Gamma-Proteobacteria *Aquicella lusitana* gen. nov., sp. nov., and *Aquicella siphonis* sp. nov. Infect Protozoa and Require Activated Charcoal for Growth in Laboratory Media

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Several isolates, belonging to two new species of the same novel genus of gamma-proteobacteria, were recovered from drilled well (borehole) and spa water at São Gemil in central Portugal. These organisms are phylogenetically most closely related to the strictly intracellular uncultured species of the genus *Rickettsiella*, which cause disease in arthropods, and to the facultatively intracellular species of the genus *Legionella*, some of which cause Legionnaires' disease and Pontiac fever. The São Gemil strains grew only on media containing charcoal, as is also true of the species of the genus *Legionella*. Unlike the vast majority of *Legionella* isolates, the new isolates did not require L-cysteine or ferric pyrophosphate for growth but like the legionellae had an absolute requirement for α -ketoglutarate. Strains SGT-39^T and SGT-56 grew consistently between 30 and 43°C, while strains SGT-108^T and SGT-109 grew between 30 and 40°C. The pH ranges for growth of these organisms were surprisingly narrow: strains SGT-39^T and SGT-56 grew between pH 6.3 and 7.3, while strains SGT-108^T and SGT-109 grew between pH 6.3 and 7.0. Both organisms proliferated in the amoeba *Hartmannella vermiformis* but did not grow in U937 human cells. Based on 16S rRNA gene sequence analysis and physiological, biochemical, and chemical analysis we describe two new species of one novel genus; one species is represented by strain SGT-39^T, for which we propose the name *Aquicella lusitana*, while strain SGT-108^T represents a second species of the same genus, for which we propose the name *Aquicella siphonis*.

Some organisms classified in the γ -subclass of the *Proteobacteria* have an intracellular life cycle, and some of them have been grown only in animals, embryonated yolk sacs, or animal cell lines. These strictly intracellular parasites include *Coxiella burnetii*, which infects several invertebrates and vertebrates, including humans, where this organism is the etiological agent of Q fever (42). The three species of the genus *Rickettsiella*, namely, *R. grylli*, *R. chironomi*, and *R. popilliae*, are also obligate intracellular parasites of arthropods such as insects, arachnids, and crustaceans (35, 42), but very little is known of their physiology and biochemical characteristics.

The species of the genus *Legionella* are, on the other hand, intracellular parasites of protozoa and animal cells that also grow on laboratory media. Some species of this genus, e.g., *L. pneumophila*, cause Legionnaires' disease or a milder form of disease known as Pontiac fever (15, 20). However, most of the 48 validly described species of the genus *Legionella* have not been implicated in disease (1, 28); they were isolated from man-made or natural aquatic environments with buffered charcoal-yeast extract-based (BCYE) media (12). The requirement of species of *Legionella* for charcoal, L-cysteine, and ferric ions is well known, but the reason for these requirements remains largely unidentified. Charcoal may protect the organisms from

oxygen radicals, to which they appear to be extremely sensitive, and, as is probably the case for cysteine, serves as an essential nutrient (16, 17). Most legionellae grow readily on BCYE, but it has been known for some time that many, if not all, species of the genus *Legionella* are, in fact, intracellular parasites of protozoans (1, 13, 36). The species *L. lytica*, for example, has been known to be an intracellular parasite of amoebae since 1956 (10, 11) but was recognized as a species of the genus *Legionella* much later (18). Recently, the new species *L. drozanskii*, *L. rowbothamii*, and *L. fallonii* were isolated from amoebae obtained from several aqueous sources, reinforcing the hypothesis that these organisms are primarily intracellular parasites of protozoans (1) or, as animal pathogens, are intracellular parasites of macrophages.

Natural geothermal areas are known to be extensively colonized by species of the genus *Legionella* (25, 40). Among the hot springs and runoffs examined were the natural vents along the shallow river bed at São Gemil in central Portugal. During routine follow-up quality control enumeration of legionellae in the water from a drilled well (borehole) and from the spa at São Gemil, required by Portuguese law, we recently isolated several organisms on BCYE containing glycine, vancomycin, polymyxin B, and cycloheximide (GVPC) with colony morphology indistinguishable from the pinkish or bluish colonies with ground-glass texture of *Legionella* species. Fatty acid analysis indicated that the isolates belonged to two distinct species that were not included in our *Legionella* fatty acid database and could belong to new species of this genus (8) or to species of

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other genera. 16S rRNA gene sequence analysis showed that the organisms represented two species of a novel genus whose closest taxonomically defined relatives are the unculturable species of the genus *Rickettsiella*.

On the basis of 16S rRNA gene sequence analysis and physiological, biochemical, and molecular genetic analyses we are of the opinion that strain SGT-39^T represents a new species of a novel genus for which we propose the name *Aquicella lusitana* (CIP 107650, LMG 21647), while strain SGT-108^T represents a second species of the same novel genus, for which we propose the name *Aquicella siphonis* (CIP 107651, LMG 21648).

MATERIALS AND METHODS

Isolation, bacterial strains, and culture conditions. Strains SGT-39^T, SGT-53, SGT-56, SGT-107, SGT-108^T, SGT-109, and SGT-110 were recovered from water samples taken from the borehole and therapeutic spa located at São Gemil in central Portugal. Water samples were transported to the laboratory at ambient temperature and filtered through 45-mm-diameter membrane filters (Gelman; Supor 200; 0.2- μ m pore size) within 12 h of sampling. The filters were placed in small sterile plastic bags containing 10 ml of the original water. The bags containing the filters were rubbed manually for a few minutes to remove organisms from the filters; one portion of the sample (0.1 ml) was directly placed on the surface of BCYE medium (12) containing GVPC (6) and spread over the entire surface. Another part of the sample was acid treated (3.9 ml of 0.2 M HCl–25 ml of 0.2 M KCl, pH 2.2) for 5 min at room temperature and then spread (0.1 ml) over BCYE-GVPC plates (2), while a third portion of the sample was heat treated at 50°C for 30 min and then spread over plates of the same medium (7). The plates were incubated at 37°C for up to 9 days. Cultures were purified by subculturing on BCYE and were maintained at –70°C in 5% (wt/vol) yeast extract with 15% (vol/vol) glycerol. Type and reference strains of *Legionella* spp. *L. pneumophila* (ATCC 43109), *L. anisa* (ATCC 35292^T), *L. oakridgensis* (ATCC 33761^T), *L. bozemanae* (ATCC 33217^T), *L. longbeachae* (ATCC 33462^T), and *L. micdadei* (ATCC 33218^T) were obtained from the American Type Culture Collection, Manassas, Va., and one *L. pneumophila* strain isolated from São Gemil, designated SGM-50, were also used for comparative purposes.

Several media were tested in an attempt to grow the SGT strains and facilitate their characterization. Common microbiological media, namely, nutrient agar, tryptic soy agar, Mueller-Hinton agar, and brain heart infusion agar (Difco), were buffered with 30 mM ACES (*N*-[2-acetamido]-2-aminoethanesulfonic acid) to pH 6.7 and were used without activated charcoal or with the addition of 2.0 g of activated charcoal liter⁻¹. Growth on Columbia III agar with 5% sheep blood (Becton Dickinson, Sparks, Md.) and chocolate agar (Becton Dickinson) was also examined. Growth was also attempted on two low-nutrient media commonly used to isolate and grow organisms from thermal sources, medium 630 (Deutsche Sammlung von Mikroorganismen und Zellkulturen; www.dsmz.de/media/med630.htm) and *Thermus* medium (www.dsmz.de/media/med878.htm), which are very similar except for the concentrations of yeast extract and tryptone (5, 44). Both media were adjusted to pH 6.7 with ACES, and growth with and without charcoal (2.0 g liter⁻¹) was examined.

Growth of these organisms on several modified forms of the basic BCYE medium was also examined; the modifications included the omission of charcoal, L-cysteine, α -ketoglutarate, and ferric pyrophosphate individually or in combination. Growth in a medium identical to BCYE where yeast extract was replaced by 3.0 g of tryptone or Casamino Acids liter⁻¹ was also examined.

A liquid medium was also devised to access growth parameters of these organisms. This liquid medium, designated BYE-L, was based on the composition of BCYE as follows. Agar (12.5 ml, 1.5%; Difco) containing charcoal (2.0 g liter⁻¹) was autoclaved in 50-ml wide-mouth screw-cap tubes. The charcoal agar was allowed to solidify, and a thin layer (2.5 ml) of autoclaved molten 1.5% agar was overlaid on the charcoal agar layer. This layer was also allowed to solidify. Over this layer 12.5 ml of the yeast extract–L-cysteine liquid medium (BCYE without charcoal) was poured, leaving a headspace of about 20 ml. The culture bottles were stored overnight to allow the BYE-L to equilibrate in all layers. The final concentration of nutrients was approximately the same as in the solidified BCYE medium. The agar layer between the liquid medium prevented the resuspension of charcoal particles in the liquid medium. The charcoal agar layer at the bottom of the tubes was necessary for growth, since none of the SGT strains

or those of the genus *Legionella* examined grew without the charcoal. The cultures were incubated at 37°C without shaking.

Morphological, biochemical, and tolerance characteristics. Cell morphology and motility were examined under phase-contrast microscopy after cultivation on BCYE. Growth in the temperature range of 22 to 50°C on BCYE and BYE-L medium was examined for 5 days. The plates were wrapped in plastic bags and incubated submerged in water baths that controlled the temperature within 0.1°C. The liquid cultures were also placed in water baths, and growth was examined by measuring the turbidity (optical density at 610 nm). The pH range for growth was determined at 37°C in BCYE and BYE-L buffered with 30 mM MES (2-[*N*-morpholino]ethanesulfonic acid) for pH values between 5.5 and 6.5, in ACES for pH values between 6.0 and 7.5, in HEPES for pH values between 6.5 and 8.2, and in TAPS (*N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid) for pH values between 7.5 and 9.0. Control media containing each buffer adjusted to pH 6.7 were used to assess possible inhibitory effects of the buffering agents.

Catalase, cytochrome oxidase, and the reduction of nitrate were determined as described by Smibert and Krieg (38). The hydrolysis of hippurate and casein was examined as described previously (41). Other enzyme activities were determined at 37°C with the API ZYM test system (BioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Anaerobic growth on BCYE, blood agar, and chocolate agar at 37°C in anaerobic chambers containing a H₂–CO₂ atmosphere (GENbox anaer; BioMérieux) was examined. Microaerobic growth in the same medium was examined with a CO₂ generator (GENbox microaer; BioMérieux). Autofluorescence under long-wave UV light (365 nm) was examined after growth on BCYE for 3 and 5 days.

Assimilation of glucose in BYE-L medium containing 5.0 g of filter-sterilized glucose liter⁻¹ was assessed. The glucose was allowed to equilibrate at room temperature for 24 h in all layers of the tubes. The tubes were inoculated to give an initial turbidity of about 0.05 (optical density at 610 nm). Two portions of the cultures were taken immediately after inoculation and frozen at –20°C for later analysis of the initial glucose concentration. Duplicate portions were also removed from the same tubes after 5 days of incubation. These samples were centrifuged, and glucose consumption in the supernatants was estimated with a glucose oxidase-peroxidase test kit (510-DA; Sigma-Aldrich).

Growth on glucose, fructose, pyruvate, succinate, malate, citrate, oxaloacetate, asparagine, glutamine, and Casamino Acids (all filter sterilized and at a final concentration of 1.0 g liter⁻¹) in BCYE medium without α -ketoglutarate was also examined.

Survival of the organisms in water maintained at 37 (as control), 48, 55, and 60°C was estimated as follows. Water from the borehole (drilled well) at São Gemil was filtered (Gelman; Supor 200; 0.2- μ m pore size), and 15 ml was dispensed in metal-capped tubes. Three-day-old cultures were resuspended in this sterile water to a turbidity equivalent to the McFarland no. 1 standard (38). The tubes were incubated in temperature-controlled water baths for up to 48 h. Samples were taken immediately after inoculation and after 1, 3, 6, 10, 24, and 48 h. Survival was determined by spreading 0.1-ml portions directly from the suspension on BCYE and from serial dilutions in sterile borehole water. CFU were determined after incubating the cultures at 37°C for 3 days.

Growth of the organisms in *H. vermiformis* and human lymphoma cells (U937). *H. vermiformis* was grown as previously described (14). Cocultures of this amoeba with strains SGT-39^T and SGT-108^T (10 ml) were prepared in 25-ml tissue culture flasks. The number of amoebae was adjusted to 1.4×10^5 ml⁻¹, and the cocultures were incubated at 35°C. Control flasks consisted of bacteria alone in the same medium. U937 cells were grown as previously described in RPMI 1640 medium supplemented with 10% fetal calf serum without antibiotics (14, 29). Cocultures were prepared as described above with U937 cells adjusted to 4×10^6 liter⁻¹. Samples were obtained immediately after the preparation of the cocultures and at appropriate time intervals within 7 days. CFU were enumerated on BCYE after incubation at 37°C for up to 7 days.

Antibiotic sensitivity. Antibiotic susceptibility of strains SGT-39^T, SGT-56, SGT-108^T, and SGT-109 to disks (BioMérieux) containing ampicillin (10 μ g), carbenicillin (100 μ g), cephalothin (30 μ g), cefazolin (30 μ g), chloramphenicol (30 μ g), ciprofloxacin (5 μ g), doxycycline (30 μ g), erythromycin (15 μ g), gentamicin (10 μ g), kanamycin (30 μ g), lincomycin (2 μ g), nalidixic acid (30 μ g), neomycin (30 μ g), ofloxacin (5 μ g), penicillin G (10 U/IE), polymyxin B (300 U/IE), rifampin (30 μ g), streptomycin (10 μ g), tetracycline (30 μ g), and vancomycin (30 μ g) on BCYE at 37°C was examined for 72 h.

Polar lipid, lipopeptide, and fatty acid composition. The cultures used for polar lipid analysis were grown in BCYE for 72 h. The harvesting of the cultures and extraction of lipids were performed as described previously (9, 32). The individual polar lipids were separated by monodimensional thin-layer chromatography (TLC) on silica gel G plates (Merck; 0.25 mm thick) with a solvent

system consisting of chloroform-methanol-acetic acid-water (80:12:15:4 by volume). Lipoquinones were extracted from freeze-dried cells, purified by TLC, and separated with a Gilson high-performance liquid chromatography apparatus by using a reverse-phase column (RP18; Spherisorb; S5 ODS2) with methanol-heptane (10:2, vol/vol) as the mobile phase and were detected at 269 nm (39).

Cultures for fatty acid analysis were grown on plates of BCYE agar incubated in sealed plastic bags at 37°C for 72 h. Fatty acid methyl esters were obtained from fresh wet biomass by saponification, methylation, and extraction as described previously by Kuykendall et al. (22) and separated, identified, and quantified with the MIS library generation software (Microbial ID Inc., Newark, Del.).

RAPD and analysis of *mip*, *dotA*, and *traA*-like genes. Crude cell lysates were used as DNA template for random amplified polymorphic DNA (RAPD) analysis as described by Wiedmann-al-Ahmad et al. (43). Amplification reactions were performed with a total volume of 50 μ l containing 1.5 U of *Taq* polymerase, 1.5 mM MgCl₂ (Pharmacia Biotech), 0.2 mM (each) deoxynucleoside triphosphate, 0.6 μ M primer OPA3 (5'-AGTCAGCCAC-3'), and 2.0 μ l of crude cell lysates. Samples were subjected to 45 cycles of amplification (Perkin-Elmer; model 240) as follows: 1 min at 94°C, 1 min at 34°C, and 2 min at 72°C, followed by a final extension step of 7 min at 94°C. The fragments were analyzed by electrophoresis in 2% agarose gel in Tris-acetate-EDTA buffer.

The isolation of chromosomal DNA from the SGT strains of *L. pneumophila* and *L. anisa* to assess the presence of *mip*, *dotA*, and *traA*-like genes was as described by Rainey et al. (33). Primers MIPF and MIPR were used to amplify part of the *mip* gene as described previously (34). Primers for amplification of a fragment of the *dotA* gene were those used by Ko et al. (21). Primers based on the *traA*-like sequence of *L. pneumophila* were used to detect the presence of this gene in the new organisms (31). A pair of degenerate primers, TF (5'-CTBGT HGGHGAYCCGSAWCAG-3') and TR (5'-GTCRBSGCATARCCGTRRTCS A-3'), was also designed based on sequence alignment of the *traA*-like gene (GenBank accession no. AF315650) from *L. pneumophila* (31) and gene homologs from *Mesorhizobium loti* (BAB48437), a *Rhizobium* sp., and *Agrobacterium tumefaciens* (23). PCR amplifications were carried out in a Perkin-Elmer GeneAmp PCR System 2400, in reaction mixtures of 50 μ l containing 100 ng of DNA from each strain, 200 ng of each primer, 10 mM Tris-HCl (pH 9.0), 2.5 mM MgCl₂, 50 mM KCl, 1 U of *Taq* DNA polymerase (Promega), and 0.2 mM (each) deoxynucleoside triphosphate. *dotA* fragments (0.4 kb), *mip* fragments (0.6 to 0.7 kb), and *traA*-like fragments (0.6 kb) were visualized after agarose gel electrophoresis. PCR conditions were as follows: initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and primer extension at 72°C for 1 min. The extension reaction in the last cycle was prolonged for 7 min.

Determination of G+C content of DNA and 16S rRNA gene sequence determination and phylogenetic analyses. The DNA for the determination of the guanine-plus-cytosine (G+C) content of the DNA was isolated as described by Cashion et al. (3). The G+C content of DNA was determined by high-performance liquid chromatography as described by Mesbah et al. (26). The extraction of genomic DNA for 16S rRNA gene sequence determination, PCR amplification of the 16S rRNA gene, and sequencing of the purified PCR products were carried out as described previously (33). Purified reaction mixtures were electrophoresed with a model 310 genetic analyzer (Applied Biosystems, Foster City, Calif.). The 16S rRNA gene sequences determined in this study were aligned against representative reference sequences of members of the *Proteobacteria* with the ae2 editor (24). The method of Jukes and Cantor (19) was used to calculate evolutionary distances. Phylogenetic dendrograms were generated with various treeing algorithms contained in the PHYLIP package (J. Felsenstein, PHYLIP [phylogenetic inference package], version 3.5.1, Department of Genetics, University of Washington, Seattle, 1993).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences for strains SGT-39^T, SGT-108^T, and SGT-109 are deposited with EMBL under accession numbers AY359282, AY359283, and AY359284, respectively.

RESULTS

Isolation of strains and physiological, tolerance, and biochemical characteristics of the SGT organisms. Organisms with a colony morphology identical to that of the species of *Legionella* were isolated on four occasions, between March and July 2000, from the drilled well (borehole) and the spa at São Gemil on BCYE-GVPC medium. The borehole, designated HDN1, had been drilled into a granitic stratification to a depth

of 91.0 m, with additional water intakes between 61 and 67 m and between 76 and 82 m. Water vents at a temperature of 48°C and pH 8.4; the two sites within the spa had water temperatures of 37 to 40°C and a pH of 8.0. The isolates were recovered from samples that were acid treated and heat treated and from untreated samples. The organisms were gram-negative nonmotile rod-shaped cells with filaments on BCYE. Colonies were whitish with a pinkish or bluish sheen and a ground-glass texture under a dissecting microscope. We did not observe bluish white or red autofluorescence in the SGT strains after 3 or 5 days of incubation at 37°C on BCYE. These organisms produced colonies that were indistinguishable from those of the species of *Legionella* and were initially believed to be isolates of this genus. Strains SGT-39^T, SGT-56, SGT-108^T, and SGT-109 grew on BCYE and required α -ketoglutarate and charcoal for growth but also grew in BCYE medium lacking L-cysteine and ferric pyrophosphate. The organisms grew on Degryse medium 162 buffered to pH 6.7 and supplemented with charcoal and α -ketoglutarate, but not in *Thermus* medium. None of the strains grew on any of the other media tested with or without charcoal. The addition of α -ketoglutarate or pyruvate to BCYE or Degryse medium 162 was required for growth of the São Gemil strains. These organisms did not grow on BCYE where these organic acids were replaced by glucose, fructose, asparagine, glutamine, succinate, malate, citrate, oxaloacetate, or Casamino Acids. Control cultures of *L. pneumophila*, *L. bozemaniae*, and *L. oakridgensis* grew on BCYE where α -ketoglutarate was replaced with pyruvate, succinate, and oxaloacetate (Table 1). Strains SGT-108^T and SGT-109, unlike strains SGT-39^T and SGT-56, grew in buffered charcoal-L-cysteine- α -ketoglutarate medium in which yeast extract was replaced by tryptone (3.0 g liter⁻¹).

Strains SGT-39^T and SGT-56 grew consistently between 30 and 43°C, while strains SGT-108^T and SGT-109 grew between 30 and 40°C, but none of the strains grew at 25 or 45°C after 7 days of incubation of BCYE plates. The pH range for growth of strains was surprisingly narrow; strains SGT-39^T and SGT-56 grew on BCYE plates between pH 6.3 and 7.3, while strains SGT-108^T and SGT-109 grew on BCYE plates between pH 6.3 and 7.0. Growth was not observed at pH 6.0 or 7.5. Some of the type strains of the genus *Legionella* examined in this study also had very narrow pH ranges for growth. For example, the pH ranges for growth of *L. anisa* (6.5 to 7.3) and *L. oakridgensis* (6.5 to 7.7) were also unexpectedly narrow (results not shown).

We also devised a liquid medium, designated BYE-L, based on the nutrient composition of BCYE, to obtain more-precise results on the growth temperature range and pH range. This medium favored the growth of several species of *Legionella* and the SGT organisms (Table 1), and the results obtained with the BYE-L medium confirmed those with BCYE agar.

The two organisms were catalase and cytochrome oxidase negative. Casein was degraded by strain SGT-108^T but was not degraded by strain SGT-39^T, and the hydrolysis of other proteinaceous substrates could not be observed on the BCYE medium because of the charcoal. Hippurate was hydrolyzed by both strains. Strains SGT-39^T and SGT-108^T had identical enzyme activities in the API ZYM tests (Table 1). The organisms did not grow under anaerobic conditions, but grew microaerobically. Nitrate was not reduced. Glucose was not as-

TABLE 1. Phenotypic characteristics that distinguish SGT strains from *Legionella* spp.

Characteristic	Result ^a for:						
	SGT-39 ^T	SGT-56	SGT-108 ^T	SGT-109	<i>L. bozemanae</i>	<i>L. oakridgensis</i>	<i>L. pneumophila</i>
Growth on:							
BCYE-INC ^b	+	+	+	+	—	+	—
BCYE with pyruvate ^c	+	+	w	w	+	+	+
BCYE with succinate ^c	—	—	—	—	+	+	+
BCYE with oxaloacetate ^c	—	—	—	—	+	+	+
BCYE with tryptone ^d	—	—	+	+	ND	ND	ND
Growth on BYE-L ^e (mean final turbidity)	0.430	0.412	0.320	0.345	0.585	0.605	0.820
Hydrolysis of:							
Casein	—	—	w	w	+	+	+
Hippurate	+	+	+	+	—	—	+
Presence of:							
α-Chymotrypsin	+	+	—	—	—	—	—
Catalase	—	—	—	—	+	+	—
Cystine arylamidase	—	—	—	—	+	+	+

^a +, positive result or growth; w, weakly positive; —, negative result or no growth; ND, not determined. Alkaline and acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, and naphthol-AS-BI-phosphohydrolase activities were present in all organisms. All strains are oxidase negative. Arbutin, esculin, and tryptophan were not hydrolysed by any of the organisms; trypsin, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, β-galactosidase, α-glucosidase, and β-glucosidase were negative in all strains.

^b BCYE without L-cysteine and ferric pyrophosphate.

^c BCYE where α-ketoglutarate was replaced with succinate, pyruvate, or oxaloacetate.

^d BCYE where yeast extract was replaced by 3.0 g of tryptone liter⁻¹.

^e Initial turbidity of 0.05 ± 0.01 at 37°C. Final turbidity was assessed after 3 days at 37°C.

simulated from BYE-L under aerobiosis, nor was acid produced when the cultures were incubated under microaerophilic or anaerobic conditions, indicating that this sugar was not fermented. The organisms were sensitive to all the antibiotics tested except vancomycin, polymyxin B, and lincomycin.

The recovery of strains SGT-39^T and SGT-108^T incubated in borehole water decreased by 29% over 48 h at 37°C. At incubation temperatures of 48 and 55°C the organisms were not recovered after 10 and 6 h, respectively, nor could the organisms be recovered after 1 h at 60°C (results not shown).

Intracellular growth in *H. vermiformis* and the human U937 cell line. The intracellular growth of strain SGT-39^T in *H. vermiformis*, estimated as the increase in CFU after 7 days incubation at 37°C, was approximately fourfold; the number of CFU increased from $5.4 \times 10^2 \pm 78 \text{ ml}^{-1}$ to $2.27 \times 10^3 \pm 183 \text{ ml}^{-1}$ after 7 days of coculture in *H. vermiformis*. The growth of strain SGT-108^T in this amoeba was similar, with CFU increasing from $2.4 \times 10^2 \pm 57 \text{ ml}^{-1}$ to $9.3 \times 10^2 \pm 150 \text{ ml}^{-1}$. Neither organism was recovered from the medium without amoebae after 3 days. Both strains became undetectable in coculture with the human lymphoma U937 cell line after 7 days of incubation.

Polar lipid, lipoquinone, and fatty acid composition. Diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylcholine dominated the polar lipid composition of the São Gemil strains. Ubiquinone 11 (Q11) was the major respiratory quinone of the SGT-39^T and SGT-108^T strains, followed by smaller amounts of Q9, Q10, and Q12 (results not shown). The predominant fatty acid of the SGT organisms was 17:0 iso, although the concentration was lower in the species represented by SGT-39^T, SGT-56, and SGT-110 (14 to 21%) than in the species represented by strains SGT-108^T and SGT-109 (38 and 40%). Anteiso fatty acids were detected in vestigial amounts in these organisms. Mono-unsaturated fatty acids, namely, 17:1 ω₉c iso, 11 methyl 18:1 ω₇c, and 19:1 ω₉c, were also the predominate acyl components

of the São Gemil organisms. Hydroxy fatty acids, such as iso 13:0 3OH, iso 15:0 3OH, and iso 19:0 3OH, were moderate fatty acid components of these organisms and were helpful in distinguishing the two species from each other (Table 2). The two species could be easily distinguished from each other by the presence of iso 19:0 3OH in strains SGT-39^T, SGT-56, and SGT-110 and the presence of 11 methyl 18:1 ω₇c in strains SGT-108^T and SGT-109. One unknown fatty acid with an equivalent chain length (ECL) of 17.495 was also a prominent component of the acyl chains of the SGT organisms but was not identified.

RAPD and *mip*, *dotA*, and *traA*-like gene analysis. RAPD analysis indicated that strains SGT-39^T, SGT-53, SGT-56, SGT-107, and SGT-110 belonged to the same clone despite having been isolated on four different occasions from the spa and the borehole. On the other hand, strains SGT-108^T and SGT-109 represented two clones isolated on one occasion from the borehole (Fig. 1). The *mip*, *dotA*, and *traA*-like genes were not encountered in the SGT strains by PCR amplification. Our results confirmed the presence of *mip* gene sequences in *L. pneumophila* (ATCC 43109), *L. pneumophila* SGM-50, and *L. anisa* (Fig. 2). The *dotA* gene was detected in the *L. pneumophila* strains examined (Fig. 2). The *traA*-like gene was detected in *L. pneumophila* but not in the new organisms (results not shown).

16 S rRNA gene sequence comparison and G+C content of DNA. Almost complete 16S rRNA gene sequences comprising 1,490 and 1,482 nucleotides were determined for strains SGT-39^T and SGT-108^T, respectively. Comparison of these sequences with representatives of the main lines of descent within the domain *Bacteria* indicated that these strains were members of the γ-subclass of the *Proteobacteria* and were most closely related to the lineage containing *Legionella* and *Rickettsiella* species (Fig. 3). The pairwise 16S rRNA gene sequence similarity between strains SGT-39^T and SGT-108^T was 94.0%. The 16S rRNA gene sequence showing highest pairwise simi-

TABLE 2. Mean fatty acid compositions of strains SGT-39^T, SGT-56, SGT-110, SGT-108^T, and SGT-109 after growth in BCYE agar

Fatty acid ^a	Fatty acid composition (%) of:				
	SGT-39 ^T	SGT-56	SGT-110	SGT-108 ^T	SGT-109
12:0 3OH	0.5	1.0	0.4	— ^f	—
i13:0 3OH	5.6	5.9	3.4	2.0	1.7
13:0 2OH	0.4	0.4	0.2	—	—
i15:0	2.6	2.7	2.3	2.0	2.1
15:0	—	—	—	0.2	0.4
i16:0	0.4	0.3	0.4	0.3	0.7
Feature 4 ^b	1.4	2.9	1.6	0.3	0.2
16:0	2.0	3.1	2.3	3.2	3.1
i15:0 3OH	0.4	0.5	0.3	4.6	4.7
i17:1 ω9c	9.5	11.1	8.8	14.3	15.0
Feature 5 ^c	0.8	0.5	0.4	0.6	0.4
i17:0	21.0	14.2	15.3	38.2	39.7
a17:0	1.1	0.9	1.2	1.4	1.2
Cyclo17:0	7.0	11.1	9.3	1.1	0.8
17:0	1.8	1.9	2.0	5.2	4.9
Unknown ^d	11.1	6.9	11.8	12.4	12.0
i18:0	0.6	0.7	0.7	0.4	0.9
Feature 7 ^e	1.8	3.4	1.8	0.6	0.2
i17:0 2OH	0.4	0.3	0.5	—	0.2
i18:1 ω5c	0.5	0.3	0.5	0.2	0.1
18:0	4.5	6.2	7.8	4.2	4.2
11 methyl 18:1 ω7c	—	—	—	2.6	2.6
i19:1 ω9c	4.5	3.2	3.3	1.1	0.7
i19:0	6.7	5.3	8.8	2.6	1.5
a19:0	0.8	0.9	1.0	0.2	0.2
Cyclo 19:0 ω8c	5.2	4.7	6.1	1.7	1.5
19:0	0.9	0.7	1.3	—	0.4
20:0	0.4	0.4	0.5	—	0.1
i19:0 3OH	6.1	6.6	5.8	—	—

^a The fatty acids shown are those present at ≥0.4% of the total in at least some of the strains.

^b Group of fatty acids including 16:1 ω7c and/or i15:2 OH.

^c Group of fatty acids including i17:1 I and/or a17:1 B.

^d Unknown fatty acid with ECL of 17.495.

^e Group of fatty acids including 18:1 ω7c and/or 18:1 ω9c and/or 18:1 ω12c.

^f —, not detected.

larity (92%) to the sequences of the new isolates was that of an unpublished environmental 16S rRNA gene sequence (GenBank accession no. AJ252618) recovered from agricultural soil in which transgenic and nontransgenic potato plants had been grown. Similarities to previously described taxa of the genera *Legionella* and *Rickettsiella* were >90%. The DNA of strain

FIG. 1. Agarose gel electrophoresis of RAPD. Lane 1, marker IV (Roche Molecular Biochemicals); lanes 2 to 8, products generated with primer OPA3 from DNA of SGT-39^T (lane 2), SGT-53 (lane 3), SGT-56 (lane 4), SGT-107 (lane 5), SGT-110 (lane 6), SGT-108^T (lane 7), and SGT-109 (lane 8).

FIG. 2. Agarose gel electrophoresis of PCR-amplified partial *mip* and *dotA* genes. Lane 1, marker IV (Roche Molecular Biochemicals); lanes 2 to 6, products generated with primers LegmipF and LegmipR (34) from DNA of *L. pneumophila* (ATCC 43109) (lane 2), *L. pneumophila* SGM-50 (lane 3), *L. anisa* ATCC 35292^T (lane 4), SGT-39^T (lane 5), and SGT-108^T (lane 6); lanes 7 to 11, products amplified with primers DL1 and DL2 (21) from DNA of *L. pneumophila* (lane 7), *L. pneumophila* SGM-50 (lane 8), *L. anisa* ATCC 35292^T (lane 9), SGT-39^T (lane 10), and SGT-108^T (lane 11).

SGT-39^T and SGT-108^T had G+C contents of 44.9 and 48.3 mol%, respectively.

DISCUSSION

The two new species represented by strains SGT-39^T and SGT-108^T comprise a novel branch within the γ-proteobacteria, whose closest relatives are the species of the genera *Rickettsiella* and *Legionella*. The species of the genus *Rickettsiella* have never been grown in artificial media, and their characteristics are largely unknown, so that comparisons of these bacteria with the São Gemil organisms cannot be made. The same is true for the organism represented by the environmental 16S rRNA gene sequence (GenBank accession no. AJ252618), which has not been cultured but, based on its relationship to the SGT organisms, could represent an additional, as yet uncultured, species of the genus proposed here. The new organisms can, however, be compared to the species of the genus *Legionella* to which they are also phylogenetically related. The new organisms, like the species of the genus *Legionella*, require activated charcoal for growth but, unlike the vast majority of species of *Legionella*, do not require L-cysteine or ferric pyrophosphate (17, 27). The São Gemil organisms have an absolute requirement for α-ketoglutarate or pyruvate which, in the *Legionella* strains examined, could be replaced by succinate and oxaloacetate. These organic acids do not serve as carbon and energy sources but appear to be involved in oxidative stress protection (30). The SGT organisms, like most isolates of the genus *Legionella*, also tolerate the acid and the heat treatments designed to reduce contaminant flora. Since they were isolated from BCYE-GVPC medium, it was not surprising that they were resistant to vancomycin and polymyxin B, used as selective agents during isolation.

Differences in the fatty acid compositions, however, indicate that the new organisms are not members of the genus *Legionella*. The new organisms have chain lengths longer than those found in *Legionella*. Moreover, one of the major fatty acids remains unknown but has not been found in any of the species of the genus *Legionella* (1, 8).

Only a few physiological and biochemical characteristics were determined because of the difficulty of growing the or-

FIG. 3. Phylogenetic dendrogram based on 16S ribosomal DNA gene sequence comparison showing the position of the SGT strains within the radiation of representative taxa of the γ -subclass of *Proteobacteria*. Scale bar, 10 inferred nucleotide substitutions per 100 nucleotides.

ganisms under the conditions required to assess many of the phenotypic parameters used to describe other species of bacteria. However, the two organisms clearly represent distinct species on the basis of the growth temperature range, pH range, and fatty acid composition. Strains SGT-39^T and SGT-56 grew consistently at 43°C, while strains SGT-108^T and SGT-109 did not. There were also reproducible differences in the pH range for growth. Strains SGT-108^T and SGT-109 grew in charcoal-based medium lacking yeast extract, indicating that they, unlike strains SGT-39^T and SGT-56 and the strains of the genus *Legionella*, did not require cofactors for growth found in yeast extract. Moreover, strains SGT-108^T and SGT-109 degraded casein, while the species represented by strain SGT-39^T did not. The differences in the fatty acid compositions of the SGT strains also indicate that these organisms represent two species.

The ability of strains SGT-39^T and SGT-108^T to grow in *H. vermiformis* represents one of the most striking characteristics of these organisms. However, these organisms, unlike strains of the genus *Legionella*, do not reach very high intracellular numbers in this protozoan. Whereas legionellae reach numbers of 10^6 CFU ml⁻¹ in this protozoan, strains SGT-39^T and SGT-108^T reach only about 10^3 to 2×10^3 CFU ml⁻¹ (14). These results lead us to speculate that the new organisms preferentially infect other protozoans, where they establish more-lethal infections, or that these organisms establish chronic infections that do not overwhelm the protozoan as rapidly as those established by legionellae. The inability of these organisms, in contrast to many legionellae, to grow in the human lymphoma U937 cell line, could indicate that the new organisms do not infect human phagocytic cells, as was proposed for isolates of *L. anisa* (14), but it is also possible that strains SGT-39^T and SGT-108^T infect other human phagocytic cell lines or, like the species of *Rickettsiella*, infect cells of other animals. Several genes have been implicated in the invasion and survival of *Legionella* spp. in macrophages and amoebae. In this study we chose to detect the *mip* gene, found in all *Legionella* spp. examined and believed to be necessary for intracellular multiplication of legionellae in macrophages and amoebae (4), the *dotA* gene, implicated in the intracellular growth of *L. pneumophila* in macrophages (37), and the *traA*-like gene, found in *L. pneumophila* (31) and viewed as important for survival in *Acanthamoeba castellanii* and *H. vermiformis*.

The failure to detect any of these genes in strains SGT-39^T and SGT-108^T could indicate that the SGT strains do not infect phagocytes or even amoebae. It is, however, possible that strains SGT-39^T and SGT-108^T have other genes for infectivity of eukaryotic cells or that the sequences of the genes examined are sufficiently different in the new organisms to be undetected with the primers used.

The very narrow pH range of the new organisms was also surprising. If we assume that the narrow pH range of the new SGT organisms in laboratory medium reflects, to a large extent, the pH range for growth in natural environments, we are led to conclude that these strains are unable to grow where shifts of pH occur frequently. Moreover, these organisms were isolated from water with pHs of 8.0 to 8.4, which would not permit the growth of the organisms. The results showing that strains SGT-39^T and SGT-108^T grow in *H. vermiformis* lead us to hypothesize that these organisms generally inhabit protozoans where the intracellular pH would be stable and around neutrality.

The growth temperature range, which is also very narrow, indicates that the organisms are unable to grow in the aquifer, where the temperature is around 48°C. Our analysis of the recovery at 48°C indicates that the organisms can survive in the São Gemil water for about 10 h, but it also indicates that these organisms (and their putative hosts) probably originate from colder water that enters the thermal water and survive during transit along the aquifer and borehole.

The new organisms from São Gemil represent a deep phylogenetic branch within the γ -*Proteobacteria* allied to organisms that are strict or facultative intracellular parasites of eukaryotes and which may cause disease in unknown hosts. Based on physiological, biochemical, and phylogenetic results we wish to present strain SGT-39 as the type strain of a new species of a novel genus, for which we propose the name *Aquicella lusitana*. We also consider that strain SGT-108^T represents a novel species phenotypically and phylogenetically distinct from SGT-39^T (94% 16S rRNA gene sequence similarity) and of the same genus, for which we propose the name *Aquicella siphonis*.

Description of *Aquicella* gen. nov. *Aquicella* (A. qui. cel'la, L. n. aqua, water; L. fem. n. cella, chamber or cell; L. fem. n. Aquicella, a cell from water). *Aquicella* forms rod-shaped cells and filaments. Cells stain gram negative. Motility and flagella are not observed. Cells do not produce spores. Colonies on

BCYE medium are whitish with a pinkish or bluish sheen and a ground-glass texture. The species of *Aquicella* have growth temperature ranges between about 30 and 45°C; the strains of this genus require neutral pH for growth. *Aquicella* is strictly aerobic and cytochrome oxidase and catalase negative. Fatty acids are branched chain. Major phospholipids are phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, and diphosphatidylglycerol. Q11 is the major respiratory quinone. *Aquicella* is chemo-organotrophic. The two species of the genus grow only on media containing activated charcoal and require α -ketoglutarate. Yeast extract may or may not be necessary for growth. The organisms grow in protozoa. The species of this genus belong to the γ -proteobacteria. The type species is *A. lusitana*.

Description of *Aquicella lusitana* sp. nov. *Aquicella lusitana* (lu.si.ta'na; L. fem. n. *lusitana*, pertaining to *Lusitania*, the Roman province in western Iberia). *A. lusitana* forms rod-shaped cells 0.4 to 0.7 μ m in width and 1.8 to 2.2 μ m in length. The optimum growth temperature is about 37°C, the maximum growth temperature is about 43°C, and growth does not occur at 45°C. The optimum pH for growth is between 6.5 and 7.3; no growth occurs at pH 6.0 or 7.5. The predominant fatty acids are 17:0 iso, an unknown fatty acid with an ECL of 17.495, and 17:1 ω 9c iso. Nitrate is not reduced to nitrite. Hippurate is hydrolyzed; casein is not hydrolyzed. The organism grows on BCYE and Degryse medium 162 adjusted to pH 6.7 and supplemented with charcoal and α -ketoglutarate and requires yeast extract for growth. It grows in the protozoan *H. vermiformis* but does not grow in the human lymphoma U937 cell line.

The mole G+C ratio of the DNA is 44.9%. This bacterium was isolated from water at the thermal spa at São Gemil. The type strain, SGT-39^T, has been deposited in the Collection of the Institute Pasteur, Paris, France, as strain CIP 107650 and in the BCCM/LMG Bacteria Collection, Ghent, Belgium, as strain LMG 21647.

Description of *Aquicella siphonis* sp. nov. *Aquicella siphonis* (si.pho'nis; L. gen. n. *siphonis*, pertaining to a tube or pipe). *A. siphonis* forms rod-shaped cells 0.4 to 0.8 μ m in width and 1.9 to 2.3 μ m in length. The optimum growth temperature is about 37°C, the maximum growth temperature is about 40°C, and growth does not occur at 43°C. The optimum pH for growth is between 6.3 and 7.0; no growth occurs at pH 6.0 or 7.3. The predominant fatty acids are 17:0 iso, 17:1 ω 9c iso, and an unknown fatty acid with an ECL of 17.495. Nitrate is not reduced to nitrite. Hippurate and casein are hydrolyzed. The organism grows on BCYE and Degryse medium 162 adjusted to pH 6.7 and supplemented with charcoal and α -ketoglutarate, and it grows on a modified BCYE where yeast extract is replaced by tryptone. It grows in the protozoan *H. vermiformis* but does not grow in the human lymphoma U937 cell line.

The mole G+C ratio of the DNA is 48.3%. This bacterium was isolated from water at the thermal spa at São Gemil. The type strain, SGT-108^T, has been deposited in the Collection of the Institute Pasteur as strain CIP 107651 and in the BCCM/LMG Bacteria Collection as strain LMG 21648.

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