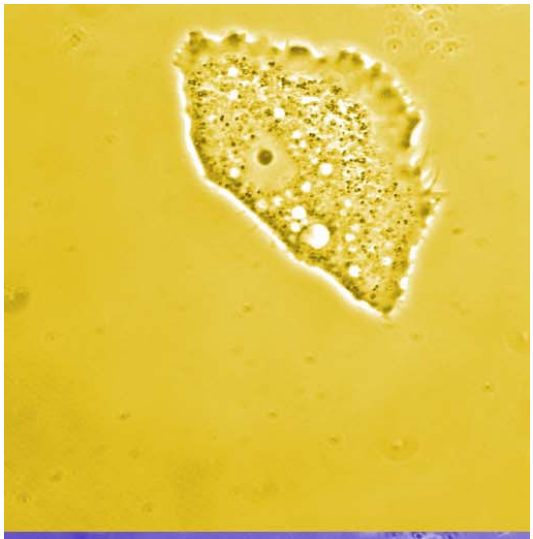

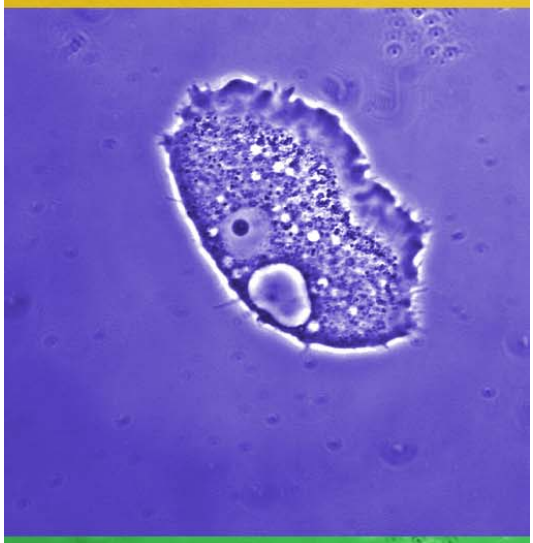

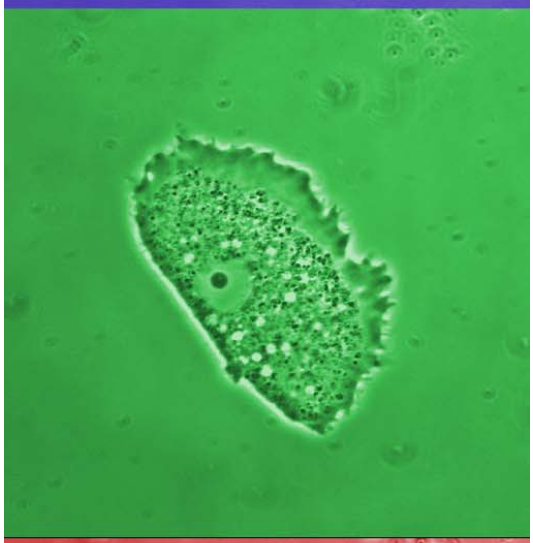

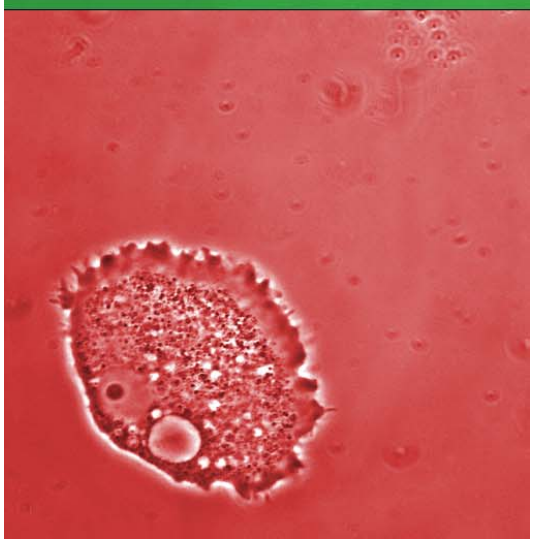

Matthias Horn

Molecular Ecology of Free-living Amoebae and Their Bacterial Endosymbionts: Diversity and Interactions

June 2001

Lehrstuhl für Mikrobiologie

Molecular Ecology of Free-living Amoebae and Their Bacterial Endosymbionts: Diversity and Interactions

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für meine Eltern

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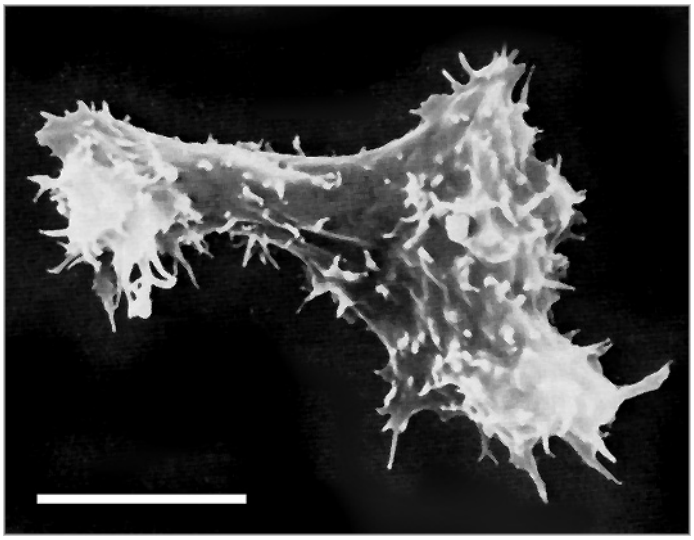
Chapter I

Introduction and Outline



Front:

Trophozoite of *Acanthamoeba castellanii*. Scanning electron micrograph.

Bar 10 µm. (From Martinez, 1985)

Free-living amoebae

Amoeba*, from the Greek "amoibe" meaning "change"*

When Antonie van Leeuwenhoek in the summer of 1674 used his self constructed microscope to have a look at a drop of water, he was the first person to observe free-living protozoa. Yet, it took another 80 years until Rösel von Rosenhof for the first time described a free-living amoeba which he called "small Proteus". Later, in 1767, Carl Linnaeus classified this amoeba as *Chaos proteus*. Today, these microorganisms have extensively been investigated and numerous free-living and parasitic amoebae have been discovered. Amoebae are unicellular motile eukaryotes that lack a cell wall and move by constantly changing the form of their bodies (a locomotion known as amoeboid movement). While parasitic amoebae are frequent commensals and pathogens of animals and men, free-living amoebae (FLA) play an important role in the environment. An introduction to diversity, biology, ecology and clinical significance of FLA is given below exemplary for members of the genus *Acanthamoeba* which were the main focus of the investigations presented in this thesis.

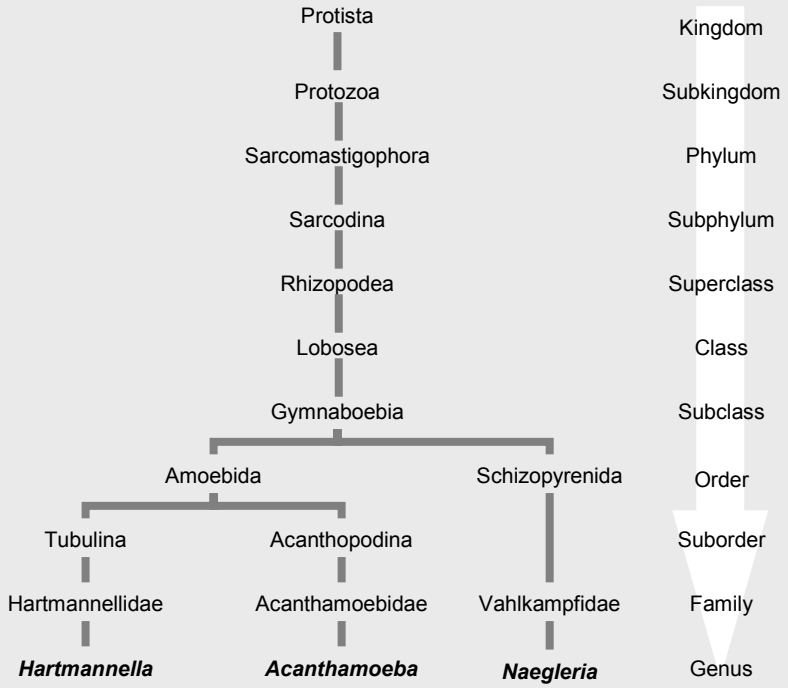


Figure 1. Taxonomic position of selected free-living amoebae (From Visvesvara, 1991).

The classification of free-living amoebae was traditionally based on morphology. Some major taxonomic groups of FLA and their position within the phylum Sarcomastigophora (amoeboflagellates) are listed in Figure 1. The genus *Acanthamoeba* was subdivided into three subgroups (I-III; Pussard and Ponds, 1977) containing more than 18 species (Page, 1976). More recently, several molecular approaches including isoenzyme pattern analysis (De Jonckhere, 1983), restriction fragment length polymorphisms (RFLP) of mitochondrial DNA (McLaughlin *et al*., 1988), and comparative 18S rDNA sequence analysis (Gast *et al*., 1996; Stothard *et al*., 1998) were used for classification of acanthamoebae. The latter method

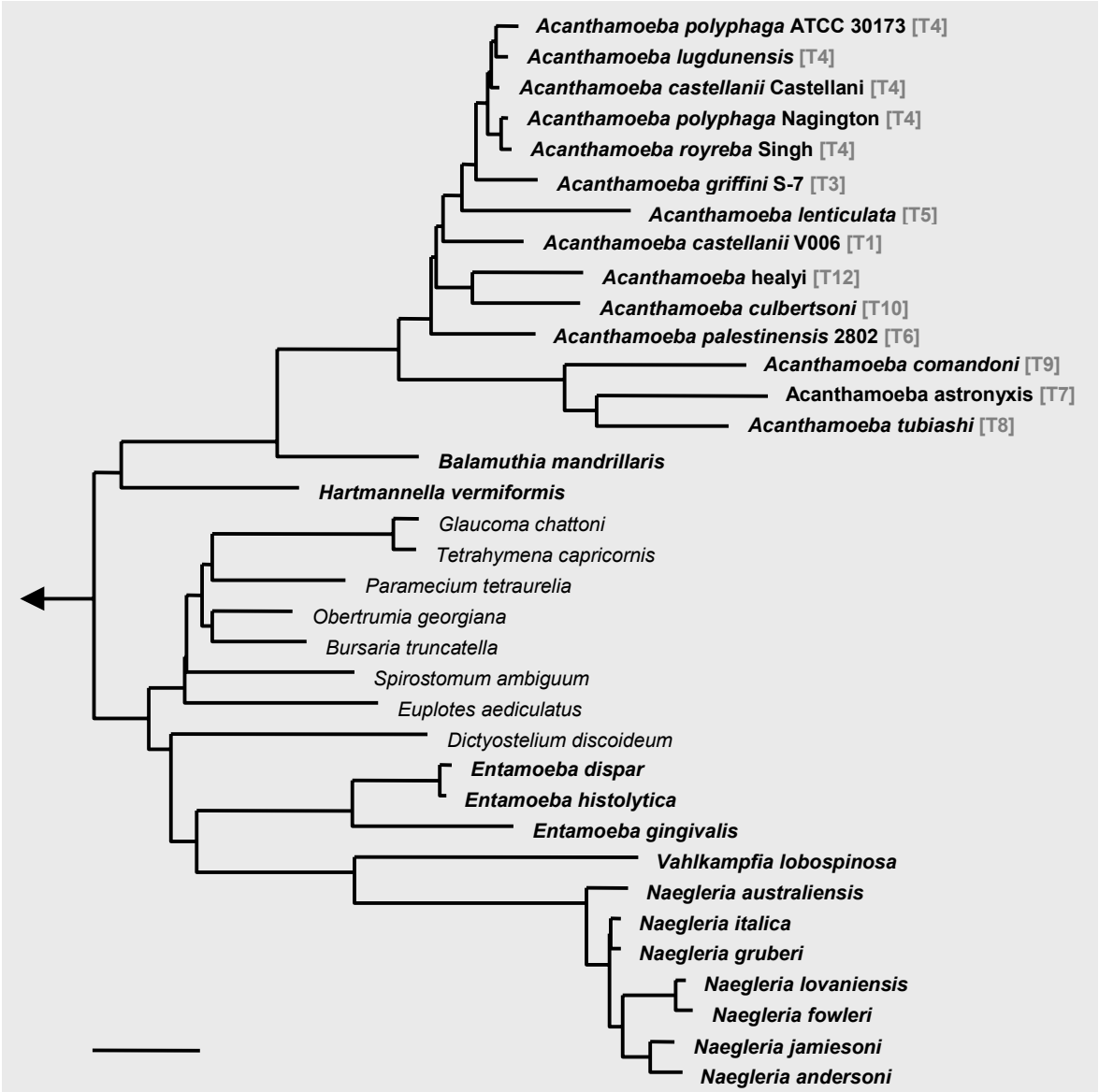


Figure 2. 18S rRNA based phylogenetic tree reflecting the relationships of major groups of free-living amoebae (bold). Representative 18S rRNA *Acanthamoeba* genotypes are noted in square brackets. Arrow, to outgroup. Bar, 10% estimated evolutionary distance.

demonstrated that the genus *Acanthamoeba* is monophyletic and led to the description of twelve 18S rDNA genotypes, each comprising amoebal strains showing at least 95% 16S rDNA sequence similarity with each other (Figure 2; Stothard *et al*., 1998). Based on these rDNA sequence data, oligonucleotide probes for the specific detection of acanthamoebae could be developed and were successfully used for the identification of amoebae by fluorescence *in situ* hybridization (Figure 3; Stothard *et al*., 1999; Horn, unpublished data).

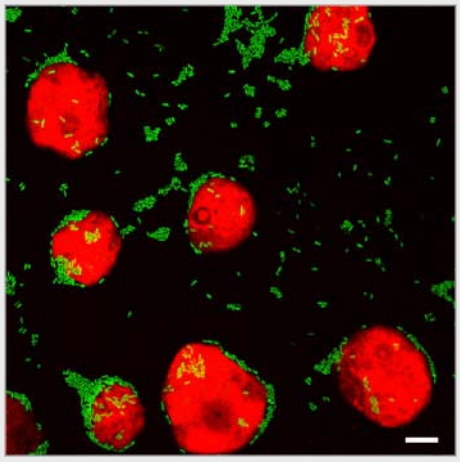


Figure 3. Identification of *Acanthamoeba* sp. by fluorescence *in situ* hybridization (FISH) using a specific probe for acanthamoebae (red) and for bacteria (green), respectively, in an artificial mixture of *Acanthamoeba* and *E. coli* pure cultures. Bar, 10 µm.

Acanthamoebae are ubiquitous free-living amoebae that are distributed worldwide, living in diverse environments. They were isolated from various water sources (freshwater, seawater, swimming pools, municipal water supplies, bottled mineral water, air-conditioning units), soil, sewage, compost, dust and the air. In addition, a number of animals, including fish, reptiles, birds, and mammals have been found to harbor acanthamoebae (Walker, 1996). Due to their bi-phasic life cycle acanthamoebae are able to survive unfavorable environmental conditions (Figure 4). The amoebal trophozoites posses an active metabolism, are able to nourish, and replicate by binary fission. Environmental stress induces the formation of metabolically inactive cysts, which show an increased resistance against dryness, heat, radiation, and disinfectants (like chlorine). In contrast to other free-living amoebae, for example *Naegleria* spp., members of the genus *Acanthamoeba* lack a flagellate stage (Page and Siemensma, 1991). Acanthamoebae live at interfaces (water-soil, water-plants, water-air) and need to be attached onto a surface during the vegetative growth phase. Like other FLA, they mainly feed on bacteria but also phagocytose fungi, yeasts, algae and other protozoa. Between 600 and 8,300 bacterial cells are necessary to produce one trophozoite (Bryant *et al*., 1982; Danso and Alexander, 1975). The grazing activity of FLA may thus produce a decline in the number of bacteria from 10^8 to 10^5 per gram of soil. Therefore FLA are considered important predators controlling microbial communities (Rodriguez-Zaragoza, 1994). Their presence stimulates the bacterial turnover of

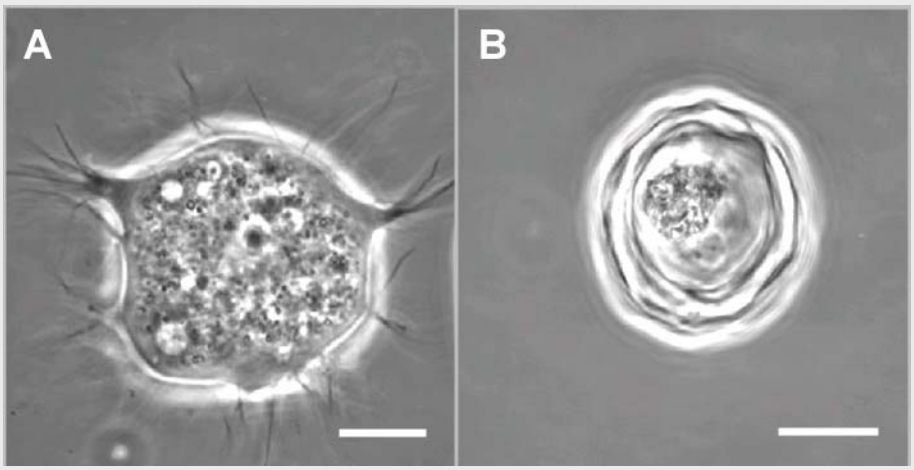


Figure 4. Life cycle of acanthamoebae. The trophozoites (A) measure between 24-56 µm in diameter and are characterized by needle-like pseudopodia (acanthopodia), one contractile vacuole, and the typical appearance of the single nucleus, which is also referred to as "bull's eye". In contrast to other FLA, *Acanthamoeba* trophozoites move very slowly. Considerable variation in cyst morphology occurs among the different species of *Acanthamoeba*. The double walled cysts (B) measure 12-30 µm in diameter and show an increased resistance in the environment. The cellulose-containing cyst wall is made up of an outer wrinkled ectocyst and an inner endocyst. Bar, 10 µm.

phosphorus and nitrogen fixation (Clarholm, 1981). In addition, a direct participation of FLA in the nitrogen cycle was suggested (Rodriguez-Zaragoza, 1994) based on the finding that *Acanthamoeba polyphaga* and *Acanthamoeba castellanii* do not need pre-synthesized purines and pyrimidines and have a highly active nitrogen metabolism (Weekers and Van der Drift, 1993).

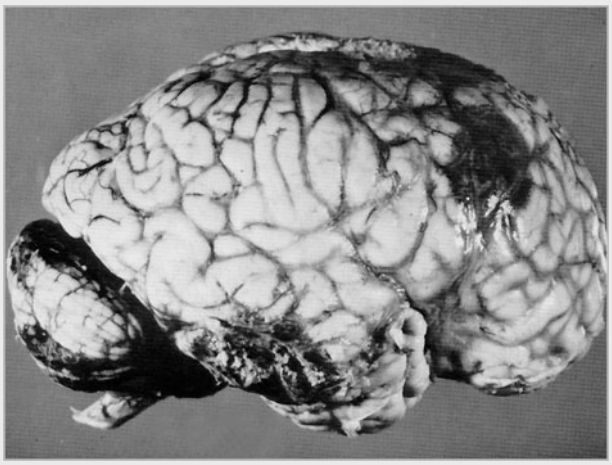


Figure 5. GAE. Lateral view of cerebral hemispheres and cerebellum of a human brain. Isolated areas of congestion are noted. (From Martinez, 1985.)

Besides their ecological significance, acanthamoebae have been recognized as opportunistic pathogens and are thus also referred to as amphizoic amoebae. They were first associated with human infection by Culbertson and co-workers in 1958 who observed the cytopathogenic effect of an *Acanthamoeba* isolate (now designated *A. culbertsoni*) on a monkey kidney tissue culture and subsequently demonstrated amoebal trophozoites in brain lesions of mice that died within a week after intracerebral inoculation with these amoebae (Culbertson *et al*., 1958; Culbertson *et al*., 1966). Since the

first case report of chronic granulomatous amebic encephalitis (GAE, Figure 5) in men in the early 1960s more than 110 cases of this fatal disease occurred worldwide (Visvesvara, 1995). While the free-living amoeba *Naegleria fowleri*, which causes a similar disease (primary amoebic encephalitis, PAM), is able to infect healthy individuals, GAE was only reported in immunosuppressed or otherwise chronically ill patients. Especially in patients with human immunodeficiency virus (HIV) or AIDS infections with acanthamoebae can also lead to ulcerative skin lesions or abscesses and may even result in systemic infections (Visvesvara, 1995).

Another, more common disease caused by acanthamoebae is *Acanthamoeba* keratitis, a painful and vision-threatening infection of the human cornea (Figure 6; Gautom *et al*., 1997). Several hundred cases have been reported worldwide and showed that the use of contact lenses is the major risk factor for *Acanthamoeba* keratitis. The invasion of the corneal epithelium by amoebal trophozoites through micro-lesions results in a characteristic 360° or partial ring infiltrate and recurrent epithelial breakdown. Untreated *Acanthamoeba* keratitis eventually leads to blindness (Gautom *et al*., 1997).

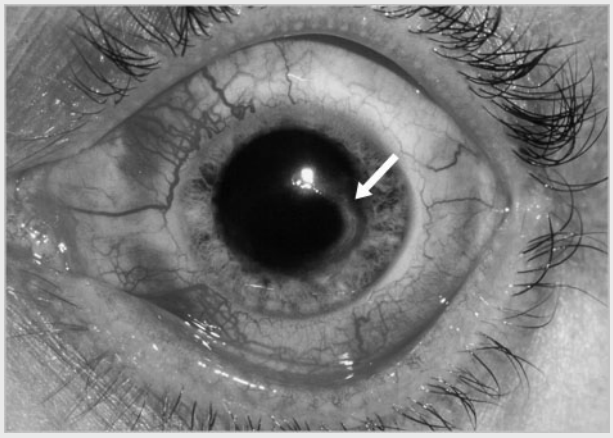


Figure 6. *Acanthamoeba* keratitis. The characteristic paracentral ring infiltrate is noted (arrow).

Facultative endosymbionts

"[...] the lysis was due to the infection of amoebae with an unknown rod-like bacterium."
W. Drozanski, 1956

Even though acanthamoebae feed on other microorganisms by phagocytosis, several bacteria are able to survive the uptake into the amoebal cell, multiply within altered phagosomes, and in some cases eventually lyse the amoebal host cell. This observation was first reported by Drozanski in 1956 who found a bacterium resembling *Pseudomonas* as agent

of a fatal infection of acanthamoebae (Drozanski, 1956). In the meantime a wide range of bacteria, including several human pathogens, have been identified as being able to thrive within *Acanthamoeba* cells (Table 1). The intra-protozoal growth has been associated with enhanced environmental survival of the bacteria, increased resistance against antibiotic substances (Barker *et al*., 1999), and in some cases with increased virulence (Cirillo *et al*., 1994, Cirillo *et al*., 1997). To cite one example, the obligate anaerobe bacterium *Mobiluncus curtisii* is able to survive in an otherwise aerobic environment by multiplication and persistence within acanthamoebae. Free-living amoebae were thus suggested as possible new sources, reservoirs and transfer mechanisms of infections caused by obligate anaerobic bacteria (Tomov *et al*., 1999).

Table 1. Facultative endosymbionts of acanthamoebae

Chlamydia pneumoniae	Essig *et al*., 1997
Listeria monocytogenes	Ly and Müller, 1990
Mycobacterium avium	Steinert *et al*., 1998
Burkholderia cepacia	Marolda *et al*., 1999
Burkholderia pickettii	Michel and Hauröder, 1997
Escherichia coli O157	Barker *et al*., 1999
Legionella pneumophila	Rowbotham, 1986
Legionella lytica	Adeleke, 1996
Pseudomonas aeruginosa	Michel *et al*., 1995
Mobiluncus curtisii	Tomov *et al*., 1999

In particular the interaction between free-living amoebae and *Legionella pneumophila*, the causative agent of a severe community-acquired pneumonia (Legionaire's disease), was subject of many studies, demonstrating, that *L. pneumophila* depends on intra-amoebal multiplication prior to the infection of men (Rowbotham, 1986). Moreover, reproduction of *Legionella* seems to be restricted to protozoan cells, since they are unable to multiply extracellularly in environmental water bodies (Abu Kwaik *et al*., 1998). Thus free-living amoebae are considered to represent natural hosts for legionellae (Atlas *et al*. 1999).

Following the attachment of *L. pneumophila* to the amoebal cell via type IV pili (competence- and adherence-associated pili, CAP), the bacteria are phagocytosed, actively retard the lysosomal fusion and rapidly multiply within the amoebal phagosome. In their stationary growth phase the legionellae trigger a cytolytic mechanism mediated by a pore-forming activity, which eventually leads to lysis of the amoebal cell and the release of vesicles filled with bacteria, which represent the infective particles for men (Berk *et al*., 1998; Gao and Abu Kwaik, 2000). Interestingly, *L. pneumophila* is also able to survive amoebal

cyst formation. Within the *Acanthamoeba* cysts they are protected against chlorine at concentrations commonly used for disinfection (Kilvington and Price, 1990).

Taking advantage of the ability of legionellae to thrive within FLA, acanthamoebae have successfully been used for the recovery of viable *L. pneumophila* from clinical and environmental samples (Rowbotham, 1986), and for resuscitation of viable but non-culturable legionellae (Steinert *et al*., 1997).

In addition to *L. pneumophila*, a number of studies reported on the occurrence of intracellular bacteria in FLA, which also lysed their amoebal hosts and thus were designated *Legionella*-like amoebal pathogens (LLAP). Most of these LLAPs were originally found in amoebal isolates from sources associated with confirmed cases or outbreaks of Legionnaire's disease (Adeleke *et al.*, 1996). In addition, one LLAP strain was recovered by amoebal enrichment from a sputum specimen of a patient with pneumonia (Rowbotham, 1993). While clinical samples from this patient were negative for *L. pneumophila* and attempts to cultivate this LLAP on media commonly used for the propagation of legionellae failed, treatment for *Legionella* infection was successful and the patient recovered. However, the role of LLAPs in respiratory disease of humans is still unclear. Although the LLAPs have initially been considered non-culturable, some of them were meanwhile successfully isolated on cell-free media (Adeleke *et al*., 1996).

Obligate endosymbionts

> "*The presence of [...] bacterial bodies free in the amoebal cytoplasm [...] as well as the aspects suggestive of cell division led the authors to consider them as integrated in the* Acanthamoeba *Sn economy, correlating them with bacterial endosymbionts."*
>
> Mara Proca-Ciobanu *et al*., 1975

In addition to the transient associations of bacteria and free-living amoebae described above, also long term relationships have been reported. An extraordinary case of symbiosis between FLA and intracellular bacteria has its origin in the mid 1960s when a culture of the *Amoeba proteus* strain D became spontaneously infected with bacteria (Jeon and Lorch,

1968). In the beginning these bacteria (designated X-bacteria) were virulent and heavily multiplied within the amoebal trophozoit, leading to almost complete effacement of the amoebal culture. During the course of a few years the average number of bacteria per amoebal cell decreased from 100,000 to 42,000. The host amoeba grew normally under standard culture conditions, and eventually became dependent on their symbionts for survival (Jeon, 1995). At that point, treatment with antibiotics failed to produce symbiont-free amoebae. Jeon and colleagues showed that the symbiont-bearing amoebae no longer produce a certain cytoplasmic protein necessary for the survival of the eukaryote. Instead, this essential enzyme activity derived from a protein produced by the X-bacteria (Choi *et al*., 1997; Jeon, 1995). These findings for the first time demonstrated the transition of virulent bacteria to obligate endosymbionts necessary for survival of their eukaryotic hosts and thereby provides evidence for the plausibility of the endosymbiotic theory for the origin of eukaryotic cells (Margulis, 1975).

The presence of natural bacterial endosymbionts in an environmental *Acanthamoeba* isolate was first noted by Proca-Ciobanu and co-workers (Proca-Ciobanu *et al*., 1975). These intracellular bacteria could not be maintained outside their amoebal host cells and were thus considered obligate endosymbionts. The first systematic survey of this phenomenon was performed by Fritsche and colleagues, who found that nearly 25% of all clinical and environmental *Acanthamoeba* isolates included in this study contained bacterial endosymbionts (Fritsche *et al*., 1993). Giemsa-, Gram-staining, and electron microscopy revealed that all symbionts possessed a Gram-negative type cell wall, while their morphology differed, suggesting that *Acanthamoeba* endosymbionts comprise a diverse taxonomic assemblage (Figure 7).

A more comprehensive characterization of these bacteria was in the following hampered due to their obligate intracellular life style. This changed dramatically with the introduction of the ribosomal RNA (rRNA) as universal phylogenetic marker molecule by Carl Woese (Woese, 1987). This approach not only allowed to create a classification system that reflects the natural affiliations between bacteria, but also enabled the culture-independent analysis of complex microbial communities (Ward *et al*., 1990). In a next step, the application of fluorescently labeled oligonucleotide probes targeting rRNA signature regions permitted the specific identification of uncultured bacteria directly within environmental or clinical specimens on a single-cell level (DeLong *et al*., 1989; Amann *et al*., 1995). The suitability of

this strategy to investigate obligate bacterial endosymbionts of protozoa was first demonstrated by Amann and co-workers who used the so called "full cycle rRNA approach" to characterize *Holospora obtusa*, a macronuclear symbiont of *Paramecium caudatum* (Amann *et al*., 1991). In a similar manner, the phylogenetic position of *Legionella*-like amoebal pathogens (LLAP) was resolved, which were indeed closely affiliated with the genus *Legionella*. Consequently, the name *Legionella lytica* was proposed for certain LLAPs (Springer *et al*., 1992; Adeleke *et al*., 1996; Hookey *et al*., 1996). Also the first obligate endosymbiont of *Acanthamoeba* spp. was recently identified using this molecular approach (as a novel member of the order *Chlamydiales*; Amann *et al*., 1997). However, the majority of *Acanthamoeba* symbionts remained uncharacterized.

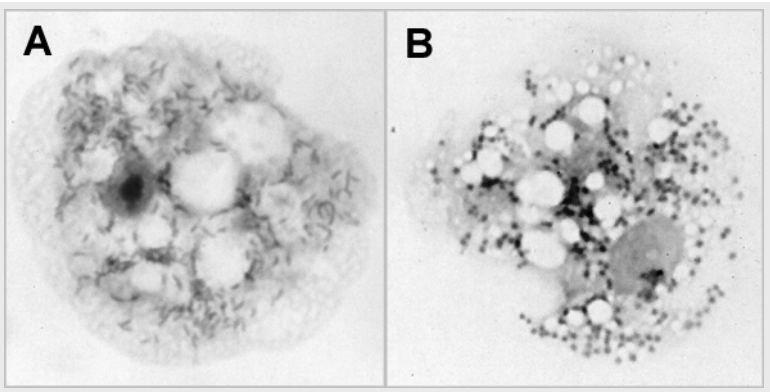


Figure 7. Rod-shaped (A) and coccoid (B) obligate endosymbionts *Acanthamoeba* spp. visualized using Giemsa-stain. (From Fritsche *et al*., 1993)

In summary, free-living amoebae have been recognized as transportation and multiplication vehicles for a variety of bacteria including important human pathogens. In addition, many FLA contain hitherto unidentified obligate endosymbionts. The presence of these multiple interactions with bacteria render amoebae a promising model system for future analysis of evolution and mechanisms of symbiosis between bacteria and eukaryotic hosts, which may help to understand the adaptations of bacteria for intracellular survival. A prerequisite for such studies is a detailed knowledge on the phylogeny, ecology and distribution of the host amoebae and their natural bacterial endosymbionts, a challenge that is met in this thesis.

Outline

The following chapters report on the investigation of bacterial endosymbionts found in environmental and clinical isolates of free-living amoebae (chapters II to VII). Amoebal strains isolated previously or obtained from the American Type Culture Collection (ATCC) mainly originated from sampling sites in North America. To obtain a geographically more balanced collection, additional amoebal strains harboring endosymbionts were recovered from environmental sources in Germany and Malaysia and adapted to axenic growth conditions. Investigations consisted of (i) attempts to culture the bacterial symbionts on cell-free media, (ii) assessment of the host range of the bacterial endosymbionts by transfer experiments to other amoebal species, (iii) electron microscopic analysis of morphology and intracellular location of the bacterial symbionts, and (iv) analysis of bacteria and host phylogeny by the full cycle rRNA approach. The latter included comparative rRNA sequence analysis, and identification of the endosymbionts directly within their host cells by fluorescence *in situ* hybridization (FISH) using specific rRNA-targeted oligonucleotide probes. In total, five different, previously not recognized evolutionary lineages of bacterial endosymbionts, which were present in both amoebal cysts and trophozoites, were identified in these studies.

Chapter II reports on the identification of rod-shaped, Gram-negative endosymbionts of *Acanthamoeba* spp. isolated in North America as novel members of the *Rickettsiales*. The *Acanthamoeba* symbionts formed a novel, deep branching evolutionary lineage within this phylogenetic group, which exclusively comprises intracellular bacterial symbionts, commensals, parasites and pathogens of arthropods, crustaceans, birds, mammals, and men.

The characterization of morphologically similar endosymbionts of acanthamoebae is described in Chapter III. These endosymbionts also grouped in the α-subclass of *Proteobacteria*, but were most closely affiliated with *Caedibacter caryophilus*, a makronuclear symbiont of *Paramecium caudatum*. Thus they were tentatively designated *Candidatus* Caedibacter acanthamoebae, *Candidatus* Paracaedibacter acanthamoebae and *Candidatus* Paracaedibacter symbiosus. Comparison of rRNA based phylogenetic dendrograms of the symbionts and their corresponding hosts suggested co-evolution between the *Acanthamoeba* hosts and the bacterial symbionts.

The *Acanthamoeba* endosymbionts analyzed in chapter IV were found to represent novel members of the β-subclass of *Proteobacteria*, a diverse phylogenetic group that in contrast to the α-*Proteobacteria* comprises only few obligate intracellular bacteria. The provisional name "*Canditatus* Procabacter acanthamoebae" gen. nov., sp., nov., pertaining to M. Proca-Ciobanu who for the first time described bacterial endosymbiont in acanthamoebae (Proca-Ciobanu *et al*., 1975) was proposed for designation of these bacteria.

Chapter V reports on an extraordinary *Acanthamoeba* isolate recovered from an eutrophic lake sediment in Malaysia. This amoebal strain was found to contain extremely large numbers of bacterial endosymbionts. Nevertheless, this symbiosis did not cause lysis of the amoebal host cells. Phylogenetic analysis placed this bacterial endosymbiont, which showed a pronounced host specificity, within the phylum *Cytophaga-Flavobacterium-Bacteroides*, and the name "*Candidatus* Amoebophilus asiaticus" was proposed for its classification.

Chapters VI and VII describe the identification of several obligate endosymbionts of *Hartmannella* and *Acanthamoeba* as novel *Chlamydia*-related bacteria, which were demonstrated by electron microscopy to possess developmental stages similar to the elementary and reticulate bodies of chlamydiae. While the order *Chlamydiales* traditionally comprised exclusively important bacterial pathogens of animals and men, the finding of these endosymbionts significantly expanded our knowledge on chlamydial diversity and raised the question of clinical significance of these microorganisms.

In order to further investigate distribution and diversity of *Chlamydia*-related bacteria in the environment, a PCR assay was developed and used for the analysis of different waste water treatment plants. The application of this PCR assay, described in Chapter VIII, provided evidence for additional genus-level diversity of environmentally occurring members of the *Chlamydiales*.

Chapter IX shortly summarizes the findings of the preceding papers. Additional results from experiments focussing on the interaction between acanthamoebae and their bacterial

endosymbionts are presented and the findings are discussed particularly with regard to the current literature.

The Appendix contains a German review article on obligate endosymbionts of *Acanthamoeba spp.* published in the journal *Biologie in unserer Zeit* (Horn and Wagner, 2001), as well as the list of publications and scientific activities, and the curriculum vitae of the author.

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Chapter II

In Situ Detection of Novel Bacterial Endosymbionts of *Acanthamoeba* spp. Phylogenetically Related to Members of the Order *Rickettsiales*

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In Situ Detection of Novel Bacterial Endosymbionts of *Acanthamoeba* spp. Phylogenetically Related to Members of the Order *Rickettsiales*

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Acanthamoebae are ubiquitous soil and water bactivores which may serve as amplification vehicles for a variety of pathogenic facultative bacteria and as hosts to other, presently uncultured bacterial endosymbionts. The spectrum of uncultured endosymbionts includes gram-negative rods and gram-variable cocci, the latter recently shown to be members of the Chlamydiales*. We report here the isolation from corneal scrapings of two* Acanthamoeba *strains that harbor gram-negative rod endosymbionts that could not be cultured by standard techniques. These bacteria were phylogenetically characterized following amplification and sequencing of the near-full-length 16S rRNA gene. We used two fluorescently labelled oligonucleotide probes targeting signature regions within the retrieved sequences to detect these organisms* in situ*. Phylogenetic analyses demonstrated that they displayed 99.6% sequence similarity and formed an independent and well-separated lineage within the* Rickettsiales *branch of the alpha subdivision of the* Proteobacteria*. Nearest relatives included members of the genus* Rickettsia*, with sequence similarities of approximately 85 to 86%, suggesting that these symbionts are representatives of a new genus and, perhaps, family. Distance matrix, parsimony, and maximum-likelihood tree-generating methods all consistently supported deep branching of the 16S rDNA sequences within the* Rickettsiales*. The oligonucleotide probes displayed at least three mismatches to all other available 16S rDNA sequences, and they both readily permitted the unambiguous detection of rod-shaped bacteria within intact acanthamoebae by confocal laser-scanning microscopy. Considering the long-standing relationship of most* Rickettsiales *with arthropods, the finding of a related lineage of endosymbionts in protozoan hosts was unexpected and may have implications for the preadaptation and/or recruitment of rickettsia-like bacteria to metazoan hosts.*

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Introduction

Members of the *Rickettsiales* comprise a diverse group of bacteria, most of which are small gram-negative rods that exist as either parasitic or mutualistic symbionts within eukaryotic cells. Beyond these common characteristics, uncertainty about their classification is mostly due to the difficulties of working with obligate intracellular bacteria. In addition to the usual genera (*Rickettsia*, *Orientia*, *Neorickettsia*, *Ehrlichia*, *Wolbachia*, *Cowdria*, and *Bartonella*, among others) historically included in the *Rickettsiales* on the basis of phenotypic and/or genotypic data (12, 36, 46, 48), a large number of "rickettsia-like" endosymbiotic bacteria that are associated with protozoa, insects and other invertebrates, and fungi are incompletely described (33). Previously, we reported the occurrence of noncultured bacterial endosymbionts in both clinical and environmental isolates of *Acanthamoeba* spp. (16). To date, 17 (22%) of 78 axenically growing *Acanthamoeba* strains we maintain contain endosymbionts, including the presence of gram-negative rods (GNR) in 17% (13 amoebic isolates) and gram-negative cocci (GNC) in 5% (4 amoebic isolates) (15). Preliminary phylogenetic analyses of three of the GNC strains revealed that they were most closely related to but distinct from the genus *Chlamydia*, while one of the GNR was shown to display affinities to the *Rickettsiales* (19). This is consistent with other recent reports describing the recovery of *Chlamydia*-like endosymbionts in *Acanthamoeba* spp. (2, 8) and an *Ehrlichia*-like endosymbiont within an isolate of *Saccamoeba* (30). The finding of protozoal endosymbionts closely related to members of the *Chlamydiales* and *Rickettsiales* adds to the diversity of bacterial lineages that adapted themselves to intracellular survival within amoebae. While the life cycles of the *Chlamydiales* and *Rickettsiales* are typically dependent upon an intracellular habitat for survival and growth, a variety of facultatively growing bacteria, most notably members of the *Legionellaceae*, also survive and multiply within amoebic hosts. Such host-symbiont interactions are thought to be critical in the epidemiology of legionellosis (3, 6, 14, 37). *Acanthamoeba* spp. are increasingly recognized as serious human pathogens responsible for keratitis, granulomatous encephalitis, and both focal and systemic disease in immunocompromised hosts, although the mechanisms of pathogenesis are poorly understood (20). Due to the recent observation of putative enhancement of cytopathogenicity of *Acanthamoeba* following acquisition of noncultured GNR and GNC bacterial endosymbionts (17) and the potential of GNC endosymbionts to directly produce human disease (8), a more detailed characterization of *Acanthamoeba* endosymbionts may be of clinical relevance. In this paper, we present details of the morphologic and phylogenetic analyses of two GNR endosymbionts infecting axenically maintained isolates of *Acanthamoeba* originally

recovered from patients with amoebic keratitis. Because these bacterial isolates could not be cultivated by standard microbiological approaches, we undertook a comparative analysis of their 16S rRNA genes to determine their phylogenetic affiliations. Fluorescently labelled oligonucleotide probes targeting signature regions within the retrieved 16S rDNA sequences were subsequently designed for *in situ* hybridization to further assist with the characterization and intracellular localization of individual bacterial cells.

Materials and Methods

Isolation and maintenance of *Acanthamoeba* strains. The techniques used for recovery and maintenance of acanthamoebae from clinical and environmental sources are described elsewhere (16, 44). Briefly, primary isolation was performed from infected human corneal tissues by using 1.5% nonnutrient agar plates seeded with live *Escherichia coli*. Subsequent incubation was performed at ambient temperature (22 to 24°C) for up to 10 days. Upon evidence of growth, clonal cultures were established by transference of a single double-walled cyst to fresh medium. The use of heat-killed *E. coli* and/or incorporation of antibiotics (penicillin, 100 mg/ml; streptomycin, 10 mg/ml; and amphotericin B, 0.25 mg/ml) in subsequent subcultures resulted in axenic growth. Clones were then adapted to growth in sterile tryptic soy-yeast extract broth. Two isolates of *Acanthamoeba* (UWC8 and UWC36) known to be infected with intracellular, rod-shaped bacteria that are readily detected by Gram, Giemsa, and fluorochrome staining methods were included in this study. General phenotypic characteristics of both endosymbiont strains, including an electron micrograph of UWC8, have been described previously (16, 18).

DNA isolation, PCR amplification, cloning, and sequencing. Amoebae and their endosymbionts were harvested from axenic cultures, washed twice with double-distilled water, and resuspended in 500 ml of an appropriate lysis buffer. UWC8 amoebae were lysed in STE buffer (2% sodium dodecyl sulfate [SDS], 10 mM EDTA, 50 mM Tris HCl [pH 8.0]) containing 0.3 mg of proteinase K per ml by incubation at 37°C for 2 h, followed by 5 min of gentle inversion at room temperature (28); UWC36 amoebae were lysed in UNSET buffer (8 M urea, 2% SDS, 0.15 M NaCl, 0.001 M EDTA, 0.1 M Tris-HCl [pH 7.5]) by incubation at 60°C for 5 min (23). The lysates were extracted twice with phenol-chloroform, and DNA was precipitated with 2 volumes of absolute ethanol. Oligonucleotide primers targeting highly conserved 16S rDNA signature regions within the domain *Bacteria* were used for PCR to obtain near-full-length bacterial 16S rRNA gene fragments. The nucleotide sequences of the forward and reverse primers used for amplification of UWC8 were, respectively, 5'-AG AGTTTGATCCTGGCTCAG-3' and 5' ACGGCTACCTTGTTACGACTT-3' (47), while those used for UWC36 were, respectively, 5'-AGAGTTTGATYMTGGCTCAG-3' (*Escherichia coli* 16S rDNA positions 8 to 27) and 5'-CAKAA AGGAGGTGATCC-3' (*E. coli* 16S rDNA positions 1529 to 1546) (9). Amplification reactions for UWC8 were performed in a 100-ml reaction volume in a programmable thermal cycler (Perkin-Elmer, Foster City, Calif.) with the GeneAmp PCR reagent kit (Perkin-Elmer) as recommended by the manufacturer. Thermal cycling consisted of 35 cycles of denaturation at 94°C for 1.5 min, annealing at 42°C for 1 min, and elongation at 72°C for 4 min, with a final elongation step at 72°C for 20 min. Amplification reactions for UWC36 were performed in a reaction volume of 50 ml in a thermal capillary cycler with reaction mixtures, including a 20 mM MgCl2 reaction buffer, prepared as recommended by the manufacturer (Idaho Technology, Idaho Falls, Idaho) with *Taq* DNA polymerase (Promega, Madison, Wis.). Thermal cycling consisted of an initial denaturation step at 94°C for 30 s followed by 30 cycles of denaturation at 94°C for 20 s, annealing at 52°C for 15 s, and elongation at 72°C for 30 s, with a final elongation step at 72°C for 1 min. Positive controls containing purified DNA from *E. coli* were included along with negative controls (no DNA added). The presence and size of the amplification products were determined by 0.8% agarose gel electrophoresis and ethidium bromide staining of the reaction product. Amplified DNA from UWC8 was purified by electrophoresis in low-melting point agarose and ligated into the cloning vector Bluescript II (Stratagene, La Jolla, Calif.), while amplified DNA from UWC36 was directly ligated into the cloning vector pCR2.1 (Invitrogen, Carlsbad, Calif.), with subsequent transformation of *E. coli* by each vector. Nucleotide sequences of the cloned DNA fragments were determined by automated

dideoxynucleotide methods with the Taq DyeDeoxy Terminator cycle-sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, Calif.) for UWC8 and the Thermo Sequenase cycle-sequencing kit (Amersham Life Science, Little Chalfont, England) for UWC36.

Phylogenetic analysis. 16S rDNA sequences were added, with the program package ARB, to the 16S rRNA sequence database of the Technischen Universität München, which encompasses about 10,000 published and unpublished homologous small-subunit rRNA primary structures (11, 26, 42). Alignment of sequences was performed with the ARB automated alignment tool. Alignments were refined by visual inspection and by secondary-structure analysis. Phylogenetic analyses were performed by applying ARB parsimony, distance matrix, and maximum-likelihood methods to different data sets. To determine the robustness of phylogenetic trees, analyses were performed both on the original data set and on a data set from which highly variable positions were removed by use of a 50% conservation filter for the members of the *Rickettsiales* (24). A check for chimeric sequences was conducted by independently subjecting the first, second, and third 513-base positions (59 to 39) to independent phylogenetic analyses.

***In situ* identification and detection of *Acanthamoeba* endosymbionts.** *Acanthamoeba* cells were harvested from 3 ml of liquid broth culture by centrifugation (200 3 *g* for 3 min), washed briefly with 1 ml of Page's saline (44), and pretreated for *in situ* hybridization (39). The specific pretreatments included (i) resuspension of amoebic cells in a 1:3 ratio of Page's saline and 4% paraformaldehyde for 12 h at 4°C, spotting of 30 ml of the cell suspension onto glass slides, and air drying; (ii) resuspension of cells in a 1:9 ratio of saturated mercuric chloride and Page's saline for 12 h at 4°C, washing with 1 ml of Page's saline, spotting of 30 ml onto slides, air drying, and dehydration by immersion in 80% ethanol for 5 to 10 s; (iii) resuspension of cells in 0.4% trichloroacetic acid in Page's saline for 15 min at room temperature followed by processing as for (ii); (iv) resuspension of cells in Page's saline followed by spotting of 50 ml of suspension on a glass slide, storing the slide in a moisture chamber for 2 h to permit natural cell attachment, immersion in 80% ethanol for 10 to 30 s, and air drying; and (v) resuspension of cells in a solution containing 0.05% (final concentration) agarose in Page's saline, spotting of 20 ml onto a slide, air drying, and immersion in 80% ethanol for 5 to 10 s. Oligonucleotide probes S-*-AcEnd-0090-a-A-18 (AcRic90) and S-*-AcEnd- 1196-a-A-18 (AcRic1196), both specific for UWC8 and UWC36 endosymbionts, were designed by using the Probedesign/Probematch tools of ARB (42); the probes were designated according to the standard proposed by Alm et al. (1). Oligonucleotides were synthesized and directly labeled with the hydrophilic sulfoindocyanine fluorescent dye Cy3 or Cy5 (Interactiva, Ulm, Germany). Optimal hybridization conditions for the probes were determined by using the hybridization and wash buffers (with and without SDS) described by Manz et al. (27). Negative control *in situ* hybridization experiments were performed with Cy3- and Cy5-labelled derivatives of the oligonucleotide probe BET42a, specific for the beta subclass of *Proteobacteria* (27). The slides were examined with a confocal laser-scanning microscope (LSM 510; Carl Zeiss, Oberkochen, Germany) with two helium-neon lasers (543 and 633 nm). Image analysis processing was performed with the standard software package delivered with the instrument (version 1.5). Staining of endosymbiont-harboring amoebic cells with 49,6-diamidino- 2 phenylindole (DAPI) after *in situ* hybridization was performed by incubation with 1 mM aqueous DAPI solution for 4 min at room temperature.

Electron microscopy. Amoebic strains in which symbioses were detected by conventional microscopy were further examined by electron microscopy, using a variation of published methods (21). Briefly, aliquots of amoebae in broth were fixed with 2% glutaraldehyde in 0.1 M cacodylate. The fixed amoebae were then pelleted in agar and embedded. Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips CM-10 electron microscope.

Nucleotide sequence accession numbers. Recovered 16S rDNA sequences were deposited in GenBank under accession no. AF069962 (endosymbiont of *Acanthamoeba* sp. strain UWC36) and AF069963 (endosymbiont of *Acanthamoeba* sp. strain UWC8).

Table 1. Overall 16S rRNA sequence similarities for the UWC8 and UWC36 endosymbionts and affiliated bacteria.

		A	B	C	D	E	F	G	H	I	J	K	L
A	*Wolbachia pipientis*												
B	*Wolbachia* sp.	99.2											
C	*Ehrlichia canis*	86.4	86.1										
D	*Cowdria ruminantium*	86.8	86.5	96.3									
E	*Ehrlichia equi*	87.2	87.0	91.8	92.0								
F	*Neorickettsia helminthoeca*	84.8	84.2	84.9	85.2	85.5							
G	*Rickettsia sibirica*	83.8	83.4	82.9	83.9	83.7	83.8						
H	*Rickettsia australis*	83.7	83.1	82.6	83.6	83.4	83.1	98.8					
I	*Rickettsia typhi*	83.9	83.5	82.6	83.3	83.9	83.4	98.4	98.1				
J	*Orientia tsutsugamushi*	82.4	81.8	82.3	83.4	83.0	82.1	90.3	89.8	90.2			
K	*Caedibacter caryophilus*	82.2	81.4	81.2	82.6	82.3	81.6	84.5	84.8	84.4	84.3		
L	**UWC36 endosymbiont**	83.4	82.8	82.7	84.7	83.7	81.7	85.5	85.6	85.5	83.4	84.5	
M	**UWC8 endosymbiont**	83.3	82.9	82.9	84.4	83.9	81.6	85.4	85.2	85.4	83.0	83.9	99.6

Results

Phylogenetic inference.

Two almost complete 16S rDNA sequences were amplified, cloned, and sequenced from two clinical *Acanthamoeba* isolates containing microscopically observable prokaryotic endosymbionts. Comparative sequence analysis revealed that the UWC8 and UWC36 endosymbiont 16S rDNA sequences were almost identical (99.6% sequence similarity) and clustered unequivocally with members of the alpha subclass of *Proteobacteria* (Table 1). Their closest neighbors included *Rickettsia australis*, *R. sibirica*, and *R. typhi*, with sequence similarities of approximately 85 to 86%. Phylogenetic analyses demonstrated that the retrieved sequences form an independent, well-separated lineage within the *Rickettsiales* (46). The neighbor-joining tree shown in Fig. 1 is based on the results of a distance matrix analysis of all available 16S rRNA sequences from representatives of the alpha subclass of *Proteobacteria* and a selection of members of the major lines of descent among the *Bacteria*. Only sequence positions that have the same nucleotides in at least 50% of all available sequences from the *Rickettsiales* were included, to reduce potential tree artifacts that may result from multiple base changes (24). To enhance clarity, several phylogenetic groups within the alpha subclass and the outgroup organisms were subsequently removed from the tree without changing its topology. The topology of the tree was further evaluated by parsimony and maximum-likelihood analyses of a variety of data sets differing with respect to the inclusion of sequence positions and outgroup reference sequences. Different tree-generating methods consistently supported deep branching of retrieved 16S rDNA sequences within the *Rickettsiales*, but an unambiguous pattern of the respective branch origins within the *Rickettsiales* could not be determined.

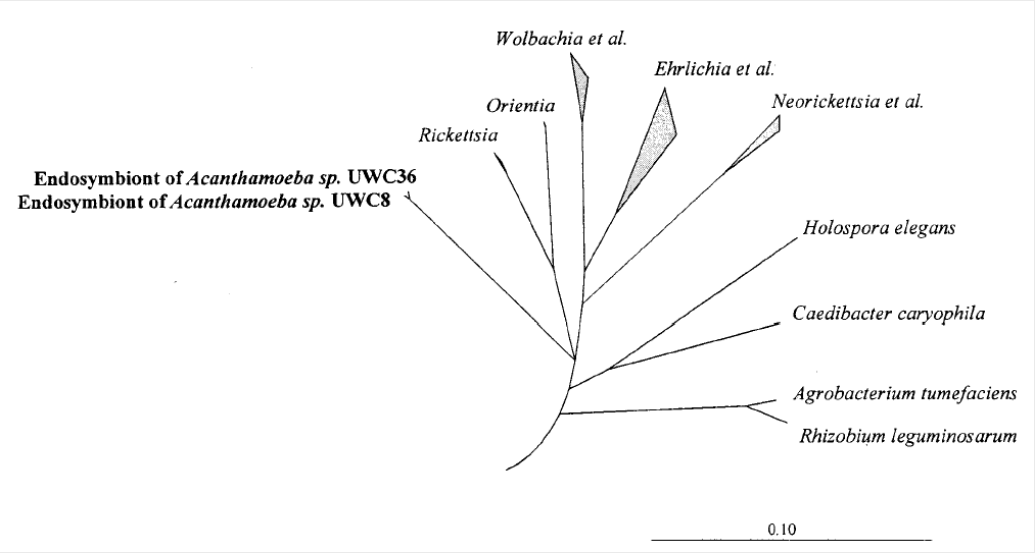

Figure 1. Neighbor-joining dendrogram showing relationships of endosymbionts of *Acanthamoeba* strains UWC8 and UWC36 to related members of the *Rickettsiales* and outgroups (the bar represents the estimated evolutionary distance). All tree-generating methods support deep branching of the retrieved 16S rDNA sequences, although an unambiguous pattern of the respective branch origins within the *Rickettsiales* could not be determined based upon the current data set, resulting in the presence of a multifurcation (24).

In situ *analysis of endosymbionts by electron microscopy and* in situ *hybridization.*

Light microscopic and ultrastructural analyses of UWC8 determined that the bacterial cells displayed a typical gram-negative cell wall, varied in shape from straight to curved, and were cytoplasmic. They often had an adjacent clear zone suggestive of capsules or slime layers (Fig. 2A and B). The symbionts varied considerably in size, being 0.3 to 0.5 mm wide by 0.8 to 2.3 mm long. The oligonucleotide probes AcRic90 and AcRic1196 were designed complementary to specific target regions shared between both retrieved 16S rRNA sequences. Both probes had at least three mismatches with respect to all other available 16S rRNA sequences (Fig. 3). Use of these probes for *in situ* detection of the endosymbionts within their eukaryotic host cells by fixation with 4% paraformaldehyde and standard hybridization methods (27) was initially hampered by amoebic cell shrinkage accompanied by an increase in autofluorescence. Similar problems were observed after the use of $HgCl_2$ and trichloroacetic acid-based fixation methods (35). Attempts to maintain amoebic cell morphology by capitalizing on their natural abilities to attach to a glass substrate, while

effective, resulted in significant disruption of the host cells upon exposure to 80% ethanol. Consequently, we implemented an additional agarose-embedding step that successfully stabilized *Acanthamoeba* cell morphology despite treatment with 80% ethanol. Exclusion of SDS from the hybridization and washing buffers in subsequent *in situ* hybridization reactions further minimized the detrimental effects to the amoebic cells and allowed the unambiguous detection of probe labeled, rodshaped endosymbionts by confocal laser-scanning microscopy (Fig. 2C and D). Numbers of endosymbionts per host cell detected by *in situ* hybridization varied from 1 to approximately 100. Simultaneous application of endosymbiont specific probes and DAPI staining verified that all DAPI-detectable endosymbiont cells were also visualized by probe-conferred fluorescence. A side effect of agarose embedding was the formation of large vacuoles within the *Acanthamoeba* cells (Fig. 2C). The optimal hybridization stringency for both endosymbiont- targeted probes was determined by the addition of formamide to the hybridization buffer in 5% increments at a constant hybridization temperature of 46°C. Probe-conferred signals increased following the addition of formamide up to 10% for probe AcRic1196 and 25% for probe AcRic90, then decreased and eventually disappeared at 20 and 50%, respectively. An increase in probe sensitivity and specificity following the addition of formamide up to an optimal concentration was reported previously and may result from better access of the probe to its target site (i.e., denaturation) or from a direct effect on the probe, such as unfolding (4). Non-specific binding of fluorescently labeled oligonucleotide probes to *Acanthamoeba* endo-symbionts was ruled out by the application of Cy3- and Cy5-labelled derivatives of probe BET42a, specific for the beta subclass of

Probe AcRic 90	**3′-CCTCAAGACGATCACCGT-5′**
Target (E.coli position 90-107)	5′-GGAGUUCUGCUAGUGGCA-3′
UWC8 endosymbiont	.....
UWC36 endosymbiont	.....
Mallomonas papillosa	...U.....A.C.....
Blepharisma americanum	.....U..AA..U.....
Neospora canium	...U.......G.AA...
Toxoplasma gondii	...U.......G.AA...
Halorubrum trapanicum	....GA....C....C..
Synura spinosa	...U.....G...C.A..
Probe AcRic 1196	**3′-TGTTAACCTGCGTTATCC-5′**
Target (E.coli position 1196-2013)	5′-ACAAUUGGACGCAAUAGG-3′
UWC8 endosymbiont	.....
UWC36 endosymbiont	.....
Methylocystis pyriformis	.....G......G.A...
Methylosinus sporium	.....G......G.AG..
Nitrobacter winogradskyi	.....G...A....AG..
Butyrovibrio vibrisolvens	....AG...A.....C..
Mycoplasma gallinaceum	....AGA.........C.
Campylobacter rectus	.....GA.........UC

Figure 3. Alignment of 16S rRNA target regions for endosymbionts of *Acanthamoeba* strains UWC8 and UWC36, along with other bacterial species displaying the smallest number of mismatches with respect to probes AcRic90 and AcRic1196.

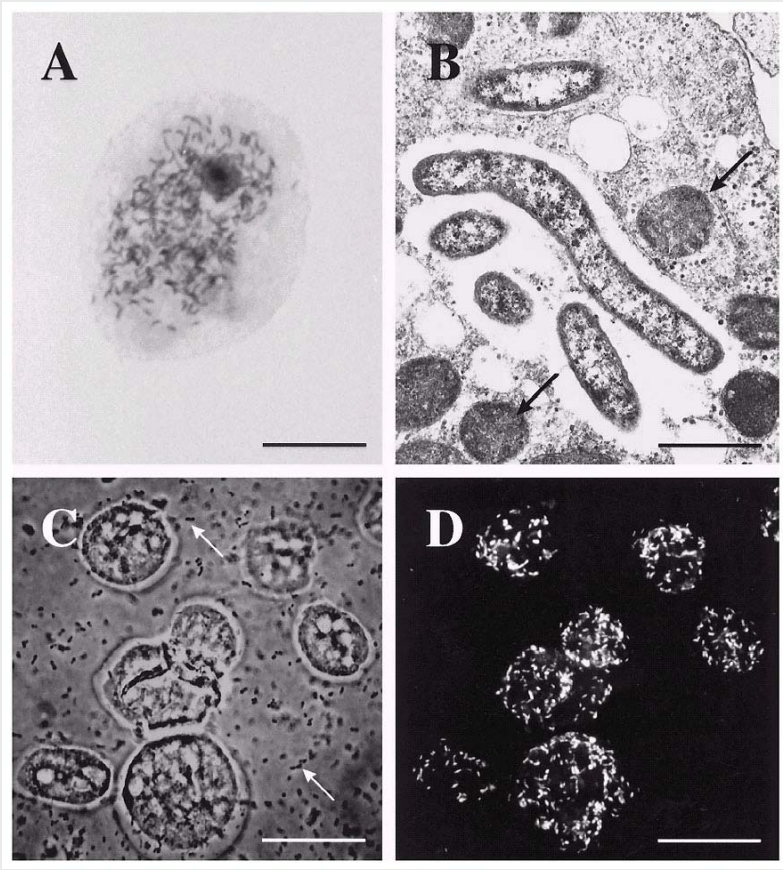

Figure. 2. (A) *Acanthamoeba* trophozoites (UWC8 isolate) naturally infected with rod-shaped bacterial endosymbionts as seen with Hemacolor stain (bar, 7 mm). (B) Electron micrograph demonstrating intracellular bacterial symbionts (UWC8 isolate) and several mitochondria (arrows) in an *Acanthamoeba* trophozoite (bar, 1 mm). (C) Phase-contrast photomicrograph of fixed *Acanthamoeba* strain UWC8 trophozoites, with numerous *E. coli* food bacteria seen in the background (arrows) (bar, 15 mm). (D) Specific fluorescent *in situ* detection of the endosymbionts of *Acanthamoeba* strain UWC8 within the same field as seen in panel C; numerous rod-shaped intracellular bacteria are recognized by using probe AcRic1196 labelled with Cy3 (bar, 15 mm).

Proteobacteria (data not shown). The specificity of the endosymbiont probes was further confirmed by the absence of detectable signals following *in situ* hybridization of para-formaldehyde-fixed municipal activated sludge with both probes (data not shown) (45). Consequently, positive hybridization reactions of the bacterial endosymbionts with the specific probes demonstrated that the retrieved *Rickettsia*-like 16S rDNA sequences did originate from the endosymbionts of *Acanthamoeba* strains UWC8 and UWC36.

Discussion

In the past, the order *Rickettsiales* served as a convenient location for the taxonomic grouping of a large variety of gramnegative bacteria that have an obligate need to develop within eukaryotic cells. A reappraisal of this concept includes proposals to remove all *Bartonella* spp. (including those reclassified from the genus *Rochalimaea*), *Afipia* spp., and

Coxiella burnetii from the order and to move *R. tsutsugamushi* to a new genus, *Orientia*. All *Ehrlichia* spp., most *Wolbachia* spp., *Neorickettsia helminthoeca*, *Cowdria ruminatum*, and *Anaplasma marginale* appear to form a series of related groups separate from the genus *Rickettsia* but clearly within the order and with a common ancestor (12, 36, 48). Within the genus *Rickettsia*, analysis of the 16S rRNA gene is proving to be less useful as a tool for evolutionary inference, with the analysis of other genes, including the one encoding citrate synthase, appearing to provide greater discriminatory potential (40). In the final analysis, strict intracellular location of gram-negative organisms can no longer be regarded as a definitive taxonomic marker only of the *Rickettsiales* (12). We applied the techniques of small-subunit ribosomal gene sequence analysis to characterize two isolates of GNR endosymbionts stably infecting isolates of *Acanthamoeba* spp. recovered from keratitis specimens. It was surprising to find that these endosymbionts were related to the *Rickettsiales*, given that most members of the order are associated with arthropods. The finding is consistent, however, with previous studies on these and other endosymbionts that demonstrate their reliance upon intracellular growth, presence of a capsule or slime layer, and typical gram-negative cell wall (16, 21, 34). While indicating emergence from a common ancestor, the endosymbiont lineage does branch deeply from other members of the order in a pattern that is not clearly established by the treegenerating methods used (Fig. 1). Such ambiguity is displayed by the presence of a multifurcation, resolution of which will be forthcoming only through analysis of other meaningful data sets, such as additional gene sequences with phylogenetic potential or an increased data set of the various rickettsial lineages (24). Use of two fluorescently labelled oligonucleotide probes targeting signature regions at the 59 and 39 ends of the generated 16S rDNA sequence data did allow us to identify all individual bacterial cells with both probes within amoebic host cells and excluded a chimeric nature of the determined 16S rDNA sequences. The finding of uncultured endosymbionts in *Acanthamoeba* spp. related to the *Rickettsiales* broadens the spectrum of bacteria known to interact with protozoa and may help to explain the appearance of host-symbiont specificity or cellular tropism known to exist with symbionts of ciliates (22, 33). Such specificity was previously demonstrated for the endosymbiont of *Acanthamoeba* sp. strain UWC8 and another uncharacterized GNR endosymbiont, which were shown to infect closely related strains of *Acanthamoeba* spp. but which failed to infect strains considered to be more distantly related, as determined by mitochondrial DNA restriction fragment length polymorphism analysis (18). Occasionally, the presence of a “killer” phenotype which appears to be dependent upon the degree of genetic relatedness of the originating and receiving hosts was observed in different

groups of protozoa: contact between genetically matched pairs results in the creation of a stable symbiosis, whereas contact between mismatched though recognized pairs may result in host cell death (18, 22, 33). Theoretically, protozoal strains capable of maintaining stable symbiotic relationships may realize a substantial selective advantage from the ability to control competing, bactivorous populations of related protozoa, which succumb to the killer phenotype following acquisition of discharged symbionts. Michel et al. also demonstrated that an *Ehrlichia*-like organism found infecting an environmental isolate of *Saccamoeba limax* was able to infect certain other strains of *Saccamoeba* but was not able to develop within isolates representing nine other amoebic genera (30). Such cellular tropism, which is usually receptor mediated, is a characteristic presumably shared by all members of the *Rickettsiales* and is an important determinant of the particular disease presentations seen in higher mammals. Many free-living soil and water protozoa mimic the role of professional phagocytes in their abilities to ingest and destroy large numbers of bacteria, and they undoubtedly serve as a natural testing ground for innumerable evolutionary experiments in intracellular survival (3, 41). The spectrum of pathogens able to survive and multiply to various degrees within acanthamoebae includes *Legionella* spp., *Burkholderia pickettii*, *Listeria monocytogenes*, *Vibrio cholerae*, *Francisella tularensis*, *Mycobacterium avium*, and *Chlamydia pneumoniae* (3, 5, 7, 10, 13, 14, 25, 29, 31, 32, 38, 40, 41, 43). For all of these organisms, acanthamoebae are potential reservoirs and vectors, due in part to their ubiquity in the environment, their resistant cyst stages, and their potential to grow in water supply, cooling, and humidification systems (7, 14, 37). The recovery of rickettsia-like 16S rRNA gene sequences from endosymbionts of *Acanthamoeba* spp. is a novel finding that broadens the spectrum of the bacterium-host relationships documented among the *Rickettsiales*. This may reflect an evolutionary divergence of the protozoan endosymbiont lineage from the other recognized rickettsial lineages at a time before their acquisition by arthropods or may represent an earlier association with protozoa, which preadapted them to life in the intracellular environment, thus facilitating their ultimate recruitment to metazoan hosts.

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Chapter III

Novel Bacterial Endosymbionts of *Acanthamoeba* spp. Related to the *Paramecium caudatum* Symbiont *Caedibacter caryophilus*

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Novel Bacterial Endosymbionts of *Acanthamoeba* spp. Related to the *Paramecium caudatum* Symbiont *Caedibacter caryophilus*

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Acanthamoebae are increasingly being recognized as hosts for obligate bacterial endosymbionts, most of which are presently uncharacterized. In this study the phylogeny of three Gram-negative, rod-shaped endosymbionts and their Acanthamoeba *host cells was analyzed by the rRNA approach. Comparative analyses of 16S rDNA sequences retrieved from amoebic cell lysates revealed that the endosymbionts of* Acanthamoeba polyphaga *HN-3,* Acanthamoeba *sp. UWC9, and* Acanthamoeba *sp. UWE39 are related to the* Paramecium caudatum *endosymbionts* Caedibacter caryophilus, Holospora elegans *and* Holospora obtusa*. With overall 16S rRNA sequence similarities between 87% and 93% to their closest relative* C. caryophilus*, these endosymbionts represent three distinct new species.* In situ *hybridization with fluorescently labeled endosymbiont-specific 16S rRNA-targeted probes demonstrated that the retrieved 16S rDNA sequences originated from the endosymbionts and confirmed their intracellular localization. We propose to provisionally classify the endosymbiont of* Acanthamoeba polyphaga *HN-3 as "*Candidatus *Caedibacter acanthamoebae", the endosymbiont of* Acanthamoeba *sp. strain UWC9 as "*Candidatus *Paracaedibacter acanthamoebae" and the endosymbiont of* Acanthamoeba *sp. strain UWE39 as "*Candidatus *Paracaedibacter symbiosus". The phylogeny of the* Acanthamoeba *host cells was analyzed by comparative sequence analyses of their 18S rRNA. While* Acanthamoeba polyphaga *HN-3 clearly groups together with most of the known* Acanthamoeba *isolates (18S rRNA sequence type 4),* Acanthamoeba *sp. UWC9 and UWE39 exhibit < 92% 18S rRNA sequence similarity to each other and other* Acanthamoeba *isolates. Therefore we propose two new sequence types (T13 and T14) within the genus* Acanthamoeba *containing, respectively,* Acanthamoeba *sp. UWC9, and* Acanthamoeba *sp. UWE39.*

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Introduction

The genus *Acanthamoeba* comprises numerous species of free-living amoebae which form double-walled cysts and are ubiquitous soil and water bactivores. Using comparative sequence analysis of nuclear 18S rDNA twelve separate but related sequence types were recognized within this genus (Stothard *et al*., 1998). In addition to being opportunistic pathogens of humans (Visvesvara, 1995), acanthamoebae serve as natural vehicles for the human pathogen *Legionella pneumophila* (Rowbotham, 1986; Berk *et al.*, 1998) and for a variety of other *Legionella*-like microorganisms (Birtles *et al.*, 1996; Michel *et al.*, 1998). Additionally, a variety of other clinically relevant bacteria, including *Chlamydia pneumoniae*, *Mycobacterium avium*, *Vibrio cholerae*, *Listeria monocytogenes* and *Burkholderia cepacia* are able, after infection in vitro, to survive and multiply for a limited time period within acanthamoebae (Ly *et al*., 1990; Michel *et al*., 1997; Steinert *et al*., 1998; Essig *et al*., 1997; Thom *et al*., 1992). Such interactions might be of clinical relevance, especially since multiplication of *Legionella pneumophila* and *Mycobacterium avium* within the amoeba host cell increases bacterial virulence (Cirillo *et al*., 1994; Cirillo *et al*., 1997). In contrast to these transient interrelationships which result in lysis of the infected amoebae, the presence of stably infecting, obligate bacterial endosymbionts has been reported for about 25% of all *Acanthamoeba* isolates (Fritsche *et al*., 1993). Using the rRNA approach, the relationship of some of these endosymbionts to the genera *Rickettsia* and *Chlamydia* have been demonstrated (Amann *et al*., 1997; Fritsche *et al*., 1999; Fritsche *et al.*, submitted). The close association of these endosymbionts to recognized human pathogens and the detection of specific antibodies to the *Acanthamoeba* symbiont termed “Hall’s coccus” (which shares more than 99% 16S rRNA sequence similarity to the *Chlamydia*-like symbiont "*Candidatus* Parachlamydia acanthamoebae") in sera from patients with pneumonia (Birtles *et al*., 1997) indicates the possible clinical significance of these "nonculturable" bacterial symbionts. In addition certain of these endosymbionts have a demonstrated potential in enhancing cytopathogenicity of acanthamoebae in vitro (Fritsche *et al.,* 1998).

To date most symbiotic relationships involving bacteria and protozoa have been analyzed using specialized staining techniques and conventional and electron microscopy. Detailed understanding of the phylogeny of the acanthamoebae and their endosymbionts, and of the probable physiological interactions between both partners will require the application of modern molecular biological techniques as has been previously demonstrated e.g. for the aphids-*Buchnera* symbiosis (Baumann *et al*., 1995; Schleper, *et al*., 1998).

In this study we analyzed two clinical *Acanthamoeba* spp. isolates (HN-3, UWC9) which have been reported to contain Gram-negative rod-shaped bacterial endosymbionts (Fritsche *et al.*, 1993; Hall *et al.*, 1985). In addition, we isolated and studied an environmental *Acanthamoeba* specimen (UWE39) bearing a morphologically similar endosymbiont. Previous attempts to culture these bacteria using different media and conditions have not been successful (Fritsche *et al.*, 1993). Although cultivation failure does not necessarily exclude the existence of a free-living stage of these bacteria, we provisionally consider them as being obligate endosymbionts. 18S and 16S rDNA sequences were retrieved for these amoebic isolates and their "uncultured" symbionts, respectively, and used for phylogenetic analysis. Fluorescent *in situ* hybridization (FISH) using endosymbiont-specific 16S rRNA-targeted oligonucleotide probes in combination with confocal laser scanning microscopy (CLSM) was applied to detect the endosymbionts within their eukaryotic host cells.

Results

Electron Microscopy

The rod-shaped endosymbionts of *Acanthamoeba polyphaga* HN-3, *Acanthamoeba* sp. UWC9, and *Acanthamoeba* sp. UWE39 measured 0.7 to 3.3 µm by 0.22 to 0.33 µm and showed a typical Gram-negative cell wall surrounded, in some cases, by an electron

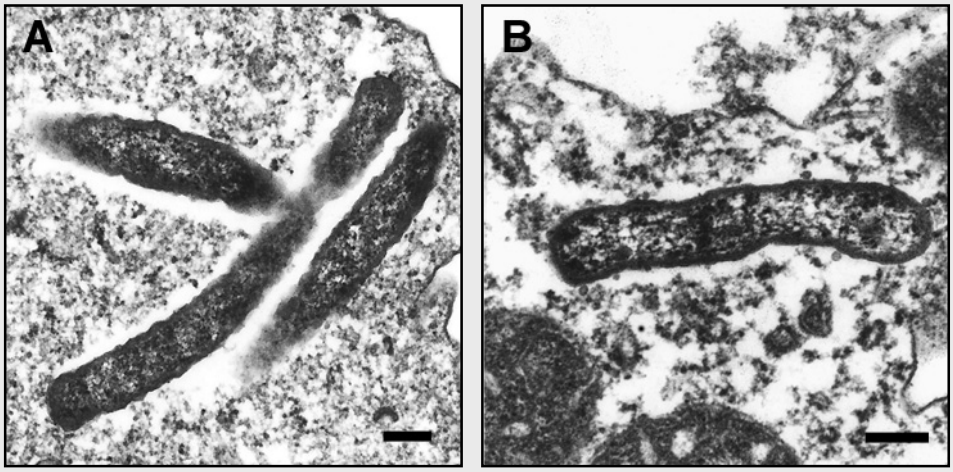

Figure 1. Electron micrographs of (A) *Candidatus* Caedibacter acanthamoebae (endosymbiont of *Acanthamoeba polyphaga* HN-3), and (B) *Candidatus* Paracaedibacter acanthamoebae (endosymbiont of *Acanthamoeba* sp. UWC9). Bacteria are intracytoplasmic and variably display a surrounding electron translucent space suggestive of a capsule or slime layer (bar = 0.2 µm).

translucent area (Figure 1). Endosymbionts were found equally distributed within the cytoplasm of both trophozoites and cysts and were not enclosed in vacuoles. No ”R-body”-like structures were observed.

16S rRNA sequence analysis of Acanthamoeba spp. Endosymbionts

Near full-length 16S rRNA gene fragments (approximately 1,400 bp) from the bacterial endosymbionts of *Acanthamoeba polyphaga* HN-3, *Acanthamoeba* sp. UWC9, and *Acanthamoeba* sp. UWE39 were successfully amplified, cloned and sequenced. Comparative sequence analysis revealed that the 16S rRNA gene sequences of all three endosymbionts were novel and showed highest similarities to members of the alpha-subclass of *Proteobacteria*, particularly to *Caedibacter caryophilus*, a macronuclear endosymbiont of *Paramecium caudatum* (Schmidt et *al*., 1987; Euzeby, 1997). The respective similarity matrix is presented in Table 1. The endosymbionts of *Acanthamoeba polyphaga* HN-3 showed the highest similarity to *Caedibacter caryophilus* (93%; Springer *et al*., 1993). This symbiont also showed moderate similarities with members of the genus *Holospora* (86%; Amann *et al*., 1991), comprising another group of nuclear symbionts of *Paramecium* sp.. Comparable similarities of the HN-3 symbiont were determined to the "NHP-bacterium" (86%; Loy *et al*., 1996), an important intracellular shrimp pathogen. The 16S rRNA sequences of the endosymbionts of *Acanthamoeba* sp. UWC9 and *Acanthamoeba* sp. UWE39 showed a similarity of 92% to each other and of 88-90% to the HN3-endosymbiont. Compared to the HN-3 endosymbiont these two endosymbionts display significantly lower overall sequence similarities to *Caedibacter caryophilus* (87-88%), the members of the genus *Holospora* (84-85%), and the "NHP-bacterium" (82-84%). The 16S rRNA gene of *Caedibacter caryophilus* has been previously reported to contain an unusual 194 bp insertion within the 5’-terminal part, which cannot be found in the mature 16S rRNA, but could be detected as fragmented rRNA within *C. caryophilus* (Springer *et al*., 1993). Despite the phylogenetic relationship of the endosymbionts of *Acanthamoeba polyphaga* HN-3, *Acanthamoeba* spp. UWC9 and UWE39 to *Caedibacter caryophilus*, they do not contain a similar insertion within their 16S rRNA genes.

Table 1. Overall sequence similarities for the retrieved 16S rRNA sequences of *Candidatus* Caedibacter acanthamoebae (endosymbiont of *Acanthamoeba polyphaga* HN-3), *Candidatus* Paracaedibacter acanthamoebae (endosymbiont of *Acanthamoeba* sp. UWC9), and *Candidatus* Paracaedibacter symbiosus (endosymbiont of *Acanthamoeba* sp. UWE39) and representative members of the α-subclass of *Proteobacteria*.

		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
A	*Agrobacterium tumefaciens*																				
B	*Bartonella bacilliformis*	93,7																			
C	*Brucella abortus*	95,2	94,6																		
D	*Rhodobacter sphaeroides*	89,8	88,7	89,8																	
E	*Wolbachia pipientis*	83,1	82,5	81,9	81,7																
F	*Ehrlichi canis*	81,9	82,7	81,9	82,1	86,4															
G	*Cowdria ruminantium*	82,4	83,0	82,3	82,5	87,0	96,4														
H	*Neorickettsia helminthoeca*	82,6	81,6	82,2	81,7	84,8	84,9	85,2													
I	*Rickettsia rickettsii*	83,8	84,9	84,3	83,4	83,6	82,8	83,6	83,3												
J	*Rickettsia typhi*	83,9	84,5	84,2	83,5	83,9	82,6	83,1	83,2	98,3											
K	*Holospora elegans*	83,5	82,1	83,2	82,1	80,4	81,1	81,3	80,2	82,5	82,9										
L	*Holospora obtusa*	83,7	82,3	83,5	81,8	80,5	81,4	81,6	80,4	82,5	82,7	98,7									
M	*Caedibacter caryophilus*	85,7	86,1	85,8	85,7	82,1	81,2	82,2	81,6	84,0	84,3	86,1	86,1								
N	*Afipia felis*	89,5	88,4	90,0	87,9	80,2	80,3	80,7	81,1	82,8	82,8	83,2	83,5	85,3							
O	*Sphingomonas capsulata*	88,6	87,5	88,5	86,6	80,0	81,0	82,0	79,9	83,8	84,1	82,0	81,4	84,9	85,3						
P	*Azospirillum lipoferum*	88,2	87,7	88,4	88,6	80,6	81,7	81,7	80,9	84,9	85,5	83,2	83,2	87,6	88,0	86,3					
Q	*Rhodospirillum rubrum*	87,0	86,4	87,5	86,6	81,1	82,6	82,6	81,3	82,7	83,0	82,0	81,7	84,6	86,6	85,9	88,6				
R	***Candidatus* Paracaedibacter acathamoebae**	85,1	85,5	85,3	85,7	81,9	82,9	83,5	82,1	84,0	84,2	84,2	84,5	86,5	85,5	84,0	86,1	83,6			
S	***Candidatus* Caedibacter acathamoebae**	86,7	86,7	86,6	86,6	81,3	81,0	82,2	80,8	84,1	84,2	85,8	85,8	93,3	84,7	84,9	87,5	85,0	87,9		
T	***Candidatus* Paracaedibacter symbiosus**	85,4	85,3	85,6	85,9	81,6	82,0	82,7	80,8	84,1	84,2	83,8	84,0	87,5	85,2	84,2	85,7	83,1	92,1	89,8	
U	"NHP-bacterium"	82,6	82,7	82,7	82,0	80,6	80,5	80,6	80,1	83,0	83,1	83,6	83,5	85,8	82,6	82,2	83,7	80,8	84,4	86,4	82,3

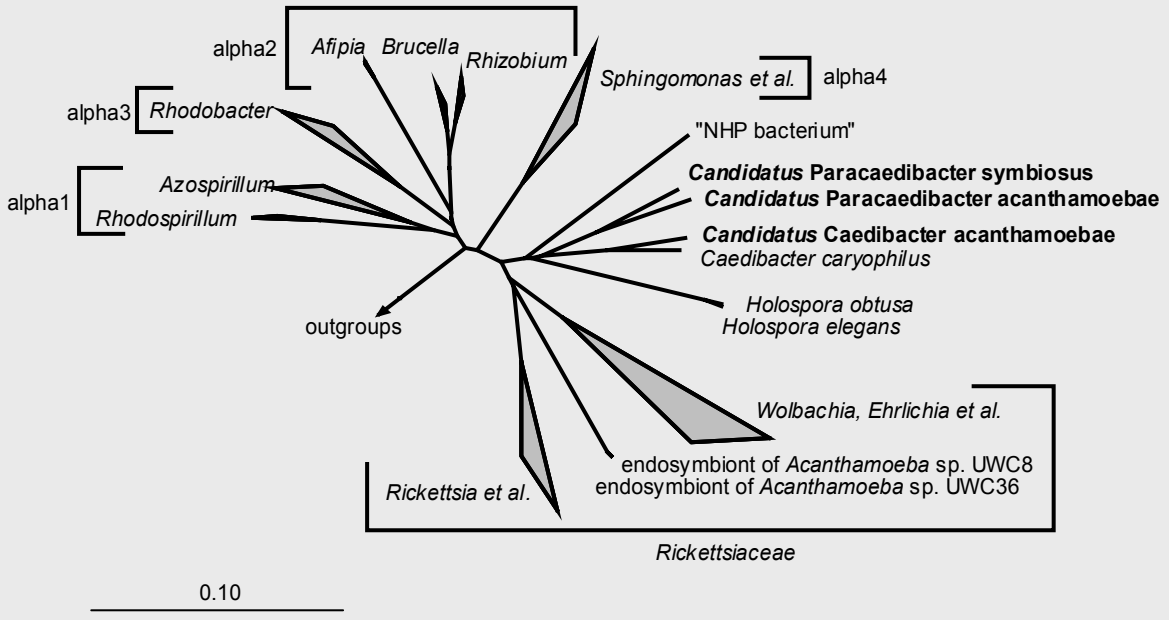

Figure 2. Neighbor joining dendrogram, based on comparative 16S rRNA analysis, showing relationships of *Candidatus* Caedibacter acanthamoebae (endosymbiont of *Acanthamoeba polyphaga* HN-3), *Candidatus* Paracaedibacter acanthamoebae (endosymbiont of *Acanthamoeba* sp. UWC9), and *Candidatus* Paracaedibacter symbiosus (endosymbiont of *Acanthamoeba* sp. UWE39) to representative members of the α-subclass of *Proteobacteria*. Bar represents 10% estimated evolutionary distance.

All applied phylogenetic treeing methods consistently demonstrated that the *Acanthamoeba* endosymbionts form a monophyletic cluster together with the intracellular shrimp pathogen "NHP-bacterium" and the *Paramecium caudatum* endosymbionts *Caedibacter caryophilus*, *Holospora obtusa* and *Holospora elegans* within the α-subclass of *Proteobacteria* (Figure 2). The sequence data also indicate a common origin of this cluster with the *Rickettsiaceae*. The HN-3 endosymbiont branches together with *Caedibacter caryophilus* within the cluster while the UWC9 and UWE39 endosymbionts form an independent lineage.

In situ *hybridization of endosymbionts*

Comparative 16S rDNA sequence analysis of the endosymbionts of *Acanthamoeba polyphaga* HN-3, UWC9, and UWE39 revealed the presence of the target site of probe Cc23a, previously designed for specific detection of *Caedibacter caryophilus* (Springer *et al*., 1993). Consistent with this finding, all endosymbionts were successfully visualized within their eukaryotic host cells by FISH using probe Cc23a (data not shown). Additionally, the oligonucleotide probes S-S-CaeAc-998-a-A-18, S-S-PcaeC9-217-a-A-18 and S-S-PcaeE39-217-a-A-18 were designed to specifically hybridize to complementary target regions on the 16S rRNA of the endosymbionts of *Acanthamoeba polyphaga* HN-3, UWC9 and UWE39, respectively. The endosymbiont-specific probes display at least two mismatches to all other available 16S rRNA sequences including the one of *Caedibacter caryophilus* (Figure 3). Optimal hybridization stringency was determined for all three probes by increasing the formamide concentration in the hybridization buffer in increments of 5 % at a constant hybridization temperature of 46°C. Probe-conferred signals of the endosymbionts remained at the same level following the addition of formamide up to 30 % for probe S-S-CaeAc-998-a-A-18 and 40% for probes S-S-PcaeC9-217-a-A-18 and S-S-PcaeE39-217-a-A-18, and then decreased rapidly. For additional verification of probe specificity, activated sludge from a municipal sewage treatment plant known to contain a high diversity of bacteria (Snaidr *et al*., 1997) was hybridized with the endosymbiont -specific probes S-S-CaeAc-998-a-A-18, S-S-PcaeC9-217-a-A-18 and S-S-PcaeE39-217-a-A-18 at the determined stringent hybridization conditions. No detectable signals were observed by inspection of 20 microscopic fields at a magnification of 630. Non-specific binding of fluorescently-labeled oligonucleotide probes to *Acanthamoeba* cell compounds and/or their endosymbionts was excluded since no signals were observed after application of FLUOS, Cy3- and Cy5-labeled derivatives of probe BET42a, specific for the β-subclass of *Proteobacteria* (data not shown). Consequently,

positive hybridization reactions of bacterial endosymbionts with the specific probes S-S-CaeAc-998-a-A-18, S-S-PcaeC9-217-a-A-18, and S-S-PcaeE39-217-a-A-18 demonstrated that the retrieved 16S rRNA sequences did originate from the endosymbionts of *Acanthamoeba* strains HN-3, UWC9 and UWE39 respectively, and furthermore confirmed their intracellular localization (Figure 4). In addition, simultaneous hybridization of the probes S-S-CaeAc-998-a-A-18, S-S-PcaeC9-217-a-A-18, and S-S-PcaeE39-217-a-A-18 with the bacterial probe EUB338 labeled with different dyes illustrated the absence of endosymbiotic bacteria negative for the respective endosymbiont-specific probes within the *Acanthamoeba* host cells (Figure 4). However, it should be stressed that these results do not exclude the possible occurrence of microheterogeneity within the different symbiont populations.

Probe S-S-PcaeC9-217-a-A-18 (3'-5')	**AGCGGTTAACTCGTCGGG**
Target (*E.coli* pos. 217; 5'-3')	UCGCCAAUUGAGCAGCCC
endosymbiont of *Acanthamoeba* sp. UWC9	----------
endosymbiont of *Acanthamoeba* sp. UWE39	----U--AG--A------
endosymbiont of Acanthamoeba polyphaga HN-3	-----UUGA---G-----
Caedibacter caryophilus	----U-UCA--U------
Azospirillum irakense	-------AG---------
Caulobacter crescentus	------U-G----G----
Mycoplana segnis	------U-G----G----
Kingella denitrificans	G---U-U----------A
Chloroflexus aggregans	A----GG-G------U-G
Neisseria macaca	G---UGU------G---G

Probe S-S-PcaeE39-217-a-A-18 (3'-5')	**AGCGATTTCCTTGTCGGG**
Target (*E.coli* pos. 217; 5'-3')	UCGCUAAAGGAACAGCCC
endosymbiont of *Acanthamoeba* sp. UWE39	----------
endosymbiont of *Acanthamoeba* sp. UWC9	----C--UU--G------
endosymbiont of *Acanthamoeba polyphaga* HN-3	----CUUGA--GG-----
Caedibacter caryophilus	------UC---UG-----
Azospirillum irakense	----C------G------
Helicobacter hepaticus	----------U-----U
Azorhizobium caulinodans	-----G-----U-G----
Nitrosomonas europaea	G----------G-G---G
Thiobacillus ferrooxidans	G------G----G----U
Rhodopseudomonas acidophila	----CG-----GUG----

Probe S-S-CaeAc-998-a-A-18 (3'-5')	**CCCTAGCGCCTCTGTTCT**
Target (*E.coli* pos. 998; 5'-3')	GGGAUCGCGGAGACAAGA
endosymbiont of *Acanthamoeba polyphaga* HN-3	----------
endosymbiont of *Acanthamoeba* sp. UWE39	-U---UAA-----UG---
endosymbiont of *Acanthamoeba* sp. UWC9	-U---UAA--G--UG---
Caedibacter caryophilus	-----UAU-----GG---
Prochlorothrix hollandica	----G----------G-U
Synechococcus sp.	----G--G-------G-U
Desulfovibrio gabonensis	----G-----U----G-U
Bacillus schlegelii	----CG---------G-U
Capnocytophaga granulosa	CC---U------G--G-G
Ruminococcus torques	---GCA---------G-U

Figure 3. Difference alignment of 16S rRNA target regions for probes S-S-CaeAc-998-a-A-18, S-S-PcaeC9-217-a-A-18 and S-S-PcaeE39-217-a-A-18.

Table 2. Overall sequence similarities for the retrieved 18S rRNA sequences of *Acanthamoeba polyphaga* HN-3, *Acanthamoeba* sp. UWC9, and *Acanthamoeba* sp. UWE39 and representative *Acanthamoeba* species (18S rRNA sequence types are given in brackets).

		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
A	*A. castellanii* Castellani [T4]																
B	*A. palestinensis* reich [T2]	92,60															
C	*A. castellanii* V006 [T1]	93,58	91,16														
D	*A.* sp. KA/E2 [T4]	98,88	92,42	93,61													
E	*A.* sp. UWC9	91,88	90,61	91,02	91,97												
F	*A.* sp. HN-3	97,88	92,70	93,51	98,20	91,91											
G	*A. healyi* [T12]	86,62	87,02	87,04	85,55	86,42	86,74										
H	*A. culbertsoni* A1 [T4]	97,94	92,48	93,45	98,08	91,79	99,28	86,54									
I	*A. palestinensis* 2802 [T6]	90,78	94,88	90,45	90,86	89,63	91,00	87,16	90,87								
J	*A. astronyxis* [T7]	81,73	80,95	81,90	81,34	80,55	81,91	78,44	81,64	80,20							
K	*A. tubiashi* [T8]	83,04	80,87	82,90	83,03	81,85	82,87	80,72	82,74	80,63	84,85						
L	*A. comandoni* [T9]	80,51	79,59	80,93	80,67	79,70	80,87	77,71	80,65	79,50	78,97	82,94					
M	*A. culbertsoni* A1 [T10]	87,73	87,17	88,98	88,04	87,01	88,31	88,59	88,07	86,78	79,90	80,60	78,84				
N	*A. hatchetti* BH-2 [T11]	94,03	91,86	92,39	94,43	91,56	94,59	86,05	94,39	90,39	81,12	82,33	80,05	88,17			
O	*A. griffini* H37 [T3]	93,20	91,97	91,84	93,01	91,29	93,20	86,14	93,20	90,91	80,09	81,61	79,85	88,63	93,51		
P	*A. lenticulata* E18-2 [T5]	89,45	86,69	87,96	89,54	87,86	89,27	84,03	89,24	86,04	77,80	79,59	77,56	84,89	88,55	87,68	
Q	*A.* sp. UWE39	91,07	89,51	90,60	91,33	92,24	91,35	86,38	91,33	88,30	81,13	82,51	80,24	86,85	89,77	89,06	86,87

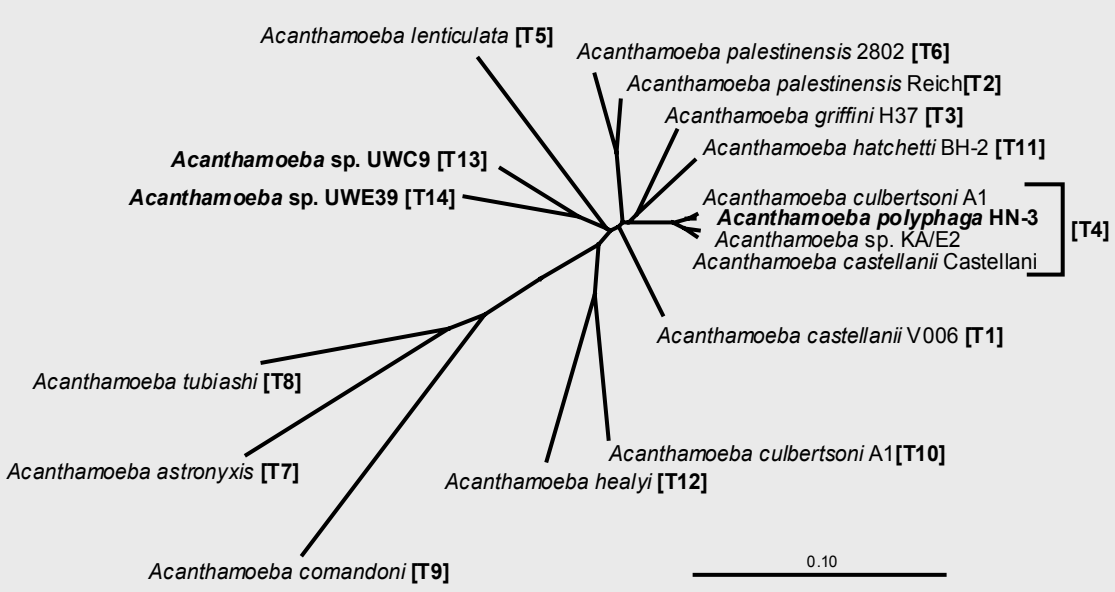

Figure 5. Neighbor joining dendrogram, based on comparative 18S rRNA analysis, showing relationships of *Acanthamoeba polyphaga* HN-3, *Acanthamoeba* sp. UWC9, and *Acanthamoeba* sp. UWE39 to representative members of the *Acanthamoeba* (bar represents 10% estimated evolutionary distance; 18S rRNA sequence types are given in brackets).

18S rRNA sequence analysis of Acanthamoeba spp. host cells

Almost the entire 18S rRNA genes (approximately 2,200 bp) of *Acanthamoeba polyphaga* HN-3, *Acanthamoeba* sp. UWC9, and *Acanthamoeba* sp. UWE39 were amplified, cloned and sequenced. Subsequent comparative sequence analysis revealed that the retrieved sequences show highest overall sequence similarities (between 81% and 98%) to other *Acanthamoeba* 18S rRNA sequences. The respective similarity matrix is shown in Table 2. While *Acanthamoeba polyphaga* HN-3 displays a very high similarity to the type species *Acanthamoeba castellanii* Castellani (98%), *Acanthamoeba* sp. UWC9 and *Acanthamoeba* sp. UWE39 show remarkable moderate sequence similarities to each other (92%) and to other 18S rRNA sequences from Acanthamoebidae (< 92%) presently deposited in public databases. Acanthamoebae typically possess extended 18S rRNA genes of approximately 2,300 bp due to the presence of additional bases in various regions designated as expansion segments (Gunderson *et al*., 1986). Additionally, group I introns have been found in two different *Acanthamoeba* species (*A. lenticulata*, *A. griffini*; Gast et *al*., 1994). The 18S rRNA genes of *Acanthamoeba polyphaga* HN-3, *Acanthamoeba* sp. UWC9 and UWE39 contain these typical expansion segments, but there is no evidence for the presence of group I introns.

Phylogenetic parsimony, neighbor joining and maximum likelihood trees clearly demonstrate the affiliation of the analyzed amoebae isolates to the genus *Acanthamoeba*. Within the genus *Acanthamoeba*, *Acanthamoeba polyphaga* HN-3 is lumped together with the majority of the *Acanthamoeba* species (sequence type 4; Stothard *et al*., 1998.), while *Acanthamoeba* sp. UWC9 and *Acanthamoeba* sp. UWE39 form two new separate but common branching lineages (Figure 5).

Discussion

Acanthamoebae are well known as hosts for a variety of bacterial endocytobionts. Parasitic and actual endosymbiotic host-endocytobiont relationships have been observed (Birtles *et al*., 1996; Fritsche *et al*., 1999). Naturally occurring endocytobionts of acanthamoebae which have been previously phylogenetically characterized are either affiliated with the genus *Legionella* (Birtles *et al*., 1996; Michel *et al*., 1998), the *Chlamydiales* (Amann *et al*., 1997; Fritsche *et al*., submitted), or the *Rickettsiales* (Fritsche *et*

al., 1999). Here we report the existence of a novel, fourth group of *Acanthamoeba* endosymbionts which are members of the α-subclass of *Proteobacteria* and are clearly affiliated to a monophyletic cluster of exclusively obligate intracellular bacteria distinct from, but related to, the *Rickettsiaceae*. This cluster comprises yet uncultured microorganisms which, interestingly, are able to infect distantly related eukaryotes including the ciliate *Paramecium caudatum* (*Caedibacter caryophilus, Holospora obtusa, Holospora elegans*), the *Acanthamoebae* spp. isolates currently under investigation, and the shrimp *Penaeus vannamei*

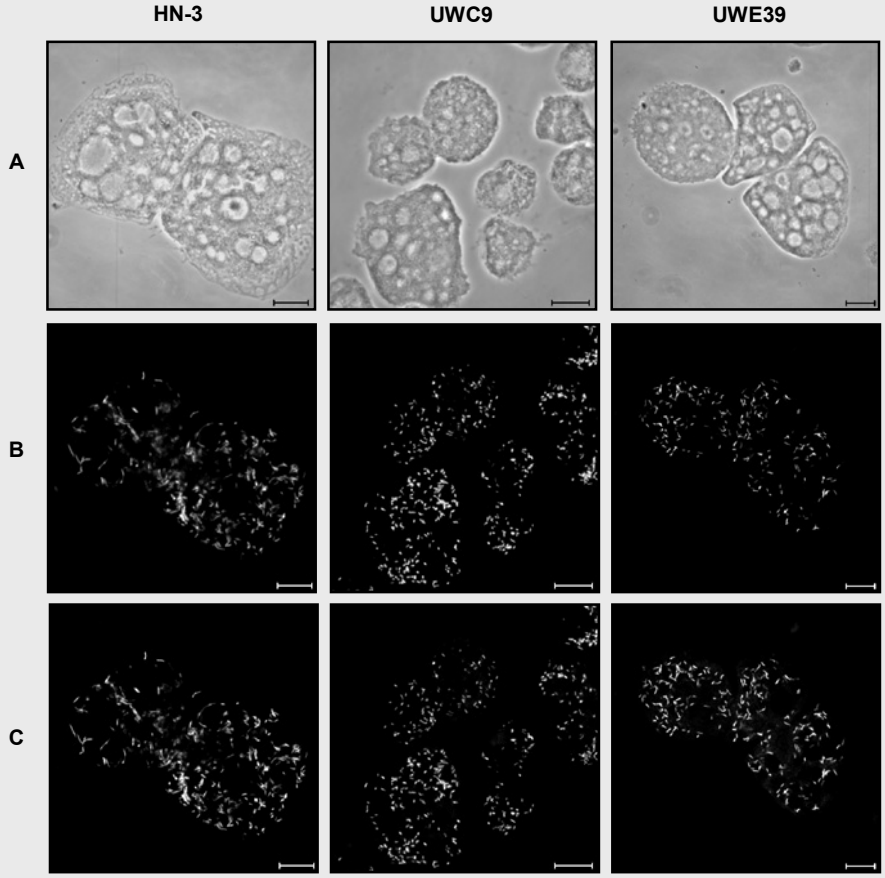

Figure 4. Confocal laser scanning microscopic analysis of *Candidatus* Caedibacter acanthamoebae (endosymbiont of *Acanthamoeba polyphaga* HN-3), *Candidatus* Paracaedibacter acanthamoebae (endosymbiont of *Acanthamoeba* sp. UWC9) and *Candidatus* Paracaedibacter symbiosus (endosymbiont of *Acanthamoeba* sp. UWE39). Row A-C: *In situ* identification of the endosymbionts within their eukaryotic host cells. Identical microscopic fields are depicted (bar = 10 µm). Row A: phase contrast, row B: FLUOS labeled oligonucleotide probe EUB338, row C: CY3-labeled endosymbiont-specific probe S-S-CaeAc-998-a-A-18, S-S-PcaeC9-217-a-A-18 and S-S-PcaeE39-217-a-A-18, respectively.

("NHP-bacterium"). Within this cluster the three *Acanthamoeba* endosymbionts are most closely related to *Caedibacter caryophilus*, previously known as kappa particle (Springer *et al*., 1993). The genus *Caedibacter* is currently defined and characterized on the basis of morphological and physiological properties (Pond *et al*., 1989): five species have been described as Gram-negative, nonsporeforming, nonmotile, facultative anaerobic, R body (refractile inclusion body) producing, obligate endosymbionts of *Paramecium tetraurelia*, *P. biaurelia*, and *P. caudatum*. Whereas *C. taeniospiralis*, *C. varicaedens*, *C. pseudomutans* and *C. paraconjugatus* are located in the cytoplasm of their respective host species, *C. caryophilus* is a macronuclear endosymbiont of *P. caudatum*. Only the latter endosymbiont species has yet been phylogenetically characterized by 16S rRNA analysis. There seems, however, to be considerable phylogenetic diversity within the genus *Caedibacter* as indicated by DNA-DNA hybridization studies (Pond *et al*., 1989.). To date it is not possible to predict whether the *Acanthamoeba* endosymbionts are closely related to those *Caedibacter* species for which no 16S rDNA sequence information is available. However, the absence of detectable R-bodies within the *Acanthamoeba* endosymbionts, and the difference in host species render this possibility unlikely. *In situ* hybridization with a specific oligonucleotide probe provided direct visual evidence that the analyzed 16S rRNA sequences were derived from the intracytoplasmatic *Acanthamoeba* endosymbionts. Considering that a 97% 16S rDNA sequence similarity threshold value has been applied for species definition of prokaryotes (Stackebrandt and Goebel, 1994), the relatively low 16S rRNA sequence similarities (<93%) of these endosymbionts to each other and to previously described bacteria suggest that they represent three distinct new species. While the 16S rDNA sequence similarity of 93% of the *Acanthamoeba polyphaga* HN-3 endosymbiont to *C. caryophilus* supports grouping it in the genus *Caedibacter*, 16S rDNA sequence similarities of the *Acanthamoeba* sp. UWC9 and UWE39 endosymbionts of below 87% to other bacteria suggests their assignment to a new provisional genus. Therefore, according to Murray and Schleifer (1994), we propose provisional classification of the endosymbiont of *Acanthamoeba polyphaga* HN-3 as "*Candidatus* Caedibacter acanthamoebae".

The short description of "*Candidatus* Caedibacter acanthamoebae" is as follows: phylogenetic position, α-subgroup of *Proteobacteria*; cultivation, not cultivated on cell-free media; Gram-reaction, negative; morphology, rod-shaped, approximately 0.7-3.3 µm in length, 0.22-0.33 µm in diameter; basis of assignment, 16S rDNA sequence (accession number AF132138) and nucleotide S-S-CaeAc-998-a-A-18 (5'-TCTTGTCTCCGCGATCCC-

3'); association and host, intracellular symbionts of *Acanthamoeba polyphaga* HN-3; mesophilic; authors, Horn *et. al*, this study.

In addition we propose provisional classification of the endosymbionts of *Acanthamoeba* sp. UWC9 and UWE39 as "*Candidatus* Paracaedibacter acanthamoebae" and "*Candidatus* Paracaedibacter symbiosus", respectively.

The short description of "*Candidatus* Paracaedibacter acanthamoebae" is as follows: phylogenetic position, α-subclass of proteobacteria; cultivation, not cultivated on cell-free media; Gram-reaction, negative; morphology, rod-shaped, approximately 1.3 - 1.7 µm in length, 0.22 - 0.24 µm in diameter; basis of assignment, 16S rDNA sequence (accession number AF132137) and nucleotide probe S-S-PcaeC9-217-a-A-18 (5'-GGGCTGCTCAATTGGCGA-3'); association and host, intracellular symbiont of *Acanthamoeba* sp. UWC9; mesophilic; authors, Horn *et. al*, this study.

The short description of "*Candidatus* Paracaedibacter symbiosus" is as follows: phylogenetic position, α-subclass of proteobacteria; cultivation, not cultivated on cell-free media; Gram-reaction, negative; morphology, rodshaped, approximately 1.3 - 1.7 µm in length, 0.22 - 0.24 µm in diameter; basis of assignment, 16S rDNA sequence (accession number AF132139) and nucleotide probe S-S-PcaeE39-217-a-A-18 (5'-GGGCTGTTCCTTTAGCGA-3'); association and host, intracellular symbiont of *Acanthamoeba* sp. UWE39 respectively; mesophilic; authors, Horn *et. al*, this study.

Traditional classification of acanthamoebae was based on trophozoit and cyst morphology (Pussard and Pons, 1977), while only recently molecular approaches, like isoenzyme electrophoretic patterns, mtRFLP analysis and 18S rRNA sequence comparison were applied (Daggett *et al*., 1985; Gautom *et al*., 1994; Stothard *et al*., 1998). The latter method led to subdivision of the genus *Acanthamoeba* into 12 sequence types. *Acanthamoeba* isolates belonging to one sequence type share a common 18S rRNA sequence similarity of at least 95% (Stothard *et al*., 1998). The majority of hitherto analyzed *Acanthamoeba* isolates belong to sequence type T4, which also contains most of the clinical isolates. The clinical isolate *Acanthamoeba polyphaga* HN-3, sharing 98% 18S rRNA sequence similarity with acanthamoebae of sequence type T4, is thus affiliated with this type. The clinical isolate *Acanthamoeba* sp. UWC9 and the environmental isolate *Acanthamoeba* sp. UWE39 are only

distantly related to sequence type T4 or other sequence types of acanthamoebae (82-92% 18S rRNA sequence similarity to all other *Acanthamoeba* species, and 92% 18S rRNA sequence similarity to each other) and thus represent two new sequence types. Therefore we propose *Acanthamoeba* sp. UWC9 and UWE39 be referred to as *Acanthamoeba* 18S rRNA sequence type T13 and T14, respectively. The two novel *Acanthamoeba* isolates as well as their endosymbionts share, according to the rRNA trees, a common ancestor and form an independent lineage within the genus *Acanthamoeba* and the *Caedibacter-Holospora*-HNP-cluster, respectively, indicating a possible coevolution of the *Acanthamoeba* hosts and their bacterial endosymbionts. We are currently further investigating this hypothesis by crossinfection studies of the endosymbionts with *Acanthamoeba* spp. representing different sequence types.

The novel finding that members of the *Caedibacter-Holospora*-HNP-Cluster are able to persistently infect *Acanthamoeba* spp. isolated from the environment and from humans suggests that these bacteria which are related to the Rickettsiaceae might be of clinical relevance directly or indirectly. Future studies will focus on detecting evidence of human seroreactivity to these bacterial agents, the abilities of these endosymbionts to enhance amoebic cytopathogenicity, and fosmid cloning and sequencing of endosymbiont genome fragments to shed more light into the function of these yet uncultured microorganisms (Schleper, *et al*., 1998).

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Experimental procedures

Isolation and maintenance of acanthamoebae. Three *Acanthamoeba* isolates (HN-3, UWC9 and UWE39) containing rod-shaped bacterial endosymbionts were analyzed in this study. *Acanthamoeba* sp. UWC9 was isolated from a contact lens case (Fritsche *et al*. 1993); *Acanthamoeba polyphaga* HN-3 was isolated from a human nasal swab and was obtained from the American Type Culture Collection (ATCC 30173); and *Acanthamoeba* sp. UWE39 was recovered from a soil sample collected in Clearwater County, Minnesota. All isolations were performed using a standard technique (Visvesvara, 1995). Briefly, primary isolation was performed using 1.5% non-nutrient agar (NNA) plates seeded with live *Escherchia coli.* Subsequent incubation was at ambient temperature (22-24 °C) for 5 days. Upon evidence of growth, a clonal culture was

established transferring a single double-walled cyst with a micromanipulator to a new agar plate. The use of heat-killed *E. coli* and incorporation of antibiotics (penicillin 100µg/ml, streptomycin 10µg/ml, and amphotericin B 0.25µg/ml) into the media in subsequent subcultures resulted in axenized growth. Each clone was then adapted to growth in sterile trypticase-soy-yeast extract (TSY) broth. Acanthamoebae were maintained in TSY broth at room temperature by replacing the broth weekly. Prokaryotic endosymbionts of the *Acanthamoeba* sp. isolates were readily detected using Gram- and Giemsa-staining.

Electron Microscopy. *Acanthamoeba* isolates were examined by electron microscopy using a variation of published methods (Hall *et al*., 1985). Briefly, aliquots of amoebae in broth were fixed with 2% glutaraldehyde in 0.1 M cacodylate. Fixed amoebae were then pelleted and embedded in agar. Thin sections were stained with uranyl acetate and lead citrate and examined with a Phillips CM-10 electron microscope.

DNA isolation, amplification of near full-length 16S and 18S rDNA, cloning and sequencing. Simultaneous isolation of DNA from the amoebae and their endosymbionts was performed using a modified UNSET procedure (Hugo *et al*., 1992): amoebae and their endosymbionts were harvested from axenic cultures by centrifugation (2000 g, 3 min), washed twice with double-distilled water, resuspended in 500 µl UNSET lysis buffer (8M Urea, 0.15M NaCl, 2% SDS, 0.001M EDTA, and 0.1M Tris/HCl at pH 7.5) and incubated at 60 °C for 5 minutes. Lysates were extracted twice with phenol-chloroform (Roth, Karlsruhe, Germany) and DNA was precipitated for 3h at -20°C with a double volume of absolute ethanol. After centrifugation (10.000 g, 10 min) at 4 °C the ethanol was removed and the pellet was washed twice with 80% ice-cold ethanol to remove residual salts. Pellet was air-dried and resuspended in 30 µl double-distilled water.

Oligonucleotide primers targeting 16S rDNA signature regions which are highly conserved within the domain Bacteria were used for PCR to obtain near full-length bacterial 16S rRNA gene fragments. The nucleotide sequences of the forward and reverse primers used for amplification were 5´-AGAGTTTGATYMTGGCTCAG-3´ (*Escherichia coli* 16S rDNA positions 8 to 27, Brosius *et al.* 1981) and 5´-CAKAAAGGAGGTGATCC-3´ (*E.coli* 16S rDNA positions 1529 to 1546). Amplification of near full-length amoeba 18S rRNA gene fragments was carried out using primers SSU1 (5'-AACCTGGTTGATCCTGCCAG-3') and SSU2 (5'-GATCCTTCTGCAGGTTCACCTAT-3') complementary to conserved target regions at both ends of the 18S rDNA (Gast *et al*., 1994). 16S rDNA and 18S rDNA amplification reactions were performed separately in a thermal capillary cycler (Idaho Technology, Idaho Falls, ID) using reaction mixtures, including 15 pM of each primer, 0.25 µg/ml BSA, 2 mM $MgCl_2$ reaction buffer and 2,5 IU Taq DNA polymerase (Promega Madison, WI). Thermal cycling was carried out as follows: an initial denaturation step at 94°C for 30 s followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 52 °C for 20 s, and elongation at 72 °C for 30 s. Cycling was completed by a final elongation step at 72 °C for 5 min. Positive controls containing purified DNA from *E. coli* and from *Tetrahymena* sp., respectively, were included along with negative controls (without DNA template). The presence and size of the amplification products were determined by agarose gel electrophoresis and ethidium bromide staining.

Amplified products were directly ligated into the cloning vector pCR2.1 or pCRII-TOPO (Invitrogen, Carlsbad, CA) and transformed into competent *E.coli* (TOP10 cells) following the instructions of the manufacturer. Nucleotide sequences of the cloned DNA fragments were determined by the dideoxynucleotide method (Sanger *et al*., 1977) by cycle sequencing of purified plasmid preparations (Qiagen, Hilden, Germany) with a Thermo Sequenase Cycle Sequencing Kit (Amersham Life Science, Little Chalfont, England) and an infrared automated DNA sequencer (Li-Cor Inc., Lincoln, Nebr.) under conditions recommended by the manufacturers. For 16S rDNA sequencing, the dye-labeled vector-specific primers M13/pUC V (5'-GTAAAACGACGGCCAGT-3') and M13/pUC R (5'-GAAACAGCTATGACCATG-3') were applied. For obtaining almost complete 18S rDNA sequences the primer Ac1138 (5'-CTCTAAGAAGCACGGACG-3') was designed and used as dye labeled derivative in addition to primers M13/pUC V and M13/pUC R.

Phylogenetic analysis. The obtained 16S and 18S rDNA sequences were added to the rDNA sequence database of the Technische Universität München (encompassing about 10,000 published and unpublished homologous small subunit rDNA primary structures) by use of the program package ARB (Strunk *et al*., unpublished; program available through the homepage of the Technische Universität München, http://www.biol.chemie.tu-muenchen.de). Two 16S rDNA clones of each endosymbiont were analyzed in this study. Alignment of the new rDNA sequences was performed by using the ARB automated alignment tool (version 2.0). The alignments were refined by visual inspection and by secondary structure analysis. Phylogenetic analyses were performed by applying the ARB parsimony, distance matrix, and maximum likelihood methods to different data sets. To determine the robustness of the phylogenetic trees, analyses were performed with and without

the application of various filtersets to exclude highly variable positions.

Oligonucleotide probes, fluorescent *in situ* hybridization, and confocal laser scanning microscopy. The following oligonucleotide probes were used: (i) EUB 338 (5'-GCTGCCTCCCGTAGGAGT-3'), targeting most but not all members of the domain *Bacteria* (Amann *et al*. 1990), (ii) BET42a (5'-GCCTTCCCACTTCGTTT-3') targeting a signature region of the 23S rRNA of the beta subclass *Proteobacteria* (Manz *et al.*, 1992), and (iii) Cc23a (5'-TTCCACTTTCCTCTCTCG-3') targeting the 16S rRNA of the *Paramecium caudatum* symbiont *Caedibacter caryophilus* (Springer *et al.*, 1993). In addition, the following oligonucleotide probes were designed using the Probedesign/Probematch tools of the ARB software package: (i) S-S-CaeAc-998-a-A-18 (5'-TCTTGTCTCCGCGATCCC-3'; probe designation according to Alm *et al.*, 1996) specific for *Candidatus* Caedibacter acanthamoebae (endosymbiont of *Acanthamoeba polyphaga* HN-3), (ii) S-S-PcaeC9-217-a-A-18 (5'-GGGCTGCTCAATTGGCGA-3') specific for *Candidatus* Paracaedibacter acanthamoebae (endosymbiont of *Acanthamoeba* sp. UWC9), and (iii) S-S-PcaeE39-217-a-A-18 (5'-GGGCTGTTCCTTTAGCGA-3') specific for Candidatus Paracaedibacter symbiosus (endosymbiont of *Acanthamoeba* sp. UWE39). In order to ensure probe-specificity, all available 16S and 23S rDNA sequences included in the ARB database were checked for the presence of the probe target sites. Oligonucleotides were synthesized and directly labeled with 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS), or the hydrophilic sulphoindocyanine fluorescent dyes Cy3 or Cy5 (Interactiva, Ulm, Germany).

For *in situ* hybridization, *Acanthamoeba* cells were harvested from 4 ml liquid TSY broth culture by centrifugation (2000 *g*, 3 min), washed twice with double distilled water, and resuspended in 0.05% agarose. 20 µl of this suspension were spotted on a glass slide, air dried and subsequently dehydrated in 80% EtOH for 10 sec. Optimal hybridization and washing conditions were determined for the endosymbiont-specific probes S-S-CaeAc-998-a-A-18, S-S-PcaeC9-217-a-A-18 and S-S-PcaeE39-217-a-A-18 by using the hybridization and wash buffers (without SDS) described by Manz *et al*. (1992). Slides were examined using a confocal laser-scanning microscope (LSM 510, Carl Zeiss, Oberkochen, Germany) equipped with two helium-neon-lasers (543 and 633 nm) and an argon-krypton-laser (488 and 568 nm). Image analysis processing was performed with the standard software package delivered with the instrument (version 2.01).

Activated sludge from the high-load aeration basin of the municipal sewage treatment plant Großlappen (München I) was fixed with 3% paraformaldehyde for 3h and stored in a 1:1 (vol/vol) 1x PBS (130 mM NaCl, 10mM sodium phosphate buffer, pH 7.2) /EtOH solution at -20 °C.

Nucleotide sequence accession numbers. The recovered 16S rDNA sequences have been deposited in GenBank under accession numbers AF132137 (*Candidatus* Paracaedibacter acanthamoebae, endosymbiont of *Acanthamoeba* sp. UWC9), AF132139 (*Candidatus* Paracaedibacter symbiosus, endosymbiont of *Acanthamoeba* sp. UWE39) and AF132138 (*Candidatus* Caedibacter acanthamoebae, endosymbiont of *Acanthamoeba polyphaga* HN-3). The 18S rDNA sequences received the accession numbers AF132134 (*Acanthamoeba* sp. UWC9), AF132136 (*Acanthamoeba* sp. UWE39) and AF132135 (*Acanthamoeba polyphaga* HN-3).

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Chapter IV

Obligate Bacterial Endosymbionts of *Acanthamoeba* spp. Related to the β-Subclass of *Proteobacteria*: Proposal of "*Candidatus* Procabacter acanthamoebae" gen. nov., sp. nov.

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Obligate Bacterial Endosymbionts of *Acanthamoeba* spp. Related to the β-Subclass of *Proteobacteria*: Proposal of "*Candidatus* Procabacter acanthamoebae" gen. nov., sp. nov.

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All obligate bacterial endosymbionts of free-living amoebae described presently are affiliated with the α-subclass of Proteobacteria, *the* Chlamydiales, *or the* Cytophaga-Flavobacterium-Bacteroides *phylum. Here we report on six rod-shaped Gram-negative obligate bacterial endosymbionts of clinical and environmental isolates of* Acanthamoeba *spp. from the United States and Malaysia. Comparative 16S rDNA sequence analysis demonstrated that the investigated endosymbionts formed a novel, monophyletic lineage within the β-subclass of* Proteobacteria *showing less than 90% sequence similarity to all other recognised members of this subclass. 23S rDNA sequence analysis of two symbionts confirmed this affiliation and revealed the presence of uncommon putative intervening sequences of 146 base pair length within helix-25 sharing no sequence homology to any other bacterial rDNA. In addition, the 23S rRNA of these endosymbionts displays one polymorphism at the target site of oligonucleotide probe BET42a which is conserved in all other sequenced β-*Proteobacteria*. Intra-cytoplasmatic localisation of the endosymbionts within the amoebal host cells was confirmed by electron microscopy and fluorescence* in situ *hybridization with a specific 16S rRNA-targeted oligonucleotide probe. Based on these findings the provisional name "*Candidatus *Procabacter acanthamoebae" is proposed for classification of a representative of the six investigated endosymbionts of* Acanthamoeba *spp. Comparative 18S rDNA sequence analysis of the* Acanthamoeba *host cells revealed their membership with either* Acanthamoeba *18S rDNA sequence type T5 (*A. lenticulata*) or sequence type T4 that comprises the majority of all* Acanthamoeba *isolates.*

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Introduction

In 1975 Proca-Ciobanu *et al*. for the first time described rod-shaped bacterial endosymbionts within free-living amoebae of the genus *Acanthamoeba* which coexisted with their hosts without lysing the amoebal cell. More recent studies demonstrated that stable bacterial endosymbiosis can be observed in about 25 % of *Acanthamoeba* isolates (Fritsche *et al*., 1993) and does also occur in other free-living amoeba like *Hartmannella vermiformis* (Horn *et al*., 2000). 16S rDNA sequence analysis has assigned all currently investigated endosymbionts either to the α-*Proteobacteria* (Fritsche *et al*., 1999; Horn *et al*., 1999; Birtles *et al*., 2000), to novel genera within the *Chlamydiales* (Amann *et al.*, 1997; Fritsche *et al*., 2000; Horn *et al*., 2000) or to the *Cytophaga-Flavobacterium-Bacteroides* phylum (Horn *et al.*, 2001).

In this report six Gram-negative rod-shaped obligate bacterial endosymbionts of clinical and environmental isolates of *Acanthamoeba* spp. were investigated. The presence of endosymbionts in four of these *Acanthamoeba* strains has previously been noticed by one of us but no attempts for symbiont identification were made (Fritsche *et al*., 1993). All symbionts analysed in the present study coexisted with their respective *Acanthamoeba* host and did not cause apparent lysis of their host cells. Previous attempts to cultivate the investigated bacterial endosymbionts using various media and culturing conditions had failed (Fritsche *et al*., 1993). Consequently, phylogenetic characterisation of these endosymbionts was performed by the full-cycle rRNA approach including comparative 16S rDNA sequence analysis, and detection of endosymbionts within the amoebic host cell by fluorescence *in situ* hybridization (FISH) using specific 16S rRNA-targeted oligonucleotide probes. In addition, the phylogenetic affiliation of the *Acanthamoeba* host cells was determined by comparative 18S rDNA sequence analysis.

Isolation of acanthamoebae

Six *Acanthamoeba* isolates (*Acanthamoeba polyphaga* strain Page 23, *Acanthamoeba* sp. strains UWC6, UWC12, UWE2, TUMSJ-226, and TUMSJ-341) containing rod-shaped bacterial endosymbionts were analysed in this study. *Acanthamoeba* sp. strains UWC6 and UWC12 have previously been isolated from corneal scrapings of *Acanthamoeba* keratitis patients (Fritsche *et al*. 1993). *Acanthamoeba polyphaga* Page 23 was isolated from freshwater, (Drummond, Wisconsin, USA; Page, 1967) and was obtained from the American

Type Culture Collection (ATCC 30871). *Acanthamoeba* sp. strain UWE2 was recovered from a soil sample collected in Clearwater County, Minnesota; and *Acanthamoeba* sp. strains TUMSJ-226 and TUMSJ-341 were isolated in this study from a eutrophic lake sediment from Malaysia. Isolations and maintenance of amoebae were performed as described previously (Fritsche *et al*., 1993). Prokaryotic endosymbionts of the *Acanthamoeba* isolates were readily detected under the microscope using Gram- and Giemsa- or DAPI-staining.

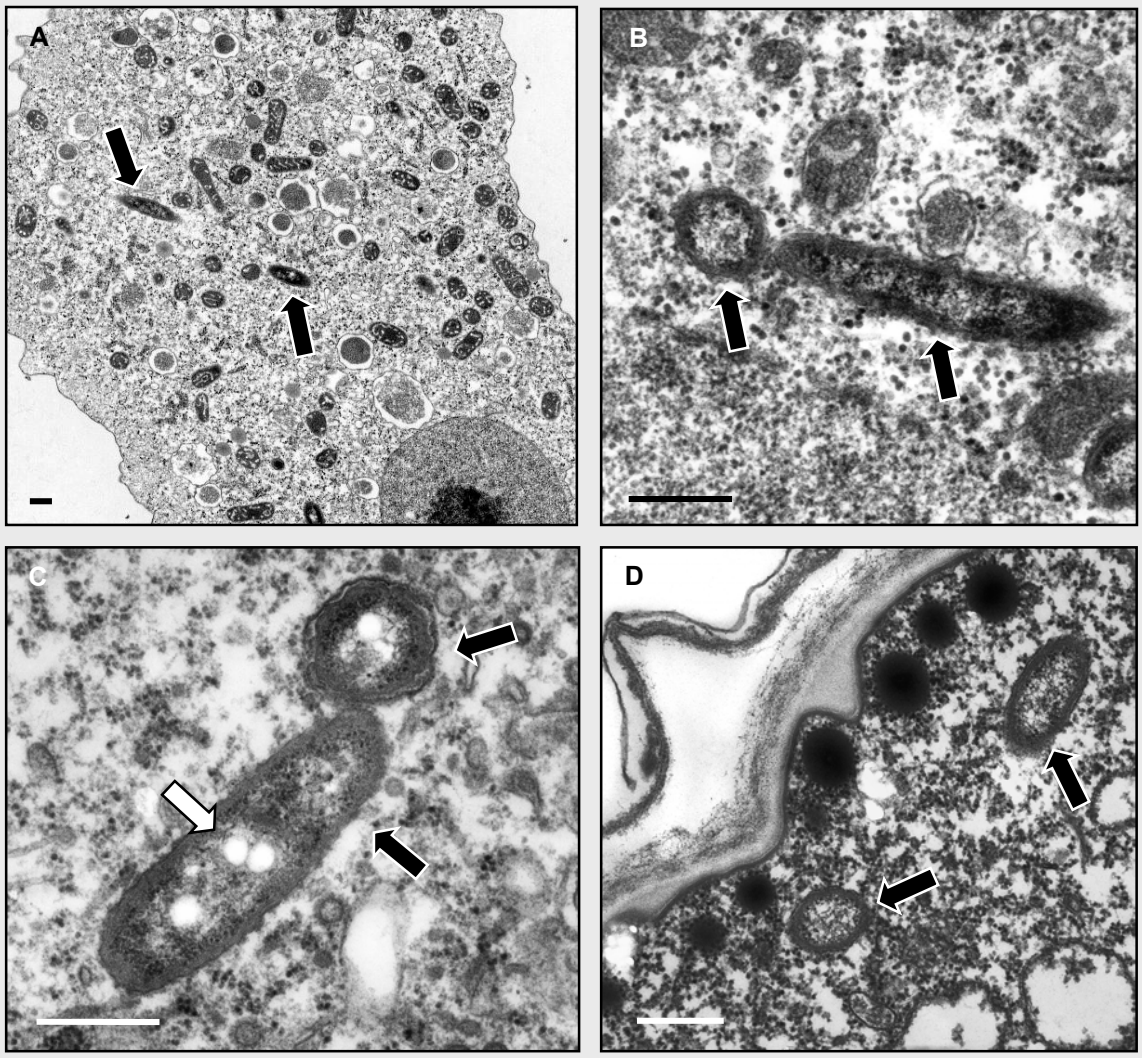

Figure 1: Electron micrographs of (A, B) "*Candidatus* Procabacter acanthamoebae" (endosymbiont of *Acanthamoeba* sp. UWC12), (C) the endosymbiont of *Acanthamoeba polyphaga* Page 23, and (D) the endosymbiont of *Acanthamoeba* sp. UWE2 showing their intracytoplasmatic localisation. Symbionts are marked with black arrows. Electron-translucent inclusions (white arrow) are present in some bacterial cells. Bars, 0.5 mm.

Morphological characterisation of bacterial endosymbionts by electron microscopy

Examination of *Acanthamoeba* isolates by electron microscopy was performed as described elsewhere (Horn *et al*., 1999) and demonstrated, that the rod-shaped endosymbionts of *Acanthamoeba polyphaga* Page 23, *Acanthamoeba* sp. strains UWC6, UWC12, and UWE2 measured 0.3 to 0.5 µm by 1 to 2 µm and showed a Gram-negative type cell wall (Figure 1). Electron-translucent spheroid structures were observed in some bacterial cells. Endosymbionts were found equally distributed within the cytoplasm of both trophozoites and cysts and were not enclosed in vacuoles or embedded in slime layers, as reported for other symbionts of acanthamoebae (Fritsche *et al*., 1998; Horn *et al*., 1999).

16S and 23S rDNA-based phylogeny of bacterial endosymbionts

Simultaneous isolation of DNA from amoebae and their endosymbionts was performed using a previously described modification of the UNSET procedure (Hugo *et al*., 1992; Fritsche *et al*., 1998). Oligonucleotide primers targeting 16S rDNA and 23S rDNA signature regions which are highly conserved within the domain *Bacteria* were used for PCR to obtain near full-length bacterial 16S and 23S rRNA gene fragments, respectively. Nucleotide sequences of forward and reverse primers used for amplification of 16S rDNA were 5′-AGAGTTTGATYMTGGCTCAG-3′ (*E. coli* 16S rDNA positions 8 to 27; Weisburg *et al.*, 1991) and 5′-CAKAAAGGAGGTGATCC-3′ (*E. coli* 16S rDNA positions 1529 to 1546). Nucleotide sequences of primers used for amplification of 23S rDNA were 5′-TCYGAATGGGGNAAC-3′ (*E. coli* 23S rDNA positions 115 to 130) and 5′-CCGGTCCTCTCGTAC-3′ (*E. coli* 23S rDNA positions 2654 to 2669). Amplification of near full-length amoeba 18S rRNA gene fragments was carried out using primers SSU1 (5’-AACCTGGTTGATCCTGCCAG-3’) and SSU2 (5’-GATCCTTCTGCAGGTTCACCTAT-3’) complementary to conserved target regions at both ends of the 18S rDNA (Gast *et al*., 1994). Amplified products were cloned into *E. coli* (TOPO TA Kit, Invitrogen, Carlsbad, CA) and sequenced.

Near full-length 16S rRNA gene sequences (approx. 1,400 bp) from bacterial endosymbionts of *Acanthamoeba polyphaga* Page 23, *Acanthamoeba* sp. strains UWC6,

UWC12, UWE2, TUMSJ-226, and TUMSJ-341 as well as near full-length 23S rRNA gene sequences (approx. 2,600 bp) from bacterial symbionts of *Acanthamoeba* sp. UWC6 and UWC12 were obtained and added to the rRNA sequence database of the Technische Universität München (encompassing about 15,000 published and unpublished homologous small subunit rRNA primary structures) by use of the program package ARB (program available at http://www.mikro.biologie.tu-muenchen.de). Alignment of retrieved rDNA sequences was performed by using the ARB automated alignment tool and refined by visual inspection and secondary structure analysis. Comparative sequence analysis revealed that the 16S rRNA genes of the six endosymbionts were novel and showed highest albeit moderate similarities with members of the β-subclass of *Proteobacteria* (less than 90%), while 16S rDNA similarity values between the respective symbionts were much higher (95 - 99%). Analysis of 23S rRNA genes of bacterial endosymbionts of *Acanthamoeba* sp. UWC6 and UWC12 confirmed their membership with the β-subclass of *Proteobacteria*. While 23S rDNA similarity values with all β-*Proteobacteria* deposited in public databases were below 88%, the 23S rDNA of the two *Acanthamoeba* symbionts was almost identical (99% sequence similarity). Subsequent phylogenetic analyses, performed by applying the distance matrix, parsimony, and maximum likelihood methods implemented in ARB to different data sets

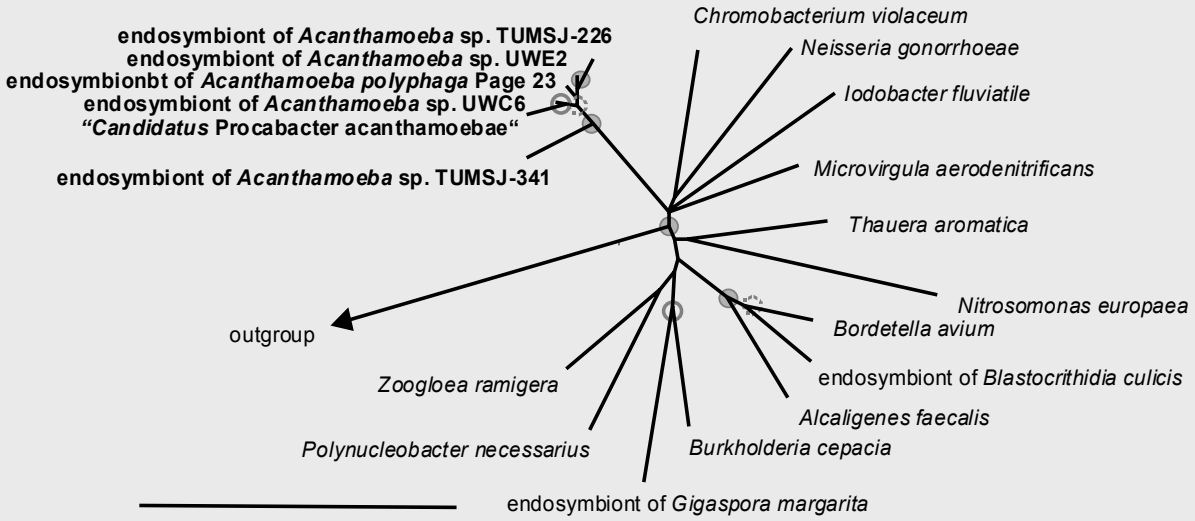

Figure 2. 16S rRNA based neighbor-joining tree reflecting the affiliation of endosymbionts of *Acanthamoeba polyphaga* Page 23, *Acanthamoeba* sp. strains UWC6, UWC12, UWE2, TUMSJ-226, and TUMSJ-341 with selected members of the β-*Proteobacteria*. Circles indicate parsimony bootstraps values (1000 replicates) of 100% (filled circles), more than 80% (open circles) and more than 50% (dashed circles). Bootstrap values below 50% were omitted. Bar indicates 10% estimated evolutionary distance.

(with and without filters excluding highly variable positions) consistently demonstrated for both 16S and 23S rDNA, that the investigated *Acanthamoeba* endosymbionts form a novel and monophyletic cluster within the β-subclass of *Proteobacteria* (Figure 2).

Putative intervening DNA sequences are present in the symbiont 23S rRNA genes

Analysis of the 23S rRNA genes of the β-proteobacterial endosymbionts of *Acanthamoeba* sp. UWC6 and UWC12 revealed the presence of 146 additional bases at position 550 (according to the *E. coli* rRNA gene numbering) which are identical in both investigated symbionts. rRNA secondary structure prediction using the free energy minimisation algorithm as implemented in the programs RNAstructure (Mathews *et al*., 1999) and mfold (Walter *et al*., 1994) demonstrated that these insertions form a stable stem-loop structure, replacing the tetraloops at helix-25 in the postulated 23S rRNA secondary structure of *E. coli* (Figure 3) and thus can be regarded as putative intervening sequences (IVSs) which are within the 23S rRNA genes typically found at helix-25 or helix-45 (Burgin *et al.*, 1990). The G+C content of these inserts matches the overall G+C content of the symbiont 23S rRNA gene (51 %). Although a primary stem of at least 14 bp, which is the postulated cleavage site (Liiv & Remm, 1998), is present in the predicted symbiont IVSs secondary structure, no significant sequence homology to any previously described rRNA gene or IVS could be found in public databases. To our knowledge, this is the first demonstration of putative IVSs in the 23S rRNA gene of members of the β-*Proteobacteria*. The presence of IVSs within rRNA genes has previously been reported for several bacterial genera of other phylogenetic

Figure 3. Structure of the putative IVS present in the 23S rDNA sequence of "*Candidatus* Procabacter acanthamoebae" (endosymbiont of *Acanthamoeba* sp. UWC12) and the endosymbiont of *Acanthamoeba* sp. UWC6.

groupings. IVSs were for example described to occur in the 23S rDNA of *Haemophilus* (Song *et al*., 1999), *Salmonella* (Pabbaraju & Sanderson, 2000), *Leptospira* (Ralph and McClelland, 1993) and in the 16S rDNA of *Helicobacter* (Linton *et al*., 1994) and *Blochmonia* (Sauer *et al*., 2000). The highly variable extended stem-loop structures formed by the IVSs are excised from the rRNA post-transcriptionally (Burgin *et al*., 1990). Interestingly, the presence of IVSs is not necessarily conserved in very closely related bacterial strains and IVSs are not always present in all copies of the rDNA operons within the same bacterium (Pabbaraju & Sanderson, 2000). A more detailed analysis of the β-proteobacterial symbiont IVSs is currently performed but was beyond the scope of the present study.

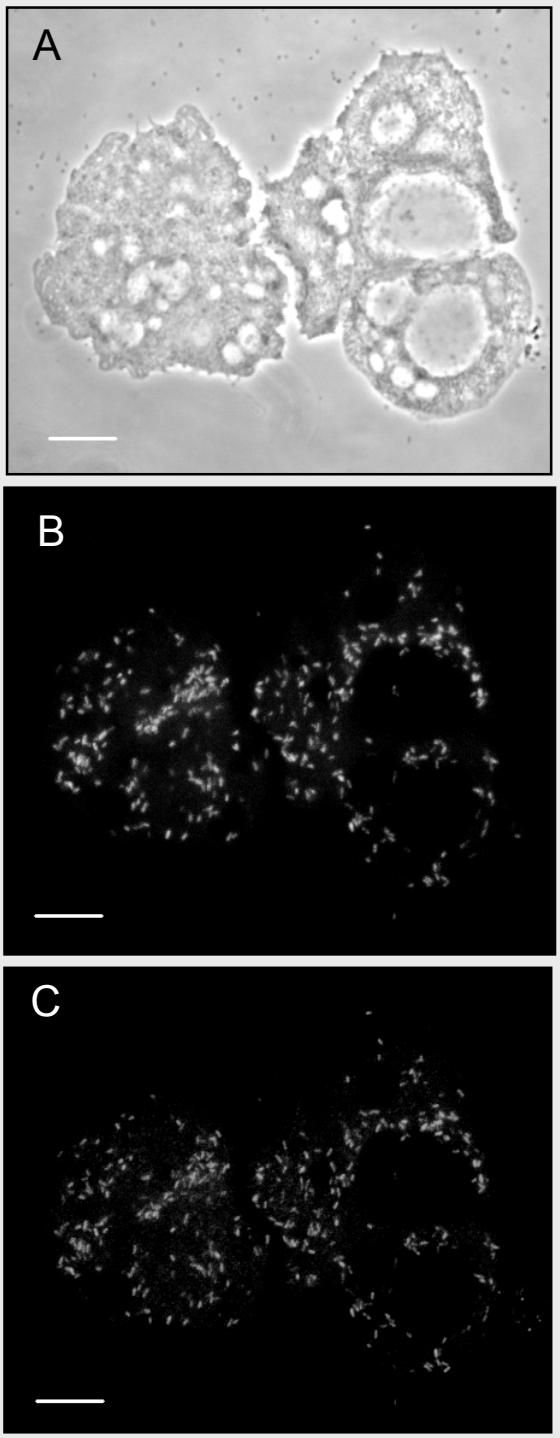

Figure 4. FISH demonstrating the intracellular localisation of "*Candidatus* Procabacter acanthamoebae" (endosymbiont of *Acanthamoeba* sp. UWC12) using bacterial probe EUB338 labelled with FLUOS (B), and endosymbiont-specific probe S-G-Proca-438-a-A-18 labelled with Cy3 (C). A phase contrast image is shown in (A). Bar, 10 µm.

In situ *detection of symbionts within* Acanthamoeba *host cells by FISH*

Bacterial endosymbionts of *Acanthamoeba polyphaga* Page 23, *Acanthamoeba* sp. UWC6, UWC12, UWE2, TUMSJ-226, and TUMSJ-341 were readily visualised by fluorescence *in situ* hybridization using the bacterial probe EUB338 (5'-GCTGCCTCCCGTAGGAGT-3'; Amann *et al*., 1990). Application of the 23S rRNA-targeted probe BET42a (5'-

GCCTTCCCACTTCGTTT-3'; Manz *et al*., 1992), previously designed to hybridize to all bacteria within the β-subclass of *Proteobacteria*, however, did result in only weak fluorescent signals. Subsequent inspection of the 23S rRNA genes of *Acanthamoeba* sp. UWC6 and UWC12 confirmed the presence of a single central strong T-U mismatch at the target site of probe Bet42a destabilising the probe-rRNA hybrids. Consequently, oligonucleotide probe S-G-Proca-438-a-A-18 (probe designation according to Alm *et al.*, 1996) was designed to specifically hybridize to a complementary target region on the 16S rRNA of all analysed endosymbionts. This probe displays at least two mismatches to all other available 16S rRNA sequences. Optimal hybridization stringency for probe S-G-Proca-438-a-A-18 was observed with 20% formamide in the hybridization buffer. Using these stringent hybridization conditions, no detectable signal was obtained when activated sludge from a municipal sewage treatment plant known to contain a high diversity of bacteria (Snaidr *et al*., 1997) was hybridized with the endosymbiont-specific probe S-G-Proca-438-a-A-18. Consequently, positive hybridization reactions of bacterial endosymbionts with specific probe S-G-Proca-438-a-A-18 demonstrated that the retrieved 16S rDNA sequences did originate from the endosymbionts of *Acanthamoeba polyphaga* Page 23, *Acanthamoeba* sp. strains UWC6, UWC12, UWE2, TUMSJ-226, and TUMSJ-341, respectively, and furthermore confirmed their intracellular location (Figure 4). In addition, simultaneous hybridization of the endosymbionts with probes S-G-Proca-438-a-A-18 and the bacterial probe EUB338 labelled with different dyes illustrated that all bacteria within the *Acanthamoeba* host cells were stained by both probes, demonstrating the absence of phylogenetically different symbionts within the respective amoebal host cells.

18S rDNA-based phylogeny of Acanthamoeba *host cells*

Comparative sequence analysis of 18S rRNA gene fragments retrieved from the amoeba host cells revealed highest sequence similarities with members of the genus *Acanthamoeba* (95-99%) and thus confirmed the morphology-based classification of the investigated host amoebae. 18S rDNA sequence similarities of more than 95% with members of the *Acanthamoeba* 18S rRNA sequence type T4 allowed us to assign the amoebal host cells *Acanthamoeba polyphaga* Page 23, *Acanthamoeba* sp. strains UWC6, UWC12, UWE2, and TUMSJ-226 to this sequence type, which comprises the majority of *Acanthamoeba* isolates including all clinical isolates (Gast *et al*., 1994). In contrast to these isolates, *Acanthamoeba* sp. TUMSJ-341 showed highest 18S rDNA similarity (95%) with members of sequence type

T5 indicative for the species *Acanthamoeba lenticulata* (Gast *et al.*, 1994). Like *A. lenticulata*, *Acanthamoeba* sp. TUMSJ-341 contains a larger 18S rDNA (3,000 bp) than all other *Acanthamoeba* species (2.200 bp) caused by a 640 bp insertion with similarities to the group I intron previously found in the small subunit ribosomal gene of *A. lenticulata* (Gast *et al.*, 1994). All applied phylogenetic treeing methods consistently confirmed the membership of the investigated *Acanthamoeba* isolates with 18S rRNA sequence types T4 and T5, respectively (Figure 5).

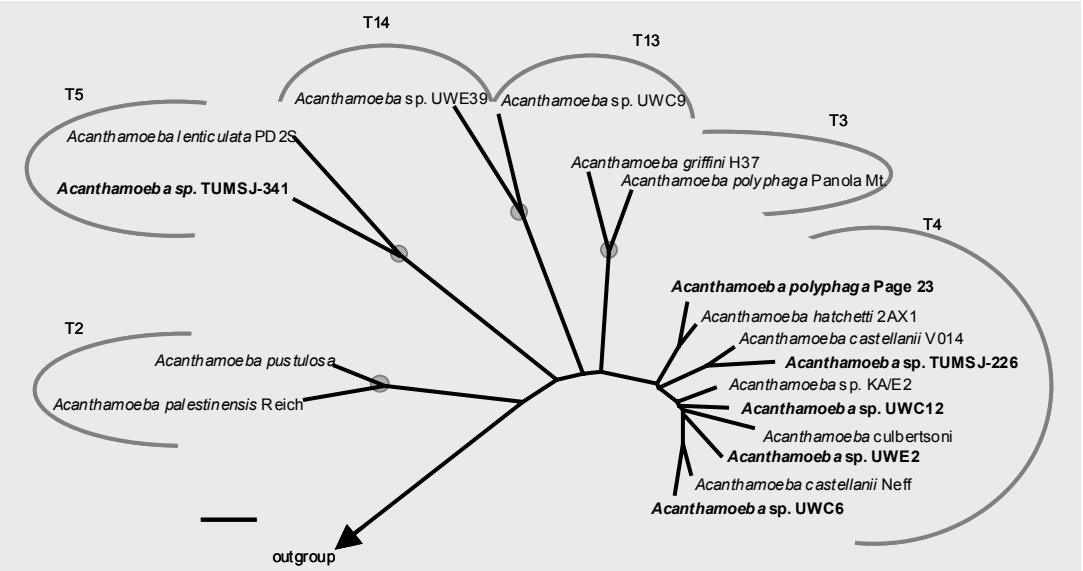

Figure 5. 18S rRNA based neighbor-joining tree reflecting the affiliation of amoebal host cells *Acanthamoeba polyphaga* Page 23, *Acanthamoeba* sp. strains UWC6, UWC12, UWE2, TUMSJ-226, and TUMSJ-341. Acanthamoeba 18S rDNA sequence types are noted. Filled circles indicate parsimony bootstraps values (1000 replicates) of 100%. Bootstrap values below 50% were omitted. Bar indicates 1% estimated evolutionary distance.

Classification of the novel β-proteobacterial endosymbionts of acanthamoebae

While endosymbiosis of bacteria within protozoa, plants or animals is widespread in nature, the majority of described bacteria with this lifestyle are members of the α- or γ-subclass of *Proteobacteria*. Currently, only four endosymbiotic microorganisms pertaining to the β-subclass of *Proteobacteria* were reported: i) *Polynucleobacter necessarius*, an endosymbiont of the hypotrichous ciliate *Euplotes aediculatus* (Springer *et al.*, 1995), ii) *Kinetoplastibacterium crithidii* and *Kinetoplastibacterium blastocrithidii*, which are obligate

endosymbionts of the insect trypanosomatids *Crithidia* sp. and *Blastocrithidia culicis*, respectively (Du *et al.*, 1994), and iii) endosymbionts of the arbuscular-mycorrhizal fungus *Gigaspora margarita* (Bianciotto *et al.*, 1996). The β-proteobacterial *Acanthamoeba* endosymbionts identified in this study and the other, distantly related presently recognised endosymbiotic bacteria within this subclass are members of different lines of descent (Figure 2) suggesting that the capability to thrive within eukaryotic host cells has evolved several times within the β-*Proteobacteria*.

Since the investigated endosymbionts of *Acanthamoeba* spp. showed a 16S rDNA sequence similarity with all other members of the β-*Proteobacteria* of less than 90% and thus could not be assigned to any recognised taxon within this subclass, we propose provisional classification of one representative, the endosymbiont of *Acanthamoeba* sp. UWC12, as "*Candidatus* Procabacter acanthamoebae" gen. nov., sp. nov. 95-99% 16S rDNA sequence similarity of the endosymbionts of *Acanthamoeba polyphaga* strain Page 23, *Acanthamoeba* sp. strains UWC6, UWE2, TUMSJ-226, and TUMSJ-341 with "*Candidatus* Procabacter acanthamoebae" suggested that they all belong to the tentative genus "Procabacter".

Description of "Candidatus Procabacter acanthamoebae" gen. nov., sp. nov.

Procabacter acanthamoebae (Pro.ca.bac.ter N.G. masc. n.; *Procabacter* pertaining to the microbiologist M. Proca-Ciobanu, who for the first time reported on rod-shaped intracellular symbionts in *Acanthamoeba*; a'canth.a.moe.bae L. gen. sing. n. of *Acanthamoeba* taxonomic name of a genus of *Acanthamoebidae*; pertaining to the name of the host amoeba, *Acanthamoeba* sp. strain UWC12, in which the organism was first discovered) The short description is as follows: Gram-negative; rod-shaped morphology; 0.3 to 0.5 µm in width, 1 to 2 µm in length; basis of assignment: 16S rDNA sequence accession number AF177427, 23S rDNA sequence accession number AF352393, 16S rRNA-targeted nucleotide probe S-G-Proca-0438-a-A-18 (5'-CGATTTCCTCCCRGACAA-3'); not cultivated on cell-free media; obligate intracytoplasmatic symbiont of *Acanthamoeba* sp. strain UWC12 isolated from a corneal scrapings of *Acanthamoeba* keratitis patients at the University of Washington Medical Center, Seattle WA, USA; artificial infection of amoebal host cells with "*Candidatus* Procabacter acanthamoebae" enhances their in vitro cytopathogenicity (Fritsche *et al*., 1998); authors: Horn *et al.*, this study.

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Chapter V

Members of the *Cytophaga-Flavobacterium-Bacteroides* Phylum as Intracellular Bacteria of Acanthamoebae: Proposal of "*Candidatus* Amoebophilus asiaticus"

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Members of the *Cytophaga-Flavobacterium-Bacteroides* Phylum as Intracellular Bacteria of Acanthamoebae: Proposal of "*Candidatus* Amoebophilus asiaticus"

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Three Gram-negative, rod-shaped bacteria that were found intracellularly in two environmental and one clinical Acanthamoeba *sp. isolates were analysed. Two endocytobionts showing a parasitic behaviour were successfully propagated outside their amoebal host cells and were subsequently identified by comparative 16S rRNA sequence analysis as being most closely affiliated with* Flavobacterium succinicans *(99% 16S rRNA sequence similarity) or* Flavobacterium johnsoniae *(98% 16S rRNA sequence similarity). One endocytobiont could neither be cultivated outside its original* Acanthamoeba *host (*Acanthamoeba *sp. TUMSJ-321) nor transferred into other amoebae. Electron microscopy revealed that the amoebal trophozoites and cysts were almost completely filled with cells of this endosymbiont which are surrounded by a host-derived membrane. According to 16S rDNA sequence analysis this endosymbiont could also be assigned to the* Cytophaga-Flavobacterium-Bacteroides *phylum, but was not closely affiliated to any recognised species within this phylogenetic group (less than 85% 16S rRNA sequence similarity). Identity and intracellular localisation of this endosymbiont was confirmed by application of a specific fluorescently labelled 16S rRNA-targeted probe. Based on these findings we propose classification of this obligate* Acanthamoeba *endosymbiont as "*Candidatus *Amoebophilus asiaticus". Comparative 18S rDNA sequence analysis of the host of "*Candidatus *Amoebophilus asiaticus" revealed its membership with* Acanthamoeba *18S rDNA sequence type T4, that comprises the majority of all* Acanthamoeba *isolates.*

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Introduction

Acanthamoebae are ubiquitous free-living amoebae that act as important predators controlling microbial communities (Rodriguez-Zaragoza, 1994) and, in addition, have been recognised as opportunistic human pathogens causing keratitis or granulomatous amoebic encephalitis (GAE; Visvesvara *et al*., 1995). Several types of interactions between acanthamoebae and bacteria leading to transient as well as long-term associations have been reported (e.g. Barker and Brown, 1994; Fritsche *et al*., 1993). Besides being a food source for amoebae some bacteria survive phagocytosis and are able to multiply as parasites within free-living amoebae. This capability, which may protect the bacteria from unfavourable environmental conditions, has for example been demonstrated for *Legionella pneumophila* (Fields, 1996), *Chlamydia pneumoniae* (Essig *et al*., 1997), *Listeria monocytogenes* (Ly & Müller, 1990), and *Mycobacterium avium* (Steinert *et al*., 1998). Even obligate anaerobic microorganisms like *Mobiluncus curtisii* can exploit amoebal hosts as transportation and protection vehicles (Tomov *et al.*, 1999). Although long-term symbiotic associations between acanthamoebae and bacteria have already been observed for the first time in the 1970s (Proca-Ciabanu *et al*., 1975), the characterisation of diversity and phylogeny of these obligate intracellular microorganisms had to await the recent development of culture-independent identification techniques. Comparative analysis of 16S rRNA sequences led to the description of four major evolutionary lineages of *Acanthamoeba* endosymbionts. Two of these lineages are within the α-subclass of *Proteobacteria* being most closely related with either the *Rickettsiales* (Fritsche *et al*., 1999a) or the paramecium symbiont *Caedibacter caryophilus* (Horn *et al*., 1999; Birtles *et al*., 2000). In addition, several acanthamoebae endosymbionts form a monophyletic assemblage within the β-subclass of *Proteobacteria* (Fritsche *et al*., 1999b). Furthermore, a number of coccoid amoebal endosymbionts were identified as novel members of the order *Chlamydiales* in which they can be assigned to at least two previously not recognised genera (Amann *et al*., 1997; Horn *et al*., 2000; Fritsche *et al*., 2000).

This report describes the investigation of three *Acanthamoeba* isolates from Germany and Malaysia harbouring intracellular bacteria. These bacteria were identified as three different members of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) phylum. Two of the investigated intracellular bacteria were parasites of the amoebae and could be successfully cultivated. The third bacterial strain could neither be maintained outside its original host nor transferred to other amoebae. Since this obligate endosymbiont of *Acanthamoeba* sp. TUMSJ-

321 has no close relative within the CFB group, the tentative name "*Candidatus* Amoebophilus asiaticus" is proposed for its classification.

Results

Isolation and electron microscopy of acanthamoebae bearing intracellular bacteria

Two *Acanthamoeba* strains were isolated previously in Germany from drinking water in a hospital (isolate RM-69; Michel *et al*., 1995) and from corneal scrapings of a keratitis patient (isolate HLA; Schmid *et al*., 1993). A third *Acanthamoeba* strain TUMSJ-321 was recovered in this study from an eutrophic lake sediment in Malaysia.

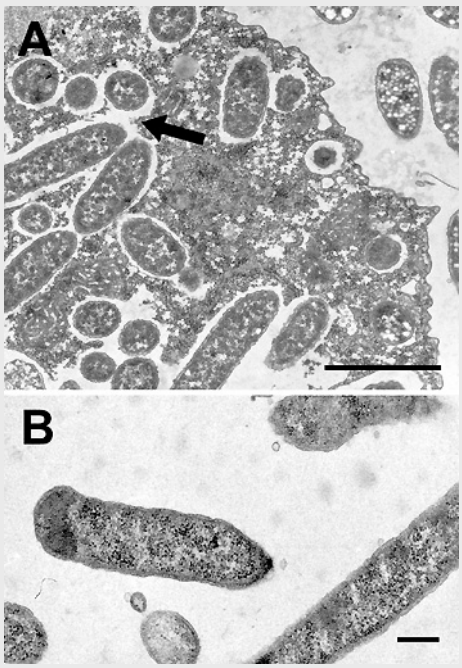

Figure 1. Electron micrographs of (A) the intracellular bacteria of *Acanthamoeba* sp. strain RM-69 within lagoon-like structures (arrow) inside their original amoebal host cell (bar, 0.5 µm), and (B) the intracellular bacteria found in *Acanthamoeba* sp. HLA outside their host (bar, 0.1 µm).

Phase contrast microscopy and DAPI-staining of the amoeba isolates readily visualised rod-shaped, intracellular bacteria within vacuoles (*Acanthamoeba* sp. HLA) or distributed throughout the cytoplasm (*Acanthamoeba* sp. strains RM-69 and TUMSJ-321). While the bacteria found within *Acanthamoeba* sp. strains HLA (also referred to as $KHLA_1$) and RM-69 (also referred to as K69i) prevented amoebal cyst-formation and showed a parasitic behaviour ultimately leading to lysis of the amoebal host cells, the endocytobiont of *Acanthamoeba* sp. strain TUMSJ-321 coexisted with its host, did not inhibit cyst formation, and did not destroy the amoebal cells.

The endoparasites of Acanthamoeba *spp. HLA and RM-69 are culturable and closely related to* Flavobacterium *sp.*

Initially, the parasitic bacteria of *Acanthamoeba* sp. strains HLA and RM-69 were characterised by electron microscopy within their host cells. Both endocytobionts posses a Gram-negative type cell wall. Unlike the intracellular bacteria of *Acanthamoeba* sp. HLA

which were located within vacuoles, the intracellular bacteria of *Acanthamoeba* sp. RM-69 were not surrounded by a membrane but were spread in the amoebal cytoplasm within lagoon-like structures resembling the intracellular occurrence of *Legionella*-like amoebal pathogens (LLAPs) in free-living amoebae (Rowbotham, 1993; Figure 1). The intracellular bacteria of *Acanthamoeba* sp. strain RM-69 were previously shown to be culturable without their eukaryotic hosts by isolation on NN-agar with a thin overlay of SCGYE-medium and subsequent transfer to liquid SCGY-medium (Müller *et al.*, 1999). Here we could demonstrate that the intracellular bacteria of the *Acanthamoeba* strain HLA are also facultative inhabitants of amoebae since they were successfully grown on Columbia blood agar. Fatty acid profiling of both facultatively intracellular bacteria indicated their affiliation with *Cytophaga* sp. (data not shown for intracellular bacteria of *Acanthamoeba* sp. HLA; see Müller *et al*., 1999 for the fatty acid profile of intracellular bacteria of *Acanthamoeba* sp. RM-69). This tentative assignment was confirmed and specified by comparative 16S rRNA gene sequence analysis. Within the CFB phylum, the intracellular bacteria of *Acanthamoeba* sp. strains RM-69 and HLA are closely related with the cultured organisms *Flavobacterium succinicans* (99% 16S

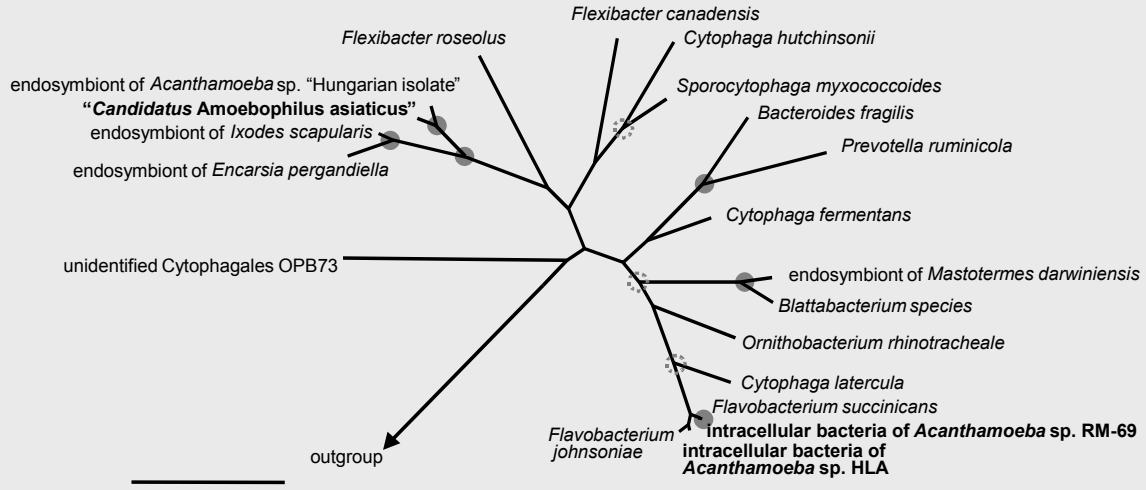

Figure 2. 16S rRNA based maximum likelihood tree reflecting the affiliation of "*Candidatus* Amoebophilus asiaticus" (endosymbiont of *Acanthamoeba* sp. TUMSJ-321) and the intracellular bacteria found in *Acanthamoeba* sp. isolates HLA and RM-69 with selected members of the *Cytophaga-Flavobacterium-Bacteroides* phylum. Circles indicate parsimony bootstraps values (1000 replicates) of 100% (filled circles) and more than 50% (dashed circles). Missing circles indicate that either the parsimony bootstrap values were below 50% or that no consistent tree subtopology was found between the parsimony and the maximum likelihood tree. Sequence accession numbers are listed in the experimental procedures section. Bar indicates 10% estimated evolutionary distance.

rRNA sequence similarity; Bernardet *et al*., 1996) and *Flavobacterium johnsoniae* (98% 16S rRNA sequence similarity; Oyaizu *et al*., 1981), respectively (Figure 2).

A novel obligate endosymbiont of acanthamoebae belonging to the CFB phylum

In contrast to the facultative intracellular bacteria of acanthamoebae described above, attempts to culture the endocytobiont of *Acanthamoeba* sp. TUMSJ-321 on a number of cell-free media (performed in two independent laboratories) failed. These findings suggest that eukaryotic host cells are necessary for the growth of these bacteria, which are therefore considered obligate endosymbionts of *Acanthamoeba* sp. TUMSJ-321.

Electron microscopic analysis revealed that the symbionts measure 0.3 to 0.5 µm in width and 1.0 to 1.5 µm in length, posses a Gram-negative type cell wall, and are present within the amoebal trophozoite in extremely large numbers replacing a significant fraction of the amoebal cytoplasm (Figure 3a). The bacteria were equally distributed throughout the cytoplasm with each of them being surrounded by a membrane (Figure 3b). The attachment of numerous ribosomes to this membrane indicates that it is derived from the rough endoplasmatic reticulum. Densely packed endosymbionts could also be observed in the cysts of *Acanthamoeba* sp. TUMSJ-321 (Figure 3c).

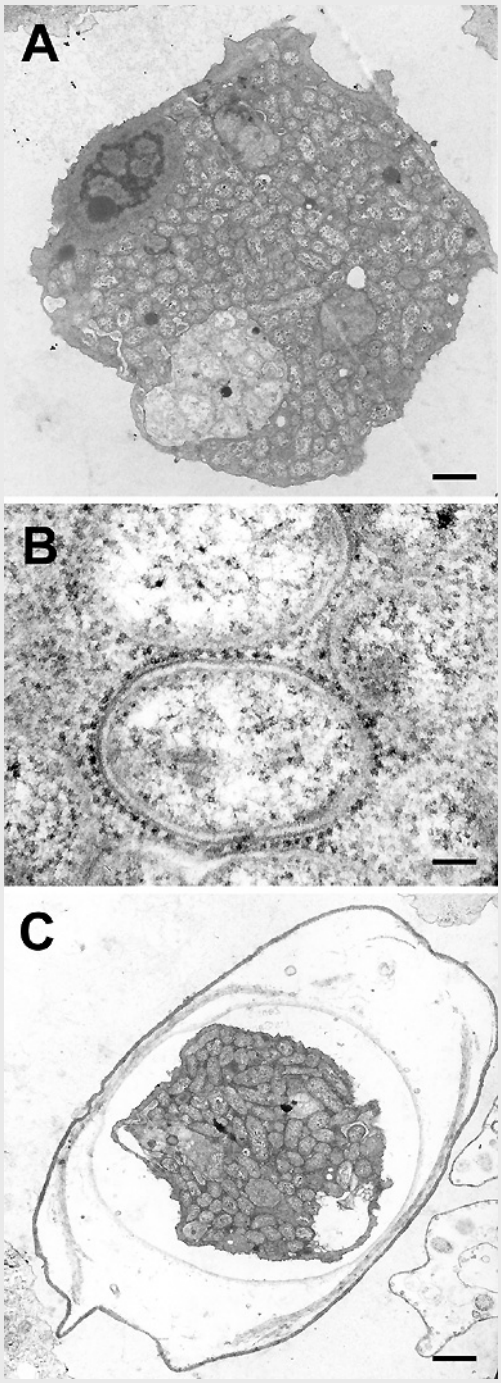

Figure 3. Electron micrographs of "*Candidatus* Amoebophilus asiaticus" (endosymbiont of *Acanthamoeba* sp. TUMSJ-321) within an amoebal trophozoite (A; bar, 1 µm). Bacteria are surrounded by host-derived membranes associated with numerous ribosomes (B; bar, 0.1 µm). "*Candidatus* Amoebophilus asiaticus" within an amoebal cyst (C; bar, 1 µm).

Near full-length 16S rDNA fragments (1,471 bp) of the obligate endosymbiont of *Acanthamoeba* sp. TUMSJ-321 were amplified, cloned and sequenced. Comparative sequence analysis supported the assignment of this symbiont to the CFB phylum. Within this group the *Acanthamoeba* symbiont showed highest but only moderate 16S rRNA sequence similarities with symbionts of (i) the tick *Ixodes scapularis* (90%; Kurtti *et al.*, 1996) and (ii) the parasitic white fly *Encarsia pergandiella* (87%). Even lower similarity values were noted with cultured representatives of the CFB phylum (less than 82%). Sequence database searches revealed, however, a higher similarity with a 16S rRNA sequence submitted recently to public databases but not published elsewhere (96%, deposited as endosymbiont of *Acanthamoeba* sp. "Hungarian isolate"; GenBank accession number AF215634). Phylogenetic analysis using distance matrix, parsimony, and maximum likelihood methods consistently demonstrated that the *Acanthamoeba* symbiont, the *Ixodes*, and the *Encarsia* symbionts form a novel monophyletic lineage within the CFB phylum (Figure 2).

The 16S rRNA sequence of the *Acanthamoeba* sp. TUMSJ-321 symbiont possesses multiple mismatches to all previously published oligonucleotide probes designed to be specific for the CFB phylum or its subgroups (Manz *et al*., 1996; Weller *et al*., 2000). Consistent with this finding fluorescence *in situ* hybridization (FISH) with probe CF319a (Manz *et al*., 1996) targeting most but not all *Cytophaga*, *Flavobacterium*, and *Flexibacter* species resulted in no detectable signal (data not shown). Consequently, the oligonucleotide

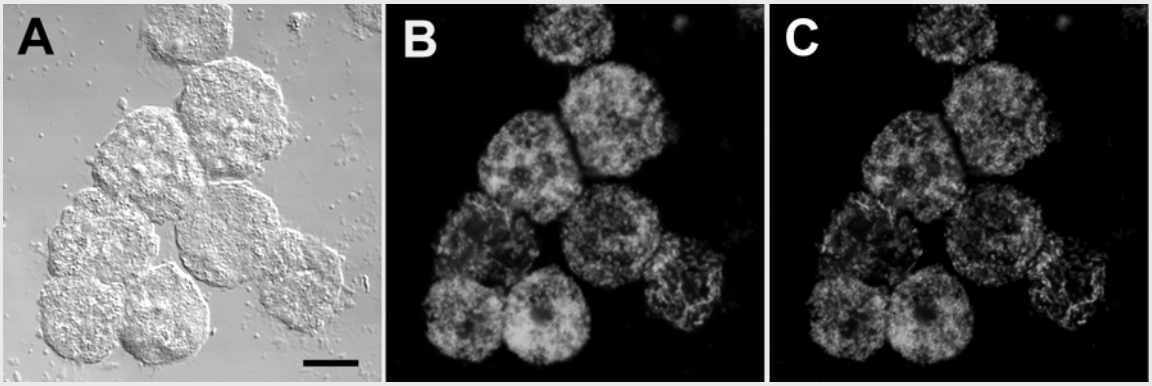

Figure 4. FISH demonstrating the intracellular localisation of "*Candidatus* Amoebophilus asiaticus" (endosymbiont of *Acanthamoeba* sp. TUMSJ-321) using bacterial probe EUB338 labelled with FLUOS (B), and endosymbiont-specific probe S-G-Aph-1180-a-A-18 labelled with Cy3 (C). Due to the dense packing of the endosymbiont within their hosts single cell resolution of the fluorescently labelled bacteria could not be achieved for all image areas. A phase contrast image is shown in (A). Bar, 10 µm.

probe S-G-Aph-1180-a-A-18 specific for the endosymbionts of *Acanthamoeba* sp. TUMSJ-321 was designed and evaluated. Fluorescence *in situ* hybridization using this endosymbiont-specific probe under stringent conditions (see Experimental procedures) in combination with confocal laser scanning microscopy successfully visualised the endosymbionts of *Acanthamoeba* sp. TUMSJ-321 within their host cells and demonstrated their intracellular localisation (Figure 4). In addition, simultaneous hybridization experiments with the bacterial probe EUB338 demonstrated that all bacteria detectable by *in situ* hybridization also hybridized with the endosymbiont-specific probe, suggesting the absence of additional, phylogenetically different bacteria within the amoebal host (Figure 4).

Host range of the obligate endosymbiont of Acanthamoeba *sp. TUMSJ-321*

Since standard cultivation techniques failed to support extracellular growth of the endosymbiont of *Acanthamoeba* sp. TUMSJ-321 the host range of this symbiont was assessed by transfection experiments using a set of related amoebal species as potential hosts including two *Dictyostelium discoideum* strains (Table 1). None of these amoebae were able to propagate the obligate endosymbiont of *Acanthamoeba* sp. TUMSJ-321. These findings are in striking contrast to other previously described bacterial endosymbionts of free-living amoebae that were able to also thrive within other amoebal host cells (Gautom *et al*., 1995; Michel, 1997; Horn *et al.*, 2000; Michel *et al*. 2000).

Table 1. Host spectrum of "*Candidatus* Amoebophilus asiaticus" (endosymbiont of *Acanthamoeba* sp. strain TUMSJ-321).

Amoeba species	Strain	Intracellular multiplication
Acanthamoeba sp.	TUMSSJ-321	+
Acanthamoeba castellanii	C3	—
Acanthamoeba sp.	HLA	—
Acanthamoeba lenticulata	45	—
Naegleria lovaniensis	Aq/9/1/40	—
Naegleria gruberi	NI_1	—
Hartmannella vermiformis	C3/8	—
Vahlkampfia ovis		—
Balamuthia mandrillaris		—
Willaertia magna	NI_4Cl_{1*}	—
Dictyostelium discoideum	$Berg_{25}$	—
Dictyostelium discoideum	Sö-R_1	—

Phylogeny of the amoebal host

Acanthamoeba *sp. TUMSJ-321*

In order to further characterise the original host (*Acanthamoeba* sp. TUMSJ-321) of the novel *Cytophaga*-related endosymbiont, the amoebal 18S rRNA gene was amplified, cloned and sequenced (2,251 bp). Comparative sequence analysis confirmed the morphology based identification of this isolate as *Acanthamoeba* sp. Using the 95% similarity threshold value for the definition of the *Acanthamoeba* 18S rRNA sequence types (Stothard *et al*., 1998) *Acanthamoeba* sp. TUMSJ-321 could be assigned to sequence type T4 comprising the majority of clinical and environmental *Acanthamoeba* isolates (Figure 5).

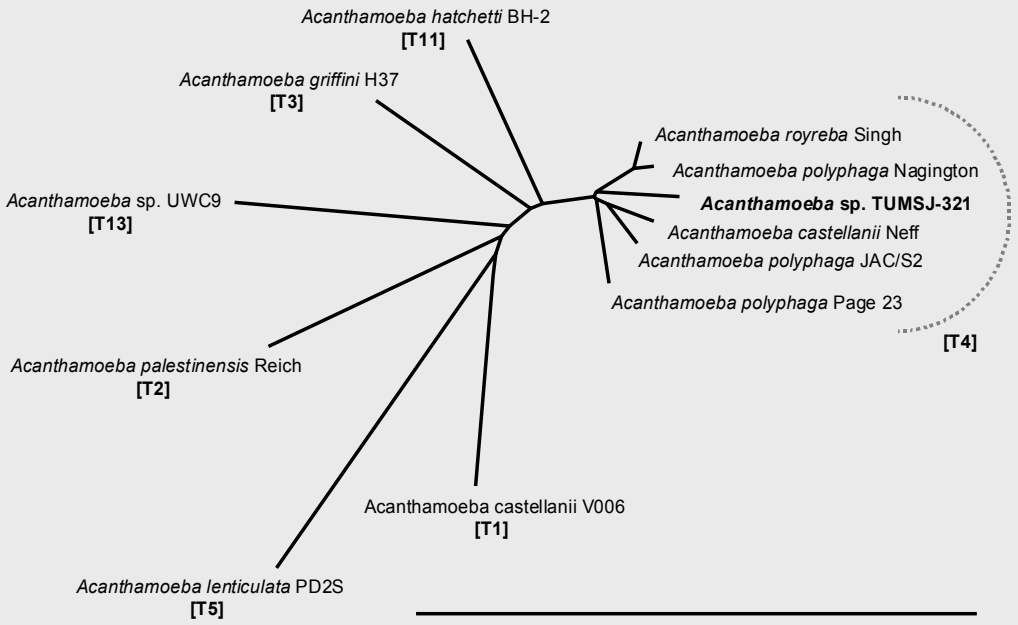

Figure 5. 18S rRNA based neighbor-joining tree reflecting the affiliation of *Acanthamoeba* sp. TUMSJ-321 (host of "*Candidatus* Amoebophilus asiaticus") with selected members of the genus *Acanthamoeba*. Appropriate *Acanthamoeba* 18S rRNA sequence types are noted (Stothard *et al*., 1998; Horn *et al*., 1999). Bar indicates 10% estimated evolutionary distance.

Discussion

The *Cytophaga-Flavobacterium-Bacteroides* phylum (Woese *et al*., 1985) was originally described as comprising gliding (the genus *Cytophaga*), pigmented (the genus *Flavobacterium*), and non-spore forming anaerobic bacteria (the genus *Bacteroides*) and has today been recognised to contain bacterial species showing an extremely broad range of

physiological and morphological properties (Reichenbach, 1991). Members of the CFB phylum were detected in soil, fresh and sea water, activated sludge, and the human gut (Weber *et al*., 2001; Glöckner *et al*., 2000; Sievert *et al*., 2000; Bond *et al*., 1995; Suau *et al*., 1999). However, there have been only few reports on symbiotic or parasitic intracellular bacteria belonging to the CFB phylum, namely the *Ixodes scapularis* symbiont (Kurtti *et al.*, 1996), and the *Blattabacteria* and relatives which occur as male-killing parasites of lady bird beetles (Hurst *et al*., 1999) or as endosymbionts in cockroaches (Bandi *et al*., 1994) and the termite *Mastotermes darwiniensis* (Bandi *et al*., 1997).

This study describes three members of the CFB phylum able to thrive within free-living amoebae of the genus *Acanthamoeba*. Two of these intracellular bacteria were successfully cultivated on cell-free media and were found to be closely related to other cultured members of this phylum (*Flavobacterium succinicans, Flavobacterium johnsoniae*). Despite being close relatives (96% 16S rRNA sequence similarity) the intracellular location of the two facultative endoparasites differed remarkably (within vacuoles or directly within the host cytoplasm). These findings strengthen the hypothesis that acanthamoebae are exploited as transitory hosts by a huge variety of both, environmental and clinically relevant bacteria (Harb *et al*., 2000). Moreover, *Flavobacterium* sp. related bacteria which were isolated from the air-cooling system of a textile-producing facility have been implicated as causative agents of a lung disease similar to hypersensitivity pneumonitis (Liebert e*t al*., 1984; Flaherty *et al*., 1984). Since free-living amoebae are abundant in those systems (e.g. Dondero *et al*., 1980) and, in addition, are known to be essential for the spread of Legionnaire's disease (caused by *Legionella pneumophila*; Atlas, 1999), the prior intracellular multiplication of *Flavobacterium* sp. in free-living amoebae might explain this outbreak.

The third investigated *Acanthamoeba* isolate contained bacteria that depend upon intracellular growth within amoebae and could neither be cultured on cell-free media nor transferred to other amoebae. Therefore these bacteria are considered to be obligate endosymbionts of *Acanthamoeba* sp. TUMSJ-321. This is also supported by the observation that symbiont-harbouring trophozoites of *Acanthamoeba* sp. TUMSJ-321 retained their capability to form viable cysts. The cysts still contained bacterial endosymbionts and when transferred to fresh media, amoebal trophozoites hatched from their cysts, proliferated, and thus founded a new population of symbiont-harbouring acanthamoebae. While comparative rRNA sequence analysis also assigned this symbiont to the CFB phylum, no close validly

described relatives within this group could be determined (less than 85% 16S rRNA sequence similarity). This does also apply to the relation of the amoeba symbiont with the *Blattabacteria*, the only recognised intracellular bacteria belonging to the CFB group (less than 78% sequence similarity). Consequently, the endosymbionts of *Acanthamoeba* sp. TUMSJ-321 could not be assigned to any validly described taxon within the CFB phylum. Keeping in mind that 16S rRNA sequence similarities between two bacteria of less than 95% are indicative of their affiliation with two different genera (Ludwig *et al*., 1998), the endosymbiont of *Acanthamoeba* sp. TUMSJ-321 most likely represents a new species of a new genus. In addition, the intracellular way of living of the *Acanthamoeba* symbiont within protozoa which is unique among members of the CFB group and its restriction to a single host warrants the classification of this microorganism into a novel genus. Therefore we propose the tentative name "*Candidatus* Amoebophilus asiaticus". for classification of the endosymbiont of *Acanthamoeba sp.* strain TUMSJ-321.

*Short description of "*Candidatus *Amoebophilus asiaticus"*

Amoebophilus asiaticus (A.moe'bo.phi.lus, pertaining to the obligate intracellular life style of the bacteria in free-living amoebae; a.si.a'ti.cus, pertaining the continent Asia where the original host was isolated). phylogenetic position: *Cytophaga-Flavobacterium-Bacteroides* phylum; Gram-negative; rod-shaped morphology; 0.3 to 0.5 µm in width, 1.0 to 1.5 µm in length; basis of assignment: 16S rDNA sequence accession number AF366581, nucleotide probe S-G-Aph-1180-a-A-18 (5'-CTGACCTCATCCCCTCTT-3'); not cultivated on cell-free media; obligate intracytoplasmatic symbiont of *Acanthamoeba* sp. strain TUMSJ-321 isolated from an eutrophic lake sediment in Malaysia; authors: Horn *et al*., this study.

In essence, this study demonstrates that certain previously not known members of the CFB phylum are parasites as well as symbionts of acanthamobae. The identification of "*Candidatus* Amoebophilus asiaticus" revealed a novel evolutionary lineage of obligate symbionts of acanthamoebae. Interestingly, "*Candidatus* Amoebophilus asiaticus" forms a monophyletic group together with bacterial symbionts of the tick *Ixodes scapularis* and the white fly *Encarsia pergandiella* within the CFB phylum indicating that members of this evolutionary lineage share the capability to form stable associations with eukaryotic cells. This is consistent with the observation that all previously detected endosymbionts of acanthamoebae are members of evolutionary lineages exclusively consisting of bacteria dependent on intracellular survival within eukaryotes (Horn *et al*., 1999; Horn *et al*., 2000;

Fritsche *et al*., 1999a; Fritsche *et al*., 1999b). The *Amoebophilus* as well as the *Caedibacter*/*Paracaedibacter* lineage (Horn *et al*., 1999) contain bacteria which can infect phylogenetically unrelated eukaryotes. Thus, the ancestor of these bacteria obviously acquired or developed adaptive features which allow its descendants to successfully infect different eukaryotic cells.

Acknowledgement

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Experimental procedures

Isolation and maintenance of *acanthamoebae*. The amoebal host strain *Acanthamoeba* sp. RM-69 was isolated previously from the drinking water system of a hospital in Koblenz (Germany) by filtering a 100 ml water sample obtained from the source water as described elsewhere (Michel *et al.*, 1995). *Acanthamoeba* sp. HLA was recovered from corneal scrapings of a keratitis patient. The amoebal host strain *Acanthamoeba* sp. TUMSJ-321 was retrieved from an eutrophic lake water sediment sample from Malaysia. Isolation and identification of these isolates was performed as described elsewhere (Michel *et al*., 1995; Horn *et al*., 2000). *Acanthamoeba* strains RM-69 and HLA were maintained on NN-agar plates (non-nutrient agar; Page, 1988) seeded with *Enterobacter cloacae*. *Acanthamoeba* sp. TUMSJ-321 was successfully axenized in fluid SCGYE-medium (containing 10 g/l casein, 2.5 g/l glucose, 5 g/l yeast extract, 1/10 foetal bovine serum, 1.325 g/l Na_2HPO_4, 0.8 g/l KH_2PO_4; De Jonckheere, 1977) by temporary addition of penicillin and streptomycin (0.2 mg ml^{-1} each). Cultures were incubated at 30°C and fresh medium was applied every 5-10 days.

Extracellular growth of acanthamoebae endocytobionts. Attempts to culture the intracellular bacteria of the investigated *Acanthamoeba* spp. isolates on cell-free media included cultivation on blood agar (Becton Dickinson, New Jersey), Casiton-agar (Biotest-Heipha, Germany), SCGYE (De Jonckheere, 1977), R2A (Reasoner & Geldreich, 1985), and media commonly used for the propagation of *Cytophaga* or *Flexibacter* species (DSMZ [German Collection of Microorganisms and Cell Cultures] media numbers 160 and 357), at incubation temperatures of 20 °C and 30 °C. Both, whole amoeba cells and filter-purified endocytobionts from lysed amoeba cells (by freeze-thawing) were transferred to the respective media. If no growth was observed after 14 days of incubation, cultures where considered negative.

Transfection experiments. Following lysis of endosymbiont bearing *Acanthamoeba* sp. TUMSJ-321 cells from 4-5 day-old cultures by freeze-thawing, the coccoid bacterial endocytobionts were filter purified (3 µm or 5 µm membrane filter). An aliquot of the resulting suspension was added to strains of different amoeba species, growing either in SCGYE-medium or on non-nutrient agar plates covered with a lawn of *Enterobacter cloacae*. The host range of the endocytobiont was investigated by transfection experiments with 9 different amoeba species (Table 1), and two strains of *Dictyostelium discoideum*. Infection of each host species was monitored by microscopic examination using phase contrast microscopy. After 21 days of incubation at 30°C or 20°C the host was considered resistant to infection if no infected cells nor any marked reduction in amoebal numbers (which may have resulted from parasitic activity of the endocytobiont) were observed.

Electron microscopy. For electron microscopical studies, the endosymbiont-harbouring trophozoites from 4-5 day-old cultures were harvested and prefixed in 2.5% glutaraldehyde in cacodylate

buffer (pH 7.2) for 2 hours. After prefixation the specimens were fixed in 1% osmium tetroxide followed by 2% uranyl acetate in aqueous solution. Subsequently specimens were dehydrated in alcohol and embedded in epoxy resin. Thin sections were stained with 1% lead citrate and examined with a Zeiss EM 910 electron microscope.

DNA isolation, amplification of 16S and 18S rDNA, cloning and sequencing. DNA from the cultured intracellular bacteria of *Acanthamoeba* sp. strains RM-69 and HLA was isolated using the FastDNA Kit (Bio 101, Carlsbad, CA, U.S.A) and a bead beater (Bio 101-FP120) following the instructions of the manufacturer. Simultaneous isolation of DNA from the *Acanthamoeba* sp. TUMSJ-321 host cells and their bacterial endosymbionts was performed using a modified UNSET procedure (Hugo *et al*., 1992) as described elsewhere (Horn *et al*., 2000). Oligonucleotide primers targeting 16S rDNA signature regions which are highly conserved within the domain *Bacteria* were used for PCR to obtain near full-length bacterial 16S rRNA gene fragments. Nucleotide sequences of forward and reverse primers used for amplification were 5´-AGAGTTTGATYMTGGCTCAG-3´ (*Escherichia coli* 16S rDNA positions 8 to 27, Brosius *et al.* 1981; Weisburg *et al.*, 1991) and 5´-CAKAAAGGAGGTGATCC-3´ (*E.coli* 16S rDNA positions 1529 to 1546). Amplification of near full-length amoeba 18S rRNA gene fragments was carried out using primers SSU1 (5'-AACCTGGTTGATCCTGCCAG-3') and SSU2 (5'-GATCCTTCTGCAGGTTCACCTAT-3') complementary to conserved target regions at both ends of the 18S rDNA (Gast *et al*., 1994). 16S and 18S rDNA amplification reactions were performed separately in a thermal capillary cycler (Idaho Technology, Idaho Falls, ID). Positive controls containing purified DNA from *Escherichia coli* or *Acanthamoeba* sp. were included along with negative controls (without DNA template). The presence and size of the amplification products were determined by agarose gel electrophoresis and ethidium bromide staining. Amplified products were directly ligated into the cloning vector pCRII-TOPO and transformed into competent *E.coli* (TOP10 cells) per instructions of the manufacturer (Invitrogen, Carlsbad, CA). Nucleotide sequences of the cloned DNA fragments were determined by the dideoxynucleotide method (Sanger *et al*., 1977) by cycle sequencing of purified plasmid preparations (Qiagen, Hilden, Germany) with a Thermo Sequenase Cycle Sequencing Kit (Amersham Life Science, Little Chalfont, England) and an automated DNA sequencer (Li-Cor Inc., Lincoln, Nebr.) under conditions recommended by the manufacturers. Dye-labelled vector-specific primers M13/pUCV (5-GTAAAACGACGGCCAGT-3') and M13/pUCR (5'-GAAACAGCTATGACCATG-3') were applied. The additional primer Ac1138 (5'-CTCTAAGAAGCACGGACG-3'; Horn *et al.*, 1999) was used to complete the 18S rDNA sequences.

Phylogenetic analysis. Sequence homology searches within the public databases DDBJ/EMBL/GenBank were performed using the BLASTn service available at the NCBI (National Centre for Biotechnology Information; Altschul *et al*., 1990). The obtained 16S rRNA sequences were added to the rRNA sequence database of the Technische Universität München (encompassing more than 15,000 published and unpublished homologous small subunit rDNA primary structures) by use of the program package ARB (Strunk *et al*., unpublished; program available through the website of the Technische Universität München, http://www.mikro.biologie.tu-muenchen.de). Alignment of the new rRNA sequences was performed by using the ARB automated alignment tool (version 2.0). The alignments were refined by visual inspection and by secondary structure analysis. Phylogenetic analyses were performed by applying the ARB parsimony, distance matrix, and maximum likelihood (fastDNAml 3.3) methods to different data sets. The maximum likelihood model employed allows for unequal expected frequencies of the four nucleotides, for unequal rates of transitions and transversions, and for different rates of change in different categories of sites (Felsenstein, 1981; Olsen et al., 1994). To determine the robustness of the phylogenetic trees, analyses were performed with and without the application of various filter sets to exclude highly variable positions.

Fluorescence *in situ* hybridization and confocal laser scanning microscopy. The following oligonucleotide probes were used: (i) EUB 338 (5'-GCT GCC TCC CGT AGG AGT-3'), targeting most, but not all members of the domain *Bacteria* (Amann *et al*. 1990; Daims *et al*., 1999), and (ii) CF319a (5'- TGG TCC GTG TCT CAG TAC-3', Manz et al., 1996) complementary to a 16S rRNA signature region of many *Cytophaga* and *Flavobaterium* species. In addition, probe S-G-Aph-1180-a-A-18 (5'-CTG ACC TCA TCC CCT CTT-3'; probe designation according to Alm *et al.*, 1996) was designed using the Probedesign/Probematch tools of the ARB software package to specifically hybridize with the endosymbionts of *Acanthamoeba* sp. TUMSJ-321. Probe S-G-Aph-1180-a-A-18 displays at least one centrally located strong mismatch with all available 16S and 23S rDNA sequences included in the ARB database. Optimal hybridization stringency was determined for this probe by increasing the formamide concentration in the hybridization buffer in increments of 10 % at a constant hybridization temperature of 46°C. Probe-conferred signals of the endosymbionts remained at the same level

following the addition of formamide up to 20 %. Further increase of stringency caused significant decrease of signal intensity (data not shown). Oligonucleotides were synthesised and directly 5'-labelled with 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS), or the hydrophilic sulphoindocyanine fluorescent dye Cy3 (Interactiva, Ulm, Germany). For *in situ* hybridization amoebal cells were harvested from 4 ml liquid broth culture by centrifugation (2000 *g*, 3 min) and washed with Page's saline. 20 µl aliquots of this suspension were spotted on a glass slide, air dried, fixed with 4% paraformaldehyde for 30 minutes at room temperature, and subsequently dehydrated in EtOH. Hybridization was performed using the hybridization and washing buffer described by Manz *et al*. (1992). Slides were examined using a confocal laser-scanning microscope (LSM 510, Carl Zeiss, Oberkochen, Germany) in combination with a helium-neon-laser (543 nm) and an argon-krypton-laser (488 nm). Image analysis processing was performed with the standard software package delivered with the instrument (version 2.01).

Nucleotide sequence accession numbers. The recovered 16S and 18S rRNA sequences have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AF366581 (16S rRNA of "*Candidatus* Amoebophilus asiaticus", endosymbiont of *Acanthamoeba* sp. TUMSJ-321), AF366582 (16S rRNA of intracellular bacteria of *Acanthamoeba* sp. HLA), AF366583 (intracellular bacteria of *Acanthamoeba* sp. RM-69), and AF366580 (18S rRNA of *Acanthamoeba* sp. TUMSJ-321, original host of "*Candidatus* Amoebophilus asiaticus").

The sequence accession number of bacteria included in Figure 2 are Y08957 (*Thermonema rossianum*), M58787 (*Flexibacter roseolus*), AB001518 (endosymbiont of *Ixodes scapularis*), AF319783 (*Encarsia pergandiella*), M58782 (*Flexibacter elegans*), AF027008 (unidentified *Cytophagales* OPB73), M58790 (*Haliscomenobacter hydrossis*), M59053 (*Flavobacterium johnsoniae*), D12673 (*Flavobacterium succinans),* M58769 (*Cytophaga latercula*), L19156 (*Ornithobacterium rhinotracheale*), X75624 (*Blattabacterium* sp.), Z35665 (endosymbiont of *Mastodermis darwiniensis*), M58766 (*Cytophaga fermentans*), AB004909 (*Prevotella ruminicola*), X83938 (*Bacteroides fragilis*), AJ310654 (*Sporocytophaga myxococcoides*), M58768 (*Cytophaga hutchinsonii*), AF215634 (endosymbiont of *Acanthamoeba* sp. "Hungarian isolate"), and M28055 (*Flexibacter canadensis*).

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Chapter VI

Neochlamydia hartmannellae gen. nov., sp. nov. (*Parachlamydiaceae*), an Endoparasite of the Amoeba *Hartmannella vermiformis*

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Neochlamydia hartmannellae gen. nov., sp. nov. (*Parachlamydiaceae*), an endoparasite of the amoeba *Hartmannella vermiformis*

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Free-living amoebae are increasingly being recognized to serve as vehicles of dispersal for various bacterial human pathogens and as hosts for a variety of obligate bacterial endocytobionts. Several Chlamydia-*like* Acanthamoeba *endocytobionts constituting the recently proposed family* Parachlamydiaceae *are of special interest as potential human pathogens. In this study coccoid bacterial endocytobionts of a* Hartmannella vermiformis *isolate were analysed. Infection of* H. vermiformis *with these bacteria resulted in prevention of cyst formation and subsequent host-cell lysis. Transfection experiments demonstrated that the parasites were not capable of propagating within other closely related free-living amoebae but were able to infect the distantly related species* Dictyostelium discoideum*. Electron microscopy of the parasites revealed typical morphological characteristics of the* Chlamydiales*, including the existence of a* Chlamydia-*like life-cycle, but indicated that these endocytobionts, in contrast to* Chlamydia *species, do not reside within a vacuole. Comparative 16S rRNA sequence analysis showed that the endocytobiont of* H. vermiformis*, classified as* Neochlamydia hartmannellae *gen. nov., sp. nov., is affiliated to the family* Parachlamydiaceae*. Confocal laser scanning microscopy in combination with fluorescence* in situ *hybridization using rRNA-targeted oligonucleotide probes confirmed the intracellular localization of the parasites and demonstrated the absence of other bacterial species within the* Hartmannella *host. These findings extend our knowledge of the phylogenetic diversity of the* Parachlamydiaceae *and demonstrate for the first time that these endocytobionts can naturally develop within amoebae of the genus* Hartmannella*.*

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Introduction

Free-living amoebae (FLA), such as *Hartmannella* spp., *Acanthamoeba* spp., *Naegleria* spp. and *Vahlkampfia* spp., are an important component of soil and water ecosystems, acting as important predators controlling bacterial populations (Rodriguez-Zaragoza, 1994). They are cosmopolitan in distribution, and can be found in fresh water, in marine waters, in soil, on plants and animals, and inside vertebrates, feeding on bacteria, fungi, yeasts, algae and other protozoa. In addition to their environmental significance, some FLA have been identified as human pathogens, causing the diseases amoebic keratitis and meningoencephalitis, and systemic infections (Visvesvara, 1995). A significant fraction of environmental and clinical FLA isolates harbour, like many other protozoa (Heckmann & Görtz, 1992; Preer & Preer, 1984), bacterial endocytobionts (Fritsche *et al*., 1993; Michel *et al*., 1995). Recent studies have begun to elucidate the phylogenetic diversity of FLA-associated endocytobionts by applying the rRNA approach. The majority of the endocytobionts identified thus far are related to bacterial genera currently recognized as important human pathogens. For example, *Legionella*-related, *Rickettsia*-related and *Chlamydia*-related organisms are known to occur in FLA (Amann *et al*., 1997; Birtles *et al*., 1996; Fritsche *et al*., 1999). In addition, several endocytobionts which group phylogenetically with the *Paramecium caudatum* symbiont *Caedibacter caryophilus* (Springer *et al*., 1993) are known to proliferate within *Acanthamoeba* spp. (Horn *et al*., 1999). Whereas the relationship between hosts and endocytobionts remains largely unexplored, there is increasing evidence that some FLA endocytobionts are of medical importance. Endocytobiont-mediated increase of *Acanthamoeba* cytopathogenicity in tissue culture suggests that these intracellular bacteria enhance FLA virulence (Fritsche *et al*., 1998). Furthermore, some of the endocytobionts have been implicated as causative agents for disease, as indicated by the presence of specific antibodies against *Chlamydia*-related endocytobionts of *Acanthamoeba* in blood from respiratory-disease patients, and by the detection of *Parachlamydia*-like 16S rDNA sequences in specimens from bronchitis patients (Birtles *et al*., 1997; Ossewaarde & Meijer, 1999). This report describes the investigation of coccoid bacterial endocytobionts of *Hartmannella vermiformis* strain A_1Hsp isolated from the water conduit system of a dental unit, by (i) transfection experiments, (ii) electron microscopy, and (iii) the rRNA approach including comparative 16S rRNA sequence analysis and fluorescence *in situ* hybridization using 16S rRNA-targeted oligonucleotide probes and confocal laser scanning microscopy. Portions of this work were presented as an abstract at the 99th General Meeting of the American Society for Microbiology in Chicago, IL, 1999.

Materials and Methods

Isolation and maintenance of *Hartmannella vermiformis*. The original amoebal host strain *H. vermiformis* strain A_1Hsp was isolated from the water conduit system of a dental unit in Lahnstein, near Koblenz, Germany, by filtering a 100 ml water sample obtained from the source water as described elsewhere (Michel *et al*., 1995). Coccoid endocytobionts recovered from this isolate were transferred to *H. vermiformis* strain OS101 on NN-agar plates (non-nutrient agar; Page, 1988) seeded with *Enterobacter cloacae*. The infected *H. vermiformis* strain OS101 could subsequently be axenized in fluid SCGYE medium (De Jonckheere, 1977) by temporary addition of penicillin and streptomycin (0.2 mg/ml each). Cultures were incubated at 30 °C and fresh medium was applied every 5-10 d. To investigate the capability of the original amoebal host strain *H. vermiformis* A_1Hsp to form cysts, amoebae were cured of their endocytobionts by treatment with rifampicin as described by Michel *et al*. (1994).

Extracellular growth of *H. vermiformis* endoparasites. Attempts to culture the *Hartmannella* endocytobionts extracellularly included cultivation on blood agar (Becton Dickinson), Casiton-agar (Biotest-Heipha) and SCGYE (De Jonckheere, 1977) at incubation temperatures of 20 and 30 °C. Both whole amoeba cells and filter-purified endocytobionts from lysed amoeba cells were transferred to the respective media. If no growth was observed after 14 d incubation, cultures were considered negative.

Transfection experiments. Following lysis of endocytobiont-infected *H. vermiformis* cells from 4-5-d-old cultures by freeze-thawing, the coccoid bacterial endocytobionts were filter-purified (1.2 µm membrane filter). An aliquot of 80 µl of the resulting suspension was added to strains of different species of FLA, growing either in SCGYE medium or on NN-agar plates covered with a lawn of *Enterobacter cloacae*. The host range of the endocytobiont was investigated by transfection experiments with 14 different strains of FLA (Table 1), and one strain of *Dictyostelium discoideum* isolated from human nasal mucosa. Infection of each host species was monitored by phase-contrast microscopy. After 21 d incubation at 30 or 20 °C the host was considered resistant to infection if no infected cells nor any marked reduction in amoebal numbers (which may have resulted from parasitic activity of the endocytobiont) were observed.

Electron microscopy. For electron microscopical studies, the heavily infected trophozoites from 4-5-d-old cultures were harvested and prefixed in 2.5% glutaraldehyde in cacodylate buffer (pH 7.2) for 2 h. After prefixation the specimens were fixed in 1% osmium tetroxide followed by 2% uranyl acetate in aqueous solution. Subsequently specimens were dehydrated in alcohol and embedded in epoxy resin. Thin sections were stained with 1% lead citrate and examined with a Zeiss EM 910 electron microscope.

DNA isolation, amplification of 16S rDNA, cloning and sequencing. Simultaneous isolation of DNA from the amoebae and their endocytobionts was performed using a modified UNSET procedure (Hugo *et al*., 1992). Amoebae and their endocytobionts were harvested from axenic cultures by centrifugation (2000 ***g***, 3 min), washed twice with double-distilled water, resuspended in 500 µl UNSET lysis buffer (8 M urea, 0.15 M NaCl, 2% SDS, 0.001 M EDTA and 0.1 M Tris/HCl at pH 7.5) and incubated at 60 °C for 5 min. Lysates were extracted twice with phenol/chloroform (Roth) and DNA was precipitated for 3 h at 20 °C with 2 vols absolute ethanol. After centrifugation (10000 ***g***, 10 min) at 4 °C the ethanol was removed and the pellet was washed twice with 80% ice-cold ethanol to remove residual salts. The pellet was air-dried and resuspended in 30 µl double-distilled water. Oligonucleotide primers targeting 16S rDNA signature regions which are conserved within the *Chlamydiales* were used for PCR to obtain near-full-length bacterial 16S rRNA gene fragments. The nucleotide sequences of the forward and reverse primers used for amplification were 5´-CGGATCCTGAGAATTTGATC- 3´ and 5´-TGTCGACAAAGGAGGTGATCCA-3´ (Pudjiatmoko *et al*., 1997). 16S rDNA amplification reactions were performed in a thermal capillary cycler (Idaho Technology) using reaction mixtures including 15 pM of each primer, 0.25 µg BSA/ml, 2mM $MgCl_2$ reaction buffer and 2.5 IU *Taq* DNA polymerase (Promega). Thermal cycling was carried out as follows: an initial denaturation step at 94 °C for 30 s followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 52 °C for 20 s, and elongation at 72 °C for 30 s. Cycling was completed by a final elongation step at 72 °C for 5 min. A negative control was performed using a reaction mixture without added DNA. Amplified products were directly ligated into the cloning vector pCRII TOPO and transformed into competent *Escherichia coli* (TOP10 cells) according to the instructions of the manufacturer (Invitrogen). Nucleotide sequences of the cloned DNA fragments were determined by the dideoxynucleotide method (Sanger *et al*., 1977) by cycle sequencing of purified plasmid preparations (Qiagen) with a Thermo Sequenase Cycle Sequencing Kit (Amersham Life Science) and an automated DNA sequencer (Li-Cor) under conditions recommended by the manufacturers. Dye-labelled vector specific primers M13/pUC V (5´-GTAAAACGACGGCCAGT-3´) and M13/pUC

R (5´- GAAACAGCTATGACCATG-3´) were applied.

Phylogenetic analysis. The 16S rDNA sequences obtained were added to the rDNA sequence database of the Technische Universität München (encompassing more than 16000 published and unpublished homologous small-subunit rDNA primary structures) by use of the program package ARB (O. Strunk and others, unpublished; program available through the homepage of the Technische Universität München, http://www.mikro.biologie.tu-muenchen.de). Alignment of the new rDNA sequences was performed by using the ARB automated alignment tool (version 2.0). The alignments were refined by visual inspection and by secondary-structure analysis. Phylogenetic analyses were performed by applying the ARB parsimony, distance matrix and maximum likelihood methods to different data sets. To determine the robustness of the phylogenetic trees, analyses were performed with and without the application of various filtersets to exclude highly variable positions.

Fluorescence *in situ* hybridization and confocal laser scanning microscopy. The following oligonucleotide probes were used: (i) EUB338 (5´-GCTGCCTCCCGTAGGAGT-3´), targeting most, but not all, members of the domain *Bacteria* (Amann *et al*., 1990; Daims *et al*., 1999), and (ii) S-S-ParaC-0658-a-A-18 (5´-TCCATTTTCTCCGTC TAC-3´), previously designed as complementary to a signature region of the 16S rRNA of the *Parachlamydia*-related endosymbionts of *Acanthamoeba* spp. strains UWC22 and TUME1 (T. R. Fritsche and others, unpublished; probe designation according to the standard proposed by Aμm *et al*., 1996). Oligonucleotides were synthesized and directly 5´-labelled with 5(6)-carboxyfluorescein- *N*-hydroxysuccinimide ester (FLUOS), or the hydrophilic sulphoindocyanine fluorescent dye Cy3 (Interactiva). For *in situ* hybridization, *Hartmannella* cells were harvested from 4 ml liquid broth culture by centrifugation (2000 ***g***, 3 min), washed twice with double-distilled water, and resuspended in 0.05% agarose. Twenty microlitres of this suspension was spotted on a glass slide, air-dried and subsequently dehydrated in 80% ethanol for 10 s. Hybridization was performed using the hybridization buffer (including 30% formamide) and the buffer washing (containing 112 mM NaCl, without SDS) described by Manz *et al*. (1992). Slides were examined using a confocal laser scanning microscope (LSM 510, Carl Zeiss) in combination with a helium-neon laser (543 nm) and an argon-krypton laser (488 nm). Image analysis processing was performed with the standard software package delivered with the instrument (version 2.01).

Results and Discussion

Coccoid endocytobionts block cyst formation in H. vermiformis

The original amoebal host strain A_1Hsp, containing coccoid prokaryotic endocytobionts, was isolated from the water conduit system of a dental unit. Phase contrast microscopic observation of the amoebae revealed morphological characteristics typical for the genera *Hartmannella* and *Cashia* (Page, 1988). Since no cysts could be observed, the amoebae were provisionally identified as members of the genus *Cashia*. Attempts to grow the amoebal isolate axenically failed. Consequently, bacterial endocytobionts were transferred from the original host strain A_1Hsp into *H. vermiformis* strain OS101, which was subsequently axenized in order to facilitate the following investigations, including evaluation of host spectrum, electron microscopy, and 16S rDNA sequencing. Interestingly, infection with the coccoid endocytobionts prevented cyst formation of *H. vermiformis* strain OS101, a phenomenon which has been previously reported for *Acanthamoeba* sp. Bn_9 and $Berg_{17}$ (and *Acanthamoeba castellanii* strain C3 after infection with *Parachlamydia acanthamoebae;*

Amann *et al*., 1997; Michel *et al*., 1994). Since the ability to form cysts discriminates the genera *Cashia* and *Hartmannella*, this observation forced us to re-evaluate the capability of the original amoebal host strain A_1Hsp to form cysts in the absence of endocytobionts and thus its identification as member of the genus *Cashia*. For this purpose, strain A_1Hsp was cured of its endocytobionts by rifampicin treatment. Despite its toxicity for most amoebae a few trophozoites survived rifampicin treatment and multiplied after transfer to a fresh NN-agar plate containing no rifampicin. Since microscopic observation demonstrated that these endocytobiont-free amoebae were now able to form cysts, strain A_1Hsp was assigned to the genus *Hartmannella* as *H. vermiformis*. At present, we can only speculate on the ecological significance of the suppression of FLA cyst formation by some endocytobionts. A possible clue might be provided by the observation that *Escherichia coli* (Steinert *et al*., 1998) is eradicated from artificially infected *Acanthamoeba* cells during cyst formation. Thus, prevention of cyst formation might be a protection mechanism for parasitic endocytobionts, which would be negatively affected by the differentiation of FLA into resting forms. This strategy would contrast with the one used by *Legionella pneumophila* (Steinert *et al*., 1998; Kilvington & Price, 1990) and several other obligate *Acanthamoeba* endocytobionts (Fritsche *et al*., 1999; Horn *et al*., 1999), which survive host cell cyst formation and thus directly benefit from cyst-mediated host resistance against unfavourable environmental conditions.

Chlamydia-*like life cycle and parasitic behaviour of the* H. vermiformis *endocytobiont*

Electron micrographs revealed a *Chlamydia*-like morphology and developmental cycle of the endocytobionts (Fig. 1). Stages showing binary fission resembling those of reticulate bodies (RBs, 0.4-0.6 µm in diameter) of *Chlamydia* could be observed. Additionally, the highly condensed coccoid stages (0.5-0.6 µm in diameter) are similar to elementary bodies (EBs) of *Chlamydia*. While RBs of the *H. vermiformis* endocytobiont clearly possess a Gram-negative type cell wall, results of electron microscopic analysis of its EBs are ambiguous. However, since the EBs of the *Hartmannella* endocytobiont showed an outer membrane, we consider them as Gram-negative. This is in noticeable contrast to the Gram-positive type of cell wall which has been observed for the *Chlamydia*-related endoparasite of *Acanthamoeba* sp. strain Bn_9 (*P. acanthamoebae*; Amann *et al*., 1997). In contrast to *Chlamydia* species and *Parachlamydia*-related endocytobionts of *Acanthamoeba* (Amann *et al*., 1997; T. R. Fritsche and others, unpublished), RBs and EBs of the *Hartmannella* endocytobiont were not surrounded by vacuoles and were thus located directly within the cytoplasm of the host cell,

indicating that the endocytobionts possess an escape mechanism from the phagosomes. Electron microscopic inspection of amoebal cells at different time points showed that massive amounts of mature EBs occurred 3-5 d after infection, and subsequently led to rupture or lysis of heavily infected trophozoites. Shedding of single mature EBs into the environment, not accompanied by host cell destruction, was, however, already observed at earlier stages of infection (Fig. 1). Since ultimately all infected *Hartmannella* trophozoites are killed by the coccoid endocytobionts, they are considered by us to be intracellular parasites. Natural stability of this host-parasite association would require an amoebal generation time shorter than the period between parasite infection and host cell lysis. The aggressive parasitic behaviour of the endocytobiont within its *Hartmannella* host suggests a limited adaptation of host and parasite caused by a relatively short evolutionary relationship. *Hartmannella* species may have only recently been infected by these parasites, suggesting their origin from another protist species. Limited adaptation of the endoparasite to the *H. vermiformis* host is also suggested by the suppression of cyst formation, which might protect the parasites from eradication but which may decrease the fitness of the association against environmental stress.

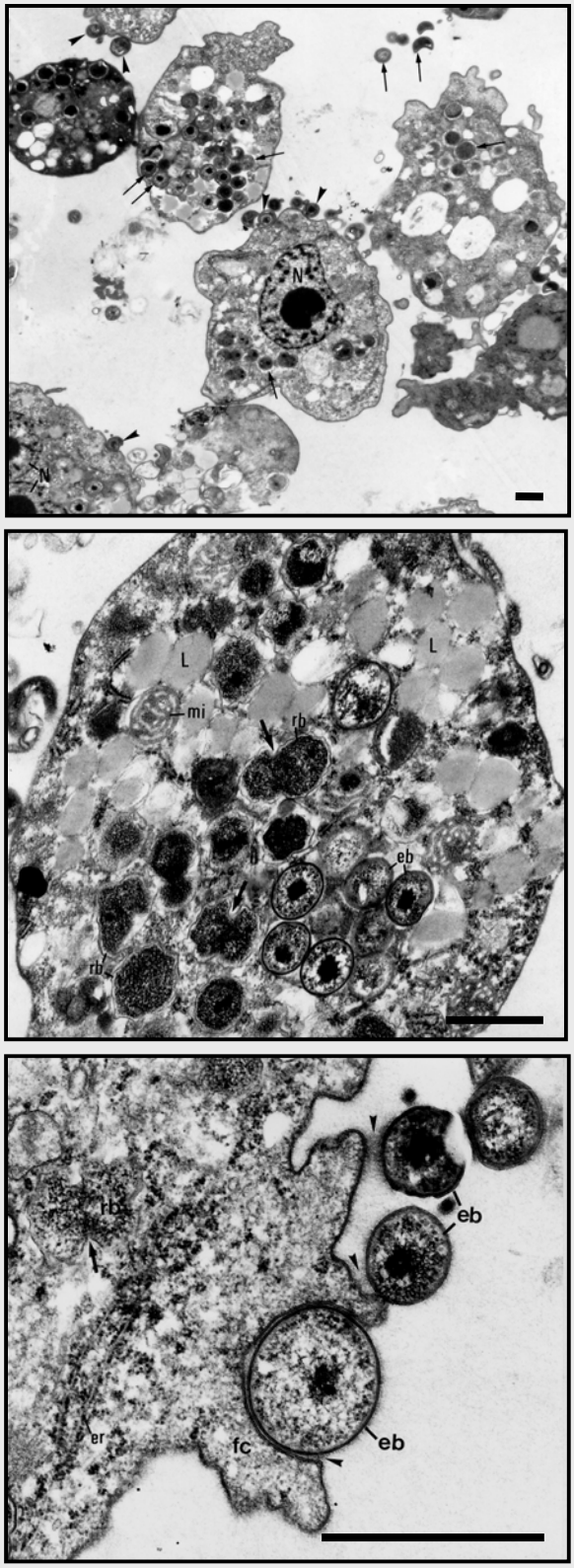

Figure 1. A. *Hartmannella vermiformis* trophozoites harbouring the *Chlamydia*-related endoparasite *Neochlamydia hartmannellae* (arrows). Free elementary bodies can also be seen outside the eukaryotic cells, some of which are attached to the amoebal cell membrane (arrowheads; magnification 8,400x). **B.** Different stages of *Neochlamydia hartmannellae* (endoparasites of *Hartmannella vermiformis* A_1Hsp) within the cytoplasm of the amoebal host cell: reticulate bodies together with elementary bodies could be observed simultaneously and do not reside within vacuoles. Arrows indicate constrictions of reticulate bodies undergoing binary fission. In contrast to *Rickettsia*- and *Caedibacter*-related *Acanthamoeba* endosymbionts (Horn *et al*., 1999, Fritsche *et al*., 1999) no electron-translucent layers surrounding the intracellularly located bacteria could be observed (magnification 26,500x). **C.** Adhesion and phagocytosis of elementary bodies of *Neochlamydia hartmannellae* by a trophozoite of *Hartmannella vermiformis*: adhesion is mediated by fine fibrinous material (arrowheads) discernible at the amoebal cellular membrane (glycocalyx) and also at the surface of the elementary bodies. One of them of exceptional size has already been engulfed partially by the amoeba forming a characteristic foodcup (fc). Within the cytoplasm a constricted stage (arrow) of a reticulate body can be seen (magnification 50,400x). eb, elementary body; rb, reticulate body; N, nucleus of the host cell; mi, mitochondrium; L, lipid granules; er, endoplasmic reticulum; bars indicate 1 μm.

Host range of the obligate endoparasite of H. vermiformis

Standard cultivation techniques failed to support extracellular growth of the *Hartmannella* endoparasites, suggesting that *Hartmannella* cells are necessary for its growth. This finding is in accordance with the obligate intracellular growth of other endocytobionts of FLA (Amann *et al*., 1997; Fritsche *et al*., 1993). The host range of the *Hartmannella* endoparasite was determined by transfection experiments encompassing a recently isolated *D. discoideum* strain and 14 different strains of FLA belonging to the genera *Hartmannella*, *Acanthamoeba*, *Naegleria* and *Willaertia* (Table 1). Except for the original host *H. vermiformis* A_1Hsp and two more *H. vermiformis* strains, only *D. discoideum* strain $Berg_{17}$ could be successfully infected. Whereas the extent of parasitic behaviour of the investigated endocytobionts varied slightly between the different *H. vermiformis* host strains, aggregation and stalk and fruiting body formation of *D. discoideum* were not disturbed by the endocytobionts and endoparasite-free spores were formed. There are remarkable differences in host range between the investigated *Hartmannella* endoparasite and the *Acanthamoeba* endoparasite *P. acanthamoebae*. The *Hartmannella* endoparasite is not able to infect the tested *Acanthamoeba* strains, including *A. castellanii* C3, which is a suitable host for *P.*

Table 1. Host spectrum of *Neochlamydia hartmannellae* (endoparasite of *Hartmannella vermiformis* strain A_1Hsp).

Amoeba species	Strain	Intracellular multiplication
Incubation at 30° C		
Acanthamoeba castellanii	C3	—
Acanthamoeba sp.	HLA	—
Acanthamoeba sp.	Renk	—
Acanthamoeba quina-lugdunensis	312-1	—
Acanthamoeba lenticulata	45	—
Acanthamoeba lenticulata	89	—
Naegleria lovaniensis	Aq/9/1/40	—
Naegleria gruberi	NI_1	—
Hartmannella vermiformis	A_1Hsp	+
Hartmannella vermiformis	OS101	+
Willaertia magna	JIII Cl	—
Willaertia magna	NI_4Cl_1*	—
Incubation at 20° C		
Acanthamoeba comandoni	Am 23	—
Acanthamoeba comandoni	Pb 30/40	—
Comandonia operculata	WBT	—
Hartmannella vermiformis	C 3/8	+
Dictyostelium discoideum	$Berg_{25}$	+

acanthamoebae (Amann *et al*., 1997). Conversely, *P. acanthamoebae* is unable to infect *H. vermiformis* strains, which serve as host for the investigated *Hartmannella* endoparasite (R. Michel, personal communication). Future studies are required to elucidate the molecular mechanism of a specific recognition system that may mediate specificity of infection.

Phylogeny and in situ *identification of the* H. vermiformis *endoparasite*

Near-full-length 16S rDNA amplicons (1529 bp) retrieved from mixed genomic DNA of amoebal hosts and bacterial endoparasites were successfully cloned and sequenced. Comparative sequence analysis revealed that the retrieved 16S rRNA sequence displayed highest similarity values with 16S rRNA sequences of members of the *Chlamydiales* (Table 2). In particular, the investigated *Hartmannella* endoparasites are moderately related to the *Acanthamoeba* parasite *P. acanthamoebae* strain Bn_9 (92%), the only validly described member of the new family *Parachlamydiaceae* (Everett *et al*., 1999). It should be noted that even higher sequence similarities of between 96.5% and 97.1% to the *Hartmannella* parasite were calculated for 16S rRNA sequences of two recently investigated *Parachlamydia*-related endosymbionts of *Acanthamoeba* (Table 2; T. R. Fritsche *et al*., unpublished). Phylogenetic analysis using distance matrix, parsimony and maximum-likelihood treeing methods provided consistent evidence for an affiliation of the endoparasites of *H. vermiformis* A_1Hsp with the *Parachlamydiaceae*. Within this family the retrieved sequence forms a monophyletic grouping with the two above-mentioned *Acanthamoeba* endosymbionts (strains UWC22 and

Table 2. Overall sequence similarities for the retrieved 16S rRNA sequence of *Neochlamydia hartmannellae* (endoparasite of *Hartmannella vermiformis* strain A_1Hsp) and representative members of the *Chlamydiales*.

		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
A	*Chlamydophila abortus* B577																
B	*Chlamydophila psittaci* 6BC	99,9															
C	*Chlamydophila felis* FP Baker	98,0	98,4														
D	*Chlamydophila caviae* GPIC	98,9	98,9	97,9													
E	*Chlamydiphila pecorum* E58	96,2	96,5	95,9	96,1												
F	*Chlamydophila trachomatis* HAR-13	95,0	95,2	94,8	95,3	95,1											
G	*Chlamydia suis* S45	94,3	94,7	94,4	94,5	94,5	97,3										
H	*Chlamydia muridarum* MoPn	95,6	95,7	95,5	95,7	95,5	98,4	97,7									
I	*Chlamydophila pneumoniae* TW-183	95,8	96,2	95,1	95,3	95,8	93,9	93,5	94,6								
J	*Parachlamydia acanthamoebae* Bn_9	86,2	86,7	86,6	86,9	86,4	86,2	87,2	87,0	87,0							
K	*Simkania negevensis* Z	83,5	83,8	83,9	83,9	83,6	84,2	84,4	84,2	83,7	88,2						
L	endosymbiont of *Acanthamoebae* sp. UWE1	85,8	85,5	84,8	85,4	84,4	84,3	85,4	85,0	85,6	93,1	85,7					
M	*endosymbiont of Acanthamoebae* sp. UWE25	86,2	86,0	85,6	86,0	85,6	85,4	85,9	86,1	86,3	92,5	85,4	93,0				
N	endosymbiont of *Acanthamoebae* sp. UWC22	86,8	86,7	86,2	86,7	86,2	85,7	86,4	86,4	86,1	91,2	85,5	92,9	91,9			
O	***Neochlamydia hartmannellae***	87,0	87,5	86,9	87,5	86,5	87,0	87,5	87,4	86,6	92,0	86,5	92,2	91,6	96,5		
P	endosymbiont of *Acanthamoebae* sp. TUME1	86,8	87,2	86,8	87,2	86,8	86,3	87,0	87,0	86,6	91,2	85,9	92,3	91,4	99,4	97,1	
Q	*Waddlia chondrophila* WSU-85-1044	84,4	84,6	84,4	84,6	84,2	84,7	84,8	84,9	84,4	87,2	84,4	87,1	87,9	87,0	87,0	87,1

TUME1; Fig. 2). Sequence analysis of 16S rDNA of the investigated endoparasites of *H. vermiformis* revealed the presence of the target site for probe S-S-ParaC-0658-a-A-18, specifically designed previously for the related *Parachlamydia*- like endosymbionts of *Acanthamoeba* sp. TUME1 and UWC22 (T. R. Fritsche *et al*., unpublished). Simultaneous fluorescence *in situ* hybridization of fixed *Hartmannella* cells with probe S-S-ParaC-0658-a-A-18 and the bacterial probe EUB338 demonstrated that all bacteria detectable by *in situ* hybridization also hybridized with the endocytobiont-specific probe, suggesting the absence of additional, phylogenetically different bacteria within the amoebal host. Confocal laser scanning microscopic analysis confirmed the intracellular localization of the endoparasites of *H. vermiformis* A_1Hsp (Fig. 3). The genetic data described herein, and the morphological similarity, are consistent with a close relationship between the endoparasite of *H. vermiformis* A_1Hsp and *P. acanthamoebae*. With a 16S rRNA sequence similarity of 92% with *P. acanthamoebae*, a *Chlamydia*-like development cycle, and the ability to multiply and survive within free living amoeba, the endoparasite of *H. vermiformis* A_1Hsp meets the main requirements for inclusion within the family *Parachlamydiaceae* (Everett *et al*., 1999). However, keeping in mind that 16S rRNA sequence similarities between two bacteria of less than 95%are indicative of their affiliation with two different genera (Ludwig *et al*., 1998), the *Hartmannella* endoparasite most likely represents a new species of a new genus, since its 16S rRNA similarity to the closest validly described relative, *P. acanthamoebae*, is 92%. In this regard, it should be noted that 16S rRNA sequence similarities of the *Hartmannella* endoparasite with two recently discovered *Parachlamydia*-related endosymbionts of *Acanthamoeba* (T. R. Fritsche and others, unpublished) are higher than 95% but below 97±1%. Nevertheless, we believe that the *Hartmannella* and the *Acanthamoeba* endocytobionts should be assigned to different genera due to their profound differences in host spectra (see above). Consequently, we propose that the endoparasite of *H. vermiformis* A_1Hsp be classified as *Neochlamydia hartmannellae* gen. nov., sp. nov.

Description of Neochlamydia *gen. nov.*

Neochlamydia (Ne.o.chla.my'di.a L. fem. n.; *Neochlamydia* referring to the modest phylogenetic relationship to the *Chlamydiaceae*). Phylogenetic position: order *Chlamydiales*, family *Parachlamydiaceae*. Members of the genus *Neochlamydia* should have a 16S rDNA that is >95% identical to the 16S rDNA of the type species, *Neochlamydia hartmannellae* strain A_1Hsp.

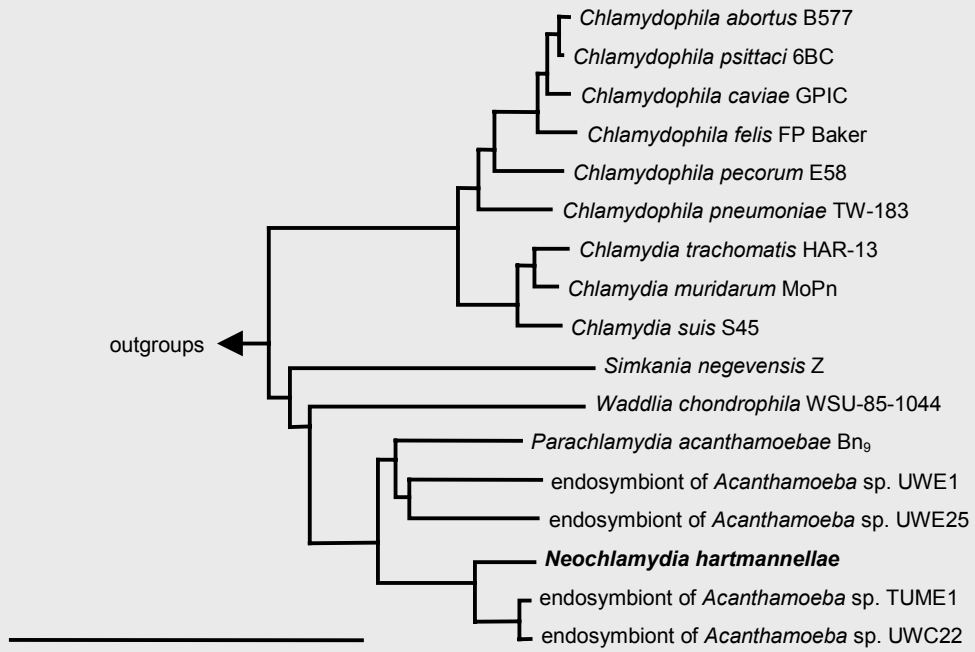

Figure 2. 16S rRNA based phylogenetic tree reflecting the affiliation of *Neochlamydia hartmannellae* (endoparasites of *Hartmannella vermiformis* A_1Hsp). The tree was obtained using the neighbour joining method. To exclude highly variable and thus phylogenetically non-informative sequence positions, only those sequence positions which are conserved in at least 50% of the deposited 16S rRNA sequences of the *Chlamydiales* were used for treeing. Nomenclature according to revised taxonomy of the *Chlamydiales* by Everett *et al*. (1999). Bar indicates 10% estimated evolutionary distance.

Description of Neochlamydia hartmannellae *sp. nov.*

Neochlamydia hartmannellae (hart'mann.el.lae. L. gen. sing. n. of *Hartmannella*, taxonomic name of a genus of *Hartmannellidae*; pertaining to the name of the host amoeba, *Hartmannella vermiformis* strain A_1Hsp, in which the organism was first discovered). Gram-negative reticulate bodies and Gram-negative elementary bodies; coccoid morphology; 0.4-0.6 μm in diameter. Basis of assignment: 16S rDNA sequence accession number AF177275, nucleotide probe S-S-ParaC- 0658-a-A-18 (5′-TCCATTTTCTCCGTCTAC- 3′). Not cultivated on cell-free media; obligate intracytoplasmatic parasite of *H. vermiformis* strain A_1Hsp and other *H. vermiformis* strains, therein preventing cyst formation. Host range: able to multiply in *D. discoideum*, but not in *Acanthamoeba* spp.; mesophilic (20-30 °C). Isolated from the water conduit system of a dental unit (Lahnstein, Germany). Type strain, A_1Hsp (ATCC 50802).

Diversity within the Chlamydiales *and clinical aspects of* Neochlamdia hartmannellae

In a more general perspective, our results and the recent identification of four *Parachlamydia*-related acanthamoebal endocytobionts (T. R. Fritsche and others, unpublished), a *Chlamydia* like bovine intracellular organism (*Waddlia chondrophila*; Rurangirwa *et al*., 1999) and a *Chlamydia*-related organism observed within tissue culture (*Simkania negevensis*; Kahane *et al*., 1999) demonstrate a previously unrecognized diversity within the *Chlamydiales*. Interestingly, the order *Chlamydiales* still exclusively comprises obligate intracellular bacteria, some of which have developed mechanisms to survive and exploit uptake by protozoa. The adaptation to intracellular growth in the ubiquitously distributed FLA could have functioned as a preadaptation of *Chlamydia*-like ancestors to survival within other host cells of higher eukaryotes, including humans; this raises the question of the clinical significance of members of the family *Parachlamydiaceae*. Few studies have addressed this important issue. Among these, Birtles *et al*. (1997) screened for the presence of specific antibodies against *Parachlamydia*-like endocytobionts of *Acanthamoeba* sp. ("Hall's coccus", displaying more than 99% 16S rDNA similarity to *P. acanthamoebae*) in blood sera from patients with pneumonia of undetermined cause, and found positively reacting sera that did not react with *Chlamydia psittaci*, *C. trachomatis* or *C. pneumoniae*. These researchers therefore suggested that `Hall's coccus' be considered potentially pathogenic for humans. Another remarkable finding was the recovery of novel C*hlamydia*-like 16S rDNA sequence fragments from specimens of respiratory-disease patients, recently reported by Ossewaarde & Meijer (1999). The sequence similarities of these

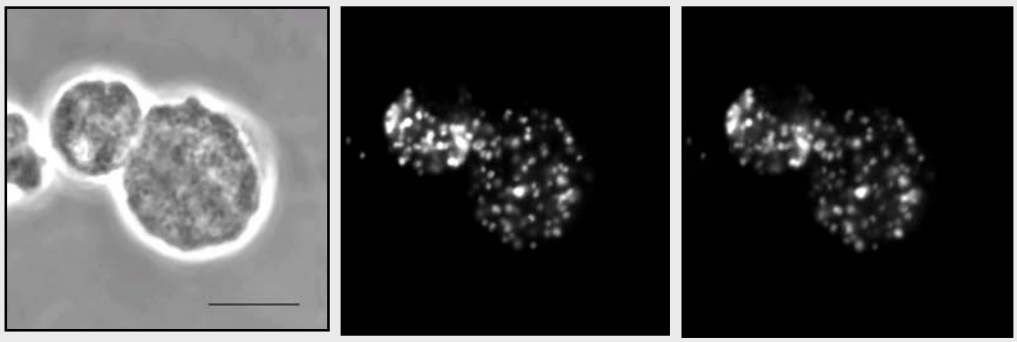

Figure 3. *In situ* detection of *Neochlamydia hartmannellae* (endoparasites of *Hartmannella vermiformis* strain A_1Hsp) using bacterial probe Eub338 labelled with FLUOS (B), and endocytobiont specific probe S-S-ParaC-0658-a-A-18 labelled with Cy3 (C). Phase contrast image is shown in A. Bar indicates 10 μm.

sequences and *N. hartmannellae* range between 72% and 84%. Reliable phylogenetic analysis of the *Chlamydia*-like sequences and the 16S rRNA sequence of *N. hartmannellae* could not be performed, due to the short length of the *Chlamydia*-like sequence fragments (approx. 220 bp). A stable tree topology could not be obtained by applying different treeing methods and data sets. Further research is needed to clarify whether the FLA endocytobionts of the family *Parachlamydiaceae* are indeed able to infect humans.

Concluding remarks

In conclusion, we have identified an obligate endoparasite of *H. vermiformis*, provisionally classified as *Neochlamydia hartmannellae*, as a new member of the family *Parachlamydiaceae*. These findings broaden our knowledge of the phylogenetic diversity within the *Chlamydiales*. Although it is too early to draw conclusions on the clinical significance of these bacteria, the detection of these organisms in FLA suggests that FLA may act as a general reservoir for *Chlamydia*-like organisms. More detailed knowledge is needed on the natural habitats, diversity, physiology and virulence of members of the family *Parachlamydiaceae*.

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Chapter VII

Phylogenetic Diversity among Geographically Dispersed *Chlamydiales* Endosymbionts Recovered from Clinical and Environmental Isolates of *Acanthamoeba* spp.

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Phylogenetic Diversity among Geographically Dispersed *Chlamydiales* Endosymbionts Recovered from Clinical and Environmental Isolates of *Acanthamoeba* spp.

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The recently proposed reorganization of the order Chlamydiales *and description of new taxa are broadening our perception of this once narrowly defined taxon. We have recovered four strains of gram-negative cocci endosymbiotic in* Acanthamoeba *spp., representing 5% of the* Acanthamoeba *sp. isolates examined, which displayed developmental life cycles typical of members of the* Chlamydiales*. One of these endosymbiont strains was found stably infecting an amoebic isolate recovered from a case of amoebic keratitis in North America, with three others found in acanthamoebae recovered from environmental sources in North America (two isolates) and Europe (one isolate). Analyses of nearly full length 16S rRNA gene sequences of these isolates by neighbor joining, parsimony, and distance matrix methods revealed their clustering with other members of the* Chlamydiales *but in a lineage separate from those of the genera* Chlamydia*,* Chlamydophila*,* Simkania*, and* Waddlia *(sequence similarities, <88%) and including the recently described species* Parachlamydia acanthamoebae *(sequence similarities, 91.2 to 93.1%). With sequence similarities to each other of 91.4 to 99.4%, these four isolates of intra-amoebal endosymbionts may represent three distinct species and, perhaps, new genera within the recently proposed family* Parachlamydiaceae*. Fluorescently labeled oligonucleotide probes targeted to 16S rRNA signature regions were able to readily differentiate two groups of intra-amoebal endosymbionts which corresponded to two phylogenetic lineages. These results reveal significant phylogenetic diversity occurring among the* Chlamydiales *in nontraditional host species and supports the existence of a large environmental reservoir of related species. Considering that all described species of* Chlamydiales *are known to be pathogenic, further investigation of intra-amoebal parachlamydiae as disease-producing agents is warranted.*

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Introduction

All members of the order *Chlamydiales* are recognized pathogens of mammals, marsupials, or birds. The ability to produce respiratory disease, among other clinical presentations, is a feature of most species within the order and is especially characteristic of infections produced by *Chlamydophila pneumoniae*, *Chlamydophila psittaci*, certain serovars of *Chlamydia trachomatis*, and the recently described species *Simkania negevensis* (20, 21, 23). The recent finding of evidence for seroconversion to antigen of *Parachlamydia acanthamoebae*, an intra-amoebal *Chlamydia*-like bacterium, in a small number of humans experiencing community-acquired pneumonia, along with the findings of novel *Parachlamydia*-related 16S rRNA sequences in respiratory specimens, peripheral blood, and aortic tissue, suggests that this and related species of protozoal endosymbionts may also be of clinical significance, warranting further investigation (2, 5, 30). Previously, we have reported on the common occurrence of uncultured bacterial endosymbionts in protozoa of the genus *Acanthamoeba* (13). While 20% of axenically growing *Acanthamoeba* isolates recovered from clinical and environmental sources were found to be host to gram-negative rod endosymbionts, 5% were host to gram-negative coccus endosymbionts; none could be cultured by standard microbiological techniques. Phylogenetic analyses of the gram-negative rod endosymbionts to date have included two lineages of alpha *Proteobacteria*: one containing *Rickettsiales*-affiliated isolates and the other containing isolates related to the *Paramecium caudatum* symbiont *Caedibacter caryophilus* (14, 18). Subsequent ultrastructural studies of the coccoid endosymbionts demonstrated the presence of a developmental cycle suggestive of a relationship to members of the *Chlamydiales* (R. Gautom, R. Herwig, and T. R. Fritsche, Abstr. 96th Gen. Meet. Am. Soc. Microbiol., abstr. R-29, p. 474).

In this paper, we present further morphologic and phylogenetic analyses of four isolates of *Chlamydiales* endosymbionts found naturally infecting *Acanthamoeba* sp. trophozoites, three of which were recovered from environmental amoebic isolates originating in North America (two isolates) and Europe (one isolate), and one which was from amoebae infecting the corneal tissues of a patient in North America. Because these bacterial isolates could not be cultivated by standard microbiological techniques, we applied the culture-independent ribosomal RNA (rRNA) approach to determine phylogenetic relatedness to each other and to other strains for which sequence data are available. Fluorescence *in situ* hybridization (FISH) with oligonucleotide probes designed to target ribosomal signature

regions was used to verify the origin of the retrieved sequences and further assist with characterization of these endosymbionts. The finding of a potentially large environmental reservoir of intracellular *Chlamydia*-like organisms has implications for the evolution of *Chlamydiales* and their preadaptation and ultimate recruitment to higher animals.

Materials and Methods

Isolation and maintenance of *Acanthamoeba* strains. The techniques used for recovery and maintenance of acanthamoebae from clinical and environmental sources have been described in detail elsewhere (13, 39). Four isolates of *Acanthamoeba* spp. were included in this study and were recovered from infected corneal tissues (UWC22), soil samples from western Washington State (UWE1 and UWE25), and municipal sewage sludge from Munich, Germany (TUME1). All isolates were found to have gram-negative cocci occurring as endosymbionts within the cytoplasm which could be readily demonstrated using Giemsa, Hemacolor (Harleco, Gibstown, N.Y.), and other appropriate bacterial stains. The general phenotypic characteristics of three of these endosymbiont strains (UWE1, UWE25, and UWC22) have been described previously (13).

Electron microscopy. Amoebic isolates containing endosymbionts were examined by electron microscopy using a variation of published methods (16). Briefly, aliquots of amoebae in broth were fixed with 2% glutaraldehyde in 0.1 M cacodylate. Fixed amoebae were then pelleted in agar and embedded. Thin sections were stained with uranyl acetate and lead citrate and examined with a Phillips CM-10 electron microscope.

DNA isolation and PCR amplification of nearly full-length 16S rDNA. UWC22, UWE1, and UWE25 amoebae in log-phase growth were pelleted, washed three times with cold (4°C) sterile distilled water, and freeze-thawed three times followed by aspiration and expulsion through a 24-gauge needle. The cell slurry was resuspended in 5 ml of cold (4°C) sterile physiologic (0.15 M) saline and gently centrifuged (5 min at 120 3 *g*). The supernatant was filtered through a 5-mm syringe filter, and the bacteria were pelleted by centrifugation (10 min at 3,000 3 *g*). Extraction of bacterial DNA was performed by standard procedures (34). Amplification of ribosomal gene sequences of these three isolates was performed with 1 mg of extracted DNA using fD1 (5' AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') broad-range eubacterial primers (40) along with standard Gen Amp reagents (Perkin-Elmer, Norwalk, Conn.) according to the manufacturer's recommendations. Thermal cycling consisted of 35 cycles of denaturation at 94°C for 1.5 min, annealing at 42°C for 1 min, and elongation at 72°C for 4 min; cycling was completed with a final elongation step of 20 min. TUME1 amoebae were harvested from axenic cultures, washed twice with double-distilled water, and resuspended in 500 ml of UNSET lysis buffer (8 M urea, 0.15 M NaCl, 2% sodium dodecyl sulfate [SDS], 0.001 M EDTA, 0.1 M Tris-HCl [pH 7.5]) at 60°C for 5 min (19). Lysates were extracted twice with phenol-chloroform, and DNA was precipitated with 2 volumes of absolute ethanol. Oligonucleotide primers targeting 16S ribosomal DNA (rDNA) signature regions that are conserved within the *Chlamydiales* were used for PCR to obtain nearly full-length bacterial 16S rRNA gene fragments of TUME1 (32). Forward and reverse primer sequences were 5'-CGGATCCTGA GAATTTGATC-3' (*Escherichia coli* 16S rDNA positions 22 to 18) and 5'-TGTCGACAAAGGA GGTGATCCA-3' (*E. coli* 16S rDNA positions 1554 to 1537), respectively. Amplification reactions were performed in a reaction volume of 50 ml in a thermal capillary cycler with reaction mixtures, including a 20 mM MgCl2 reaction buffer, prepared as recommended by the manufacturer (Idaho Technology, Idaho Falls, Idaho) with *Taq* DNA polymerase (Promega, Madison, Wis.). Thermal cycling consisted of an initial denaturation step at 94°C for 30 s, followed by 30 cycles of denaturation at 94°C for 20 s, annealing at 50°C for 15 s, and elongation at 72°C for 30 s, with a final elongation step at 72°C for 1 min. Positive controls containing purified DNA from *E. coli* were included along with negative controls (no DNA added). The presence and size of all amplification products were determined by agarose gel electrophoresis and ethidium bromide staining.

Cloning and sequence analysis. Amplified DNA from UWC22, UWE1, and UWE25 was purified by electrophoresis in low melting-point agarose and ligated into the cloning vector Bluescript II (Stratagene, La Jolla, Calif.), while amplified DNA from TUME1 was ligated directly into the cloning vector pCR2.1 (Invitrogen, Carlsbad, Calif.), with subsequent transformation of *E. coli* by each vector. The nucleotide sequences of the cloned DNA fragments were determined by automated dideoxynucleotide methods with the *Taq* Dye Deoxy Terminator cycle sequencing kit (Applied

Biosystems, Foster City, Calif.) for UWC22, UWE1, and UWE25 and the Thermo Sequenase cycle sequencing kit (Amersham Life Science, Little Chalfont, England) for TUME1.

Phylogenetic analysis. Obtained sequences were added to the 16S rRNA sequence database maintained at the Technische Universita¨t Mu¨nchen (encompassing about 16,000 published and unpublished homologous small-subunit rRNA primary structures). Alignment of the new sequences was performed using the program package ARB and its automated alignment tool (O. Strunk and W. Ludwig, www.biol.chemie.tu-muenchen.de/pub/ARB/), with refinement of positioning by visual inspection and by secondary-structure analysis. The ARB parsimony, distance matrix, and maximum-likelihood treeing methods, combined with and without use of filters which exclude highly variable regions, were applied to different data sets.

Oligonucleotide probes and FISH. Oligonucleotide probes $Bn9_{658}$ (a probe described previously [2]), specific for *P. acanthamoebae* and targeting *E. coli* positions 658 to 675) and $C22_{658}$ (designed from sequence data derived in this study from the endosymbiont infecting *Acanthamoeba* sp. isolate UWC22) were used to differentiate between two groups of endosymbionts (Table 1). Probe $C22_{658}$ is designated S-*-ParaC-0658-a-A-18 according to the standard proposed by Alm et al. (1). Both probes were synthesized and directly labeled with the hydrophilic sulfoindocyanine dye Cy3 or Cy5 (Interactiva, Ulm, Germany). For *in situ* hybridization studies, infected amoebic isolates UWC22, UWE25, and TUME1 were washed with Page's saline (39), and living cells were allowed to adhere to six-well slides for 3 h, followed by a single 10-s dip in sterile distilled water, a single 10 s dip in 80% ethanol for fixation and dehydration, and air drying. Amoebae were hybridized at 46°C with the fluorescently labeled probes according to published methods (35), but without SDS in the hybridization and washing buffers. Optimal hybridization stringency conditions were determined for each probe, including formamide concentrations used in the hybridization buffer (25). Positive and negative controls were carried out with probes Eub338 (targeting most members of the domain *Bacteria*) and Bet42a (specific for the beta subgroup of proteobacteria) (25). Slides were examined with a confocal laser scanning microscope (LSM 510; Carl Zeiss, Oberkochen, Germany) equipped with two HeNe lasers (543 and 633 nm) and optical sectioning capabilities. Image analysis processing was performed with the standard software package delivered with the instrument (version 1.5).

Nucleotide sequence accession numbers. The recovered 16S rDNA sequences have been deposited in GenBank under accession numbers AF083616 (endosymbiont of *Acanthamoeba* sp. UWC22), AF083614 (endosymbiont of *Acanthamoeba* sp. UWE1), AF083615 (endosymbiont of *Acanthamoeba* sp. UWE25), and AF098330 (endosymbiont of *Acanthamoeba* sp. TUME1).

Results

Morphologic analyses

As seen by light microscopy, the organisms are coccoid in appearance and stain gram negative. Use of Giemsa and related stains revealed that they are dispersed throughout the cytoplasm and do not form discrete inclusions, although small clusters and morulae are occasionally noted (Fig. 1A). By electron microscopy, large numbers of cocci are seen in each amoebic trophozoite and display a developmental life cycle typical of *Chlamydiales*, consisting of smaller electron-dense forms (elementary bodies) and larger dividing forms (reticulate bodies) (Fig. 1B and C). Rather than growing as discrete intravacuolar inclusions, the bacteria are found dispersed throughout the cytoplasm but appear to be surrounded by vacuolar membranes which insinuate themselves around each bacterial cell (Fig. 1C). Some bacteria are found in food vacuoles but appear to be in various stages of disintegration; these

forms may have been nonviable bacteria released from other amoebae previously and phagocytosed. When present in amoebic cysts, the bacteria appear to form inclusions and lack evidence of a developmental cycle, suggesting that they are in a resting state (Fig. 1D).

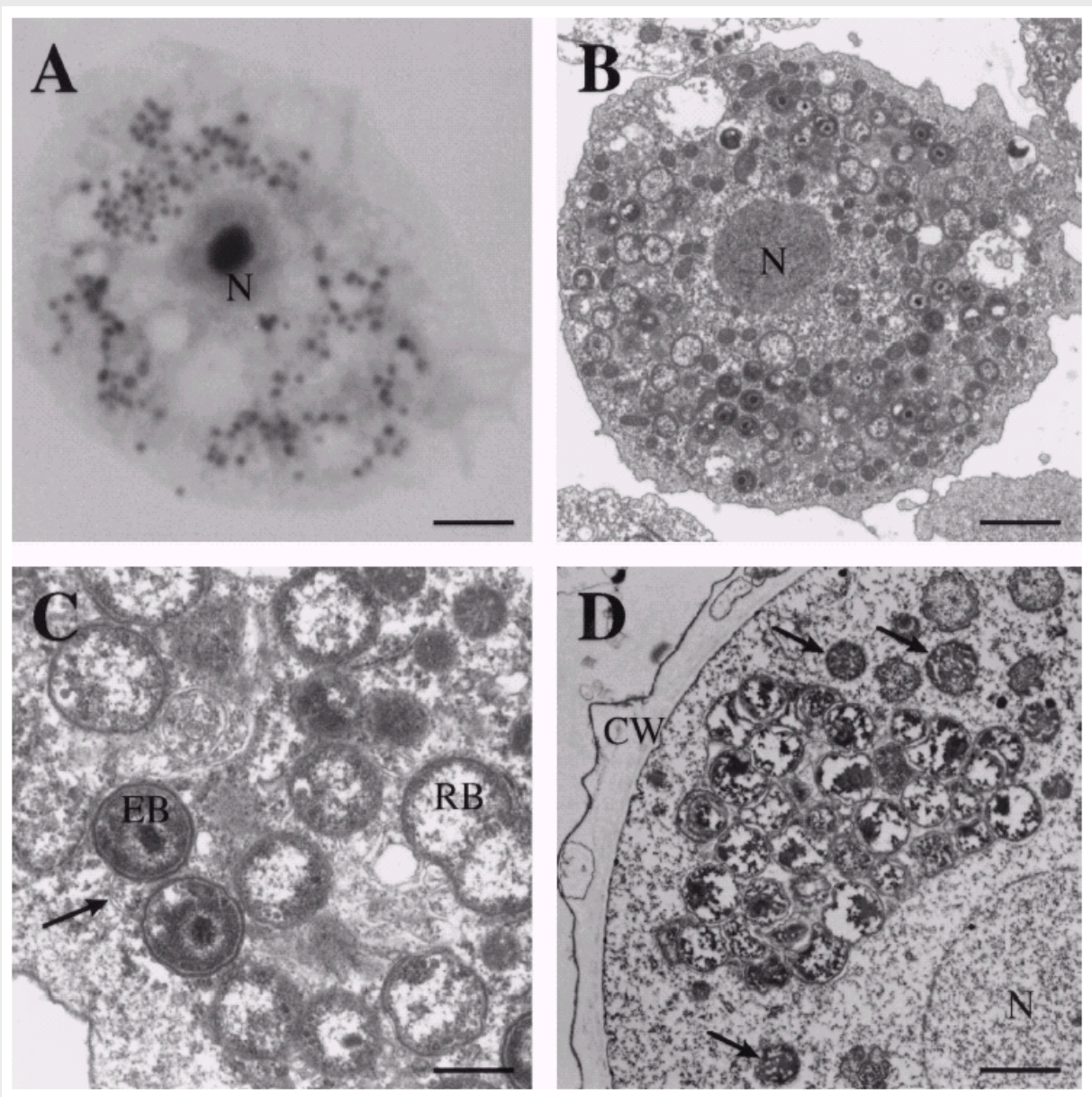

Figure 1. (A) *Acanthamoeba* sp. trophozoite (strain UWE25) infected with coccoid bacterial endosymbionts as seen using Hemacolor stain. N, nucleus. Bar, 7 mm. (B) Low-power electron micrograph of an *Acanthamoeba* trophozoite; note numerous bacteria scattered throughout the cytoplasm in various stages of differentiation. Bar, 1 mm. (C) High-power view of intracellular bacteria; note developmental stages (elementary bodies [EB] and reticulate bodies [RB]) typical of *Chlamydiales* and the presence of vacuolar membranes (arrow) between bacteria and surrounding each bacterium. Bar, 1 mm. (D) *Chlamydia*-like bacterial inclusion seen in a cyst of *Acanthamoeba*. The bacteria appear to be inactive, with an absence of developmental stages. CW, cyst wall. The arrows indicate mitochondria. Bar, 1 mm.

Comparative sequence results and phylogenetic analysis

Nearly full-length 16S rDNA sequences from the four endosymbiont strains were amplified, cloned, and sequenced. Comparative sequence analysis using parsimony, distance matrix, and maximum-likelihood treeing methods revealed that the four clustered unequivocally with other members of the *Chlamydiales* but formed a lineage with *P. acanthamoebae* and, more distantly, *S. negevensis* and *Waddlia chondrophila* that is distinct from *Chlamydia* and *Chlamydophila* spp. (Fig. 2). Further analysis of tree topology revealed the presence of two lineages of intra-amoebal endosymbionts: one contains the closely related isolates UWC22 and TUME1 (99.1% sequence similarity), and a second includes UWE1, UWE25, and *P. acanthamoebae* (sequence similarities of 91.5 to 93.2%). All four isolates warrant inclusion, along with *P. acanthamoebae*, in the recently proposed family *Parachlamydiaceae* and may represent up to three new genera and species (Table 1) (11, 36). With sequence similarities of 83.5 to 88.2% and 84.2 to 87.9% to all other *Chlamydiales*, *S. negevensis* and *W. chondrophila*, respectively, would appear to reside in separate families, as has been proposed (11).

Table 1. Overall sequence similarities for retrieved 16S rRNA sequences of endosymbionts of *Acanthamoeba* sp. Strains UWE1, UWE25, UWC22, and TUME1 and representative members of the *Chlamydiales*

		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
A	***Chlamydophila abortus* B577**															
B	***Chlamydophila psittaci* 6BC**	99,7														
C	***Chlamydophila felis* FP Baker**	98,0	98,4													
D	***Chlamydophila caviae* GPIC**	98,9	98,9	97,9												
E	***Chlamydophila pecorum* E58**	96,2	96,5	95,9	96,1											
F	***Chlamydia trachomatis* HAR-13**	95,0	95,2	94,8	95,3	95,1										
G	***Chlamydia suis* S45**	94,3	94,7	94,4	94,5	94,5	97,3									
H	***Chlamydia muridarum* MoPn**	95,6	95,7	95,5	95,7	95,5	98,4	97,7								
I	***Chlamydophila pneumoniae* TW-183**	95,8	96,2	95,1	95,3	95,8	93,9	93,5	94,6							
J	***Parachlamydia acanthamoebae* Bn9**	86,2	86,7	86,6	86,9	86,4	86,2	87,2	87,0	87,0						
K	***Simkania negevensis* Z**	83,5	83,8	83,9	83,9	83,6	84,2	84,4	84,2	83,7	88,2					
L	***Parachlamydia* sp. UWE1**	85,8	85,5	84,8	85,4	84,4	84,3	85,4	85,0	85,6	93,1	85,7				
M	***Parachlamydia* sp. UWE25**	86,2	86,0	85,6	86,0	85,6	85,4	85,9	86,1	86,3	92,5	85,4	93,0			
N	***Parachlamydia* sp. UWC22**	86,8	86,7	86,2	86,7	86,2	85,7	86,4	86,4	86,1	91,2	85,5	92,9	91,9		
O	***Parachlamydia* sp. TUME1**	86,8	87,2	86,8	87,2	86,8	86,3	87,0	87,0	86,6	91,2	85,9	92,3	91,4	99,4	
P	***Waddlia chondrophila* WSU-85-1044**	84,4	84,6	84,4	84,6	84,2	84,7	84,8	84,9	84,4	87,2	84,4	87,1	87,9	87,0	87,1

FISH analysis

The oligonucleotide probe $Bn9_{658}$, specific for *P. acanthamoebae*, fully matched the corresponding sequence in endosymbionts from *Acanthamoeba* sp. strains UWE1 and UWE25, whereas probe $C22_{658}$ was designed to recognize the endosymbionts present in *Acanthamoeba* sp. strains UWC22 and TUME1 (Fig. 3). These probes displayed two

mismatches between each other and at least three mis-matches to other members of the *Chlamydiales* and other bacteria. The probe $Bn9_{658}$ gave strong signals when applied to the endosymbiont of *Acanthamoeba* sp. strain UWE25 and no signal when applied to the endosymbionts of strains UWC22 and TUME1, whereas probe $C22_{658}$ gave strong signals when applied to the endosymbionts of UWC22 and TUME1 and no signal when applied to the endosymbionts of UWE25 (Fig. 4). Both probes performed optimally at a formamide concentration of 30% when applied separately, determined on the basis of a formamide dilution series (25). Because the two probes act as competitors for the same hybridization site, differentiation of the two groups of endosymbionts in mixed cultures using both probes simultaneously was demonstrated at a formamide concentration of 10% in the hybridization buffer and a hybridization temperature of 46°C. The optimal formamide concentration for the horseradish peroxidase-labeled probe $Bn9_{658}$ had been described previously as 40% at a hybridization temperature of 35°C (2). Horseradish peroxidase cannot be used at the higher temperature, requiring a higher concentration of formamide to achieve the same hybridization stringency.

Probe $Bn9_{658}$	3′-CATCCGCCTCTTTTGCCT-5′
Target (*E.coli* position 658-676)	5′-GUAGGCGGAGAAAACGGA-3′
Candidatus Parachlamydia acanthamoebae	.....
UWE1 endosymbiont	.....
UWE25 endosymbiont	.....
UWC22 endosymbiont	....A.....U...
TUME1 endosymbiont	....A.....U...
Chlamydia trachomatis	....AU.....G...
Chlamydia psittaci	....AU.....G...
Mycobacterium avium	.....U.....G.A.C.
Eubacterium timidum	.C...A..G....G....
Mycobacterium paratuberculosis	.....U.....G.A.C.
Ruminococcus gravus	..C..A.AG.....

Probe $C22_{658}$	3′-CATCTGCCTCTTTTACCT-5′
Target (*E.coli* position 658-676)	5′-GUAGACGGAGAAAAUGGA-3′
UWC22 endosymbiont	.....
TUME1 endosymbiont	.....
Candidatus Parachlamydia acanthamoebae	....G.....C...
UWE1 endosymbiont	....G.....C...
UWE25 endosymbiont	....G.....C...
Chlamydia trachomatis	.....U.....G...
Chlamydia psittaci	.....U.....G...
Chlamydia pneumoniae	A....U..G.....G...
Corynebacterium xerosis	.....U.....G.A.C.
Mycobacterium marinum	.....U.....G.A.C.
Orientia tsutsugamushi	....U..CGA.....

Figure 3. Difference alignment of the 16S rRNA region of intra-amoebal *Chlamydiales* targeted by probes $Bn9_{658}$ and $C22_{658}$ with their corresponding *E. coli* positions; other bacterial species showing 2 to 4 nucleotide mismatches are also noted. Dots represent nucleotide matches.

Discussion

Phylogenetically, members of the *Chlamydiales* comprise a unique bacterial assemblage which is highly divergent from other groups within the domain *Bacteria* in the 16S rDNAbased universal tree. Among the *Bacteria*, rDNA sequence analysis has revealed their closest relatives to be members of the *Planctomycetales*, a group of free-living, mostly aquatic bacteria which also display developmental cycles (10). While little is known about the evolution of traditional *Chlamydiales* species, the recent description of the *Parachlamydiaceae* occurring as endosymbionts in protozoa, specifically *Acanthamoeba* spp., has measurably broadened the possible evolutionary origins of the group (2, 11). Of the six isolates of *Parachlamydiaceae* described in the literature for which partial or nearly full-length 16S rDNA sequencing has been performed, including the four presented here, sequence dissimilarities suggest the existence of up to four genera and species; two of the isolates studied previously appear to be highly (99% sequence similarity) related (2, 5). The finding of additional related endosymbionts in these and other protozoa would therefore not be surprising, given the species diversity and geographic range noted to date, and suggests that a large environmental reservoir for them may exist. We conclude that the phylogenetic diversity of the *Chlamydiales* is extensive but is underrepresented in the literature as a result of bias towards the study of species recognized as being medically and economically important.

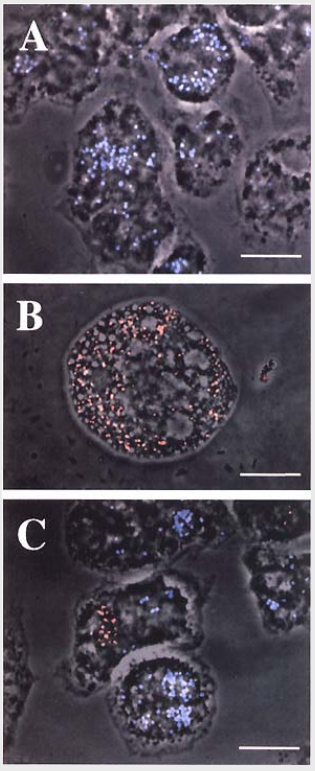

Figure 4. FISH reactions demonstrating the application of specific oligonucleotide probes to the detection of intracellular *Chlamydia*-like endosymbionts of *Acanthamoeba* spp. (A) Positive reaction of the Cy-5 (blue)-labeled probe $Bn9_{658}$ with the endosymbionts of UWE25 amoebae. (B) Positive reaction of the Cy-3 (red)-labeled probe $C22_{658}$ with the endosymbionts of UWC22 amoebae. (C) Positive reactions with probes Bn9658 (blue) and $C22_{658}$ (red) and their abilities to simultaneously differentiate between the UWC22 and UWE25 endosymbionts following a 3-h cocultivation of infected amoebic trophozoites. In some cases bacteria released from one amoebic isolate are seen to have been taken up by the other amoebic isolate during cocultivation. Bars, 15 mm.

Considering that all described species of *Chlamydiales* are known to be pathogenic and capable of producing respiratory tract disease, among other clinical presentations, analysis of the intra-amoebal forms for pathogenic potential should be stressed as well. Recently, seroepidemiologic evidence has been presented that "Hall's coccus," an endosymbiont of acanthamoebae which appears to be identical to *P. acanthamoebae*, may be responsible for some cases of community-acquired pneumonia (5). Other evidence comes from the amplification of *Chlamydiales* 16S rDNA fragments (216 to 224 bp) in respiratory tract specimens from humans with pulmonary disease, an aortic specimen, and peripheral blood mononuclear cells using broad-range primers designed to detect any member of the order (29). While sequences characteristic of *Chlamydophila pneumoniae* (three specimens) and *Chlamydia trachomatis* (one specimen) were recovered from the 42 respiratory specimens examined, four sequences (two respiratory, one blood, and one aortic tissue) were also detected which group in the second major *Chlamydiales* lineage, which includes the *Simkaniaceae* and *Parachlamydiaceae*. Because *S. negevensis* (Fig. 2) has been reported to cause both community-acquired pneumonia in adults and acute bronchiolitis in infants (20, 23), further analysis of the *Parachlamydiaceae* as disease-producing agents should be undertaken. Acanthamoebae and other free-living protozoa are uniquely positioned ecologically to support the dissemination of environmental respiratory pathogens. Able to colonize water supply, cooling, and humidification systems, they also serve as amplification

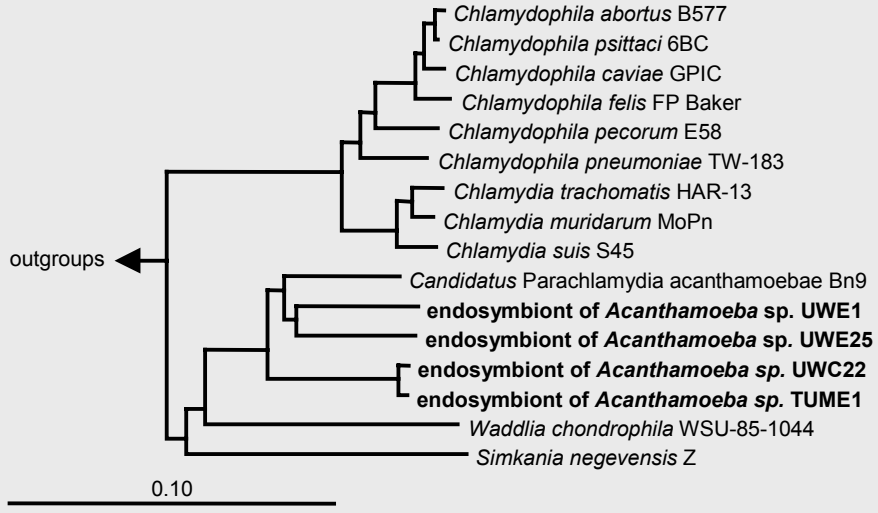

Figure 2. Neighbor-joining dendrogram showing phylogenetic relationships of the coccoid endosymbionts of *Acanthamoeba* sp. isolates UWC22, TUME1, UWE1, and UWE25 to other members of the *Chlamydiales* and outgroups (bar represents estimated evolutionary distance).

vehicles and possibly reservoirs for *Mycobacterium avium* and a variety of *Legionella* spp.; even *C. pneumoniae* has been shown to survive and replicate within acanthamoebae (8, 9, 12, 27, 29, 33, 37). Such intraprotozoal multiplication followed by aerosolization and inhalation of bacterium-laden vesicles has been proposed as a mechanism to explain the epidemiology of legionellosis and the apparent lack of case-to-case spread (4, 6).

The ubiquity of acanthamoebae in the environment, the presence of a resistant cyst stage, and their ability to support growth of a variety of intracellular pathogens make them prime suspects in the epidemiology of respiratory disease caused by other organisms as well. Many free-living protozoa mimic the role of professional phagocytes in their abilities to ingest and destroy large numbers of bacteria. Such bacterium-protozoan interactions have undoubtedly provided selective pressure resulting in the emergence of environmental species capable of escaping intracellular destruction, which are known to include *Legionella* spp., *Burkholderia pickettii*, *Listeria monocytogenes*, *Vibrio cholerae*, and *M. avium*, among others (3, 7, 24, 26, 37, 38). The adaptation of legionellae and *M. avium* to an intracellular existence in free-living protozoa may also have been a driving force in the evolution of virulence mechanisms which permit their survival within pulmonary macrophages (3, 22, 37). While many species of facultative bacteria develop in and ultimately lyse their protozoal host, a variety of obligate endosymbionts of ciliates and amoebae are well known to stably infect their host, suggesting the occurrence of longer-term coevolution (15, 17, 31). The *Parachlamydia*-like endosymbionts described here also appear to be well adapted to their natural amoebic hosts and have been maintained in continuous cultivation for several years. The presence of these bacteria in amoebic cysts in an apparent nondividing resting stage (Fig. 1D) further supports the stable symbiotic nature of the relationship. Considering that, until recently, all *Chlamydiales* were considered to be pathogenic, the finding of a potentially large environmental and geographically dispersed reservoir of related organisms which are adapted to stable intracellular growth in specific hosts provides an important link in the evolution of the order. The existence of a pool of protozoal symbionts stably adapted to the intracellular milieu may have provided the genetic material from which recruitment to vertebrates accidentally occurred, with the attendant appearance of pathogenic properties. Such recruitment would be facilitated by the frequent occurrence of these endosymbionts in *Acanthamoeba* spp. (and perhaps other protists), the ubiquity of acanthamoebae in the environment, and the ease with which these amoebae transiently colonize the respiratory tract of humans and animals (28).

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Chapter VIII

Evidence for Additional Genus-Level Diversity of *Chlamydiales* in the Environment

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Evidence for Additional Genus-Level Diversity of *Chlamydiales* in the Environment

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The medically important order Chlamydiales *has long been considered to contain a few closely related bacteria which occur exclusively in animals and humans. This perception of diversity and habitat had to be revised with the recent identification of the genera* Simkania*,* Waddlia*,* Parachlamydia*, and* Neochlamydia *with the latter two comprising endosymbionts of amoebae. Application of a newly developed PCR assay for the specific amplification of a near full length 16S rDNA fragment of these novel* Chlamydia*-related bacteria on activated sludge samples revealed the existence of at least four additional, previously unknown evolutionary lineages of* Chlamydiales *(each showing less than 92 % 16S rRNA sequence similarity with all recognized members of this order). These findings suggest that some waste water treatment plants represent reservoirs for a diverse assemblage of environmental chlamydiae, a discovery which might also be of relevance from the viewpoint of human public health.*

Introduction

Until recently the order *Chlamydiales* consisted of the single family *Chlamydiaceae* exclusively containing obligate intracellular bacteria. Based on comparative sequence analysis of different macromolecules Everett and co-workers [1, 2] reclassified these pathogens into nine species belonging to two different genera (*Chlamydophila* and *Chlamydia*) within the family *Chlamydiaceae*. Recently, intracellular bacteria showing developmental stages similar to the reticulate and elementary bodies of the *Chlamydiaceae* were observed by electron microscopy (i) in free-living amoebae [3, 4], (ii) in a contaminated cell culture [5], and (iii) in an aborted bovine fetus [6]. Based on comparative 16S rRNA sequence analysis these bacteria were found to represent the four new genera *Neochlamydia* [7], *Parachlamydia* [1, 8, 9], *Simkania* [1], and *Waddlia* [10] within the order *Chlamydiales*.

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From a phylogenetic but also from a medical point of view the expanding diversity of the *Chlamydiales* deserves attention. All members of the *Chlamydiaceae* are well recognized human or animal pathogens causing e.g. sexually transmitted (*C. trachomatis*), or respiratory diseases (*C. pneumoniae*). Furthermore, they were associated with intrinsic asthma [11] and cardiovascular disease [12] in men. Despite them only recently being discovered, there is accumulating evidence suggesting clinical importance also for the novel *Chlamydia*-related bacteria [13-15]. The assumption, that these bacteria are capable to thrive within humans is also supported by the PCR-mediated retrieval of several of their 16S rDNA sequences from clinical specimens of respiratory disease patients [16, 17]. The goal of this study was to evaluate the presence and diversity of *Chlamydia*-related bacteria in waste water treatment plants (WWTP). These systems contain an enormous diversity of protozoa, including free-living amoebae, and thus might represent a previously overlooked reservoir for environmental chlamydiae. For this purpose, a new PCR assay targeting the novel members of the *Chlamydiales* (but excluding members of the *Chlamydiaceae*) was developed. In contrast to a previously published diagnostic PCR test which amplifies a short, approx. 200 bp region of the 16S rDNA [16], the new PCR assay generates 16S rDNA amplificates of sufficient length for reliable phylogenetic analysis.

Materials and methods

Primer design and PCR. Primers were designed using a comprehensive 16S rRNA database (comprising over 15,000 aligned sequences, including all published 16S rRNA sequences of members of the *Chlamydiales*) and the ARB software package (available at http://www.arb-home.de).
Amplification reactions were performed in a total volume of 50 µl using a thermal capillary cycler (Idaho Technology, Idaho Falls, Idaho) with reaction mixtures including 2 mM $MgCl_2$, 250 pM dNTP, 1 pM of forward and reverse primer, 2.5 U Taq DNA polymerase, BSA buffer as recommended by the manufacturer, and 100 ng template DNA.

Environmental samples, DNA isolation, cloning and sequencing. Activated sludge samples from eight different municipal and industrial WWTPs in Germany were investigated. DNA was isolated and purified as described elsewhere [18]. Products amplified from environmental samples with the newly developed PCR assay were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Nucleotide sequences of cloned DNA fragments were determined by cycle sequencing of purified plasmid preparations with a Thermo Sequenase Cycle Sequencing Kit (Amersham Life Science, Little Chalfont, England), dye-labelled vector-specific primers and an automated DNA sequencer (Li-Cor Inc., Lincoln, Nebr.) under conditions recommended by the manufacturers.

Phylogenetic analysis. The obtained 16S rRNA sequences were added to the rRNA sequence database of the Technische Universität München by use of the program package ARB. Alignment of the new rRNA sequences was done automatically by using the respective ARB tool. The alignments were refined by visual inspection and by secondary structure analysis, and chimera checks were conducted. Phylogenetic analyses were performed by applying the parsimony, distance matrix, and maximum likelihood methods implemented in ARB to different data sets. To determine the robustness of the phylogenetic trees, analyses were carried out with and without the application of filter sets to exclude highly variable positions (conserved in less than 50% of all bacteria or the *Chlamydiales*, respectively).

Results and discussion

The oligonucleotide primers PCf (5‘-TCAGATTGAATGCTGAC-3‘, *E. coli* 16S rDNA positions 24-40) and PCr (5‘-TTNNGGATTTGCTTCACC-3‘, *E. coli* 16S rDNA positions 1270-1287) were designed to specifically amplify a large fragment of the 16S rDNA of the novel members of the *Chlamydiales* (while excluding the *Chlamydiaceae*) and showed at least two mismatches to all non-target 16S rDNA sequences currently available in public databases. Optimal PCR conditions using the two newly designed primers were determined with purified template DNA of suitable target (endosymbiont of *Acanthamoeba* sp. UWE25, belonging to the family *Parachlamydiaceae*) and non-target organisms (*C. pneumoniae*, *C. trachomatis*, *Listeria monocytogenes*) by varying the annealing temperature between 45 °C and 62 °C. Specific amplification was observed if thermal cycling consisted of an initial denaturation step at 94 °C for 4 min followed by 30 cycles of denaturation at 94 °C for 20 s, annealing at 60 °C for 45 s, and elongation at 72 °C for 90 s, with a final elongation step at 72 °C for 7 min. Using these parameters, PCR with DNA of the *Chlamydia*-related endosymbiont of *Acanthamoeba* sp. UWE25 resulted in amplified fragments of the expected length (approximately 1,200 bp), while all negative controls yielded no product (data not shown).

The genomic DNA recovered from each of the eight activated sludge samples was initially used for PCR together with primers targeting 16S rDNA signature regions which are highly conserved within the domain *Bacteria* [19] in order to demonstrate that the extracted DNA was of sufficient quality for PCR. While this broad range 16S rDNA-PCR resulted in amplificates from all eight WWTPs, the application of the *Chlamydiales*-specific PCf/PCr primer set produced a fragment of the expected size only from the industrial WWTP Plattling (Germany) connected to a rendering plant (data not shown). The retrieved PCR product was cloned and the insert sequences of 20 clones were determined completely.

Comparative sequence analyses of the recovered 16S rDNA clones assigned them to four novel, previously unknown evolutionary lineages within the *Chlamydiales.* In the following these lineages are referred to as the “environmental chlamydiae lineage” (ECL) ECL I, ECL II, ECL VI, and ECL VII (Figure 1). If a 95% 16S rRNA sequence similarity threshold value is applied for the separation of genera within the order *Chlamydiales*, as suggested by Everett [1], each of the four novel lineages represents a previously not recognized genus. In detail, ECL I and ECL II cluster together with the *Parachlamydiaceae*

(87-90%, and 91% sequence similarity, respectively), and ECL VI forms a common branch with *Simkania negevensis*, the only described member of the genus *Simkania* (88% sequence similarity). ECL VII represented by a single sequence is clearly monophyletic with the *Chlamydiaceae* (87-89% sequence similarity). In addition to the four evolutionary lineages identified in this study, three other lineages, ECL III-V; exclusively comprising unusual chlamydiae can be defined applying the 95% 16S rRNA similarity criterion to previously published 16S rDNA sequences [1, 7, 8, 9, 10, 17]. We thus suggest to provisionally subdivide the order *Chlamydiales* into the validly described genera and seven environmental chlamydiae lineages (ECL I to VII, Figure 1).

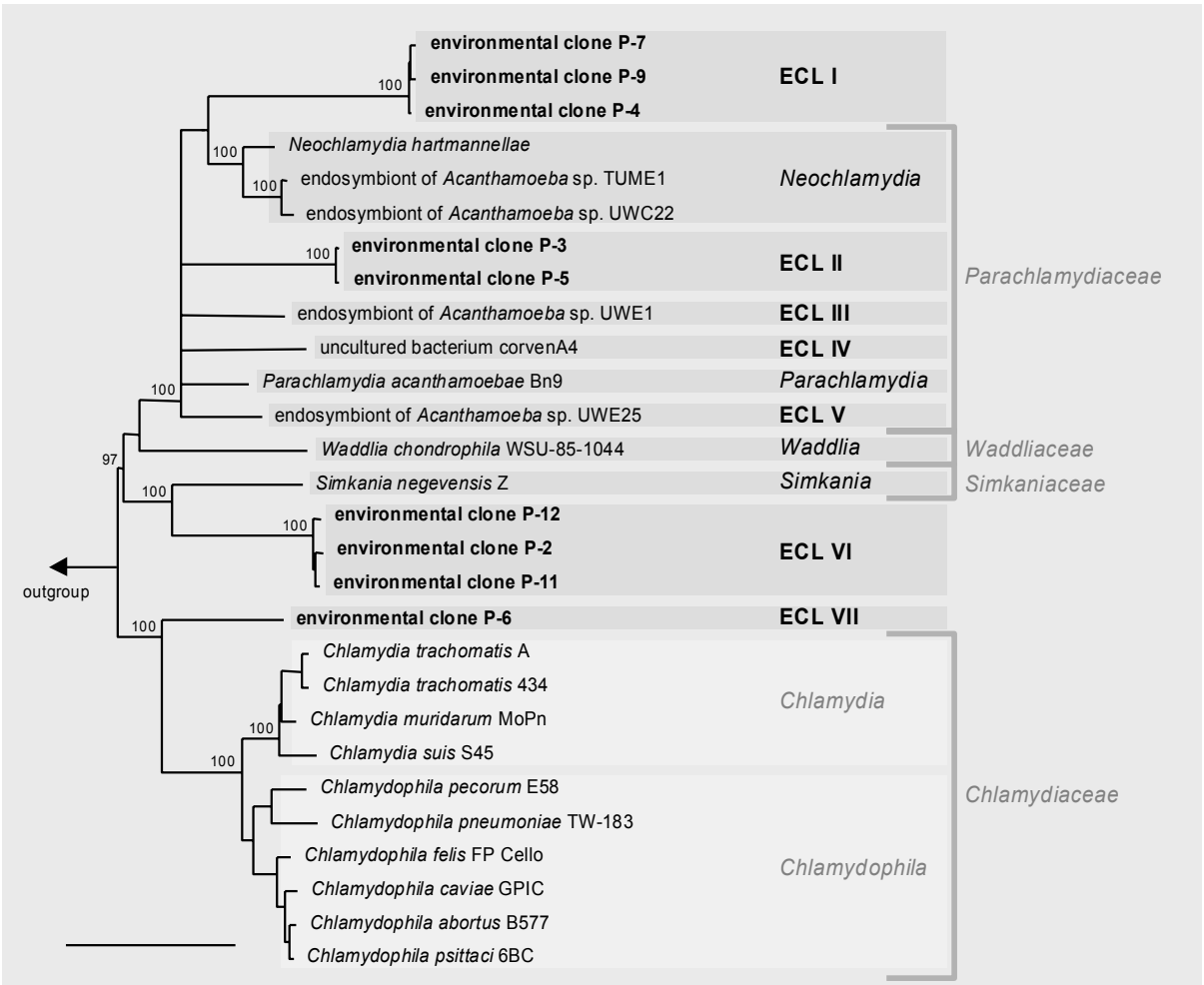

Figure 1. Consensus tree showing phylogenetic relationships of environmental chlamydiae as derived from comparative 16S rRNA sequence analysis. Multifurcations are shown for those lineages for which the branching order could not be unambiguously resolved. Parsimony bootstrap values (100 resamplings) larger than 90 % are noted. Environmental chlamydiae clusters (ECL I to VII) were defined using the 95 % 16S rRNA sequence similarity threshold for the definition of a genus within the *Chlamydiales* [7]. Only three out of a total of 14 highly similar (>98%) clones are shown for ECL VI. Family assignment of the sequences was based on a 90% 16S rRNA similarity criterion [7]. Bar, 10% estimated evolutionary distance.

Several additional partial 16S rDNA sequences (approximately 200 bp in length) most similar with the rDNA of members of the *Chlamydiales* were deposited at GenBank (GenBank accession numbers AF097184-AF097200, and AY013394-AY013474), most of them not having been published elsewhere [16]. Naturally, it is not possible to calculate overall 16S rRNA similarity values for those short sequences with other 16S rDNA sequences of the *Chlamydiales* (which is a prerequisite for the application of the 95% threshold value for the definition of a genus, or the ECL defined above). Furthermore, due to their low information content, application of different treeing methods failed to retrieve any consistent branching order for these sequences, which consequently may or may not be affiliated with the ECL. However, similarity values calculated based on comparison of the 200 available nucleotide positions demonstrated, that none of the novel *Chlamydia*-related sequences reported in this study is identical (or highly similar, i. e. more than 95% sequence similarity) with the rRNA sequences found in other investigations.

In conclusion, we demonstrated a previously not recognized genus-level diversity within the *Chlamydiales* in the environment. Given the capability of all validly described members of this order to infect and multiply within eukaryotic cells, future studies should attempt to identify the natural host(s) of the novel environmental chlamydiae and the potential of these bacteria to invade humans.

Acknowledgements

We gratefully acknowledge Dr. A. Essig (Universität Ulm, Germany) for kindly providing purified reference DNA of *C. pneumoniae* and *C. trachomatis*. This study was supported by Deutsche Forschungsgemeinschaft grant WA 1027/2-2 to M.W and a grant from the BMB+F in the framework of the CAPNET program.

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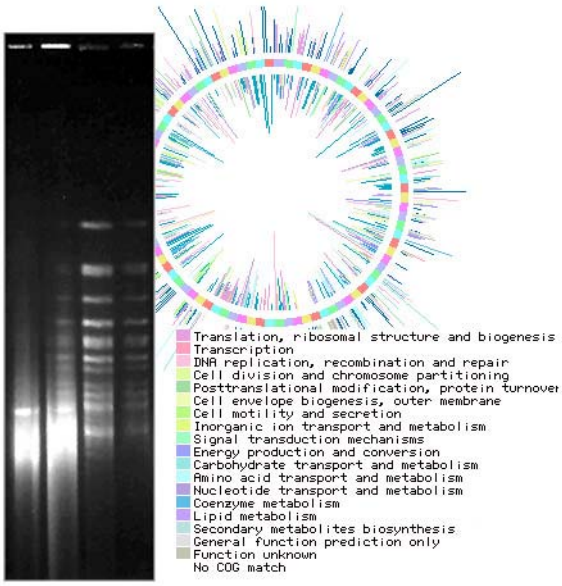
Chapter IX

Discussion and Future Perspectives



Front:

Sma I restriction digest of genomic DNA from *Acanthamoeba* sp. C1 (lane 1), *Acanthamoeba* sp. C1 containing *Parachlamydia* sp. E25 (lane 2), purified *Parachlamydia* sp. UWE25 (lane 3 and 4) separated by pulsed field gel electrophoresis (Horn, in preparation). Background, protein coding genes distribution map of *Chlamydia trachomatis* (From NCBI, Entrez Genome).

A definition of symbiosis

> *„a phenomenon in which dissimilar organisms live together, or symbiosis“*
> Heinrich Anton de Bary, 1879

The term symbiosis was created by Heinrich Anton de Bary (1831-1888) during his work on the nature of lichens and originally referred to organisms living together (De Bary, 1879). Using this broad definition, De Bary did not consider the effects of the symbiotic association (whether beneficial or harmful) on its partners. Later, symbiosis was redefined and equated with mutualism implying that both partners benefit from the symbiotic relationship. This definition turned out to be problematic, since it became obvious that the mechanisms underlying mutualism (both partners benefit), commensalism (one partner benefits and the other is neither harmed nor benefits), parasitism (one of the partners benefits on the expense of the other) and pathogenicity are quite similar (Hentschel *et al*., 2000; Paracer and Ahmadjian, 2000). Our current view of symbiosis resuscitated De Bary's definition (Paracer and Ahmadjian, 2000) which includes all forms of relationships between organisms: commensalism, mutualism, and parasitism. Pathogens are defined as entities that produce disease conditions in their host (Read, 1994).

This chapter addresses several aspects of the intimate relationship between bacteria and free-living amoebae using De Bary's broad definition of symbiosis. These aspects include (i) consequences of the phylogenetic diversity of bacterial endosymbionts of acanthamoebae, (ii) the occurrence of co-evolution between the bacterial symbionts and their eukaryotic hosts, (iii) a summary of our knowledge on the interactions between amoebal hosts and bacterial symbionts, and (iv) a discussion of promising future approaches to investigate these symbiotic associations.

Diversity of bacterial endosymbionts of free-living amoebae

The endosymbionts of free-living amoebae described in the preceding chapters represent a phylogenetically diverse group of obligate intracellular bacteria. Overall, six groups of *Acanthamoeba* symbionts (including the previously described *Legionella*-like amoebal pathogens, LLAP) can be differentiated (Figure 1). Two groups are within the α-subclass of *Proteobacteria*, one lineage is within the β-subclass of *Proteobacteria*, and one within the γ-subclass of *Proteobacteria*. Several endosymbionts are novel members of the order *Chlamydiales* and one endosymbiont belongs to the phylum *Cytophaga-Flavobacterium-Bacteroides*.

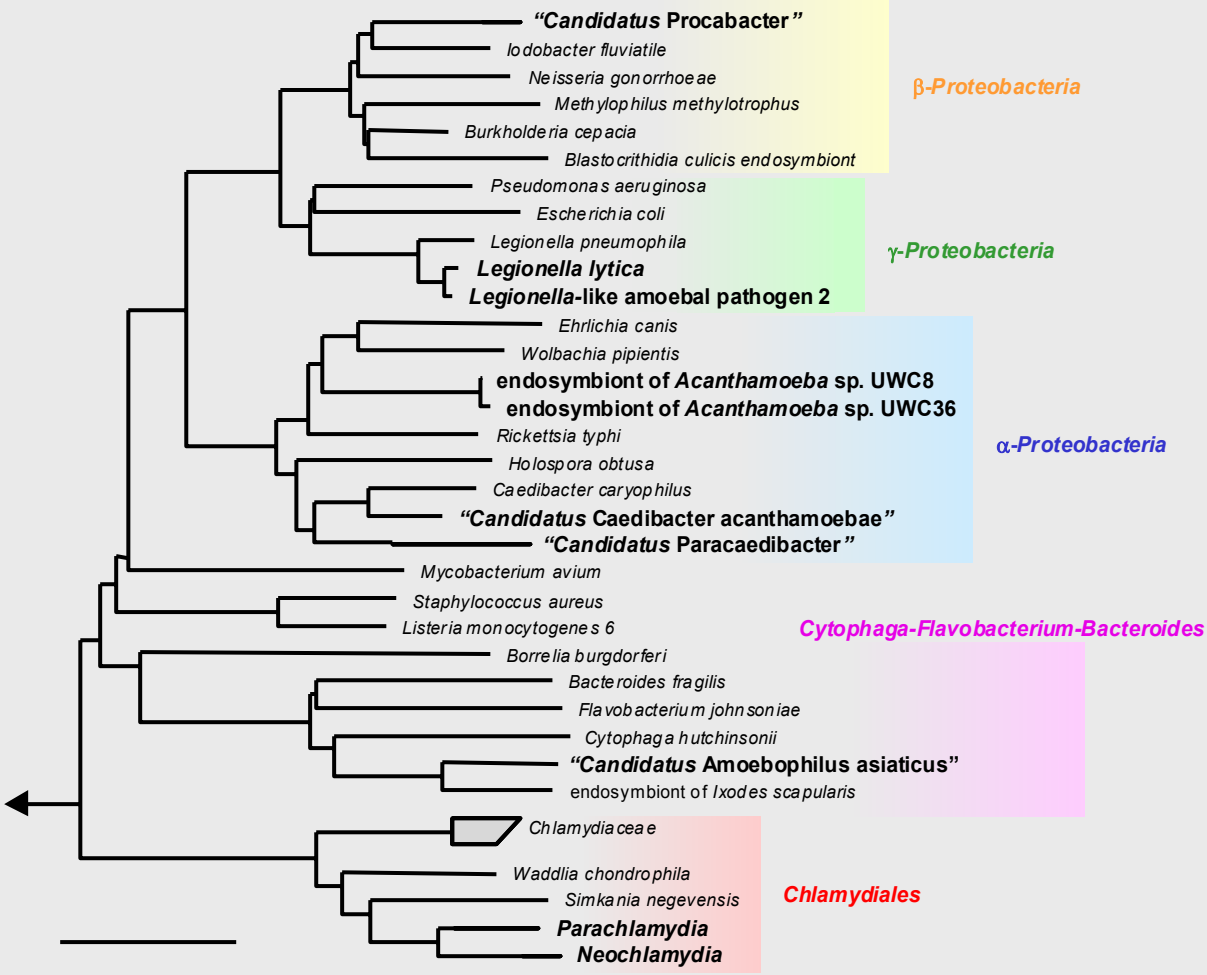


Figure 1. 16S rDNA based neighbor joining dendrogram demonstrating the affiliation of bacterial symbionts of *Acanthamoeba* spp. with their closest relatives. Arrow, to outgroup. Bar, 10% estimated evolutionary distance.

The occurrence of phylogenetically different symbionts of FLA is in striking contrast to other symbiotic associations, like e.g. the *Buchnera*-aphids symbiosis in which the prokaryotic partners represent closely related groups of organisms (Baumann *et al*., 1995). Thus, it seems unlikely that the ability to exploit amoebal cells as ecological niche was acquired only once by the ancestor of the six symbiont lineages, since this scenario implicates that the sister groups of the *Acanthamoeba* endosymbionts lost their ability to survive intracellularly in protozoa. Consequently, the ability to infect eukaryotic host cells most probably developed multiple times during evolution. This hypothesis is also supported by the finding that five of the six symbiont lineages are closely related with other intracellular bacteria. Consequently, the strategies of the amoebal endosymbionts for intracellular survival might differ between the six evolutionary lineages.

Although in total only 28 bacterial endosymbionts of acanthamoebae have been identified so far (Figure 2; Amann *et al*., 1997; Fritsche *et al*., 1999; Horn *et al*., 1999; Birtles *et al*., 2000; Horn *et al*., 2000; Fritsche *et al*., 2000; Horn *et al*., 2001a; Horn, *et al*. 2001b), there is accumulating evidence, that at least some of the symbiont groups are geographically widely distributed (Figure 2). For example, *Chlamydia*-related endo-symbionts have been found in amoebal strains isolated in North America, Europe and Asia (Figure 2). A more comprehensive collection of symbiont bearing amoebal strains from diverse sampling sites is necessary to more precisely understand their global distribution. Future efforts to isolate and characterize additional amoebae and their bacterial symbionts will also help to answer the question, whether additional symbiont diversity beyond the six evolutionary lineages identified so far exists.

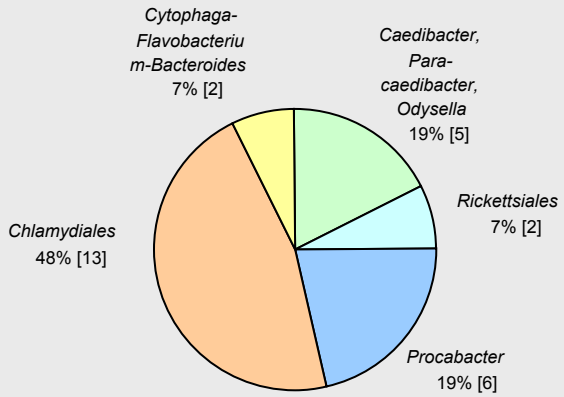


Figure 2. Relative numbers of identified bacterial endosymbionts of acanthamoebae in the five major evolutionary lineages. Absolute numbers are given in brackets.

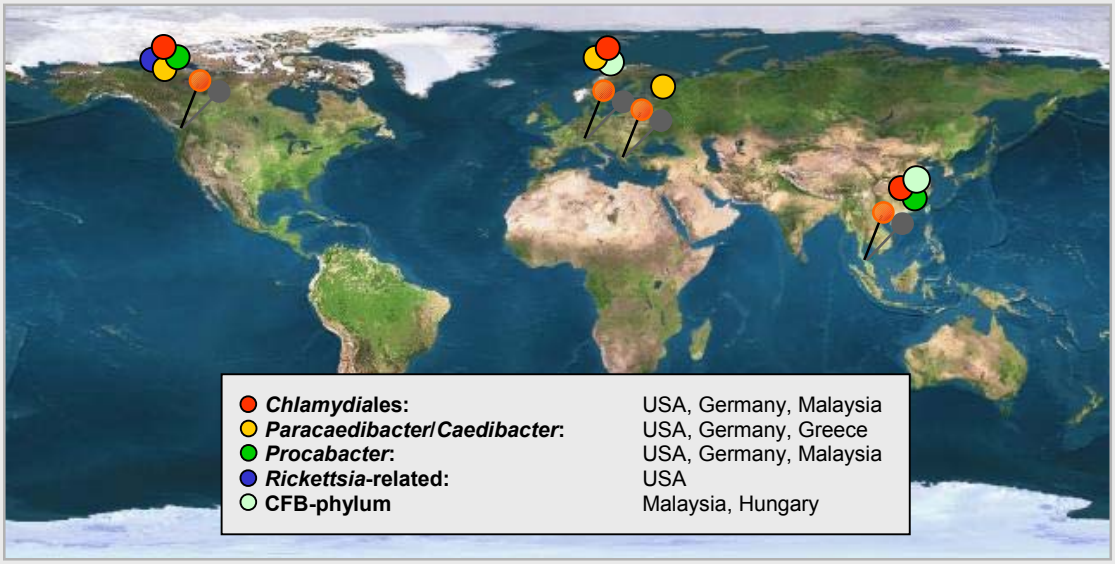


Figure 3. Geographical distribution of bacterial endosymbionts of acanthamoebae

Neochlamydia, Parachlamydia, *and the expanding diversity within the* Chlamydiales

Among the six groups of *Acanthamoeba* endosymbionts identified, one group deserves special attention – the symbionts most closely affiliated with the genus *Chlamydia*. Members of the genus *Chlamydia* are obligate intracellular bacteria. Their unique developmental cycle differentiates them from all other microorganisms. The chlamydial elementary bodies (EB) are adapted to extracellular survival and can infect eukaryotic cells. Within these host cells the EB differentiate to reticulate bodies (RB), which are metabolically active and replicate within the host cytoplasm, forming characteristic intracellular inclusions. Eventually, the RB become EB, which are released from the host cell and initiate another infection cycle. In this manner all recognized *Chlamydia* species cause a wide range of human and animal diseases (Schachter and Stamm, 1999). The genus *Chlamydia* until recently represented the single genus within the single family *Chlamydiaceae* of the bacterial phylum (or order) *Chlamydiales*. Therefore, the discovery of *Chlamydia*-related bacteria as endosymbionts of free-living amoebae (Hall, 1985; Michel *et al*., 1994; Amann *et al*., 1997; Horn *et al*., 2000; Fritsche *et al*., 2000; see chapters VI and VII), in an aborted bovine fetus (Rurangirwa *et al*., 1999), or as spontaneous infection of a cell culture (Kahane *et al*., 1998), and the detection of *Chlamydia*-like rDNA sequences in environmental and clinical samples (Ossewaarde *et al*., 1999; Horn and Wagner 2001; Corsaro *et al*., 2001; see chapter VIII) dramatically changed our view of chlamydial diversity and distribution. The identification of these organisms lead

to the creation of three new families (*Parachlamydiaceae*, *Simkaniaceae*, and *Waddliaceae*) comprising at least four new genera (*Parachlamydia*, *Neochlamydia*, *Simkania*, and *Waddlia*) within the order *Chlamydiales* (Everett *et al*., 1999; Rurangirwa *et al*., 1999; Horn *et al*., 2000). The recent finding of a novel *Chlamydia*-like microorganism as spontaneous infection of *Acanthamoeba castellanii*, that also showed the characteristic chlamydial developmental cycle, but was about 50% larger than previously described *Chlamydia*-related endosymbionts of acanthamoebae (Michel *et al*., 2001) indicates that also significant morphological variability exists within the phylum *Chlamydiales*.

The widespread occurrence of *Chlamydia*-related bacteria in FLA isolated from environmental and clinical samples (in the following also referred to as "environmental chlamydiae") and the well-known pathogenicity of their closest relatives raises the question of the clinical significance of *Chlamydia*-like amoebal symbionts. Several recently published studies provide preliminary indications for an association of environmental chlamydiae with human disease (Birtles *et al*., 1997; Ossewaarde *et al*., 1999; Kahane *et al*., 1998). In order to more thoroughly investigate this important question, two projects were initiated focussing on the occurrence of *Chlamydia*-related bacteria in clinical specimens. Blood sera from patients with respiratory disease of unknown cause will be analyzed for the presence of specific antibodies against environmental chlamydiae, and in an alternative approach, sputum samples of pneumonia patients will be screened for *Chlamydia*-like 16S rDNA sequences using the PCR assay described in chapter 9. In addition, the capability of *Chlamydia*-related endosymbionts to infect and thrive within different animal and human cell lines will be investigated. These experiments will provide a basis for further analysis of the possible role of *Chlamydia*-related bacteria as new emerging pathogens.

Co-evolution between Caedibacter caryophilus-related endosymbionts and their amoebal hosts

Chapter III described the identification of three Gram-negative, rod-shaped bacteria that reside directly within the cytoplasm of their *Acanthamoeba* hosts as being most closely affiliated with the *Paramecium* symbiont *Caedibacter caryophilus*. Consequently, the names *Candidatus* Caedibacter acanthamoebae, *Candidatus* Paracaedibacter acanthamoebae, and *Candidatus* Paracaedibacter symbiosus have been proposed for classification of these endosymbionts (Horn *et al*., 1999). Meanwhile, a similar endosymbiont of acanthamoebae

was found by Birtles and co-workers, who suggested the provisional name *Candidatus* Odyssella thessalonicensis (Birtles *et al*., 2000), and an additional symbiont was detected in an amoeba isolate from a waste water treatment plant, which is most closely related with *Candidatus* Paracaedibacter symbiosus (*Paracaedibacter* sp. TUMK2.3 in Figure 3; Horn, in preparation).

Comparison of rDNA based dendrograms of these endosymbionts and their protozoan host cells revealed a consistent branching order, indicating a close relationship between the phylogeny of the symbionts and their hosts species (Figure 3). This process of co-speciation might be a consequence of co-evolution between *Caedibacter caryophilus*-related bacteria and their hosts. Comparison of symbiont and host phylogeny also suggested that the ancestor of the *C. caryophilus*-related endosymbionts lived within an amoebal progenitor and was then transferred into a *Paramecium* host. This scenario however implies that those *Acanthamoeba* strains which do not harbor *Caedibacter*-related bacteria must have lost these endosymbionts during their evolutionary history. The uptake of R-body-coding mobile genetic elements by the *C. caryophilus*-symbiont might have taken place after the transfer into the *Paramecium* host cell, for example by co-infection of the host with different *Caedibacter* species (Preer *et al*., 1974). This hypothesis is also supported by the observation that *C. caryophilus* strain C220 quickly lost its ability to produce R-bodies under laboratory conditions (Schmidt *et al*., 1987; Schmidt *et al.,* 1988) and that no R-bodies were present in the *C. caryophilus*-related endosymbionts of acanthamoebae (Horn *et al.*, 1999). However, co-evolution also implies a reciprocal influence taking place between the two partners, a process which still has to be demonstrated for this symbiotic relationship. No evidence for co-evolution was found for other *Acanthamoeba* endosymbiont lineages.

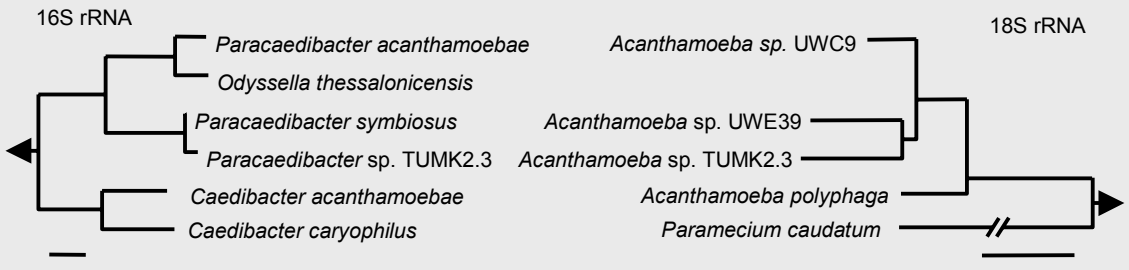


Figure 3. rDNA based dendrograms reflecting the phylogeny of *Caedibacter caryophilus*-related endosymbionts (left) and their *Acanthamoeba* or *Paramecium* host cells (right). Arrows, to outgroup. Bars, 1% estimated evolutionary distance.

Interactions between acanthamoebae and their bacterial endosymbionts

With the exception of LLAPs, the described symbiont lineages can not be maintained on cell-free media. This failure might be due to the lack of adequate culturing methods, but it appears more likely that obligate interactions between the symbiotic bacteria and their amoebal host cells exist. Several findings point to a certain specificity of this intimate relationship. Gautom and Fritsche observed that the *Rickettsia*-related symbionts, *Procabacter* spp., and *Parachlamydia* sp. UWE25 are able to infect certain *Acanthamoeba* host species while no symbiosis could be established with other *Acanthamoeba* strains (Table 1; Gautom and Fritsche, 1995; Fritsche *et al*., 1998). Limited host ranges were also noted for *Neochlamydia hartmannellae*, *Parachlamydia acanthamoebae* and *Endocytophaga acanthamoebae*, suggesting the presence of a specific recognition system between the amoebae and their symbionts (Amann *et al*., 1997; Horn *et al*., 2000; Horn *et al*., 2001b; see chapters V and VI).

Table 1. Transmissibility of bacterial endosymbionts between isolates of *Acanthamoeba* spp.; nd, not determined. (From Gautom and Fritsche, 1995; Fritsche *et al*., 1998; modified)

		Endosymbiont				
Amoebic	**mtDNA**	***Rickettsia*-related**	***Procabacter* sp.**			***Parachlamydia* sp.**
strain	**group**	**UWC8***	**UWC6***	**UWC12**	**UWE2**	**UWE25**
UWC1	I	+	+	+	+	+
UWC10	II	+	+	nd	nd	nd
UWC5	IV	-	-	nd	nd	nd
UWC4	VI	-	+	+	+	+
UWC7	nd	nd	nd	+	+	-
UWC13	nd	nd	nd	+	+	-

*Host amoebae belong to mtDNA fingerprint group II.

While the bacteria obviously benefit from their eukaryotic hosts, only little is known about the effect of the symbiosis on the amoebae. In order to test, whether the host amoebae depend on their bacterial endosymbionts, we performed curing experiments with (i) the original hosts of *Paracaedibacter acanthamoebae*, the *Parachlamydia*-related symbiont TUME1, and *Parachlamydia* sp. UWE25 and (ii) newly-established symbiotic pairs between these endosymbionts and an amoebal strain without natural symbionts. The antibiotic treatment used successfully eradicated endoymbionts from the artificially infected amoebae, but failed to kill the bacteria inside their original host cells (Table 2; Harzenetter and Horn,

unpublished), suggesting the presence of multiple interactions between acanthamoebae and their bacterial symbionts, and indicating a dependency on both sides.

Two other studies showed, that the infection of *Hartmannella vermiformis* with the amoebal endosymbiont *Neochlamydia hartmannellae* induced an enhanced amoebal fission rate (R. Michel, personal communication), and that the presence of *Parachlamydia* sp. UWE25 significantly increased the *in vitro* cytopathogenicity of its amoebal host (Fritsche *et al*., 1998) in tissue culture experiments, indicating that also the amoebal hosts might benefit from these symbiotic relationships.

Table 2. Curing experiments

Host	**Symbiont*/Infected with°**	**Curing** (15 µg/ml Rifampicin)
Acanthamoeba sp. UWC9	*Candidatus* Paracaedibacter acanthamoebae*	-
Acanthamoeba sp. UWC1	*Candidatus* Paracaedibacter acanthamoebae°	+
Acanthamoeba sp. TUME1	*Parachlamydia*-related symbiont TUME1*	-
Acanthamoeba sp. UWC1	*Parachlamydia*-related symbiont TUME1°	+
Acanthamoeba sp. UWC1/E25	*Parachlamydia* sp. UWE25°	+

A more detailed analysis of symbiont-host interactions might be possible by infection experiments using a suitable model host that can, in contrast to acanthamoebae, be genetically modified. The soil amoeba *Dictyostelium discoideum* ("slime mold"), for example, was shown to be an useful infection model for several facultative and obligate intracellular bacteria, e.g. the pathogens *Legionella pneumophila* and *Mycobacterium avium* (Hägele *et al*., 2000; Solomon *et al*., 2000; Skriwan *et al*., 2001). *D. discoideum* is haploid, can be genetically modified, its genome is currently sequenced, and there are already several mutants available suited to study host-symbiont interactions. Since *Parachlamydia*, *Neochlamydia*, and *Procabacter* are able to multiply within these amoebae (Skriwan *et al*., 2001), *D. discoideum* might represent a suitable model system for the future.

Non-mitochondrial ATP/ADP transporter proteins

The observations summarized in the previous section demonstrated that the interactions between acanthamoebae and their bacterial symbionts are manifold. However, the molecular basis of this symbiosis is still unclear. A conceivable approach to investigate these interactions is to evaluate whether already known mechanisms used by other obligate intracellular bacteria can also be detected for the FLA symbionts. Rickettsiae and chlamydiae, which are moderately affiliated with some *Acanthamoeba* endosymbionts (Fritsche *et al*., 1999; Amann *et al*., 1997; Horn *et al*., 2000; Fritsche *et al*., 2000, see chapters II, VI, and VII), for example, are known to posses special ATP/ADP transporter proteins which exchange bacterial ADP for ATP from the host cell cytoplasm (Williamson *et al*., 1989; Tjaden *et al*., 1999; Winkler and Neuhaus, 1999). By this means the intracellular bacteria can live as energy parasites using the ATP of their eukaryotic hosts. The membrane associated ATP/ADP transporter proteins have also been reported in plastids of *Arabidopsis thaliana* and *Solanum tuberosum* (Möhlmann *et al*., 1998; Tjaden *et al*., 1998) and can easily be differentiated from mitochondrial ATP/ADP transporter proteins, which catalyze the reverse reaction (Table 3).

Table 3. Mitochondrial and non-mitochondrial and ATP/ADP transporter proteins.

	mitochondrial	**bacterial**
occurrence	mitochondria, chloroplasts	heterotrophic plastids, *Rickettsia* sp., *Chlamydia* sp.
location	membrane	membrane
function	ATP export, ADP import	ATP import, ADP export
molecular weight	30-34 kDa	56-62 kDa
sequence	approx. 300 aa	489-589 aa
transmembrane domains	6	12
inhibitors	atractyloside, bongkrekic acid	

Using two primer sets designed on the basis of known nucleotide sequences of genes coding for non-mitochondrial ATP/ADP transporter, the *Acanthamoeba* endosymbionts identified in chapters II to VIII were investigated for the presence of homologous genes (Horn, in preparation). Application of these primers in PCR resulted in amplified fragments of the expected length for *Parachlamydia* sp. UWE25, *Neochlamydia* sp., TUME1, *Caedibacter acanthamoebae, Paracaedibacter symbiosus*, and the *Rickettsia*-related symbiont of *Acanthamoeba* sp. UWC36, while no amplificates were obtained for *Procabacter* sp. and *Amoebophilus asiaticus*. Sequencing of the retrieved amplificates demonstrated the presence of motifs conserved among known non-mitochondrial ATP/ADP translocases. Consistent

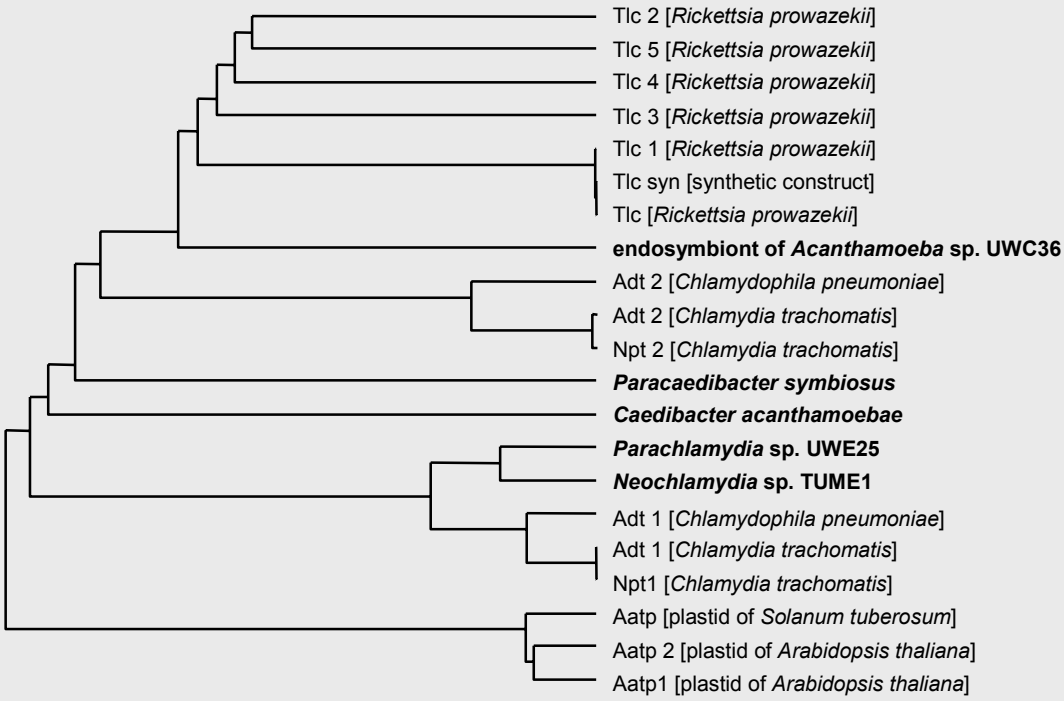


Figure 4. Dendrogram showing the affiliation of the putative ATP/ADP transporters of the *Acanthamoeba* endosymbionts with non-mitochondrial ATP/ADP transporters of rickettsiae, chlamydiae, and plastids. The tree is based on the respective amino acid sequences and was calculated using the maximum parsimony method.

with this finding, the deduced amino acid sequences showed highest similarities with known ATP/ADP transporter proteins (38-60%). Comparative sequence analysis revealed that the putative ATP/ADP transporters of *Neochlamydia* and *Parachlamydia* were indeed most closely related with ATP/ADP transporter proteins of *Chlamydia pneumoniae* and *Chlamydia trachomatis*, while the putative ATP/ADP transporter of the *Rickettsia*-related symbiont of *Acanthamoeba* sp. UWC36 grouped together with recognized ATP/ADP translocases of *Rickettsia prowazekii*. No close relationship or consistent branching was found for the putative transporters of *Caedibacter acanthamoebae* and *Paracaedibacter symbiosus* (Figure 4).

Transmembrane prediction analysis showed that the profiles of the putative *Neochlamydia* and *Parachlamydia* ATP/ADP transporters were highly similar with the transmembrane profiles of chlamydial ATP/ADP transporters, indicating that the proteins under investigation are indeed located in the membrane (Figure 5).

In addition, cDNA from *Acanthamoeba* sp. UWE25 containing *Parachlamydia* sp. UWE25 was extracted and the 5' terminal sequence of the putative ATP/ADP transporter gene of this endosymbiont was retrieved by 5'-RACE (rapid amplification of cDNA ends; Frohman *et al*., 1988), demonstrating that the putative nucleotide transporter is actually transcribed (Horn, in preparation). In order to analyze *in situ* function and substrate specificity these putative ATP/ADP transporters eventually have to be expressed in heterologous systems. By means of such experiments it was demonstrated that only one of the two nucleotide transport proteins of *Chlamydia trachomatis* catalyzes ATP and ADP transport in an exchange mode (Npt1) while the other transporter is a nucleosidetrisphosphate/H+ symporter (Npt2) responsible for anabolic reactions (Tjaden *et al*., 1999).

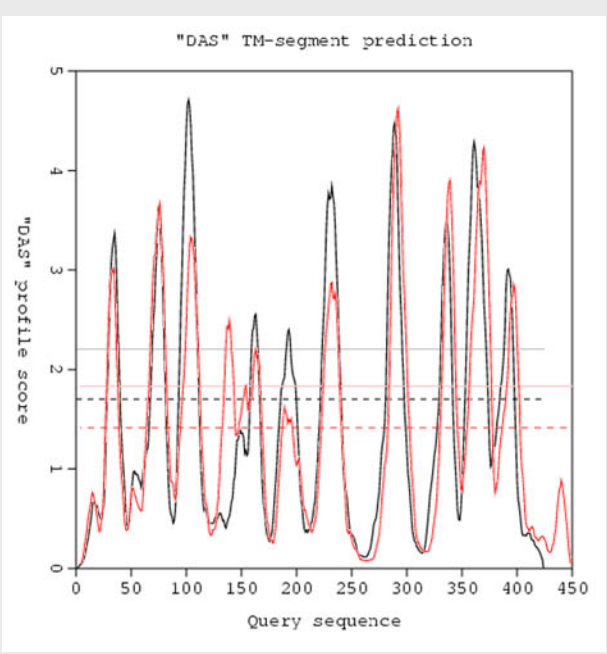


Figure 5. Transmembrane prediction profile of the putative ATP/ADP transporter of *Parachlamydia* sp. UWE25 (black) and *Chlamydia trachomatis* (Npt 1, red).

In summary, these preliminary experiments indicate that ATP/ADP transporter proteins are present in three of the six *Acanthamoeba* endosymbiont lineages. The symbionts could thereby thrive as energy parasites, exploiting their amoebal host cells by importing ATP from the host cytoplasm in exchange for ADP. Non-mitochondrial ATP/ADP transporters are apparently not restricted to rickettsiae and chlamydiae, but might represent a more general strategy of obligate intracellular bacteria. Therefore, these molecules are potential novel drug targets for antibiotics against intracellular bacteria.

Future perspective: genomics and beyond

Investigating the biology of obligate intracellular bacteria is a major challenge for microbiologists which almost certainly will provide new insights into the evolution of pathogenicity as well as organelle formation. The recent progress in genome sequencing may help to approach this task. Craig Venter's suggestion of "a new strategy for genome sequencing" in 1996, the so called shotgun approach (Venter *et al*., 1996), was the starting point for the initiation of more than 300 genome projects, with about 50 microbial genomes being completed so far. The genome sequence of the bacterial symbiont of aphids *Buchnera* sp. APS, for example, provided interesting insights into the biology of this obligate endosymbiont and demonstrated that *Buchnera* lacks genes for cell-surface components (including lipopolysaccharides and phospholopids) and genes involved in defense of the cell (Shigenobu *et al*., 2000). Consequently, *Buchnera* is considered completely symbiotic and viable only in its limited niche, the bacteriocyte (Shigenobu *et al*., 2000). Furthermore, the presence of genomic features similar to eukaryotic organelles suggested that these bacteria may represent an intermediate between symbiont and organelle (Andersson, 2000).

Members of the genera *Chlamydia* and *Chlamydophila* have also been subject of intensive genomic research. Six chlamydial genome sequences have been completed so far (Stephens *et al*., 1998; Kalman *et al*., 1999; Shirai *et al*., 2000; Read *et al*., 2000) and their analyses complemented and expanded much of the information acquired in the pregenomic era. These studies provided additional evidence for the general reduction in biosynthetic machinery and confirmed that chlamydiae are auxotrophic for many precursors (Rockey *et al*., 2000). Genome sequence analysis also confirmed that chlamydiae do encode functional glucose-catabolizing enzymes, which can be used for generation of ATP (Ilifee-Lee and McClarty, 2000; Rockey *et al*., 2000). Apparently, chlamydiae are able to produce their own ATP and reducing power, and are not solely energy parasites (see also above).

Buchnera and chlamydiae are only two representative examples for the potential of the genomic approach to get new insights into the biology of obligate intracellular bacteria. Based on the findings presented in this thesis ,the *Acanthamoeba* endosymbiont *Parachlamydia* was chosen for whole genome analysis in a project funded by the BMB+F (German Ministry for Science and Education) as part of the network program "Kompetenznetz Pathogenomik ". The project will start summer 2001 and aims for a comparative analysis of the *Parachlamydia* genome with published genomes of its pathogenic relatives, *C. pneumoniae* and *C.*

trachomatis. Preliminary experiments using restriction enzyme analysis and pulsed field gel electrophoresis estimated a genome size of 1.6 to 1.8 Mb for *Parachlamydia* sp. UWE25, which indicates that the *Parachlamydia* genome is about 60% larger than the genomes of *C. pneumoniae* and *C. trachomatis* (Horn, unpublished). The comparative analysis of theses genomes will help to extend our knowledge on the evolution and the mechanisms of pathogenicity within the medically important order *Chlamydiales* and will provide the basis for a more comprehensive understanding of the biology of environmental chlamydiae. Future approaches will include analysis of the transcriptome (the totality of transcribed gene) and the proteome (the totality of expressed protein) of *Parachlamydia* symbionts under different environmental conditions.

Concluding remarks

The studies presented in this thesis provide a basis for continuing efforts to investigate the symbiosis between free-living amoebae and their bacterial endosymbionts. Such analyses might help to understand the process of adaptation of bacteria to an intracellular way of life in eukaryotic host cells. Long before humans evolved, these adaptations might have represented the first steps in the development of microorganisms pathogenic for men.

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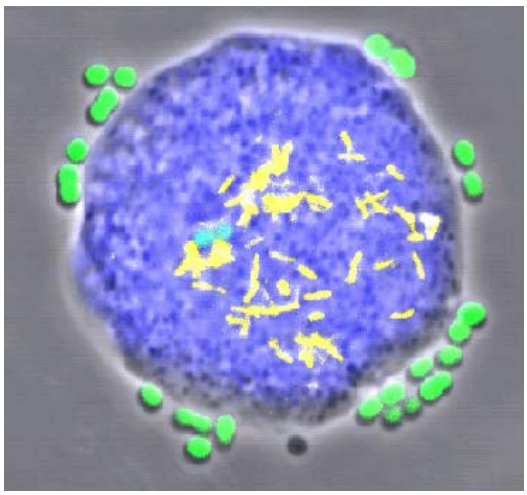
Chapter X

Summary

Zusammenfassung



Front:
In situ identification of *Acanthamoeba* sp. and their bacterial endosymbionts by fluorescence *in situ* hybridization.

Summary

Acanthamoebae are ubiquitous free-living amoebae that play an important role as predators controlling microbial communities. In addition, acanthamoebae have been recognized as opportunistic human pathogens. Despite being a food source several bacteria are able to survive the uptake and temporarily multiply in free-living amoebae. Furthermore, one fourth of *Acanthamoeba* isolates contain unknown obligate intracellular bacterial symbionts. The symbiosis between these bacteria and their amoebal host cells was subject of this thesis.

The obligate endosymbionts of nineteen isolates of free-living amoebae were assigned to five novel evolutionary lineages by means of the full cycle rRNA approach, including comparative rRNA sequence analysis and fluorescence *in situ* hybridization. Four of these lineages appeared to be cosmopolitan in distribution since phylogenetically almost identical strains were found in *Acanthamoeba* isolates from geographically distant sources. Interestingly, these novel lineages are most closely related to other obligate intracellular bacteria including several important human pathogens.

Two rod-shaped Gram-negative endosymbionts formed a novel lineage within the *Rickettsiales*, a diverse group within the α-subclass of *Proteobacteria* comprising recognized pathogens of animal and humans as well as a number of insect symbionts. Several other, rod-shaped Gram-negative endosymbionts, for which the provisional genus "*Candidatus* Procabacter" was proposed, were members of a previously not recognized evolutionary lineage within the β-subclass of *Proteobacteria*. Another endosymbiont, tentatively classified as "*Candidatus* Amoebophilus asiaticus", was identified as a novel member of the phylum *Cytophaga-Flavobacterium-Bacteroides*.

Several *Acanthamoeba* endosymobionts were identified as being related to the paramecium symbiont *Caedibacter caryophilus* and were therefore grouped into the new genus „*Candidatus* Paracaedibacter". Comparison of the phylogenetic affiliation of the *Caedibacter*-affiliated endosymbionts and their amoebal host cells suggested co-evolution between these symbionts and their *Acanthamoeba* hosts.

Several coccoid endosymbionts of *Acanthamoeba* were demonstrated to represent at least two new genera (designated *Parachlamydia* and *Neochlamydia*) within the *Chlamydiales*, indicating a previously underestimated genus-level diversity within this medically important order. Development and application of a PCR assay specific for these novel members of the *Chlamydiales* demonstrated that the genus-level diversity of chlamydiae in the environment is yet not fully discovered.

First indications for a molecular basis of the intimate association between free-living amoebae and their bacterial endosymbionts are provided by the detection of genes coding for putative non-mitochondrial ATP/ADP transporter proteins in members of three of the five endosymbiont lineages, suggesting that these bacteria can thrive as energy-parasites within their *Acanthamoeba* host cells.

The analysis of the symbiosis between free-living amoebae and their intracellular bacteria might eventually help to understand the process of adaptation of bacteria to an intracellular way of life. Long before humans evolved, these adaptations might have represented the first steps in the development of microorganisms pathogenic for humans.

Zusammenfassung

Acanthamöben sind weit verbreitete, frei lebende Amöben denen als Räuber eine wichtige Rolle bei der Kontrolle mikrobieller Gemeinschaften zukommt, die jedoch auch als opportunistische Krankheitserreger des Menschen gelten. Verschiedene Bakterien, wie beispielsweise der Erreger der Legionärs-Krankheit, *Legionella pneumophila*, können sich dem Verdauungsmechanismus der Acanthamöben entziehen und vermehren sich vorübergehend innerhalb der Amöben. Zusätzlich beherbergen etwa ein Viertel aller Acanthamöben dauerhaft bislang unbekannte bakterielle Endosymbionten. Die Untersuchung der Symbiose zwischen diesen Bakterien und ihren Amöbenwirten war Gegenstand dieser Arbeit.

Die obligaten Endosymbionten von neunzehn Isolaten frei lebender Amöben konnten mit Hilfe des vollständigen rRNS-Ansatzes durch vergleichende rDNS-Sequenzanalyse und Fluoreszenz-*in-situ*-Hybridisierung fünf neuen Entwicklungslinien zugeordnet werden. Vier dieser Entwicklungslinien scheinen weltweit verbreitet zu sein, da nahezu identische Symbionten in Amöbenisolaten aus geographisch weit entfernten Regionen gefunden wurden. Diese neuen Entwicklungslinien weisen interessanterweise höchste Verwandtschaft mit anderen obligat intrazellulären Bakterien auf, darunter bedeutende Krankheitserreger von Mensch und Tier.

Zwei der untersuchten Endosymbionten bilden eine tiefzweigende Entwicklungslinie innerhalb der *Rickettsiales*, eine diverse Gruppe innerhalb der α-Unterklasse der Proteobakterien, die sowohl Krankheitserreger als auch eine Vielzahl an Symbionten verschiedener Insekten umfaßt. Mehrere Endosymbionten, für die der Gattungsname „*Candidatus* Procabacter“ vorgeschlagen wurde, gehören einer neuen Entwicklungslinie innerhalb der β-Unterklasse der Proteobakterien an. Ein weiterer Endosymbiont wurde als neues Mitglied des Phylums *Cytophaga-Flavobacterium-Bacteroides* identifiziert und erhielt den vorläufigen Namen „*Candidatus* Amoebophilus asiaticus“.

Eine Gruppe obligater Endosymbionten der Acanthamöben ist mit dem Paramecien-Endosymbionten *Caedibacter caryophilus* verwandt und wurde vorläufig als „*Candidatus*

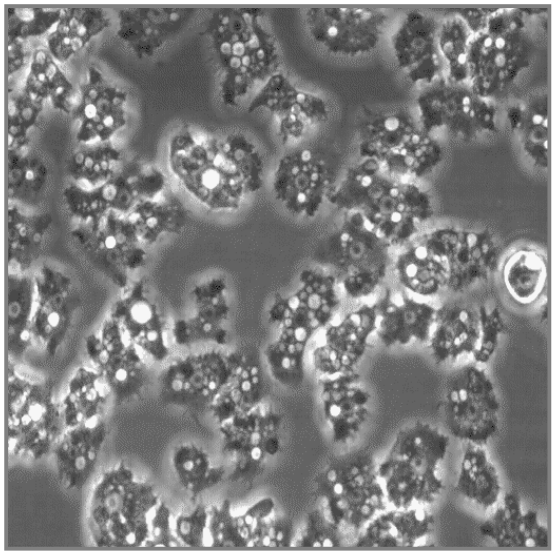
Paracaedibacter“ klassifiziert. Ein Vergleich der Abstammungsgeschichte dieser Symbionten mit der ihrer Wirtszellen zeigte, dass bei der Entwicklung dieser Symbiose Koevolution eine Rolle gespielt haben könnte.

Eine Reihe coccoider Endsymbionten gehört den neuen Gattungen *Neochlamydia* und *Parachlamydia* innerhalb der medizinisch bedeutenden Ordnung *Chlamydiales* an. Weiterführende Untersuchungen mittels eines für diese neuartigen Chlamydien spezifischen PCR-Tests zeigten, dass die Diversität und Verbreitung dieser obligate intrazellulären Bakterien in der Umwelt bislang weitgehend übersehen wurde.

Verschiedene Untersuchungen legen die Existenz spezifischer Wechselwirkungen zwischen frei lebenden Amöben und ihren symbiontischen Bakterien nahe. So läßt der Nachweis von für nicht-mitochondrielle ATP/ADP Transporterproteine codierenden Genen in einigen der hier untersuchten Symbionten vermuten, dass diese Symbionten ein Leben als Energieparasiten führen können - eine mögliche Ursache für die beobachtete Abhängigkeit der Bakterien von ihren Wirtszellen.

Die Untersuchung der Symbiose zwischen frei lebenden Amöben und ihren intrazellulären, bakteriellen Symbionten wird zu einem besseren Verständnis der Anpassung von Bakterien an intrazelluläres Überleben in phagozytischen, eukaryontischen Zellen führen. Möglicherweise stellten diese Adaptationen - lange vor der Entwicklung des Menschen - die ersten Schritte in der Evolution humanpathogener Mikroorganismen dar.

Appendix



Front:
Axenic *Acanthamoeba* culture, phase contrast image.

Dasein im Verborgenen

Bakterien, die in Acanthamöben leben

MATTHIAS HORN | MICHAEL WAGNER

Frei lebende Amöben der Gattung Acanthamoeba stehen in vielfältigen Wechselbeziehungen mit Bakterien: Acanthamöben ernähren sich beispielsweise von Mikroorganismen oder dienen als „Vermehrungsvehikel" für humanpathogene Bakterien. Einige Acanthamöben beherbergen in ihrem Zellinneren Bakterien, mit denen sie stabile Lebensgemeinschaften eingehen. Erst der Einsatz moderner molekularbiologischer Techniken führte zur Entdeckung bisher unbekannter Bakterien – die nicht nur Protozoen, sondern möglicherweise auch den Menschen infizieren.

Acanthamöben sind weltweit verbreitete, frei lebende Protozoen (bewegliche, zellwandlose Einzeller), die sich von Bakterien, Hefen und Pilzen ernähren. Sie können aus Erde, Staub, Süßwasser, Salzwasser und Trinkwasser isoliert werden. Ihr zweiphasiger Lebenszyklus (Abbildung 1) ermöglicht ein Überdauern ungünstiger Lebensbedingungen: Als Cysten sind sie resistent gegen Trockenheit, Hitze, Strahlung und viele antibiotische Agenzien. Das Trophozoiten-Stadium dagegen dient der vegetativen Vermehrung.

Acanthamöben sind Räuber und beeinflussen die Größe und Zusammensetzung mikrobieller Lebensgemeinschaften im Boden. Darüber hinaus gelten sie auch als potentiell humanpathogen: Beisielweise können Acanthamöben durch ungenügend gereinigte Kontaktlinsen ins Auge gelangen und dort eine schwere Hornhautentzündung (Keratitis) hervorrufen (Abbildung 2). Acanthamöben verursachen zudem bei immuninsuffizienten Patienten eine spezielle Form der Gehirnentzündung – die granulomatöse

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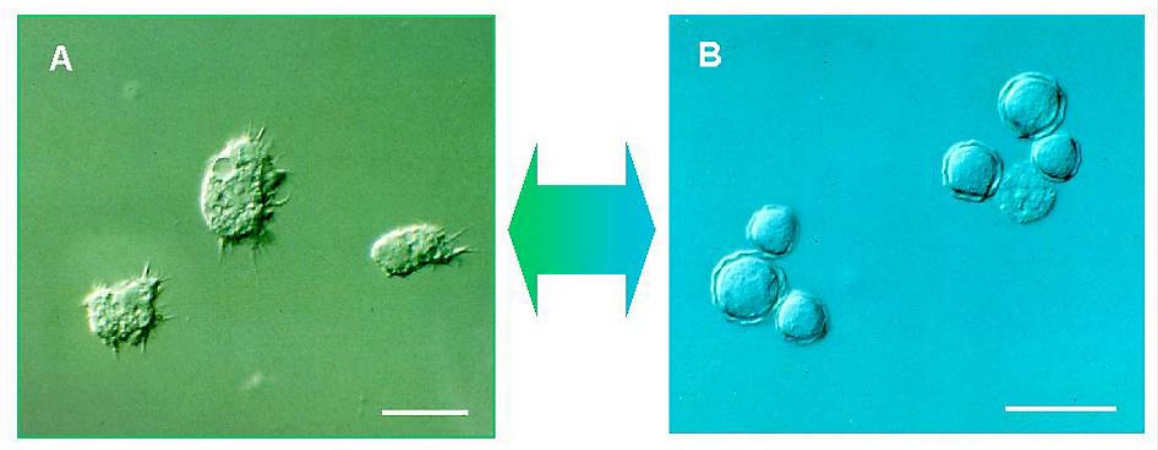

Abbildung 1. Der zweiphasige Lebenszyklus der Acanthamöben. Die Trophozoiten (A) der Acanthamöben besitzen einen aktiven Stoffwechsel und vermehren sich durch Zweiteilung. Ungünstige Umweltbedingungen induzieren die Bildung von doppelwandigen, widerstandsfähigeren Cysten (B), die einen stark eingeschränkten Stoffwechsel aufweisen. (Der Balken entspricht 20 µm.)

Amöbenenzephalitis (GAE, Abbildung 3) – und lösen im Extremfall systemische Infektionen aus. Während gegen die meist letale GAE keine effiziente Therapie zur Verfügung steht, kann die Acanthamöben-Keratitis bei rechtzeitiger Diagnose erfolgreich mit Antibiotika (beispielsweise mit Neomycin) behandelt werden.

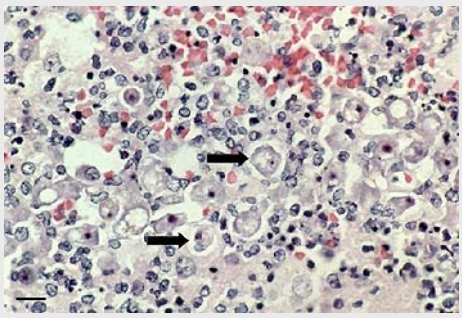

Abbildung 3. Granulomatöse Amöbenencephalitis (GAE). Insbesondere bei immuninsuffizienten und immunsupprimierten Patienten können Acanthamöben systemische Infektionen sowie eine schwere Entzündung des Gehirns – die so genannte granulomatöse Amöbenencephalitis – verursachen. Hier dargestellt ist ein gefärbtes Hirngewebepräparat, in dem deutlich die Trophozoiten der Acanthamöben zu erkennen sind (Pfeile).

Bereits 1956 beobachtete Drozanski Bakterien, die sich innerhalb von Acanthamöben vermehren können [4]. Inzwischen ist bekannt, dass das Vorkommen von intrazellulären Bakterien in Acanthamöben weit verbreitet ist.

Acanthamöben übertragen humanpathogene Bakterien

Acanthamöben ernähren sich von Mikroorganismen. Einige humanpathogenen Bakterien nutzen diese Tatsache allerdings für ihre eigenen Zwecke indem sie die Acanthamöben als Transport- und Vermehrungsvehikel verwenden [11]: Die Acanthamöben nehmen diese pathogenen Bakterien genauso wie alle anderen „Futter-

Mikroorganismen“ durch Phagozytose auf. Die pathogenen Bakterien sind jedoch in der Lage, sich dem Verdauungsmechanismus der Acanthamöben zu entziehen und sich innerhalb der Amöbenzelle zu vermehren. Dies führt in der Regel zur Zerstörung der Amöbe und somit zur Freisetzung der intrazellulären Bakterien.

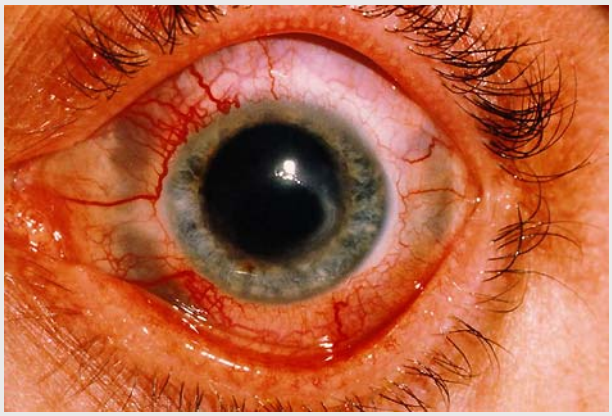

Abbildung 2. Acanthamöben-Keratitis. Acanthamöben können eine schwer wiegende Entzündungen der Hornhaut des Auges hervorrufen. Vorwiegend übertragen durch den Gebrauch ungenügend gereinigter Kontaktlinsen, gelangen die Acanthamöben ins Auge, dringen über kleinste Verletzungen in die Hornhaut ein und können sich dort vermehren. Es bildet sich ein für die Acanthamöben-Keratitis charakteristisches Ringinfiltrat. Unerkannt und folglich unbehandelt führt die Infektion der Hornhaut des Auges mit Acanthamöben zur Erblindung.

Acanthamöben spielen auf diese Weise eine entscheidende Rolle bei der Infektion des Menschen – beispielsweise mit *Legionella pneumophila*, dem Erreger der so genannten Legionärskrankheit. Die Vermehrung der Legionellen innerhalb der Acanthamöben führt zur Bildung und Freisetzung von Membranvesikeln, die mit Legionellen gefüllt sind. Einer Hypothese von Rowbotham [17] zufolge wird erst durch diesen Mechanismus eine für den Menschen infektiöse Dosis an *Legionella pneumophila* erreicht. Zusätzlich konnte in Laborexperimenten gezeigt werden, dass Legionellen, die sich zuvor in Acanthamöben vermehrt hatten, aus bisher nicht geklärten Gründen eine erhöhte Virulenz gegenüber humanen Epithelzellen aufweisen.

Aufgrund dieser Erkenntnisse gelten Acanthamöben, die feuchte Umgebungen als Habitat bevorzugen, heute als Umweltreservoir und natürliche Wirte von Legionellen. Die ausgeprägte Resistenz der Amöbencysten gegenüber Chlor und anderen antibakteriellen Substanzen bietet den Legionellen dabei zusätzlichen Schutz und könnte so wesentlich zu Ausbrüchen von Atemwegserkrankungen beitragen, die durch Legionellen verursacht werden [6]. Die Tatsache, dass Acanthamöben dem Erreger der Legionärskrankheit als Wirt dienen, macht man sich heute auch in der medizinischen Diagnostik zu Nutze: Klinisches Probenmaterial wird mit Acanthamöben inkubiert, anschließend werden die Amöben auf intrazelluläre Legionellen untersucht.

Neben Legionellen können sich auch eine Reihe anderer humanpathogener Bakterien, wie Escherichia coli H157/O7 (auch bekannt als enterohämorrhagische *E. coli*, EHEC), *Chlamydia pneumophila*, *Listeria monocytogenes*, *Burkholderia cepacia* und *Mycobacterium avium* in Acanthamöben vermehren. Für die meisten dieser Bakterien ist jedoch noch nicht geklärt, welche Rolle diese Fähigkeit für ihr Überleben in der Umwelt und bei der Infektion des Menschen spielt.

Acanthamöben besitzen bakterielle Endosymbionten

Acanthamöben können nicht nur eine zeitlich begrenzte Assoziation mit humanpathogenen Bakterien eingehen, sondern auch verschiedenen Bakterien dauerhaft als Wirt dienen. Ein Viertel der aus klinischen Proben oder aus der Umwelt isolierten Acanthamöben beherbergen solche bakteriellen Endosymbionten [7]. Diese intrazellulären Bakterien können mit den zurzeit zur Verfügung stehenden Kultivierungsmethoden nicht ohne ihre Wirtszellen im Labor vermehrt werden und gelten folglich als obligate Endosymbionten. Einfache Färbemethoden wie Giemsa-, Gram- oder DAPI-Färbung sowie elektronenmikroskopische Untersuchungen zeigen, dass diese Bakterien einen Großteil der Amöbenzelle ausfüllen können (Abbildung 4). Neben Gram-negativen, stäbchenförmigen Bakterien wurden auch Gram-negative Kokken in verschiedenen Acanthamöben-Isolaten beobachtet. Die Identität dieser Bakterien konnte jedoch lange Zeit nicht aufgeklärt werden, da eine traditionelle Identifizierung und Klassifizierung anhand physiologischer Merkmale aufgrund ihrer obligat intrazellulären Lebensweise nicht möglich war.

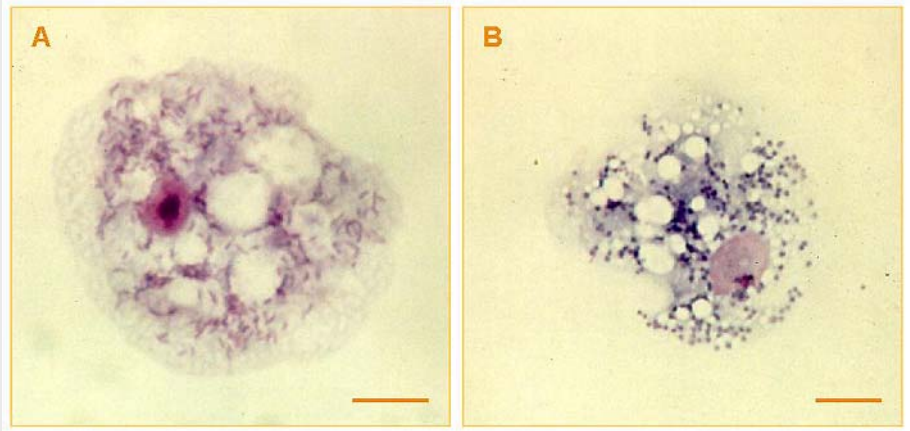

Abbildung 4. Visualisierung der bakteriellen Endocytobioten der Acanthamöben. Die intrazellulären Endosymbionten der Acanthamöben können durch einfache Färbemethoden wie der Giemsa-Färbung sichtbar gemacht werden. Dabei sind stäbchenförmige Endosymbionten (A) sowie kokkoide Endosymbionten (B) zu erkennen. (Der Balken entspricht 5 µm.)

Dies änderte sich erst mit der Einführung der molekularen Taxonomie in der mikrobiellen Systematik: Basierend auf vergleichender Sequenzanalyse von Makromolekülen, die in allen Lebewesen vorkommen, werden hierbei Aussagen über die genetische Verwandtschaft der Bakterien abgeleitet und ihre Evolutionsgeschichte (Phylogenie) rekonstruiert. Heute wird hierzu üblicherweise die 16S ribosomale Ribonukleinsäure (16S-rRNS), die Teil der kleinen Untereinheit des bakteriellen Ribosoms (Ort der Proteinbiosynthese) ist, als phylogenetischer Marker verwendet [19]. Auch bei nicht kultivierbaren Bakterien lassen sich die für die 16S-rRNS kodierenden Gene mittels der Polymerase-Ketten-Reaktion (PCR) vervielfältigen (amplifizieren). Anschließend werden die amplifizierten Gene durch die Klonierung in *Escherichia coli* vereinzelt, sequenziert und phylogenetisch analysiert. Somit kann die Position vorher unbekannter Bakterien im Stammbaum des Lebens bestimmt werden. Mit Hilfe der so gewonnen Daten können für den untersuchten Mikroorganismus spezifische, gegen die 16S-rRNS gerichtete Gen-Sonden entworfen werden. Nach Markierung dieser kurzen, künstlich hergestellten DNS-Stücke mit einem Fluoreszenzfarbstoff ist ein kultivierungsunabhängiger Nachweis der Bakterien *in situ* (das heißt vor Ort in der untersuchten Probe) mittels Fluoreszenz-*in-situ*-Hybridisierung (FISH) möglich (Abbildung 5). Durch Verwendung dieses molekularbiologischen Methodenarsenals gelang es im Lauf der letzten Jahre, die Identität einer Reihe obligater Endosymbionten von Acanthamöben zu entschlüsseln (Abbildung 6).

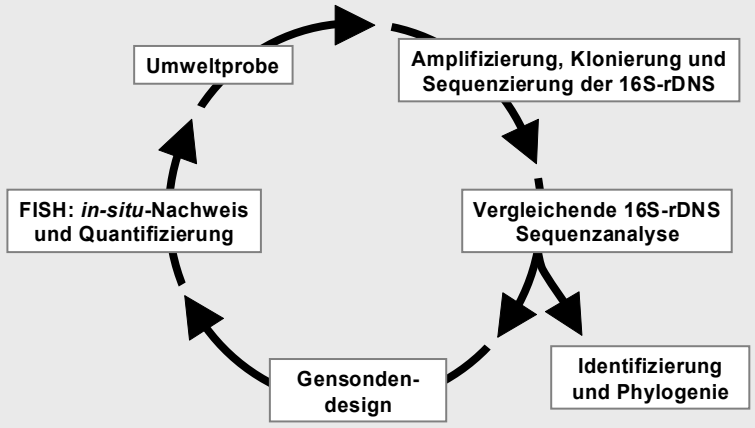

Abbildung 5. Schematische Darstellung der kultivierungsunabhängigen Untersuchung von Mikroorganismen. Ausgehend von der Umweltprobe wird eine 16S-rDNS-Genbank erstellt. Die vergleichende Analyse der darin enthaltenen Sequenzen mit einer umfassenden 16S-rDNS-Datenbank erlaubt die phylogenetische Charakterisierung der in der Umweltprobe vorhandenen Organismen. Basierend auf diesen Daten können anschließend 16S-rRNS-gerichtete Gensonden entworfen werden. Deren Einsatz in der Fluoreszenz-*in-situ*-Hybridisierung (FISH) ermöglicht schließlich den spezifischen Nachweis der Bakterien direkt in der Umweltprobe.

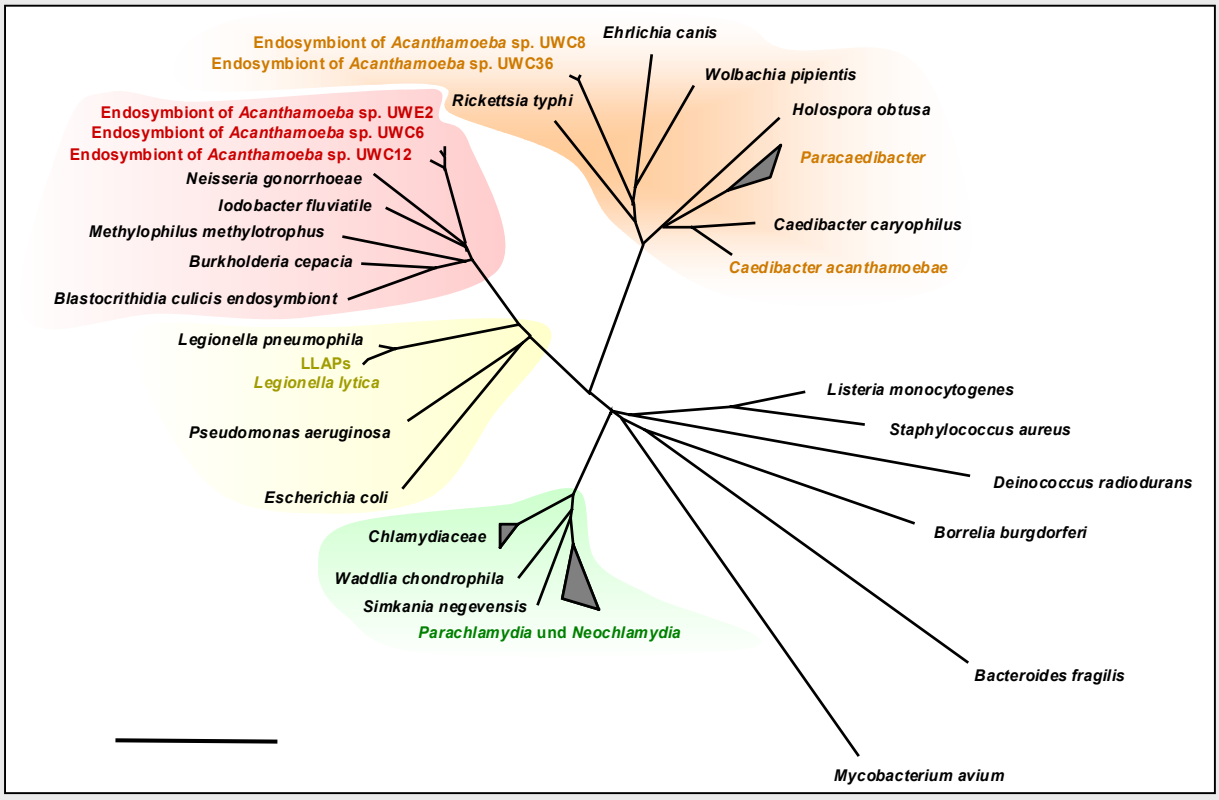

Abbildung 7. Stammbaum der intrazellulären Bakterien der Acanthamöben. Vier der heute bekannten Endosymbiontengruppen gehören zur Klasse der Proteobakterien. Während die „*Legionella* like amoebal pathogens“ (LLAP) Mitglieder der γ-Unterklasse (gelb unterlegt) sind, gehören die Rickettsien-verwandten Endosymbionten sowie *Caedibacter acanthamoebae*, *Paracaedibacter acanthamoebae* und *Paracaedibacter symbiosus* der α-Unterklasse an (orange unterlegt). Eine weitere Gruppe von Endosymbionten konnte der β-Unterklasse (rot unterlegt) zugeordnet werden. Die fünfte Gruppe bildet innerhalb der *Chlamydiales* (grün unterlegt) eine eigene Entwicklungslinie. (Der Balken entspricht 10 % geschätzter phylogenetischer Distanz.)

Legionellen-ähnliche Endoparasiten

Bereits im Jahr 1956 hatte Drozanski Gram-negative, stäbchenförmige Bakterien in Amöben entdeckt, die aus dem Boden isoliert worden waren. Diese Bakterien vermehrten sich intrazellulär in ihren Wirtszellen und führten schließlich zur Zerstörung der Amöbenzelle. Aufgrund des parasitischen Verhaltens dieser Endocytobionten und ihrer morphologischen Ähnlichkeit zu Bakterien der Gattung *Legionella* wurden sie als „*Legionella* like amoebal pathogens“ (LLAP) bezeichnet. Heute sind mehr als ein Dutzend verschiedene LLAPs bekannt, die neben Acanthamöben auch andere frei lebende Amöben als Wirtszellen nutzen können.

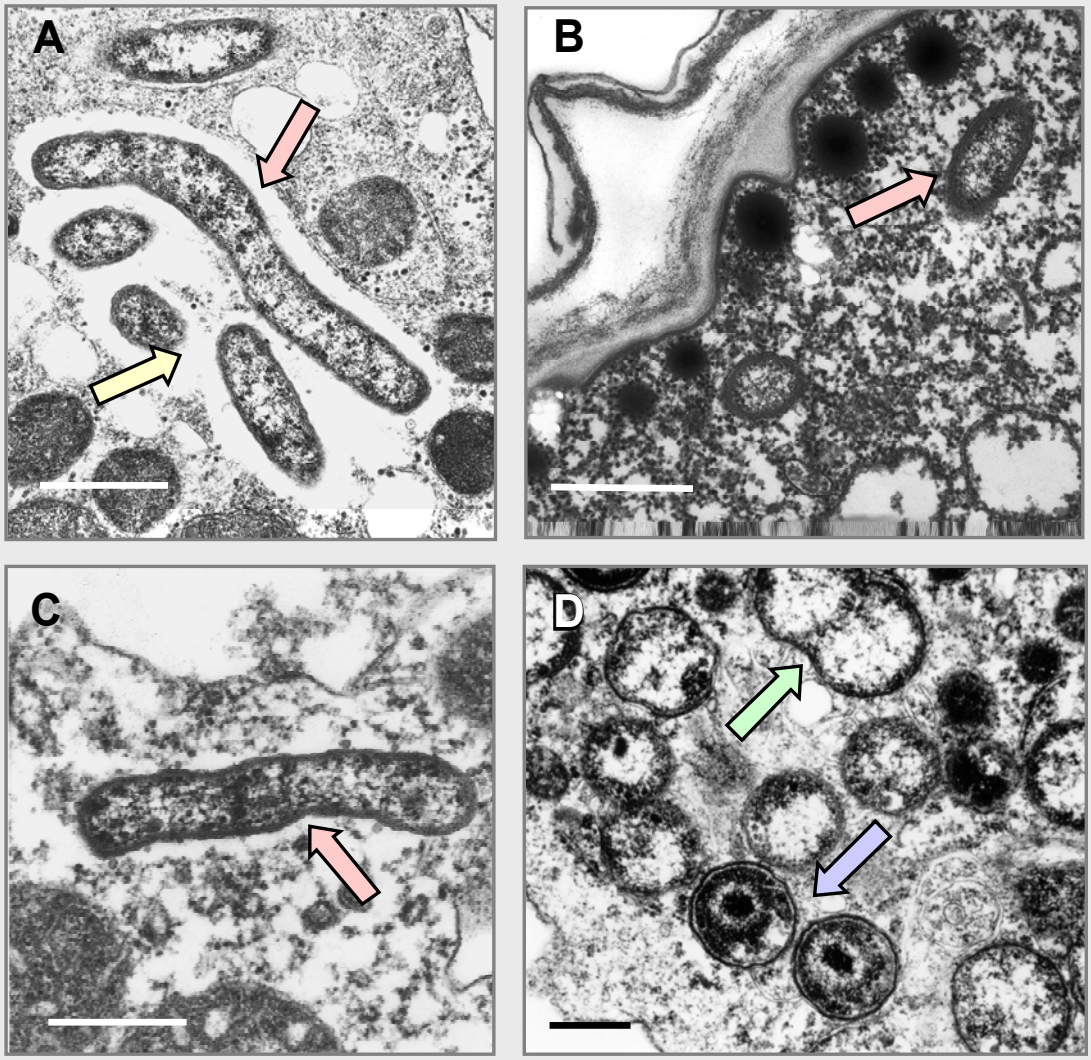

Abbildung 8. Elektronenmikroskopische Untersuchungen zeigen die intracytoplasmatische Lokalisation der (A) Rickettsien-verwandten Endocyobionten, (B) Symbionten der Gattung *Procabacter* und (C) von *Paracaedibacter acanthamoebae* (Bakterien, rote Pfeile; zum Teil umgeben von einer Schleimhülle, gelber Pfeil). Die Chlamydien-verwandten Symbionten (D) hingegen sind von einer Membran umgeben und treten in zwei verschiedenen Entwicklungsstadien auf, die den Retikularkörperchen (grüner Pfeil) beziehungsweise den Elementarkörperchen (blauer Pfeil) der Chlamydien ähnlich sind. (Der Balken entspricht 0,5 µm.)

Erst knapp 40 Jahre nach der ersten Beschreibung dieser Mikroorganismen konnte mit Hilfe von vergleichenden 16S-rRNS-Analysen gezeigt werden, dass die LLAPs tatsächlich zur Gattung *Legionella* gehören [18]. Der von Drozanski beschriebene LLAP trägt heute den Namen Legionella lytica (Abbildung 7). Während LLAPs lange Zeit als nicht kultivierbar galten, gelang deren Anzucht in jüngster Zeit erstmals auch außerhalb ihrer Wirtszellen durch Modifikation einer Methode zur Kultivierung von Legionellen [1].

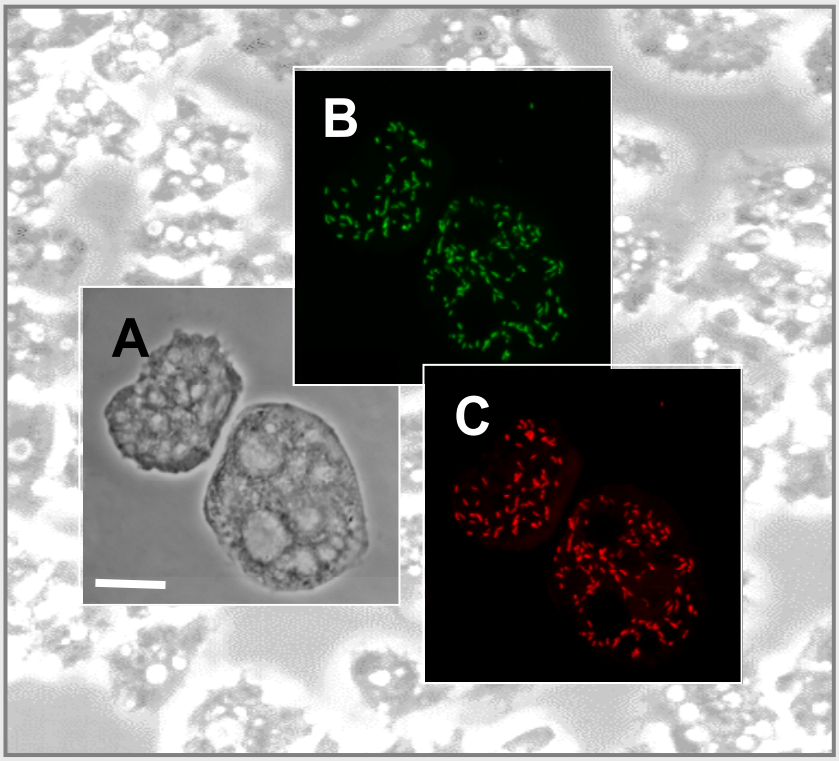

Abbildung 6. *In-situ*-Identifizierung der Endosymbionten der Acanthamöben. Der Nachweis der Endosymbionten der Acanthamöben mit spezifischen 16S-rRNS-gerichteten Gensonden mittels FISH ermöglicht eine Visualisierung und Identifizierung der Endosymbionten direkt in der Amöbenzelle – hier gezeigt für *Paracaedibacter acanthamoebae*. Durch die gleichzeitige Verwendung verschiedenfarbig markierter Sonden unterschiedlicher Spezifität – hier einer bakteriellen Sonde in grün (B) und einer für *Paracaedibacter acanthamoebae* spezifischen Sonde in rot (C) – wird deutlich, dass die Amöbenwirtszelle *P. acanthamoebae* als einzigen Endosymbionten beherbergt. (A: Phasenkontrast-aufnahme; B, C: Fluoreszenzaufnahmen des gleichen mikroskopischen Feldes. Der Balken entspricht 10 µm.)

Während Vertreter der Gattung *Legionella* bereits seit langer Zeit als Erreger von Atemwegserkrankungen beim Menschen bekannt sind, deuten neuere Erkenntnisse darauf hin, dass zumindest einige LLAPs ebenfalls humanpathogen sein könnten. So gelang beispielsweise die Isolierung von LLAP-3 aus einer Sputumprobe eines Patienten, der an Lungenentzündung erkrankt war. Die Probe wurde mit Acanthamöben koinkubiert, nachdem alle zuvor durchgeführten Untersuchungen auf herkömmliche Erreger von Atemwegserkrankungen ergebnislos verlaufen waren. Im Blut dieses Patienten wurde eine erhöhte Konzentration an Antikörpern gegen LLAP-3 nachgewiesen. Der Patient wurde

schließlich erfolgreich mit einer üblicherweise bei Legionellen-Infektionen angewendeten Antibiotika-Therapie behandelt [1].

Verschiedene serologische Studien der letzten Jahre ergaben weitere indirekte Hinweise auf eine Beteiligung der LLAPs an der Entstehung von Atemwegserkrankungen beim Menschen. Bis heute fehlen jedoch geeignete diagnostische Methoden für eine routinemäßige Untersuchung klinischen Probenmaterials auf LLAPs. Die Aufklärung der Rolle der LLAPs bei Erkrankungen der Atemwege bleibt darum eine Herausforderung für die Zukunft.

Mit den Rickettsien verwandt

Im Gegensatz zu den LLAPs können die Mehrzahl der Endosymbionten der Acanthamöben nicht außerhalb ihrer Wirtszellen kultiviert werden. Dazu zählen beispielsweise zwei obligate Endosymbionten, die zu der α-Unterklasse der Proteobakterien gehören. Innerhalb dieser Unterklasse sind diese Endosymbionten am nächsten mit Mitgliedern der Ordnung *Rickettsiales* verwandt. Sie bilden innerhalb der *Rickettsiales* jedoch eine eigenständige, vorher unbekannte Entwicklungslinie und weisen eine nur relativ geringe genetische Verwandtschaft mit anderen Vertretern dieser Ordnung auf (Abbildung 7, [9]).

Die Rickettsien-verwandten Endosymbionten der Acanthamöben leben direkt im Cytoplasma der Wirtszelle. Sie sind von einer Schleimhülle umgeben, deren Bedeutung noch nicht geklärt ist (Abbildung 8 A). Alle bisher bekannten Mitglieder der Ordnung *Rickettsiales* sind kleine Gram-negative, stäbchenförmige Bakterien. Sie vermehren sich innerhalb eukaryontischer Zellen und pflegen parasitische bis symbiontische Beziehungen zu ihren Wirten – darunter Protozoen, Insekten und Wirbeltiere. Einige Vertreter der *Rickettsiales* lösen beim Menschen zum Teil schwere, durch Arthropoden übertragene Infektionskrankheiten aus. Ob die in den Acanthamöben vorkommenden neuartigen Vertreter der *Rickettsiales* ebenfalls humanpathogen sind, ist Gegenstand aktueller Untersuchungen.

Koevolution zwischen Endosymbionten und Acanthamöben

Eine andere Gruppe obligater Endosymbionten der Acanthamöben, ähneln morphologisch den Rickettsien-verwandten Endosymbionten. Die fünf Mitglieder dieser Gruppe sind ebenfalls Gram-negative, stäbchenförmige Bakterien und leben direkt im

Cytoplasma der Acanthamöben (Abbildung 8 C). Tatsächlich bestätigte die phylogenetische Untersuchung dieser Bakterien, dass sie ebenfalls den α-Proteobakterien zugeordnet werden können (Abbildung 7). Innerhalb dieser Unterklasse sind diese Endosymbionten jedoch am engsten mit *Caedibacter caryophilus* verwandt – einem obligaten Symbionten des „Pantoffeltierchens“ *Paramecium caudatum*. *Caedibacter caryophilus* lebt im Makronukleus dieser weit verbreiteten Protozoen [12]. Somit nutzen sowohl *C. caryophilus* als auch die *C. caryophilus*-verwandten Symbionten der Acanthamöben – für die kürzlich die Namen *Paracaedibacter* sp. beziehungsweise *Odyssella thessalonicensis* vorgeschlagen wurden – eine ähnliche ökologische Nische.

Grundsätzlich stellt sich bei allen symbiontischen Beziehungen die Frage nach Stabilität und Spezifität der Interaktionen zwischen Symbiont und Wirt: Je angepasster die Lebensweisen von Symbiont und Wirt aneinander sind, desto größer wird auch die gegenseitige Abhängigkeit sein. Über einen längeren Zeitraum der Evolution gesehen, kann diese Abhängigkeit dazu führen, dass Symbiont und Wirt sich gegenseitig beeinflussen und gemeinsam entwickeln – ein Prozess, der als Koevolution bezeichnet wird. Unter Koevolution versteht man demzufolge eine reziproke (gegenseitige) evolutionäre Änderung der Eigenschaften (respektive der Gene) zweier oder mehrerer interagierender Lebensformen (Arten oder Populationen).

Der Begriff der Koevolution wurde bereits 1964 von Ehrlich und Raven im Rahmen einer Untersuchung der Diversität von Schmetterlingen und deren Futter-Pflanzen geprägt. Bis heute konnte Koevolution in so unterschiedlichen Bereichen wie bei der Entwicklung des

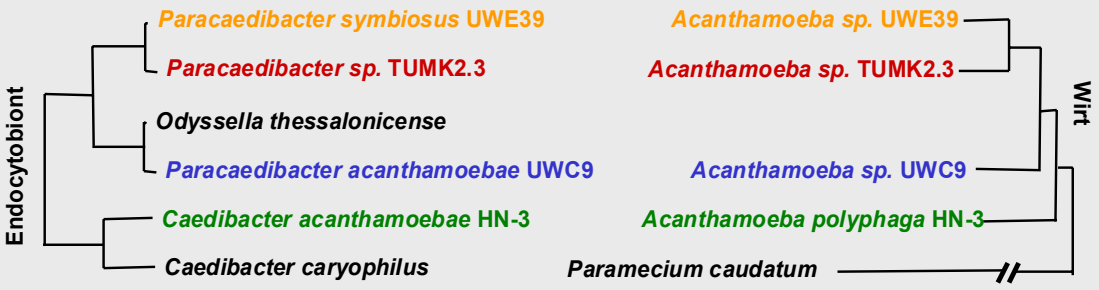

Abbildung 9. Koevolution der *Caedibacter*-verwandten Endosymbionten mit ihren Acanthamöben-Wirten. Die Gegenüberstellung der Stammbäume der Wirtszellen (rechts) und deren Endosymbionten (links) liefert Hinweise auf eine gemeinsame Entwicklungsgeschichte (Koevolution) von Wirt und Symbiont.

Mutualismus zwischen Ameisen der Gattung *Pseudomyrmex* und Akazien, sowie zwischen Leuchtbakterien und ihren Wirten nachgewiesen werden.

Beruht auch die Entwicklung der Symbiose zwischen Acanthamöben und ihren Endosymbionten auf dem Mechanismus der Koevolution? Um diese Frage zu beantworten, bietet sich zunächst ein Vergleich der Phylogenie der Symbionten und ihrer Wirte an. Bei der Gegenüberstellung der Stammbäume der *Caedibacter*-verwandten Symbionten und der entsprechenden Amöbenwirtszellen fällt tatsächlich eine erstaunliche Parallelität auf (Abbildung 9), wie man sie beim Auftreten von Koevolution erwarten würde. Die phylogenetische Analyse legt zudem nahe, dass eine horizontale Übertragung von einem Vorläufer des Acanthamöben-Symbionten *Caedibacter acanthamoebae* HN-3 auf einen Urahn von *Paramecium caudatum* stattgefunden hat. Obwohl diese Analyse eine wesentliche Komponente der Untersuchung einer Lebensgemeinschaft auf Koevolution darstellt, ist sie dennoch kein ausreichender Beleg für deren Existenz. Letztlich müsste gezeigt werden, dass es während der Entwicklung der Symbionten in den Amöbenwirten tatsächlich eine reziproke Beeinflussung beider Partner gegeben hat. Diese gegenseitige Abhängigkeit während der Entwicklung einer Symbiose könnte beispielsweise durch die Untersuchung funktioneller Aspekte der Interaktion zwischen Symbiont und Wirt belegt werden.

Neue Entwicklungslinie innerhalb der β-Proteobakterien

Auch die dritte Gruppe obligater Endosymbionten lebt direkt im Cytoplasma der Acanthamöben. Diese Endosymbionten sind jedoch nicht von einer Schleimhülle umgeben und weisen charakteristische Einlagerungen in der Nähe der Zellpole auf (Abbildung 8 B). Diese Bakterien gehören nicht zur Untergruppe der α-Proteobakterien, sondern bilden eine eigene, vormals unbekannte phylogenetische Entwicklungslinie innerhalb der β-Proteobakterien (Abbildung 7). Ihre Verwandtschaft zu anderen Mitgliedern der β-Proteobakterien ist so gering, dass für diese Endosymbionten die neue Gattung *Procabacter* vorgeschlagen wurde [14]. Die Entdeckung endosymbiontisch lebender β-Proteobakterien verdient besondere Aufmerksamkeit, da in dieser Entwicklungslinie kaum Bakterien mit obligat intrazellulärer Lebensweise bekannt sind.

Neuartige, Chlamydien-verwandte Bakterien

Die bisher erwähnten Endosymbionten der Acanthamöben besitzen alle eine stäbchenförmigen Morphologie und gehören der Klasse der Proteobakterien an. Im Gegensatz dazu ist eine weitere Gruppe von Endosymbionten der Acanthamöben unter dem Lichtmikroskop als kleine, Gram-variable Kokken zu erkennen. Elektronenmikroskopische Untersuchungen dieser kokkoiden Endosymbionten zeigen eine auffällige morphologische Ähnlichkeit mit Chlamydien – medizinisch bedeutsamen, obligat intrazellulären Bakterien, die früher den Viren zugeordnet wurden. Ebenso wie die Chlamydien scheinen diese Endosymbionten zwei Lebensstadien zu besitzen: Neben den vegetativen, der intrazellulären Vermehrung dienenden Retikularkörperchen können so genannte Elementarkörperchen gebildet werden, die speziell an das Überleben in extrazellulärem Milieu angepasst sind (Abbildung 8 D). Wie man heute weiß, sind diese Acanthamöben-Symbionten tatsächlich entfernt mit den klassischen Chlamydien verwandt (Abbildung 7). Sie tragen den Gattungsnamen *Parachlamydia* [2]. Inzwischen ist eine Vielzahl unterschiedlicher Parachlamydien in Acanthamöben nachgewiesen worden (Abbildung 10, [3, 10]). Der selben Entwicklungslinie zuzuordnen ist auch das Bakterium *Neochlamydia hartmannellae* [13], das in der frei lebenden Amöbengattung *Hartmannella* vorkommt sowie die Bakterien *Waddlia chondrophila* und *Simkania negevensis*, die in einem abgestorbenen Rinderfötus, beziehungsweise als bakterielle Kontamination einer humanen Zellkultur erstmals in Erscheinung traten.

Bisher unbekannte Krankheitserreger?

Offenbar scheinen Chlamydien-ähnliche Bakterien in unserer Umwelt wesentlich weiter verbreitet zu sein als bisher angenommen. Zudem wurde die Diversität innerhalb der Ordnung Chlamydiales, die bis vor kurzem aus nur einer Gattung (*Chlamydia*) bestand, bisher dramatisch unterschätzt (Abbildung 10). So ist in naher Zukunft sicherlich mit der Entdeckung und Beschreibung weiterer Chlamydien-verwandter Bakterien zu rechnen. Da alle klassischen Chlamydien ernst zu nehmende Krankheitserreger für den Menschen darstellen, ist dies von ganz besonderer Bedeutung. So ist *Chlamydophila pneumoniae* (früher *Chlamydia pneumoniae*) ein weit verbreiteter Erreger von Atemwegserkrankungen, der in jüngster Zeit auch mit der Entstehung von Arteriosklerose in Zusammenhang gebracht wurde.

Chlamydia trachomatis hingegen zählt zu den am häufigsten durch sexuellen Kontakt übertragenen Bakterien. Neben Geschlechtskrankheiten kann *C. trachomatis* Bindehautentzündungen sowie eine entzündliche Augen-krankheit, das Trachom, auslösen. Vor allem in den Entwicklungsländern stellt das Trachom eine der häufigsten Erblindungsursachen dar. Diese pathogenen Bakterien sind mit den in Protozoen vorkommenden Para- und Neochlamydien verwandt. In diesem Zusammenhang stellt sich die Frage, ob auch diese „Umweltchlamydien" bislang nicht erkannte Krankheitserreger bei Mensch und Tier sind. Die Beantwortung dieser Frage erscheint dringend, da in jüngster Zeit erste Hinweise auf eine Beteiligung von „Umwelt-chlamydien" an Atemweger-krankungen des Menschen gefunden wurden. So ergab die serologische Untersuchung von mehr als 400 Patienten, die an Lungenentzündung unbekannter Ursache erkrankt waren, dass einige von ihnen einen erhöhten Antikörperspiegel gegen Parachlamydien aufwiesen [3]. Zudem konnten mittels eines spezifischen PCR-Nachweisverfahrens Chlamydien-ähnliche 16S-rDNS-Sequenzen in menschlichem Lungengewebe, arteriosklerotischem Gewebe und Blut detektiert werden, die größere Ähnlichkeit zu den „Umwelt-chlamydien" als zu den klassischen Chlamydien aufweisen [16].

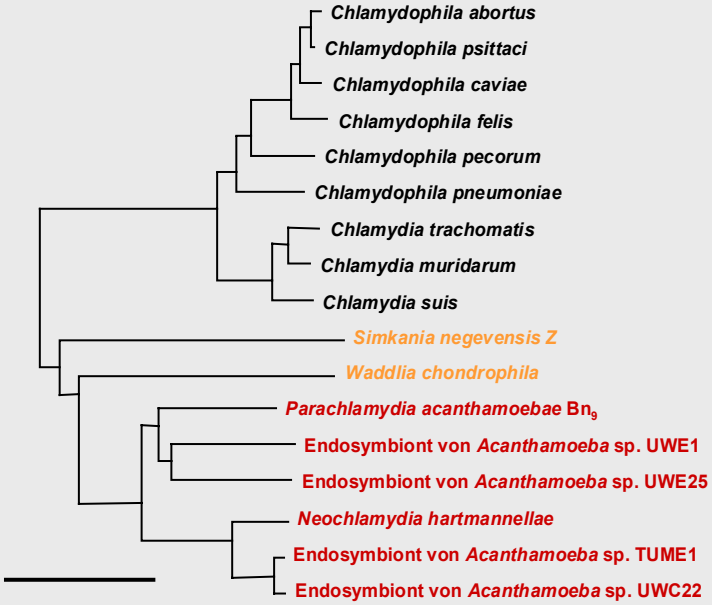

Abbildung 10. Phylogenie der Chlamydien. Alle klassischen Chlamydien (schwarz) sind Krankheitserreger bei Menschen und Tieren. Mit der Entdeckung von Chlamydien-verwandten Mirkoorganismen als Endosymbionten in Acanthamöben wurde erstmals deutlich, dass Mitglieder dieses Phylums auch in der Umwelt zu finden sind (rot). Es gibt erste Indizien, die auf eine Pathogenität dieser Umweltchlamydien hindeuten. (Der Balken entspricht 10 % geschätzter phylogenetischer Distanz.)

In diesem Zusammenhang darf auch der mit den Para- und Neochlamydien verwandte Mikroorganismus *Simkania negevensis* nicht unerwähnt bleiben, der in jüngster Zeit aufgrund serologischer Daten in Zusammenhang mit Bronchitis bei Kleinkindern und Erwachsenen gebracht wurde [15]. Obwohl alle bisherigen Studien nur indirekte Hinweise

auf eine Beteiligung der „Umweltchlamydien“ an der Entstehung von Atemwegserkrankungen liefern, sollten sie ernst genommen werden, da für mehr als die Hälfte aller Pneumonien beim Menschen heute noch kein Erreger ermittelt werden kann. „Umweltchlamydien“ könnten neben Viren für zumindest einen Teil dieser Atemwegsinfektionen verantwortlich sein.

Zusammenfassung und Ausblick

Die molekulare Untersuchung der Lebensgemeinschaften zwischen Acanthamöben und ihren bakteriellen Endosymbionten zeigte, dass sich mindestens vier verschiedene, bislang unbekannte Bakteriengruppen auf das Überleben in diesen Protozoen spezialisiert haben. Die meisten der Endosymbionten scheinen geographisch weit verbreitet zu sein, da sie in Acanthamöben-Isolaten aus verschiedenen Kontinenten nachgewiesen werden konnten. Ob die bis heute identifizierten Endosymbiontengruppen alle die tatsächlich in der Natur vorkommenden Acanthamöben-Symbionten repräsentieren, ist Gegenstand laufender Untersuchungen. Neben der Erfassung der Diversität von Bakterien, die intrazellulär in Acanthamöben vorkommen, werden sich zukünftige Forschungsprojekte unserer Arbeitsgruppe vor allem mit einer eingehenden Charakterisierung der Interaktionen zwischen Acanthamöben und ihren Endosymbionten befassen, über die bislang nur einzelne Beobachtungen vorliegen. So gelingt es in den meisten Fällen nicht, Acanthamöben durch Behandlung mit Antibiotika von ihren bakteriellen Endosymbionten zu befreien. Weiterhin lassen Kreuz-Infektionsversuche vermuten, dass ein spezielles Erkennungssystem für die Spezifität der Bakterien-Amöben-Interaktion sorgt. Dabei steigert interessanterweise die künstliche Infektion endosymbiontenfreier Acanthamöben mit natürlichen Endosymbionten anderer Acanthamöben die Cytopathogenität der Acanthamöben gegenüber humanen Zellkulturen [8]. Letztendlich wird ein funktionelles Verständnis dieser Wechselwirkungen Einblicke in die Genetik der beteiligten Partner erfordern. Hierbei bieten sich vor allem Genom-, Transkriptom- und Proteomstudien an. Dies erscheint besonders interessant, da von derartigen Untersuchungen neue Erkenntnisse über die Anpassung von Bakterien an ein intrazelluläres Überleben in phagozytischen, eukaryontischen Zellen erwartet werden. Möglicherweise stellten diese Adaptationen – lange vor der Entwicklung des Menschen – die ersten Schritte in der Evolution humanpathogener Mikroorganismen dar.

Summary

One fourth of Acanthamoeba isolates studied contain obligate bacterial endosymbionts. These intracellular bacteria have recently been assigned to four different, previously unknown phylogenetic lineages within the *Proteobacteria* and the *Chlamydiales*. The symbiotic association of these amoebae and their bacterial endosymbionts might be a valuable model system for the analysis of bacterial adaptations and mechanisms for intracellular survival. In addition, *Chlamydia*-related amoebal endosymbionts have been implicated as causative agents for respiratory disease suggesting that these protozoa may be sources of new emerging pathogens.

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Glossar

Cyste. Hier: Überdauerungsstadium der Acanthamöben.

Trophozoit. Hier: Vermehrungsfähiges Lebensstadium der Acanthamöben.

DAPI-Färbung. Methode zur Anfärbung von DNS. Der Farbstoff (DAPI, 2-(4-Amidinophenyl)-6-Indolcarbamidin-dihydrochlorid) bindet an die DNS und fluoresziert bei Anregung im UV-Bereich bläulich.

Giemsa-Färbung. Gebräuchliche Methode zur Anfärbung von eukaryontischen Zellen und Gewebeproben mit Hilfe einer Azur-Eosin-Methylenblau-Lösung.

Mutualismus. Wechselbeziehung zwischen zwei Lebewesen, die für beide förderlich, für einen der Partner aber lebensnotwendig ist.

Sputum. Ausgehustetes Bronchialsekret; enthält normalerweise Zellen des Lungengewebes, Staubteilchen und eventuell Mikroorganismen; bei Patienten mit Atemwegerkrankungen makroskopisch verändert.

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1. **Fritsche, T. R., Horn, M., Seyedirashti, S., Gautom, R. K., Schleifer, K.-H. & Wagner, M. (1999).** *In situ* detection of novel bacterial endosymbionts of *Acanthamoeba* spp. phylogenetically related to members of the *Rickettsiales*. *Appl Environ Microbiol* **65**, 206-212.
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2. **Horn, M., Fritsche, T. R., Gautom, R. K., Schleifer, K.-H. & Wagner, M. (1999).** Novel bacterial endosymbionts of *Acanthamoeba* spp. related to the *Paramecium caudatum* symbiont *Caedibacter caryophilus*. *Environ Microbiol* **1**, 357-367.
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4. **Fritsche, T. R., Horn, M., Wagner, M., Herwig, R. P., Schleifer, K.-H. & Gautom, R. K. (2000).** Phylogenetic diversity among geographically dispersed *Chlamydiales* endosymbionts recovered from clinical and environmental isolates of *Acanthamoeba* spp. *Appl Environ Microbiol* **66**, 2613-2619.
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10. **Hu J, Limaye AP, Fritsche TR, Horn M, Juretschko S, & Gautom R. (2001).** Direct detection of *Legionellae* in respiratory tract specimens using fluorescence *in situ* hybridization. In *Legionella* - Proceedings of the 5th International Conference, ASM Press, in press.

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1. **Horn, M.** Phylogenetic affiliations of uncultured bacterial endosymbionts occuring in *Acanthamoeba* spp. Fall Meeting British Columbia Parasitologists, Nanaimo, B.C., Canada, October 1999.

2. **Horn, M., Fritsche, T. R., Michel, R. & Wagner, M.** Obligate bacterial endocytobionts of *Acanthamoeba* spp. Third International Congress on Symbiosis (TICS 2000), Marburg, Germany, August 2000.

3. **Horn, M.** Molecular ecology of free-living amoebae and their bacterial endosymbionts. Molecular Microbial Ecology Workshop, Genoscope, Evry, France, March 2001.

4. **Horn, M., Michel, R., Linner, T., Harzenetter, M., Neuhaus, E., Fritsche, T. R., & Wagner, M.** Obligate bacterial endosymbionts of free-living amoebae: diversity and interactions. IXth International Meeting on the Biology and Pathogenicity of Free-living Amoebae, Paris, France, July 2001.

5. **Horn, M., Fritsche, T. R., & Wagner, M.** Bacterial endosymbionts of free-living amoebae: diversity and interactions. XI International Congress of Protozoology ICOP, Salzburg, Austria, July 2001.

6. **Horn, M., Fritsche, T. R., & Wagner, M.** Bacterial endosymbionts of free-living amoebae: diversity and interactions. 9th International Symposium on Microbial Ecology, Amsterdam, The Netherlands, August 2001.

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1. **Horn, M., Wagner, M., Fritsche, T. R. & Schleifer, K.-H. (1998)**. Phylogenetic studies on Acanthamoeba and nonculturable bacterial endosymbionts using 18S and 16S rDNA sequence analysis. (1998). Jahrestagung der Vereinigung für Allgemeine und Angewandte Mikrobiologie e.V. (VAAM), Frankfurt a. M., Germany, March 1998.

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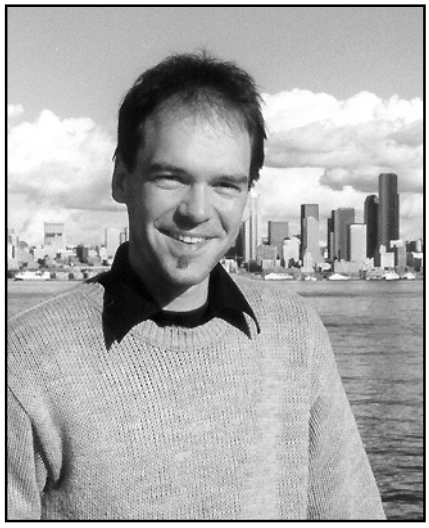
10. **Adamczyk, J., Beier, C., Daims, H., Horn, M., Juretschko, S., Klein, M., Lee, N., Lehner, A., Loy, A., Purkhold, U., Schlatter, B., Schmid, M., Schmid, M., Walcher, M., and Wagner, M. (2000).** Molecular microbial ecology - exploring the unseen majority on planet earth. BioLog 2000, 2. Münchner Kongress für Biowissenschaften, München, Germany, November 2000.

Curriculum Vitae



19.11.1971	born in Karl-Marx-Stadt, Germany.
1979 - 1983	Grundschule, Fürstenfeldbruck, Germany
1983 - 1991	Viscardi-Gymnasium, Fürstenfeld-bruck, Germany. Abitur 1991.
1992 – 1998	Technische Universität München, to major in Microbiology. Dipl.-Biol. Univ. 1998
1998 – 2001	Lehrstuhl für Mikrobiologie, Technische Universität München, Germany, Ph.D. student.

Teaching activities

19.10.1998 - 23.10.1998: International FISH Course 98. Seminar at the Lehrstuhl für Mikrobiologie, Technischen Universität München, Germany.

22.02.2000: *In situ* analysis of complex microbial communities. Lecture at the „Adhesion-Agregation des Microorganismes - 8eme Seminaire“, Faculté de Pharmacie, Nancy, France.

06.10.2000: Gentechnik. Seminar for 11th grade, Gymnasium Laufen, Germany.

14./15.03.2001: Molecular microbial ecology: the rRNA approach and beyond. From phylogeny to molecular diagnostics of cultured and non-cultured bacteria. Workshop, Genoscope, Evry, France.

Awards

1998: DGMH Grant for a research visit in Seattle, WA, U.S.A., Stiftung der Deutschen Gesellschaft für Hygiene und Mikrobiologie e.V. (DGHM).

2001: FEMS Young Scientist Grant, Federation of European Microbiological Societies.

Membership in scientific organizations

since 1998: Vereinigung für Allgemeine und Angewandte Mikrobiologie e.V. (VAAM)

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