Acidiphilium angustum sp. nov., Acidiphilium facilis sp. nov., and Acidiphilium rubrum sp. nov.: Acidophilic Heterotrophic Bacteria Isolated from Acidic Coal Mine Drainage

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Acidophilic heterotrophic bacteria recovered from samples of water and sediment collected from acidic mine drainage streams were compared nutritionally, genetically, and morphologically. All 37 bacterial strains examined were rod shaped, motile, gram negative, and strictly aerobic, utilized citric acid and Tween 80 as sole carbon sources, and were unable to grow at or above pH 6.0. The ultrastructure of representative strains was not markedly different from that of gram-negative bacteria. Differences among the strains were evident in cell size (4.2 by 0.6 to 1.2 by 0.6 μm), pigmentation (when present), and nutritional faculties (the carbon sources suitable for growth of individual strains ranged from 8 to 20 of the 32 compounds tested). The guanine-plus-cytosine base composition of eight typical strains ranged from 63 to 68 mol%. All of the strains exhibited primary characteristics of the recently described genus Acidiphilium; however, important differences between our strains and the type species Acidiphilium cryptum suggested that new Acidiphilium species should be described. No significant deoxyribonucleic acid-deoxyribonucleic acid homology was found between five acidophilic heterotrophic strains and A. cryptum. Furthermore, no significant deoxyribonucleic acid-deoxyribonucleic acid homology was evident between the acidophilic heterotrophs and six Thiobacillus species. The bacteria which we studied could be divided into three groups based on genetic and nutritional characteristics. We propose the following three new species: Acidiphilium rubrum (type strain, strain OP [= ATCC 35905]), Acidiphilium angustum (type strain, strain KLB [= ATCC 35903]), and Acidiphilium facilis (type strain, strain PW2 [= ATCC 35904]).

Obligate acidophily is now a well-recognized bacterial trait that is not restricted to a particular group (12). Acidophiles are represented among heterotrophs (e.g., Bacillus acidocaldarius), archaea (e.g., Sulfolobus acidocaldarius and Thermoplasma acidophilum), and autotrophs (e.g., Thiobacillus ferroxidans). Recently, acidic mineral sulfide environments have been the source of several strains of mesophilic, acidophilic, heterotrophic bacteria (3, 8, 20). Harrison (4) has described and designated mesophilic, acidophilic heterotrophs recovered from cultures of Thiobacillus ferroxidans and other acidic mineral sulfide environments as members of a new genus, Acidiphilium. In this report we describe the taxonomic position of acidophilic heterotrophs which we isolated and the relationship of these organisms to other bacteria isolated from similar sources.

MATERIALS AND METHODS

Bacteria. The bacterial strains examined in this study were isolated as previously described (20) from acidic waters discharged from abandoned coal mines in central Pennsylvania.

Media and growth experiments. Nutritional and physiological characterization of strains was performed in duplicate as described previously (20). The majority of the nutritional and physiological tests were performed by using a basal salts medium modified as necessary for individual experiments.

This medium was prepared by adding the following reagent chemicals to distilled water: (NH₄)₂SO₄, 0.15 g/liter; KCl, 0.15 g/liter; KH₂PO₄, 0.15 g/liter; MgSO₄·7H₂O, 3.36 g/liter; CaCl₂, 0.97 g/liter; Al₂(SO₄)₃·18H₂O, 2.25 g/liter; and MnSO₄·H₂O, 0.12 g/liter. The substrates used for characterization are indicated below. The medium containing amino acids, carbohydrates, or ferrous iron were filter sterilized; other media were heat sterilized (121°C, 15 min). Alcohols were aseptically transferred to sterilized salt solutions from stock solutions. Unless indicated otherwise, experiments were performed with media adjusted to pH 3.0 with 3 N H₂SO₄ prior to sterilization and were incubated at 20°C.

Utilization of single carbon sources was assessed after a minimum of six transfers in similar media. When growth was slight, the experimental result was considered negative. Culture turbidity was monitored by measuring absorbance at 550 nm.

Cytological tests. Tests for Gram reaction, polyphosphate (Neisser stain [17]), and lipid inclusions (Burdon [7]) were performed by using washed cells at pH 7.0.

Ultrastructural studies were performed by using logarithmic-phase cells grown in 0.1% citric acid-basal salts medium. Negative staining was accomplished by using samples placed on Parlodion-carbon-coated 200-mesh grids for 5 min. The excess sample was removed, and the grids were rinsed several times with distilled water before staining for 1 min with 2% phosphotungstic acid at pH 7. All electron microscopy was performed with a model EM-300 electron microscope (Phillips Electronic Instruments, Inc., Mahwah, N.J.) operated at an accelerating voltage of 80 kV.

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DNA. Bacterial strains were cultivated in 0.1% citric acid–basal salts medium and harvested during the logarithmic phase of growth, and the deoxyribonucleic acid (DNA) was extracted by using the method of Marmur (15) and lysozyme and sodium lauryl sulfate to disrupt the cells. The guanine-plus-cytosine (G+C) contents of the DNAs of the strains PW1, PW2, PW2 (T = type strain), AWB, and BBW were determined by using the buoyant density method by M. Mendel. Extraction and determination of the G+C contents of the DNAs of strains PW2, AWB, KLB, and OPT by the thermal melting point method were performed by A. P. Harrison, Jr. The method used to determine DNA homology has been described previously (5). Homology was determined by using single-stranded radiolabeled reference DNA and unlabeled test DNA reassociated at 67°C. S1 nuclease was used to digest unassociated single-stranded DNA, and the reassociated double-stranded DNA was precipitated with cold trichloroacetic acid, collected on a membrane filter, and radioassayed to determine the degree of reassociation.

Lipids. A preliminary comparison of the general nature of the lipid patterns of strains KLB, PW1, and AWB, *Acidiphilium cryptum*, and heterotrophically grown *Thiobacillus acidophilus* was carried out by extracting 1.0 g (dry weight) of late-log-phase cells with chloroform-methanol-water (1:2:0.8, vol/vol), using the procedure of Bligh and Dyer (1). The total lipids were fractionated into neutral lipid and polar lipid classes on a column (1 by 10 cm) of silicic acid by elution with 10 column volumes of chloroform for neutral lipids and with methanol for polar lipids (glycolipids plus phospholipids).

Thin-layer chromatography of the polar lipids was performed on 0.25-mm layers of Silica Gel H developed in chloroform-methanol-water (65:25:4, vol/vol). Components were detected by exposure to I2 vapors or by charring after the plates were sprayed with 50% methanolic H2SO4. The spray reagent of Vaskovsky and Kostetsky (19) was used to detect phospholipids, and 0.2% ninhydrin in water-saturated n-butanol was used to detect free amine groups.

Fatty acid methyl esters were released from the polar lipids by acid methanolysis in 1 N anhydrous methanolic HCl at 100°C for 18 h. Fatty acid methyl esters were extracted into *n*-hexane and analyzed by gas-liquid chromatography. Analyses were conducted with a Hewlett-Packard model 402 gas chromatograph equipped with flame ionization detectors and a model 3370A digital electronic integrator. Glass columns (1.8 m by 4 mm) packed with 5% OV-11 or 10% SP-2100 were used at a temperature of 205°C. Fatty acid methyl esters and derivatives were identified by comparison with appropriate standards, as described elsewhere (16, 21).

**RESULTS AND DISCUSSION**

Little information exists regarding the microbial ecology of mineral sulfide environments. Historically, emphasis has been placed on the chemolithotrophic bacteria (i.e., *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*) (11). However, it is apparent that heterotrophic bacteria suited for survival in mineral acid regimes are present and diverse in their characteristics. Understanding and controlling mineral dissolution are contingent upon determining the ecological interactions of participating bacteria.

All of the strains examined in this study were gram negative, rod shaped, strictly aerobic, motile, heterotrophic, acidophilic (growth at pH 2.5, but not at pH 6.0), catalase positive, oxidase positive (washed cell pellets), nondenitrifying, nonencapsulated, nonsporforming, and pteridin 0/129 resistant, contained sudanophilic and polyphosphate granules, and were not halotolerant (7.5% NaCl). The cells exhibited a pleomorphic tendency at varied pH values and in the presence of different carbon sources.

Nutritional profile and size (Table 1) distinguished our strains from the type species of the genus *Acidiphilium*, *A. cryptum*. Furthermore, the G+C contents of our strains were lower than the values reported for *A. cryptum* (68 to 70 and 63 to 67 mol% G+C, respectively). The levels of DNA–DNA homology of strains PW2, AWB, OPT, and KLB with *A. cryptum* (Lht2 were 14% or less (Table 2) (A. P. Harrison, Jr., personal communication). There is no apparent justification for considering our bacteria strains of *A. cryptum*. However, the bacteria described in this paper are members of the genus *Acidiphilium* and are all acidophilic, gram-negative, motile, mesophilic, aerobic, rod-shaped organisms that cannot utilize acetate as a sole energy source but readily utilize citrate and other organic compounds and have DNA G+C contents between 63 and 70 mol%.

At least 36 of the 37 isolates examined grew on citric acid, aspartic acid, Tween 80, and one or more dilute (0.1, 0.2, or 1% (wt/vol) complex media containing tryptone, peptone, Casamino Acids, yeast autolyzate, or Casitone. The carbon sources or complex media which did not support growth of any isolate included acetic acid, formic acid, lactic acid, oxalic acid, glycolic acid, glycine, oxaloacetic acid, methanol, butanol, starch, tributyrin, Trypticase soy broth, and nutrient broth. Although glucose was not utilized as a sole carbon source by all isolates, a medium consisting of 0.01% (wt/vol) yeast autolysate in glucose-basal salts supported the growth of all isolates.

**TABLE 1. Differentiating characteristics of *Acidiphilium* species isolated from acidic coal mine drainage**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>A. angustum</em></th>
<th><em>A. rubrum</em></th>
<th><em>A. faciis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>G + C content</td>
<td>67</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Mean cell size</td>
<td>2.9 by 0.8</td>
<td>2.2 by 0.6</td>
<td>1.8 by 0.7</td>
</tr>
<tr>
<td>Population doubling time (h)</td>
<td>11</td>
<td>14</td>
<td>3.3</td>
</tr>
<tr>
<td>Pigmentation</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Inhibition by acetate</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Urea hydrolysis</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>H2S production from cysteine</td>
<td>-</td>
<td>-/+</td>
<td>+/+</td>
</tr>
<tr>
<td>Growth on:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>-</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
<td>-/+</td>
<td>+/+</td>
</tr>
<tr>
<td>Ethanol</td>
<td>+</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>L-Malate</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>cis-Aconitate</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>-</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>Fumarate</td>
<td>-</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>Glutamate</td>
<td>-</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>-</td>
<td>+/+</td>
<td>+/+</td>
</tr>
</tbody>
</table>

* G+C content of the DNA of the type strain.
* Tested in shaking cultures at 27 to 30°C in 0.1% (wt/vol) glucose–0.01% (wt/vol) yeast autolysate–basal salts medium (pH 3.0).
* More than 80% of all strains positive; –, more than 80% of all strains negative; +/+, more than 50% of all strains positive; –/–, between 20 and 80% of all strains positive.
* Glucose was tested as a sole carbon source. All strains grew in glucose-based salts when yeast autolysate was added.
Although physiologically similar, our isolates could be empirically divided into two major groups and a subgroup on the basis of nutritional faculties (Table 1). Shuttleworth et al. (18) investigated the intermediary metabolism of *A. cryptum* and several isolates used in this study (strains AWB, KLB∩, OP∩, PW2∩, and QBP). All of the bacteria examined apparently utilized the pentose phosphate and Entner-Doudoroff pathways for glucose catabolism and lacked the Embden-Meyerhoff-Parnas pathway. Group 1 consisted of 13 isolates which grew on three or fewer of the seven organic acids examined, were pigmented (9 of 13 isolates), and could not hydrolyze urea. Group 2 consisted of 24 isolates which grew on four or more of the same seven organic acids, were not pigmented, and generally hydrolyzed urea (22 of 24 isolates). The group 2 isolates were subdivided (subgroup 2a) on the grounds that eight isolates liberated H2S from cysteine and did not hydrolyze urea. Details of individual strain characteristics have been reported elsewhere (20).

Ten representative isolates examined by electron microscopy (strains AWB, PW1, PW2∩, KLB∩, OP∩, JPB, QBP, EECE, DPW, and TTO) exhibited polar to subpolar flagella and typical gram-negative outer membrane morphology marked by continuous convolution. Cell swelling was most evident among the group 1 isolates. Thin sections of cultures exhibiting swelling revealed the presence of electron-transparent material (Fig. 1) which was presumed to be polyhydroxybutyrate. Examination of the ultrastructure of representative isolates revealed the presence of cellular components (polyribosomes, cell membrane, and cell wall) typical of gram-negative bacteria.

The group 2 and subgroup 2a isolates examined had identical DNA G+C contents (65 ± 0.4 mol%); the DNA G+C contents of group 1 strains were 63 mol% (strain OP∩) and 67 mol% (strain KLB∩). The versatility of substrate utilization was far less for the group 1 isolates than for the group 2 strains. Group 2 isolates PW1, PW2∩, AWB, and BBW grew on 18 of the 32 substrates tested. Group 1 isolates KLB∩ and OP∩ grew only on 8 and 9 of the same 32 substrates, respectively.

Representative isolates (strains AWB, BBW, PW2∩, KLB∩, and OP∩) of the groups and the subgroup were selected for genetic and membrane lipid characterization. A preliminary analysis of the nature of the membrane lipids of representative isolates in the collection *Acidiphilium angustum* and *Acidiphilium facilis* revealed a pattern that is not typical of gram-negative, nonacidophilic, aerobic bacteria. The polar lipids of all isolates contained the same four phospholipids (Rf 0.34, 0.50, 0.63, and 0.82) when they were examined by thin-layer chromatography. In addition, all of the isolates contained two amino lipids (Rf 0.70 and 0.59) which accounted for about 40% of the total polar lipids. The principal fatty acids of the polar lipids consisted of straight-chain C14:0, C16:0, and C18:1 fatty acids, cyclopropane C15:0 fatty acids, and C14:0, C16:0, and C18:0 β-hydroxy fatty acids. In addition, the polar lipid and fatty acid profile of *A. cryptum* was identical to the lipid profiles of the representative isolates from the collection, indicating close taxonomic relationships. Furthermore, with the exception of an additional minor phospholipid, the same lipids were found in the facultative autotroph *Thiobacillus acidophilus*.

Acidophily is probably a definitive characteristic, involving basic modifications of the cell membrane essential to survival at high hydrogen ion concentrations. A preliminary comparison of the acidophilic heterotrophs and *A. cryptum* with respect to membrane lipid pattern revealed identical compositions. In addition, the amino lipids, β-hydroxy fatty acids, and cyclopropane fatty acids present in the heterotroph membranes resembled the lipid composition of the autotrophic thiobacilli, *Thiobacillus thioparus* and *Thiobacillus ferrooxidans* (9, 10, 13, 14). However, these latter taxa contain only one ornithine-based amino lipid (10), whereas the heterotrophic acidophiles contain two as-yet-unidentified amino lipids as major constituents. Furthermore, autotrophic acidophilic thiobacilli possess ubiquinone Q-8 (9), and the heterotrophic acidophiles and *A. cryptum* contain Q-10 as the major lipoquinone (2). The lipid compo-

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**TABLE 2. Comparative nucleic acid homologies for acidophilic, heterotrophic bacteria and *A. cryptum* and *Thiobacillus* spp.**

<table>
<thead>
<tr>
<th>Acidophilic, heterotrophic test strain</th>
<th>% Homology with the following reference strains:*a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>A. cryptum</em> Lhe12</td>
</tr>
<tr>
<td>A. facilis PW2∩</td>
<td>5*</td>
</tr>
<tr>
<td>A. facilis AWB</td>
<td>2</td>
</tr>
<tr>
<td>A. rubrum OP∩</td>
<td>14</td>
</tr>
<tr>
<td>A. angustum KLB∩</td>
<td>2</td>
</tr>
</tbody>
</table>

* The G+C contents of the DNAs of the reference strains were as follows: *A. cryptum* Lhe12, 68 to 70 mol%; *Thiobacillus ferrooxidans* m-1, 65 mol%; *Thiobacillus acidithiooxidans* 7P, 63 to 64 mol%; *Thiobacillus versutus* A-2, 68 mol%; *Thiobacillus peromelobatis* ATCC 2337C, 66 mol%; *Thiobacillus novellus* ATCC 8093, 68 mol%; and *Thiobacillus intermedius* ATCC 15466, 67 mol%.

* Level of homology between test strain DNA and tritiated reference strain DNA.

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**FIG. 1.** Thin section of *A. angustum* KLB∩, showing electron-transparent material. Bar = 1 μm.
sition of the heterotrophic acidophiles appears to be similar to that of Thiobacillus thiooxidans or Thiobacillus ferrooxidans and very similar to that of the facultative autotroph Thiobacillus acidophilus, which also contains lipoquinone Q-10 (9).

All of the isolates examined for DNA homology (strains PW2\textsuperscript{7}, AWB, OP\textsuperscript{9}, and KLB\textsuperscript{9}) exhibited less than 20% homology (Table 2) with reference DNA from A. cryptum Lhet2, Thiobacillus acidophilus 7p, Thiobacillus versutus, Thiobacillus perometabolus ATCC 23370, Thiobacillus novellus ATCC 8093, Thiobacillus intermedius ATCC 15466 and Thiobacillus ferroxidans m-1 (Harrison, personal communication). Homology data were not available for isolate OP\textsuperscript{9} and Thiobacillus acidophilus, Thiobacillus versutus, or Thiobacillus novellus.

The members of the genus Thiobacillus are nutritionally, genetically, and physiologically diverse (5, 6, 11); however, all of these organisms can derive energy from reduced iron or sulfur compounds. The heterotrophic strains which we studied are incapable of autotrophic or mixotrophic activity and share little DNA homology with the Thiobacillus species tested. The genus Acidiphilium consists of obligately acidophilic, heterotrophic bacteria isolated from mineral sulfide environments (4). We have assigned our isolates to Acidiphilium, recognizing differences between them and the only previously described species, A. cryptum. It appears that our acidophilic isolates represent three new species.

We propose the three new species described below. The type strains have been deposited with the American Type Culture Collection. The descriptions of the type strains are the same as those for the species.

**Acidiphilium rubrum** sp. nov. *Acidiphilium rubrum* (ru'brum. L. adj. rubrum red colored). In addition to the characteristics given previously for the genus, *A. rubrum* is distinguished by a reddish violet pigmentation of colonies and broth cultures, a cell size of approximately 2.2 by 0.6 \(\mu m\) when the organisms are grown in citric acid-based salts medium, polar flagellation, growth inhibition by acetate, and a lack of nutritional versatility. L-Malate and \(\alpha\)-ketoglutarate support growth; however, cis-acconitate, glycerol, lactose, fumarate, pyruvate, and ethanol do not support growth. The G+C content of the DNA is 63 mol\%. The type strain is strain PW2 (= ATCC 35904).

**Acidiphilium angustum** sp. nov. *Acidiphilium angustum* (an.gus'tum. L. adj. angustum limited, with respect to nutritional versatility). In addition to the characteristics given previously for the genus, *A. angustum* is distinguished by a pink pigmentation of colonies and broth cultures, cells which are often swollen and distended, a cell size of approximately 2.9 by 0.8 \(\mu m\), polar flagellation, growth inhibition by acetate, and a lack of nutritional versatility. No growth occurs on \(\alpha\)-ketoglutarate, cis-acconitate, glutamate, glycine, lactose, succinate, L-malate, pyruvate, or fumarate. Ethanol and glycerol support growth. The G+C content of the DNA is 67 mol\%. The type strain is strain KLB (= ATCC 35905).

**Acidiphilium facilis** sp. nov. *Acidiphilium facilis* (fa'ci.lis. L. adj. facilis ready, quick, with respect to growth). In addition to the characteristics given previously for the genus, *A. facilis* is distinguished by white or light brown colonies, a cell size of approximately 1.8 by 0.7 \(\mu m\), cells which are often observed in chains and small flocs, polar flagellation, a lack of acetate inhibition, nutritional versatility, and more rapid and luxuriant growth than the other species of Acidiphilium. Urea is hydrolyzed, and glucose, glycerol, lactose, cis-acconitate, glutamate, succinate, L-malate, fumarate, \(\alpha\)-ketoglutarate, and ethanol are all readily utilized for growth. The type strain is strain PW2 (= ATCC 35904).

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We thank M. Mandel for determining the DNA G+C contents of the strains. Appreciation is also extended to A. P. Harrison, Jr., for determining DNA homologies and G+C contents and for his encouragement and consultation during preparation of the manuscript.

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