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Obligate Intracellular Bacterial Parasites of Acanthamoebae Related to *Chlamydia* spp.

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The phylogeny of obligate intracellular coccoid parasites of acanthamoebae isolated from the nasal mucosa of humans was analyzed by the rRNA approach. The primary structures of the 16S and 23S rRNA molecules of one strain were determined in almost full length. In situ hybridization with a horseradish peroxidase-labeled oligonucleotide probe targeted to a unique signature site undoubtedly correlated the retrieved 16S rRNA sequence to the respective intracellular parasite. This probe also hybridized with the second strain, suggesting a close relationship between the two intracellular parasites. Comparative sequence analysis demonstrated a distinct relationship to the genus *Chlamydia*. With 16S rRNA similarities of 86 to 87% to the hitherto-sequenced *Chlamydia* species, the intracellular parasites are likely not new species of this genus but representatives of another genus in the family of the *Chlamydiaceae*. Consequently, it is proposed to provisionally classify the endoparasite of *Acanthamoeba* sp. strain Bn_9 as "*Candidatus* Parachlamydia acanthamoebae." From an epidemiological perspective, the results suggest that small amoebae could be environmental reservoirs and vectors for a variety of potentially pathogenic bacteria including members of the *Chlamydiaceae*.

Two strains of acanthamoebae (Bn_9 and $Berg_{17}$) harboring obligate intracellular parasites of coccoid shape were isolated from the nasal mucosa of two female volunteers (23, 24, 26). The behavior and morphology of the parasites of strain Bn_9 have been described in detail recently (23). Living acanthamoeba cells are necessary for the growth of this tiny intracellular parasite (diameter, 0.4 to 0.6 μm) since none of 19 nutrient media evaluated supported growth (23). Similar to the intracellular parasites of Bn_9, two developmental stages were visualized for the endoparasites of strain $Berg_{17}$ by electron microscopy. While the multiplication stages showing binary fission have thin cell walls and stain gram negative, the more numerous infectious stages have very thick cell walls (Fig. 1) and stain gram positive. The host range of the intracellular parasites was found to be restricted to the genus *Acanthamoeba*. Interestingly, the mode of the host-parasite interrelationship varied considerably for different strains of acanthamoebae (22, 32).

Since these phenotypic and behavioral features did not match those of any bacteria hitherto described as endosymbionts of protozoans (15, 30), the phylogenetic affiliation of the endoparasitic strains remained uncertain. We had utmost interest in the identification and classification of the strains, especially since they had been isolated from humans. However, classical methods based on biochemical or physiological traits were not applicable due to our failure to obtain pure cultures. The minimal phenotypic information obtained by microscopy and staining techniques was sufficient for neither reliable identification nor for a sound classification. Today, rRNA-based techniques offer a cultivation-independent approach to the identification in situ and phylogeny of obligate endosymbionts (1, 2). The full rRNA approach is based on a two-step procedure. First, rRNA sequences are retrieved in a cultivation-independent way and then analyzed comparatively. In the second step, in situ hybridization with oligonucleotide probes targeted to selected signature sites of the retrieved sequences can be used to correlate the retrieved sequences to defined individual cells. In the last years, this approach has been applied several times (for a review, see reference 1). On one occasion, it was used to demonstrate that *Sarcobium lyticum* (9), another obligate intracellular parasite of small amoebae, is closely related to *Legionella pneumophila* (37). Here, we applied the same approach to reveal the phylogenetic affiliation of the unusual coccoid endoparasites of acanthamoebae isolated from the nasal mucosa.

MATERIALS AND METHODS

Isolation. Details on the isolation of infected acanthamoebae have been published before (23). Briefly, the acanthamoebal strains Bn_9 and $Berg_{17}$ were isolated from the nasal mucosa of two female volunteers with a sterile cotton swab soaked in sterile distilled water. After transfer to nonnutrient agar plates by the method of Page (29), the parasitized trophozoites multiplied readily on the plate surface, grazing on *Enterobacter cloacae* as food bacteria. Parasitized amoebae of this kind were isolated only once. Ten subsequent attempts of reisolation failed.

Axenic cultures. The host amoeba of strain $Berg_{17}$ was identified as *Acanthamoeba mauritaniensis* by morphology of cysts as described by Page (28). It could be transferred directly to axenic SCGYE medium (7) if treated with penicillin and streptomycin (0.2 mg/ml each) to kill the accompanying bacteria but not the intracellular parasites. Since the host strain of Bn_9 parasites did not grow on SCGYE medium, the parasites had to be liberated from their host cells by rupture and then cocultivated on nonnutrient agar plates with endosymbiont-free *Acanthamoeba castellanii* C_3 known to grow readily on SCGYE medium. Seventy-two hours later, *A. castellanii* trophozoites, revealing unequivocal signs of infection, were successfully transferred to SCGYE medium treated by the same antibiotics as those mentioned above. Amoebae were grown and treated in tubes. For mass propagation, they were transferred to 25-ml cell culture flasks (Greiner, Nürtingen, Germany).

Preparation of parasite cells. *A. castellani* cells harvested from the axenic culture at the exponential phase of growth were submitted to freeze-thawing to disrupt infected trophozoites liberating the intracellular infectious stages. This suspension was passed through a Minisart NML filter (Sartorius, Göttingen, Germany) with a pore size of 1.2 μm before the filtrate was used for further studies.

Sequence analysis. Modifying an earlier protocol (36), small aliquots of the filter-purified Bn_9 parasites were pelleted by centrifugation, stored in a −20°C freezer, and later used directly for the amplification of the almost-full-length

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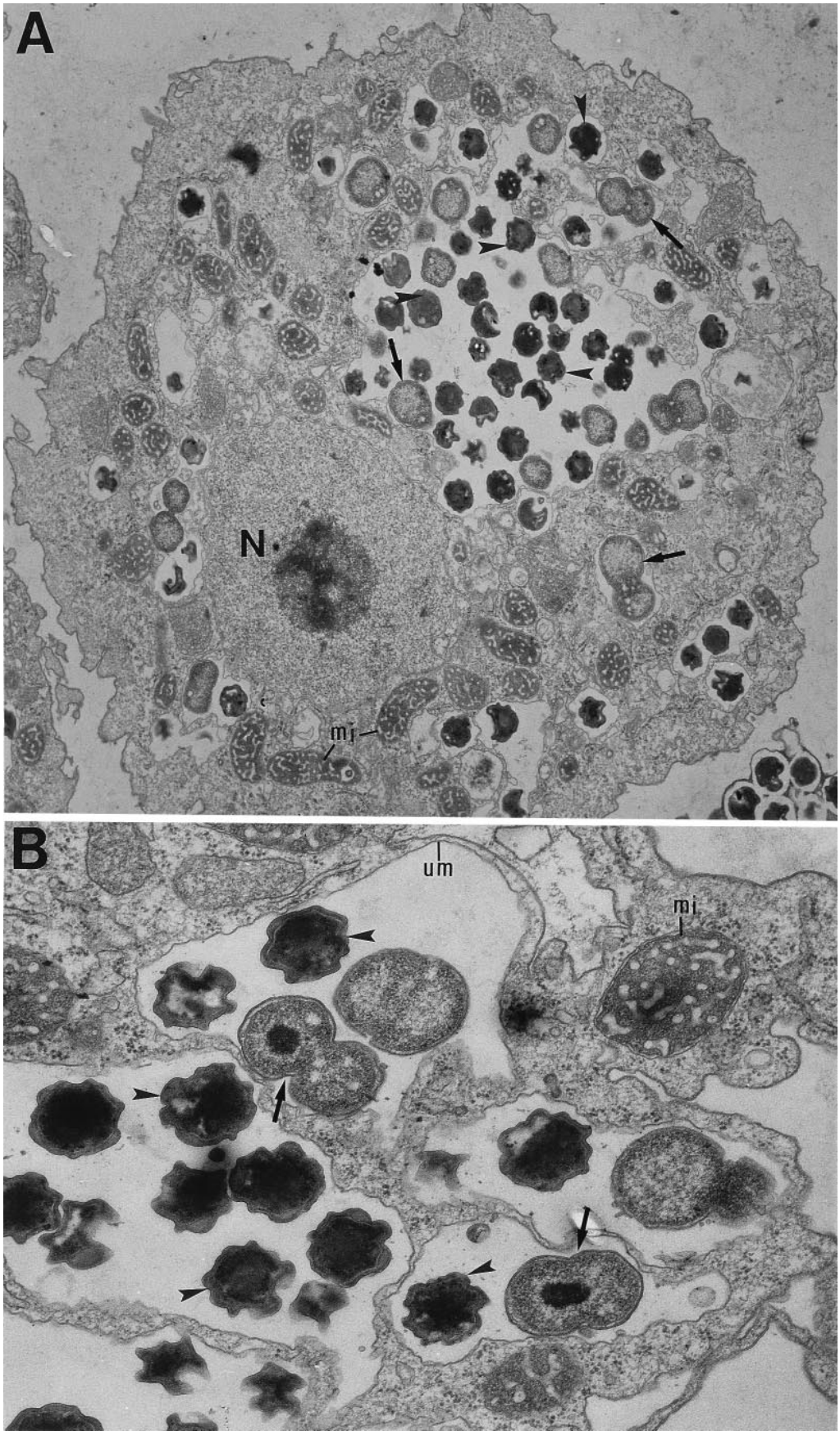

FIG. 1. Trophozoite of *A. mauritaniensis* $Berg_{17}$ with one large and several small vacuoles containing different developmental stages of the obligate intracellular parasites. Some of the thin-walled gram-negative stages show binary fission (arrows). The gram-positive condensed infective stages (arrowheads) are more numerous. Magnifications, ca. 7,400 (A) and 28,000 (B). N, nucleus of the host amoeba; mi, mitochondria; um, unit membrane.

TABLE 1. Overall rRNA sequence similarities for the parasite of *Acanthamoeba* sp. strain Bn_9 and all other members of the domain *Bacteria*[a]

Phylogenetic group	% Sequence similarity to:						
	16S rRNA of:						23S rRNA of Bn_9
	Bn_9	Z	*C. trachomatis*	*C. psittaci*	*C. pneumoniae*	Clones	
Bn_9							
Z	88.2						
C. trachomatis	86.3	84.0					
C. psittaci	86.2	83.3	95.3				
C. pneumoniae	87.0	83.7	94.2	95.7			
Clones	74.0	72.7	72.5	72.2	72.2		
Planctomycetes	72.5	71.7	71.6	71.2	71.5	71.3	70.7
Cyanobacteria	74.2	73.3	73.9	73.8	74.4	72.1	67.8
Cytophaga-Flexibacter-Bacteroides	70.8	70.3	70.4	70.3	70.6	69.3	69.9
Green sulfur bacteria	73.3	72.1	73.1	72.5	72.3	72.5	70.3
Flexistipes	75.0	73.8	73.8	73.2	73.5	72.3	
Spirochetes	73.7	72.5	72.4	72.3	73.1	71.0	70.3
Fibrobacter	72.8	72.2	73.2	72.8	73.1	72.0	
Nitrospira	73.8	73.1	73.2	72.8	72.8	74.3	70.6
Proteobacteria	75.1	73.4	74.2	73.8	74.2	72.6	71.0
Gram-positive; high G+C	75.0	74.8	74.3	74.3	74.7	74.0	67.8
Fusobacteria	73.8	72.7	73.2	72.6	73.5	70.6	71.9
Gram-positive; low G+C	75.2	73.8	74.0	74.0	74.7	72.8	72.3
Deinococci	72.3	72.1	73.3	72.6	73.1	73.0	69.8
Green nonsulfur bacteria	71.6	71.4	73.2	71.5	71.2	73.8	70.1
Thermotogales	71.3	70.8	70.8	70.3	70.7	70.8	69.8
Aquifex	70.3	69.9	69.5	69.2	69.1	71.1	66.2

[a] All available at-least-70%-complete (in comparison with the homologous sequences from *E. coli*) 16S and 23S rRNA primary structures were included for calculation. Mean values are given for the cluster of clones (17) as well as for the major phylogenetic groups (phyla) of the domain *Bacteria.*

rRNA operon by the PCR. PCR fragments covered 16S rRNA position 8 to 5S rRNA position 49 (*E. coli* numbering [6]). The ribosomal DNA (rDNA) was sequenced directly with the T7 sequencing kit of Pharmacia (Freiburg, Germany) by using rRNA gene-specific primers (18, 33, 36). The 23S and 16S sequences were submitted to the EMBL Data Library and assigned the accession numbers Y07555 and Y07556, respectively. The new 16S rDNA sequence was added to an alignment of about 6,000 published and unpublished homologous small-subunit rRNA primary structures (20, 39) by use of the alignment tool of the ARB program package (38). Phylogenetic analyses were performed by applying distance matrix, maximum parsimony, and maximum likelihood methods on different data sets by use of the respective tools of the ARB program and Gary Olsen's fastDNAml (20). Maximum parsimony analyses were done on the complete data base of small-subunit rRNA sequences, whereas distance matrix and maximum likelihood analyses were based on all available homologous primary structures of members of the *Chlamydia* branch and selected representatives of all other known major bacterial lines of descent. The data sets varied with respect to the reference sequences as well as alignment positions included. Positional variabilities of the individual alignment positions were determined with the respective tools of the ARB package and used as a criterion to successively remove highly variable positions from the data set. This was done to recognize and minimize treeing artifacts resulting from alignment errors, data base inconsistencies, and false identities in highly variable regions resulting from multiple base changes.

In situ hybridization. For in situ hybridization, whole infected amoebae grown as described above were washed and resuspended in phosphate-buffered saline. They were fixed by the addition of 1 volume of ethanol as described by Roller et al. (34). Cells were immobilized on gelatin-coated slides by air drying and subsequently dehydrated by immersing the slides in 50, 80, and 96% ethanol for 3 min each.

Amino-linked oligonucleotides (MWG Biotech, Ebersberg, Germany) were labeled with tetramethylrhodamine (4) and horseradish peroxidase (3) as described before. Probes EUB338 (4) and ACA (40) targeted to 16S rRNA signatures of most members of the domain *Bacteria* and the genus *Acinetobacter*, respectively, were used as positive and negative controls together with the endoparasite-specific oligonucleotide.

Hybridizations with tetramethylrhodamine-labeled oligonucleotides were performed by standard protocols (4). Cells were hybridized with horseradish peroxidase-labeled oligonucleotides for 90 min at 35°C in 10 μl of a buffer containing 0.9 M NaCl, 0.01% sodium dodecyl sulfate, 20 mM Tris-HCl (pH 8), 0 to 50% formamide, and 2% blocking reagent (Boehringer GmbH, Mannheim, Germany) as described by Amann et al. (3). Subsequently, the slides were washed at 35°C for 20 min in a buffer containing 900 to 19 mM NaCl, 0.01% sodium dodecyl sulfate, 20 mM Tris-HCl, and 5 mM EDTA (pH 8). Whereas the stringency in the hybridization buffer was increased by the addition of formamide, the stringency in the washing buffer was increased by lowering the NaCl concentration as described by Manz et al. (21). Slides were rinsed shortly with distilled water, air dried, and covered with substrate solution (0.5 mg of diaminobenzidine per ml prepared from a stock solution of 25 mg/ml in dimethyl formamide; 0.6% H_2O_2 in phosphate-buffered saline) for 5 min at room temperature. Finally, the slides were rinsed with distilled water and mounted in glycerol. Stained endoparasite cells were visualized by brownish substrate precipitates with a Zeiss Axioplan microscope and bright-field illumination. Monochrome photographs were taken with Kodak Tmax400 film.

Electron microscopy. Trophozoites of *A. mauritaniensis* infected with $Berg_{17}$ intracellular parasites were harvested from 4-day-old axenic cultures fixed in cacodylate-buffered 2.5% glutaraldehyde, postfixed in 1% OsO_4, and stained with uranyl acetate and lead citrate. Sections were examined with a model 10A transmission electron microscope (Carl Zeiss, Jena, Germany).

RESULTS

Sequence analysis. Without prior DNA extraction, rDNA fragments covering almost the complete rRNA operon could be amplified from the intracellular parasite preparation of strain Bn_9. The fragments could be sequenced directly. This suggested that one bacterial species was dominant in the analyzed cell suspension. Comparative sequence analysis revealed that the newly obtained 16S rRNA sequence is, with 88.2% similarity, most closely related to that of organism Z, a parasite of tissue cultures previously not validly described (16). It is with 86 to 87% overall sequence similarity also related to the members of the genus *Chlamydia*, whereas similarities to 16S rRNA sequences of other members of the *Bacteria* range only between 70 and 75% (Table 1). A phylogenetic tree based on 16S rRNA sequences clearly demonstrates that the retrieved sequence together with that of organism Z forms an independent lineage within the chlamydial branch of the bacterial tree (Fig. 2).

The results of the comparison of the retrieved full-length 23S rDNA (Table 1) are in line with the 16S rRNA results since the data, in the absence of other publically available 23S

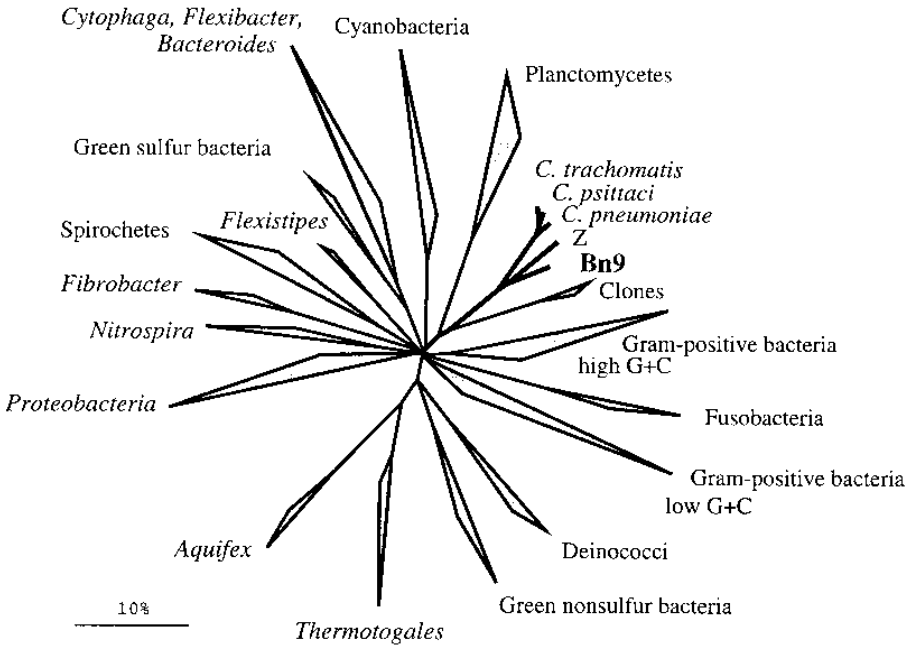

FIG. 2. 16S rRNA-based phylogenetic tree reflecting the relationship of the intracellular parasites of *Acanthamoeba* sp. strain Bn_9 and the genus *Chlamydia*. Z refers to the 16S rRNA of organism Z (16). Clones refers to the clone sequences obtained from an Australian terrestrial environment by Liesack and Stackebrandt (17). The bar indicates 10% estimated sequence divergence.

rRNA sequences of chlamydiae (8), support a phylum status of *Chlamydia* and relatives.

In situ hybridization. The oligonucleotide Bn_9658 5′-TCCG TTTTCTCCGCCTAC-3′ was designed complementary to a characteristic region of the retrieved 16S rRNA homologous to *E. coli* 16S rRNA positions 658 to 675 (6). A recent probe check (June 1996) on more than 6,000 full and partial 16S rRNA sequences confirmed that the oligonucleotide has three mismatches to the sequences of members of the genus *Chlamydia* and more than three mismatches to all other available 16S rRNA sequences. Number, quality, and location of the mismatches in the probe target region of selected reference organisms are shown in Fig. 3. A tetramethylrhodamine-labeled oligonucleotide did not yield unambiguous in situ identification of the small parasitic cells in strain Bn_9 due to relatively strong autofluorescence of *A. castellanii* host cells. Consequently, a nonfluorescent in situ hybridization was performed. In this assay, horseradish peroxidase-labeled oligonucleotides are hybridized and subsequently detected by the intracellular precipitation of diaminobenzidine (3). Bright-field microscopy revealed brownish precipitates in small coccoid cells which were present in various numbers per host cell (Fig. 4A). When the stringency of hybridization was gradually raised at a constant temperature of 35°C by adding formamide to the hybridization in 10% increments, the probe-conferred signal remained unchanged up to a 40% formamide concentration. With an addition of 50% formamide, the signal became very weak and disappeared completely at 60%. Fixed reference cells (e.g., *E. coli* and *Acinetobacter calcoaceticus*) did not bind detectable levels of probe Bn_9658 in hybridization buffers containing 30% or more formamide. Hybridization of fixed infected acanthamoebae with horseradish peroxidase probe EUB, serving as a

Probe Bn_9658	**3′-CATCCGCCTCTTTTGCCT-5′**
Target (*E. coli* position 658-675)	5′-GUAGGCGGAGAAAACGGA-3′
Acanthamoeba sp. strain Bn_9 endosymbiont	.....
Chlamydia psittaci (M13769)	....AU.....G...
Chlamydia trachomatis (M59178)	....AU.....G...
Planctomyces staleyi ATCC 27377	.GGAUA.AG.U..G....
Planctomyces limnophilus ATCC 43296^T	.AG.CA..GUGU.CG.A.
Eubacterium timidum ATCC 33093	.C...A..G....G....
Mycobacterium gadium ATCC 27726	......AU....G.A...
Mycobacterium celatum ATCC 51131	.....U......G.A.C.
Synechocystis sp. PCC 6308	N.......U.G....U..
Clostridium aurantibutyricum NCIMB 10659	.C...A..G....GU...
Thermotoga maritima DSM 3109	..U..U....G...G..G

FIG. 3. Difference alignment of 16S rRNA target regions. The *E. coli* position of the probe target site is given as described in Brosius et al. (6). M13769 and M59178 are EMBL accession numbers.

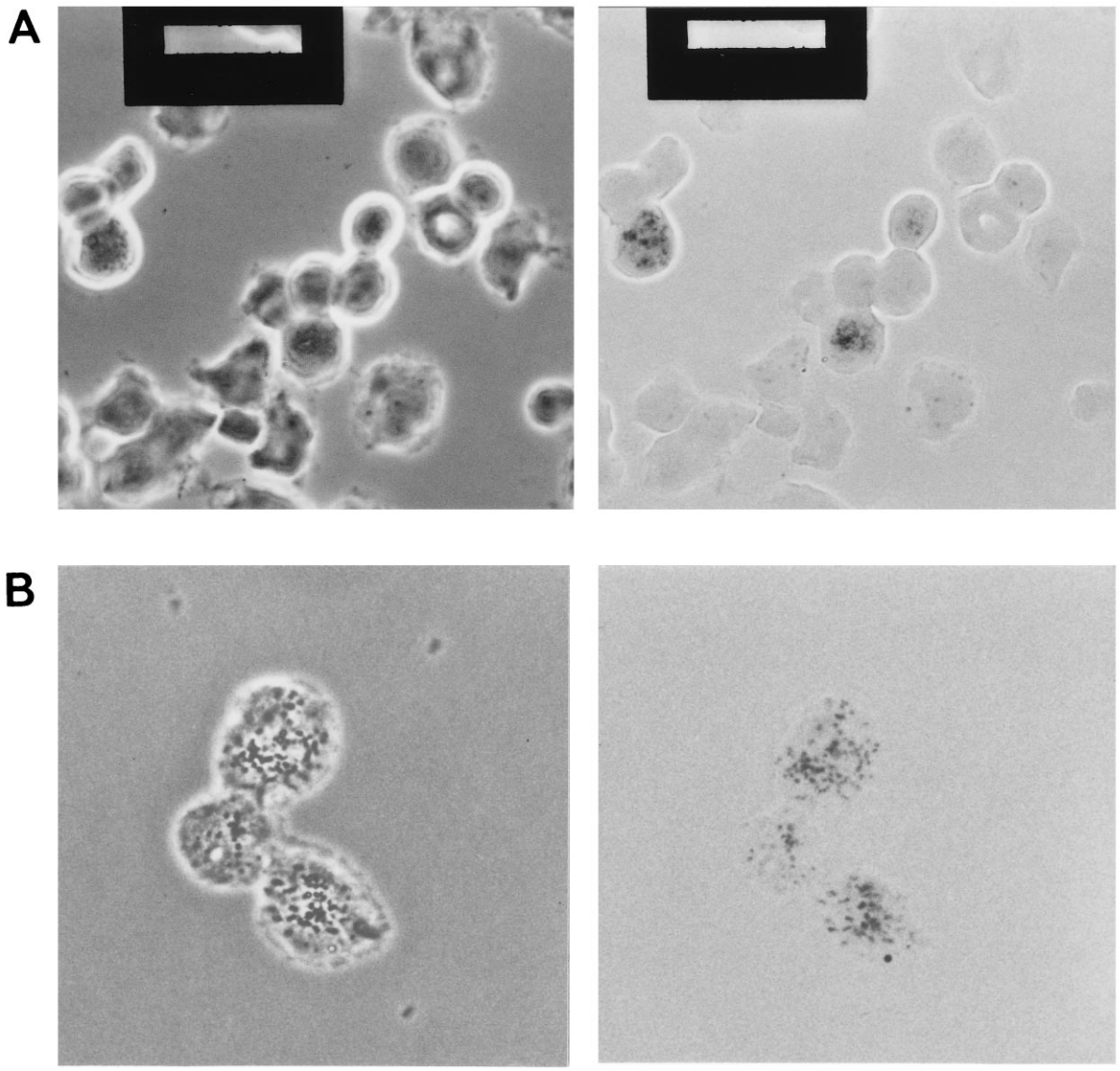

FIG. 4. In situ detection of chlamydia-related intracellular parasites in acanthamoebae. Ethanol-fixed samples were hybridized at 35°C in 30% formamide with the horseradish peroxidase-labeled probe Bn_9658, and probe-conferred enzyme was subsequently visualized by diaminobenzidine precipitation. Note the small coccoid precipitates. Left panels, phase contrast; right panels, bright field for selective visualization of precipitate. (A) *Acanthamoeba* sp. strain Bn_9; (B) *A. mauritaniensis* $Berg_{17}$. Magnification, ×1,000. Bar, 20 μm.

positive control, and with probe ACA, serving as a negative control, was as expected (data not shown). These results demonstrate specific probe hybridization and thereby corroborate the assignment of the retrieved 16S rRNA sequence to the intracellular parasites of the *Acanthamoeba* sp. strain Bn_9.

Interestingly, probe Bn_9658 also hybridized with the intracellular parasites of *A. mauritaniensis* $Berg_{17}$ even at 40% formamide (Fig. 4B). The fact that the binding of probe Bn_9658 to these parasites is as stable as it is to those of strain Bn_9 is strong evidence for the presence of a fully complementary target site. The occurrence of an identical rRNA signature in both parasites indicates a close evolutionary relationship between the two strains.

DISCUSSION

Obligate endosymbionts are commonly observed in protozoans (15, 30). For *Acanthamoeba*, approximately one of four isolates from both environmental and medical samples contains bacterial endosymbionts (11). It is well established that not only can legionellae proliferate in acanthamoebae (5, 12), but the hosts can even be used to isolate *L. pneumophila* from clinical and environmental samples (35). Therefore, it was not surprising when in an earlier study the rRNA approach demonstrated that *Sarcobium lyticum*, a well-described and long-known intracellular parasite of small amoebae, is closely related to the genus *Legionella* (37). The retrieval of a chlamydia-related 16S rRNA sequence was therefore originally quite surprising. The subsequent retrieval and comparative analysis (8) of a 23S rRNA sequence from the same parasite yielded consistent results (Table 1). Whereas *Legionella* is a genus within the gamma subclass of the class *Proteobacteria* (19), the chlamydiae are an independent major line of descent of the domain *Bacteria* with a distant relationship to the order *Planctomycetales*. Also, a group of cloned sequences retrieved from

an Australian terrestrial environment appear to represent microorganisms deeply branching from the chlamydial lineage (17). However, the significance of the common root of chlamydiae and this group, as well as the significance of the root between the chlamydiae and planctomycetes, is quite low.

In situ hybridization with a specific oligonucleotide probe was applied to definitively link the retrieved sequence to the observed small coccoid endoparasites. The microscopic results demonstrated beyond any doubt that the chlamydial sequences originated in the obligate intracellular parasites of *Acanthamoeba* sp. strain Bn_9. This intracellular parasite and the microorganism Z are probably the first examples of endoparasites that will eventually give greater phylogenetic depth to the chlamydial branch of the *Planctomyces-Chlamydia* phylum which, until recently, just included three species of the genus *Chlamydia*. In line with the argument of Kahane et al. (16) on organism Z, we believe that the 16S rRNA data of Bn_9 parasites will, upon further characterization, justify its classification in a new genus in the family *Chlamydiaceae*. The 16S rRNA sequence similarity of organism Z and the intracellular parasite of strain Bn_9 of only 88.2% does not support lumping both bacteria in one genus.

Therefore, according to Murray and Schleifer (27), we propose provisional classification of the intracellular parasite of *Acanthamoeba* sp. strain Bn_9 as "*Candidatus* Parachlamydia acanthamoebae." This should indicate that a hitherto-uncultured bacterium originally isolated with a strain of *Acanthamoeba* sp. has been partially characterized by molecular techniques. The short description of "*Candidatus* Parachlamydia acanthamoebae" is as follows: phylogenetic position, *Chlamydiaceae*; cultivation, not cultivated on cell-free media, only on Vero cell lines; Gram reaction, variable; morphology, coccoid with a diameter of approximately 0.5 μm; basis of assignment, 16S rDNA sequence (accession number Y07556) and oligonucleotide probe Bn_9658 5′-TCCGTTTTCTCCGCCTAC-3′; association and host, intracellular parasites of *Acanthamoeba* sp. strain Bn_9; mesophilic; authors, Amann et al., this study.

The presence of the probe Bn_9658 target site in the intracellular parasites of *A. mauritaniensis* $Berg_{17}$ was not unexpected since a close relationship to the intracellular parasite of *Acanthamoeba* sp. strain Bn_9 had already been indicated by gas chromatographic fatty acid profiling (26). Even without direct evidence from the sequencing of its 16S rRNA, the $Berg_{17}$ parasite is with high probability another member of the new chlamydial group represented by the intracellular parasites of strain Bn_9.

The results of this study supplement other recent findings. Marre and coworkers have shown that after controlled infection, *A. castellani* served as a host for *Chlamydia pneumoniae* (10). The chlamydiae persisted and multiplied in the *A. castellanii* for at least 2 weeks. This laboratory study suggested that small amoebae could be environmental hosts for chlamydiae. Our results strongly support this theory.

In two independent studies, Michel et al. (25) described intracellular *Ehrlichia*-like bacteria in different species of *Saccamoeba* and Gautom and coworkers (14) reported retrieval of both *Chlamydia*- and *Rickettsia*-related 16S rRNA sequences from *Acanthamoeba* isolates in the United States. The results of the two studies are consistent since the genus *Ehrlichia* is part of the order *Rickettsiales*, a monophyletic group of intracellular parasites within the alpha subclass of the class *Proteobacteria*. Obviously, not only chlamydiae and legionellae but also members of the order *Rickettsiales* are able to survive and even multiply in the phagosomes of small amoebae. It has been known before that the small amoeba might be an important environmental reservoir and vector for Legionnaires' disease. Our results point towards a more general role of small amoeba in the epidemiology of diseases.

In this respect, it is interesting to note that studies on the transmissibility of bacterial endosymbionts between different *Acanthamoeba* isolates indicated a specific recognition system between host and symbiont (13). Symbiont-free strains from the same mitochondrial DNA *Eco*RI fingerprint group of acanthamoebae readily formed stable symbioses, whereas infection of members of other fingerprint groups resulted in the death or encystation of the infected cells (13). One of us also encountered differences in the host-parasite relationships for different strains of acanthamoebae (22). Growth was observed for cultures of *A. castellani* C_3 infected to 100% with either Bn_9 or $Berg_{17}$. Under good environmental conditions, the multiplication of the host cells balanced that of the parasites and a stable host-parasite ratio was maintained. As soon as the growth rate of the acanthamoebae was reduced, e.g., due to changes in the medium, they were killed by the parasites. To date, we have not found an acanthamoebal strain that forms a stable endosymbiosis with the parasites of strain Bn_9 or $Berg_{17}$; however, it might exist. Likely, the resistant acanthamoebal strains that are able to grow when hosting intracellular bacteria do not just serve as passive reservoirs for multiplication but actually gain a selective advantage over other susceptible populations of protozoa by the acquisition of what would then be true endosymbionts. Their function might be similar to that of *Caedibacter* endosymbionts in paramecia. Here, paramecium strains that carry *Caedibacter* bacteria have a killer trait. Obviously, they release some of their endosymbionts into the environment, which upon ingestion kill sensitive paramecia (31). In situ hybridization probes, such as those used in this study, are ideal tools to investigate in the future the environmental abundance and localization as well as the epidemiological significance of bacteria that might be both endosymbionts of small amoebae and potential pathogens of humans.

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