universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's thesis

Characterization of an intranuclear symbiont of *Naegleria clarki*

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien 2019 / Vienna 2019

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 066 830
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Molekulare Mikrobiologie, Mikrobielle Ökologie und Immunbiologie
Betreut von / Supervisor:	Univ.-Prof. Dr. Matthias Horn

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1 Summary

Forms of intracellular symbioses in protist cells are diverse. They have been described for various bacterial species, but also for differing niches inside their eukaryotic hosts. Especially amoeba frequently serve as hosts to members of the bacterial phylum *Chlamydiae*, which is comprised solely of obligate intracellular bacteria well known for their potential as human and veterinary pathogens. While they mostly invade their hosts cytoplasm and thrive within modified host-derived vacuoles, they are rarely seen colonizing other intracellular niches. In fact only one such case is known within the whole phylum. This bacterium, termed *Chlamydiales* bacterium Pn, has been found located around the nucleolus of the free-living amoebae *Naegleria clarki*. Here I investigated morphology and physiology of both the symbiont Pn and its host during continuous co-culturing, as well as at the initial stages of infection. Additionally this study offers a comparison of the two frequently used amoeba culturing methods, either involving growth on a bacterial food source, or nutrient rich, axenic medium. Results of this comparison indicate changes in the *N. clarki* physiology, but also a major shift from growth enhancing to growth decreasing effects of the symbiont Pn towards its host, upon transition from bacteria-fed to nutrient rich medium. Investigations of the symbiont Pn, using fluorescence *in situ* hybridization (FISH) show a divergence from other chlamydial species, not only concerning its unique niche, but also by the detectability of the metabolically less active extracellular stages, which is unusual for bacteria of the phylum. For an amoeba infecting *Chlamydiae* species, Pn shows a short developmental cycle of only 48 hours, ultimately leading to a stable, chronic infection of the amoeba population with the symbiont. Taken together this study represents an important starting point for further, deeper investigations of a unique chamydial intranuclear symbiosis, but also shows the importance in assessing the effect of different amoeba culturing methods on the host-symbiont relationship.

2 Zusammenfassung

Die Formen intrazellulärer Symbiosen in protistischen Zellen sind vielfältig. Sie wurden fr verschiedene Bakterienarten, aber auch für verschiedene Nischen innerhalb ihrer eukaryontischen Wirte beschrieben. Besonders Amöben dienen häufig als Wirte für Mitglieder des Bakterienphylums *Chlamydiae*, welches ausschlielich aus obligat intrazellulären Bakterien besteht, die für ihr Potenzial als Human- und Veterinärpathogene bekannt sind. Während sie meist in das Zytoplasma ihrer Wirte eindringen und in modifizierten, aus dem Wirt abstammenden Vakuolen gedeihen, werden sie selten als Kolonisatoren anderer intrazellulärer Nischen gesehen. Tatsächlich ist nur ein solcher Fall im gesamten Phylum bekannt. Dieses Bakterium, genannt *Chlamydiales* Bakterium Pn, wurde um den Nukleolus der freilebenden Amöbe *Naegleria clarki* herum gefunden. Hier untersuchte ich die Morphologie und Physiologie sowohl des Symbionten Pn als auch seines Wirtes während kontinuierlicher Co-Kultur, sowie in den Anfangsstadien der Infektion. Zusätzlich bietet diese Studie einen Vergleich der beiden häufig verwendeten Amöbenkulturmethoden, entweder mit Wachstum auf einer bakteriellen Nahrungsquelle oder nährstoffreichem, axenischem Medium. Die Ergebnisse dieses Vergleichs deuten auf Veränderungen in der *N. clarki*-Physiologie hin, aber auch auf eine deutliche Verschiebung von wachstumsfördernden zu wachstumsvermindernden Effekten des Symbionten Pn auf seinen Wirt, beim Übergang vom bakteriellen zum nährstoffreichen Medium. Untersuchungen des Symbionten Pn mittels Fluoreszenz-*in-situ*-Hybridisierung (FISH) zeigen eine Aweichung von anderen Chalmydialarten, nicht nur hinsichtlich seiner einzigartigen Nische, sondern auch hinsichtlich der Nachweisbarkeit metabolisch weniger aktiver extrazellulärer Stadien, die fr Bakterien des Phylums ungewöhnlich ist. Für eine amöbeninfizierende *Chlamydiae*-Art zeigt Pn einen kurzen Entwicklungszyklus von nur 48 Stunden, was letztlich zu einer stabilen, chronischen Infektion der Amöbenpopulation mit dem Symbionten führt. Zusammengenommen stellt diese Studie einen wichtigen Ausgangspunkt für weitere, tiefer gehende Untersuchungen einer einzigartigen chamydialen intranukleären Symbiose dar, zeigt aber auch die Bedeutung für die Beurteilung der Wirkung verschiedener Amöbenkulturmethoden auf die Wirts-Symbiontenbeziehung.

3 Introduction

3.1 General and intranuclear symbioses

Symbiosis, a term coined in 1868 by Simon Schwenderer and later formulated into the broad concept of "unlike named organisms living together" by Anton de Bary in 1879 [4]. The definition of this concept, describing close proximity of the partaking organisms as a 'must', for many only implies a mutualistic association, while others in a more general approach include additional broad categories, differentiating symbiotic parasitism, commensalism and mutualism [73]. The nature of these associations however is not confined by distinct boundaries and might shift in response to developmental, environmental or behavioral modifications [64]. Parasitism involves a durable interspecific relationship of a parasite living at the expense of a host [42] during at least one stage of its life cycle [27]. Mutualism implies an exchange of long duration, that is beneficial, but not necessarily in equal measure, to all benefiting partners of the symbiosis [64]. This type of association has also been described as a highly developed division of labour, producing a superorganism [73]. A typical example of such an association is the intracellular symbiosis between aphids (Homoptera, Aphididae) and the Gamma proteobacterium *Buchnera aphidicola* [46]. This association, which is supposed to have started 200-250 million years ago [54], has led to parallel divergence and cospeciation, leaving the *Buchnera* symbiont dependent on nutrients and protection from the aphid host and the host in dependence on essential nutrients provided by the symbiont [46, 47]. The definition of commensalism is somewhat more problematic. It applies to symbionts that benefit from an indirect diversion of energy from the host, without an apparent cost for the latter [16]. A frequently cited example is the human commensal *Staphylococcus aureus*, which can asymptomatically inhabit the human skin. Although benefits to commensals can also be given in other forms such as transportation or shelter. However, the term commensalism indicates a relationship that is difficult to prove, as affected parameters, generally easy to find for parasitic or mutualistic relationships (e.g. fecundity, body mass, etc.), are parts of a broad continuum and hard to pinpoint in commensal relationships [98].
Endosymbiotic relationships between amoebae and prokaryotic cells occur commonly in nature and have emerged from multiple branches of the bacterial tree of life [72]. The majority of the so called amoeba-resisting bacteria (ARB), that have been recovered by amoeba coculture, reside directly in the host cytoplasm or in host-derived vacuoles [68]. Establishing this kind of intracellular niche usually involves recruitment of host ribosomes, endocytic and secretory pathways to the inclusion membrane, ultimately forming a specialized organelle for the bacteria to replicate in [18]. Occasionally endosymbionts can be found located directly within other cellular organelles, including chloroplasts [78, 96], mitochondria [74] and the endoplasmic reticulum [91]. However the majority of organelle inhabiting isolates have

been described colonizing the nuclear compartment of either amoebae [48, 52, 75, 80, 84, 93], other protists [86], or higher eukaryotes (including arthropods, marine invertebrates and even mammalian cells [79]). As the nucleus is rich in substrates (such as proteins, nucleic acids and nucleoside triphosphates) required for basic cellular functions like bacterial DNA replication, transcription and translation, it offers an attractive niche for intracellular bacteria away from cytosolic defense systems. Descriptions of nuclear invasion strategies have been reported for groups of bacteria, including *Holospora* spp. and *Nucleicultrix amoebiphila*, which, after escape from the phagosome, either make use of a specialized actin-binding invasion tip [40, 71], or enter during disintegration of the nuclear envelope during host mitosis [80]. Entry without prior escape from phagosomes was described for nuclear symbionts of *Euglena* [85] and the amoeba symbiont *Ca. Berkiella cookevillensis* [21]. Once the intranuclear niche is established, reports are still missing on how intranuclear bacteria are able to thrive inside the nucleus. Likewise, the question of whether mechanisms such as take-up of nutrients and maintenance of host cell integrity and nuclear functions are shared among all intranuclear bacteria, is yet to be resolved. Intranuclear symbiosis seems to also hold a role for lateral transfer of genome fragments between host and symbiont. As of yet, only Sato *et al.* (2014) [75] have found strong suggestions of lateral gene transfer (LGT), by showing 16S rRNA pseudogenes of a verrucomicrobial intranuclear symbiont present in the host *Trichonympha* genome. However, for cytoplasmic symbionts LGT has been reported several times, including *Wolbachia* and their invertebrate hosts [28], symbionts of deep-sea hydrothermal vent mussels [76] and several symbionts of amoebae [10].

3.2 The free-living amoeboflagellate *Naegleria*

The genus *Naegleria* constists of unicellular, uninucleate free-living protists with a heterotrophic life-style. They can be commonly found in both aerobic and microaerobic environments of freshwaster and moist soils, with temperature tolerance up to 45^{o}C in some species [24]. They were first described more than a century ago in 1899 by the viennese scientist Franz Schardinger in his study of an *Amoeba lobosa* that was able to transform into a flagellated form [77]. Only later, in 1912 was the genus name *Naegleria* suggested by Alexeiff [2]. In 1985 the genus, as part of the family *Vahlkampfiidae*, was included in the newly established class of Heterolobosea by Page and Blanton [59]. The class comprises a small group of amoebae, amoeboflagellates and flagellates, that includes ca. 140 described species and unites unicellular Schizopyrenida with Acrasida that form multicellular bodies [61]. Until the late 1960s this genus was considered to be of no medical relevance and was used mostly as a model system for transformation into flagellates [34, 44, 97]. However, in 1970 a certain *Naegleria* species was found to be involved with the for humans fatal brain disease primary amoebic encephalitis (PAM) [19]. The disease had originally been attributed to amoeba of the genus *Acanthamoeba* in 1966 by Malcom Fowler [30], who was the first to describe cases of the disease in Australia and after whom the disease causing species *Naegleria fowleri* was later named. This finding sparked a greater interest in the genus in general, leading, besides investigation of pathogenic strains *N. fowleri* and later found *N. australiensis* and *N. italica* [24], to studies on life-cycle [94], cellular organization [31, 37], whole genome analyses [32]

and the role as possible pathogen carriers of free-living *Naegleria* [20].
The typical life-cycle of *Naegleria* consists of three distinct morphological stages, the amoeboid or trophozoite stage, the flagellate stage and the cyst, representing a resting stage [61](Figure 1). For *Naegleria clarki* the trophozoite represents the predominant stage, constituting its reproductive phase and the starting point for differentiation into the other two stages. Cysts may arise after a culture reaches stationary phase, e.g. when bacteria serving as a food source are no longer present [5]. Flagellates arise when trophozoites are washed off from a surface (e.g. agar) into a liquid medium, constitute an important stage for escape from adverse environmental conditions and dispersal in the environment [34]. The close relative of *N. clarki*, *N. gruberi* has been used as a model system for studying flagellation [33], providing well established protocols for their transformation into flagellates, although several *Naegleria* species have been shown to vary significantly in their ability and efficiency to transform [26], or have lost this ability entirely after prolonged cultivation [60].

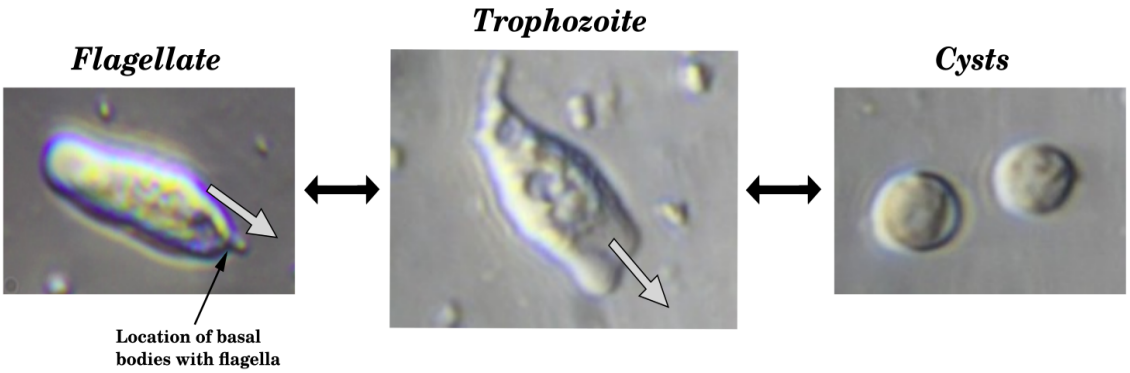

Figure 1. Life-cycle stages of the amoeboflagellate *Naegleria clarki*. At the center the reproductive trophozoite stage (average length of 15 μm) is shown, which can transform either into the similarly sized, highly motile flagellate stage (left), or the protective resting stage as a cyst (right, average diameter od 10 μm)).Grey **arrows** indicate the direction of movement.

All currently known *Naegleria* species possess one nucleus, with a central, prominent nucleolus, which contains 3000-5000 copies of a 14kb circular plasmid, carrying 18S, 23S and 5.8S ribosomal RNA genes, which cannot be found on the chromosomes [60]. Cellular division is always preceded by a closed nuclear division (also termed promitosis), with nuclear membrane and nucleolus remaining intact throughout the entire process [95]. Sexual reproduction within the whole class of Heterolobosea, although discussed for several heterolobosean genera, has not yet been fully resolved. In 1992 Pernin *et al.* [65] with their analysis of a large number of *Naegleria lovaniensis* isolates, on several polymorphic loci, provided strong evidence for sexual reproduction in the species. Later, meiosis-associated genes were discovered in *N. gruberi* [32] and two key genes involved in gamete and nucleus fusion (HAP2 and GEX1) were shown to be present in the whole class of Heterolobosea [88]. *Acrasis rosea* is an amoeboid organism of the family *Acrasidae* within the Heterolobosea, that has been shown to aggregate and form a multicellular, spore-producing structure, called a sorocarp, which represents the presumed sexual stage of the amoeboflagellate [14]. The induction of this multicellular life-cycle has once also been shown for *A. spelaea* [15], a member of the *Allovahlkampfia*, which is a sister genus to *Naegleria*. Members of both these

families *Acrasidae* and *Vahlkampfiidae*, also share the limax morphology, meaning they are consistently monopodial, lack recognizable stacked Golgi bodies and a closed mitosis [15]. Still, despite increasing evidence for a sexually reproductive stage within the genus *Naegleria*, except for this study, neither sorocarpic structures, nor any other form of mating in amoeba or flagellate stage, have been observed.

Endosymbionts of *Naegleria* spp. Beside their role as human pathogens, the clinical relevance of free-living *Naegleria* as hosts and vehicles of possible human pathogens has been known since Rowbotham in 1980 first confirmed the ability of *Legionella pneumophila*[1] to infect and grow within vacuoles of *Acanthamoebae* and *Naegleria* spp. [69]. Since mechanisms, allowing bacteria to escape from phagocytosis and digestion by free-living amoeba (FLA), are believed to be similar among most bacterial endosymbionts, it is not surprising to find many of them capable of infecting several different phagocytic host organisms, stemming from far separated phyla in the eukaryotic tree of life (Figure 2) [7].

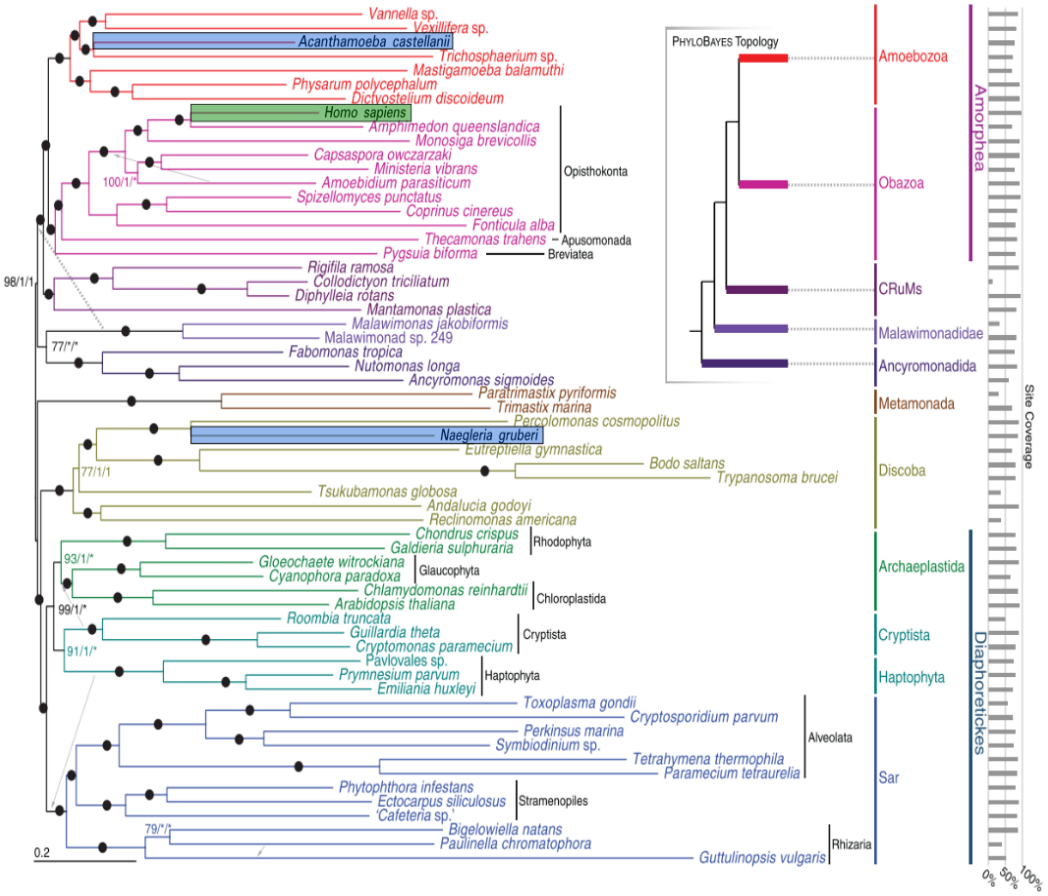

Figure 2. Maximum Likelihood tree, inferred from 351 proteins, showing the phylogenetic relationship of 61 eukaryotic species, modified from Brown *et al.* (2018) [13]. *Acanthamoeba castellanii*, belonging to the Amoebozoa is much more closely related to *Homo sapiens* (highlighted in green) as part of the sisterclade Obazoa, than to *Naegleria gruberi*, belonging to the Discoba. Both *A. castellanii* and *N. gruberi* (highlighted in blue) represent two phylogenetically far separated genera, that have a similar bacteria grazing life-style, occupy similar habitats and are suscepitble to infection with several endosymbionts of the same species [7].

[1]The caustive agent of legionnaires' disease

Naegleria species are, besides *Acanthamoeba*, *Hartmannellae* and *Vermamoebae*, among the most commonly isolated fresh-water amoebae [90]. While *Acanthamoeba* and *Vermamoebae* frequently harbour endosymbionts, only few bacterial species have been found replicating in *Naegleria* species. These include *Legionella*-like organisms (order Legionellales), *Stenotrophomonas maltophilia* (order Xanthomonadales), *Acidovorax temperans* (order Burkholderiales), *Flavobacterium johnsoniae* (order Flavobacteriales) and some *Chlamydiae* (order Parachlamydiales) [7].

In 1999 an amoeba, isolated from a garden pond (Bad Hönningen, Germany), later assigned to the species *Naegleria clarki* [93], was found harbouring two different endosymbionts at the same time [53]. Of these two bacterial strains, one was found replicating inside the hosts' cytoplasm (termed 'Pc'), inhibiting host transition into the cyst stage, while the other (termed 'Pn') was found located inside the hosts' nucleus, around the prominent nucleolus [93]. The strain *N. clarki* N-DMLG, containing both endosymbionts, was cured from the cytoplasmic symbiont population in 2005 by Walochnik *et al.* [93], reinstating the hosts' ability to form cysts, producing the strain N-DMLG-Pn (subject of this study), which still contained the intranuclear Parachlamydiales symbiont (classification based on personal communication with Stephan Köstlbacher and Astrid Collingro (unpublished); Figure A.8). The strain Pn was described by [53, 93] as unable to grow on any synthetic media and, using transmission electron micriscopy (TEM), it was described to be of a coccoid morphology and dividing by binary-fission without the formation of septa (Figure 3). Since then, however experimental investigation of this *Naegleria* isolate and its endosymbiont populations has more or less ceased, until this study.

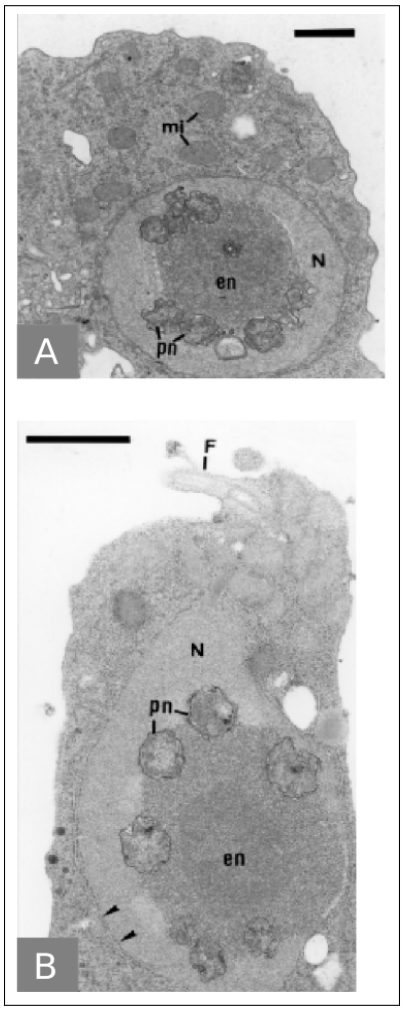

Figure 3. TEM image, adapted from Walochnik *et al.* (2005) [93], showing *Naegleria clarki* strain N-DMLG-Pn. (A) - trophozoite harbouring the intranuclear endocytobiotic population (pn) located at the interphase between nucleolus (en) and karyoplasm (N - nucleus, mi - mitochondria. Scale bar 1 μm). (B) - flagellate stage harbouring the intranuclear population (pn) (F - flagellum, N - nucleus. Arrowheads - nuclear membrane). Scale bar 1 μm.

3.3 The phylum *Chlamydiae*

Chlamydiae constitute a monophyletic group of diverse bacteria, that all share a dependence on eukaryotic host cells for their reproduction [50]. Their obligate intracellular lifestyle is facilitated by a characteristic developmental cycle consisting of two morphologically and physiologically distinct stages, the elementary body (EB) and the reticulatebody (RB) [1] (Figure 4). EBs function mainly to survive the extracellular environment and to infect new host cells. Upon infection, the EB differentiates to an RB, that can multiply inside a host-derived vacuole [8]. The colonized phagocytic compartment is modified to generate a specialized vacuole, called an inclusion. Several *Chlamydiae*-derived proteins are conserved in all chlamydial lineages and include a type III secretion system (a virulence factor) and the ADP-ATP translocase (a transporter involved in energy parasitism) [66].

Organisms of this phylum were first described in 1907 [36] and, until the end of the 1940s, had been categorized as human pathogens of the lymphogranuloma-trachoma-psittacosis group [43]. In 1947 this group was shown to be separate from 'true viruses' and suggested to be in a position between the bacteria and viruses, with the genus name *Chlamydia* [43]. Moulder in 1966 [55], with the advent of electron microscopy, recognized and classified *Chlamydiae* as bacteria, mainly due to their posession of DNA, RNA, ribosomes and a cell wall similar to Gram-negative bacteria. Today the phylum is known as part of the PVC superphylum (*Planctomycetes, Verrucomicrobia, Chlamydiae*) [92] and consists of at least eight families within the single class of *Chlamydiia*[2], which is separated into the two orders of *Chlamydiales* and *Parachlamydiales*. The order *Chlamydiales*, which until recently constituted the only order within the class, contains the family *Chlamydiaceae* and the candidatus family *Candidatus Clavichlamydiaceae*. The family of *Chlamydiaceae* is made up of 13 species belonging to the genus *Chlamydia* and still remains the most widely studied family of the order [22]. It consists of primary human pathogens, known for example to cause genital tract infections or trachoma (*Chlamydia trachomatis*) and respiratory tract infections (*Chlamydia pneumo-*

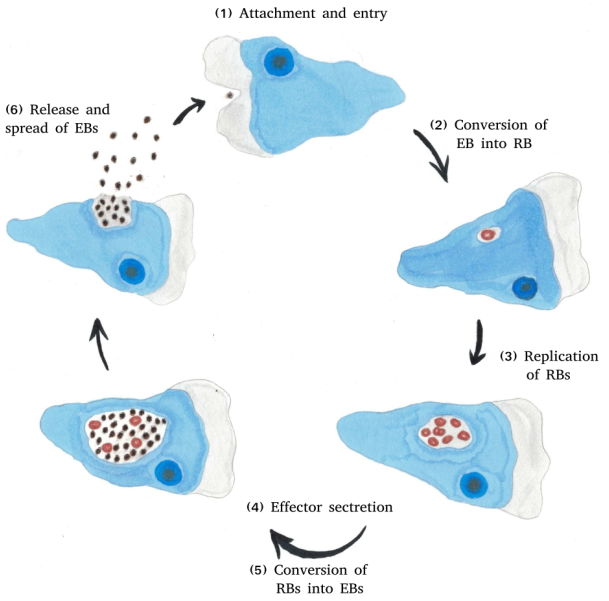

Figure 4. Schematic representation of the biphasic life-cycle of *Chlamydiae*. The major events in the developmental cycle are: **(1)** attachment and uptake of infectious particles (EBs) usually via phagocytosis; **(2)** conversion of the EB into the replicating stage (RB); **(3)** replication of the bacteria inside the intracellular inclusion; **(4)** secretion of effector molecules, modifying the inclusion and secretion/uptake of molecules; **(5)** conversion of RBs back into EBs prior to escape from the host cell; **(6)** escape from the host cell either by lysis of the inclusion and host cell membranes, or by exocytosis without lysis of the host (not shown) [1].

[2]Recent reevaluation, based on evolutionary relationships among organisms of the bacterial tree of life, has placed the class *Chlamydiia* under the phylum *Verrucomicrobiota* [62]. However, as this as of yet remains the only such study, pointing to this conclusion, previous assumptions, placing the *Chlamydiia* in the separate phylum *Chlamydiae*, are assumed for this study.

niae), but also of animal pathogens, that pose risks for livestock and potential zoonotic transmission [22]. In addition to medically important chlamydiae, found in recent years to only be the 'tip of the iceberg' in terms of diversity within the phylum [89], a much larger number of chlamydial taxa can be found in the order *Parachlamydiales*. This order contains the families *Parachlamydiaceae*, *Criblamydiaceae*, *Simkaniaceae*, *Rhabdochlamydiaceae* and *Waddliaceae* and the candidatus families *Ca. Parilichlamydiaceae* and *Ca. Piscichlamydiaceae* [66]. Families of this order have often collectively been referred to as '*Chlamydia*-like organisms (CLOs) or 'environmental chlamydia', although these terms might be misleading, as the host-range of members of the order is ranging from warm-blooded terrestrial vertebrates to fish, reptiles and amphibians to eukaryotic microorganisms such as amoeba [89].

3.4 Aims of this study

This study was intended as a characterization of the interaction between the unique intranuclear symbiont *Chlamydiales* bacterium Pn and its amoebal host *Naegleria clarki*. As a first part, this investigation was focused around identifying length and time-points of key developmental stages of the Pn infectious cycle and further determining the progression and modes of transmission to the surrounding culture cells, using fluorescence *in situ* hybridization (FISH) with a Pn-specific probe. In addition, the impact of chronic infection with the symbiont was determined by utilizing a continuous host-symbiont co-culture (the strain *N. clarki* N-DMLG-Pn; Table 4). Nucleus and cell sizes, but also rates of flagellation between infected and uninfected individuals were compared.
The second part of investigation was focused initially around establishing an axenic infection system for later use in transcriptomics experiments, as the hitherto used monoxenic method of cultivation hinders such experiments through its intrinsic contamination by food-bacteria. However, as first infection experiments in such axenized host cultures showed an instable interaction between host and symbiont, further tests were designed around explaining this change in interaction stability. Differences were investigated on the one hand by comparing infection success and transmission under axenic conditions and on the other hand by comparing growth of infected and uninfected host cultures, using axenic and monoxenic cultivation under different temperatures.
Lastly, a possible multicellular stage in the life-cycle of *N. clarki* was also investigated for the two different aposymbiotic strains N-DMLG0 and CCAP 1518/14, as well as the symbiotic strain N-DMLG-Pn (Table 4).

4 Materials and Methods

4.1 Equipment

Equipment	*Company*
Axioplan 2 imaging (Epifluorescence microscope)	Carl Zeiss MicroImaging GmbH, Jena, Germany
Leica TCS SP8 (Confocal laser scanning microscope)	Leica Microsystems GmbH, Wetzlar, Germany
Leica LIGHTNING (image information extraction)	Leica Microsystems GmbH, Wetzlar, Germany
inoLab® pH Level 1 (pH meter)	Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany
Water purification system MILLI-Q® biocel	Millipore GmbH, Vienna, Austria

Table 1. Technical equipment used for experimental work.

4.2 Software

Software	*Developer*
Rstudio Version 1.1.456	2009-2018 RStudio, Inc.
Leica LAS X	Leica Microsystems GmbH, Wetzlar, Germany
Leica LAS X Core	Leica Microsystems GmbH, Wetzlar, Germany
Inkscape v0.92	https://www.inkscape.org

Table 2. Software used for data collection and analysis.

4.3 Consumables

Consumable	*Company*
Reaction tubes (15 ml, 50 ml) IsoporeTM	Greiner Bio-One GmbH, Frickenhausen, Germany
Isopore^{TM} polycarbonate membrane filters (0.22 m pore size, 25 mm diameter, black)	Millipore GmbH, Vienna, Austria
Parafilm® M laboratory film	American National Can Company, Chicago, IL, USA
VWR® Disposable Serological Pipets (2ml, 10ml, 25ml, 50ml)	VWR International bvba, B-3001 Leuven
1.5 ml and 2 ml reaction tubes	Greiner Bio-One GmbH, Frickenhausen, Germany
175 cm^2 Cell Culture Flask	Biologix® Group Limited, Shandong, China
25 cm^2 Cell Culture Flask, Nunclon^{TM} Delta Surface	Thermo Fisher Scientific, Roskilde, Denmark
Syringe filter, cellulose acetate (0.2 μm, 1.2 μm and 5 μm)	Sartorius Stedim Biotech GmbH, Göttingen, Germany

Syringe Omnifix® single use, sterile (various sizes)	B. Braun Meslungen AG, Meslungen, Germany
Multiwell dishes, Nunclon™ Delta Surface (6, 12 and 24 wells)	Thermo Fisher Scientific, Roskilde, Denmark
Sterican® needles, various sizes	B. Braun Meslungen AG, Meslungen, Germany
Low Retention Manual filter Pipette tips, various sizes	Biotix Holdings Inc., The Hague, Netherlands
Low Retention Manual Pipette tips, various sizes	Biotix Holdings Inc., The Hague, Netherlands
Cell spatula 240mm and 360mm	TPP Techno Plastic Products AG, Trasadingen, Switzerland
Teflon-coated Microscope slides	Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany
Microscope slide	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
FAST-READ 102® (Cell counting chamber)	BIOSIGMA S.r.l., Cantarana, Italy
Polycarbonate membrane (0.2μm)	EMD Millipore, Billerica, MA, USA
2ml Lysing Matrix E	MP Biomedicals Germany GmbH, Eschwege, Germany

Table 3. Consumables used for experimental work

Strains	*Relevant characteristics*	*Source*
Naegleria clarki	wild-type (CCAP 1518/14)	CCAP
Naegleria clarki N-DMLG0	wild-type, strain cured of *Chlamydiales bacterium* Pn and Pc	unpublished
Naegleria clarki cured(2019)	wild-type, strain cured of *Chlamydiales bacterium* Pn	this study
Naegleria clarki N-DMLG-Pn	wild-type, strain contains *Chlamydiales bacterium* Pn, but cured of the cytoplasmic bacterium Pc	Walochnik *et al.* 2005
Acanthamoeba castellanii - NEFF	wild-type	ATCC 50373
Acanthamoeba castellanii - UWE25	wild-type, strain contains the symbiont *Protochlamydia amoebophila* UWE25	ATCC PRA-7
Escherichia coli JW5503	Δ(*araD-araB)567*, Δ*lacZ4787*(::rrnB-3), λ^-, Δ*tolC732::kan*, *rph-l*, Δ*(rhaD-rhaB)568*, *hsdR514*	*E. coli* Genetic Stock center

Table 4. Strains used throughout this study

4.4 Fluorescence *in situ* hybridization (FISH)

4.4.1 Fixation of Amoeba samples

Fixantion of amoebae samples was done similar to what has been described elsewehere [67]. Culture cells were detached from the culture flask surface by scraping, using a cell spatula. Resulting cells suspensions were poured into sterile reaction tubes and harvested by centrifugation at 10000g for 10 minutes at RT. After resuspension in appropriate amounts of PAS, 20 μl were applied to 2 wells (per sample) on a teflon-coated microscopy slide. After 20 minutes, amoebae cells were considered sufficiently attached to the slide and the supernatant was removed. Samples were then incubated with 20 μl of 4% (w v^{-1}) formaldehyde (Rotipuran, Cat. No 4979.1) fixative for 15-20 minutes. The formaldehyde was removed after fixation, followed by an immediate, short washing step with 20 μl Milli-Q (MQ) water. Slides were dried at 37^{o}C. The slides were dehydrated, using a 50%, 75%, 96% ethanol series (each for 3 minutes). Slides prepared in this way were used directly for FISH or stored at -20^{o}C.

4.4.2 Fixation of bacteria samples

Fixation of bacterial cell was based on the standard fixation protocol according to Amann *et al* [3]. Cells were harvested by centrifugation at 15550g for 15 minutes at 4^{o}C. The cell pellett was resuspended in 1ml PAS and 10-100 μl were transferred into a separate reaction tube and mixed with 3 volumes of 4% formaldehyde. This mixture was then incubated for 1-2 hours at 4^{o}C and either directly used for determination of bacterial cell numbers, or centrifuged again, resuspended in 50% ethanol and stored at -20^{o}C.

Probe	*Target Organism*	*Sequence*	*Dye*	*Source*
PN440	*Chlamydiales bacterium* Pn	CTACTCTTGTTCTCAGGC	Cy3	Biomers
Chls523_II - DOPE	*Chlamydiales*	CTTCCGTATTACCGCAGC	Cy3	Biomers
EUB338_I - DOPE	most Bacteria	GCTGCCTCCCGTAGGAGT	Fluos	Biomers
EUB338_II - DOPE (SBACT_P 338)	*Planctomycetales*	GCAGCCACCCGTAGGTGT	Fluos	Biomers
EUB338_III - DOPE (SBACT_V 338)	*Verrucomicrobiales*	GCTGCCACCCGTAGGTGT	Fluos	Biomers
EUK516	Eukarya	ACCAGACTTGCCCTCC	Cy5	Biomers
NAEG1088	*Naegleria*	GTGGCCCACGACAGCTTT	Cy5	Biomers
Acant412a	*Acanthamoeba*	ACTCTTATCGAGCGCCTG	Cy5	Biomers

Table 5. Oligonucleotide probes used for FISH

4.4.3 Hybridization and DAPI staining

Oligonucleotide probes (Table 5) used for FISH were checked for specificity, using the Testprobe tool from ARB-SILVA (www.arb-silva.de). Further analysis of the retrieved data was done, using the R program packages "data.table" and "ggplot2" (www.r-project.org). Finally, probes were also checked for specificity on fixed *Acanthamoeba* cells, uninfected, or infected with the bacterium *Protochlamydia amoebophila* UWE25, as well as all available infected and uninfected *Naegleria clarki* strains (Table 4). Hybridization of probes to the corresponding target sites was done, using the hybridization and washing buffers described elsewhere [3]. If not stated otherwise, the formamide concentration of the hybridisation buffer was 25%. The slides were incubated in a closed, hybridisation buffer saturated environment at 46^{o}C in the dark for 90-120 minutes. Afterwards the slides were washed in prewarmed (48^{o}C) washing buffer for 15min. The last step consists of a hyper stringent 2 second washing step in ice-cold MQ water, followed by immediate air-compressed drying and storage at -20^{o}C, or further staining with 4=,6=-diamidino-2-phenylindole (DAPI, 1 μg/ml PAS) and microscope analysis. Prior to embedding the slides with Citifluor (Agar-Scientific, Stansted, UK), samples were incubated with DAPI for approximately 3 minutes.

4.5 Harvesting of *Naegleria clarki* and *Chlamydiales bacterium* Pn

4.5.1 Harvesting of Amoebae

Culture cells were detached from the culture flask surface by scraping, using a cell spatula. Resulting cells suspensions were poured into sterile reaction tubes, in sizes fitting experimental requirements. Amoebae were obtained by centrifugation at 10000g for 10 minutes at room temperature (RT). The supernatant was decanted and the cell pellet re-suspended in

either desired amounts of culture medium, or a fixative for downstream FISH analysis.

4.5.2 Harvesting of extracellular *Chlamydiales bacterium* Pn

Naegleria clarki cultures, containing >95% of cells infected with *Chlamydiales bacterium* Pn, were scraped with a cell spatula to bring symbiont cells into solution. Before harvesting, amoeba cells were left for 20 minutes to reattach to the culture flask surface. Then the supernatant was taken out and filtered through 1.2 μm into a 50ml Greiner tube to obtain a host-cell free symbiont cell suspension. This suspension was then further concentrated by centrifugation at 15550g for 15 minutes at RT and re-suspended in 1-2 ml of PAS medium. Storage of harvested symbionts was tested by storing them for 1-5 days either in PAS at RT, or 4^{o}C, or resuspended in 1ml SPG (SPG; 3 g*l^{-1} Saccharose (Sigma-Aldrich), 0.0208 g*l^{-1} KH2PO4 (Sigma, Lot. SLBT6311), 0.0612 g*l^{-1} Na2HPO4*2H20 (Sigma-Aldrich, S0876), 0.03 g*$^{-1}$ glutamic acid) at -80^{o}C, or in 10% DMSO (Sigma-Aldrich, 276855) at -80^{o}C; and was only recorded possible in PAS at RT or 4^{o}C for several hours, without loss of infectivity.

4.5.3 Harvesting of intracellular *Chlamydiales bacterium* Pn

Naegleria clarki cultures, containing >95% of cells infected with *chlamydiales bacterium* Pn, were scraped with a cell spatula to bring symbiont cells into solution. Before harvesting, Amoeba cells were left to reattach to the culture flask surface for about 20 minutes. Then the supernatant was discarded and replaced with PAS. Then the culture cells were scraped again, the cell suspension transferred into 50ml tubes and the cells harvested at 10000g for 10 minutes at RT. The resulting cell pellett was resuspended in 1ml PAS and transferred into a 2ml Lysing Matrix E tube and vortexed (at 26krpm) for 2 minutes. The resulting cell suspension was screened for sufficient lysis of host cells by transferring 5-10 μl of the suspension onto a microscopy slide, which is directly examined, using light-microscopy, and later, after drying of the suspension also by DAPI staining and analysis under an Epi-fluorescence microscope. The rest of the suspension was diluted to a volume of 10 ml and first filtered through a 5 μm syringe-filter, then through a 1.2 μm syringe-filter. This suspension was further concentrated again by centrifugation at 15550g for 15 minutes at RT and re-suspended in 1 ml of PAS medium. Storage of harvested symbionts was tested as described above (section 4.5.2) and was also only possible at RT or 4^{o}C for several hours, without loss of infectivity.

4.6 *Acanthamoeba* cultivation

Acanthamoeba castellanii NEFF with or without *Protochlamydia amoebophila* UWE25, or *Chlamydiales bacterium* Pn (Table 4) 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^{o}C. Cultures were maintained in cell culture flasks (Nunclon delta-surface, Thermoscientific, St Leon Rot, Germany), the medium was renewed around every seven days by removing the supernatant and adding 10ml of TSY. Parallel to culturing in TSY, NEFF cultures without endosymbionts, or containing *Chlamydiales bacterium* Pn, were also cultivated in Pages amoebic saline (PAS; 120mg*l^{-1} NaCl, 4mg*l^{-1} MgSO*7H2O, 4mg CaCl2*2H2O, 142mg*l^{-1}

Na2HPO4, 136mg*l^{-1} KH2PO4). Cultures were also 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 10ml or 50ml of PAS, depending on culture flask size. Additionally, cultures were fed an *E. coli* tolC^{-} strain (Table 4), together with an ampicillin addition of 5 μg/ml, every three to four days (from here on together referred to as the "*E. coli* supplement"). Cultures were regularly screened by fluorescence *in situ* hybridization and DAPI staining (1 μg/ml) to exclude contamination.

4.7 *Naegleria* cultivation

Symbiont-free *Naegleria clarki* (CCAP 1518/14) (Table 4) were obtained from the Culture Collection of Algae and Protozoa (CCAP; Ambleside, United Kingdom) in modified changs medium II [MCII, 10 g*l^{-1} Casein digest (Casitone, cat. No 225930), 1.325g*l^{-1} Na_2HPO_4*2H_2O (Sigma-Aldrich, S0876), 0.8g*l^{-1} KH_2PO4 (Sigma, Lot. SLBT6311), 5g Yeast extract (Oxoid LP0021), 2.5g*l^{-1} D-glucose (Roth, cat. No X997.2), 100ml*l^{-1} Sterile foetal calf serum (Gamma-Irradiated, added aseptically after serilization)], which was replaced by fresh MCII medium every 3-4 days (Culture from hereon referred to as '*N. clarki* CCAP'). Later, this culture was inoculated in PAS. The symbiont-free *Naegleria clarki* strain N-DMLG0 (Table 4) was first cultured by the non nutrient agar (NNA) plate culture method according to Page (1988). Briefly, samples were inoculated on NNA plates, coated with an *E. coli* tolC^{-} (Table 4) lawn, and incubated at 30^{o}C. Using a sterile razor blade, patches of Agar containing trophozoites, identified using phase-contrast microscopy, were cut out and transferred into culture flasks, containing liquid PAS. The strain *Naegleria clarki* containing *Chlamydiales bacterium* Pn (*N. clarki*_PN) was taken from an existing lab stock (Table 4) and has been kept in PAS. Media of all PAS grown cultures were renewed at least every seven days by replacing the supernatant with fresh PAS, supplemented with an *E. coli* tolC^{-} strain (Table 4), together with an ampicillin addition of 5 μg/ml. All *Naegleria* cultures were also cultivated in a serum-casein- glucose-yeast extract medium [SCGYEM, 1.325g*l^{-1} Na_2HPO_4*2H_2O (Sigma-Aldrich, S0876), 5g*l-1 KH_2PO4, Yeast extract (Oxoid, LP0021), 2,5ml NaOH (1M), 2.5g*l^{-1} D-glucose, 100ml*l^{-1} Sterile foetal calf serum (Gamma-Irradiated, added aseptically after serilization)], which was renewed every 7 days with fresh SCGYEM (see: section 4.9). Cultures were regularly screened by fluorescence *in situ* hybridization and DAPI staining (1 μg/ml) to exclude contamination.

4.8 Preparation of *E. coli* tolC^{-} as supplement for monoxenic cultures

An *E. coli* tolC^{-} (Table 4) aliquot from a stock, frozen in liquid Nitrogen was thawed and grown on a Lysogeny Broth-Agar plate (LB-Agar, 10 gl^{-1} Peptone (Roth, Cat. No 2365.3), 5 gl^{-1} Yeast extract (Oxoid, LP0021), 5 gl^{-1} NaCl (Roth, Cat. No 3957.2), 15 gl^{-1} Agar (Sigma-Aldrich, Lot. BCBW0385)) by incubation over night at 37^{o}C. From this plate single colonies were transferred into liquid LB medium (LB, 10 gl^{-1} Peptone (Roth, Cat. No 2365.3), 5 gl^{-1} Yeast extract (Oxoid, LP0021), 5 gl^{-1} NaCl (Roth, Cat. No 3957.2)) and again incubated over night at 37^{o}C. Then the cells were harvested by centrifugation at 10000g

for 10 minutes at RT and resuspended in amounts of PAS to reach an OD_{600} of 30. Cells were stored at 4^{o}C until use.

4.8.1 Heat-inactivation of *E. coli* tolC$^-$

After harvesting and concentrating *E. coli* tolC$^-$ (Table 4) cells to an OD_{600} of 30, aliquots of 1 ml were transferred into 1.5 ml reaction tubes. The tubes were then placed into a heat block, pre-heated to 80^{o}C, at 159 rpm (rotations per minute) for 15 minutes. Cells were stored at 4^{o}C until use.

4.9 Selection for growth in axenic SCGYE-medium

Uninfected *N. clarki* N-DMLG0 and CCAP 1518/14 (Table 4) culture cells in PAS were detached and 2 ml of unquantified culture were transferred 4 times per culture into new 25 cm^2 cell culture flasks, prefilled with 8 ml SCGYEM, that was supplemented with 50 μl of heat-inactivated *E. coli* (Table 4). After 4-5 days of incubation at 30^{o}C, the cultures were scraped again to detach cells and, in duplicates, 100 μl per replicate for each culture were placed into 2 wells of a 24-well plate, that contained 900 μl of SCGYEM, without any *E. coli* supplement. This well-plate was incubated for 3 days at 30^{o}C. The increase in amoeba numbers over 3 days ('Amoeba gowth') inside the well plates was determined by counting amoeba in 10 random sight fields in each of the wells under a phase-contrast microscope at 40x magnification on days 0 and 3 and calculating the difference, shown in equation 1, where N3 is the cell count at time t (3 days) and N0 is the initial cell count of t0.

$$Amoeba\ growth = \frac{ln\frac{N3}{N0}}{t} \tag{1}$$

From these calculations, the 2 best *N. clarki* N-DMLG0 and the 2 best *N. clarki* CCAP replicates, in terms of growth, were selected and used to start 2 new 25 cm^2 cell culture flask replicates in SCGYEM + 50 μl of heat-inactivated *E. coli*, while discarding the other 2 along with the 24-well plate, leaving again 4 replicates per culture. The flask incubation for 5 days, followed by growth assessment and subsequent duplication of the best growing replicates was repeated for each culture at least until amoeba growth was >1.
For the infected *N. clarki* culture N-DMLG-Pn (Table 4), adaptation to the axenic medium was established by harvesting infected amoebae from two 25 cm^2 cell culture flasks and transferring the harvested cells into fresh 25 cm^2 cell culture flasks, containing liquid SCGYE-medium, supplemented with heat-inactivated *E. coli*. This method of cultivation was maintained for approx. 5 weeks, while gradually decreasing the amounts of heat-killed *E. coli* from an OD_{600} of 0.15 in the beginning, to an OD_{600} of 0.06 after 4 weeks and entirely dropping the supplement after 5 weeks of incubation, when the culture cells could be stably maintained in the nutrient medium alone.

4.10 Determination of cell concentrations

4.10.1 Determination of amoeba cell concentration

Culture cells were detached from the culture flask surface by scraping, using a cell spatula. 7 μl of the cell suspension were transferred to a cell counting chamber. The number of amoeba cells per ml was determined by counting amoeba in at least 4 of the ten small grids within the chamber. The total count was divided by four (or the number of counted, small grids) and multiplied with 10^4 .

4.10.2 Determination of bacterial cell numbers

Extracellular or intracellular *chlamydiales bacterium* Pn cells were harvested as described above (sections 4.5.3 and 4.5.2). 10-100 μl of the resulting cell suspension were fixed for 20 minutes at RT, or 2 hours at 4^oC in 4% formaldehyde (Rotipuran, Cat. No 4979.1) solution. After fixation the solution was diluted in 10 ml of PAS and collected on a polycarbonate filter, using a vacuum-filter-tower. Filters were subsequently incubated with 300-400 μl of DAPI (10 μg/ml) for 5 minutes. The average number of bacteria was determined by counting 10 random spots, using an epifluorescence microscope at 100x maginifaction (Equation 2).

$$cells/\mu l = \frac{15126 * number\ of\ cells}{counted\ spots * initial\ volume} \tag{2}$$

4.11 Infection experiments

Extracellular or intracellular *chlamydiales bacterium* Pn cells were freshly purified from amoeba cultures grown in 175 cm^2 cell culture flasks as already described above (sections 4.5.3 and 4.5.2). Symbiont-free amoebae were harvested as described above (section 4.5.1) and 10^5 cells per ml were seeded into individual wells of a well plate, or into 25 cm^2 cell culture flasks, at least one day prior to infection. This was followed the next day by the addition of harvested symbiont cells, using either a multiplicity of infection (MOI) suitable to the individual infection experiment, or unknown amounts of harvested cells. The final volume inside wells was reached by addition of the growth medium SCGYEM, or PAS (PAS supplemented with *E. coli* $tolC^-$ and 5μg/ml Ampicillin). Well plates were subsequently centrifuged at 1500 g for 15 min at RT. If not stated otherwise, infections were synchronized by taking off the culture supernatant 2 hours post infection (h.p.i.), washing twice with PAS and then adding fresh culture medium. Termination of each infection experiment was done at varying time points, fitting the individual experiments. All infection and control experiments were performed in biological duplicates. Efficiency of infections was assessed by FISH and DAPI staining. Infected cells were distinguished into 2 broad categories. Amoeba containing one or more, single Pn cells in the cyto- or nucleoplasm, that appear to not have started replication yet, were considered to be of 'low' infection. Amoebae containing many intranuclear Pn cells, that appear to be almost, or entirely colonizing the host's nucleolus, were considered to be of 'high' infection.

4.12 Induction of the flagellate stage in *Naegleria clarki*

The ability of *Naegleria clarki* cells, uninfected or infected with *chlamydiales bacterium* Pn, to transform into flagellates was tested in the standard procedure with slight derivations according to [34]. This was done by incubating *Naegleria* cells in 4 biological replicates and equal cell densities on separate NNA plates, containing an *E. coli* tolC- (table 4) lawn. Plates were incubated either on 20 or 30^{o}C. When plates reached complete colonization with *Naegleria* cells, they were washed thoroughly with 2ml Tris buffer (2mM TRIS-hydrochlorid (Roth, Cat. No 9090.2) in ddH_2O, pH 7.5). Cell suspensions were then transferred into 2 ml reaction tubes and centrifuged two times, once at 5000g, once at 2500g for 10 minutes each, at RT, resuspending in 1ml Tris buffer every time. Amoeba cell density was determined, as described above (section 4.10.1) and Amoeba were again seeded in equal densities in 25 cm^2, filled with 5-10 ml Tris buffer and incubated on 30 ^{o}C. Transformation of *Naegleria* cells during the experiment was measured by scraping cells from the flask surfaces at varying timepoints and counting only trophozoites in a cell counting chamber. For determination of the end- trophozoite and flagellated cell numbers, the supernatant of each flask was collected (harbouring mostly flagellated cells), then flasks were refilled to their initial volume, using Tris buffer, trophozoite cells were then scraped from the flasks surfaces and the supernatant again collected from each flask. These cell suspensions were then centrifuged at 10000g, for 10 minutes at RT, resuspended in 100μl PAS and mixed with formaldehyde to a final concentration of 3% fixative and incubated for 2 hours at 4^{o}C. After fixation, cells were harvested again by centrifugation at 10000g, for 10 minutes at RT and resuspended in 250 μl of phosphate buffered saline (PBS; 40g*l^{-1} NaCl (Roth, cat. No 3957.2), 9g*l^{-1} Na_2HPO_4*$2H_2O$ (ROTH, cat. No 4984.1), 1.2g*l^{-1} KH_2PO_4 (Sigma, Lot. SLBT6311), 1g KCl (Millipore, CAS-No 7447-40-7)), then mixed with equal amounts of 100% ethanol and stored at -20^{o}C. Quantification of fixed trophozoite and flagellated cells was done in cell counting chambers, cells were later used for FISH analysis.

4.13 Comparison of growth between infected and uninfected *Naegleria clarki* cultures

Growth comparison between *Naegleria clarki* cells uninfected or infected with *chlamydiales bacterium* Pn was tested under axenic (SCGYE-medium) and monoxenic (medium PAS + *E. coli* tolC$^-$) conditions, both on agar-plates and in liquid cultures. Liquid infected and uninfected cultures were inoculated in 4 biological replicates in a 12- well plate with 10^4 cells per ml at 20 and 30^{o}C. Relative cell density was determined after differing timepoints (depending on growth medium and growth speed of the cultures), by counting 10 random sight fields per replicate under a 40x phase-contrast microscope, or by scraping cells from the flasks surface and counting amoeba cells in a counting chamber. Plate cultures were also inoculated in 4 biological replicates, seeded with 10^5 cells per plate and inoculated at 20 and 30^{o}C. Cell density was determined and compared 2-3 days later. Trophozoites were washed in excatly the same fashion thoroughly from each plate, using 2ml PAS per plate, and counting amoeba cells after collecting cell suspensions in 2ml reaction tubes. The percentage of infected cells of each culture was determined before and after the experiment by FISH. Analysis of the data was done in Rstudio [70], using packages ggplot2 and data.table.

Normality of retrieved data was assessed visually via P-P plot (probability-probability plot) and Q-Q plot (quantile-quantile plot) and tested through the Shapiro-Wilk test, as recommendet by *Ghasemi* & *Zahediasl* (2012) [35], using R packages dplyr and ggpubr.

4.14 Quantification of cell and nucleus sizes

Previously FISH (probes: NAEG1088, EUB338I-III and PN440) and DAPI stained samples from continuously Pn infected *N. clarki*, asynchronous cultures were captured by confocal laser scanning microscopy and analysed with the image analysis tool LAS X Core by Leica. At least 100 infected and 100 uninfected amoebae were measured in terms of nuclear diameter and overall cell size (measured along 2 axis, Figure 5). Normality of retrieved data was assessed visually via P-P plot (probability-probability plot) and Q-Q plot (quantile-quantile plot) and tested through the Shapiro-Wilk test, as recommended by *Ghasemi* & *Zahediasl* (2012) [35], using R packages dplyr and ggpubr.

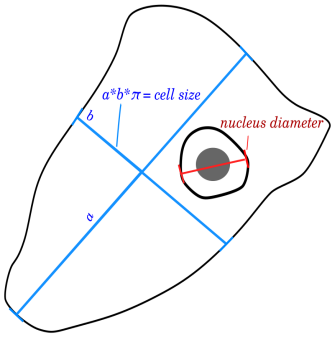

Figure 5. Schematics of a *Naegleria* cell. Nucleus diameter was determined, using fixed and DAPI stained amoebae. Cell size was crudely determined by calculating an ellipse from the long (a) and short (b) axis, that can usually be determined in *Naegleria* amoebae.

4.15 Temperature curing of a symbiotic *N. clarki* culture

The curing experiment was conducted by transferring 1 ml of unquantified, already axenically growing, infected cultures, described in section 5.5.1, into new 25 cm^2 culture flasks, containing 9 ml of SCGYE-medium and incubating them at 30^{o}C. The amount of infected cells per culture was monitored weekly for the first three weeks, the last time-point was measured ten days after that, using FISH. For the first two weeks of incubation, the culture medium was replaced once per week, at which point samples were also taken for FISH analysis. After the second week, low amounts (100 μl of uncounted amoeba suspension) of each culture were taken and split weekly into 3 wells of a 6-well plate. When no infected amoeba could be detected via FISH, the cultures were maintained either in SCGYEM, or monoxenic PAS medium.

5 Results

5.1 Examination of *Chlamydiales* bacterium Pn infection in its *Naegleria clarki* host

5.1.1 Stages of the Pn infection cycle

Monitoring of the infection of *Acanthamoeba castellanii* (done in a previous study [83]) and *Naegleria clarki* using FISH, showed that Pn cells are taken up individually by phagocytosis and only start replication once they reach their intranuclear destination. As early as 2 hours post infection (hpi) Pn is not only detectable in the cytoplasm, but half of all infected cells already show bacteria located inside the nucleus (Figure 6, 2 hpi). Following invasion of the nucleus, the symbionts start replicating within the first 24h, yet undergo only few replications within that time. At this point around four to several more bacteria were present per nucleus, presumably depending on the number of bacterial cells, that initially entered the nucleus (Figure 6, 24 hpi). A clearly visible localization of Pn around the hosts' nucleolus could be found 48 hpi where Pn cells already start to leave the intranuclear compartment again (Figure 6, 48 hpi, arrows), marking the completion of the Pn developmental cycle. Succeeding the end of this cycle, Pn cells start not only to infect new amoeba cells, but also to have an enlarging effect on the hosts' nucleus, visible at 72 hpi (Figure 6, 72 hpi). The increase in host nucleus sizes can be seen as the transition to a chronically infected culture, where average infected nucleus volume exceeds uninfected nucleus volume by around 14%. Up until this point horizontal transmission is only achieved by individually exocytosed bacterial cells, that are taken up by uninfected amoebae nearby. Lysis of host cells, caused by the intranuclear symbiont, only occurs occasionally at an as of yet unknown, later timepoint.

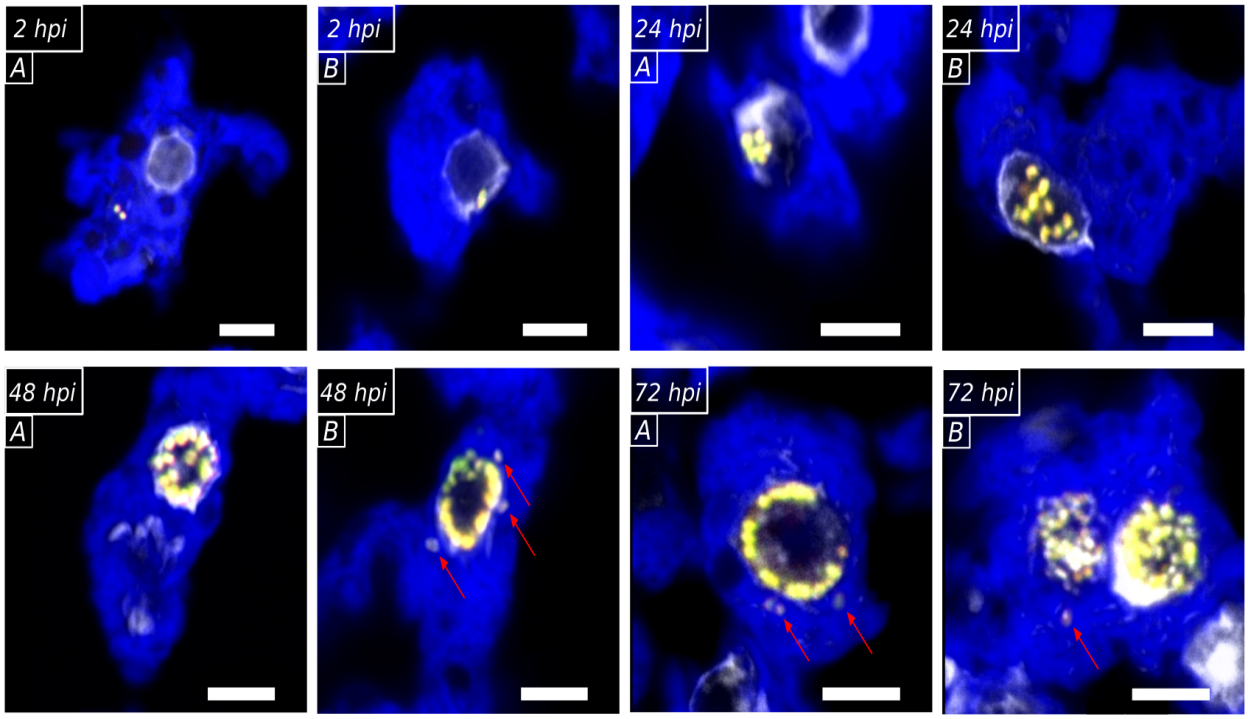

Figure 6. Infection cycle of *Chlamydiales* bacterium Pn in its *N. clarki* host. Infection was monitored over a course of 72 hours. Visualization was done via FISH, using probes NAEG1088 (**blue**), EUB338I-III (**green**) and PN440 (**red**); cells were additionally stained with DAPI (**grey**). Pn bacteria enter amoeba cells individually (**2 hpi A**) and are able to reach the nucleus (**2 hpi B**) within 2 hours post infection (hpi); within 24 hpi they undergo only a few replication cycles (depending on how many cells entered the nucleus at 2 hpi, more (**24 hpi A**) or less (**2 hpi B**) cells can be seen inside the nucleus at this stage); at 48 hpi the surface of the nucleolus is usually entirely covered with bacteria (**48 hpi A**) and cells already start to leave the intranuclear compartment ((**48 hpi B**), **arrows**), marking the end of the infection cycle; 72 hpi the sizes of host cell nuclei start to increase (**72 hpi A**), exocytosis of Pn happens already regularly ((**72 hpi A and B, arrows**) and newly infected cells can already be seen (not shown). Visualization was done, using confocal laser scanning microscopy. Bars indicate 5μm.

5.1.2 Pn infection progression in a *Naegleria* culture

Two *N. clarki* cultures were monitored from 2 hours up to 9 days after infection with *Chlamydiales* bacterium Pn to determine both speed of dispersal and morphological changes within the infected cultures, during the first few replication cycles of Pn. To achieve this, two *N. clarki* cultures were directly infected in 25 cm^2 cell culture flasks with an approximate MOI of 260 [3]. A steep drop in infected cells was known to occur within the first 24 hours of infection (see section 5.3). Therefore, the stringency of infection synchronization[4] at 2 h.p.i. was reduced to only carefully taking off the supernatant and replacing it with fresh medium. This low-stringency washing produced a slightly asynchronous infection, leading to around 27% of cells containing a 'low' (definition according to section 4.11) number of symbionts at

[3]Note, MOIs used for infections in cell culture flasks are not comparable to ones used in 12-, or 24- well plates, as the flask surface to volume ratio differs in a way, making relatively greater surface area available in culture flasks, than in well plates. Thus, making MOIs above 100 possible in culture flasks, without the severe effects, described in section 5.3.

[4]Washing residual infectious particles off the culture cells, to prevent further infections.

2 days p.i.. The initial number of infected cells in both cultures started out at around 80% of cells being low infected (Figure 7). The aforementioned drop in infected cell numbers at 48 h.p.i. left only about 10% of infected host cells with actively dividing, intranuclear Pn populations, defined as 'high' (definition according to section 4.11) infected cells (Figure 7). After completion of the first developmental cycle of Pn within the first 48 hours, the number of infected amoebae rose rapidly until day 5 to around 60% of host cells containing a 'high' infection and around 25% containing a 'low' infection (Figure 7). Within the next 4 days (until 9 days p.i.), the number of amoeba showing a 'high' infection reached 100%, leaving no visible new infections (Figure 7).

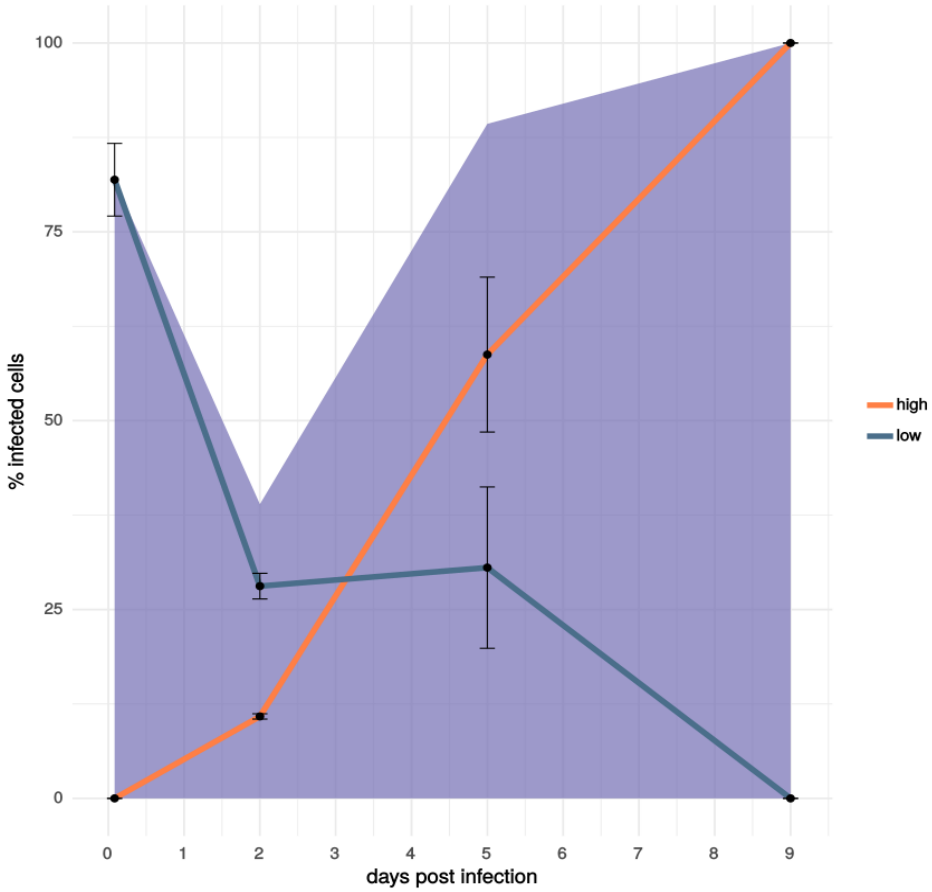

Figure 7. Progression of a *Chlamydiales* bacterium Pn infection in a *N. clarki* culture over the course of 9 days. Purple area indicates the total number of infected amoeba. Percentage of amoeba showing a fresh, or 'low' infection (only few, single Pn cells inside the cytoplasm, or nucleus) is indicated by the darkblue line. Percentage of amoeba showing an established, or 'high' infection (nucleus containing actively replicating Pn cells, most often already located around the nucleolus), is indicated by the yellow line. Errorbars show the standard deviation from the means of two replicates.

5.1.3 Effects of Pn infection on host- nucleus and cell sizes

To obtain more insight into the effect infection with *Chlamydiales* bacterium Pn has on the *Naegleria* host, two parameters were looked into more closely. For one, since Pn colonizes the nucleus, or more specifically, localizes around its hosts' nucleolus [53], the impact on host nucleus sizes was investigated. Graphically depicted in Figure 8, are the mean nucleus sizes of either infected, or uninfected host cells. 100 individuals of infected and uninfected amoebae were measured, as described in section 4.14. Nucleus sizes of infected host cells are on average 4.38 μm $\pm$0.78 in diameter, significantly exceeding those of uninfected ones, with an average size of 3.76 μm $\pm$0.46, by 0.6 μm (determined by a student's t-test, p=2.537e-10).

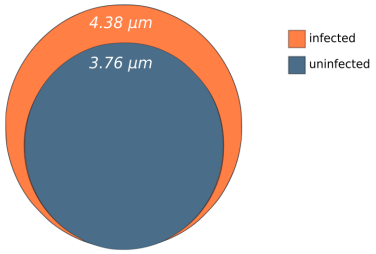

Figure 8. Graphical depiction of average symbiotic, or aposymbiotic *N. clarki* nucleus sizes. 100 individuals' nuclei of either uninfected amoeba, or amoeba chronically infected with *Chlamydiales* bacterium Pn, were measured post fixation in 4% formaldehyde. Analysis was done on previously aquired FISH- and DAPI- stained images, using the LAS X Core image analysis software by Leica. Statistical significance between infected and uninfected nucleus sizes was determined by a student's t-test (p = 2.537e-10).

The same is not true for the impact of infection with Pn on host cells sizes. Using a similar principle of analysing 100 infected and 100 uninfected host cells (see section 4.14), that had been previously fixed and FISH stained, were assessed two-dimensionally on microscopy slides. This showed, that the average infected host cell (ellipse size about 456 μm^2) does not statistically differ from the average uninfected host cell (ellipse size about 448 μm^2), as assessed by a Mann-Whitney-Wilcoxon test (p= 0.5634).

On occasion, individual cells of an infected host population show extremely enlarged nuclei of up to 10 μm, filled entirely with symbiont cells. This phenotye starts to occurr within infected *N. clarki* populations around 9 days p.i., as shown in Figure 9, and continues to occurr with rising frequency over longer periods of incubation time, but usually not affecting more than 3% of the infected host population.

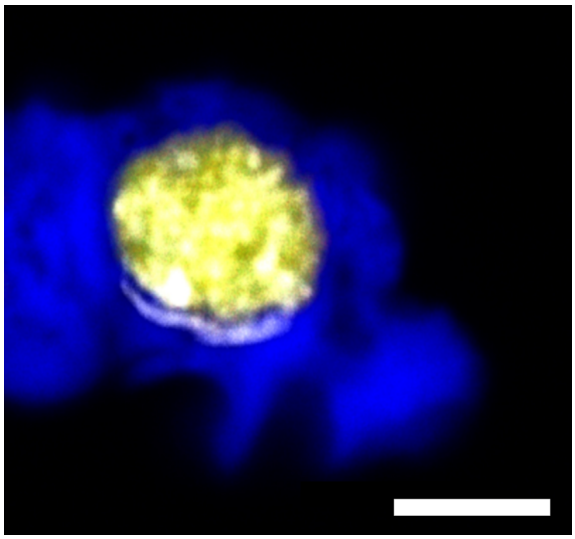

Figure 9. *N. clarki* amoeba with an enlarged nucleus (6.5 μm), filled completely with symbiont cells, 9 days p.i.. Visualization was done via FISH, using probes NAEG1088 (blue), EUB338I-III (green) and PN440 (red); cell was additionally stained with DAPI (grey). Visualization was done, using confocal laser scanning microscopy. Scale bar indicates 5 μm.

5.1.4 Pn in the *Naegleria clarki* lifecycle

The life-cycle stages of *N. clarki*, especially the cyst and flagellate stages, are able to contribute to dispersal and long-term survival under inept environmental conditions of not only the amoeboflagellate itself, but also of any endosymbiont populations, that are able to occupy these stages [72]. Therefore, involvement and possible effects *Chlamydiales* bacterium Pn might have on the life-cycle stages of its host were investigated. Both the trophozoite and the cyst stage are naturally found in monoxenically grown *N. clarki* cultures. Hence, in order to visualize Pn infection within those two host-lifecycle stages, continuously infected cultures were investigted via FISH, using two different fixation methods. For trophozoites fixation could be done by a short incubation period, leaving amoeba to attach to microscopy slide surfaces, followed by a short, on-slide fixation (described in detail in section 4.4.1). For cyst stage fixation, a method standard for bacteria, not relying on cell attachment to microscopy slide surfaces, was used (described in section 4.4.2). Figure 10 shows Pn cells still located inside the host cell, around the hosts' nucleolus, after host encystation. Figure 11 shows the location typical for Pn, around the hosts' nucleolus. However, as already described in a previous short study [83], the nuclear infection load has increased with prolonged coculturing periods. Infected cultures originally showing only few individual cells on host nucleolus surfaces, now have increased to a complete covering of the nucleolus surface as a new, standardly seen, stable phenotype. For infected amoebae, showing this newer phenotype (shown in Figure 11), a calculation (3), based on Newton's kissing problem[5] (solved by Schuette and van der Waerden in 1953 [82]), yielded around 195 cells of Pn being able to surround the average *N. clarki* nucleolus at the same time, assuming a nucleolus diameter of 3 μm (recorded nucleolus diameter, measured by Pn circle size, in this study, ranged between 3 and 4 μm) and a diameter of 500 nm for Pn. When assuming a nucleolus diameter of 4 μm already 323 cells could fit on the nucleolus surface.

[5]Equation kindly provided by Emanuel Schwarzhans.
R ... Radius of the nucleolus
r ... radius of the bacterium

$$bacteria\ per\ nucleolus = \frac{2}{1 - cos[asin(\frac{1}{\frac{R}{r}+1}]} \quad (3)$$

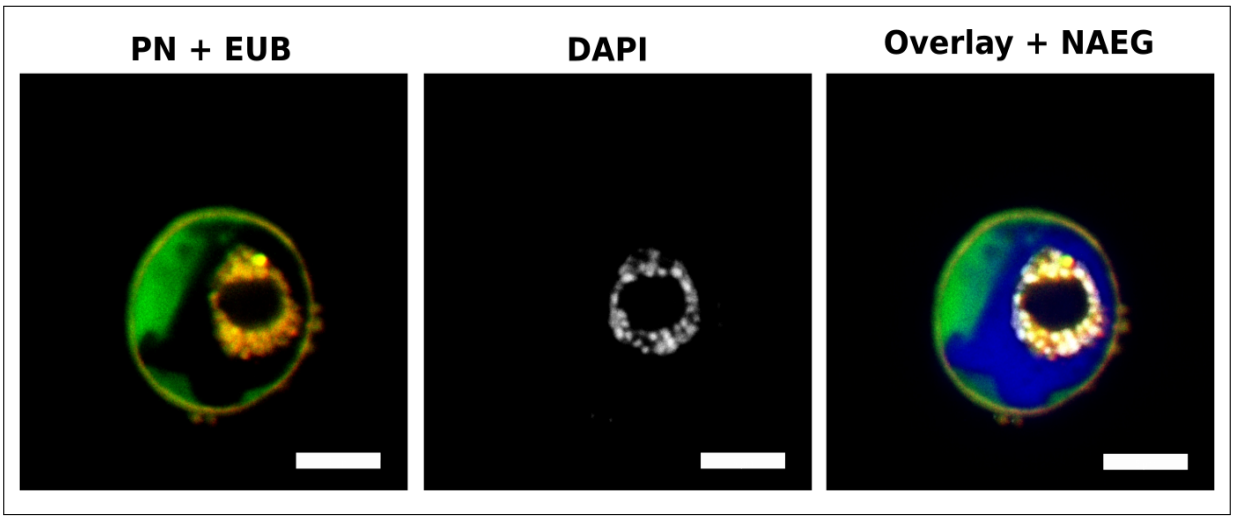

Figure 10. ***N. clarki*** **cyst, infected with Pn.** A host culture infected with *Chlamydiales* bacterium Pn was fixed, using the fixation method described in section 4.4.2 and visualized via FISH (Probes: **NAEG**1088 (blue), **EUB**338I-III (green) and **PN**440 (red)) and **DAPI** (grey) staining. This fixation method enabled the capture of non attached cyst and flagellate stages. Images were obtained, using confocal laser scanning microscopy. Scale bars indicate 5 μm.

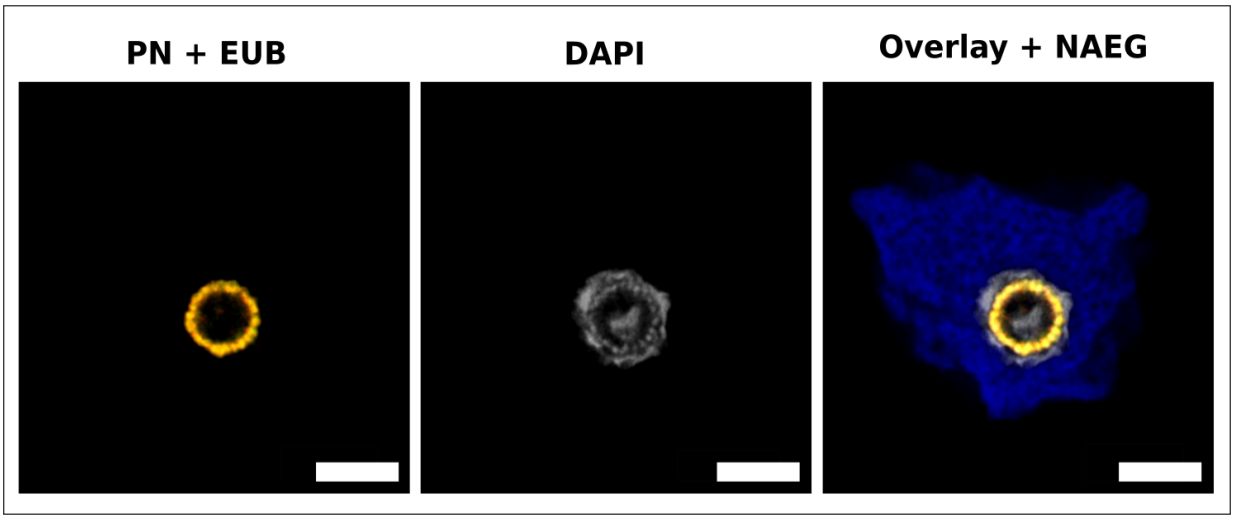

Figure 11. ***N. clarki*** **trophozoite, infected with Pn.** A host culture infected with *Chlamydiales* bacterium Pn was visualized using FISH. Bacteria can be seen in orange (overlap of **EUB**338I-III (green) and **PN**440 (red)). **DAPI** (grey) staining was used to visualize the amoebal nucleus and also to stain bacteria. The host was visualized in blue (**NAEG**1088). Images were obtained, using confocal laser scanning microscopy. Scale bars indicate 5 μm.

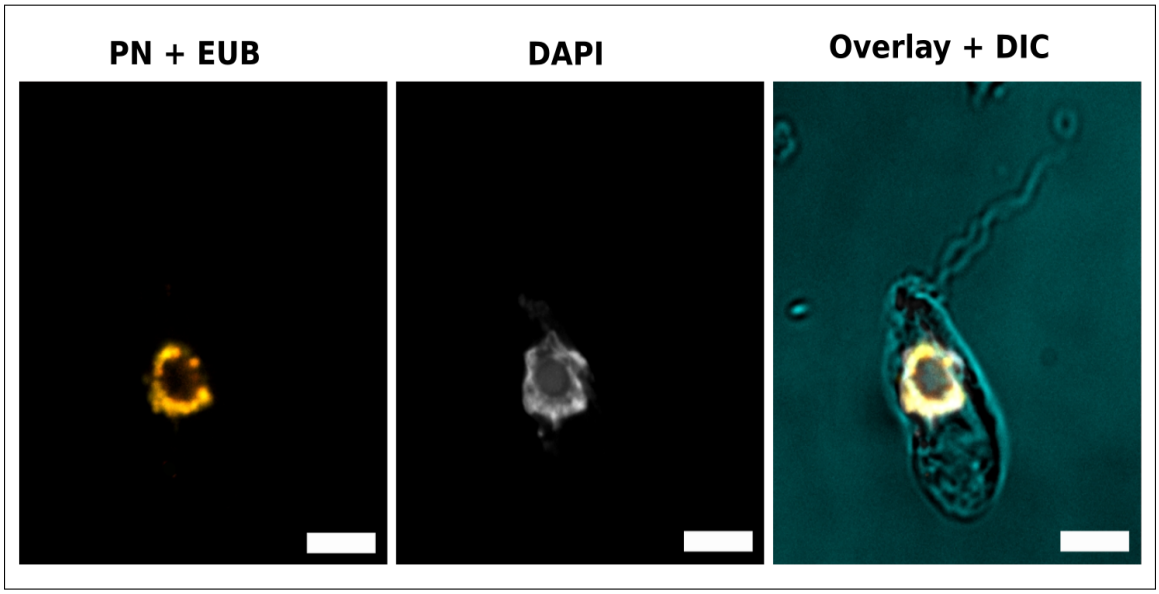

Figure 12. ***N. clarki*** **flagellate, infected with Pn.** A *Chlamydiales* bacterium Pn infected host population was used to isolate flagellates for visulaization via FISH, as described in section 4.12 and fixed according to section 4.4.2 to enable the capture of nonattached cells. Bacteria cells are visible in orange (overlap of **EUB**338I-III (green) and **PN**440 (red)) and in grey via **DAPI** staining, also highlighting the amoebal nucleus. To visualize flagella, differential inteference contrast (**DIC**) was used (cyan). Images were obtained, using confocal laser scanning microscopy. Scale bars indicate 5 μm.

Following a *Naegleria* transformation protocol based on Fulton and Dingle (1967) [34], described in section 4.12, Pn infected host cultures were first transformed and then fixed for downstream FISH analysis. Fixation of transformed cultures was done, utilizing the same fixation method used for fixation of cysts, described in section 4.4.2. Figure 12 shows one of these Pn infected, transformed host cells. In addition to verifying the hosts ability to transform into the flagellate stage, while maintaining infection with Pn, a quantification of transformation efficiency was attempted, comparing infected with cured (2019) host populations. This was done, using 4 replicates of freshly transformed infected and uninfected host populations and separately collecting fractions of unattached and attached cells of each replicate (described in detail in section 4.12). After fixation of collected samples (as described above for flagellates), cell concentration of each fraction was determined. The collected data, shown in Figure 13, did not reveal a statistically significant difference between naïve and infected amoeba. Yet, the

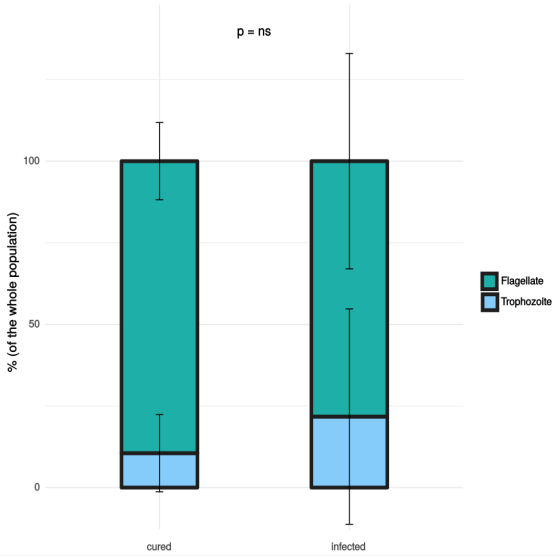

Figure 13. Mean difference in flagellate transformation efficiency of infected and cured (2019) ***N. clarki*** **cultures.** *N. clarki* cultures were transformed, as described in section 4.12 and quantified. Within each replicate, values stemming from flagellate and trophozoite fractions, were separately compared to the total cell volume of each sample, to obtain fractions of 100. Errorbars represent the standard deviation from the means of 4 replicates. Statistical analysis between infected and cured populations was done, using a student's t-test (ns, not significant).

variation of flagellate numbers between replicates increased notably in the presence of the symbiont.

5.2 Modes of Pn transmission

5.2.1 Release of Pn from the host cell

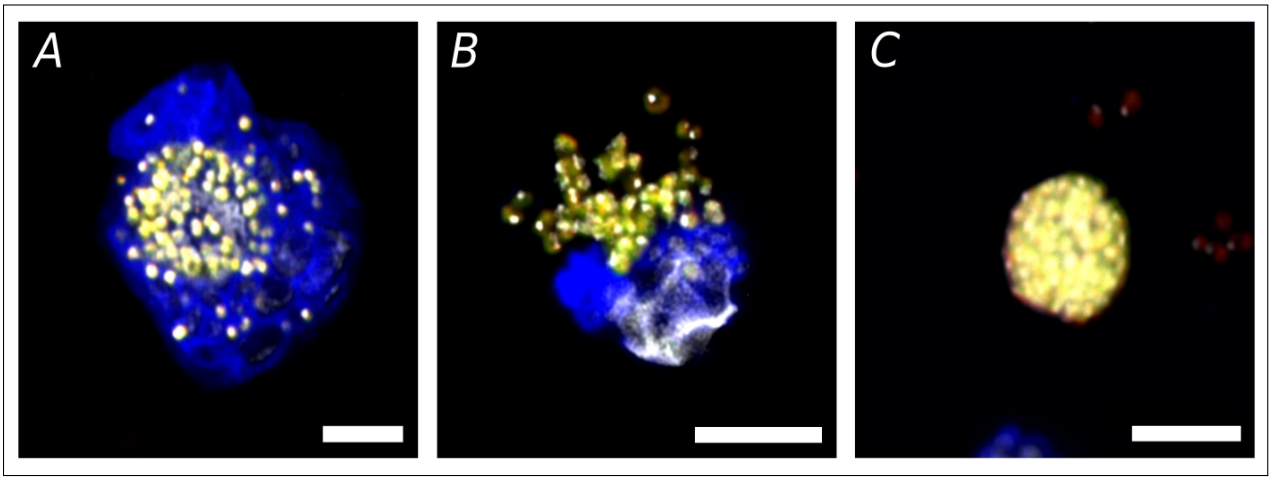

Figure 14. Exit of Pn from the host cell, either by exocytosis, or lysis. *Chlamydiales* bacterium Pn cells have been visualized during exit of their *N. clarki* host cell, either exiting via exocytosis **(A)**, or lysis **(B)** of the host, using FISH (Probes: NAEG1088 (blue), EUB338I-III (green), PN440 (red)) and DAPI (grey) staining. Occasionally host cell lysis happens without nuclear lysis (**C**). Visualization was done, using confocal laser scanning microscopy. Scale bars indicate 5 μm.

Infectious Pn particles have been observed, using FISH, to follow two different strategies of host exit. One being by lysis of the host cell, the other being by continuous release via exocytosis, without lysis of the host cell (Figure 14, A and B). Host cell lysis may also happen while the nuclear membrane stays intact (Figure 14, C), which was previously also described for the intranuclear amoeba symbiont *Ca. Nucleicultrix amoebiphila* [80].
Released particles stay infective only for a few hours after release, without a decrease in infectivity. Attempts to store isolates of Pn particles showed a complete loss of infectivity, when stored in SPG, or DMSO at -80^{o}C already for periods of 1-5 days.

5.2.2 Vertical transmission of Pn

Chlamydiales bacterium Pn in its original *N. clarki* host is transmitted to daughter cells during host cell division. It is able to remain on nucleolar surfaces throughout the entire process of the closed karyokinesis of its host *N. clarki*, without passage through, or contact with the hosts' cytoplasm. This was found during investigations of asynchronous *N. clarki* cultures, chronically infected with Pn, using FISH. Cell cycle stages were crudely determined post *in situ* hybridization on captured images, according to characteristic features of dividing

Naegleria nuclei, described by Charles Walsh (2012) [95]. Figure 15 (pre nuclear division) shows an example of an infected host cell during metaphase. Condensed host chromosomal DNA locates in the gap forming between the two separating nucleoli prior to chromosome segregation, during which Pn usually stays located on the nucleolar surface. Colonization of the extending gap between the dividing nucleoli has also been recorded (Figure A.6). As host cells progress through anaphase and teplophase (Figure 15, nuclear division), the two nucleolar masses, still entangled, elongate, while the host chromosomal DNA is already almost, or entirely separated and at the two poles of the dividing nuclei. Pn still localizes on the nucleolar surfaces at this time and is likely being passively distributed amongst the two newly forming nuclei. After nuclear division (Figure 15, post nuclear division) the two daughter nuclei, still located in the same host cell, both contain populations of Pn.

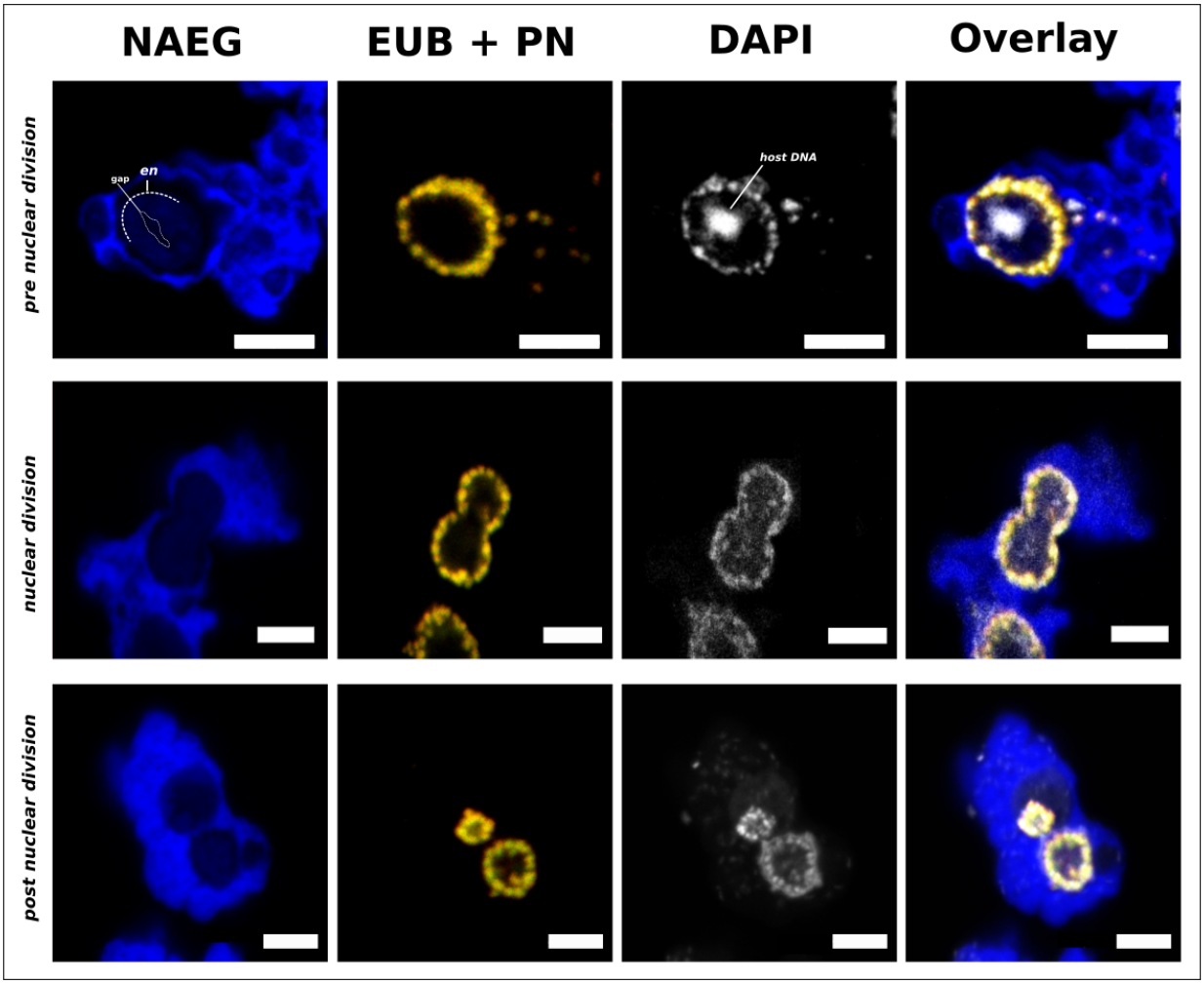

Figure 15. Vertical transmission of Pn in the *Naegleria* nuclear division, visualized using FISH and **DAPI** (grey) staining. Pn infected *N. clarki* cells were captured pre-, mid- and post- nuclear division, preceding cell division. The *Naegleria* nucleus and nucleolus (**en**) do not undergo disintegration during cell division [95]. During metaphase (**pre nuclear division**), the nucleolus forms a **gap**, in which the condesed host chromosomes locate (**host DNA**). Then nucleus and nucleolus elongate, while host chromosomes are separated during ana- and telophase (**nuclear division**). Finally both nuclei and nucleoli separate (**post nuclear division**) and cytokinesis starts. Pn cells, visible in orange (Probes: **EUB**338I-III (green) and **PN**440 (red))), localise on the nucleolar surface during the entire process of nuclear division, dispersing to both daughter nuclei of the *Naegleria* (**NAEG**1088 (blue)) host cell. Visualization was done, using confocal laser scanning microscopy. Scale bars indicate 5 μm.

5.3 Exploring effects of different multiplicities of infection (MOI) on infection success and host fitness

To investigate the effect of different MOIs on the number of host cells that remain infected (contain an established intranuclear infection) after 24-48 h.p.i., several experiments were conducted, using MOIs ranging from 5 to 75 (Figure 16). MOIs of up to 30 generally lead to at most 1% of established infections in host cells after 48 hours, eventhough initial numbers after 2 hours show up to 28% infected cells. Numbers of established infections after 48 hours start to rise at an MOI of 50, ranging between 1 and 10 % and increase to 11-18 % at an MOI of 75 (Figure 16). Next the yield of infected cells (percentage of established infections at 48 hpi stemming from initally infected cells at 2hpi) across different MOIs was considered. This showed that MOIs up to 30 yield no greater than 5% of established infections. At MOI 50 this yield rises to around 10% and already reaches 20% at an MOI of 75. MOIs greater than 75 however lead to massively lysing and or severly compromised host cells already at 2 h.p.i. (Figure 17), so that this rise in established infections cannot be exploited much further.

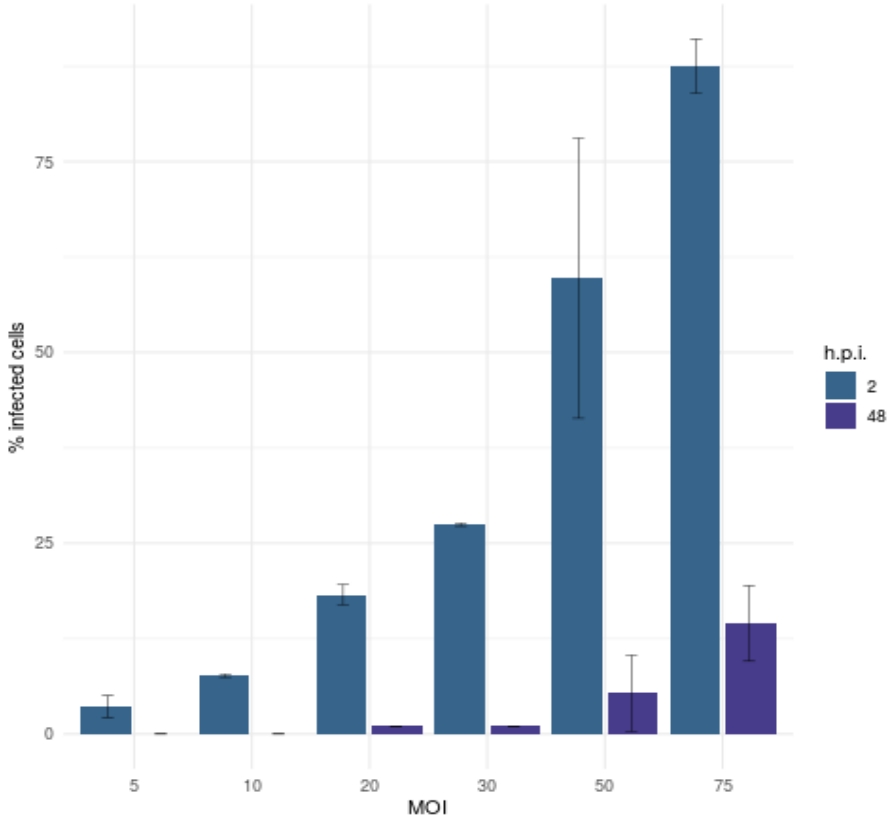

Figure 16. Effect of multiplicities of infection (MOIs) ranging from 5 to 75 on the number of *Chlamydiales* bacterium Pn infected *N. clarki* cells. Blue bars represent the number of infected cells 2 hours post infection (h.p.i.), darkblue bars represent numbers 48 h.p.i.. Each MOI and timepoint represents at least 2 biological replicates and their standard deviation from the means, shown as errorbars. Results were not all obtained in the same experiment, however all tests followed the same experimental setup.

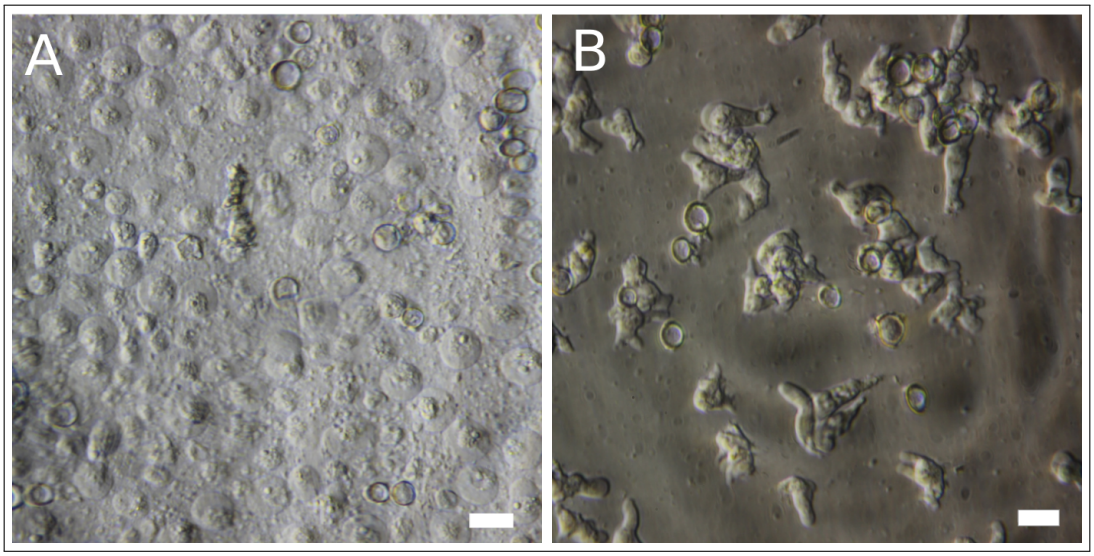

Figure 17. Impact of MOI 100 on a *N. clarki* population at 2 hours p.i., as visualized by light microscopy. **(A)** Host cells 2 hours after infection with *Chlamydiales* bacterium Pn at an MOI of 100. Cells appear rounded and provide little contrast against the culture medium background. **(B)** Host population continuously infected with Pn, provided as comparison. Scale bars indicate 20 μm.

The numbers of infected cells at 2 hpi, shown in Figure 16 remain unchanged by the process of synchronizing infections (i.e. washing residual Pn cells from the supernatant, see section 4.11). Neither do variations in timepoints of synchronization up to 4 hpi(ap. Figure A.1), or the addition of food bacteria before- or after infection synchronization (ap. Figure A.2) affect these numbers in any way. The percentages of infected hosts with symbionts that can already be found located inside the nucleus at 2 hpi were found to be 51 and 55% at MOIs 50 and 75 respectively (ap. Figure A.3). Hence, the numbers of host cells, already containing intranuclear symbionts at 2 hpi also do not provide a more accurate prediction of the 10 and 20% that actually establish an infection later on.

5.4 Evidence for sorocarp formation in *Naegleria clarki*

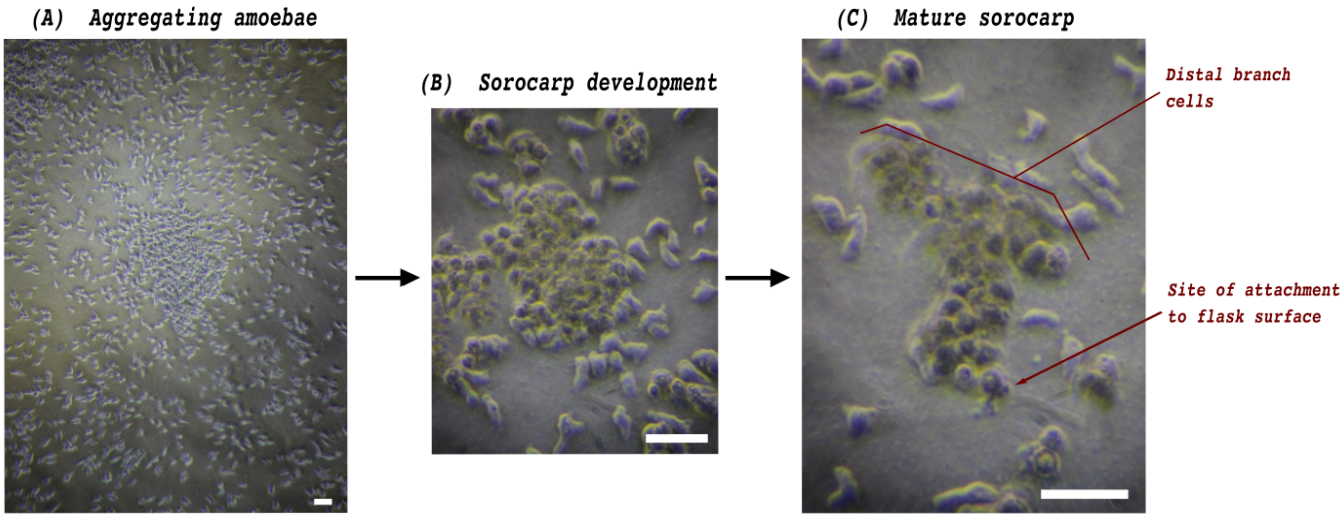

Figure 18. Sorocarp formation in *N. clarki*. *N. clarki* cultures of the strains N-DMLG0, N-DMLG-Pn and CCAP 1518/14 (Table 4) were grown in a liquid, monoxenic medium. Images depict the strain N-DMLG0. Upon depletion of food bacteria and a sufficient number of amoeba covering the culture flask surface, trophozoite amoebae may start to aggregate **(A)**; amoebae cease to aggregate and form mounds, that show cyst development in the center of the mounds **(B)**; within minutes an upward shape (mature sorocarp) is built, that is anchored, through a single attachment site, to the flask surface **(C)**. Scale bars indicate 50 μm.

To date sorocarp formation within the class of *Heterolobosea* is only known for the family of *Acrasidae* and has also been documented once for the species *Allovahlkampfia spelaea* of the family *Vahlkampfiidae* [14], but despite several indications of a sexual life-style [32, 88], sorocarp formation was never shown for the genus *Naegleria*. In this study, the uninfected strains *N. clarki* N-DMLG0 and CCAP 1518/14 and the infected strain N-DMLG-Pn (Table 4), were regularly monitored with lightmicroscopy, during cultivation in liquid monoxenic PAS medium. All three strains regularly showed the formation of multicellular structures upon depletion of the bacterial food source and sufficient cell density[6]. This process begins with an aggregation phase of amoebae within a certain circumcircle migrating towards a central point (Figure 18, A). Then, aggregation ceases and accumulated amoebae go on to form mounds (Figure 18, B), which appear to encyst in the center of the mound, possibly forming the first basal stalk cells. This mound quickly, within a matter of minutes, grows into an upward shape (here referred to as a 'mature sorocarp'), that is anchored, usuall through a single, small attachment site, to the culture flask surface (Figure 18, C). At the same time, movement of the most distal cells of the sorocarp branches can be observed.

[6]Specific concentration threshold not known, however sufficient amoeba in trophozoite stage have to be close to each other, as shown in Figure 18 for 'Aggregating amoebae'.

5.5 The Pn - *Naegleria* symbiosis under axenic conditions

5.5.1 Establishing symbiotic and aposymbiotic, axenic *Naegleria* cultures

Culturing of amoeba in axenic media has been a common practice since the 1960s [12, 17, 41], as axenically growing amoebae have proven useful for a variety of applications. Most notably, proteomics and transcriptomics experiments are rendered nearly impossible with interfering effects from the presence of food bacteria. Therefore, experiments were initiated to utilize infected and uninfected, *E. coli* - free, axenically growing host cultures. Three different *N. clarki* host strains (the two uninfected strains N-DMLG0 and CCAP 1518/14, and the infected strain N-DMLG-Pn; Table 4) were axenized, using different methods of axenization for infected and uninfected populations. Additionally, to be able to compare chronically infected (as opposed to freshly infected) *N. clarki* cultures to isogenic, uninfected host populations, curing of axenically growing, infected host cultures was attempted.

Selection for growth of different aposymbiotic *N. clarki* strains in axenic SCGYE-medium. Replicates of the two monoxenically growing, uninfected strains CCAP 1518/14 and N-DMLG0 (Table 4) were strategically selected for growth in axenic SCGYE-medium. As an intermediate stage between monoxenic and axenic cultivation, we made use of heat-inactivated *E. coli* as a food supplement, similarly to what has been described before for transition of *Acanthamoeba* into axenic media [56]. Four biological replicates of each strain were incubated in SCGYEM at 30^{o}C over the course of approximately 8 weeks (described more in detail in section 4.9). For each replicate of each strain, growth was determined every 7-8 days, from which the two best performing replicates were subcultured, or 'split', to produce two more replicates. For the strain CCAP, this selection process was ended after the fourth split of replicates. At this point the cultures showed a roughly 4 times increased growth rate, than before the experiment, although growth in the axenic medium was already visible at the beginning of the experiment (Figure 19). Amoebae of the strain N-DMLG0, never having been exposed to axenic medium, showed growth only after the fifth split. The experiment for this strain however was continued until the seventh split, to ensure stably growing cultures (Figure 19).

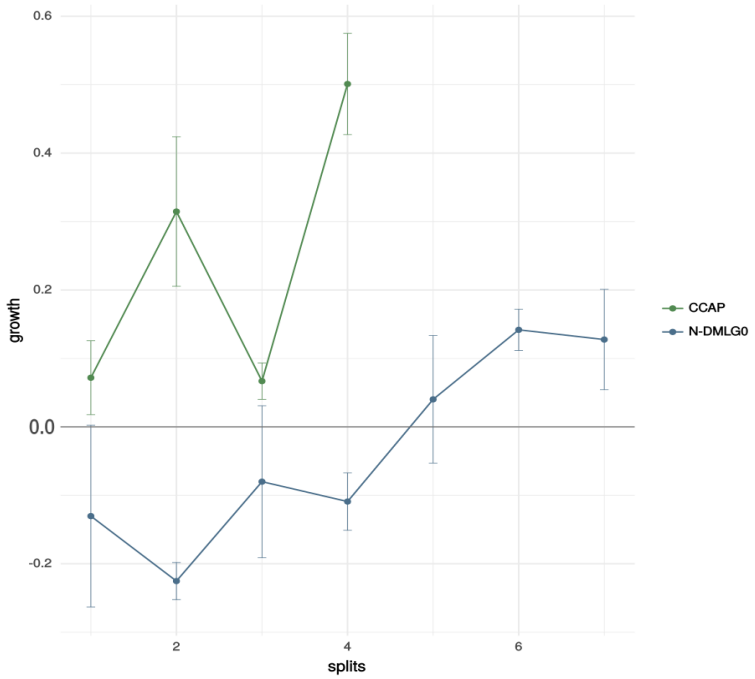

Figure 19. Selection for growth of aposymbiotic *N. clarki* strains CCAP 1518/14 and N-DMLG0 (Table 4) in SCGYE-medium. Cultures were maintained for 4-5 days in 25 cm^2 cell culture flasks, containing SCGYEM, supplemented with heat-inactivated *E. coli*. Each measurement represents the calculated growth rate (see: section 4.9) of 4 replicates of each strain after 3 days of growth under axenic conditions, without any *E. coli* supplement. From each strain, the 2 best performing cultures, in terms of growth, were taken to establish 4 new replicates in 25 cm^2 cell culture flasks. Therefore, each 'split' represents 7-8 days of incubation. Errorbars show the standard deviation from the means of four replicates.

Establishing axenic growth in a symbiotic *N. clarki* culture. In order to achieve infection of *N. clarki* host cultures under entirely bacteria-free conditions, infectious Pn particles have to be isolated from an axenic culture. Therefore, the strain *N. clarki* N-DMLG-Pn (Table 4), which contains a stable number of >99% infected host cells when maintained monoxenically, was used for axenization. As the numbers of infected host cells from this strain had previously been shown to recede, when cultures were grown above 30^{o}C (Michels *et al.*, unpublished), infected cultures were not included in the above described selection process, used for uninfected amoebae (section 5.5.1). Instead, host cultures of the strain N-DMLG-Pn, were incubated at room temperature in 25 cm^2 cell culture flasks (20 - 21^{o}C), containing axenic SCGYE-medium, which was supplemented with low amounts of ampicillin (5μg/ml) and heat-inactivated *E. coli*. Cultures were monitored using FISH over a total of 8 weeks, during which time no significant decrease from the initial >95% of infected cells could be registered (data not shown). After 5 weeks of incubation, the heat-killed *E. coli* supplementation was entirely stopped and cultures were only maintained by weekly exchanges of the growth medium. Around this time, an increase in cell size and an ability to grow into densely populated monolayers, without the formation of sorocarps (that are usually built at higher cell densities, see section 5.4), could be seen within these newly axenized cultures (Figure 20). To ensure that no *E. coli* was left in the culture flasks, part of each culture was transferred to 175 cm^2 cell culture flasks, containing SCGYEM. These 'upscaled' cultures were monitored for 3 weeks, during which time the number of infected cells was maintained at 93-99% (data not shown).

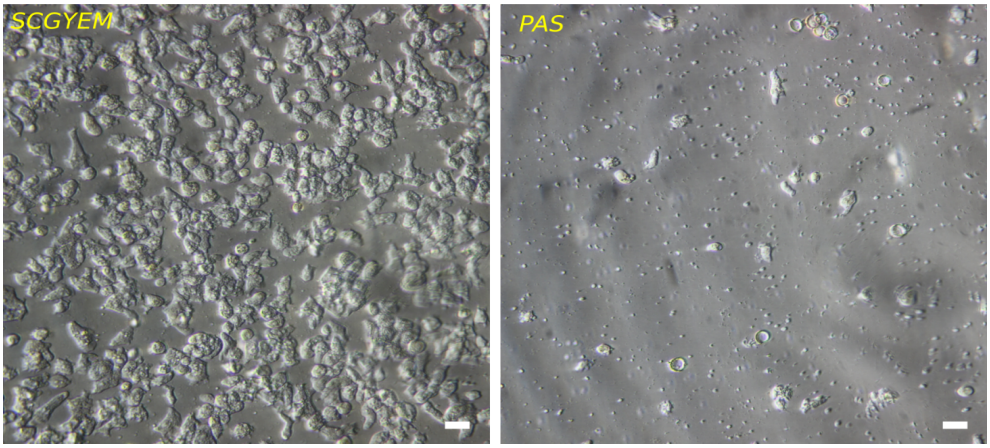

Figure 20. Cell culture morphology before and after axenization in SCGYEM. Left **'SCGYEM'** culture, shows *N. clarki* N-DMLG-Pn (Table 4) cells, infected with *Chlamydiales bacterium* Pn, 4 weeks after transfer into the axenic SCGYE-medium. Cells show an increase in size and densely populate culture flask surfaces, without the formation of sorocarps. Right **'PAS'** culture cells show a replicate of the same culture kept under monoxenic conditions. Scale bars indicate 25 μm.

Curing of a symbiotic *Naegleria clarki* culture. Curing of the axenized *N. clarki* strain N-DMLG-Pn (Table 4) was achieved by exploiting two interaction properties of the Pn - *Naegleria* symbiosis, observed during previous experiments. For one, axenically growing, infected amoebae, produce about 80% less extracellular Pn cells and have a reduction, sometimes a complete cessation in phagocytosis, as compared to monoxenically growing *Naegleria* (both described in section 5.5.2). Second, monoxenically growing *Naegleria* have a 1.7 to 1.9 fold increased growth, depending on whether they are infected or uninfected, at 30^{o}C, as compared to 20^{o}C, or room temperature (described in section 5.5.3). Hence, the curing experiment was done at 30^{o}C, using the already axenized, infected cultures, described above in section 5.5.1.

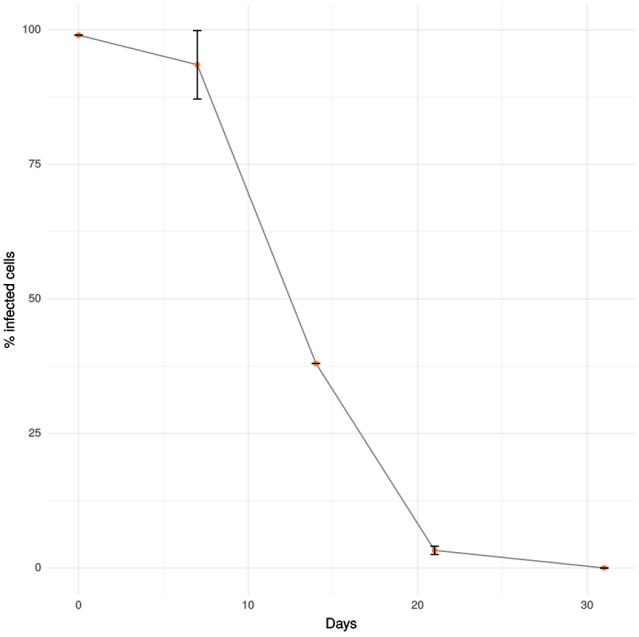

Figure 21. Successive loss of symbiotic *Chlamydiales* bacterium Pn cells in initially fully infected *N. clarki* cultures. *N. clarki* N-DMLG-Pn (Table 4) amoebae growing axenically in SCGYE-medium were incubated at 30^{o}C over the course of 32 days. Growth medium was exchanged weekly. Until week 2, points shown in the graph represent 2 replicates, after that they represent 6 replicates. Errorbars show the standard deviation from the means of 2-6 replicates.

Curing of the initially 2 replicates, used for this experiment, and quantification of infected cells for each replicate and time-point was done as described in section 4.15. Figure 21 charts the drop in infected host cells from the initial 99% to about 93% and then to 38% (∼62% drop) within the first 2 weeks. After week 2, when cultures were split into greater volumes, infected cells dropped from 38% to about 4% (almost 90% drop). The following split at week 3 resulted in no detectable infected cells, via FISH, after 10 days of incubation.

5.5.2 Exploring effects of host adaptation to axenic and monoxenic media on infection transmission

To test the general susceptibility of the axenically growing *N. clarki* strain CCAP 1518/14 (Table 4) to infection with *Chlamydiales* bacterium Pn, previously axenized host cultures (section 5.5.1) of the strain were inoculated with unquantified amounts of symbiont cells and monitored over the course of approximately 4 months, using FISH. As the experiment was performed without synchronization of the infection, cultures showed approximately 90% of infected host cells (Figure 22, A), within the first 3 days of infection. However, over the course of the following month, the number of infected host cells gradually decreased to 50% and finally after 3 and 1/2 months (105 days) to only 10% (Figure 22, B).

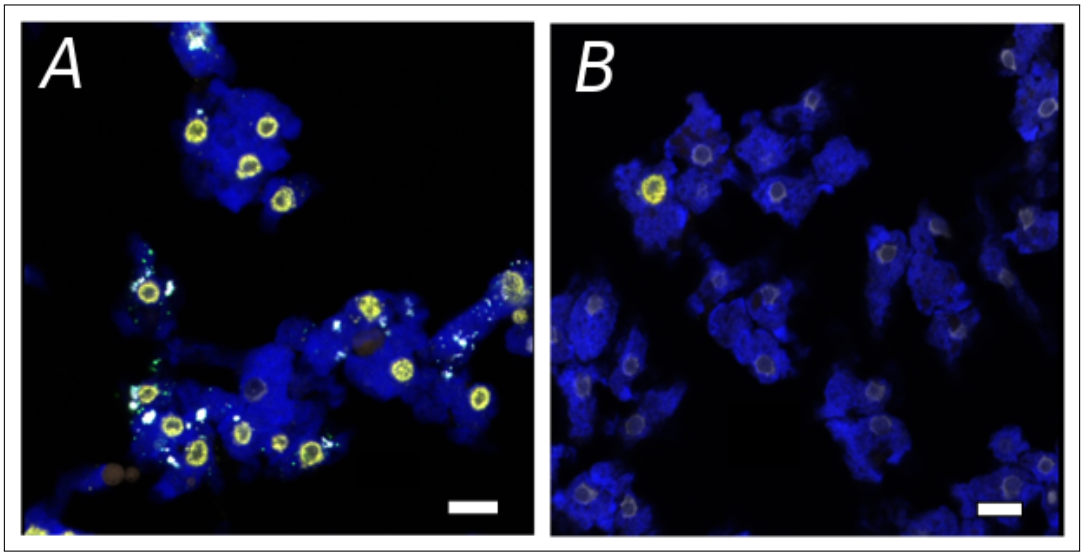

Figure 22. Infection and long-time state of infection in an axenic *Naegleria* culture. Axenically growing *N. clarki* host cultures of the strain CCAP 1518/14 (Table 4), were infected with unknown amounts of *Chlamydiales* bacterium Pn symbiont cells. Cultures were monitored over the course of approx. 4 months, using FISH (Probes: NAEG1088 (blue), EUB338I-III (green) and PN440 (red)) and DAPI (grey) staining. **(A)** Representative image of axenically growing amoeba, 3 days p.i.. Cultures contained around 90% infected host cells. After 1 month of coculturing, the number of infected cells dropped to around 50% (not shown). **(B)** Representative image of infected cultures, after 3.5 months (105 days). Only about 10% of all amoeba still remained infected. Images were obtained by confocal laser-scanning microscopy. Scale bars represent 10 μm.

Since monoxenically growing host populations do not show this gradual loss of infection over time, the resulting, approx. 10% infected, axenic host cultures were split into 6 replicates, 3 of which were continued in axenic SCGYE-medium, the other 3 were incubated in monoxenic medium (PAS plus *E. coli*). After one week of incubation, there was a significant increase of infected cells in the monoxenically growing populations, that was not present in the axenic amoeba cultures, as determined by FISH (Figure 23).

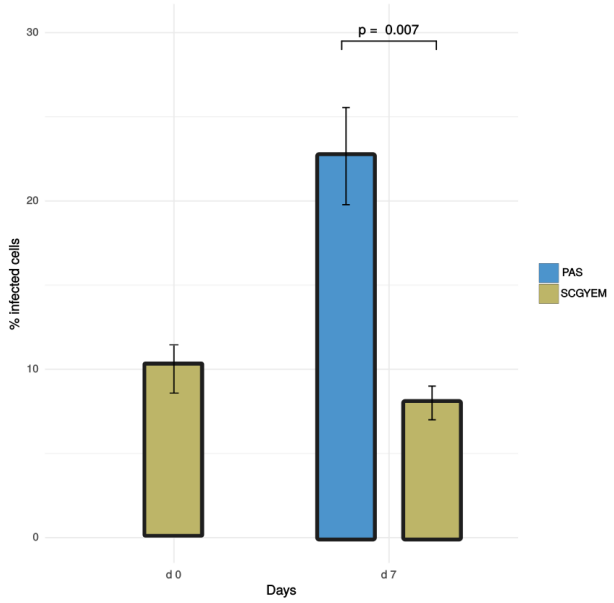

Figure 23. Infection recovery after transfer from axenic to monoxenic medium. Axenically growing *N. clarki* N-DMLG-Pn (Table 4) cultures were cocultured with the symbiotic *Chlamydiales* bacterium Pn over the course of about 4 months, after which the number of infected cells dropped from around 90% to about 10% (d 0). These cultures were, transferred to 'fresh' 25 cm^2 cell culture flasks, containing either the initial, axenic growth medium **SCGYEM**, or the monoxenic **PAS**, supplemented with *E. coli*, in 3 replicates per growth medium. Their standard deviation from the means is shown as errorbars. After 1 month of incubation at RT, the number of infected cells was quantified again, using FISH (d 7). Statistical significance between axenic and monoxenic cultures at 'd 7' was determined by a student's t-test.

Gradual loss of infection after axenization of infected amoeba. The axenization process, described in section 5.5.1, shows how the strain *N. clarki* N-DMLG-Pn (Table 4) was axenized and able to maintain 93-99% of infected host cells over the course of two months, even through a drastic increase in population size, by 'upscaling' from 25 to 175 cm^2 cell culture flasks. Eventually though, after 5 months of axenic coculturing, these cultures also decreased to around 25% infected cells.

At the same time, not mentioned in section 5.5.1, axenization in SCGYEM of these cultures was attempted without fetal calf serum supplement (which would usually be added to this particular medium) to show a possible connection of this supplement to infection-loss over time. However, cultures without this supplement steadily decreased in attached trophozoite numbers (interpreted as a sign of stressed amoeba cells), leading to less densely populated cultures (ap. Figure A.4), until, after 20 days of incubation, almost no amoeba cells could be found at all in the medium.

Symbionts are unable to infect long-term axenically cultivated naïve host cultures. At 2 months post axenization, when these cultures still contained above 93% infected cells, extracellular Pn cells were isolated to quantify their production under axenic conditions. This showed that significantly less (on average 80% less) Pn cells could be harvested, than from monoxenically growing cultures, as determined by a student's t-test on 2 replicates per growth medium ($p = 0.016$). When these Pn isolates were then used to attempt infection in the previously axenized strain *N. clarki* N-DMLG0 (section 5.5.1), using an MOI

of 4, no infection of host cells was observed. Control experiments done in parallel showed, that Pn cells isolated from monoxenically growing host populations, using the same MOI, did not infect the axenized strain N-DMLG0 either. Following experiments, either increasing the MOI to 20, adding food bacteria to the medium (Figure A.2), or using monoxenic PAS instead of SCGYEM as growth medium during infection experiments, showed the same result of no host cells being infected. A final experiment, reverting the same N-DMLG0 culture back into monoxenic medium three days prior to infection with Pn (isolated from monoxenic cultures), did show consumption of food bacteria, but again no infection of host cells with either MOI 5 or 20. However, no control experiments could be performend, that show the ability of these Pn isolates to infect a continuously monoxenic culture of the same strain, as none was available at the time.

Intracellular and exrtacellular Pn do not differ in infectivity. The results of an infectivity assay performed shortly afterwards, on such a monoxenically growing culture of the strain N-DMLG0, do show the infectivity of Pn cells, stemming from the same infected, monoxenic cultures as were used before (Figure 24). This experiment also shows no significant difference between the infectivity of extracellularly, or intracellularly harvested Pn cells. Infectivity of intracellularly isolated Pn cells, stemming from axenic cultures was later also confirmed, using an MOI of 100 on a *N. clarki* N-DMLG0 culture (ap. Figure A.1). This however, was not compared to the same amounts of similarly isolated Pn cells, stemming from monoxenic cultures.

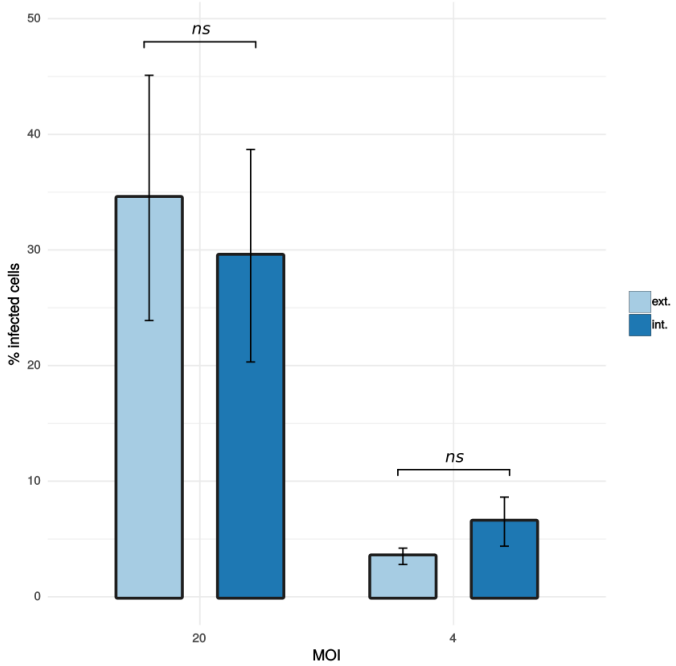

Figure 24. Infectivity comparison of intracellularly and extracellularly isolated Pn cells. *Chlamydiales* bacterium Pn was isolated either from culture supernatant (**ext.**, acc. to section 4.5.2), or from lysed *N. clarki* N-DMLG-Pn (Table 4) cultures (**int.**, acc. to section 4.5.3) and used to infect monoxenically growing *N. clarki* N-DMLG0 cultures at MOIs of 4 and 20. Percentages of infected cells were quantified 48 hpi, using FISH. Errorbars show the standard deviation from the means of two replicates. Statistical significance of infectivity between extracellular and intracellular Pn isolates was determined by a student's t-test (ns, not significant).

5.5.3 Growth comparison between infected/non-infected *Naegleria clarki* under different conditions

To quantify the effect that infection with Pn has on *N. clarki* growth rate, growth experiments, using multiple, different conditions were designed. The non-nutrient-Agar (NNA) plate culture method, according to Schardinger (1899) [77], closest represents 'natural' growth conditions of amoeba grazing on biofilm. Therefore, both the freshly cured (2019, section 5.5.1) and the symbiont-free N-DMLG0 (Michels *et al.* unpublished, Table 4) strains were compared to the infected host strain, they were originally cured from, using the NNA plate culture method both at 20^{o}C and 30^{o}C. The second temperature of 30^{o}C, was selected to detect a possible effect of *N. clarki* temperature adaptation to temperatures up to 37^{o}C [24]. However, as infected as well as uninfected *N. clarki* lab-strains did not survive the transition to the more elevated temperature of 37^{o}C, 30^{o}C, a temperature known to be suitable for and to increase growth speed of available lab-strains, was chosen to perform growth experiments. Additionally, growth comparisons were also performed on axenic SCGYEM-Agar plates, also at 20^{o}C and 30^{o}C and in liquid SCGYEM, only at 20^{o}C.

When considering the axenic growth experiments, visualized in Figure 25 and in Figure 26 (left), no statistically significant difference could be measured between the amounts of either infected, or uninfected amoeba that grew in liquid medium, or on agar plates. Under both conditions however, uninfected amoeba had, in total numbers, produced a 1.3 fold (at 20^{o}C, after 4 days in liquid SCGYEM) and 2 - 2.2 fold (at 20^{o}C and 30^{o}C respectively, after 2 days on SCGYEM-agar plates) higher numbers of amoeba. However, infected cultures at this time only contained around 25% infected cells. The cultures used to perform these experiments were afterwards transferred into liquid, monoxenic medium where they were maintained for two weeks, at which point they contained >99% infected cells. The growth experiment, visualized in Figure 26 (right), shows a replication of the previously performed plate-growth experiment, but under monoxenic conditions. Under these conditions, no statistically significant difference could be measured either. However, this time, in total numbers, infected amoeba produced 1.9 - 1.3 fold (at 20^{o}C and 30^{o}C respectively, after 2 days on NNA plates) higher numbers of amoeba.

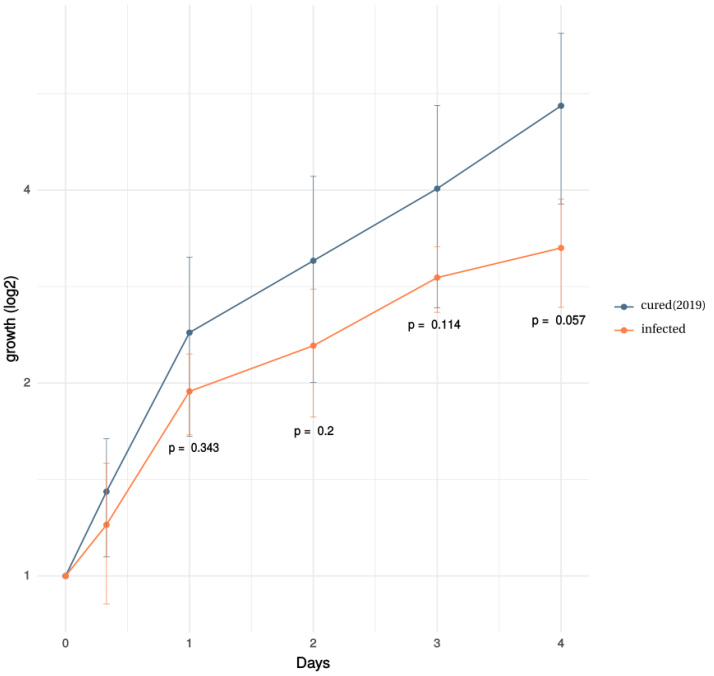

Figure 25. Growth comparison between recently cured(2019) and infected amoeba cultures. *N. clarki* N-DMLG-Pn (Table 4) cultures that had been axenized and either cured, or just maintained in axenic SCGYE-medium, were grown over a total of 4 days in liquid SCGYEM at 20^{o}C. Quantification of amoeba was done by counting amoeba in 10 random sight fields at 40x magnification, mean numbers are represented as **'growth'** on the y-axis. Errorbars show the standard deviation from 4 replicates. Statistical significance was calculated, using a student's t-test.

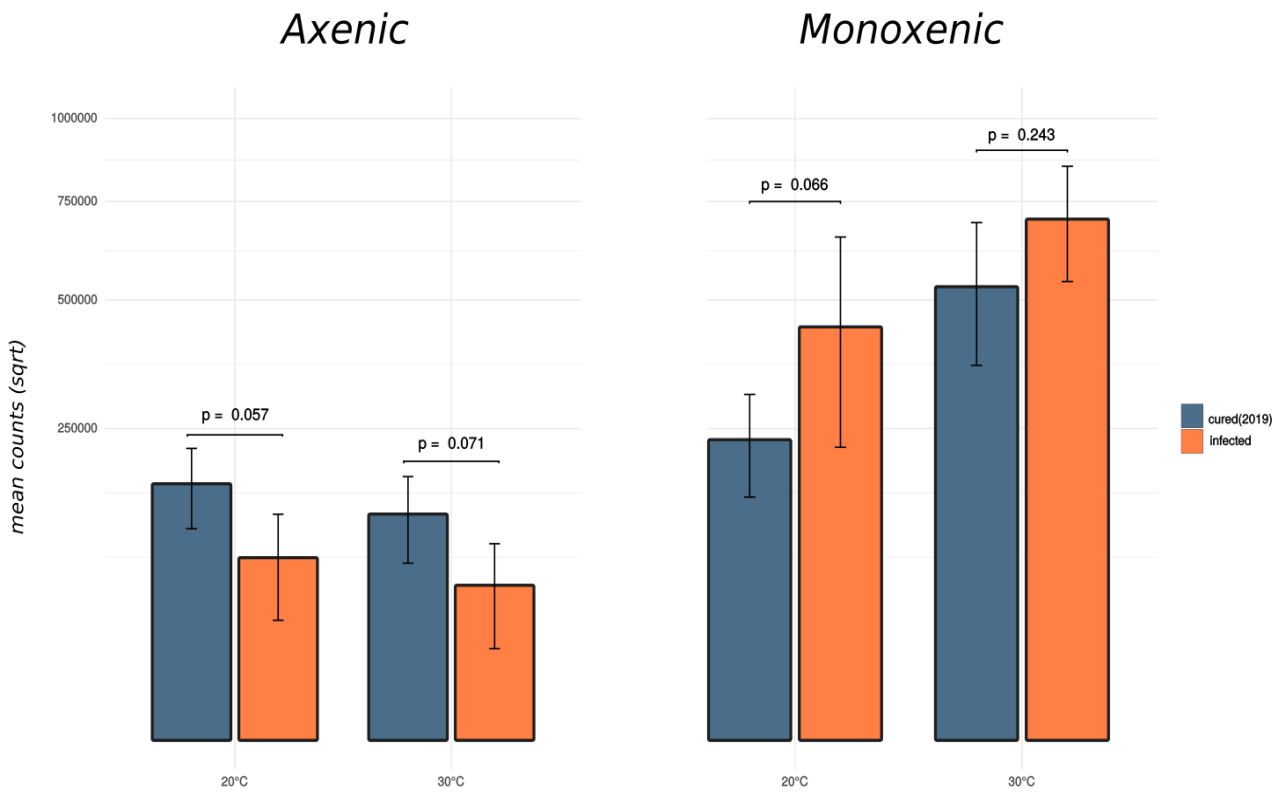

Figure 26. Growth comparison between recently cured (2019) and infected amoeba cultures. *N. clarki* cultures, that had been axenized and either cured from *Chlamydiales* bacterium Pn, or just maintained axenically, were grown on SCGYEM plates (**Axenic**) for two days, at which time infected cultures contained around 25% infected cells. The same cultures were, after a two-week transition period in liquid, monoxenic PAS medium, grown on NNA plates (**Monoxenic**) for two days, at which time infected cultures contained >99% infected cells. Monoxenic, cured cultures at this time also showed infection with Pn in 1-2 % of all culture cells. Growth experiments were conducted at either 20^{o}C, or 30^{o}C. Errorbars show the standard deviation from the means of the four replicates. Cells were quantified after 2 days, by washing cells from the plates and counting amoeba with a cell counting chamber, mean numbers are represented as **'growth'** on the y-axis. Statistical significance between infected and cured cultures was determined by a student's t-test.

Figure 27 shows the visualization of this shift from uninfected cultures producing a higher total number of amoeba in axenic media, to infected cultures producing a higher total number of amoeba in monoxenic media. It depicts a calculation of the difference of higher versus lower mean values at each condition (medium and temperature).
An analysis, calculating the difference of the amount of amoeba that grew within the same culture on either monoxenic, or axenic medium is visualized in Figure 28 and was tested for statistical significance, using a Mann-Whitney-Wilcoxon test. This shows, that cured (2019) populations show similar growth in both media at 20^{o}C (p= 0.343), but at 30^{o}C this strain shows 4 times increased growth (p= 0.029) in the monoxenic medium compared to its axenic counterpart. For infected host populations this effect also holds true, but is much more pronounced. Already at 20^{o}C the infected strain shows a 5-fold increase in growth (p= 0.003), rising to an almost 12-fold increase at 30^{o}C (p= 0.029). The trend of infected host populations producing a higher total number of amoeba in monoxenic media, could also be shown for the long-term, continuously infected strain *N. clarki* N-DMLG-Pn and its cured counterpart *N. clarki* N-DMLG0 (ap. Figure A.5). However the curing of strain N-DMLG-Pn lays several years in the past, hampering comparability of the two strains.

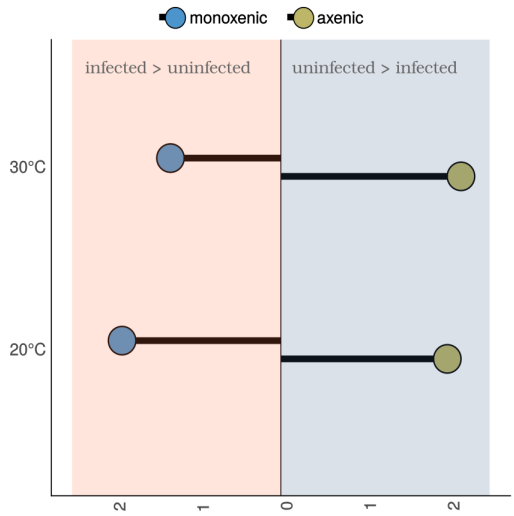

Figure 27. Mean difference in growth between infected and uninfected *N. clarki* cultures after growth on either axenic SCGYEM-Agar plates, or monoxenic NNA plates. *N. clarki* cultures, that had been axenized and either cured from *Chlamydiales* bacterium Pn, or just maintained axenically, were grown in 4 replicates each, for 2 days on SCGYEM-agar plates, or, after a transition period back to monoxenic medium, on NNA plates, either at 20^{o}C or 30^{o}C (see text). Pointranges of **monoxenic** and **axenic** values show the ratio of higher versus lower mean values, whereby values on the left indicate greater numbers of infected host cells (under monoxenic conditions) and values on the right indicate greater numbers of uninfected host cells (under axenic conditions).

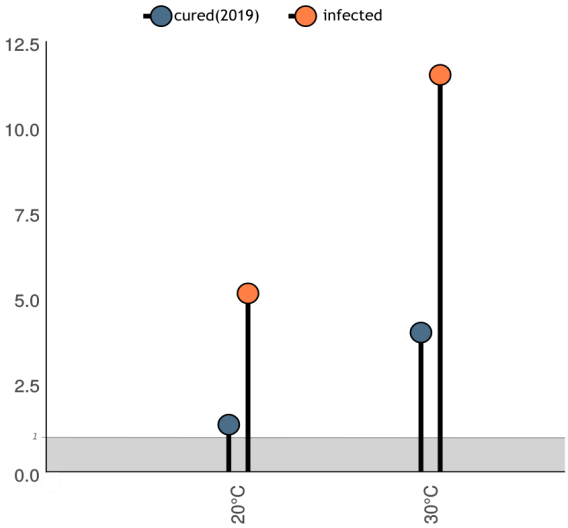

Figure 28. Relative increase in growth of cured and infected *N. clarki* populations, after transfer from axenic to monoxenic medium. The axenized strains *N. clarki* **cured (2019)** and the *Chlamydiales* bacterium Pn **infected** strain *N. clarki* N-DMLG-Pn (established see sections 5.5.1 and 5.5.1) were grown first on axenic agar medium and after a transition period also on monoxenic agar medium (see text). The relative increase in growth (shown as fold-increase on the y-axis) of each strain grown monoxenically compared to the same strain grown axenically was caluculated both for cured and infected strains at 20^{o}C and 30^{o}C and statistically analysed, using a Mann-Whitney-Wilcoxon test. The grey area below 1 on the y axis indicates no difference, or decrease in growth, between the two media conditions. Cured populations show similar growth under both media conditions at 20^{o}C (p= 0.343). However at 30^{o}C the same strain grows up to 4 times (p= 0.029) the amount of its axenic counterpart. For infected host populations this effect is even more pronounced, leading to a 5-fold increase (p= 0.003) already at 20^{o}C, that rises up to an almost 12-fold increase at 30^{o}C (p= 0.029).

6 Discussion

6.1 The unique life-cycle of a chlamydial intranuclear symbiont

So far, only few symbionts have been described, that colonize the nucleus of their host cells. One, *Ca. Nucleicultrix amoebiphila* (*Alphaproteobacteria*), colonizes the nucleus of its *Vermamoeba* sp. host and was also found capable of infecting the nucleus of *Acanthamoeba castellanii* [80]. Two species forming the novel genus *Ca. Nucleococcus* (*Verrucomicrobia*), termend *Ca. N. trichonymphae* and *Ca. N. kirbyi* , were found colonizing the nucleus of the termite gut resident *Trichonympha agilis* [75]. The bacterium *Ca. Berkiella cookevillensis* (*Gammaproteobacteria*) was isolated from amoebae grazing on biofilm of a cooling tower and found colonizing not only the nucleus of *Acanthamoeba polyphaga*, but also mammalian and non-phagocytic cell lines [21]. This is the first report, characterizing an intranuclear symbiont of amoeba, that belongs to the phylum *Chlamydiae*.
Investigations of the life-cycle of the symbiont Pn during this study, showed a time period of under 48 hours from entry into the host cell until the first release of infectious particles (Figure 6). This reflects the typical duration of 30-72 hours, described for *Chlamydiae* infecting humans and other animals [57], but is significantly shorter than the 4-15 days described for most members of the *Parachlamydiales* [45, 49, 87]. While at least 2 rounds of bacterial replication may happen within the first 24 hours of infection, localization around the hosts nucleolus only occurs during the following 24 hours. It is within this timeframe of 24-48 hpi, that symbiont numbers inside the nucleus increase to an extent that enables a complete colonization of the nucleolar surface, allowing attachment of at maximum around 200-320 (for nucleolus sizes of 3-4 μm) bacterial cells at the same time. Symbiont cells can be seen just outside the nucleus in the host cytoplasm at 48 hpi, which is followed by exit of host cells by extrusion of the infectious particles (Figure 6 arrows), leading to reinfection of new hosts, that is visible already within the following 24 hours (until 72 hpi, not shown). Whether symbiont cells undergo the typical chlamydial developmental cycle of primary differentiation from EB to RB after entry into the host and secondary differentiation back into EBs after 48 hours still remains to be investigated by TEM. Typical traits of EBs include a low ribosome content and a thick cell wall [29], which in the past have made their visualization difficult and often times only possible through DAPI staining. However, detection of the symbiont Pn has proven easily possible with FISH at any stage of its lifecycle, including extracellular stages found in the host culture supernatant (Figures 6 and A.7), similarly to what has been described for the member of the *Simkaniaceae, S. negevensis* [45]. In addition to this, Pn cells have also been observed to lack any significant difference in infectivity between intracellular and extracellular stages (Figure 24), which is again similar to what has been described for *S. negevensis* [45] and might indicate no necessity for maturation of infectious particles.

6.2 Pn is capable of horizontal and vertical transmission

Exit and entry are critical steps in the infectious cycles of intracellular pathogens. For *Chlamydiae* redifferentiation from the replicating RB back into an EB is associated with release from the host within versicles (i.e. extrusion) or by lysis of the host cell [39]. The ability of extrusion of infectious EBs, that leaves behind a viable infected host cell has been shown to be a broadly conserved exit mechanism within *Chlamydiae* [99] and was argued to be a key concept in their ability to avoid elimination within the host and cause a chronic infection [38].
In its original host, Pn is able to facilitate horizontal transmission, using either extrusion from, or lysis of the host, but also accomplishes vertical transmission during host cellular division. Figure 29 depicts the transmission modes of Pn, observed during this study, as well as a phenotype, consituting a possible intermediate stage before host lysis, which shows host nuclei of up to 10 μm in diameter, entirely filled with symbiont cells. Although lysis events have been occasionally recorded in the form of cellular debris, or on FISH images, *N. clarki* cultures, that have been in co-culture with the symbiont Pn for a long time (e.g. the strain N-DMLG-Pn, Table 4), have shown stable maintenance of amoeba populations, which suggests a low frequency of host lysis events. Extrusion of Pn particles on the other hand, may be observed in the majority of cells of a continuously-infected host culture at any given time and are thereby considered to constitute the predominant way of exit for Pn. This is especially true when considering a fresh infection, where in fact exit by extrusion appears to be the only way of escape from the host for Pn, until at least 72 hours post infection.

In addition to transmission by exit and reinfection, Pn cells are capable of vertical transmission during host mitosis. The exceptional process of cellular division in *Naegleria* and infact all heterolobosean species, allows a promitosis without disintegration of neither the nucleus, nor the nucleolus [60]. During this study, intranuclear Pn populations have been shown to passively being distributed amongst parting nucleolar masses, by solely remaining on the nucleolar surface of its host (Figure 15). This ability might represent a key factor in the maintenance of symbiosis with Pn, enabling the symbiont to maintain an infected host population, while actively replicating, without the need to revert to metabolically less active states (such as the chlamydial EB) and the necessity to constantly reinfect new host cells.

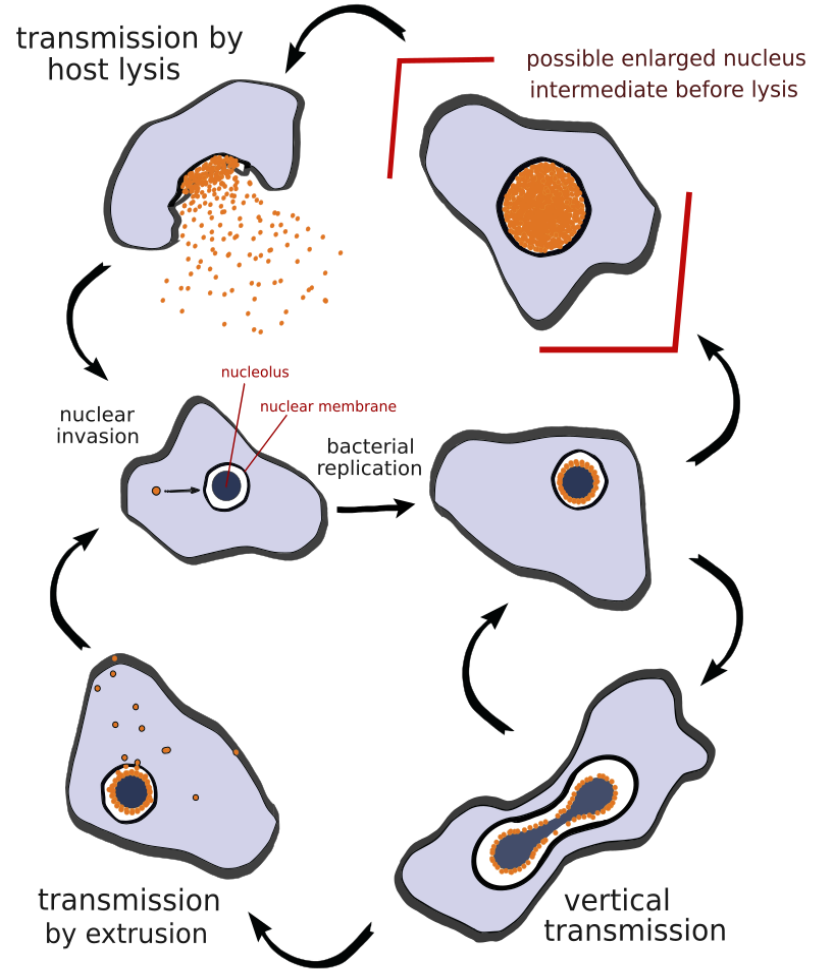

Figure 29. Transmission modes of *Chlamydiales* bacterium Pn in its *N. clarki* host. The symbiont Pn shows 3 routes of propagation within its *Naegleria* host. Horizontal transmission is either facilitated by extrusion of infectious particles, or by lysis of the host. Increased nucleus size and total colonization of the intranuclear compartment has been observed in infected cells, possibly constituing a phenotype preceding lysis.

6.2.1 Infection success and propagation of the symbiont Pn

Intracellular endosymbiosis is a particularly intricate relationship to establish between the partners. It involves invasion by the endosymbiont, survival and replication despite host defenses and ultimately an ability of the symbiont to spread to new hosts. The intranuclear symbiont Pn, although capable of infecting its *N. clarki* host horizontally, requires high MOIs of 50 to 75, to reliably infect a lasting fraction of 10-20% of a host cuture under laboratory conditions. Infection experiments, using MOIs from 5 to 75 (Figure 16), have shown that initial infection rates, at 2 hpi are many times higher than established infections, at 48 hpi. The drop in infected cells between 2 and 48 hpi, although dramatic for all MOIs that were tested, showed a more profound drop of 95% for MOIs below 30, compared to the average 89% drop for MOI 50, or the 83% drop for MOI 75. When infection experiments were executed, infections were synchronized after 2 hours, by washing off residual symbiont cells that remained in the supernatant, with a high stringency. This high stringency of synchronization however was shown not to be causal to the loss of infected cells, as an infection experiment, using a low-stringency synchronization, which still permitted infections after 2 hpi, also showed a drop of around 90% from initial infections at 2 hpi to established infections at 24 hpi (Figure A.3). Success of entry into the host cell seems to largely rely on the hosts ability to phagocytose infectious particles (shown in section 5.5.2), rather than on active entry mechanisms of the symbiont. As shown in Figure A.3 at least half of all infected amoeba show symbionts inside the nucleus within the first two hours of infection, but many of these Pn particles do not establish growth within the intranuclear compartment. Pn particles, although initially infective, seem to be rather sensitive, not only to possible host defenses, but also to environmental stressors. This sensitivity was noticed for one, when storage of intra- or extracellularly isolated Pn particles was only possible for a few hours in PAS medium at room temperature, or 4^oC, but also, when those particles, could not be retained by suction onto 0.2 μm polycarbonate filters for counting of bacterial cells, without the use of formaldehyde fixation prior to blotting, as the particles seem to have lysed due to the pressure coming from the vacuum pump.

At MOIs reaching 100, host cells seem unable to cope with the high load of symbionts, as increasing MOI also means that more particles enter a single host cell at the same time. This leads to host cells detaching from the culture flask surface, becoming more transparent and ultimately to die within the first few hours of infection (Figure 17).

Despite the big difference between success of entry into the host and establishment of the intranuclear niche, the symbiont Pn is able to fully infect a monoxenic host population in under two weeks. This could be shown in an experiment, where the progression of infection in host cultures was monitored over the course of 9 days (Figure 7). At the end of the first developmental cycle at 48 hpi, the infected host cultures exhibited around 10% established, intranuclear infections and around 40% fresh infections. After this, extrusion and spread of infectious particles facilitated the infection of the entire host population in another 7 days. During these 9 days, several signs for the transition into a chronic infection with Pn could be observed. For one, lysis events start to happen between days 5 and 9, although the exact strating point remains unknown. In addition, host nucleus sizes of infected amoeba start

to increase, the longer they remain in culture. This phenotype of increase in infected host nuclear diameter from about 3.8 to 4.4 μm, has been recorded for the strain N-DMLG-Pn (Table 4), a chronically infected strain of *N. clarki*. At 9 days post infection a phenotype, showing nuclei filled entirely with Pn cells, increasing nucleus sizes from 5 μm up to 10 μm, also starts to appear (as assessed by confocal microscopy, which shows no visible nucleolus in suchlike colonized nuclei, Figure 9). This phenotype is seen in around 3% of cells in a chronically infected host population and might represent a stage prior to host lysis.

6.3 Infection with Pn persists during *N. clarki* life-cycle stages.

For most FLAs the production of cysts and for many also the production of flagellated life-cycle stages, poses an integral part for their capacity to survive adverse environmental conditions and to disperse within their environment [60]. As the trophozoite stage remains the predominant stage for feeding and propagation of most FLAs, but also the most easily accessible one to study in the lab, it represents the stage most commonly described as targeted by intracellular endosymbionts. For Pn the trophozoite stage of *N. clarki* is likely the only way of entry into the amoeba. As *N. clarki* feed and divide only in this stage [25], it is also the most important stage in terms of metabolic acitvity and progeny production for the symbiont Pn. After transformation into the flagellate stage, the amoeba remains infected intranuclearly without apparent phenotypic change of the symbiosis (Figure 12). However, when rates of host transformation of infected and uninfected individuals were compared, amoeba cultures appeared affected by infection with Pn, which lead to higher variance in transformation rates amongst infected culture replicates, compared to uninfected ones (Figure 13), although a method clearly distinguishing flagellates from trophozoites would be needed to confirm this trend.
The ability to maintain infection through encystation of amoebae has been argued to be especially relevant from an ecological and epidemiological point of view, as it might be even more effective as shelter and vector for ARBs than the trophozoite stage [51]. During this study the symbiont Pn has been shown to retain its intranuclear localization (Figure 10) more or less unaffected by the encystation of its host. As uninfected cysts have not yet been found in chronically infected host cultures, the ability for expulsion of the entire symbiont population (as described for *L. pneumophila* in *A. castellanii* [58]) is arguably missing. However, for how long this interaction stably persists and whether the symbiont population after excystment continues to be viable, has not been analysed in this study and remains to be investigated.
Overall these results indicate, that Pn is not only able to infect a common soil and freshwater amoeba, indicating a possible wide distribution in the environment, but also its ability to disperse and survive harsh environmental conditions along with its host.

6.4 Infection success depends on the hosts adaptation to the growth medium

The main goal of axenizing the two uninfected strains *N. clarki* N-DMLG0 and CCAP 1518/14, and the infected strain *N. clarki* N-DMLG-Pn (Table 4), was to establish an infection protocol for later transcriptomics experiments, uninfluenced by food bacteria. After selective axenization of the two strains N-DMLG0 and CCAP, both were found susceptible to infection with Pn, directly after axenization. This however turned out to be an unstable interaction, quickly reducing infected cells from an initial 90% to only 10% within less than 4 months of coculturing (Figure 22). Continuous efforts to produce a more stable infection, showed that axenization of the fully infected strain N-DMLG-Pn, produces a longer initial maintenance of infected cells, remaining above 90% for the first 5 weeks, and a slower decrease to 25% within 5 months of coculturing. Yet the amounts of extracellular infectious particles that could be isolated from these cultures were found to be 80% less than ones similarly isolated from monoxenic cultures, although similar amounts of infectious particles could still be isolated intracellularly. An additional, interesting finding showed, that the loss of infected cells could be stopped and even reversed by simply transferring the axenically growing culture cells into a monoxenic growth medium (Figure 23). Further investigations of the hosts adaptation to axenic medium, showed that the axenized strains N-DMLG0 and CCAP, initially found susceptible to infection with Pn, were, after 6 months of axenic cultivation, no longer susceptible to any used amounts of symbiont cells. An addition of food bacteria to the axenic medium, or even exchange of the growth medium with monoxenic PAS up to 3 days prior to infection did not change the hosts susceptibility to infection, even though consumption of added food bacteria was visible.
A change in behavior upon cultivation in axenic medium has been recorded previously for *Naegleria* amoeba. Such studies mostly describe a difference in nutrient requirements between pathogenic and non-pathognic *Naegleria*, but also a visible change in appearance, as axenized *Naegleria* loose their slug or limax type morphology, migrate less and fill with smaller vacuoles that contain culture medium [23, 81]. However, a decrease or cessation in phagocytosis, as is very likely the reason for the shown inability of Pn to infect axenized *N. clarki* culture cells, has not been suggested prior to this study. For future transcriptomics experiments this signifies that, although sufficient infectious particles can be isolated intracellularly from axenic host cultures, axenized uninfected strains should be relatively recently axenized and regularly monitored for levels of phaocytosis with for example latex beads (as described by [6]), as *N. clarki* become less susceptible to infection with Pn, the longer they remain in axenic culture. For future symbiosis research on *N. clarki* and very probably the whole genus *Naegleria*, this implies an important change in the biology of the amoeba, likely gradually changing the mode of food consumption from phagocytosis to pinocytosis.

6.5 Cultivation in axenic medium affects the host-symbiont relationship

Bacteria have developed various strategies to affect the host physiology, cell cycle and gene expression programme for their own benefit, very often by targeting the nucleus by the use of nuclear modulation molecules, with the most direct way of influence being simply by invading the nucleus [11]. A first step in assessing the influence a symbiont has on its host is to compare growth rates between infected and uninfected populations, which gives an indication of possible costs for the host in associating with the symbiont. Up to now intranuclear infection has only been shown to affect host culture growth for the *Paramecium biaurelia* symbiont *Holospora caryophila*, through biotic factors, like host growth phase and different strains of host or symbiont [9]. Yet, observations during this study, have indicated, that the symbiont's influence on its host may be strongly associated with abiotic factors such as growth temperature, which has previously been reported to impact the relationship of *A. castellanii* and its parasite *L. pneumophila* [58], or growth medium, a factor taken much less into consideration. Experiments comparing growth between infected and uninfected populations during this study, were only performed using long-term co-cultivated host-symbiont cultures (i.e. the strain *N. clarki* N-DMLG-Pn; Table 4) and its cured counterparts (the strains *N. clarki* N-DMLG0 and cured(2019); Table 4), to exclude effects of a relatively fresh infection on naïve amoeba populations. The previously axenixed strains of infected (N-DMLG-Pn) and cured (cured(2019)) amoeba cultures were grown on axenic SCGYEM-Agar plates at 20^oC and 30^oC. This experiment indicated that the symbiont Pn might be able to negatively influence the hosts growth rate at either temperature (Figure 26). For *N. clarki* host populations that were grown on a bacterial lawn, using the monoxenic NNA plate-culture method, association with the symbiont produced a trend towards elevated growth, when compared to the cured(2019) or N-DMLG0 populations (Figures 26 and A.5). Additionally, the total growth of cultures was increased after transition into monoxenic medium, but was also strongly influenced by growth temperature. For example, the strain cured(2019) showed similar growth in both axenic and monoxenic media at 20^oC , but at 30^oC shows a 5 times elevated growth in the monoxenic medium, compared to the axenic one (Figure 28). The infected strain N-DMLG-Pn shows a 5 times increased growth in monoxenic media already at 20^oC, that rises up to an almost 12 times increase in growth at 30^oC (Figure 28).

A positive influence on the division rate of the host can distinctly increase the dispersal of symbionts within amoeba populations by vertical transmission, especially when nutrient availability and space are not limited and the host is in the exponential growth phase. Interestingly this property is only exploited by the symbiont, when the host is grown on bacteria as food source. Axenic host cultivation has, during this study, been shown in several instances to hamper the propagation of Pn within a host population, by decreasing expulsed infectious particles and phagocytosis thereof. An increase in growth rate seems a likely adaptation of the symbiont to increase the number of chronically infected individuals within a host population. Yet, a shift into the opposite effect, decreasing host growth rates caused solely by a change to axenic cultivation, seems more difficult to comprehend and might represent an important example for the manufacturing of artefacts under laboratory conditions.

6.6 Conclusion

This study represents a description of a unique chlamydial intranuclear symbiont of *N. clarki*. It shows how the symbiont is able to reach its intranuclear destination within the first two hours of infection and complete an entire developmental cycle, leading to release of infectious particles within 48 hours of infection, making this one of the shortest developmental cycles known for a member of the *Parachlamydiales*. It is distinct from most other members of the *Chalmydiae* not only by its intranuclear localization, but also considering the fragility of its particles. This leads to difficulty when filtering, or storing them, but likely also contributes to the unusual detectability of extracellular stages via FISH and possibly even to the difficulties in establishing replication after the symbionts have reached the intranuclear niche. The symbiosis is also maintained during transition of the host into different life-cycle stages. The most important one being the cysts stage, securing the symbionts ability to distribute and survive much longer in the environment together with its host. This maintenance of infection through different host life-cycle stages is also suspected, but has not been shown, for the newly described sorocarpic developmental stage of *N. clarki*.

Lysis of host cells may happen occasionally, yet extrusion of infectious particles constitutes the most important way of horizontal transmission. While vertical transmission on the other hand remains an important way for continuing a chronic infection, which can be more stably maintained in an amoeba population. These chronic infections lead to changes in the hosts' physiology over time, mostly by increasing nucleus size, but also by affecting growth and flagellate transformation rates.

Growth medium and temperature have been shown to play a major role in the relationship of host and symbiont, during this study. For the host, axenization changes the mode of food consumption from phagocytosis to pinocytosis. This leads to an inability of Pn to infect its natural host, as it likely relys on passive uptake by the amoeba. In addition, extrusion of infectious particles decreases dramatically upon axenization of infected cultures, making vertical transmission the most important way of infection transmission under axenic conditions. Furthermore, infected populations appear to have a growth disadvantage in axenic medium, which can be entirely reversed when cultures are transferred into monoxenic medium, inducing a trend towards enhanced growth for infected populations. Additonally, cultivation at elevated temperatures was also shown to induce increased host population growth in different ways between infected and uninfected monoxenic host cultures.

This study represents an investigation into many different aspects of a unique symbiont-host relationship, considering not only both the symbiont and host life-cycles, but also abiotic factors that may affect microbe-amoeba interactions.

7 Acknowledgements

I would like to thank Prof. Matthias Horn, who provided me with the opportunity to work on an interesting topic for my master's thesis, which sparked my interest to continue my scientific career in the fields of symbiosis and protistology. Together with the extended symbiosis work group, they provided a supportive work environment, giving me a lot of feedback, but also lending me a hand in the lab whenerver I needed help.

This is especially true for my direct supervisor Stephan Köstlbacher. He not only shared his personal work space with me in the lab, but also at his desk, which I cannot appreciate enough, it made my life as a masterstudent immensely more bearable and easy. He also took a lot of time and care whenever I needed to discuss the project and provided excellent diversion along with other members of DOME, whenever the project took frustrating turns.

I would also like to thank all members of DOME, that helped me with Equipment, or took care of cultures or lab-duties while I was gone, but also those that kept the lab going.

Additionally I want to thank Patrick Arthofer, who helped me in the lab a lot, but was in general a great help and very fun to be around.

At this point I also have to include everyone who regularly or irregularly played table soccer with me. It kept me warm in winter and even warmer in summer and made me very happy, mostly.

Furthermore, I would like to thank Joachim and my brother Emanuel. Emanuel took the time to come up with an equation I could use in my thesis. Joachim provided a lot of fun diversion, but also well placed criticism I needed. Both were very helpful during my entire time at the university and provided a constant source for discussions at the breakfast table.

A Appendix

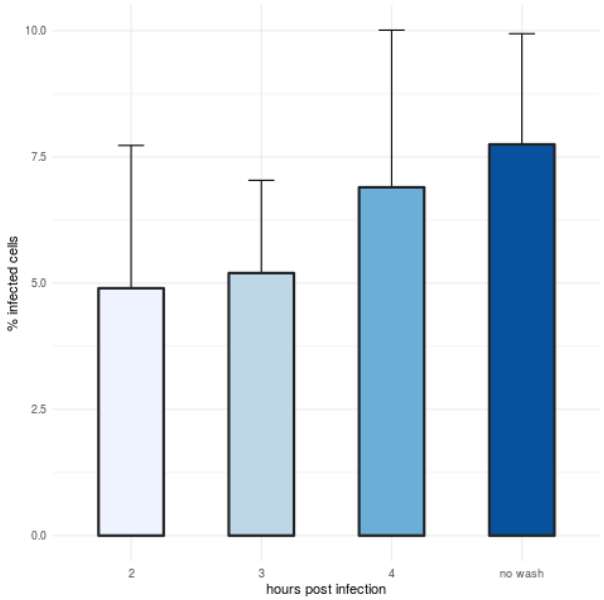

Figure A.1. Effect of differeces in timepoints of infection synchronization. *N. clarki* cultures, growing monoxenically, were infected with intracellularly isolated *Chlamydiales bacterium* Pn cells, stemming from an axenic *N. clarki* N-DMLG0 culture, at an MOI of 100. Infection synchronization was done, as described in section 4.11, at 2, 3 and 4 hours post infection (h.p.i.). Two replicates were left without synchronization ('no wash'), as control. Infected cells were quantified 72 h.p.i., using epi-fluorescence microscopy on FISH labeled samples. Errorbars show the standard deviation from the means of two replicates. Statistical significance was not given between any of the conditions. This was, tested, using a student's t-test.

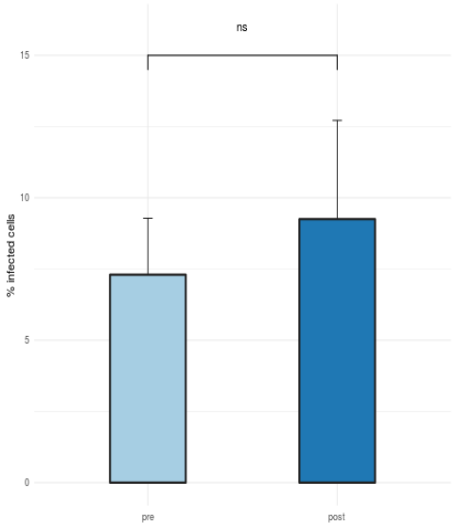

Figure A.2. Effect of addition of food bacteria pre-, or post- infection synchronization. *N. clarki* cultures, growing monoxenically, were infected with *Chlamydiales bacterium* Pn at an MOI of 50. *E. coli* food bacteria were added either **'pre'**, or **'post'** infection synchronization. Infected cells were quantified 24 h.p.i., using epi-fluorescence microscopy on FISH labeled samples. Errorbars show the standard deviation from the means of two replicates. Statistical significance was tested, using a Mann-Whitney-Wilcoxon Test (ns, not significant).

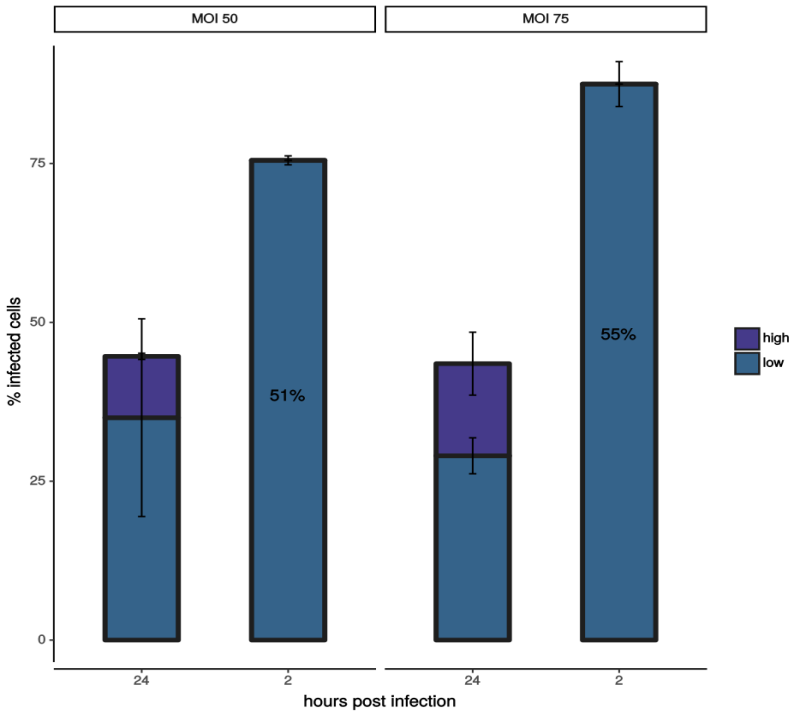

Figure A.3. Low stringency infection synchronization of *N. clarki* cultures 2 hours post infection with Pn. Infection synchronization of in infections, using an MOI of either 50, or 75, was done by carefully taking off the entire culture supernatant and replacing it with fresh, monoxenic medium. The later timepoint 24 hpi was chosen instead of 48 hpi, to reduce the chance of replication of bacteria that infected amoeba after 2 hpi. The number of infected cells was determined at 2 and 24 hpi, using FISH and epifluorescence microscopy. Amoeba cells showing a fresh infection (i.e. only few, individual cells located in the nucleus, or cytoplasm) are termed **'low'**, ones showing an established infection (i.e. intranuclearly located symbiont cells, that have seemingly undergone several rounds of replication) are termed **'high'**. Numbers inside bars show **percentages** of infected amoeba with intranuclearly located symbionts at 2 hpi. Errorbars show the standard deviation from the means of two replicates.

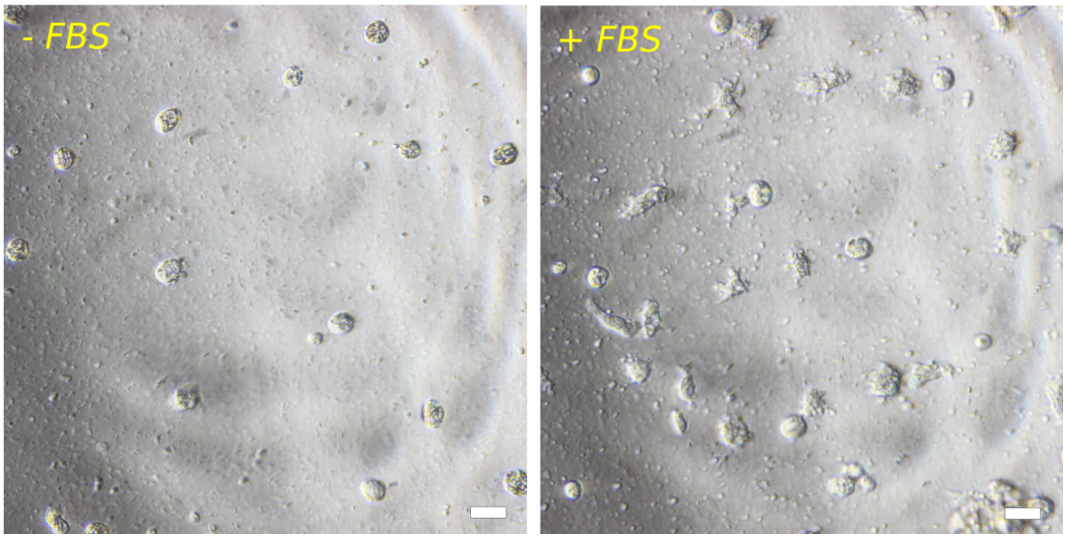

Figure A.4. Cell culture morphology during axenization in SCGYEM with and without fetal bovine seum (FBS). Left **'-FBS'** culture, shows *N. clarki* cells, infected with *Chlamydiales bacterium* Pn, 18 days after transfer into the axenic SCGYE-medium, without FBS supplement. Few attached trophozoites remain, total amount of amoeba in the cultures steadily decreases. Right **'+FBS** culture shows amoeba orginating from the same infected culture, 18 days after transfer into axenic SCGYE-medium, containing FBS. Cells already show an increase in size and are usually found attached to culture flask surfaces. Scale bars indicate 20 μm.

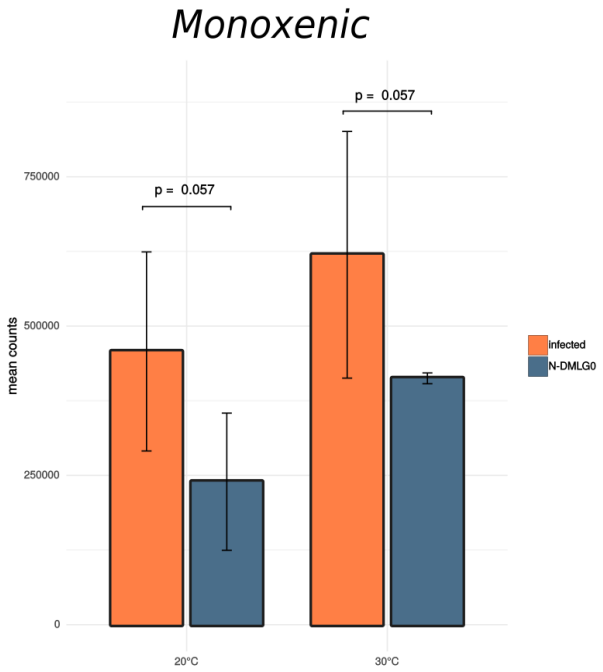

Figure A.5. Growth comparison between cured (N-DMLG0) and infected amoeba cultures. The strain *N. clarki* **N-DMLG0** (Table 4), that was cured several years ago and its corresponding, *Chlamydiales bacterium* Pn **infected** host culture N-DMLG-Pn (from which N-DMLG0 was originally cured) were grown at either 20, or 30^{o}C on NNA plates, covered with an *E. coli*-lawn. Infected cultures contained >99% infected cells at the time. Errorbars show the standard deviation from the means of four replicates. Statistical significance between infected and cured cultures was determined using a Mann-Whitney-Wilcoxon test.

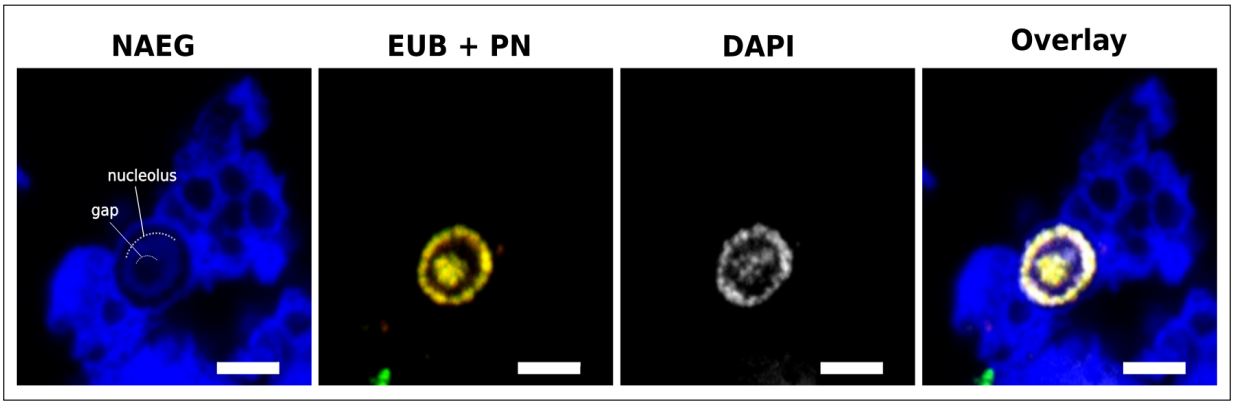

Figure A.6. Colonization by Pn of the nucleolar gap formed during initial stages of the *Naegleria* promitosis. *Naegleria* undergo a closed karyokinesis prior to cytokinesis [95]. In this initial division, the nucleolus first forms a gap, where host chromosomes accumulate for separation. During this process, *Chlamydiales bacterium* Pn cells, generally localizing around the nucleolus, can sometimes colonize this gap. Images were obtained, using confocal laser scanning microscopy. Scale bars indicate 5 μm.

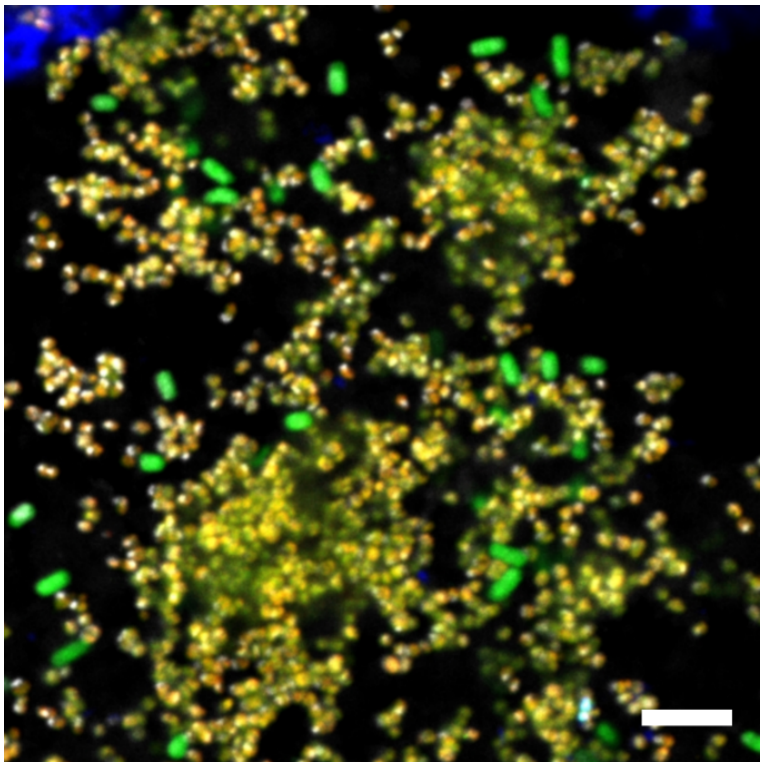

Figure A.7. FISH image of a *N. clarki* culture, infected with the symbiont Pn. Amoeba can be seen in **blue** (NAEG1088). Symbiont cells can be seen in **orange** (overlay of EUB338I-III (green) and PN440 (red)). *E. coli*, added as a food supplement can be seen in **green**. Cells were additionally stained by DAPI **(grey)**. Images were obtained, using confocal laser scanning microscopy. Scale bars indicate 5 μm.

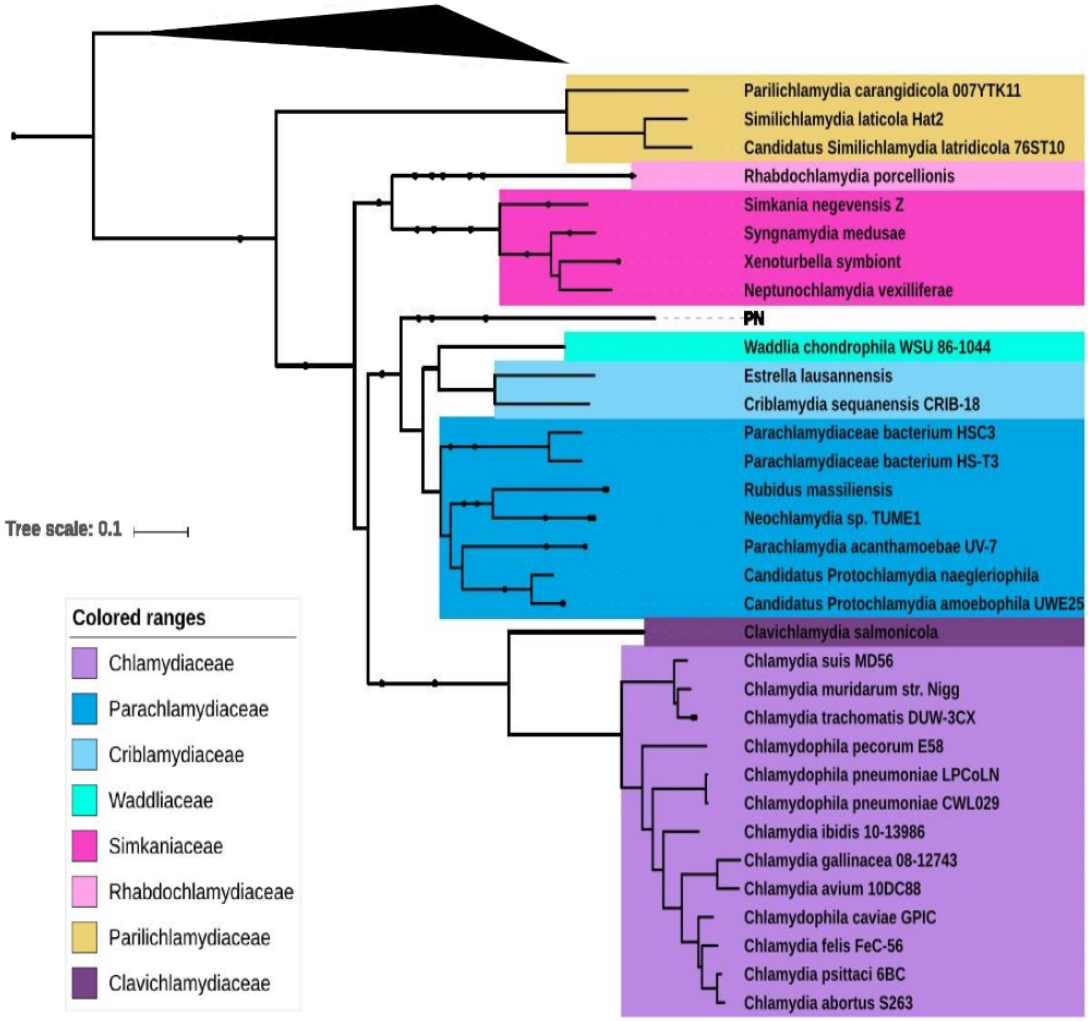

Figure A.8. Phylogenetic tree of eight *Chlamydiae* families based on the general bacterial marker set CheckM by Parks *et al.* (2015) [63], provided by S. Köstlbacher and A. Collingro (unpublished). The symbiont Pn is highlighted in bold and shown as closest related to the chlamydial family *Parachlamydiaceae*.

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