased, as the time in culture of the cured amoebae increased. The relative change of the PDT, infected cultures at 20 °C had an average decrease in the PDT of 3.487 % (standard deviation: ± 0.941 %). At 30 °C the effect is stronger with a decrease in PDT of 24.232 % (standard deviation: ± 0.526 %).

Temperature	Time in culture	Host state	r_m	PDT [d]
20 °C	2 months	infected	0.34	2.04
20 °C	2 months	uninfected	0.44	1.58
20 °C	4 months	infected	0.355	1.95
20 °C	4 months	uninfected	0.453	1.53
30 °C	2 months	infected	0.52	1.33
30 °C	2 months	uninfected	0.581	1.19
30 °C	4 months	infected	0.69	1.01
30 °C	4 months	uninfected	0.763	0.908

Table 5.1: r_m and PDT of infected and uninfected amoebae at 30 °C and 20 °C, respectively.

5.3 Host range in higher eukaryotic cells

To investigate the host range of *P. acanthamoebae* UWC12 in higher eukaryotic cells, two easily accessible cell lines representing distinct stages in the development of eukaryotic immune response were chosen. Macrophage like Schneider 2 insect cell line (obtained from *Drosophila melanogaster* by I. Schneider (1972)) and human macrophage THP-1 cell line (Auwerx 1991) were used in the following assays.

5.3.1 Infection assay with macrophage like S2 insect cell line

The infection assay on the S2 cell line was conducted as described in 4.12.2. After 24 hpi all s2 cells were infected with *P. acanthamoebae*. At this timepoint many host cells were still viable but highly

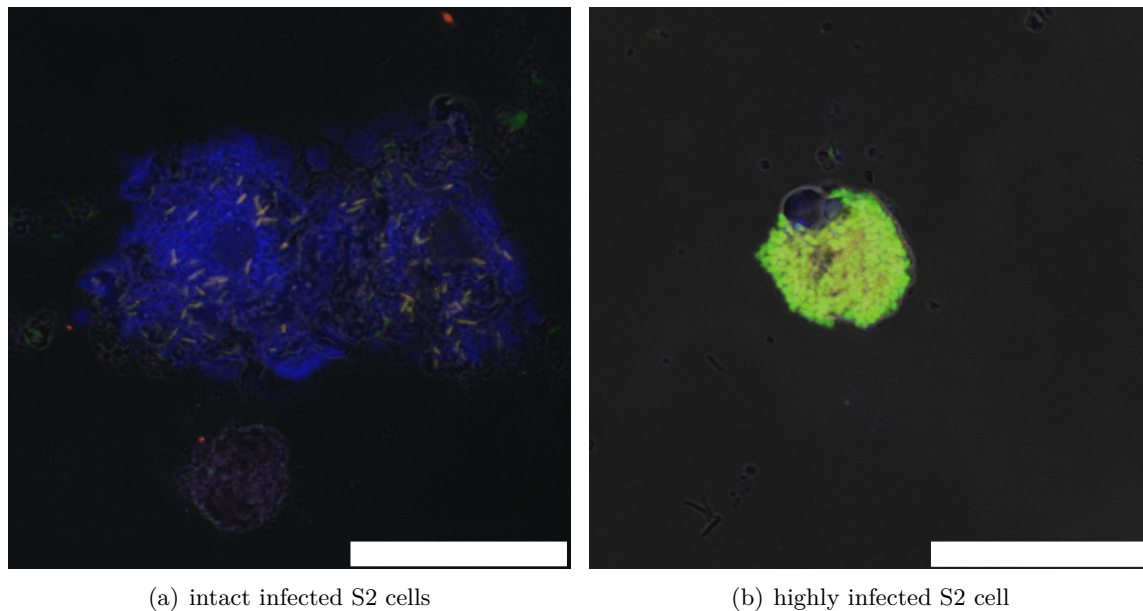


Figure 5.7: Infection of the S2 insect cell line. Specific FISH staining of *P. acanthamoebae* UWC12 cells (yellow) and general eukaryote staining (blue) for S2 cells, combined with differential interference contrast at 24 hpi. (a) Intact infected S2 cells at 24 hpi represented the minority of eukaryotic cells. (b) Most of the host cells were highly infected with the endosymbiont and did seem to lyse, or in transition to cell lysis. Scalebar: 25 μ m.

infected, but many cells were already lysed or in the process of cell lysis (Fig. 5.7). After 48 hpi most host cells were lysed and the endosymbiont cells were abundant in the supernatant.

5.3.2 Infection assay with macrophage THP-1 cell line

The infection of the THP1 cell line was conducted as described in 4.12.3. One day post infection a small proportion of approximately one percent of the host cells was infected with endosymbiont cells. *P. acanthamoebae* cells displayed an untypical morphology of small filaments, which seemed to be unicellular (Fig. 5.8). Upon further observation along the infection cycle the number of endosymbiont cells per infected THP1 cell did not change, but the unicellular filaments were elongated. After three days, the proportion of infected THP-1 host cells had decreased indicating the inability of those cells to divide and spread the infection. Host cell lysis was not observed in the infection assay.

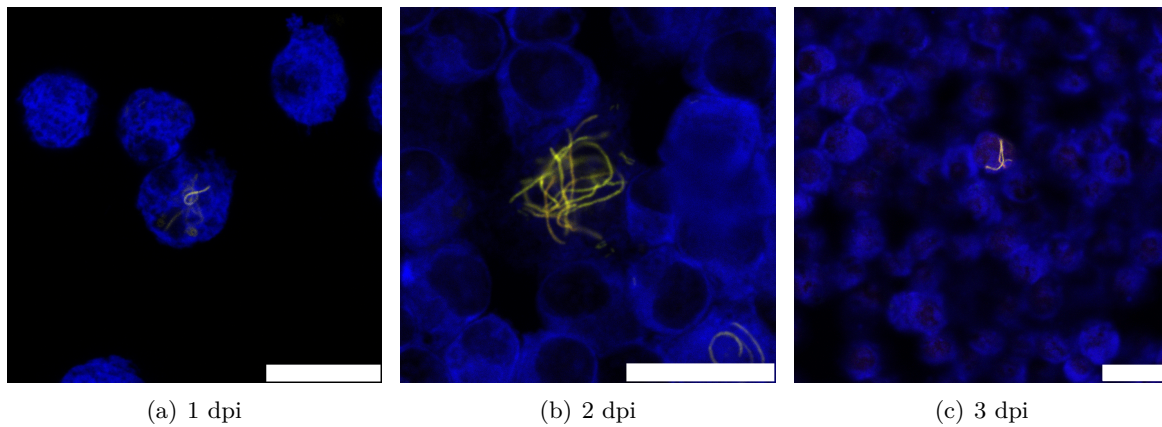


Figure 5.8: Infection of the THP-1 human cell line. Specific FISH staining of *P. acanthamoebae* UWC12 cells (yellow) and general eukaryote staining (blue) for THP-1 cells. (a) 24 hpi a small proportion of the host cells were infected with *P. acanthamoebae* which were exhibiting a filamentous morphology. (b-c) At 2 and 3 dpi respectively the proportion of infected host cells decreased. Infected cells carried long filaments of endosymbiont cells. Scalebar: 25µm.

5.4 Establishing host-free cultivation

5.4.1 Soft—agar cultivation assay

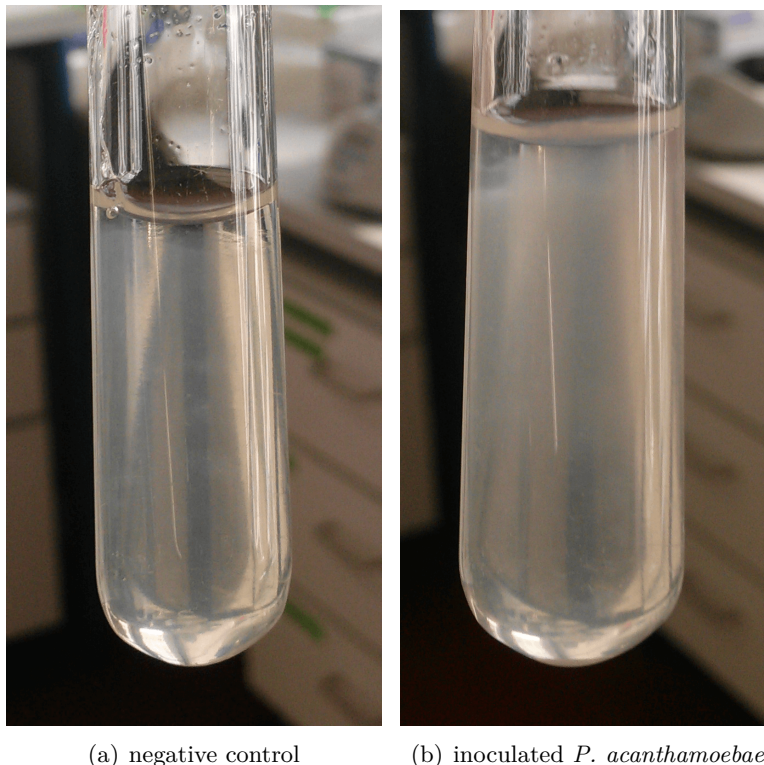
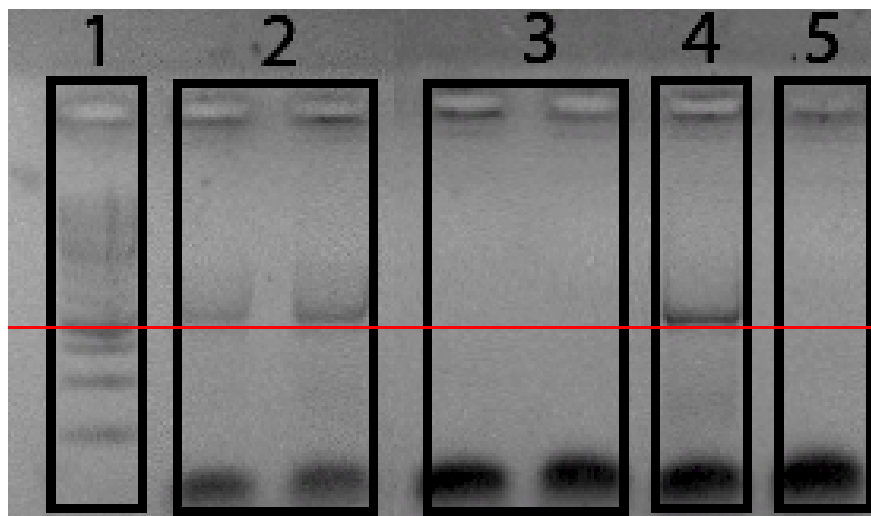


Figure 5.9: Incubation of *P. acanthamoebae* in SM soft agar tubes. Soft agar slants using Schneider medium after 30 days of incubation at 27 °C. (a) The negative control does not show a change of turbidity. (b) Slants inoculated with *P. acanthamoebae* show turbidity within the upper half of the slant.

To test potential atmospheric growth conditions and media for axenic *P. acanthamoebae* cultivation, softagar slants with an inherent oxygen gradient were prepared as described in 4.14.1. After 30 days of incubation at 27 °C turbidity could be observed in Schneider medium based slants inoculated with *P. acanthamoebae*, while negative controls without endosymbiont inoculation did not show any change in transparency (Fig. 5.9). For PYG based incubations no turbidity could be observed (data not shown).

In order to determine the identity of the bacterium causing the change in turbidity in the medium total DNA was extracted from the incubations. General bacterial primers targeting the 16S rRNA gene (616V and 1492R) were used to generate PCR product of the sample. As visualized on an agarose gel, 16S genes were amplified from the DNA preparations of inoculated SM softagar, whereas no PCR product was gained in negative controls (Fig. 5.10). Sequencing of the purified and pooled PCR product provided a perfect match to *P. acanthamoebae* UWC12.

For visualization of bacteria within the agar a modified liquid FISH protocol (4.14.1) was used (Fig. 5.11). The FISH signal was in general weak, nonetheless specific, and overlaid with the DAPI staining of DNA. Clusters of endosymbiont cells could be observed with a diameter of approximately



1: 1 kb ladder 2: Inoculation symbiont 3: Inoculation negative 4: PCR positive 5: PCR negative

Figure 5.10: PCR product of *P. acanthamoebae* DNA extracted from soft agar. 1 % agarose gel (TBE buffer) of Schneider medium based softagar slant total DNA run at 80 V for 1 h using general primers targeting the bacterial 16S rRNA gene (616V-1492R). The red line indicates a fragment size larger than 1 kb. PCR product was only present in the incubations inoculated with *P. acanthamoebae* UWC12 (Box 2) and the PCR positive control.

15 μ m. The soft agar slant was almost exclusively anaerobic after 30 days of incubation, when measuring with an oxygen probe (data not shown).

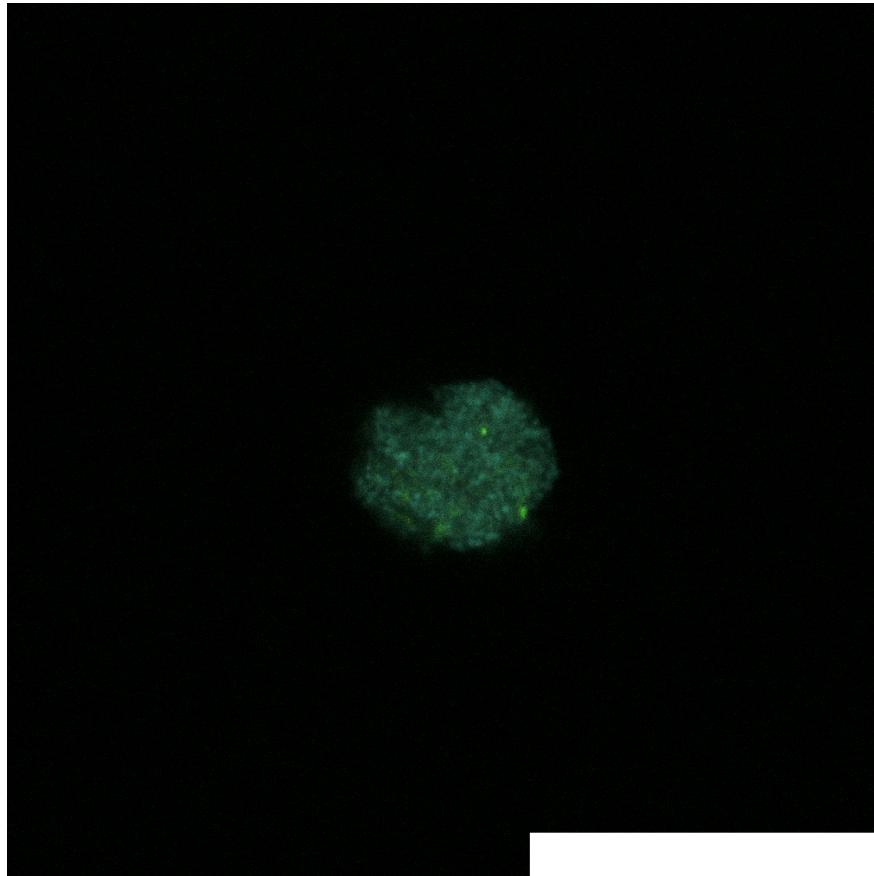


Figure 5.11: FISH staining of the SM soft agar incubations. FISH staining of bacteria (green), *P. acanthamoebae* UWC12 (red), eukaryotes (blue), and DNA staining with DAPI (gray) of a Schneider medium based softagar slant inoculated with *P. acanthamoebae* UWC12. The FISH signal is relatively weak, probably due to a lack of activity. After 30 days the soft agar slant was mostly anoxic. Scalebar: 25 μ m.

5.4.2 Liquid medium cultivation assay

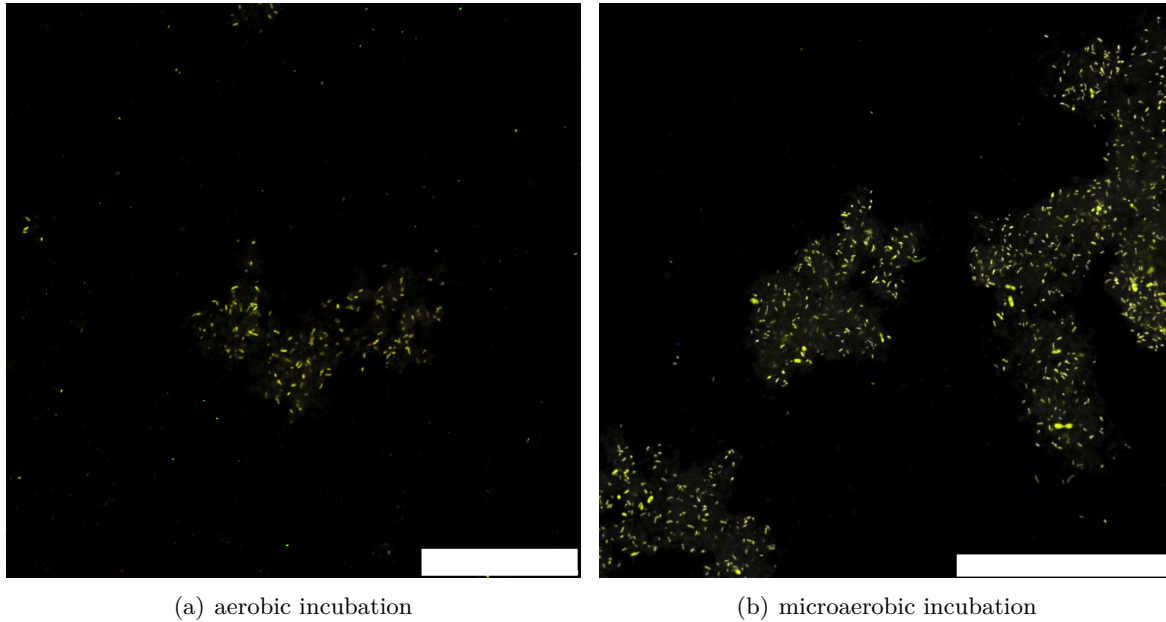


Figure 5.12: FISH staining of the liquid incubation assay (Schneider medium). Bacteria (green), *P. acanthamoebae* UWC12 (red), eukaryotes (blue), and DNA staining with DAPI (gray) of liquid Schneider medium inoculated with *P. acanthamoebae* UWC12 was performed. (a) Under aerobic conditions flocks of endosymbiont cells in an extracellular matrix can be observed, which is also true for microaerobic conditions (b). Under microaerobic conditions the number and size of the flocks is higher and signal intensity of the cells is stronger. Scalebar: 50 μm

Liquid cultivation in Schneider medium was attempted after the promising results of the softagar incubations. After 16 days under aerobic or microaerobic incubation conditions respectively, flocks of *P. acanthamoebae* cells could be observed (Fig. 5.12). Under both incubation conditions *P. acanthamoebae* formed flocks of cells. The abundance and signal intensity of the flocks was higher under microaerobic conditions.

Next, we aimed to investigate the infectivity of axenically incubated *P. acanthamoebae* under aerobic and microaerobic conditions. An infection assay using axenic endosymbionts of the liquid Schneider medium incubation in the natural host *Acanthamoeba* sp. UWC12 was conducted (Fig. 5.13). Infectivity of the endosymbiont was still present after aerobic or microaerobic incubation, respectively. The proportion of infected amoebae and the magnitude of infection per cell was higher when using microaerobically incubated endosymbiont cells.

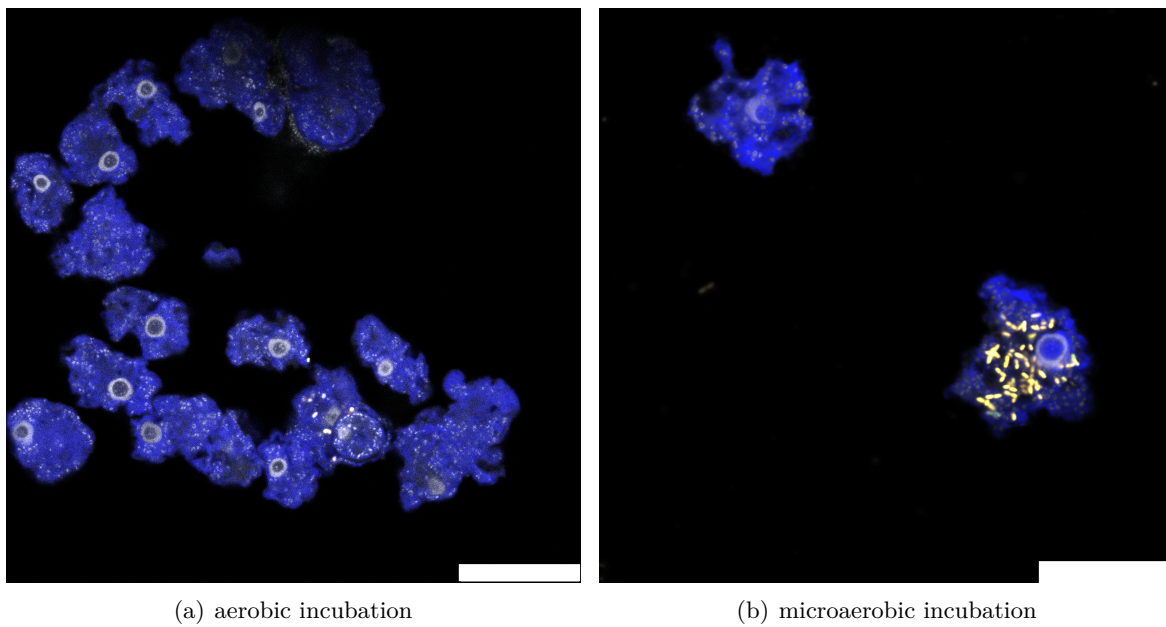


Figure 5.13: Infectivity assay of axenically cultivated endosymbionts. FISH staining of amoebae infected with *P. acanthamoebae* after host-free incubation in Schneider medium. Bacteria (green), *P. acanthamoebae* UWC12 (red), eukaryotes (blue), and DNA staining with DAPI (gray) of liquid Schneider medium with *P. acanthamoebae* UWC12 under aerobic (a) or microaerobic (b) conditions. Endosymbiont cells were potent to infect amoebae after incubation in both atmospheric conditions. The magnitude of infection (endosymbiont cells per host cell) and the infection rate of the culture was higher under microaerobic conditions. Scalebar: 25µm.

5.5 Quantification of *P. acanthamoebae* using qPCR

Due to the low cell density in axenic liquid cultures and the flock formation of *P. acanthamoebae* a qPCR approach was chosen for quantification.

5.5.1 Evaluation of established general 16S rDNA gene assay

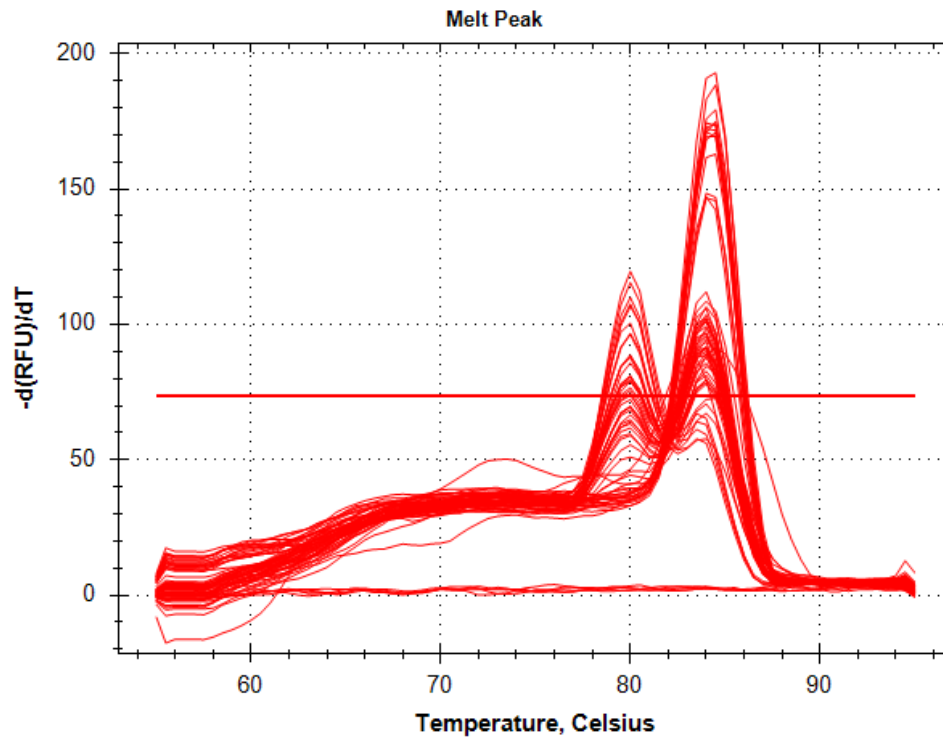
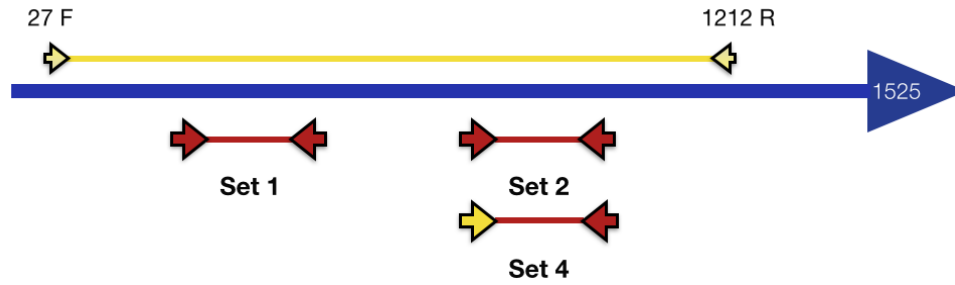


Figure 5.14: Melting curves of general 16S rRNA gene. General bacterial and archaeal 16S rDNA primer set (1389Fmix and 1492R) melting curves. The x-axis depicts the temperature gradient, while the y-axis represents the difference of relative fluorescence units in relation to the difference of temperature. Two distinct peaks can be observed at 80 °C and approximately at 84 °C. The second peak was considered to represent the endosymbiont 16S rRNA gene product since it is also present in the qPCR standard product.

Primers 1389Fmix and 1492R targeting a region in the 16S rRNA gene shared by most bacteria and archaea were tested to quantify a dilution series of extracted genomic DNA of *P. acanthamoebae* UWC12 of a running infected amoebae culture as described in 4.15.4.1. The relation of the quantified dilution series was linear (data not shown). When inspecting the melting curves of the PCR products heterogeneous melting peaks were present at 80 °C and 84 °C, respectively (5.14). exclusively the peak at 84 °C is found in the qPCR standard product and therefore belongs to the targeted 16S rRNA gene fragment. Melting peak analysis of genomic DNA of endosymbiont free *Acanthamoebae* sp. UWC12 showed that indeed the peak at 80 °C represents an amplified DNA fragment of amoebal origin (data not shown).

5.5.2 Evaluation of a specific qPCR standard and primers for relative 16S rDNA gene copy number quantification



(a) location of the primer sets at the 16S rRNA gene

Trivial name	Primer F	Primer R	Fragment length (bp)
Set 1	117	271	160
Set 2	583	744	183
Set 3	584	1212	647
Set 4	584	744	182

(b) Primer pairs used

Figure 5.15: Map of primer binding sites on the 16S rRNA gene of *P. acanthamoebae* UWC12.

(a) The blue line with an arrow head represents the full length 16S rRNA gene of the endosymbiont from the 5' to 3' end. Small arrows depict primers and their orientation on the 16S rRNA gene. Yellow arrows represent the primers which are generally used in standard PCR assays. Red arrows represent primers designed specifically for qPCR (Tsao 2014, unpublished). (b) Trivial names of primer pairs, their corresponding forward and reverse primers, and the theoretical fragment length are shown. Set 3 is not graphically depicted in (a) as it is a combination of primer 584F and 1212R which was already evaluated for *P. acanthamoebae* specific 16S rDNA targeting PCR. It was used as a positive control in the evaluation assays.

As the general 16S rDNA amplifying primers are not specific for *P. acanthamoebae* rDNA obtained from *Acanthamoeba* sp. UWC12, primer sets that have already been designed (but never used before) by Tsao (2014, unpublished) were tested for specific amplification of the desired fragment. Since quantitative PCR assays are very sensitive, evaluation of the most specific primer pair was essential for further experiments (5.15). For evaluation it was necessary to obtain a large amount of an almost full length rRNA gene of the endosymbiont. A standard template was designed using a general forward primer targeting most bacteria (27F) and a reverse primer specific to *P. acanthamoebae* (1212R) were used. This primer choice guaranteed that all designed primer sets can bind the standard template. After cloning the fragment, the linear M13 PCR product (4.15.5) was used as a template for primer evaluation.

Primer sets were evaluated using the designed standard as a template at an annealing temperature of 64 °C and an elongation time of 50 s (see the protocol at table ??). As a positive control to ensure the integrity of the standard, additionally primer set 3 was used, which consists of already evaluated specific primers (Fig. 5.16). It was not used for qPCR as it has a too large fragment size for appropriate quantification (647 bp). Primer sets 1 and 2 PCRs were visualized on agarose gel yield faint bands. The set 1 fragment has a theoretical length of 160 bp but is located at approximately 250 bp. Similarly primer set 2 should have a fragment length of 183 bp, but the band is closer

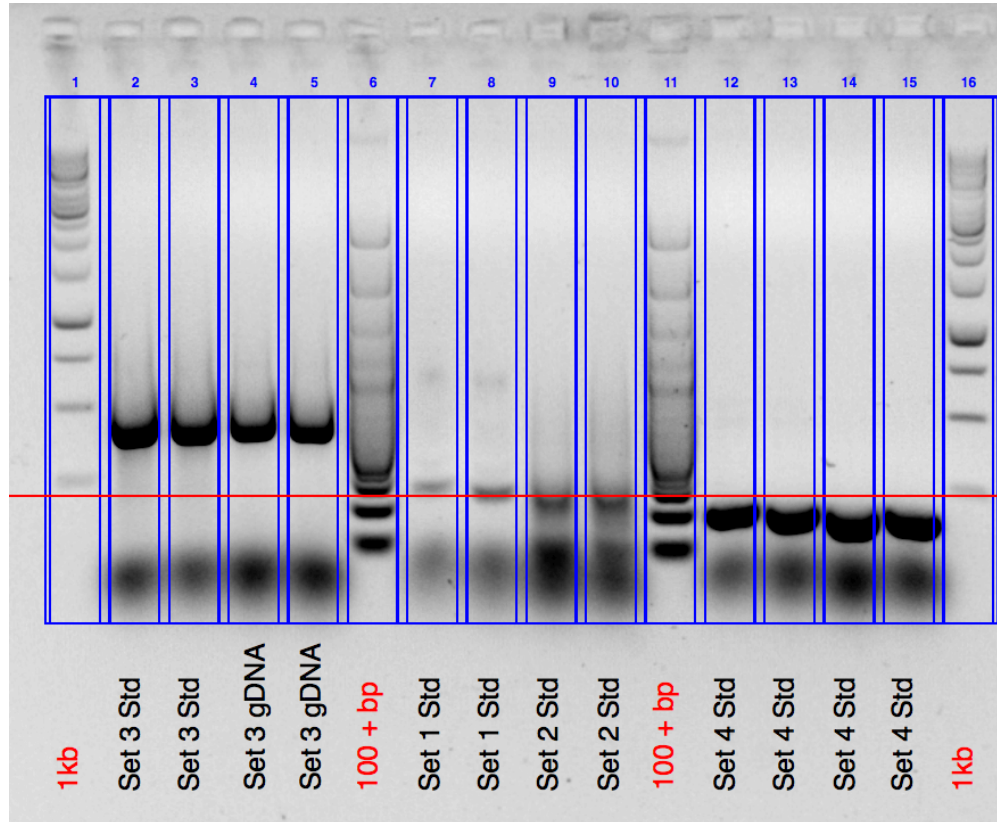


Figure 5.16: Fragment sizes of primer tests. 1.25 % agarose gel (TBE buffer) of primer sets 1, 2, and 3 on the designed *P. acanthamoebae* 16S rDNA standard run at 80 V for 45 min. As a positive control, primer set 3 was used. PCR annealing temperature was at 64 °C and elongation time was 50 seconds. The red line indicates a fragment length of approximately 250 bp and red text depicts the DNA ladders used. Set 3 was additionally run on genomic DNA. For primer set 4 the fragment length should be 182 bp and the band lies slightly below 200 bp as to be expected.

located to 250 bp and is not distinct but rather fuzzy. The primer set 4 band of the fragment lies slightly below the 200 bp marker, which corresponds to the theoretical length of 182 bp. The band is distinct and sharp, therefore the only candidate for a quantitative assay.

Further inspection of the sets was done using the specific melting peaks of the amplified DNA template (Fig. 5.17). These results validated what was already seen in the gel electrophoretic analysis. The only candidate fitting for a quantitative assay was primer set 4, which showed a distinct melting peak at 84 °C. Set 3, which consisted of already evaluated primers was included as a control. It has a distinct melting peak and shows clear gel bands, yet it has a too large fragment size for qPCR (647 bp).

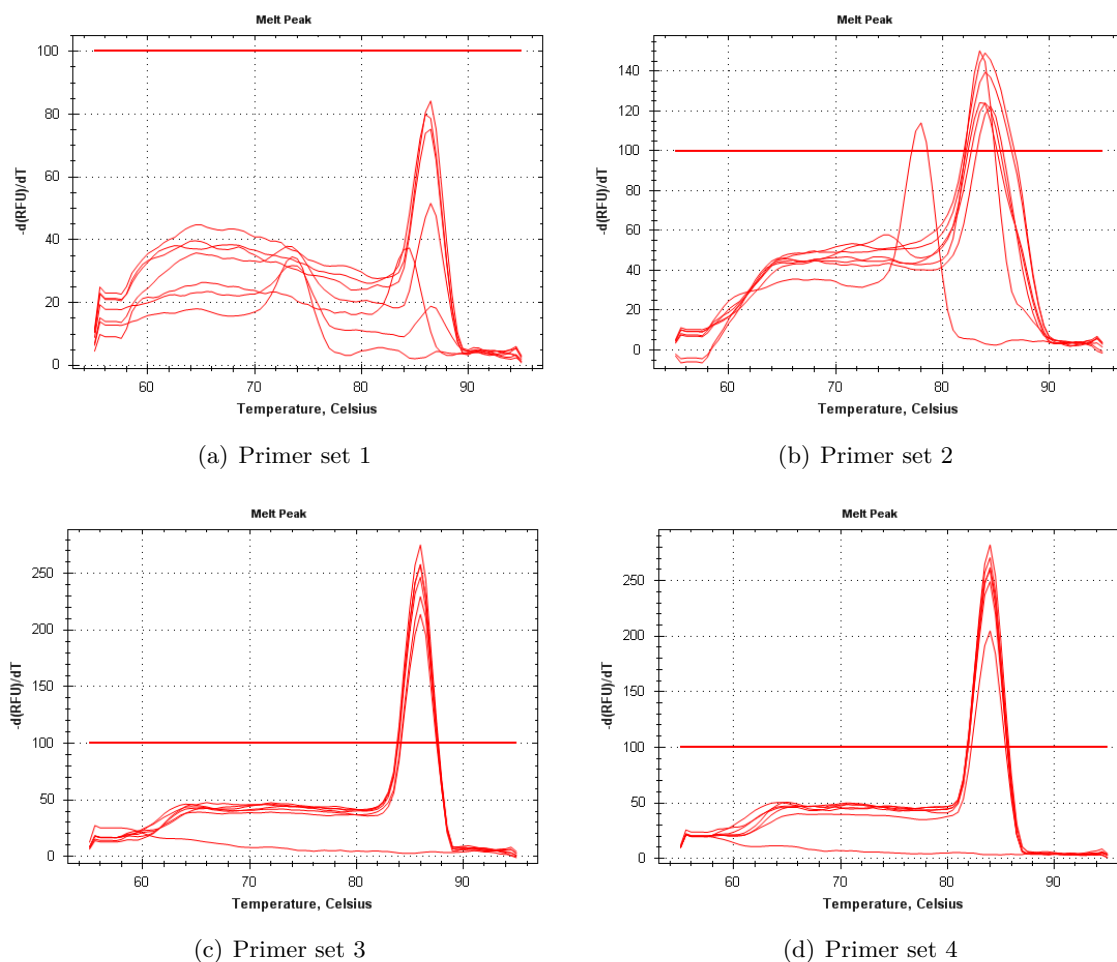


Figure 5.17: Melting curves of qPCR primer sets. Evaluation of primer sets 1 through 4 using the distinct melting curves of the amplified fragments. The x-axis depicts the temperature gradient, while the y-axis represents the difference of relative fluorescence units in relation to the difference of temperature. (a) Primer set 1 shows a very low signal, as it does not reach the threshold indicated by the red bar after 40 cycles. Furthermore there is not one distinct, but multiple small peaks. (b) Primer set 2 also does not show one distinct peak, but fuzzy peaks at around 84 °C. (c) The positive control for the assay shows a clear melting peak at approximately 86 °C. (d) Set 4 shows a clear melting peak at 84° C.

5.5.3 DNA isolation test

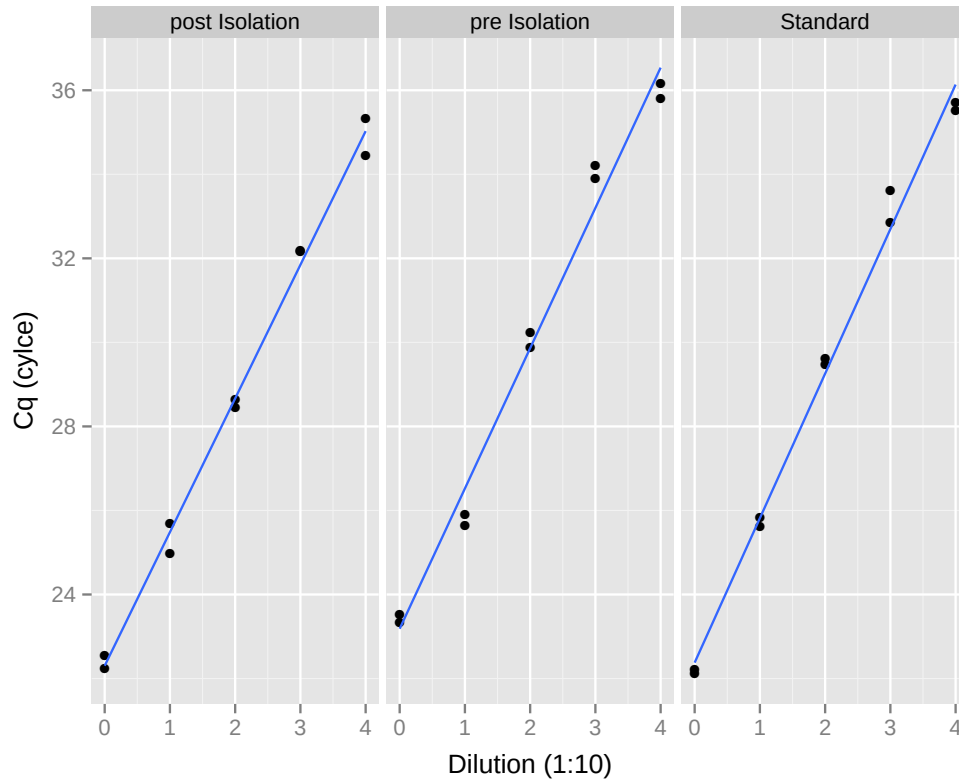


Figure 5.18: DNA isolation efficiency. Graph of the quantification of a dilution series of a sample diluted before DNA isolation (pre isolation), after DNA isolation (post isolation), and of the qPCR standard. The x-axis depicts numerically how depicts the dilution frequency (in 1:10 steps) starting from undiluted (0). The y-axis depicts the quantification cycle (Cq), meaning the elongation cycle at which the amplification fluorescence exceeded the background fluorescence of the template.

DNA extraction for qPCR was achieved with the DNeasy Blood and Tissue Kit (QIAGEN) and the workflow was adapted as described in 4.15.2.1. To check the efficiency and reproducibility of the qPCR assay with primer set 4 and the DNA extraction, a 10^{-1} dilution series of dense sample before DNA extraction with medium or after DNA extraction with buffer AE was performed. These dilution series were compared to a 10^{-1} dilution series of the qPCR standard (Fig. 5.18). The increase in Cq values is linear for all dilutions. This is supported by equation for the linear graph best fit by linear regression (Tab. 5.2). The slope of their equations varies faintly and they all fit nicely to the linear model with coefficients of determination (r^2) > 0.98 .

Sample	Intercept	Slope	r^2
post Isolation	22.3	3.18	0.995
pre Isolation	23.2	3.34	0.984
Standard	22.4	3.44	0.993

Sample	Intercept	Slope	r^2
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Table 5.2: Intercept, slope, and r^2 of the linear regression fit to the dilution series.

5.5.4 Preliminary growth curve in liquid SM

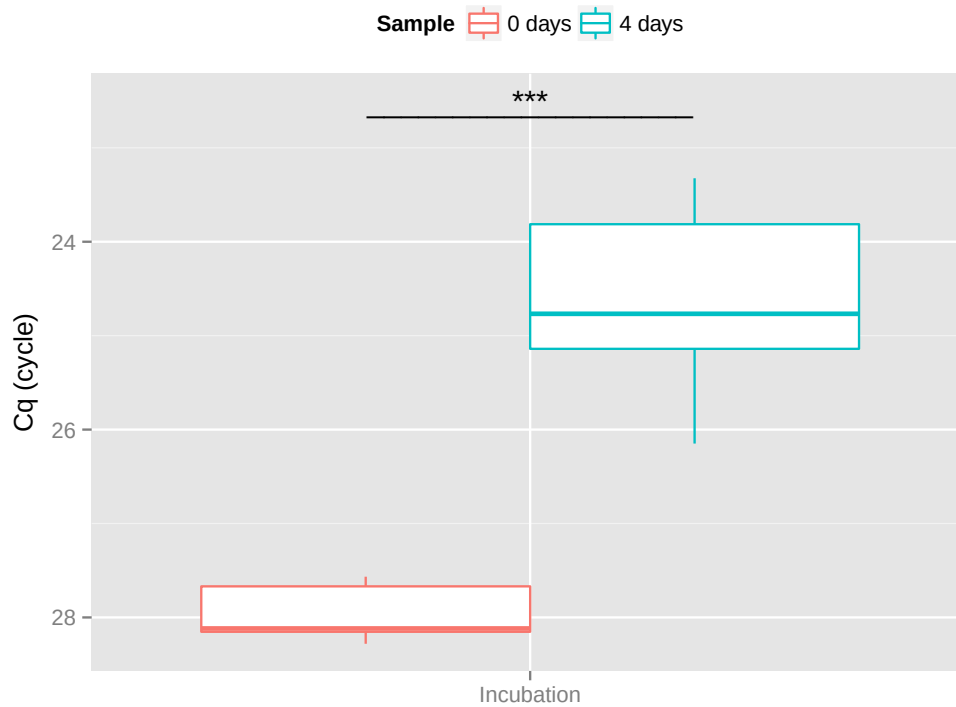


Figure 5.19: Axenic endosymbiont growth. Box plot of the quantification of axenic growth of *P. acanthamoebae* UWC12. The y-axis depicts the quantification cycle (Cq), meaning the elongation cycle at which the amplification fluorescence exceeded the background fluorescence of the template. The x-axis shows the positive control, and the incubation at timepoint 0 and after 4 days, respectively. Shown is the median biological duplicates and technical triplicates and their quantiles. Three asterisks indicate a p-value < 0.001 (students t-test).

Axenic endosymbiont growth was quantified using the established specific qPCR assay. Two timepoints were taken to make an quantify *P. acanthamoebae* growth. After 4 days of incubation in liquid SM (as described in 4.14.2), the quantification cycle of the qPCR was significantly lower (5.19). This corresponds to a significant increase of target template, which indicates an increase of endosymbiont cells.

5.6 *P. acanthamoebae* biofilm formation under aerobic conditions

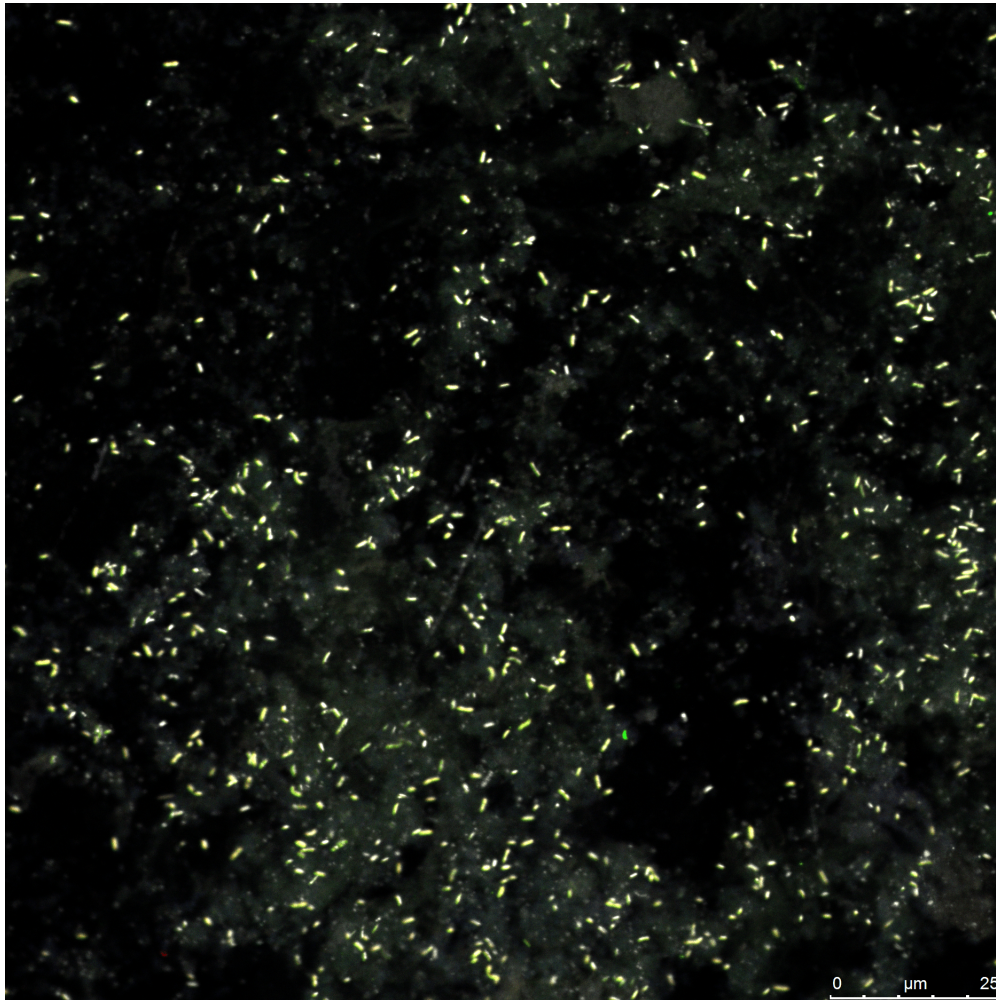


Figure 5.20: Biofilm formation of *P. acanthamoebae* under aerobic conditions. FISH staining of the axenic endosymbiont incubation assay of bacteria (green), *P. acanthamoebae* UWC12 (red), eukaryotes (blue), and DNA staining with DAPI (gray) of liquid Schneider medium inoculated with *P. acanthamoebae* UWC12 and kept under aerobic conditions. A biofilm containing endosymbiont cells is formed and was scraped from the bottom of the cultivation flasks after 16 days of incubation.

When incubated for 16 days under aerobic conditions, *P. acanthamoebae* UWC12 forms a biofilm at the bottom of containers used for cultivation. This was first analysed using FISH (Fig. 5.20). To test for a significant difference between biofilm formation under aerobic and microaerobic conditions a crystal violet based staining assay was used (4.16.2). Biofilm formation is significantly higher under aerobic conditions compared to microaerobic conditions (Fig. 5.21) with p-values < 0.001.

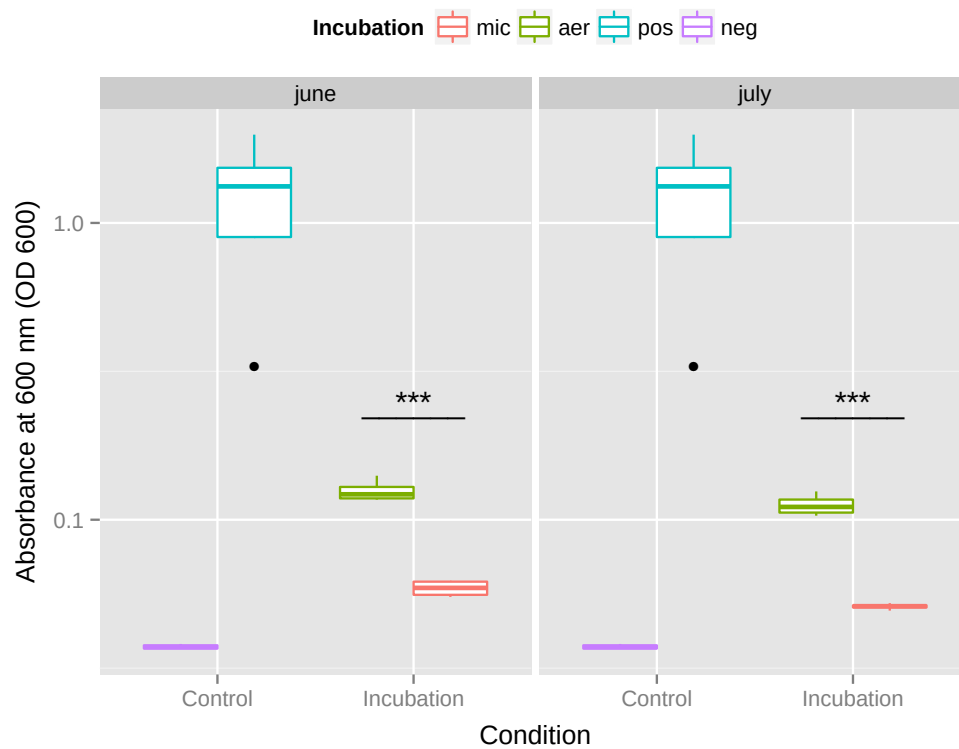


Figure 5.21: Colorimetric quantification of biofilm formation using a crystal violet assay. Box plot depicting biofilm formation of the of *P. acanthamoebae* UWC12 incubated under aerobic (aer) or microaerobic (mic) conditions compared to *Pseudomonas aeruginosa* as a positive control (pos) and sterile medium as a negative control (neg). The y-axis depicts the absorbance of the crystal violet stained biofilm at a wavelength of 600 nm. Biological quadruplicates and technical duplicates were used for analysis. The assay was conducted twice (june and july). Three asterisks indicate a p-value < 0.001 (students t-test).

Discussion

6.1 Impact of *P. acanthamoebae* on the natural host *Acanthamoeba* sp.

6.1.1 *P. acanthamoebae* relies on phagocytic uptake to invade the host, has low infectivity potential, and is non-lytic in its natural host

When conducting an infection assay the initial infection rate is at 60 % of the host population (Fig. 5.2). A substantial drop in infection rate occurs between two hpi and one dpi by 10 %. This indicates an inability to establish an infection in every initially infected host cell. Infected is in this case defined as the presence of an endosymbiont cell within a host cell. Intuitively one would expect a low initial infection rate, followed by a steady increase. One conclusion of this observation would be a weak adaptation of the endosymbiont to hijack the host cell. Additionally one has to keep in mind that the infection conditions in the lab do not reflect optimal infection conditions in the environment. If one assumes phagocytic uptake of *P. acanthamoebae* instead of an active invasion the first conclusion of a lack of adaptation to host cell infection is supported. Infecting eukaryotic cells of a higher organization level is possible for *P. acanthamoebae* when looking at S2 and MHP1 cells, respectively. (Fig. 5.7, Fig. 5.8). However, this ability seems to be limited to host cells with phagocytic properties, as *P. acanthamoebae* is not able to infect HeLa cells (Tsao 2013, unpublished). These findings hint to the necessity of passive uptake of the endosymbiont to invade host cells. It was shown that the phagocytic uptake of 1.0 μm latex beads by *A. castellanii* starts after five minutes and is saturated after two hours, meaning in this case that > 95 % of amoebae incorporated at least one bead (Avery, Harwood, and Lloyd 1995). Phagocytic activity seems to be cell cycle dependent, as it is threefold higher in large amoebae (later stage) than in small amoebae (newly divided) (Avery, Harwood, and Lloyd 1995). Additionally non-pathogenic *Escherichia coli* K12 get lysed and absorbed by *A. castellanii* within 24 h (Alsam et al. 2006).

It is therefore not surprising to see a drop of infection rate between 2 hpi and 1 dpi of *P. acanthamoebae* in *Acanthamoeba* sp. UWC12 if phagocytic uptake is assumed. Until two hpi bacterial cells are readily taken up, leading to a high infection rate in the host population. At two hpi a washing step is performed, therefore excluding an additional uptake of bacterial cells from the supernatant. Bacterial cells unable to infect get degraded and after an additional 22 hours a lower infection rate is observed. This decline is exclusive for lowly infected cells, which suggests, that if multiple *P. acanthamoebae* infect one cell, the infection is stable. Between one and three dpi the infection rate of the host population hardly increases, but rather the fraction of highly infected cells increases. This indicates the replication of bacteria intracellularly and an absence of extracellular bacteria able to infect new host cells. Further down in the infection assay, the total infection rate steadily increases until the culture is fully infected at 12 dpi. During the whole infection assay, host cell lysis

could not be observed. A propidium iodide staining assay revealed that *P. acanthamoebae* UWC12 does not seem to be lytic for *Acanthamoeba* sp. UWC12 at 20 or 30 °C when comparing an infected population to an uninfected control (data not shown).

6.1.2 Endosymbiont mediated increase of host generation time is lower at elevated temperatures

Amoebal endosymbionts related to chlamydiae and rickettsiae are known to be energy parasites, as they use ATP/ADP translocases to transport host derived ATP into their cytoplasm in exchange for bacterial ADP (Schmitz-Esser et al. 2004). Additionally these bacteria also encode transporters to import nucleotides (Tjaden et al. 1999; Audia and Winkler 2006). *Procabacter acanthamoebae* UWC12 does not encode an ATP/ADP translocase in its genome (Tsao 2014, unpublished), nonetheless it has to receive nutrients from its host to sustain metabolism and anabolism. Even in the establishment history of mitochondria as cell organelles it has been proposed, that they were initially energy parasites, drawing from the host ATP pool (Wang and Wu 2014). It has been shown for *Legionella pneumophila* growing in *Legionella* containing vacuoles of *A. castellanii*, that most amino acids are provided by the host except for Ala, Asp, and Glu (Schunder et al. 2014).

When conducting a growth comparison assay of *Acanthamoeba* sp. UWC12 infected/ not infected with *P. acanthamoebae* UWC12, retarded growth can be observed in endosymbiont infected amoebae populations (Fig. 5.5). Intuitively this effect should be caused by the nutrient uptake of the endosymbiont out of the host cytoplasm. This observation does not necessarily indicate a parasitous property of the symbiosis. The impact of the endosymbiont on its host doubling time is weaker at an elevated temperature (30 °C) than at usual the culture temperature of 20 °C, which suggests a temperature dependence of the effect (Tab. 5.1). A linear relation of the square root of growth rates to an increased temperature has been shown for bacterial cultures in a certain temperature range (Ratkowsky et al. 1982). Assuming this finding to be generally true for unicellular organisms in a constrained temperature range the decrease in amoeba PDT at 30 °C, when compared to 20 °C can be explained. For *Holospora obtusa* or *Holospora elegans* infected *Paramecium caudatum* it has been shown that the mRNA levels of the chaperones *hsp60* and *hsp70* were elevated, thereby enhancing their resistance to heat shock events (Hori and Fujishima 2003; Hori, Fujii, and Fujishima 2008). It is not yet known if the effect is actively influenced by the endosymbionts, or a general stress response to an infection. It has to be kept in mind, that *H. obtusa* and *H. elegans* inhabit the host nucleus, while *P. acanthamoebae* thrives in the cytoplasm. If an increased heat shock resistance due to increased levels of at least *hsp70* is a general stress response to infection, a balancing effect could be achieved. By improving the proper folding of proteins at an elevated temperature, the nutrient drainage by *P. acanthamoebae* could be mitigated and the weakened increase of PDT by infected *Acanthamoeba* sp. UWC12 populations could be explained.

An important component of the growth assay was to use a cured *Acanthamoeba* sp. UWC12 population as the point of departure (4.11.2). Hori, Fujii, and Fujishima (2008) reported retarded growth of aposymbiotic *P. caudatum* cured of *H. elegans* with penicillin, when compared to originally uninfected cultures for one year. In previous growth rate studies of the *P. acanthamoebae* - *Acanthamoeba* sp. UWC12 system, amoebae formerly cured of their symbiont were compared to a sub population of an infected running culture (Köstlbacher 2013, unpublished). At 30 °C, cellcount data suggested increased growth rates for amoebae infected with *P. acanthamoebae*. For the curing process rifampicin was used due to multiple antibiotic resistances of the endosymbiont (Tsao 2013, unpublished). Even though rifampicin should not inhibit the eukaryotic RNA polymerase (Campbell

et al. 2001), it has been used for the treatment of primary amoebic meningoencephalitis (Visvesvara, Moura, and Schuster 2007). Additionally, when establishing adequate rifampicin concentrations for amoebae curing, toxicity for amoebae has been observed at concentrations $> 100 \mu\text{g ml}^{-1}$. Therefore a negative effect of the curing procedure on the aposymbiotic host cells seems viable. As discussed above, the PDT of infected amoebae is in general increased. The difference in PDT between infected and uninfected amoebae at 30°C is almost neglectable, which points out the importance of using one cured starting population for comparison. Otherwise the retarded growth of the cured aposymbiotic population would mimic a positive effect of the endosymbiont on amoebal growth.

6.1.3 High persistence in host free medium might link to metabolic virility

In the environment extracellular endosymbiont persistence might be a desirable feature to acquire new host cells. Environmental chlamydiae use a biphasic lifestyle including metabolically almost inactive elementary bodies (EBs) and actively intracellularly dividing reticulate bodies (RBs) (AbdelRahman and Belland 2005). *Coxiella burnetii* the causative agent of human Q-fever shows two morphological cell types, a replicating, large cell variant (LCV) and a non-replicating, small cell variant (SCV) (McCaul and Williams 1981). For *P. acanthamoebae* no biphasic morphological differentiation could be observed. When incubated under similar conditions, EBs of *Parachlamydia acanthamoebae* strain Bn9, *Parachlamydia acanthamoebae* strain Hall's coccus, *Protochlamydia naegleriophila*, and *Waddlia chondrophila* maintained host infectivity for over 10 weeks (Coulon et al. 2012). With a maintenance of infectivity for 6 days under similar conditions, *P. acanthamoebae* UWC12 can not compete with quiescent cell variants of other symbionts. Yet the lack of such extracellular cell stages indicates that *P. acanthamoebae* can maintain its infectivity either through some kind of dormancy or a residual metabolism is possible within the axenic medium (TSY). In the study of Coulon et al. (2012) PYG was used as a nutrient rich medium for incubation. PYG is more complex than TSY and is phosphate buffered, which increases the stability of the pH and nutrient availability. This difference in incubation medium further highlights the feat of maintaining infectivity, as TSY is a less complex and unbuffered medium. By FISH signal only, the intensity of *P. acanthamoebae* seemed to decrease in a linear manner. This observation was not measured and statistically tested, yet it correlates with the decrease of infectivity, hinting at a steady decrease of endosymbiont fitness. Coupled to the high metabolic capabilities of *P. acanthamoebae*, a partial use of the complex medium TSY is suggested.

6.2 Inability to replicate within THP-1 cellline

It has been stated that the manipulation of the autophagic responses does not suffice to successfully inhabit the cytosol, rather pathogens have to interfere with many other aspects of host cell signalling to promote their survival (Ray et al. 2009). When the THP-1 cellline is infected with *P. acanthamoebae* UWC12, filamentous forms of the bacterium can be observed in the host cytosol. A similar observation has been made for macrophages infected with *Salmonella typhimurium*, which had elevated expression levels of the murine cathelicidin-related antimicrobial peptide, which leads to long filamentous bacteria (Rosenberger, Gallo, and Finlay 2004). Furthermore it is suggested that the cytosol provides only a limited subset of nutrients and challenges the endosymbiont with an organized system of pathogen recognition mechanisms and responses in mammal cells (Ray et al. 2009). At least a subset of *P. acanthamoebae* UWC12 manages to escape the phagosome upon

uptake and take residence in the host cytosol. However *P. acanthamoebae* does not have the tools to deal with the innate immune response of mammalian cells.

6.3 Towards host free cultivation of *Procabacter acanthamoebae*

In infection assays in *Acanthamoeba castelanii* NEFF and *Acanthamoeba* sp. UWC1 host cell lysis was not observed by light microscopic analysis (data not shown). The outcome of the infection assay in the S2 cellline was surprising, as host cell lysis was so abundant that it was easily assessable with a light microscope. The observation of a high abundance of extracellular endosymbiont cells coupled to the intensively populated host cells (Fig. 5.7) led to the hypothesis that *P. acanthamoebae* UWC12 is particularly fond of the Schneider medium used for S2 cell cultivation. The secondary intracellular symbiotic bacterium *Sodalis glossinidius* was isolated as a pure culture by incubating it under microoxic conditions in the medium of *Aedes albopictus* (tiger mosquito) cell line C6/36 (Dale and Maudlin 1999). Schneider medium is a very rich medium that contains all of the amino acids *P. acanthamoebae* is auxotrophic for. Amino acids are intensively taken up from the host cell by *Listeria monocytogenes* in murine macrophages and *Legionella pneumophila* in *Acanthamoeba castelanii*, respectively (Eylert et al. 2008; Schunder et al. 2014).

C. burnetii encodes cytochrome oxidases of cytochrome bd with a high affinity for O₂, which is used in microaerobic conditions (Omsland et al. 2009). Intuitively microoxic conditions are appropriate for most endosymbionts, as tissue oxygen concentrations are in general low (Braun et al. 2001). *Procabacter acanthamoebae* UWC12 also encodes cytochrome bd that seems to have been horizontally acquired from γ -*Proteobacteria* in addition to cytochrome o (Tsao 2014, unpublished). At first microoxic conditions could only be acquired through an oxygen gradient present in a softagar setup using Schneider medium and PYG as a basis, respectively. The increased turbidity coupled to sequencing and FISH images provided further evidence that *P. acanthamoebae* UWC12 is actually growing in Schneider medium softagar. Images obtained by modified liquid FISH of softagar incubations (4.14.1) showed weak signals of specific probes in the endosymbiont aggregates, but a strong DAPI signal (Fig. 5.11). Samples were only taken after 30 days of incubation and oxygen measurements with a micro probe showed, that the agar was already almost exclusively anoxic (data not shown). If one would in this case correlate ribosome content to activity (Poulsen, Ballard, and Stahl 1993), it could be assumed that *P. acanthamoebae* aggregates were left barely active due to a lack of oxygen. By FISH no other bacteria could be observed, reinforced by the sequencing results of the pooled general 16S PCR product that yield an unambiguous *P. acanthamoebae* UWC12 hit. When incubated in liquid Schneider medium, bacteria in the supernatant formed flocks under aerobic and microaerobic conditions. In *Pseudomonas aeruginosa* autoaggregation can be induced through detergent stress (Klebensberger et al. 2009). In *Pseudomonas syringae* aggregate formation was observed and it was inferred that it was a response to environmental stress (Monier and Lindow 2003). It can yet not be excluded that the aggregation of *P. acanthamoebae* in liquid Schneider medium is stress related, due to suboptimal cultivation conditions. Especially under microaerobic conditions, FISH signals were intense after 16 days which as discussed above, in case of *P. acanthamoebae*, hints at cell activity. These results are complemented by the qPCR results of the two point growth assay in liquid SM, which showed clear axenic growth within 4 days (5.5.4). Additionally when conducting an infection assay with axenically cultivated bacteria, both atmospheric incubation conditions were still infectious, with the advantage at the side of microaerobically incubated *P. acanthamoebae* (Fig. 5.13).

Many endosymbiotic (or rather human pathogenic) bacteria can form or thrive in biofilms like

Neisseria meningitidis, *Burkholderia pseudomallei*, *Francisella tularensis*, and *Legionella* spp. (Yi et al. 2004; Sawasdidoln et al. 2010; Hoek 2013; Lau and Ashbolt 2009). For *Francisella tularensis* and *Legionella* spp. biofilm association is hypothesized to increase persistence in the environment (Hoek 2013; Lau and Ashbolt 2009). For *Burkholderia pseudomallei* a correlation of biofilm formation and antimicrobial resistance has been drawn (Sawasdidoln et al. 2010). Apart from pathogens, multiple reasons for biofilm formation have been proposed, which span from aggregation and adhesion to the export of genetic information (Flemming and Wingender 2010). *P. acanthamoebae* UWC12 produces significantly more biofilm when incubated under aerobic conditions. Nonetheless aggregate formation can be observed in the supernatant under aerobic and microaerobic conditions respectively. As *P. acanthamoebae* was until now exclusively found as an intracellular symbiont of *Acanthamoeba* spp. and biofilm formation was only observed in the lab. An alternative hypothesis to persistence against environmental stresses could be overflow metabolism as the source of extracellular polymer formation that stems from incomplete oxidation of excess substrate, which is excreted as partially oxidized products (Teixeira De Mattos and Neijssel 1997).

6.4 Establishment of an adequate DNA extraction and qPCR assay for quantification of *P. acanthamoebae* growth

The initial plan was to use a simplified DNA extraction using bead beating as described by Omsland et al. (2009). Using the general 16S rDNA gene assay (see 4.15.4.2) on cell extracts of a preliminary *P. acanthamoebae* growth assay yielded inconsistent copy numbers for different centrifugation settings used for bead purification (data not shown). The standard qPCR assay using primer pair 1389F/1492R for general bacterial and archaeal 16S rDNA quantification (optimized by Pester et al. 2010) did not show the 16S peak in the product melting peak, but also an additional peak at 80 °C. The *P. acanthamoebae* cells used for cultivation were host derived and upon performing qPCR on aposymbiotic *Acanthamoeba* sp. UWC12 this peak could be clearly assigned to the amoebae. The specific *Procabacter acanthamoebae* primer evaluation step identified primer pair 584F-744R as the way to go. It showed clear bands on the newly designed linear rDNA standard and was used to evaluate the modified DNA extraction protocol based on the DNeasy Blood and Tissue Kit (QIAGEN) as shown in Fig. 5.18.

6.5 Outlook

A first attempt to grow *P. acanthamoebae* UWC12 in the liquid SM was successful. The newly established DNA extraction-qPCR assay did measure growth within 4 days of incubation. Still the axenic growth of the endosymbiont is low, probably due to sub optimal nutrient conditions in the medium. In soft agar incubations stronger growth could be observed with the formation of the large clusters of *P. acanthamoebae*. Schneider medium was diluted in the softagar incubations with PAS (1:3 v v⁻¹) to enable autoclaving with agar. Combined with the overflow hypothesis by Teixeira De Mattos and Neijssel (1997) it could indicate that pure Schneider medium is too concentrated in nutrients or that it is lacking a component present in PAS. Growth could be enhanced by diluting the medium, An addition that should be supplemented to the medium is cysteine as it has been reported to be essential for endosymbionts as *Francisella tularensis*, *Legionella pneumophila*, and *Coxiella burnetii* (P. Larsson et al. 2005; Ewann and Hoffman 2006; Omsland et al. 2009). Also fetal bovine serum, which is often used in cell culture should be tested. The next step would be

establishing growth on agar plates to establish clonal work, which is later on essential for genetic work.

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