

## Isolation of an Amoeba Naturally Harboring a Distinctive *Legionella* Species

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**There are numerous in vitro studies documenting the multiplication of *Legionella* species in free-living amoebae and other protozoa. It is believed that protozoa serve as host cells for the intracellular replication of certain *Legionella* species in a variety of environmental settings. This study describes the isolation and characterization of a bacterium initially observed within an amoeba taken from a soil sample. In the laboratory, the bacterium multiplied within and was highly pathogenic for *Acanthamoeba polyphaga*. Extracellular multiplication was observed on buffered charcoal yeast extract agar but not on a variety of conventional laboratory media. A 16S rRNA gene analysis placed the bacterium within the genus *Legionella*. Serological studies indicate that it is distinct from previously described species of the genus. This report also describes methods that should prove useful for the isolation and characterization of additional *Legionella*-like bacteria from free-living amoebae. In addition, the characterization of bacterial pathogens of amoebae has significant implications for understanding the ecology and identification of other unrecognized bacterial pathogens.**

The number of species in the genus *Legionella* has increased dramatically since the original identification of *Legionella pneumophila* (9, 24). Members of this genus are widespread in natural settings as well as certain environments created as a result of human activity, such as cooling towers, various plumbing fixtures, and dental units (2, 5–8, 19). *L. pneumophila* and occasionally other legionellae found in these settings continue to be associated with sporadic episodes of respiratory illness in humans.

Efforts to understand the ecology of *L. pneumophila* and its distribution in the environment has led to an unexpected finding. In vitro studies demonstrated that *L. pneumophila* can use protozoa, such as free-living amoebae, as host cells for intracellular replication (1, 4, 14, 28, 34). Furthermore, some intracellular events following infection, such as the appearance of ribosomes and mitochondria in proximity to the membrane-enclosed bacteria, are common to both amoebae and human mononuclear phagocytes infected with *L. pneumophila* (1, 14, 26, 28). The host cells are subsequently lysed as a result of intracellular replication of the bacteria; therefore, *L. pneumophila* is a pathogen not only of human cells but also of amoebae. The multiplication of bacteria in amoebae, resulting in degeneration of the nuclei and lysis of the host cells, has been known for nearly 90 years (27). In recent years, however, interest in interactions between bacteria and free-living amoebae has increased. This is in part a reflection of studies of *Legionella* bacteria and amoebae.

Additional undescribed *Legionella* species that are pathogens of common free-living amoebae were reported in 1993 (31). As a group, they were originally described as *Legionella*-like amoebic pathogens (LLAPs) because they were capable of multiplying in the cytoplasm of amoebae, but they were difficult to cultivate on media designed to support the growth of *Legionella* species. These LLAPs, isolated in Europe, were taken from a variety of environmental sources, and one was a

clinical isolate from an individual with persistent pneumonia (31). A recent phylogenetic analysis of their 16S rRNA genes (rDNAs) suggested that these isolates are members of the family *Legionellaceae* and that they may represent five new species in the genus *Legionella* (3). In a report by Drozanski in 1991, an obligate intracellular bacterial parasite of free-living amoebae was initially described and named *Sarcobium lyticum* (12). Subsequent analysis of the 16S rDNA of the bacterium suggested that it was a member of the genus *Legionella* but that it was different from previously described members of this genus (32). Additional studies showed that it could occasionally be cultured on buffered charcoal yeast extract (BCYE) agar (20) and that it exhibited a positive reaction with a Remel (Augusta, Ga.) *Legionella* Poly-ID kit, which consists of pooled immune sera to 22 species in the genus *Legionella*. Recently, it was proposed to transfer this bacterium to the genus *Legionella* as *Legionella lytica* comb. nov. (25).

There are numerous laboratory studies documenting the multiplication of *Legionella* in amoebae. Corroborating studies of amoebae naturally harboring *Legionella* from environmental sources have been lacking, with the exception of a report by Harf and Montel where *L. pneumophila* was identified in culture lysates of amoebae originally isolated from river waters (23). Our study describes the isolation and characterization of a bacterium (LLAP-14) initially observed within an amoeba taken from a soil sample by light microscopy. 16S rDNA analysis of the bacterium indicates that it should be included within the genus *Legionella*. Serological studies indicate that it is distinct from previously described species of *Legionella*. Also described in this study are methods useful in the visualization and isolation of bacterial amoebic pathogens. These techniques should prove helpful in future studies directed toward bacteria that have parasitic or symbiotic relationships with amoebae.

### MATERIALS AND METHODS

**Initial isolation and cultivation of the bacteria.** A small moist soil sample of approximately 0.2 g was placed in the center of a nonnutrient agar (15 g of agar/liter of H<sub>2</sub>O) plate that had been streaked with heat-killed *Escherichia coli* in an X configuration, which promoted the multiplication and accumulation of amoebae in a defined area along the line of bacteria. The *E. coli* had been

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cultivated 24 h in Trypticase soy broth (TSB) and pelleted by centrifugation. After addition of the soil sample, the plate was sealed with parafilm and incubated at room temperature (25°C). After 48 h the plate was examined at magnifications of  $\times 100$  and  $\times 400$  for the presence of amoebae infected with bacteria. Several amoebae were distinguished by the presence of many motile intracellular bacteria within amoebic cytoplasm. A scalpel was used to remove a plug of agar (3 by 3 mm) containing an infected amoeba. The agar plug was transferred to a 25-cm<sup>2</sup> tissue culture flask (Becton Dickinson, Franklin Lakes, N.J.) containing a monolayer of *Acanthamoeba polyphaga* (ATCC 30461) in spring water (Carolina Biological, Burlington, N.C.) that had been sterilized by autoclaving. A confluent monolayer had been formed by growing the amoebae in the flask containing TSB at 37°C for 48 h. Subsequently, the TSB was poured off and the adherent amoebae were washed with sterile spring water. Five milliliters of spring water was added prior to the addition of the agar plug with the infected amoeba. The replacement of the nutrient-rich TSB with nutrient-poor spring water served to greatly diminish the multiplication of contaminating bacteria before the amoeba pathogen was in pure culture. The preparation was incubated at room temperature for 72 h.

**Obtaining a pure culture of the bacteria.** A plate of sterile nonnutrient agar was streaked with heat-killed *E. coli* in an X configuration. Then amoebae were concentrated by tapping a 25-cm<sup>2</sup> tissue culture flask containing a monolayer of cells in TSB to dislodge the adherent cells, pelleting the cells by centrifugation, and then resuspending the cells in approximately 1 ml of the culture supernatant. Next, 0.1 ml of the concentrated suspension of amoebae ( $10^5$  cells) was placed in the center of the X-shaped streak of *E. coli* and allowed to dry for 2 to 3 h at room temperature. One hundred microliters of the lysate (lysed amoebae resulting from intracellular replication of the bacterial pathogen) from a coculture which still contained other indigenous bacteria was placed directly on top of the amoebae and incubated for 2 to 3 days at room temperature. The infected amoebae were allowed to migrate across the sterile surface of the plate away from any contaminating bacteria. Within 48 to 72 h, infected amoebae could be observed in the peripheral areas of the plate away from contaminating bacteria. Small plugs of agar (3 by 3 mm) containing infected amoebae were cut out with sterilized tools under aseptic conditions and transferred to fresh monolayers of amoebae in spring water. This process not only promoted the initial isolation and culture of the amoebic pathogen (LLAP-14) but also allowed separation of the bacteria from contaminating bacteria in the original soil sample.

**Giemsa stain procedure and electron microscopy.** Infected amoebae were Giemsa stained to determine if the bacteria initially accumulated within vacuoles or if the bacteria were free in the cytoplasm of the amoebae. Cytospins of amoebae cocultures were prepared with whole and lysed cells from 24-, 48-, and 72-h cocultures. The samples were spun at  $700 \times g$  for 6 min in a Shandon (Pittsburgh, Pa.) Cytospin-3. The samples were fixed in methanol for 3 min prior to staining. The Giemsa stain was prepared by adding two drops of Triton X-100 and 1 ml of stain (EM Diagnostic Systems, Gibbstown, N.J.) to 45 ml of deionized water. The slides were stained for 1 h and then rinsed with deionized water. For electron microscopy, aliquots of infected amoebae were fixed for 2 h in 0.1 M cacodylate buffer (pH 7.4) containing 3% glutaraldehyde and 1% osmium tetroxide. Cells were then washed in 0.1 M cacodylate buffer and dehydrated through a series of ethanols. Subsequently, preparations were embedded in epon-araldite resin and stained with 0.5% lead citrate and uranyl acetate (2%). Sections were examined with a Zeiss model 109 electron microscope.

**Growth on laboratory media.** When the bacteria were in monoxenic coculture with *A. polyphaga*, we performed studies to assess the ability of the bacteria to grow independently of amoebae. Aliquots of the cocultures were plated onto Trypticase soy agar (TSA), blood agar, and BCYE differential and selective agars (Becton Dickinson) which are designed to support the growth of legionellae. The plates were incubated at room temperature (25°C), at 30°C, and at 37°C for up to 14 days.

**Serological testing.** The bacteria were tested for reaction against *Legionella*-specific immune sera with a Remel *Legionella* Poly-ID kit. In addition, the bacteria were evaluated by slide agglutination with 17 pools of rabbit antiserum representing 41 species and 64 serogroups of *Legionella* at the Centers for Disease Control and Prevention, Atlanta, Ga. (CDC).

**Amplification of 16S rDNA.** Genomic DNA was extracted from the bacteria cells with a Qiagen (Chatsworth, Calif.) blood kit. PCRs were carried out on the 16S rDNA with the eubacterial primers (obtained from the CDC) 8 forward (5' AGTTTGATCCCTGGCTCAG 3') and 1510 reverse (5' GGTTACCTGTGTTAC GACTT 3'). Each reaction mixture contained 2.0  $\mu$ l of the DNA template, 0.25  $\mu$ l (1.25 U) of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, Ind.), 200  $\mu$ M each deoxynucleoside triphosphate (Boehringer Mannheim), 5.0  $\mu$ l of  $10 \times$  *Taq* buffer (Boehringer Mannheim), and 1.0  $\mu$ l each of the forward and reverse primers at a concentration of 50 pmol/ $\mu$ l. The reaction mixtures were then brought to a volume of 50  $\mu$ l with sterile distilled water. Amplification was carried out with a Perkin-Elmer (Norwalk, Conn.) model 9600 thermal cycler. The cycling of the program involved an initial 2-min hold at 94°C followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s. Purification of the DNA fragments was accomplished by using the Wizard PCR Preps DNA purification system (Promega Corp., Madison, Wis.).

**DNA sequence analysis.** The *Taq* Dye Deoxy Terminator cycle sequencing kit (Perkin-Elmer, Foster City, Calif.) was used to perform fluorescence-based dideoxy-chain termination sequencing reactions on the purified PCR products.

TABLE 1. Eubacterium- and *Legionella*-specific 16S rDNA primer sequences

Eubacterium- or <i>Legionella</i> -specific primer <sup>a</sup>	Sequence <sup>b</sup>
<b>Eubacterium specific</b>	
8 f .....	AGT TTG ATC CTG GCT CAG
104 f .....	GGA CGG GTG AGT AAC ACG TG
357 f, r .....	TAC GGG AGG CAG CAG
460 f .....	TAC CTG GGA AGA TAA TGA CGG
530 f, r .....	CAG CAG CCG CGG TAA TAC
652 f, r .....	GAT ATT CGG AGG AAC ACC AGT GGC
690 f, r .....	GTG AAA TGC GTA GA
734 f .....	TTA GAT ACC CTG GTA GTC CAC GCC
790 f, r .....	ATT AGA TAC CCT GGT AG
980 r .....	TTG CTT CGA ATT AAA CCA C
981 f, r .....	CCC GCA ACG AGC GCA ACC C
1100 f .....	CAA CGA CGC CAA CCC T
1230 r .....	CAT TGT AGC ACG TGT GTA
1390 r .....	CGG TGT GTA CAA CGC CC
1510 r .....	GGT TAC CTT GTT ACG ACT T
<b><i>Legionella</i> specific</b>	
1 f .....	CAC ATG CAA GTC GAA CGG CAG
300 f .....	AGA CAC GGT CCA GAC TCC TAC
370 r .....	GTG CTT TAC AAC CCT CAG GCC
500 f .....	AGC GTT AAT CGG AAT TAC TGG
610 r .....	TCC ACT ACC CTC TCC CAT AC
740 f .....	GAT TAG ATA CCC TGG TAG TC
850 r .....	TTG AGT TTT AAT CTT GCG AC
934 f .....	GCA CAA GCG GTG GAG CAT G
969 f .....	AAC GCG AAG AAC CTA ACT AG
1100 r .....	CGG CAG TCT CCT TAG AGT TG
1283r .....	CTA GCG ATT CCG ACT TCA TG
1321 r .....	TTG CAG ACT CCA ATC CGG AC
1368 r .....	ACA TGC TGA TTC GCG ATT AC
1400 r .....	AAC GTC CCC CCG AAG GTT AG

<sup>a</sup> f, forward; r, reverse; f, r, both forward and reverse.

<sup>b</sup> Written 5' to 3'.

The reaction mixtures contained 8.0  $\mu$ l of Ready Mix (A Dye-C Dye-G Dye-T Dye Terminator, dTTP, dATP, dTTP, dCTP, Tris-HCl [pH 9.0], MgCl<sub>2</sub>, thermal stable pyrophosphatase, AmpliTaq DNA polymerase), 1.0  $\mu$ l (3 pmol/ $\mu$ l) of one of 35 different eubacterium- or *Legionella* 16S-protein-specific primers obtained from the CDC (Table 1), and 1.5 to 2.5  $\mu$ l (0.2  $\mu$ g/ $\mu$ l) of template and were brought to a volume of 20  $\mu$ l with sterile deionized distilled water. Extension products of each sample were purified with a Centri-sep column (Princeton Separations, Inc., Adelphi, N.J.). The products of the sequencing reactions were separated with a 4.25% denaturing acrylamide gel in a model ABI-377 (Perkin-Elmer) automated DNA sequencer. The sequences were edited and assembled to give a contiguous sequence with the University of Wisconsin Genetics Computer Group sequence analysis program.

**Phylogenetic analysis.** A phenogram was established with 54 species of bacteria, including *Legionella*, LLAPs, and the outgroup *Coxiella burnetii*. The analysis was based on a 1,303-nucleotide region by the neighbor-joining method contained in the Phylogeny Inference Package (PHYLIP), version 3.5 (13). Homology values (percentages) between LLAP-14 and various species of *Legionella* and LLAPs were calculated as  $1 - 3/4 [1 - e^{-4/3(\text{distance value})}]$ , where  $e$  is the base of the natural logarithm. The distance values were calculated with the Jukes-Cantor model in the DNADist program contained in PHYLIP.

**Nucleotide sequence and American Type Culture Collection accession numbers.** A 1,474-bp nucleotide sequence of the 16S rDNA has been submitted to GenBank under accession no. U66104, and the bacterium was designated LLAP-14. The bacterium has been deposited at the American Type Culture Collection, Rockville, Md., under accession no. 700313.

## RESULTS

**Initial identification of the amoeba pathogen.** After 72 h of incubation at room temperature, amoebae in the soil sample had moved onto the *E. coli* and were actively multiplying. When viewed at magnifications of  $\times 100$  and  $\times 400$  amoebic

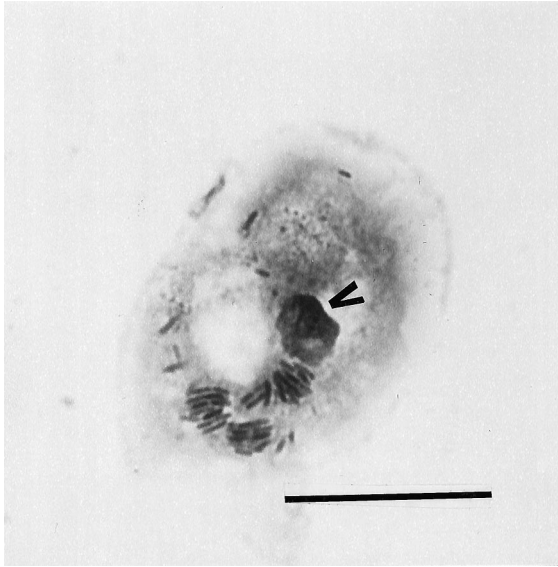


FIG. 1. Giemsa stain showing the occurrence of bacteria in vacuoles after 24 and 48 h of incubation at 25°C. Characteristic morphological features of the amoeba host cell, such as the nucleus (arrowhead), were intact. Bar, 20  $\mu\text{m}$ .

cell structures such as the nucleus and vacuoles were clearly visible. Several amoebae were distinctly swollen and contained numerous bacteria that were highly motile. In addition, the host cell nucleus was not apparent and the vacuole containing

the bacteria occupied most of the amoebic cytoplasmic space. Transferring an amoeba to a monolayer of *A. polyphaga* resulted in an increased number of the bacteria in the culture supernatant, although contaminating bacteria were still present. Subsequently, LLAP-14 was obtained in pure culture with amoebae.

**Microscopic examination of the bacterial lytic cycle.** During the first 24 h of incubation of the amoeba coculture at room temperature, intracellular bacteria were not observed by Giemsa staining. Between 24 and 48 h, Giemsa staining revealed that the bacteria initially accumulated within vacuoles (Fig. 1). At this point in the lytic cycle amoebae retained their ability to adhere to the surface of the flask and retained internal morphological features, such as the nucleus. At 48 h most amoeba trophozoites contained intracellular bacteria, as determined by Giemsa staining and phase-contrast microscopy. The amoeba host cells frequently contained two or three vacuoles filled with the highly motile bacteria. An additional feature was the alignment of mitochondria in proximity to the vacuoles containing bacteria (Fig. 2). Seventy-two to 96 h after inoculation, the infected cells lacked defined organelle structures and the amoebae were no longer adherent to the surface of the flask. Typically, the bacteria remained motile through the end of the lytic cycle and only the cytoplasmic membrane of an amoeba host cell remained intact (Fig. 3). Following lysis of amoeba trophozoites, the bacteria remained viable for at least 7 days, as determined by reinfection of fresh monolayers of amoebae. Approximately  $2.5 \times 10^4$  CFU per ml could be recovered on BCYE after completion of the lytic cycle in amoebae. No intracellular multiplication was observed at 37°C.

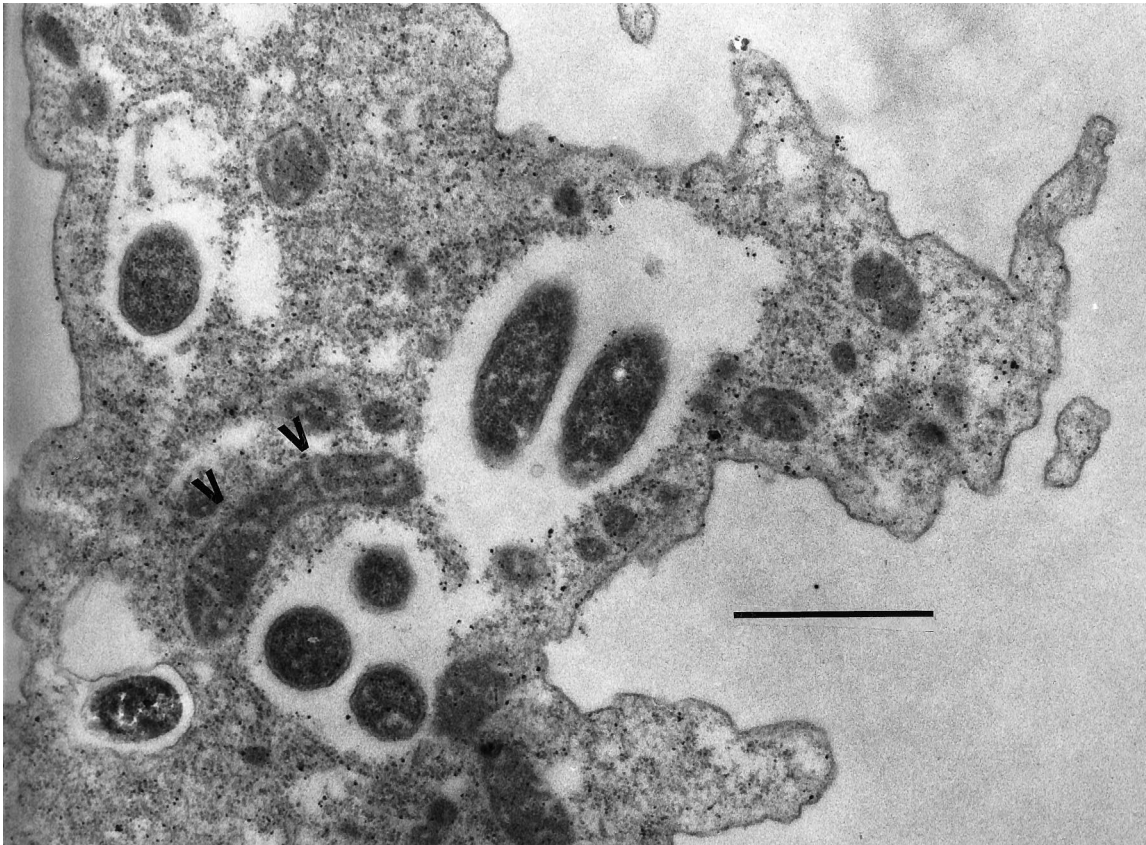


FIG. 2. Alignment of mitochondria (arrowheads) to a vacuole containing bacteria during the early stages (24 to 48 h) of the lytic cycle. Bar, 1.0  $\mu\text{m}$ .

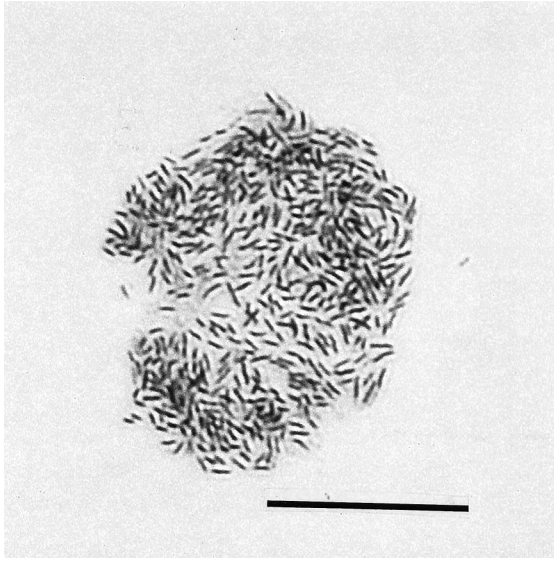


FIG. 3. After 72 h, intracellular multiplication of bacteria was accompanied by loss of the amoeba host cell infrastructure, such as the nucleus and well-defined vacuoles. The bacteria remained bound by the amoeba cytoplasmic membrane. Subsequently, the membrane ruptured, which released the bacteria to infect adjacent amoebae, beginning a new lytic cycle. Bar, 20  $\mu$ m.

**Multiplication on bacteriological media.** The bacteria failed to multiply on TSA and blood agar. Growth was observed, however, on BCYE differential and selective agars at room temperature and 30°C. No growth was observed at 37°C. The colonies exhibited a gray, cut-glass appearance typical of other legionellae. Initial recovery of the bacteria from amoeba co-cultures on BCYE differential and selective agars required 1 to 2 weeks at room temperature and 30°C. When agar-grown colonies were subcultured, the required incubation period decreased to 5 days after several passages. Substantial growth occurred on the BCYE differential medium, which does not contain antibiotics, while diminished growth occurred on the BCYE selective medium, which contains the antibiotics vancomycin and anisomycin. Confirmation that the agar-grown colonies were of the originally isolated bacterium (LLAP-14) was based on re infectivity of the bacteria in *A. polyphaga* and the absence of growth on TSA and blood agar.

**Serology.** No positive reactions were observed when cultures were tested with the Remel *Legionella* Poly ID kit and all 17 pools of *Legionella* antisera maintained at the CDC.

**Phylogenetic analysis.** The phenogram based on 16S rDNA sequences clearly demonstrates that LLAP-14 merits inclusion within the genus *Legionella*, being most closely related to *Legionella shakespearei* (Fig. 4). The homology percentage comparisons showed that LLAP-14 was 97.0% homologous to *L. shakespearei*, 95.5 to 97% homologous to all four species within the same cluster, and 94.2 to 95.8% homologous to all LLAPs and *S. lyticum* (Table 2). Overall, LLAP-14 was between 92.0 and 97.0% homologous to all members of the genus *Legionella*.

**DISCUSSION**

To more completely define the genus *Legionella*, it may be pertinent to identify bacteria that occur naturally within free-living amoebae. The significance of the interaction between *Legionella* species and amoebae is also supported by the suggestion that the description for the genus *Legionella* in *Bergey's*

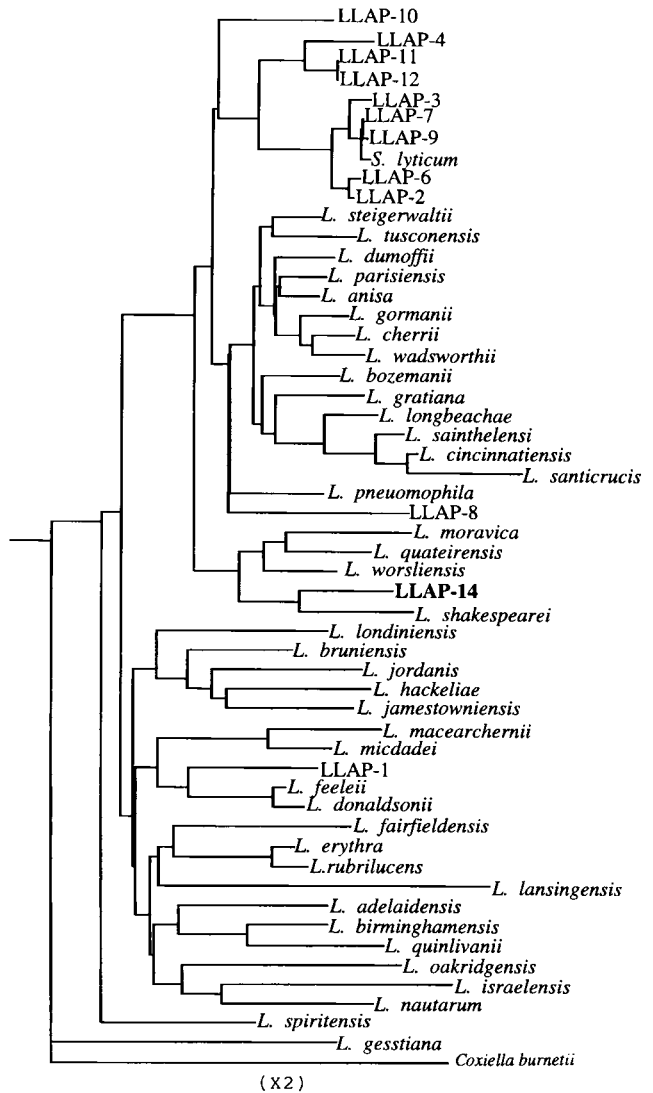


FIG. 4. 16S rDNA-based phenogram reflecting the relationship between LLAP-14 and all other well-resolved species in the genus *Legionella* and in the outgroup *C. burnetii*. Analysis was based on a 1,303-bp region by the neighbor-joining method.

*Manual of Systematic Bacteriology* (26a) be amended to include the following statement: "some legionellae appear to be primary obligate intracellular parasites of amoebae and exhibit little or no growth on current laboratory media" (3). Documentation of naturally occurring bacteria within amoebae strengthens the assumption that certain members of the genus *Legionella* use amoebae for intracellular replication in natural settings. Additional studies of bacterial amoeba pathogens as described in this report will promote our understanding of the genus *Legionella* in both naturally occurring and human-made environments.

The bacterium described in this investigation is a facultative intracellular parasite of the free-living amoeba *A. polyphaga* and proliferates only on media designed to support *Legionella* species. During the early stages of amoebic infection, the alignment of host cell organelles such as mitochondria in proximity to bacteria in enclosed vacuoles is reminiscent of that observed with *L. pneumophila* in both amoebae and macrophages (1, 14,

TABLE 2. 16S rDNA gene homology (%) between members of the genus *Legionella* and other LLAPs

Legionella sp. or LLAP	% Homology <sup>a</sup> with:																
	LLAP-2	LLAP-3	LLAP-4	LLAP-6	LLAP-7	LLAP-8	LLAP-9	LLAP-10	LLAP-11	LLAP-12	LLAP-14	<i>S. lyticum</i>	<i>L. pneumophila</i>	<i>L. shakespearei</i>	<i>L. worliensis</i>	<i>L. quateirensis</i>	<i>L. monavica</i>
LLAP-1	94.1	93.6	93.9	94.0	93.8	94.1	93.7	94.4	94.1	94.1	<b>94.2</b>	93.7	93.8	93.3	94.2	93.6	93.5
LLAP-2		98.8	96.9	99.8	99.4	95.4	99.3	96.6	97.4	97.4	<b>95.8</b>	99.2	95.8	95.3	95.4	95.3	94.1
LLAP-3			96.9	99.0	99.5	94.9	99.5	96.3	97.1	97.1	<b>95.4</b>	99.4	95.8	94.9	95.1	94.9	93.9
LLAP-4				97.0	97.2	95.1	97.1	96.1	98.5	98.5	<b>95.7</b>	97.1	95.5	95.4	95.3	94.7	93.9
LLAP-6					99.3	95.1	99.2	96.8	97.3	97.3	<b>95.7</b>	99.2	95.7	95.1	95.2	95.1	93.9
LLAP-7						95.1	99.9	96.5	97.4	97.4	<b>95.8</b>	99.9	95.9	95.1	95.3	95.3	94.3
LLAP-8							94.9	95.0	95.7	95.7	<b>94.3</b>	94.9	96.1	94.0	94.6	94.0	93.2
LLAP-9								96.4	97.3	97.3	<b>95.7</b>	99.8	95.8	95.0	95.2	95.2	94.2
LLAP-10									96.4	96.4	<b>95.7</b>	96.4	96.7	94.9	96.8	96.5	95.0
LLAP-11										100.0	<b>95.8</b>	97.2	96.4	95.3	96.2	95.4	94.2
LLAP-12											<b>95.8</b>	97.2	96.4	95.3	96.2	95.4	94.2
LLAP-14												<b>95.7</b>	<b>95.2</b>	<b>97.0</b>	<b>96.0</b>	<b>96.3</b>	<b>95.5</b>
<i>S. lyticum</i>													95.8	95.0	95.1	95.2	94.2
<i>L. pneumophila</i>														95.1	96.6	96.5	95.3
<i>L. Shakespearei</i>															95.9	96.4	95.3
<i>L. worliensis</i>																97.8	96.5
<i>L. quateirensis</i>																	97.0

<sup>a</sup> Percentages were rounded to the nearest 1/10. Boldface denotes LLAP-14 homology percentages.

26, 28). The multiplication and accumulation of the bacteria in amoebae and the corresponding loss of amoeba intracellular structures are features commonly observed in in vitro studies of *Legionella*-infected amoebae. The probability that this bacterium represents a member of the genus *Legionella* was confirmed by the phylogenetic analysis of its 16S rDNA. The 92.0 to 97.0% homology of LLAP-14 compares well with the 16S rDNA sequence identity (90.2 to 99.1%) that exists between the 39 validly described members of the genus (3). The 16S rDNA sequence over 1,303 bases was distinctive (maximum homology, 97.0%), and it may represent a new species in the genus *Legionella*. Sequence analysis of 16S rDNA was useful for establishing relationships; however, it should be used with caution to conclusively denote species identity (18). Species distinction of LLAP-14 and the LLAP-type bacteria would be best addressed by DNA-DNA hybridization studies of members of the genus *Legionella* (33). Based on serological analysis, the lack of reactivity with a battery of immune sera to previously described *Legionella* species additionally suggests that the bacterium LLAP-14 is an undescribed member of the genus *Legionella*.

The potential of *Legionella* bacteria that are also amoebic pathogens to cause disease in humans is best demonstrated with *L. pneumophila*. Infection of human cells with *Legionella* species was shown to be related to the ability of the bacteria to infect protozoa (15). It has been suggested that this ability may serve to enhance the virulence of *Legionella* and have importance in the pathogenic mechanism of the bacteria (10, 17, 21). For example, *Legionella* species such as *L. parisiensis* and *L. jamestowniensis* were originally not known to cause disease in humans. In vitro studies, however, demonstrated multiplication in macrophage-like cells (29). This result supported the concept that they could be human pathogens, which was recently confirmed when *L. parisiensis* was associated with pneumonic illness (30). One line of evidence indicates that multiplication of *Legionella* species in amoebae and the inability to multiply in human phagocytes does not preclude the possibility of causing human illness. For example, *Legionella anisa* readily

multiplied in the amoeba *Hartmannella vermiformis* but not in human monocytes or U937 cells (16). However, *L. anisa* has been shown to cause Pontiac fever, which results from exposure to high levels of nonviable bacterial cells or bacteria unable to multiply in lung phagocytes (16). The inability of LLAP-14 to multiply at 37°C under laboratory conditions suggested that it would not be a highly virulent pathogen of humans or other endothermic animals, although the potential for exposure and disease cannot be discounted at this time. Bacteria in the genus *Legionella* infect a range of eukaryotic cells. This includes cells of the monocytic cell lineage, numerous amoeba species of different genera, and some protozoan ciliates as well (14, 26, 29). It is thus likely that the host range of LLAP-14 extends beyond the infected amoebae in the original soil sample and the laboratory cultures of *A. polyphaga*. Establishment of the host range of this bacterium and related *Legionella*-like amoebic pathogens would contribute to characterization and classification to species level. Multiplication in cells of monocytic lineage might also help establish any clinical relevance. These wide-ranging future studies would likely lead to descriptive amendments for the genus *Legionella*.

Finally, increased scientific awareness along with methods useful for the isolation and characterization of amoebic pathogens may serve to promote studies identifying any role these bacteria have on the distribution and occurrence of free-living amoebae in natural settings. Historically, the little information known about amoeba population dynamics has been based on observations of physical factors, food availability, and possible predation by micro- and macroinvertebrates. Protozoa such as amoebae are the significant predators of bacteria in both soil and aquatic environments (11, 22, 35). Amoebic consumption of prokaryotes is believed to play an important role in nutrient cycling and to be of practical importance for agriculture. The influence that these newly described amoebic pathogens may have on free-living amoebae in natural settings is presently open to speculation. The understanding of relative occurrence and host range of amoeba pathogens can provide insight into alternative means by which populations of amoebae are

influenced by bacteria which invade and destroy their host cells.

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