Applied and Environmental Microbiology, Sept. 2008, p. 5822–5831 Vol. 74, No. 18
0099-2240/08/$08.00+0 doi:10.1128/AEM.01093-08

Diversity of Bacterial Endosymbionts of Environmental *Acanthamoeba* Isolates▽†

Stephan Schmitz-Esser, Elena R. Toenshoff, Susanne Haider, Eva Heinz, Verena M. Hoenninger, Michael Wagner, and Matthias Horn*

Department of Microbial Ecology, University of Vienna, 1090 Vienna, Austria

Received 15 May 2008/Accepted 11 July 2008

Free-living amoebae are frequent hosts for bacterial endosymbionts. In this study, the symbionts of eight novel environmental *Acanthamoeba* strains isolated from different locations worldwide were characterized. Phylogenetic analysis revealed that they were related to one of four evolutionary lineages of amoeba symbionts recognized previously. This study provides evidence for the existence of only a small number of phylogenetically well-separated groups of obligate intracellular endosymbionts of acanthamoebae with global distribution.

Free-living amoebae are widespread protozoa, including phylogenetically diverse genera like *Acanthamoeba*, *Hartmanella*, and *Naegleria*. They occur in various habitats, including soil, water, and the air (37, 47), and in many engineered environments, like water supplies and air-conditioning units (42). Free-living amoebae are opportunistic pathogens, causing keratitis or encephalitis, and important predators of prokaryotic and eukaryotic microorganisms with a great influence on microbial community composition (37, 47). By grazing on microbes, free-living amoebae also contribute to plant growth, soil mineralization, and nutrient cycles (9, 11, 47).

Apart from being a food source of free-living amoebae, some bacteria are able to survive phagocytosis and multiply within amoebae. The association between these bacteria and their amoeba hosts can be either transient (in the case of facultative intracellular bacteria) or stable (in the case of obligate intracellular bacteria). A wide range of well-known bacterial and eukaryotic pathogens are able to infect amoebae and exploit them for multiplication (25, 33, 43). Free-living amoebae may, thus, serve as environmental reservoirs and vectors for the transmission of pathogenic bacteria to humans (2, 5) and might represent evolutionary training grounds facilitating the adaptation of bacteria to survival within eukaryotic cells (15, 26, 29, 43, 44).

Stable associations of bacteria with amoebae leading to long-term symbiotic interactions were described for members of four evolutionary lineages within the *Alphaproteobacteria* (7, 20, 30, 57), the *Betaproteobacteria* (27, 31), the *Bacteroidetes* (32, 57), and the *Chlamydiae* (3, 8, 21, 27, 34). The different lifestyles of these obligate intracellular bacteria—either directly in the amoeba cytoplasm or enclosed in host-derived vacuoles—suggest fundamentally different mechanisms of host-cell interactions. However, with the exception of chlamydia-related amoeba symbionts (22–24, 28, 29), our knowledge about obligate intracellular symbionts of amoebae is still scarce. In this study, novel *Acanthamoeba* strains and their symbionts were analyzed.

In total, 10 different amoeba strains were isolated from soil and lake sediment samples from Austria, Tunisia, and Dominica, using nonnutrient agar plates seeded with live or heat-inactivated *Escherichia coli* or *Saccharomyces cerevisiae* as described previously (Table 1) (27). Amoeba isolates were adapted to axenic culture and tentatively classified as *Acanthamoeba* spp. based on morphological criteria characteristic for this genus (cell size, contractile vacuole, needle-like pseudopodia, and appearance of the nucleus) (45). Out of these 10 isolates, 8 contained intracellular bacteria as revealed by staining with the fluorescent DNA dye 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI). Isolates EI1, EI2, and EI6 harbored coccoid bacteria, whereas isolates EI3, EI4, EI5, 5a2, and EIDS3 contained rod-shaped bacteria (Table 1). The two *Acanthamoeba* isolates without intracellular bacteria were not analyzed further.

Simultaneous isolation of DNA from amoeba hosts and their bacterial endosymbionts was performed as described previously (27). The 18S rRNA genes were amplified using primers targeting conserved 18S rRNA gene regions (see Table S1 in the supplemental material), cloned using the Topo TA kit (Invitrogen Life Technologies), and sequenced on an ABI 3130 XL genetic analyzer using the BigDye Terminator kit v3.1. For each isolate, three to six clones were analyzed and found to be identical (99.8 to 100% sequence similarity). The software Pintail (4) indicated that the obtained sequences were not chimeric.

All 18S rRNA sequences showed highest sequence similarity with members of the genus *Acanthamoeba* (96.6 to 99.7%); similarity values to other genera were below 90% (Table 1). Using the 95% similarity threshold value for the definition of *Acanthamoeba* 18S rRNA sequence types (51), the *Acanthamoeba* sp. isolates EI1, EI2, EI3, 5a2, EIDS3, and EI6 could be assigned to the sequence type T4, and *Acanthamoeba* sp. isolates EI4 and EI5 could be assigned to sequence type T2. Consistently, phylogenetic analysis using the ARB software package (41) revealed well-supported relationships of the new amoeba isolates with the genus *Acanthamoeba* and the geno-

* Corresponding author. Mailing address: Department of Microbial Ecology, University of Vienna, Althanstr. 14, 1090 Vienna, Austria. Phone: 43 1 4277 54393. Fax: 43 1 4277 54389. E-mail: horn@microbial-ecology.net.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 18 July 2008.

TABLE 1. Amoeba isolates and their symbionts analyzed in this study

Acanthamoeba sp. isolate and ATCC no.	Source	Growth medium and optimal temp	16S rRNA GenBank accession no. of symbiont	18S rRNA GenBank accession no. of amoeba host	Highest 16S rRNA sequence similarity to[a]:	Highest 18S rRNA sequence similarity to[a]:
EI1 PRA-227	Soil; Vienna, Austria	TSY, 20°C	AM408788	AM408796	*Parachlamydia* sp. isolate Hall's coccus (99.5%; AF366365)	*Acanthamoeba castellanii* (99.6%; M13435)
EI2 PRA-226	Soil; lower Austria	TSY, 20°C	AM408789	AM408797	*Protochlamydia amoebophila* UWE25 (98.9%; AF083615)	*Acanthamoeba castellanii* 4CL (98.9%; AF260724)
EI3 PRA-225	Rainforest soil; Dominica	TSY, 20°C	AM408790	AM408798	"*Candidatus* Paracaedibacter acanthamoebae" (99.7%; AF132137)	*Acanthamoeba* sp. KA/MSS7 (99.6%; AY173015)
EI4 PRA-224	Garden soil; Vienna, Austria	PYG, 20°C	AM408791	AM408799	"*Candidatus* Amoebophilus asiaticus" TUMSJ-321 (98.3%; AF366581)	*Acanthamoeba polyphaga* OX-1 (96.6%), AF019051
EI5 PRA-223	Desert sand, Matmata, Tunisia	TSY, 20°C	AM408792	AM408800	"*Candidatus* Procabacter acanthamoebae" Page23 (97.3%; AF177425)	*Acanthamoeba pustulosa* (98.0%; AF019050)
EI6 PRA-222	Soil; Schneeberg, lower Austria	TSY, 20°C	AM408793	AM408801	*Parachlamydia* sp. isolate UV-7 (98.9%; AJ715410)	*Acanthamoeba castellanii* (99.3%; M13435)
EIDS3 PRA-221	Alkaline lake sediment; Darscho Lacke, Burgenland, Austria	PYG, 30°C	AM408794	AM408802	"*Candidatus* Amoebophilus asiaticus" TUMSJ-321 (99%; AF366581)	*Acanthamoeba* sp. isolate MZOR (99.7%; DQ103890)
5a2 PRA-228	Lake sediment; Lake Neusiedl, Burgenland, Austria	PYG, 30°C	AM408795	AM408803	"*Candidatus* Amoebophilus asiaticus" TUMSJ-321 (99.3%; AF366581)	*Acanthamoeba royreba* Oak Ridge ATCC 30884 (98.8%; U07417)

[a] Data within parentheses are sequence similarities and GenBank accession numbers, respectively.

types T2 and T4 (Fig. 1). *Acanthamoeba* sequence types correlate roughly with morphological groupings and also seem to be in concordance with antigen profiles (38). Bacterial symbionts have been identified previously in *Acanthamoeba* strains belonging to sequence types T4, T5, and T13 (30, 31); whether the presence of bacterial symbionts is in any way correlated with host sequence types is, however, an open question due to the limited data available. The eight *Acanthamoeba* isolates containing endosymbionts were deposited in the American Type Culture Collection (Table 1).

In order to identify the bacterial endosymbionts of the recovered *Acanthamoeba* isolates, their near-full-length 16S rRNA gene sequences (1,388 to 1,549 bp) were amplified (see Table S1 in the supplemental material) and cloned. For each symbiont, three to six clones were sequenced and found to be identical (99.9 to 100% sequence similarity); the software Pintail indicated that the obtained 16S rRNA sequences were not chimeric. Comparative sequence analysis revealed that all sequences are highly similar to previously described obligate endosymbionts of free-living amoebae (Table 1).

Three of the identified symbionts (in isolates EI1, EI2, and EI6) showed highest 16S rRNA sequence similarity (98.9 to 99.5%) to members of the *Parachlamydiaceae* (Table 1) and thus belong to the genera *Parachlamydia* and *Protochlamydia* within this family, according to the proposed taxonomy of *Chlamydiae* (13, 17, 39). Hereafter, these bacteria are accordingly referred to as *Parachlamydia* sp. isolate EI1, *Parachlamydia* sp. isolate EI6, and *Protochlamydia* sp. isolate EI2.

Three *Acanthamoeba* endosymbionts (in isolates EI4, 5a2, and EIDS3) showed highest 16S rRNA sequence similarity to a group of amoeba symbionts within the *Bacteroidetes* (98.3 to 99.3%) (Table 1), whose only described representative is "*Candidatus* Amoebophilus asiaticus" TUMSJ-321 (32). With the exception of a group of arthropod symbionts related to "*Candidatus* Cardinium hertigii" (58), similarity of these bacteria to other members of the *Bacteroidetes* was below 85%. These symbionts were thus named "*Ca*. Amoebophilus" EI4, "*Ca*. Amoebophilus" 5a2, and "*Ca*. Amoebophilus" EIDS3.

The endosymbiont of *Acanthamoeba* isolate EI3 was most similar to the alphaproteobacterial *Acanthamoeba* symbiont "*Candidatus* Paracaedibacter acanthamoebae" UWC9 (99.7% sequence similarity) (Table 1) (30); similarity to other members of the *Alphaproteobacteria* was significantly lower (83 to 92%). The endosymbiont of *Acanthamoeba* isolate EI3 is therefore tentatively referred to as "*Candidatus* Paracaedibacter" EI3.

The endosymbiont of *Acanthamoeba* sp. isolate EI5 had highest similarity with a group of betaproteobacterial endosymbionts of free-living amoebae, particularly with "*Candidatus* Procabacter acanthamoebae" Page23 (97.3%) (Table 1) (27, 31); similarity to other members of the *Betaproteobacteria* was below 90%. This symbiont was provisionally named "*Candidatus* Procabacter" EI5.

All applied treeing methods used to resolve phylogenetic relationships of the newly identified endosymbionts consistently showed the endosymbionts' affiliation with their respective most-similar sequences, forming stable monophyletic lineages of symbiotic bacteria with high bootstrap and TREE-

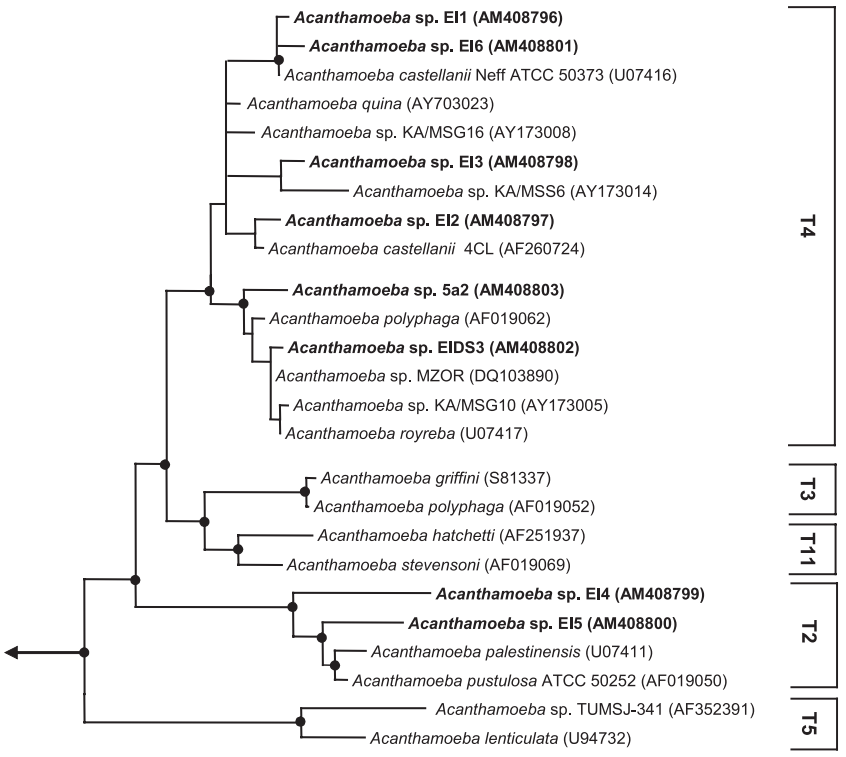


FIG. 1. Phylogenetic relationships of *Acanthamoeba* host cells. An 18S rRNA-based TREE-PUZZLE tree (HKY nucleotide substitution model) (52) is shown. A filter considering only positions which are conserved in at least 50% of all amoebal 18S rRNA sequences was used for tree calculations. Selected *Acanthamoeba* 18S rRNA sequence types (51) are indicated. Black dots represent nodes with TREE-PUZZLE support and PHYLIP maximum parsimony bootstrap values (1.000 resampling) (18) greater than 80%. GenBank accession numbers are given in parentheses. The arrow indicates toward the out-group. The bar at the bottom represents 10% of the estimated evolutionary distance.

PUZZLE support within the *Proteobacteria*, the *Chlamydiae*, and the *Bacteroidetes* (Fig. 2).

In order to demonstrate the intracellular location of the bacterial symbionts within their *Acanthamoeba* hosts, fluorescence in situ hybridization (FISH) in combination with confocal laser scanning microscopy was performed. Amoebae were harvested from axenic cultures by centrifugation (4,000 × *g*; 5 min) and washed with 1× Page's saline (45). After resuspension in 100 μl of 1× Page's saline, 20-μl aliquots of amoebic suspension were incubated on glass slides for 20 min to allow for attachment of amoebae and fixed with 20 μl of 4% paraformaldehyde for 20 min at room temperature. Hybridization was carried out as described elsewhere (14).

Symbiont-specific probes were selected using probeBase (see Table S1 in the supplemental material) (40) and applied for FISH under the recommended conditions. Positive hybridization reactions for all eight endosymbionts with the specific probes Bn9-658, Aph1180, Proca438, and CC23a were obtained and confirmed the 16S rRNA-based identification and the intracellular location of these symbionts (Fig. 3). Furthermore, the simultaneous hybridization with symbiont-specific probes and the universal bacterial probe set EUB-Mix labeled with different dyes showed that all bacteria within the *Acanthamoeba* cells were stained by both symbiont-specific probes and EUB-Mix, demonstrating the presence of only a single symbiont phylotype within the respective *Acanthamoeba* hosts (Fig. 3).

The ultrastructure and intracellular niche of the bacterial symbionts within their amoeba host cells were further investigated by transmission electron microscopy. For this analysis, one representative of each evolutionary lineage was selected (Fig. 4). Amoebae were harvested from axenic cultures and directly fixed with 2% glutaraldehyde in 1× Page's amoebic saline for 1 h at room temperature, followed by fixation with 2% osmium tetroxide for 1 h at room temperature and dehydration in an ascending series of acetone. Subsequently, samples were embedded in Spurr resin (Sigma-Aldrich) with polymerization at 60°C for 8 to 12 h. Ultrathin sections were stained with 1% uranyl acetate for 4 min and 0.3% lead citrate for 2 min and examined with a Zeiss CEM 902 transmission electron microscope.

Parachlamydia sp. isolate EI1 showed morphological forms typical of chlamydial developmental stages, consisting of electron-dense elementary bodies and electron-translucent reticulate bodies (1, 13, 21, 24, 35, 56). The diameters of the elementary and reticulate bodies were 0.4 to 0.6 μm and 0.6 to 0.9 μm, respectively (Fig. 4A). The reticulate, but not elementary,

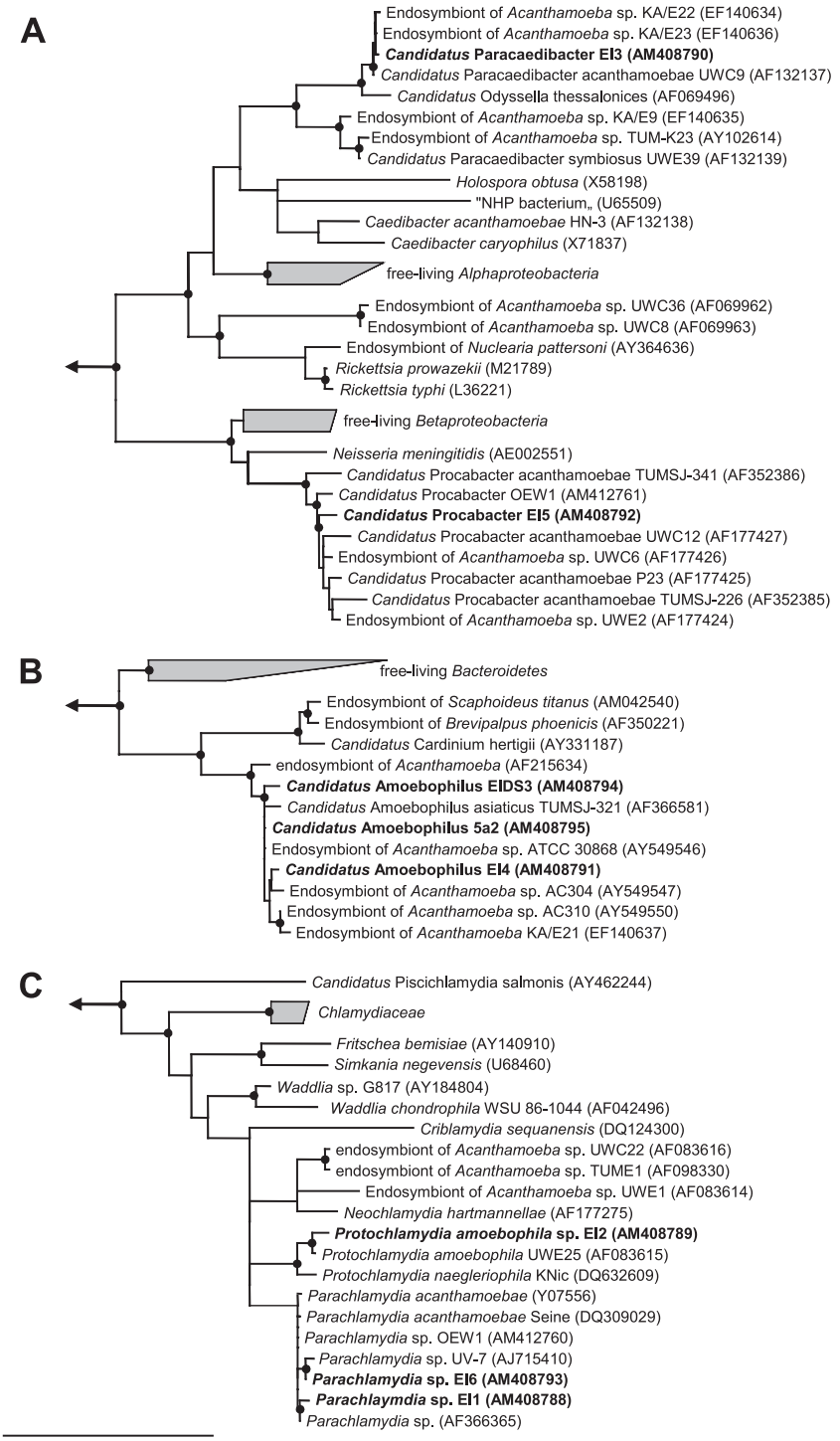
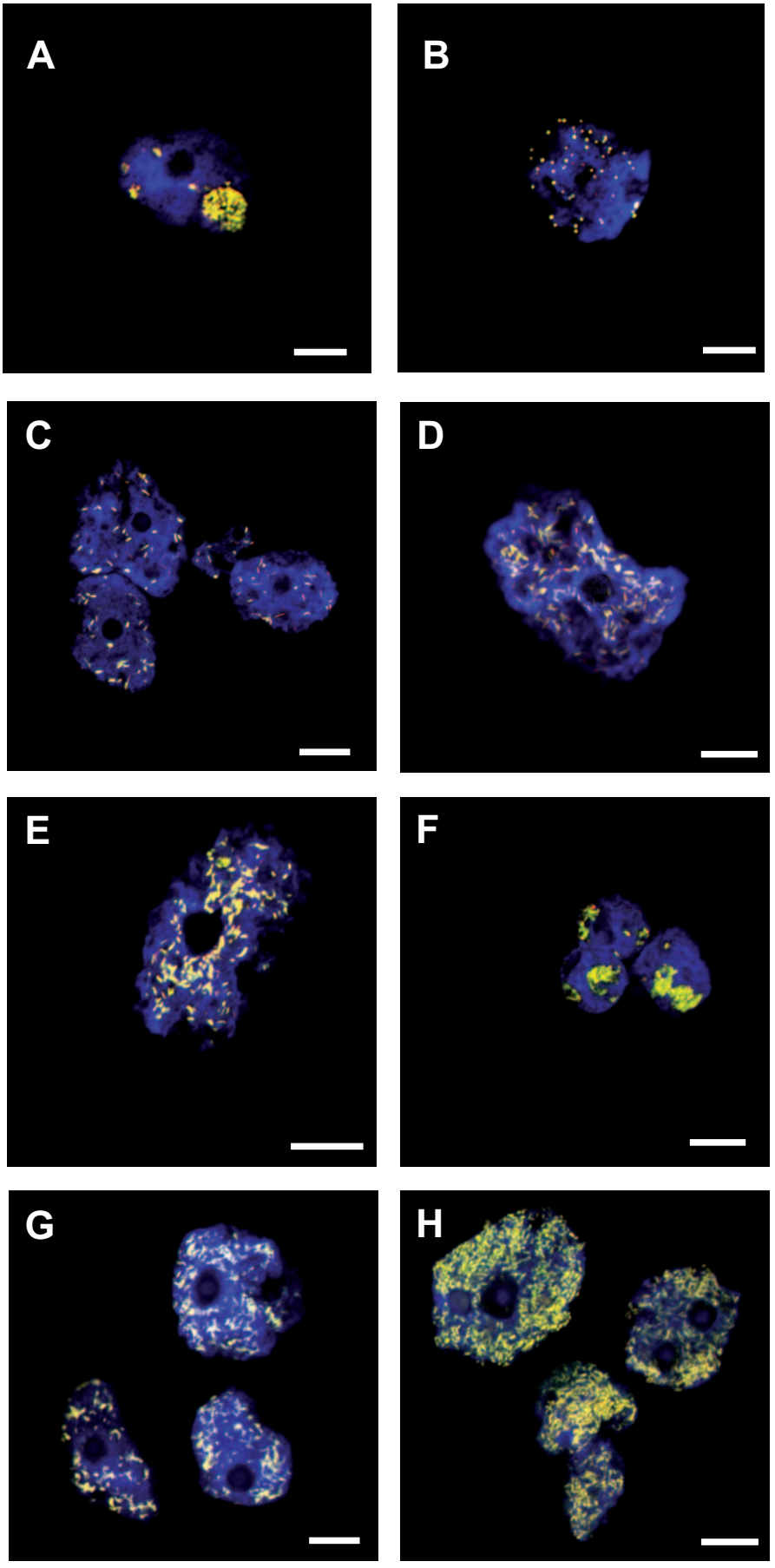


FIG. 2. Phylogenetic relationships of *Acanthamoeba* symbionts. 16S rRNA-based trees calculated using the TREE-PUZZLE algorithm (HKY nucleotide substitution model) (52) are shown for the proteobacterial symbionts (A), the *Bacteroidetes* symbionts (B) and the chlamydial symbionts (C). A filter considering only positions which are conserved in at least 50% of all *Bacteria* strains was used for tree calculations. Black dots represent nodes with TREE-PUZZLE support and PHYLIP maximum parsimony bootstrap values (1.000 resampling) (18) greater than 80%. GenBank accession numbers are given in parentheses. Arrows indicate toward the out-groups. The bar at the bottom of the figure represents 10% of the estimated evolutionary distance.

bodies were observed undergoing binary fission. Furthermore, *Parachlamydia* sp. isolate EI1 resided in large vacuoles resembling the host-derived inclusion characteristic for known chlamydiae (19).

"*Ca*. Amoebophilus" EI4 was rod-shaped (0.3 to 0.5 μm in diameter and 0.7 to 1.4 μm in length) and appeared equally spread throughout the host cytoplasm (Fig. 4B). An association with ribosome-studded host membranes was not as obvi-


A
B
C
D
E
F
G
H

ous for "*Ca*. Amoebophilus" EI4 as it was for other "*Ca*. Amoebophilus asiaticus" strains (32, 57).

"*Ca*. Paracaedibacter" EI3 had a rod-shaped morphology (0.2 to 0.4 μm in diameter and 0.9 to 1.4 μm in length). These bacteria seemed to be located directly in the host cell cytoplasm, not enclosed in vacuoles but surrounded by an electron-translucent space, indicating a capsule or slime layer similar to that of "*Ca*. Paracaedibacter acanthamoebae" UWC9 and other similar strains (Fig. 4C) (7, 30, 57).

The betaproteobacterial "*Ca*. Procabacter" EI5 exhibited rod-shaped morphology (0.3 to 0.4 μm in diameter and 0.8 to 1.3 μm in length) and was equally distributed in the host cytoplasm (Fig. 4D). Interestingly, "*Ca*. Procabacter" EI5, similar to another *Procabacter*-related amoeba symbiont described recently ("*Candidatus* Procabacter" OEW1) (27), was enclosed by a membrane, which contrasts with the original description of its closest relatives, "*Ca*. Procabacter acanthamoebae" strains Page23, UWC12, and UWE2, that were found directly in the cytoplasm (31).

In light of the ubiquity of acanthamoebae and the numerous reported transient associations between facultative intracellular bacteria and amoebae, it was surprising that all symbionts of the new *Acanthamoeba* isolates investigated in this study were related to any of the four known groups of obligate amoeba endosymbionts (Fig. 2) (3, 7, 8, 20, 21, 31, 32, 34, 57). This is even more remarkable as none of the *Acanthamoeba* isolates analyzed here originated from a location sampled previously (Table 2). In fact, for each phylogenetic group of symbionts, amoeba hosts were recovered from different habitats and different locations worldwide. The proteobacterial symbionts, for example, were found in amoebae from America, Europe, Africa, and Asia. This indicates a global distribution of only a small number of phylogenetically distinct groups of amoeba symbionts.

Despite the existence of only a few major evolutionary lineages of amoeba symbionts, there is a considerable diversity within some of these lineages. The alphaproteobacterial and the chlamydial symbionts comprise at least four different genera each (Table 2). In addition, two of the bacterial symbionts identified in this study, "*Candidatus* Amoebophilus" EI4 and "*Candidatus* Procabacter" EI5, showed a 16S rRNA sequence similarity below the recently proposed thresholds for the discrimination of bacterial species of 98.6 or 98.7% (36, 50) and, thus, represent novel species within the tentative genera "*Ca*. Amoebophilus" (at least three species in total) and "*Ca*. Procabacter" (at least four species), respectively (Table 2). This species-level diversity is further supported by differences in ultrastructure and subcellular location observed in this study compared to those in previous reports (27, 31, 32, 57).

One possible explanation for the observed limited phylogenetic diversity of bacterial endosymbionts of *Acanthamoeba* species might be a potential bias introduced by the isolation procedures and the adaptation to axenic culture conditions. The use of nonnutrient agar plates with *E. coli* or *Enterobacter aerogenes* as the food source is currently the standard procedure for isolation of free-living amoebae and was used to recover phylogenetically diverse amoebae (37, 48, 49). From the eight *Acanthamoeba* isolates analyzed in this study, six belong to *Acanthamoeba* sequence type T4 (Fig. 1), which is the most abundant genotype in the environment and also comprises most of the pathogenic *Acanthamoeba* isolates (37, 49, 55), while two belong to sequence type T2. This shows that there is considerable phylogenetic diversity among the isolates obtained with the method applied in this study. However, although unlikely, we cannot exclude that, for some unknown reason, amoebae containing certain types of symbionts are selected for by our isolation procedure. In this context, it seems interesting that the amoeba harboring "*Ca*. Procabacter" EI5, which is most different from known amoeba symbionts, was recovered from nonnutrient agar plates with *Saccharomyces cerevisiae* instead of *E. coli* as the food source. One possibility for isolating free-living amoebae harboring novel bacterial endosymbionts might therefore be to use alternative food sources during isolation.

Another possibility for the discovery of novel intracellular bacteria has been described recently. Cocultivation of environmental samples with (symbiont-free) amoebae was successfully used to identify obligate or facultative intracellular bacteria and to grow them in a surrogate *Acanthamoeba* host (13, 46, 53, 54). This technique is, by far, less time consuming than the isolation of amoebae and the adaptation to axenic culture conditions by using traditional methods. However, the cocultivation approach bears the disadvantage that the identity of the original host (which does not necessarily have to be an amoeba) remains unknown.

In concert with previous reports (3, 7, 20, 21, 30–32, 34, 57), this study provides evidence for the existence of only a limited number of phylogenetically different groups of obligate bacterial endosymbionts of *Acanthamoeba* spp., showing a global distribution. This might suggest that adaptation of bacteria to long-term intracellular symbiosis with acanthamoebae has originated only a few times during evolution. The ongoing genome projects of *Parachlamydia acanthamoebae* UV7, "*Candidatus* Amoebophilus asiaticus" 5a2, and *Acanthamoeba castellanii* Neff will help to

FIG. 3. Identification and intracellular localization of *Acanthamoeba* symbionts by FISH. Probes EUK516 labeled with Cy5 (and shown in blue), targeting most *Eukarya*, and EUB-Mix labeled with Fluos dye (green), targeting most *Bacteria* strains, were used in all experiments in combination with Cy3-labeled symbiont-specific probes (red) (Table 2); the combined signal from bacterial and symbiont-specific probes appears yellow. At least three independent experiments were performed and ≥100 individual *Acanthamoeba* host cells were examined, all of which were infected; representative confocal laser scanning micrographs are shown. (A) *Parachlamydia* sp. isolate EI1 in *Acanthamoeba* sp. isolate EI1 (probe Bn9-658). (B) *Protochlamydia* sp. isolate EI2 in *Acanthamoeba* sp. isolate EI2 (probe Bn9-658). (C) "*Candidatus* Paracaedibacter" EI3 in *Acanthamoeba* sp. isolate EI3 (probe Cc23a). (D) "*Candidatus* Amoebophilus" EI4 in *Acanthamoeba* sp. isolate EI4 (probe Aph1180). (E) "*Candidatus* Procabacter" EI5 in *Acanthamoeba* sp. isolate EI5 (probe Proca438). (F) *Parachlamydia* EI6 in *Acanthamoeba* sp. isolate EI6 (probe Bn9-658). (G) "*Candidatus* Amoebophilus" EIDS3 in *Acanthamoeba* sp. isolate EIDS3 (probe Aph1180). (H) "*Candidatus* Amoebophilus" 5a2 in *Acanthamoeba* sp. isolate 5a2 (probe Aph1180). The white bars in the bottom right corner of each panel represent 10 μm.

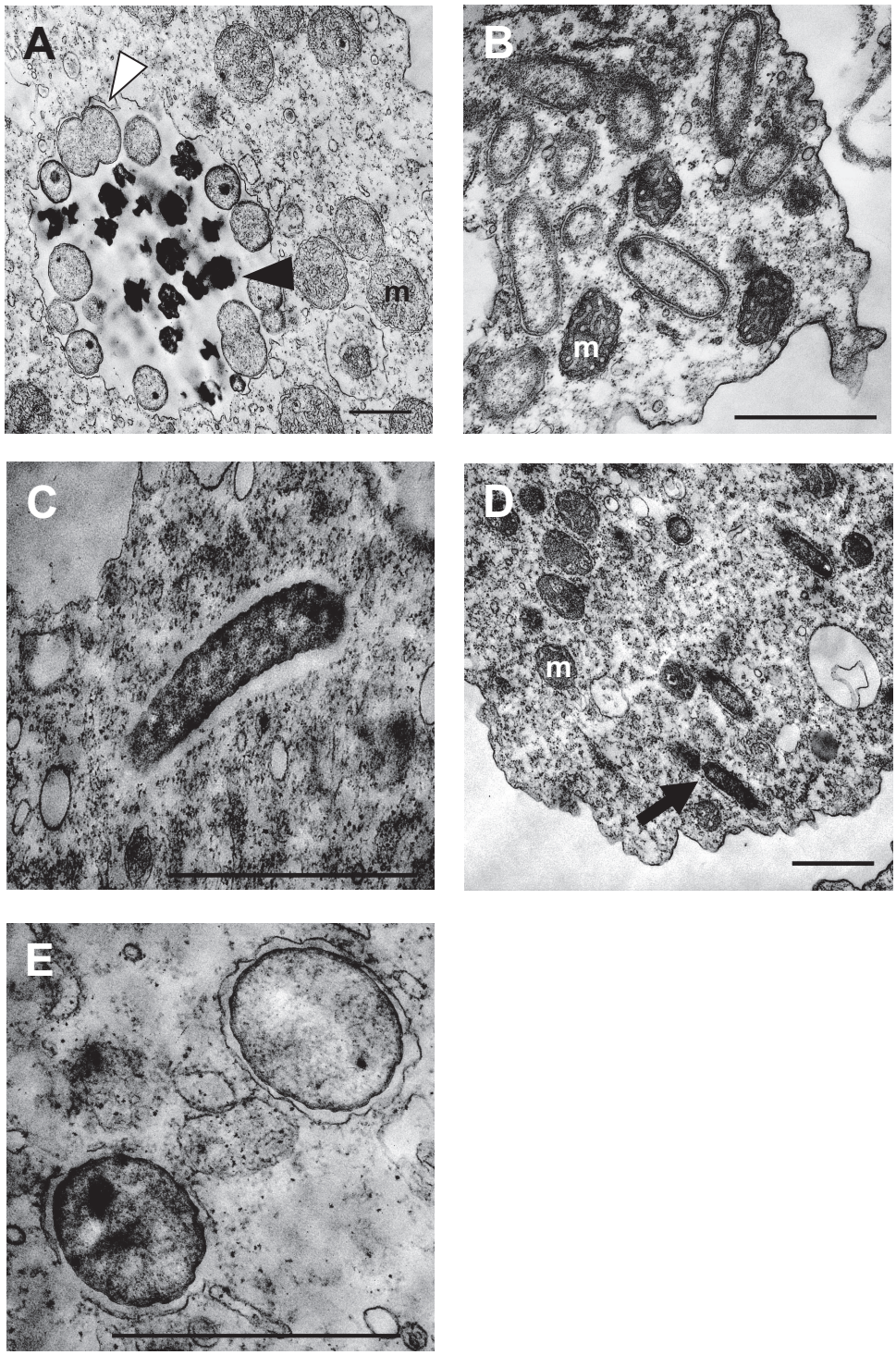


FIG. 4. Ultrastructure of symbionts within *Acanthamoeba* host cells. Representatives from each phylogenetic group of symbionts are shown. (A) *Parachlamydia* sp. isolate EI1. Elementary (black arrowhead) and reticulate (white arrowhead) bodies within the chlamydial inclusion can be seen. (B) "*Candidatus* Amoebophilus" EI4. (C) "*Candidatus* Paracaedibacter" EI3. An electron-translucent space, indicative of a capsule or slime layer, surrounding "*Candidatus* Paracaedibacter" EI3 is clearly visible. (D) "*Candidatus* Procabacter" EI5 is surrounded by a membrane (black arrow). (E) *Protochlamydia* sp. isolate EI2. Each *Protochlamydia* sp. isolate EI2 cell is surrounded by an inclusion membrane. Mitochondria are labeled "m." The lengths of bars in the bottom right corner of each panel represent 1 μm.

TABLE 2. Overview of recognized obligate intracellular symbionts of free-living amoebae

Bacterial lineage	Amoeba symbiont designation[a]	Country of origin[b]	Source habitat	GenBank 16S rRNA accession no.	Reference or source
Alphaproteobacteria	"*Candidatus* Paracaedibacter acanthamoebae" UWC9	USA	Contact lens case	AF132137	30
	"*Candidatus* Paracaedibacter" EI3	Dominica	Rainforest soil	AM408790	This study
	Endosymbiont of *Acanthamoeba* sp. isolate KA/E23	South Korea	Human corneal tissue	EF140636	57
	Endosymbiont of *Acanthamoeba* sp. isolate KA/E22	South Korea	Human corneal tissue	EF140634	57
	"*Candidatus* Odyssella thessalonicensis"	Greece	Water from air conditioner	AF069496	7
	"*Candidatus* Paracaedibacter symbiosus" E39	USA (MN)	Soil	AF132139	30
	Endosymbiont of *Acanthamoeba* sp. isolate TUMK-23	Germany	Activated sludge	AY102614	6
	Endosymbiont of *Acanthamoeba* sp. isolate KA/E9	South Korea	Human corneal tissue	EF140635	57
	Caedibacter acanthamoebae HN-3	USA	Nasal swab	AF132138	30
	Endosymbiont of *Acanthamoeba* sp. isolate UWC8	USA	Human corneal tissue	AF069963	20
	Endosymbiont of *Acanthamoeba* sp. isolate UWC36	USA	Human corneal tissue	AF069962	20
	Endosymbiont of *Nuclearia pattersoni*	Czech Republic	Gills (roach [*Rutilus rutilus*])	AY364636	16
	"*Candidatus* Procabacter acanthamoebae" UWC12	USA	Human corneal tissue	AF177427	31
	"*Candidatus* Procabacter" Page23	USA (WI)	Freshwater	AF177425	31
	"*Candidatus* Procabacter" TUMSJ-341	Malaysia	Lake sediment	AF352386	31
	"*Candidatus* Procabacter" TUMSJ-226	Malaysia	Lake sediment	AF352385	31
	"*Candidatus* Procabacter" UWC6	USA	Human corneal tissue	AF177426	31
	"*Candidatus* Procabacter" UWE2	USA (MN)	Soil	AF177424	31
	"*Candidatus* Procabacter" EI5	Tunisia	Desert sand	AM408792	This study
	"*Candidatus* Procabacter" OEW1	Austria	Saline lake sediment	AM412761	27
Bacteroidetes	"*Candidatus* Amoebophilus asiaticus" TUMSJ-321	Malaysia	Lake sediment	AF366581	32
	Endosymbiont of *Acanthamoeba* sp. isolate KA/E21	South Korea	Human corneal tissue	EF140637	57
	"*Candidatus* Amoebophilus" EIDS3	Austria	Alkaline lake sediment	AM408794	This study
	"*Candidatus* Amoebophilus" EI4	Austria	Soil	AM408791	This study
	"*Candidatus* Amoebophilus" 5a2	Austria	Lake sediment	AM408795	This study
Chlamydiae	*Protochlamydia amoebophila* UWE25	USA (WA)	Soil	AF083615	13
	Protochlamydia naegleriophila KNic	Germany	Freshwater aquarium water	DQ632609	10
	"*Candidatus* Protochlamydia" EI2	Austria	Soil	AM408789	This study
	Endosymbiont of *Acanthamoeba* sp. isolate UWE1	USA (WA)	Soil	AF083614	21
	Parachlamydia acanthamoebae Bn9	Germany	Nasal swab	Y07556	3
	Parachlamydia acanthamoebae Berg17	Germany	Nasal swab	AM941720	3
	Parachlamydia sp. isolate Hall's coccus[a]	USA (VT)	Water from humidifier	AF366365	8
	Parachlamydia sp. isolate EI1	Austria	Soil	AM408788	This study
	Parachlamydia sp. isolate EI6	Austria	Soil	AM408793	This study
	Parachlamydia sp. isolate UV-7	Germany	Activated sludge	AJ715410	12
	Parachlamydia sp. isolate Seine	France	Freshwater (Seine river)	DQ309029	53
	Parachlamydia sp. isolate OEW1	Austria	Saline lake sediment	AM412760	27
	Neochlamydia hartmannellae	Germany	Water from water conduit	AF177275	34
	Endosymbiont of *Acanthamoeba* sp. isolate TUME1	Germany	Activated sludge	AF098330	21
	Endosymbiont of *Acanthamoeba* sp. isolate UWC22	USA	Human corneal tissue	AF083616	21
	Criblamydia sequanensis	France	Freshwater (the Seine)	DQ124300	53

[a] *Parachlamydia* sp. isolate Hall's coccus, *Parachlamydia* sp. isolate UV-7, *Parachlamydia* sp. isolate Seine, and *Criblamydia sequanensis* were obtained by cocultivation with *Acanthamoeba* sp. isolates.
[b] USA, United States of America.

understand similarities and differences between these symbionts and the interactions with their *Acanthamoeba* hosts, as well as the role of free-living amoebae as evolutionary training grounds for facultative intracellular bacteria.

Nucleotide sequence accession numbers. 18S and 16S rRNA gene sequences of *Acanthamoeba* isolates and their symbionts, respectively, were deposited in the EMBL/DDBJ/GenBank data libraries under accession numbers AM408788 to AM408803 (Table 1).

Work in the laboratory of M.H. and M.W. was funded by grants from the Austrian Science Fund FWF (Y277-B03 and P16566-B14) and the University of Vienna (research focus project FS573001). S.S.-E. is supported by FWF grant P19252-B17.

We gratefully acknowledge Waltraud Klepal and the team of the Ultrastructure Laboratory (University of Vienna) and Kilian Stoecker for advice and assistance with electron microscopy and confocal laser scanning microscopy, respectively. We are grateful to Christian Baranyi for excellent technical assistance.

REFERENCES

1. **Abdelrahman, Y. M., and R. J. Belland.** 2005. The chlamydial developmental cycle. FEMS Microbiol. Rev. **29:**949–959.
2. **Albert-Weissenberger, C., C. Cazalet, and C. Buchrieser.** 2007. *Legionella pneumophila*—a human pathogen that co-evolved with fresh water protozoa. Cell. Mol. Life Sci. **64:**432–448.
3. **Amann, R., N. Springer, W. Schonhuber, W. Ludwig, E. N. Schmid, K. D. Muller, and R. Michel.** 1997. Obligate intracellular bacterial parasites of acanthamoebae related to *Chlamydia* spp. Appl. Environ. Microbiol. **63:**115–121.
4. **Ashelford, K. E., N. A. Chuzhanova, J. C. Fry, A. J. Jones, and A. J. Weightman.** 2005. At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. Appl. Environ. Microbiol. **71:**7724–7736.
5. **Barker, J., and M. R. W. Brown.** 1994. Trojan horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment. Microbiology **140:**1253–1259.
6. **Beier, C. L., M. Horn, R. Michel, M. Schweikert, H. D. Gortz, and M. Wagner.** 2002. The genus *Caedibacter* comprises endosymbionts of *Paramecium* spp. related to the *Rickettsiales* (*Alphaproteobacteria*) and to *Francisella tularensis* (*Gammaproteobacteria*). Appl. Environ. Microbiol. **68:**6043–6050.
7. **Birtles, R. J., T. J. Rowbotham, R. Michel, D. G. Pitcher, B. Lascola, S. Alexiou-Daniel, and D. Raoult.** 2000. '*Candidatus* Odyssella thessalonicensis' gen. nov., sp. nov., an obligate intracellular parasite of *Acanthamoeba* species. Int. J. Syst. Evol. Microbiol. **50:**63–72.
8. **Birtles, R. J., T. J. Rowbotham, C. Storey, T. J. Marrie, and D. Raoult.** 1997. Chlamydia-like obligate parasite of free-living amoebae. Lancet **349:**925–926.
9. **Bonkowski, M.** 2004. Protozoa and plant growth: the microbial loop in soil revisited. New Phytol. **162:**617–631.
10. **Casson, N., R. Michel, R. D. Mueller, and G. Greub.** 2008. *Protochlamydia naegleriophila* as etiologic agent of pneumonia. Emerg. Infect. Dis. **14:**168–172.
11. **Clarholm, M.** 2002. Bacteria and protozoa as integral components of the forest ecosystem—their role in creating a naturally varied soil fertility. Antonie van Leeuwenhoek **81:**309–318.
12. **Collingro, A., S. Poppert, E. Heinz, S. Schmitz-Esser, A. Essig, M. Schweikert, M. Wagner, and M. Horn.** 2005. Recovery of an environmental *Chlamydia* strain from activated sludge by co-cultivation with *Acanthamoeba* sp. Microbiology **151:**301–309.
13. **Collingro, A., E. R. Toenshoff, M. W. Taylor, T. R. Fritsche, M. Wagner, and M. Horn.** 2005. '*Candidatus* Protochlamydia amoebophila,' an endosymbiont of *Acanthamoeba* spp. Int. J. Syst. Evol. Microbiol. **55:**1863–1866.
14. **Daims, H., K. Stoecker, and M. Wagner.** 2005. Fluorescence in situ hybridization for the detection of prokaryotes, p. 213–239. *In* A. Osborn and C. Smith (ed.), Advanced methods in molecular microbial ecology. Bios-Garland, Abingdon, United Kingdom.
15. **Darby, A. C., N.-H. Cho, H.-H. Fuxelius, J. Westberg, and S. G. E. Andersson.** 2007. Intracellular pathogens go extreme: genome evolution in the *Rickettsiales*. Trends Genet. **23:**511–520.
16. **Dykova, I., M. Veverkova, I. Fiala, B. Machackova, and H. Peckova.** 2003. *Nuclearia pattersoni* sp. n. (Filosea), a new species of amphizoic amoeba isolated from gills of roach (*Rutilus rutilus*), and its rickettsial endosymbiont. Folia Parasitol. (Praha) **50:**161–170.
17. **Everett, K. D., R. M. Bush, and A. A. Andersen.** 1999. Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms. Int. J. Syst. Bacteriol. **49:**415–440.
18. **Felsenstein, J.** 1989. PHYLIP—Phylogeny Inference Package (version 3.2). Cladistics **5:**164–166.
19. **Fields, K. A., and T. Hackstadt.** 2002. The chlamydial inclusion: escape from the endocytic pathway. Annu. Rev. Cell Dev. Biol. **18:**221–245.
20. **Fritsche, T. R., M. Horn, S. Seyedirashti, R. K. Gautom, K. H. Schleifer, and M. Wagner.** 1999. In situ detection of novel bacterial endosymbionts of *Acanthamoeba* spp. phylogenetically related to members of the order *Rickettsiales*. Appl. Environ. Microbiol. **65:**206–212.
21. **Fritsche, T. R., M. Horn, M. Wagner, R. P. Herwig, K. H. Schleifer, and R. K. Gautom.** 2000. Phylogenetic diversity among geographically dispersed *Chlamydiales* endosymbionts recovered from clinical and environmental isolates of *Acanthamoeba* spp. Appl. Environ. Microbiol. **66:**2613–2619.
22. **Greub, G., J.-L. Mege, and D. Raoult.** 2003. *Parachlamydia acanthamoeba* enters and multiplies within human macrophages and induces their apoptosis. Infect. Immun. **71:**5979–5985.
23. **Greub, G., J. L. Mege, J. P. Gorvel, D. Raoult, and S. Meresse.** 2005.Intracellular trafficking of *Parachlamydia acanthamoebae*. Cell. Microbiol. **7:**581–589.
24. **Greub, G., and D. Raoult.** 2002. Crescent bodies of *Parachlamydia acanthamoeba* and its life cycle within *Acanthamoeba polyphaga*: an electron micrograph study. Appl. Environ. Microbiol. **68:**3076–3084.
25. **Greub, G., and D. Raoult.** 2004. Microorganisms resistant to free-living amoebae. Clin. Microbiol. Rev. **17:**413–433.
26. **Harb, O. S., L.-Y. Gao, and Y. A. Kwaik.** 2000. From protozoa to mammalian cells: a new paradigm in the life cycle of intracellular bacterial pathogens. Environ. Microbiol. **2:**251–265.
27. **Heinz, E., I. Kolarov, C. Kastner, E. R. Toenshoff, M. Wagner, and M. Horn.** 2007. An *Acanthamoeba* sp. containing two phylogenetically different bacterial endosymbionts. Environ. Microbiol. **9:**1604–1609.
28. **Horn, M.** Chlamydiae as symbionts in eukaryotes. Annu. Rev. Microbiol., in press.
29. **Horn, M., A. Collingro, S. Schmitz-Esser, C. L. Beier, U. Purkhold, B. Fartmann, P. Brandt, G. J. Nyakatura, M. Droege, D. Frishman, T. Rattei, H. W. Mewes, and M. Wagner.** 2004. Illuminating the evolutionary history of chlamydiae. Science **304:**728–730.
30. **Horn, M., T. R. Fritsche, R. K. Gautom, K. H. Schleifer, and M. Wagner.** 1999. Novel bacterial endosymbionts of *Acanthamoeba* spp. related to the *Paramecium caudatum* symbiont *Caedibacter caryophilus*. Environ. Microbiol. **1:**357–367.
31. **Horn, M., T. R. Fritsche, T. Linner, R. K. Gautom, M. D. Harzenetter, and M. Wagner.** 2002. Obligate bacterial endosymbionts of *Acanthamoeba* spp. related to the *beta-Proteobacteria*: proposal of '*Candidatus* Procabacter acanthamoebae' gen. nov., sp. nov. Int. J. Syst. Evol. Microbiol. **52:**599–605.
32. **Horn, M., M. D. Harzenetter, T. Linner, E. N. Schmid, K. D. Muller, R. Michel, and M. Wagner.** 2001. Members of the *Cytophaga-Flavobacterium-Bacteroides* phylum as intracellular bacteria of acanthamoebae: proposal of '*Candidatus* Amoebophilus asiaticus.' Environ. Microbiol. **3:**440–449.
33. **Horn, M., and M. Wagner.** 2004. Bacterial endosymbionts of free-living amoebae. J. Eukaryot. Microbiol. **51:**509–514.
34. **Horn, M., M. Wagner, K. D. Muller, E. N. Schmid, T. R. Fritsche, K. H. Schleifer, and R. Michel.** 2000. *Neochlamydia hartmannellae* gen. nov., sp. nov. (*Parachlamydiaceae*), an endoparasite of the amoeba *Hartmannella vermiformis*. Microbiology **146:**1231–1239.
35. **Kahane, S., N. Kimmel, and M. G. Friedman.** 2002. The growth cycle of *Simkania negevensis*. Microbiology **148:**735–742.
36. **Keswani, J., and W. B. Whitman.** 2001. Relationship of 16S rRNA sequence similarity to DNA hybridization in prokaryotes. Int. J. Syst. Evol. Microbiol. **51:**667–678.
37. **Khan, N. A.** 2006. *Acanthamoeba*: biology and increasing importance in human health. FEMS Microbiol. Rev. **30:**564–595.
38. **Köhsler, M., B. Leitner, M. Blaschitz, R. Michel, H. Aspock, and J. Walochnik.** 2006. ITS1 sequence variabilities correlate with 18S rDNA sequence types in the genus *Acanthamoeba* (Protozoa: Amoebozoa). Parasitol. Res. **98:**86–93.
39. **Kuo, C.-C., M. Horn, and R. S. Stephens.** The order *Chlamydiales*. *In* B. Hedlund, N. R. Krieg, W. Ludwig, B. J. Paster, J. T. Staley, N. Ward, and W. B. Whitman (ed.), Bergey's manual of systematic bacteriology—the *Planctomycetes*, *Spirochaetes*, *Fibrobacteres*, *Bacteriodetes* and *Fusobacteria*, 2nd ed., vol. 4, in press. Springer, New York, NY.
40. **Loy, A., F. Maixner, M. Wagner, and M. Horn.** 2007. probeBase—an online resource for rRNA-targeted oligonucleotide probes: new features 2007. Nucleic Acids Res. **35:**D800–D804.
41. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. Konig, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K. H. Schleifer.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32:**1363–1371.
42. **Marshall, M. M., D. Naumovitz, Y. Ortega, and C. R. Sterling.** 1997. Waterborne protozoan pathogens. Clin. Microbiol. Rev. **10:**67–85.
43. **Molmeret, M., M. Horn, M. Wagner, M. Santic, and Y. Abu Kwaik.** 2005. Amoebae as training grounds for intracellular bacterial pathogens. Appl. Environ. Microbiol. **71:**20–28.
44. **Ogata, H., B. La Scola, S. Audic, P. Renesto, G. Blanc, C. Robert, P. E. Fournier, J. M. Claverie, and D. Raoult.** 2006. Genome sequence of *Rickettsia bellii* illuminates the role of amoebae in gene exchanges between intracellular pathogens. PLoS Genet. **2:**e76.
45. **Page, F. C.** 1976. An illustrated key to freshwater and soil amoebae. Freshwater Biological Association, Ambleside, United Kingdom.
46. **Pagnier, I., D. Raoult, and B. La Scola.** 2008. Isolation and identification of amoeba-resisting bacteria from water in human environment by using an *Acanthamoeba polyphaga* co-culture procedure. Environ. Microbiol. **10:**1135–1144.
47. **Rodríguez-Zaragoza, S.** 1994. Ecology of free living amoebae. Crit. Rev. Microbiol. **20:**225–241.
48. **Schuster, F. L.** 2002. Cultivation of pathogenic and opportunistic free-living amebas. Clin. Microbiol. Rev. **15:**342–354.
49. **Schuster, F. L., and G. S. Visvesvara.** 2004. Free-living amoebae as oppor-

tunistic and non-opportunistic pathogens of humans and animals. Int. J. Parasitol. **34:**1001–1027.
50. **Stackebrandt, E., and J. Ebers.** 2006. Taxonomic parameters revisited: tarnished gold standards. Microbiol. Today **November 2006:**6–9.
51. **Stothard, D. R., J. M. Schroeder-Diedrich, M. H. Awwad, R. J. Gast, D. R. Ledee, S. Rodriguez-Zaragoza, C. L. Dean, P. A. Fuerst, and T. J. Byers.** 1998. The evolutionary history of the genus *Acanthamoeba* and the identification of eight new 18S rRNA gene sequence types. J. Eukaryot. Microbiol. **45:**45–54.
52. **Strimmer, K., and A. von Haeseler.** 1996. Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. Mol. Biol. Evol. **13:**964–969.
53. **Thomas, V., N. Casson, and G. Greub.** 2006. *Criblamydia sequanensis*, a new intracellular *Chlamydiales* isolated from Seine river water using amoebal co-culture. Environ. Microbiol. **8:**2125–2135.
54. **Thomas, V., K. Herrera-Rimann, D. S. Blanc, and G. Greub.** 2006. Biodiversity of amoebae and amoeba-resisting bacteria in a hospital water network. Appl. Environ. Microbiol. **72:**2428–2438.
55. **Walochnik, J., A. Obwaller, and H. Aspock.** 2000. Correlations between morphological, molecular biological, and physiological characteristics in clinical and nonclinical isolates of *Acanthamoeba* spp. Appl. Environ. Microbiol. **66:**4408–4413.
56. **Ward, M. E.** 1988. The chlamydial developmental cycle, p. 71–95. *In* A. L. Barron (ed.), Micobiology of chlamydia. CRC Press, Boca Raton, FL.
57. **Xuan, Y. H., H. S. Yu, H. J. Jeong, S. Y. Seol, D. I. Chung, and H. H. Kong.** 2007. Molecular characterization of bacterial endosymbionts of *Acanthamoeba* isolates from infected corneas of Korean patients. Korean J. Parasitol. **45:**1–9.
58. **Zchori-Fein, E., S. J. Perlman, S. E. Kelly, N. Katzir, and M. S. Hunter.** 2004. Characterization of a '*Bacteroidetes*' symbiont in *Encarsia* wasps (Hymenoptera: Aphelinidae): proposal of '*Candidatus* Cardinium hertigii.' Int. J. Syst. Evol. Microbiol. **54:**961–968.