Acidiphilium cryptum gen. nov., sp. nov., Heterotrophic Bacterium From Acidic Mineral Environments

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Acidiphilium gen. nov. is comprised of gram-negative, aerobic, mesophilic, rod-shaped bacteria that grow in lean organic media between pH 1.9 and 5.9 but not at pH 6.1. Small amounts of organic substrates are required for growth, but high concentrations inhibit growth, especially the organic digests used in conventional bacteriological media. Sodium acetate is strongly inhibitory at a concentration of 0.01%. Endospores are not formed, and some strains are motile by means of one polar flagellum or two lateral flagella. The guanine-plus-cytosine content of the deoxyribonucleic acid varies between 68 and 70 mol%. Some catalase is formed, but the oxidase reaction is very weak or absent. This genus differs from *Pseudomonas*, *Chromobacterium*, *Flavobacterium*, and other soil heterotrophs by its requirement for a high concentration of hydrogen ions and its sensitivity to many organic substrates. It differs from *Pseudomonas* also by its lack of a strong oxidase reaction and especially by its inhibition by acetate. It is easily distinguished from acidophilic thiobacilli by its inability to utilize sulfur and ferrous iron as sources of energy and by the high guanine-plus-cytosine content of deoxyribonucleic acid. The type species is *Acidiphilium cryptum*, and the type strain of this species is strain Lhet2 (=ATCC 33463).

Heterotrophic, mesophilic bacteria with requirements for high acidity have been isolated from allegedly pure cultures of *Thiobacillus ferrooxidans* (6). These heterotrophs cannot use elemental sulfur or ferrous iron as a source of energy; instead, they utilize organic matter formed by the autotroph *T. ferrooxidans* (B. W. Jarvis, M.S. thesis, University of Missouri, Columbia, 1979). Heterotrophic bacteria identical to the organisms isolated from *T. ferrooxidans* cultures were isolated on very dilute Trypticase soy glucose medium at pH 2 directly from acidic coal refuse in Missouri. Bacteria phenotypically similar to these organisms have also been isolated from coal mine water in Pennsylvania (P. L. Wichlacz, M.S. thesis, Pennsylvania State University, University Park, 1980) and from mine drainage elsewhere (10). Other isolations of these bacteria have been described previously (6). In this report I describe these acidophilic heterotrophs in greater detail and propose names for them.

MATERIALS AND METHODS

Media. The bacteria were cultivated in a medium containing the following (per liter): $(\text{NH}_4)_2\text{SO}_4$, 2 g; KCl, 0.1 g; K_2HPO_4 , 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; dehydrated Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.), 0.1 g; and glucose, 1 g. The pH was adjusted to 3 with 1 N H_2SO_4 . The glucose was autoclaved separately as a 10% solution, and after cooling a suitable amount was added aseptically to the

other autoclaved ingredients (pH 3). This medium without the organic ingredients was designated basal salts medium; when it contained Trypticase soy and glucose, it was designated TS-glucose medium. When solid media were desired, the liquid media were gelled with agar (12 g/liter). The agar and glucose (double strength) and the other ingredients (pH 3; double strength) were autoclaved separately at 15 lb/in² for 15 min and then mixed after cooling. In growth studies with different organic substrates, the organic compounds were sterilized separately (autoclaved or filtered as necessary in each case) and then added aseptically to the basal salts medium. When the substrate being tested would have affected the pH, the appropriate correction was made; the pH was monitored in these experiments to ensure that it did not influence the results.

Isolation of bacteria, maintenance of stock cultures, and test strains used. Several strains of acidophilic heterotrophs were isolated in enrichment cultures by using 100-ml quantities of TS-glucose liquid medium with the pH adjusted to 2 in 0.5-liter Erlenmeyer flasks. About 1 g of soil was added to each flask, and the flask was incubated at 28°C on a rotary shaker. After several days, material from the flask was streaked onto TS-glucose agar, and individual colonies were selected. After preliminary tests to ensure purity, stock cultures were prepared on TS-glucose agar slants, which were stored in a refrigerator; transfers were made monthly. Other strains were acquired from allegedly pure cultures of *T. ferrooxidans* (6) and from an artificial coal spoil (5). I performed preliminary tests to confirm that these isolates were strict heterotrophs (i.e., that they were not able to utilize either sulfur or ferrous iron as an energy source), that they

grew between pH 1.9 and 5.9 but not at pH 6.1, and that they did not grow on conventional peptone media at low pH values. From this collection of isolates, I selected seven representative strains for this study (Table 1).

Diagnostic tests. Catalase was detected by using a 3% hydrogen peroxide solution that was added directly to TS-glucose agar cultures. *Staphylococcus aureus* and *Streptococcus faecalis* served as positive and negative controls, respectively. Growth on TS-glucose agar was also used for the oxidase test, which was performed by the *para*-aminodimethylaniline disk method. *Pseudomonas fluorescens* and *Neisseria* strains served as positive controls, and *Escherichia coli* and *Enterobacter aerogenes* served as negative controls. Growth, pH, and temperature studies were performed on the same medium. Heat tolerance was determined by using growth from a 1-week-old TS-glucose agar culture suspended at a high density (5×10^8 cells per ml) in liquid TS-glucose medium in small test tubes (11 by 100 mm) in a 67°C water bath. Glucose utilization was measured by the phenol-sulfuric acid colorimetric method of sugar analysis (3). Cell numbers were assayed by plating diluted samples onto TS-glucose agar and were also determined by microscopic counts, using phase-contrast microscopy and a Petroff-Hausser bacterial counting chamber. Antibiotic susceptibilities were determined by the disk method on TS-glucose agar plates.

The type of flagellation was determined by the Leifson staining method (1) and also by electron microscopy. Two drops of a thick cell suspension were mixed with 4 drops of 2% phosphotungstic acid and 1 drop of 0.1% bovine serum albumin. This mixture was deposited on carbon-coated copper grids (300 mesh), and the preparation was examined with an RCA model EMU-3F transmission electron microscope.

Examination of DNA. The bacteria were cultivated, the deoxyribonucleic acid (DNA) was prepared, and the DNA homology study was performed as described previously (6). Purified DNA was diluted in $0.1 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). The guanine-plus-cytosine (G+C)

content of the DNA was determined by the DNA melting point method in $0.1 \times \text{SSC}$; a Gilford thermoprogrammer, a multiple-sample recorder, and a Beckman model DU spectrophotometer were used. To determine the melting point, the diluted DNA was dialyzed together with DNA from *E. coli* strain B as a reference. The best estimate for the G+C content of *E. coli* B DNA is 51.0 ± 0.25 mol% (12; M. Mandel, personal communication). Using this value and the experimental melting point for *E. coli* B DNA, the results were normalized to *E. coli* B DNA by using the equation of Mandel and Marmur (9).

RESULTS

Morphology. Living cells of these heterotrophic bacteria grown in TS-glucose liquid medium were rods which measured 0.3 to 0.4 by 0.6 to 0.8 μm . The cells of strains 13598het and KG-4p tended to be shorter than the cells of the other strains. When they were grown in liquid Trypticase soy-citrate medium (TS-glucose medium but with 0.1% sodium citrate in place of glucose), the cells of all strains were longer than when they were grown in TS-glucose medium. For example, strain Lhet2 was approximately 1.3 μm long in Trypticase soy-citrate medium. Below pH 2.0, the cells of all of the strains were shorter. Strains m-3 and m-5 appeared to be cocci and formed short chains, thus resembling streptococci. All strains were gram negative, and all strains except 13598het and KG-4p were motile; none formed endospores. Electron microscopy revealed that one strain, strain Lhet2, had numerous pili.

Flagellation. An examination of strain Lhet2 by electron microscopy demonstrated that most of the flagellated cells had two flagella and that these flagella were attached laterally (Fig. 1A and B). However, some cells (the longer cells in the cultures) had a single polar flagellum (Fig. 1C). The strain shown in Fig. 1 was also examined with an oil immersion lens after the cells were stained with Leifson flagellum stain. Under an optical microscope, all of the flagellated cells appeared to be monotrichous. To prepare the smear, a drop of cell suspension was allowed to flow down the surface of a glass slide as the slide was tilted. The current created by this procedure or the movement of the cells themselves as they swam from the drying edge of the smear apparently caused the two lateral flagella to lie close together or to become entangled, so that when they were coated with mordant and stain, they appeared to be one flagellum. Moreover, the flagella were so thickened and the cells were so small under the optical microscope that it was not possible to observe whether the flagellum was polar or lateral. (*Proteus* and *Escherichia* cultures served as peritrichous controls, and a

TABLE 1. Sources and habitats of the acidophilic heterotrophic bacteria studied

Strain	Source and/or habitat	Reference
Lhet2	From a culture of <i>T. ferrooxidans</i> ; coal mine water from Pennsylvania	
13598het	From <i>T. ferrooxidans</i> ATCC 13598; copper mine dump effluent, Bingham Canyon, Utah	
m-5	Coal strip mine refuse, Calloway County, Mo.	5
m-3	Coal strip mine refuse, Calloway County, Mo.	5
Dhet1	Coal strip mine refuse, Randolph County, Mo.	
Dhet2	Coal strip mine refuse, Randolph County, Mo.	
KG-4p	Derived from KG-4; isolated by Shafia and Wilkinson from a culture of <i>T. ferrooxidans</i> ; copper mine effluent, Utah	13

FIG. 1. Electron micrographs of the acidophilic heterotroph strain Lhet2. Bars = 1 μ m.

Pseudomonas culture served as a monotrichous control with the Leifson stain.) Motility was examined by phase-contrast microscopy. Strain Lhet2 in TS-glucose liquid medium was actively motile. Moreover, the cells were sticky. When cells collided with the Petroff-Hausser slide or with the underside of the cover glass, they usually adhered to it; they spun as they tried to free themselves. Many motile cells lay horizontally, and one end led the other as they swam. Other cells stood vertically, and viewed on end they looked like cocci; they swam sideways (in a direction perpendicular to their long axes) with a vigorous wobble. However, when they adhered to the slide or cover glass, the rod shape was confirmed because the cells tended to assume positions in the same plane as the horizontal surface to which they were stuck. Motility was minimal at pH extremes (i.e., below pH 2.2 and above pH 4.5).

Requirement for oxygen. All of the strains

examined were aerobic. Cells were inoculated into 30 ml of molten semisolid (0.6% agar) medium and dispensed into large test tubes (18 by 160 mm). Growth occurred only at the surface of the agar and to a depth of 3 mm.

Responses to pH and temperature. All seven strains grew between pH 1.9 and 5.9 in TS-glucose liquid medium (100 ml in shaken flasks at 30°C). Slight differences in optimum pH values (as determined by growth rate) have been observed and reported elsewhere for these organisms (6), but all grew well between pH 2.2 and 5.2. No growth occurred at pH 6.1. At pH 1.7 all of the strains lost viability, as determined by plate counts, although a few cells remained viable after 3 days. All seven strains grew slowly below 20°C and fastest between 35 and 41°C, but they did not grow at 47°C. Strain Lhet2 divided every 6 h at 25°C during the exponential growth phase in TS-glucose liquid medium on the rotary shaker. At 67°C, five of the seven strains were killed within 2 min (the viable population as determined by plate count decreased from 5×10^8 to $<10^1$ cells per ml); strains m-3 and m-5 were killed within 6 min at this temperature. This degree of heat sensitivity confirmed the observation that endospores are not formed by these organisms.

Response to organic substrates. All seven strains responded similarly with respect to growth on media containing Trypticase soy, glucose, and sodium acetate. Table 2 shows the responses of strain Lhet2 to these compounds. The best cell yield was obtained on media containing 0.01% Trypticase soy and glucose, whereas 0.05% Trypticase soy and 0.01% sodium acetate were inhibitory. Strain KG-4p yielded 2×10^8 cells per ml in TS-glucose medium; otherwise this strain responded the same as strain Lhet2. The growth rate of strain Lhet2 in liquid TS-glucose medium on the rotary shaker at 25°C was 0.17 division per h during exponential growth. A glucose concentration above 0.1% was inhibitory; with 0.2% glucose, the cells enlarged but did not divide. Growth in cultures was too sparse to permit an accurate measurement of glucose utilization. To demonstrate that strain Lhet2 could consume glucose, cells were harvested from glucose agar and suspended in basal salts medium containing 220 μ g of glucose per ml to a concentration of 5×10^8 cells per ml (plate count). After 38 h at 35°C on the rotary shaker, the population doubled, and 200 μ g of glucose per ml was consumed. Many organic compounds could replace glucose in the defined medium and could support the growth of all seven strains. These compounds (at concentrations of 0.1%) included arabinose, asparagine,

TABLE 2. Cell yields of strain Lhet2 in different media^a

Time of incubation (days)	Cell yield (cells per ml) of strain Lhet2 in basal salts medium containing:						
	No addition	0.1% Glucose	0.1% Glucose + 0.01% acetate	0.01% Trypticase soy	0.05% Trypticase soy	0.01% Trypticase soy + 0.1% glucose	0.01% Trypticase soy + 0.1% glucose + 0.01% acetate
0	1×10^5	1×10^5	1×10^5	1×10^5	1×10^5	1×10^5	1×10^5
4	$<10^6$	1×10^7	$<10^6$	3×10^7	$\sim 10^6$	1×10^8	$<10^6$

^a Cultures (100 ml) were grown in 0.5-liter Erlenmeyer flasks at 28°C on the rotary shaker. Assays were performed by using phase-contrast microscopy and a Petroff-Hausser bacterial counting chamber. The assay at zero time (inoculum) was made on the inoculum suspension (from a culture in basal salts medium containing 0.01% Trypticase soy), correcting for dilution upon addition to the test media. A 100-ml flask culture was barely turbid to the unaided eye at a cell population of $\sim 10^7$ cells per ml. Plate counts confirmed these results with strain Lhet2 and equivalent results with the other six strains.

glycerol, lactose, maltose, mannitol, raffinose, ribose, sodium tartrate, and sucrose. The following compounds did not support growth: cysteine, glycine, sodium glutamate, and sodium lactate. Interestingly, sodium thiosulfate not only did not support growth but also prevented growth in glucose. Growth was measured by plate counts.

DNA analyses. The G+C contents of the DNAs of five of the strains were as follows (as determined by the melting point method): strain KG-4p, 68.1 mol%; strain m-5, 70.0 mol%; strain 13598het, 70.0 mol%; strain Dhet1, 69.5 mol%; and strain Lhet2, 69.8 mol%. As determined by the buoyant density method, the G+C content of strain Lhet2 DNA was 69.4 mol% (Mandel, personal communication). The results of a DNA homology study on these organisms have been described previously (6), and it was shown that strains Lhet2, 13598het, m-5, and Dhet1 form a homology group with at least 90% homology to Lhet2 DNA as reference DNA. Strain KG-4p showed 34% homology with Lhet2 and thus is somewhat less closely related to strain Lhet2 than the other strains are.

Other traits. All seven strains were inhibited by aureomycin and chloramphenicol, but none was inhibited by erythromycin, neomycin, streptomycin, or penicillin. The sparse growth so characteristic of these bacteria in liquid media was also observed on TS-glucose agar. Thin, translucent growth appeared in 2 to 3 days at 37°C. If the agar culture was kept for several weeks at 20°C, the growth became opaque and white or pinkish white. At 37°C, colonies required 5 to 7 days to attain a diameter of 0.2 to 0.5 mm. Strain Lhet2 was examined for salt tolerance. In liquid TS-glucose medium at 30°C on the rotary shaker, the cell yield in medium containing 3% NaCl was one-half that obtained under the same conditions in the absence of NaCl, and no growth occurred with 7% NaCl.

All seven strains were weakly catalase positive and, compared with *Pseudomonas* and *Neisseria* species as controls, produced very weak oxidase reactions, if they produced reactions at all.

DISCUSSION

The acidophilic heterotrophic bacteria described here are widely distributed; they have been isolated directly from (or in *T. ferrooxidans* cultures derived from) copper sulfide ores and pyritic coal and effluents (Table 1) from the East, Midwest, and Rocky Mountain regions of the United States. Similar, perhaps identical bacteria have been found associated with *T. ferrooxidans* in peat from an acidic bog in Soviet Europe (17) and in association with other cultures of *T. ferrooxidans* elsewhere (8, 14). Thus, these bacteria represent a common, albeit elusive, group deserving general recognition and a name. The two most striking properties of these bacteria are their high acidophilism and their low tolerance toward organic compounds. They grow at a lower pH than other nonsporulating obligate heterotrophs (7). However, they cannot be detected with conventional media, even at low pH values. For example, Trypticase soy at a concentration of 0.05% (far below the 2.7% recommended by the manufacturer) is inhibitory. The acidophilism of these organisms is shared by some thiobacilli; however, their inability to utilize sulfur or ferrous iron with or without an organic substrate differentiates them from thiobacilli (6). Indeed, not only the lack of DNA homology with *T. ferrooxidans* (6) but also the G+C contents of the DNAs of these organisms distinguish them from *T. ferrooxidans*. Thus, strains Lhet2 and 13598het, which were isolated from *T. ferrooxidans* cultures (Table 1), have G+C contents of 69.8 and 70.0 mol%, respectively, whereas *T. ferrooxidans* strains

have values of 57.2 and 59.5 mol%, as determined by the melting point method in this study. The G+C values of these organisms also differentiate them from *Thiobacillus acidophilus* (G+C content, 62.9 to 63.2 mol% [4]). This *Thiobacillus* species utilizes either sulfur or glucose as a source of energy.

An acid-tolerant heterotroph isolated from coal mine waters was described under the name *Flavobacterium acidurans* (11). This bacterium is yellow, grows in conventional media (0.1% yeast extract plus 0.1% tryptone [equivalent to Trypticase]), does not grow at 37°C or below pH 4.5 but does grow at pH 6.5, and has a G+C content of 66.4 mol% in its DNA (11). All of these traits distinguish it from the acidophilic bacteria described here. The flavobacteria are not inhibited by organic compounds, nor are they acidophilic (16).

The flagellation of one of my isolates, strain Lhet2, is similar to that reported for some species of *Chromobacterium* (15). However, chromobacteria are not acidophilic and grow in conventional peptone digests and extracts. Furthermore, they are characteristically violet or purple. The acidophilic heterotrophs have little in common with pseudomonads, which characteristically are not acidophilic, are tolerant of organic matter, and utilize acetate rather than being inhibited by it (2). Indeed, it appears that no bacteria with the properties of the acidophilic heterotrophs described here have been described and named previously. Therefore, I propose the generic name *Acidiphilium* gen. nov. (A.ci.di.phi'li.um. M.L. noun *acidum* an acid; Gr. adj. *philus* loving; M.L. neut. n. *Acidiphilium* acid lover) for these organisms. For the name of the type species, *Acidiphilium cryptum* sp. nov. (cryp'tum. Gr. adj. *kryptos* hidden; M.L. adj. *cryptus* hidden). At this time, the genus *Acidiphilium* contains this one species. Further research may place other species within this genus. Strain Lhet2 is the type strain of *A. cryptum* and has been deposited with the American Type Culture Collection, under the accession number ATCC 33463. A combined description of the new genus and species follows.

Acidiphilium gen. nov. Gram-negative, mesophilic, aerobic, rod-shaped bacteria. Motile and nonmotile strains occur; motile cells have one polar flagellum or two lateral flagella. Growth occurs in lean organic media between pH 1.9 and 5.9 but not at pH 6.1. In basal salts medium containing glucose, 0.01% Trypticase soy stimulates growth, whereas 0.05% Trypticase soy inhibits growth. Sodium acetate at a concentration of 0.01% is strongly inhibitory. In defined media (basal salts medium containing a variety

of organic compounds), growth is sparse. Although enrichment with 0.01% Trypticase soy enhances growth, yields of more than 2×10^8 cells per ml seldom encountered. On agar media at 35°C, colonies 0.2 to 0.5 mm in diameter form after 1 week. Growth is slow at temperatures below 20°C, fastest between 35 and 41°C, and absent at 47°C. Endospores are not formed and cells die rapidly at 67°C. These bacteria are catalase positive, but the oxidase reaction is very weak or absent. Cells in basal salts liquid medium containing glucose measure 0.3 to 0.4 by 0.6 to 0.8 μ m, but cell size and shape are influenced by the kind and concentration of organic substrate and by physical conditions, such as pH. The G+C content of the DNA varies between 68 and 70 mol%. The type species is *A. cryptum* sp. nov., and the type strain of *A. cryptum* is Lhet2 (=ATCC 33463). The descriptions of the type species and the type strain are the same as the description given above for the genus.

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REPRINT REQUESTS

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