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Characterization of *Thiobacillus thioparus* LV43 and Its Distribution in a Chemoautotrophically Based Groundwater Ecosystem

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Bacterial strain LV43 was previously isolated from a floating microbial mat located in Movile Cave, the access point to a chemoautotrophically based groundwater ecosystem in southern Romania. This gram-negative, rod-shaped organism grows autotrophically through the oxidation of thiosulfate and sulfide, but it does not grow heterotrophically. Strain LV43 grows over a pH range of 5.0 to 9.0, with an optimum near 7.5 at 28°C. The pH of the medium decreased from 7.5 to 6.5 during growth on thiosulfate. Carbon isotope fractionation values for strain LV43 were within the previously reported range of fractionation values for the overall floating microbial mat in Movile Cave and were similar to values reported for chemoautotrophic sulfur-oxidizing strains of *Thiobacillus neapolitanus* and *Thiomicrospira* sp. The 16S rRNA gene sequence of strain LV43 was determined, and phylogenetic analysis indicated that strain LV43 was most closely related to *Thiobacillus thioparus* and the uncultured bacterial strain Strip2, which is represented by a 16S rRNA clone obtained by direct PCR from the Stripa research mine in Sweden. This identification of strain LV43 is supported by its G+C content of 62%, which is within the range reported for strains of *T. thioparus*. Fluorescently labeled polyclonal antibodies specific for strain LV43 were used to locate and enumerate this strain at different locations in Movile Cave and in nearby surface-water and groundwater sources. Strain LV43 was found only at aerobic, neutral-pH sites within the cave. Strain LV43 was also found outside Movile Cave in surface waters and in groundwater believed to intercept the same sulfurous aquifer as Movile Cave.

Movile Cave, the access point to a unusual groundwater ecosystem in southern Romania, contains a large and diverse community of both terrestrial and aquatic animals (23). Nearly all terrestrial and aquatic communities, including all other known cave communities, are based on energy and organic carbon that ultimately originated from photosynthesis. Exceptions to this generalization, however, are the animal communities associated with deep-sea hydrothermal vents (7). The food base for these communities includes carbon fixation by chemoautotrophic bacteria and methane oxidation by methylotrophic bacteria, with these bacteria including both symbiotic and free-living forms (9, 17). Like that of the hydrothermal vents, the food web of Movile Cave has recently been shown to be based on primary production by chemoautotrophic microorganisms (26).

Thermal waters in the submerged portions of Movile Cave are buffered by limestone (water pH = 7.3) and contain significant amounts of H_2S (8 to 10 mg liter^{-1}), CH_4 (4 to 6 mg liter^{-1}), and NH_4^+ (4 to 6 mg liter^{-1}), all of which are potential sources of energy for aerobic bacteria (25). Several of these submerged passages contain air pockets (air bells), in which atmospheric composition is enriched in CO_2 (3 to 4%) and CH_4 (1 to 3%) and depleted in O_2 (10 to 11%) (23). The water surface within the air bells, as well as portions of the air bell wall above the water, is covered by microbial mats (24). Microscopic examination suggests that the floating mat consists of filamentous *Beggiatoa* spp. and fungi, as well as associated unicellular bacteria (21, 24). Incubation of the floating mat with [^{14}C]bicarbonate resulted in the incorporation of significant amounts of radiolabel into biomass (23). Stable-isotope analysis of inorganic carbon (e.g., $CaCO_3$, CO_2, HCO_3^-) and organic carbon (e.g., floating mats and different populations of animals) in the cave indicates that the primary source of organic carbon for this ecosystem is chemoautotrophic sulfur oxidizers (26).

Prior attempts to isolate chemoautotrophic sulfur-oxidizing bacteria from Movile Cave utilized several different enrichment media inoculated with water and floating mat samples from the cave (21). Most of the enrichment cultures were dominated by small rod-shaped bacteria, one of which, strain LV43, was obtained by direct isolation and briefly described earlier (21). Ribulose-1,5-bisphosphate carboxylase/oxygenase, an enzyme central to the autotrophic growth of many bacteria which gain energy from the oxidation of reduced sulfur compounds, was isolated from strain LV43 and shown to have significant in vitro activity. As part of our overall objective of obtaining a detailed understanding of the microbial food base of this ecosystem, the aims of the present study were to isolate additional chemoautotrophic sulfur oxidizers, to further characterize strain LV43, and to determine its distribution both in Movile Cave and in other surface-water and groundwater environments nearby.

MATERIALS AND METHODS

Isolation and growth characteristics of S oxidizers. Liquid enrichment cultures for sulfur-oxidizing bacteria were prepared as described by Kuenen et al. (10) and inoculated with floating-mat and water samples from the cave. Media were incubated at 28°C for 6 to 8 days; then transfers to fresh media were made. In subsequent studies, the mineral medium (pH 7.5) for *Thiobacillus novellus* described by Kuenen et al. (10) was used. When needed, the medium was solidified with 2% Noble agar.

The ability of strain LV43 to grow autotrophically on reduced sulfur compounds was tested by supplementing the mineral medium with one of the following compounds: 40 mM sodium thiosulfate, 3 mM sodium sulfite, 0.1% (wt/vol) elemental sulfur, 5 mM sodium metabisulfite, 5 mM sodium dithionite, 0.1% (vol/vol) mercaptoethanol, and 0.1% L-cysteine. Elemental sulfur was prepared by steaming as described by Postgate (19). Growth on sulfide was determined by inoculating a 0.2% agar mineral medium placed over a 1.5% agar layer

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containing 8 mM sodium sulfide (18). The ability to grow anaerobically in the presence of nitrate was tested by using the mineral medium (pH 7.5) for *Thiobacillus denitrificans* described by Kuenen et al. (10). In all cases inoculated cultures were incubated at 28°C for 7 days.

Growth under heterotrophic conditions was monitored for 10 days at 28°C with mineral medium without thiosulfate amended with several organic compounds: yeast extract, sodium acetate, sodium citrate, L-glutamine, L-glutamic acid, L-cysteine, D-sorbitol, mannitol, L-arabinose, D-cellobiose, D-fructose, D-glucose, lactose, D-mannose, and D-xylose. Each compound was added at a concentration of 0.2% (wt/vol) for carbohydrates and 0.1% (wt/vol) for organic acids, alcohols, and yeast extract. The stimulation of LV43 growth on sodium thiosulfate (40 mM) by the above organic compounds was also tested with the same substrate concentrations.

The effect of pH on the growth rate of strain LV43 was determined with mineral medium, amended with 40 mM thiosulfate, over a pH range of 5.0 to 9.0. Cell growth in three replicate flasks was monitored at 24-h intervals by acridine orange staining and direct microscopic counting (1). The growth rate during logarithmic growth was determined for each pH value.

Growth of strain LV43 and the concurrent disappearance of thiosulfate was determined in mineral medium (pH 7.5) containing 40 mM thiosulfate over a 9-day period at 28°C. Three replicate flasks containing 250-ml cultures were sampled at 24-h intervals. Numbers of cells were determined by using acridine orange staining as described above. Thiosulfate concentrations were determined by the standard iodometric method (5) after centrifugation (at 12,000 × *g* for 15 min at 4°C) of 5 ml of the culture medium. Chemical oxidation of thiosulfate during the experiment was determined by using three control flasks containing no cells. The pHs of the inoculated and uninoculated media were also monitored during this experiment.

GC content. Total genomic DNA was isolated from strain LV43 and reference strain *Escherichia coli* DH5α according to the method of Marmur (15). The guanosine plus cytosine content of strain LV43 was determined by the thermal melting method (8).

16S rRNA sequencing and phylogenetic analysis. Total genomic DNA was isolated from strain LV43 after centrifuging of log-phase cultures and washing in 0.85% NaCl. DNA was isolated from the cell pellet by the procedure of Giovannoni et al. (4) with the following modifications: proteinase K was used at 200 μg/ml; nucleic acids were extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol); and the DNA was precipitated and washed with ethanol, redissolved in water, and stored at −20°C.

The nearly full-length 16S rDNA of strain LV43 was amplified by PCR using the 8F and 1492R primers described by Lane et al. (11) and the protocol described by Haddad et al. (6). The PCR procedure was carried out in a thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) under the following conditions: denaturation at 94°C for 50 s, primer annealing at 45°C for 50 s, and chain extension at 72°C for 140 s for a total of 30 cycles. A control tube containing all the reagents except for template DNA was used with every set of reaction mixtures. PCR-amplified fragments were examined by low-melt agarose gel electrophoresis. A band of the appropriate size was excised, purified with the Wizard PCR Prep DNA purification system (Promega, Madison, Wis.), and sequenced in both directions by using oligonucleotide primers complementary to highly conserved regions in the 16S rRNA gene sequence (11) and an automated DNA sequencer (model 373A; Applied Biosystems, Foster City, Calif.). The sequence was submitted to the CHECK CHIMERA program of the Ribosomal Database Project (RDP) at the University of Illinois to detect possible chimeric artifacts (13).

A preliminary analysis of the 16S rRNA gene sequence from strain LV43 was obtained by using the similarity rank program of the RDP (14). To determine the phylogeny of strain LV43, we conducted maximum-likelihood and parsimony analyses of its 16S rDNA sequence together with sequences of 11 members of the β subdivision of the class *Proteobacteria* (β-*Proteobacteria*) obtained from the RDP. Sequences of the α-proteobacterium *T. novellus* and the γ-proteobacterium *Thiobacillus ferrooxidans* were used as outgroups to root the tree. All sequences were manually aligned on the basis of conserved features of primary and secondary structures. The number of nucleotide positions used in the alignment was 1,465, with the first nucleotide corresponding to position 18 and the last corresponding to position 1482 according to *E. coli* 16S rRNA numbering. Phylogenetic trees were inferred by the maximum-likelihood method as implemented by PHYLIP, version 3.5c (3), and by heuristic parsimony analyses of the same sequences as implemented by PAUP, version 3.1 (28). Because the heuristic search does not explore all possible topologies to find the shortest tree, we repeated the search 100 times for each parsimony analysis. Each search was initiated with a different randomly constructed starting topology, thus minimizing the possibility that the algorithm would find a local parsimony rather than the universal optimum for a particular data set. Random stepwise addition of taxa was used in both maximum-likelihood and parsimony analyses.

Carbon isotope fractionation by LV43. To carry out stable-isotope ratio analysis (SIRA) of strain LV43 biomass, 250-ml flasks containing 100 ml of mineral medium amended with 40 mM sodium thiosulfate were inoculated with 1 ml of LV43 culture and incubated in the lake room of Movile Cave (Fig. 1) for 14 days. After incubation LV43 cells were collected by centrifugation and sent to the Water Research Center, University of Alaska—Fairbanks, for carbon SIRA. Cave atmosphere SIRA of CO_2 was previously determined (26). Values of δ (per mille) were calculated as $\delta^{13}C = [(R_{sample} - R_{standard})/R_{standard}] \times 10^3$ where R is $^{13}C/^{12}C$. Standards for carbon were Pee Dee belemnite. Precision of measurements was 0.2‰ or better.

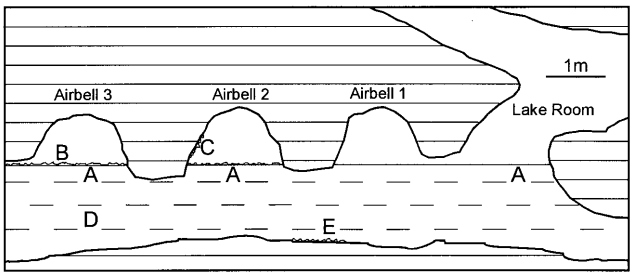

FIG. 1. Cross section of Movile Cave. Sampling sites are indicated by letters: A, surface water; B, floating mat; C, wall mat; D, deep water; E, submerged mat.

Serology and enumeration of LV43. A strain-specific polyclonal antibody was prepared for strain LV43 and tested for specificity as described by Schmidt et al. (27). The antibody did not cross-react with the other neutrophilic *Thiobacillus* strains tested. Samples of mat and water from different locations in the cave (Fig. 1), as well as nearby surface-water and groundwater samples from outside the cave (Fig. 2), were collected and fixed with paraformaldehyde (4% final concentration). A minimum of three samples were taken from each location. Immunofluorescent enumeration of bacteria was carried out as described by Moawad and coworkers (16). Water samples (1 to 5 ml) or mat samples (1 to 5 ml of supernatant resulting from shaking 0.1 g of mat with 0.85% NaCl and 2-mm-diameter glass beads at 150 rpm for 30 min) were filtered through a 0.2-μm-pore-size black polycarbonate filter. After staining with the LV43-specific fluorescent antibody, a total of 50 microscopic fields per filter were counted and numbers were converted to cells per milliliter of water or per gram (wet weight) of mat.

Nucleotide sequence accession number. The 16S rRNA sequence of strain LV43 is available from GenBank under accession no. AF005628.

RESULTS

Enrichment of S oxidizers. The use of several different enrichment media for neutrophilic, chemoautotrophic thiosulfate and sulfide oxidizers resulted in growth dominated by small gram-negative rods in all cultures. Most of these strains reacted positively with the fluorescent antibody prepared against the previously isolated strain LV43 (data not shown). Therefore, further studies focused on the characterization and ecology of this strain.

Cultural characteristics of LV43. Seven-day-old colonies of strain LV43 on mineral medium plates were round, white, rough, convex, and 0.8 mm in diameter. When the organism

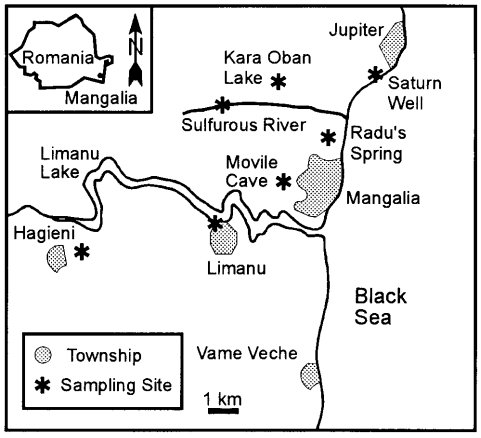

FIG. 2. Map of Mangalia region. Surface and groundwater sampling sites are indicated by asterisks.

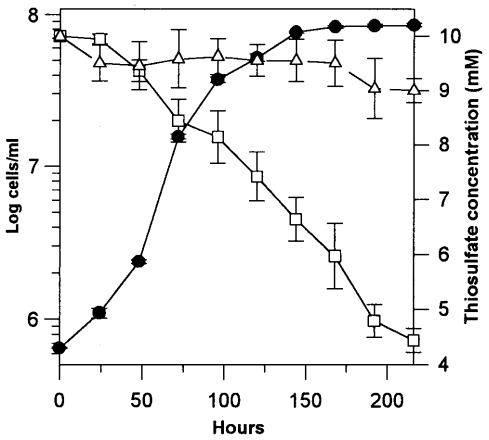

FIG. 3. Growth of strain LV43 with thiosulfate at 28°C and pH 7.0. The disappearance of thiosulfate from the culture medium was monitored at the same time. Mean values of three replicates at each time point were used; error bars represent standard deviations. Symbols: ●, LV43 cell density; □, oxidation of thiosulfate by strain LV43; △, chemical oxidation of thiosulfate.

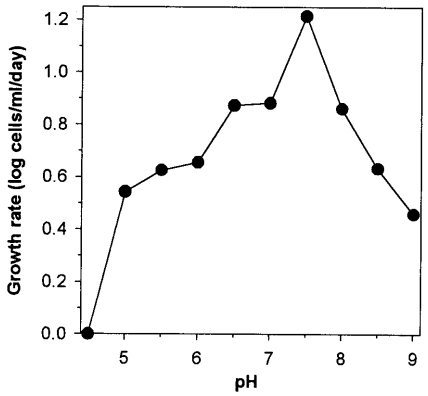

FIG. 4. Effect of pH on autotrophic growth rate (log cells per milliliter per day) of strain LV43 at 28°C. Cells were enumerated microscopically after staining with acridine orange. Mean values of three replicates at each time point were used.

was grown in liquid culture, a thin visible film developed on the surface of the medium after 5 days. During the same period, the growth medium also became cloudy white. Microscopic observation showed the presence of small particles of what appeared to be elemental sulfur in the medium. With phase-contrast microscopy strain LV43 appears as a motile rod, approximately 0.9 by 1.8 μm.

Autotrophic growth of strain LV43 was supported by thiosulfate and sulfide but not by the other reduced sulfur compounds. Strain LV43 could not grow heterotrophically on any of the organic compounds tested. The addition of organic compounds to the mineral medium containing thiosulfate did not increase the growth rate of strain LV43.

The kinetics of cell growth and thiosulfate utilization are shown in Fig. 3. The maximum cell yield of LV43 was approximately 10^8 cells/ml and was accompanied by an approximately 50% loss of thiosulfate from the medium. Some chemical oxidation of thiosulfate was measured in the cell-free controls, but this loss was significantly less than the rate of biological oxidation. During the 9-day period of this experiment, the pH decreased from 7.5 to 7.3 in the uninoculated medium and from 7.5 to 6.5 in the inoculated medium. Strain LV43 was able to grow over a pH range of 5.0 to 9.0, with an optimum pH near 7.5 (Fig. 4). Strain LV43 was not able to grow anaerobically, in the presence of nitrate, in mineral medium with thiosulfate.

Molecular characterization of LV43. The guanosine plus cytosine content of strain LV43 was 62 mol%.

The 16S rRNA gene sequence of LV43 was sent to the RDP for similarity analysis, resulting in placement of this strain within the β-*Proteobacteria* group. Maximum-likelihood analysis of 16S rRNA gene sequences from strain LV43 and 11 other members of β-*Proteobacteria*, rooted with the γ-proteobacterium *T. ferrooxidans*, resulted in the tree shown in Fig. 5. Strain LV43 was most closely related to the Strip2 gene sequence isolated from an uncultured bacterium obtained from the Stripa Mine in south central Sweden (2). The LV43 and the Strip2 sequences both formed a tight cluster with the *Thiobacillus thioparus* sequence. Using maximum parsimony analysis or substituting the α-proteobacterium *T. novellus* as the outgroup resulted in trees with congruent topologies (data not shown).

Carbon isotope fractionation by LV43. SIRA of strain LV43 incubated in the cave lake room for 7 days resulted in a $\delta^{13}C$ of −50.1‰ (per mille). Cave atmospheric CO_2 SIRA was previously determined as a $\delta^{13}C$ of −21.3‰ (26), resulting in a carbon fractionation value for LV43 of −28.8‰.

Distribution of the isolated strain in the cave. Analysis of water and mat samples collected from different locations within the cave (Fig. 1) by using the strain-specific fluorescent antibody showed that strain LV43 was present in the highest numbers in the floating mat, with somewhat lower numbers found in the surface-water samples collected from air bells 2 and 3, and in the lake room (Table 1). No LV43 cells were detected in samples taken from the air bell wall mats, the submerged mats, or the deep water.

Among the surface-water and groundwater sources sampled outside Movile Cave, strain LV43 was found only in the Limanu Spring, Radu's Spring, and Hagieni Spring. Strain LV43 was not found in the Sulfurous River, Saturn Well, or Kara Oban Lake.

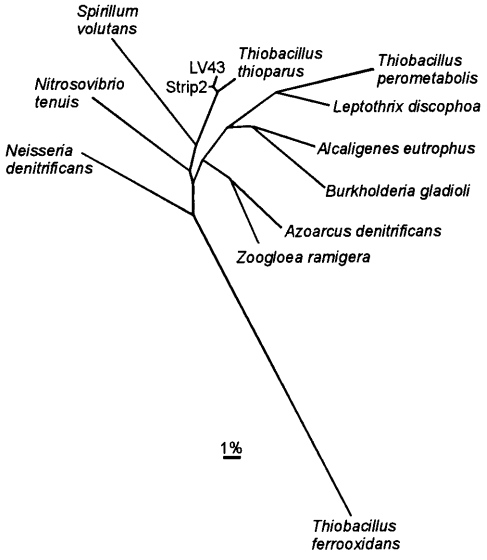

FIG. 5. Maximum-likelihood tree for 12 species in the β-*Proteobacteria* and an out-group microorganism, the α-proteobacterium *T. novellus*. The scale bar corresponds to 0.01 nucleotide substitution per sequence position.

TABLE 1. Distribution of LV43 inside and outside Movile Cave

Location	Cell count (cells/ml [SD])
Movile Cave	
Surface water (lake room)	2.1×10^4 (7.9×10^3)
Surface water (air bell 2)	2.5×10^4 (2.2×10^3)
Surface water (air bell 3)	2.4×10^4 (1.9×10^3)
Floating mat	4.7×10^4 (2.9×10^3)
Deep water[a]	0
Submerged mat	0
Wall mat	0
Other surface-water and groundwater sources	
Radu's Spring	6.2×10^2 (1.4×10^2)
Limanu Spring	1.1×10^3 (3.3×10^2)
Sulfurous River	0
Hagieni Spring	2.6×10^2 (5.7×10^1)
Saturn Well	0
Kara Oban Lake	0

[a] Sixty centimeters below water surface.

DISCUSSION

The present attempts to enrich for neutrophilic sulfur oxidizers resulted in the dominant growth of gram-negative rods, most of which reacted positively with the fluorescently labeled polyclonal antibody prepared against the previously isolated strain LV43. As a result, we carried out a more detailed examination of strain LV43 and determined its occurrence in Movile Cave and in nearby aquatic environments.

Strain LV43 did not grow under heterotrophic conditions, at least with the organic carbon sources tested here. Instead, its growth requires the oxidation of reduced, inorganic sulfur compounds. The addition of several different organic compounds to the thiosulfate-containing mineral medium did not stimulate or inhibit growth. Strain LV43 was able to use thiosulfate and sulfide during aerobic growth. Physiologically and morphologically, strain LV43 is clearly an obligately chemolithoautotrophic bacterium belonging to the genus *Thiobacillus.*

The neutral-pH preference of strain LV43 is not surprising considering the enrichment conditions used, as well as the environment from which it was isolated. Measured pH values of the cave water are near 7.0 due to the buffering capacity of the limestone walls (22). During the 9-day growth curve experiment (Fig. 3), strain LV43 decreased the pH of the culture by one unit. In addition, elemental sulfur particles accumulated in the medium. Therefore, strain LV43 appears mainly to oxidize thiosulfate to sulfur but not further to sulfate, at least over the time interval and under the conditions tested here. These characteristics of LV43 are in agreement with the observations that Movile Cave receives a high input of H_2S and the resulting elemental sulfur may be washed out of the system by the water flow (22).

The −28.8‰ (per mille) carbon isotope fractionation value for LV43 incubated under in situ conditions falls within the previously reported −26-to-−31 range of overall fractionation by the floating mat in Movile Cave (26). This fractionation value for LV43 is also similar to that reported for chemoautotrophic growth of sulfur-oxidizing strains of *Thiobacillus neapolitanus* and *Thiomicrospira* sp. (20), although these measurements were made under laboratory conditions. Carbon fractionation values measured in situ for sulfur-oxidizing bacteria may be of use in determining the food web relationships of other ecosystems that have at least a partially chemoautotrophic base.

Phylogenetic analysis of the 16S rRNA gene sequence of strain LV43 closely associated this strain with *T. thioparus* and the Strip2 bacterium. This classification is supported by the G+C content of strain LV43, which is within the ranges reported for *T. thioparus*, *Thiobacillus acidophilus*, *Thiobacillus prosperus*, and *Thiobacillus albertis* (10). The last three strains, however, are distinguishable from *T. thioparus* and strain LV43 by their acidophilic growth preferences.

The 16S rRNA gene sequence of the Strip2 bacterium was originally obtained by PCR amplification of the bacterial population that grew on glass slides incubated in groundwater from a former iron ore research mine in south central Sweden (2). The Strip2 strain was not isolated in pure culture, so nothing is known about its metabolic capabilities. In fact, the authors were unable to culture any of the organisms whose 16S rRNA genes were amplified by PCR (2). The close association between LV43 and Strip2 may be due to the similar environmental conditions (i.e., both are aerobic, mesothermal, neutral-pH groundwater systems containing hydrogen sulfide) from which they were isolated. Just as the location of strain LV43 was restricted to aerobic regions of Movile Cave, so Strip2 gene sequences were found only in the upper aerobic sampling sites of the bore hole, suggesting the preference of this uncultured organism for aerobic habitats (2).

The microscopic examination of the distribution of LV43 at different sites in Movile Cave showed that the strain is present in surface-water samples and the floating mat but is absent from the deep-water samples, the submerged mat, and the wall mat (Table 1). This finding is consistent with the phenotypic characterization of LV43 and the chemistry of the cave. The cave water becomes anaerobic within 12 cm of the water surface (22), explaining the absence of LV43 from the submerged mat and the deep-water samples. Moreover, the measured pH values of the air bell walls and wall mat are typically 2.0. These low pH values suggest that the wall mats are a suitable environment for acidophilic strains of *Thiobacillus* rather than for neutrophilic strains of *T. thioparus* such as LV43. The activity of as yet uncultured acidophilic *Thiobacillus* strains is also supported by the presence of $CaSO_4$ crystals on the limestone air bell walls (22).

Similarly, LV43 cells were also found in water sources located near Movile Cave but were not seen in several sources further north of Movile Cave (Fig. 2). This distribution correlates well with carbon and oxygen stable-isotope data, as well as water chemistry data, that indicate that the sulfurous aquifer intercepting Movile Cave extends at least 10 km west of Mangalia but is rather narrow, not extending far south of Limanu or north of Movile Cave (22).

One limitation of culture-based ecological studies in microbiology is that the isolated and characterized strains may represent "weedy" species that grow fast in enrichment cultures but play a minor role in the original environment. The percent of the total bacterial population represented by strain LV43 was less than 0.1% of the total planktonic bacterial count of 6.3×10^7 cells/ml reported earlier (21). Measurements of total planktonic bacteria in Movile Cave did not vary significantly over a 4-year period (22). LV43, however, may be only one strain of many neutrophilic, chemoautotrophic sulfur oxidizers in Movile Cave. Although several attempts have been made to isolate other chemoautotrophic sulfur-oxidizing bacteria, strain LV43 is the only one obtained so far. Since sulfur-oxidizing bacteria do not form a monophyletic group (12), a group-specific oligonucleotide probe targeting the rRNA cannot be designed for ecological studies. It may, however, be possible to design a probe specific for the *T. thioparus* group that contains Strip2 and LV43.

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