Acidophilic, Heterotrophic Bacteria of Acidic Mine Waters

PAUL L. WICHLACZ AND RICHARD F. UNZ*

Department of Civil Engineering, The Pennsylvania State University, University Park, Pennsylvania 16802

Received 1 December 1980/Accepted 2 March 1981

Obligately acidophilic, heterotrophic bacteria were isolated both from enrichment cultures developed with acidic mine water and from natural mine drainage. The bacteria were grouped by the ability to utilize a number of organic acids as sole carbon sources. None of the strains were capable of chemolithotrophic growth on inorganic reduced iron and sulfur compounds. All bacteria were rod shaped, gram negative, nonencapsulated, motile, capable of growth at pH 2.6 but not at pH 6.0, catalase and oxidase positive, strictly aerobic, and capable of growth on citric acid. The bacteria were cultivatable on solid nutrient media only if agarose was employed as the hardening agent. Bacterial densities in natural mine waters ranged from approximately 20 to 250 cells per ml, depending upon source and culture medium. Ferric hydrates and stream vegetation contained from 1,500 to over 7 × 10⁶ cells per g.

Acid-laden waters, which discharge from abandoned and active coal mines, characteristically contain excessive total dissolved solids, pronounced mineral acidity, and have low pH values. The chemical nature of acidic drainages favors colonization by a diversity of acid-intolerant life forms. Consequently, these environments have been referred to as "extreme."

Most of the research on the microbiology of acidic mine waters has focused on the chemolithotrophic action of the iron- and sulfur-oxidizing thiobacilli. *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* have been implicated as contributors to the formation of the major pollutants contained in acidic coal mine drainage. However, published accounts exist on the recovery of sundry heterotrophic bacteria from acidic mine waters (7, 14, 15, 25); albeit, limited attention has been directed to the question of acidophily among the isolated organisms. Langworthy (16) has cautioned against the possible misconceptions that could develop about the ecology of bacteria recovered from low pH environments but which grow only at circumneutral pH values in the laboratory. Information is essentially lacking on the existence and distribution of heterotrophic bacteria which reside in the acidic mine water environment and are capable of proliferation at the low pH values present.

Limited success in the recovery of large numbers of heterotrophic bacteria from acidic mine drainages has hampered investigations on the ecology of these organisms (14). Interest in the microbiology of low pH regions has extended recently to the effect of acid rain on soils and water bodies (1, 13).

The present study entailed a search for heterotrophic bacteria with potential for survival and reproduction at the depressed pH values typically encountered in acidic mine drainages. Some insight is provided on the characteristics and numbers of truly acidophilic, heterotrophic bacteria of the mine water environment.

**MATERIALS AND METHODS**

**Sample sites.** Two acidic mine drainage sites in central Pennsylvania were investigated over a 15-month period (September 1977 to December 1978). Only fresh discharges, unadulterated by alkaline surface waters, were sampled. Proctor 2 drainage originates at an abandoned deep mine in Hollywood, Pa., and flows underground approximately 180 m before erupting to become a tributary of Tyler Run. Hawk Run, which is located near Philipsburg, Pa., forms primarily from deep mine drainage discharged at borehole 3 and, secondarily, from the overflow at borehole 1. Actual sampling took place in the vicinity of the overflow of Proctor 2 abandoned mine and the Hawk Run boreholes, as well as the confluence of the discharges from the boreholes in Hawk Run.

**Sample collection and preparation.** Mine waters and residues were collected in heat-sterilized, screw-capped, Nalgene plastic cups, transported on ice, and processed within 6 h after arrival at the laboratory. Residues, e.g., gelatinous ferric hydrates (stream bed "yellow boy" and the solids adhering to the discs of a rotating biological contactor involved in experimental treatment of Proctor 2 mine drainage) and crude *Euglena* sp. biomass, were homogenized by slow mechanical blending in a Waring blender. The suspensions were used as inocula for enrichment cultures and bacterial isolation and enumeration experiments. Dry weight determinations were made on samples stored overnight at 103°C and cooled to constant weight.

**Culture media.** All culture media were prepared with distilled water. Media used in enrichment culture
experiments and many of the bacterial isolation and
coloration exercises contained the following
mineral salts (in grams per liter): (NH4)2SO4, 0.15;
KCl, 0.15; K2HPO4, 0.15; MgSO4·7 H2O, 3.36; CaCl2,
0.97; Al2(SO4)3·18 H2O, 2.25; and MnSO4·H2O, 0.12.
Culture media containing amino acids and carbohy-
drates were filter sterilized by passage through 0.2-µm
membrane filters (Millipore Corp., Bedford, Mass.).
Other media were heat sterilized at 121°C for 15 min
unless otherwise noted. All culture media employed in
enrichment, isolation, and bacterial coloration
work were adjusted to pH 3.0 with 12 N H2SO4 unless
stated otherwise.

Solid versions of enrichment media were prepared
with one of the following agars (in grams per liter):
Noble agar (Difco Laboratories, Detroit, Mich.), 12;
Ion agar no. 2 (Colab Laboratories, Glenwood, III.), 10;
purified agar (Difco), 14; agar (Difco), 15; and agarose
(Miles Laboratories, Inc., Elkhart, Ind.), 10. Agar sus-
pensions were heat sterilized and combined aseptically
with other ingredients to produce complete culture
media.

In addition to the above, other media employed in
the study were (i) ferrous iron-mineral salts medium
of Manning (18) in unmodified form or altered by
adding to a liter of medium 100 ml of combined hot
and cold extracts (1:1, vol/vol) of residues obtained
from the rotating biological contactor, (ii) tryptone-
glucose-beef extract-yeast extract medium of Dungan
et al. (7) with pH adjusted to 4.0, and (iii) mineral salts
of Olem and Umz (20) solidified with Ion agar no. 2.

Enrichment cultures. Enrichment cultures were
started by transferring 5 ml of mine water or homog-
enized solids to 50 ml of a culture medium contained
in a 125-ml Erlenmeyer flask. Duplicate enrichment
cultures were prepared for every carbon source and
inoculum tested. Carbon sources used in the prepara-
tion of enrichment culture media are given in the
results of enrichment culture experiments. Enrich-
ment cultures were incubated for up to 4 weeks on a
gyratory shaking machine (110 rpm) at 10, 20, and
28°C. Cultures were examined microscopically at 3-
day intervals.

Isolation of bacteria. Axenic recovery of bacteria
was attempted by (i) dispersion-serial dilution of ma-
ture enrichment cultures, wherein cells present in the
highest dilution exhibiting turbidity in fresh culture
medium were repeatedly subjected to the dilution
process (minimum of three passages) and, subse-
quently, plated on a solid culture medium, and (ii)
direct spread plating of enrichment culture and fresh
environmental samples. Plates were incubated at 20°C
for up to 28 days.

Preparation of inocula for strain characteri-
zation. Cells were washed twice in sterile, distilled
water (pH 3.0) after intermittent centrifugation at
1,110 × g for 10 min. Standard test tubes (outer
diameter, 16 mm) containing 5 ml of culture medium
received 0.05 ml of a turbid suspension of washed cells.

Characterization of strains. Morphology of cells
was determined with the aid of a Zeiss Universal
microscope (Carl Zeiss, Inc., New York, N.Y.)
equipped with Neofluar phase objectives. Cell dimen-
sions were determined by measuring the photographic
images of cells against a stage micrometer photo-
graphed at the same magnification (x1,250). At least
20 cells were measured per strain. Gram staining and
the staining of cell inclusions were performed on
washed cells at pH 7.0.

Catalase, capacity to develop at pH values of 2.6
and 6.0, susceptibility to the vibriostat, 0.129 (5.0 µg
of 2,4-amino-6,7-diisopropyl pteridine per liter), and
denitrification (0.25 g of KNO3 per liter) were deter-
mined in mineral salts and citric acid (0.5 g/liter).
Growth of strains at pH 2.6, but not at pH 6.0, was
considered a positive expression of the property of
acidophily.

Kovacs oxidase and iron porphyrins (5) were tested
on concentrated cell mass washed with acidic dis-
tilled water (pH 3.0).

Tolerance to ferrous iron was evaluated on mineral
salts-succinic acid (1.0 g/liter) and 3.0 g of Fe(II) per
liter supplied as FeSO4·7 H2O.

Tolerance to 7.5% NaCl, capacity to produce H2S
(detectable with lead acetate paper) from cysteine (0.1
liter), and ability to hydrolyze urea (0.1 g/liter) were
tested in yeast autolysate (0.1 g/liter). Evidence of
urea hydrolysis was taken as a change in the pH of
cultures from a value of 3.0 (control) to at least 5.0.

Acid production from glucose and glycerol was en-
visioned as a decrease in the measured pH of cultures
by at least 0.15 pH units.

Utilization of starch and Tween 80 were tested in
media solidified with agarose.

Reagent grade carbon sources were employed in
nutritional studies. Organic acids were tested at 0.5 g/
liter; other carbon sources were tested at 1.0 g/liter.

Potential of strains for chemolithotrophic growth
was determined in mineral salts containing one of the
following energy sources (in grams per liter): precipi-
tated sulfur, 10.0; sodium thiosulfate, 10.0; and ferrous
iron, 1.5. Energy sources were sterilized separately and
combined with autoclaved mineral salts to complete
media. Cultures were incubated for a minimum of 60
days.

Utilization of carbon and energy sources was visibly
scored on a growth-no growth basis.

RESULTS

Mine water characteristics. The chemical
properties of the acidic mine drainages, except
borehole 3 water, have been published elsewhere
(21). Briefly, Proctor 2 mine water had an ap-
preciably greater iron and aluminum content
[Fe(III), 121 ± 31 µg/ml; Fe(II), 146 ± 69 µg/ml; and
Al(III), 57.5 ± 1.6 µg/ml] and lower pH (mean value of
2.74) than did either Hawk Run [Fe(III), 9.6 ± 2.8 µg/ml; Fe(II), 53.2 ± 2.0 µg/
ml; Al(III), 8.6 ± 0.4 µg/ml; and mean pH of
(3.42) or borehole 1 overflow [Fe(III), 6.9 ± 2.4
µg/ml; Fe(II), 62.8 ± 8.8 µg/ml; and Al(III), 6.2
± 1.4 µg/ml; and mean pH of 3.77]. Mineral
analyses were not conducted on the overflow
from borehole 3, however, a tributary to Hawk
Run, which is derived from the borehole, con-
tained Fe(III), 11.4 ± 5.6 µg/ml; Fe(II), 32.8 ±
4.3 µg/ml; Al(III), 10.1 ± 2.3 µg/ml; and mean
pH of 3.15. Temperature of all mine waters
varied little from 10°C over the study period,
with extremes of 7 and 11°C. Since sampling was performed in the immediate vicinity of subsurface discharges, except for Hawk Run proper, little opportunity existed for serious thermal modification of mine waters by contact with other surface drainage or the atmosphere.

**Isolation of bacteria from enrichment cultures and natural sources.** Characteristics of enrichment cultures and strain designations of the respective bacterial isolates are given in Table 1. Most of the mature enrichment cultures contained bacteria; however, the presence of molds and yeasts often interfered with the routine recovery of axenic bacteria. Complex substrates and amino acids, but not temperature of incubation and the concentration of substrates used, appeared to be most stimulatory to fungus development.

Iron-oxidizing bacteria could be recovered readily from enrichment cultures and mine waters; however, only acidophilic, heterotrophic bacteria were sought in this study. Certain of the bacteria isolated from enrichment cultures, regardless of inoculum source, possessed similar morphological and colonial properties. Selection of specific bacteria for additional characterization was based on apparent dominance and frequency of occurrence among bacterial colonies cultivated by plating enrichment cultures.

A total of 75 strains of microorganisms were obtained from enrichment cultures and natural sources by the various isolation methods employed. Microscopic observations revealed that 14 of the isolates were yeasts, and these were discarded. Two green colonies, which developed under low-level fluorescent lighting on solidified mineral salts without organic substrates, proved to be euglenoids and were identified as *Euglena mutabilis* (17). All remaining organisms in the collection were bacteria, of which 22 were lost upon further subculture, leaving 37 working strains. Of the 37 bacteria, 4 strains were recovered by plating axenic cultures of organisms purified by dispersion-dilution of enrichment cultures, and 23 strains were isolated by plating enrichment cultures directly (Table 1). Another 10 strains were obtained from among colonies appearing on countable enumeration plates inoculated with environmental specimens (see Table 3). Citric and malic acids were selected as carbon sources for the recovery of bacteria directly from natural sources after it was discovered that these two organic acids supported growth of a majority of strains isolated from enrichment cultures. Important in the course of isolation work was the observation that no solidifying agent, other than agarose, permitted development of bacteria. Attempts to isolate acidophilic, heterotrophic bacteria from mine waters by employing methods or culture media of other workers were unsuccessful.

**Characteristics of isolated bacteria.** The following morphological and physiological characteristics were shared by all of the surviving 37 strains: cells single or as diplobacilli, rounded to slightly pointed ends, gram negative, strictly aerobic, actively motile, nonencapsulated, aerobic, and malic acids. Modal values of cell width and length of strains actively growing on citric acid (0.5 g/liter) ranged from 0.5 to 1.1 μm and 1.0 to 4.2 μm, respectively. Cellular morphology appears to be affected by the available carbon source (Fig. 1). Sudanophilic cellular inclusions, presumably poly-β-hydroxybutyric acid, were present in cells of many strains, especially those cultured on organic acids.

Most strains produced colonies in 14 to 30 days which were convex, entire, and 1.0 mm or less in diameter. Nineteen strains formed shades of brown and pink pigments which were enhanced in complex, nitrogenous culture media.

Although all strains grew when supplied citric acid as a sole carbon source, certain cultures developed better than others. For example, using absorbance measurements (optical density at 450 nm) as an indication of relative cell densities,
values of less than and greater than 0.5 were recorded for 11 and 26 strains, respectively, after incubation for 30 days at 20°C. All strains attained maximum cell densities before 30 days, and the utilization of citric acid resulted in a mean increase in the pH of culture fluids from 2.6 to 4.05 (standard deviation = 0.3 pH units).

The 37 strains were divided into two major groups based on an ability to grow on organic acids supplied as sole carbon sources (Table 2). Group 1 organisms were capable of growth on only three or less of the seven organic acids which supported any of the strains. Group 2 bacteria utilized at least four of the seven organic acids and a subgroup (2a) distinguished eight organisms capable of forming H₂S from cysteine. Two other organic acids, glycolic and oxalacetic acids, were not utilized by any strains.

Of the 11 strains previously indicated to have developed poorly on citric acid, 10 belonged in group 1, and 7 of these were isolated directly from environmental samples. Possibly, preex-
Table 2. Organic acids utilized by acidophilic, heterotrophic bacteria

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain code</th>
<th>Citric</th>
<th>Malic</th>
<th>Succinic</th>
<th>cis-Aconitic</th>
<th>ε-Keto-glutaric</th>
<th>Fumaric</th>
<th>Pyruvic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>KLB</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PMA</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>QBP</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>FP</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>NW</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>JPB</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>OP</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>TOR</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PLP</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>HW</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CCP</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>HHY</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>ZZZ</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>MA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>MW</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>EEB</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>SSO</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>SW</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>DDW</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>DO</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>EEEC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PW1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PW2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>SAW3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>SAW5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>YYB</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>GBW</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>AAAW</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>BBBW</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>2a</td>
<td>GGW</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>NNW</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>QWW</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>TTO</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>RRO</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>AAW</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>AWB</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>BBW</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

a Organic acids tested in static culture at 0.5 g/liter, pH 3.0, and 20°C.
b Bacterial groups were designated on the following basis: group 1 strains utilized three or less of seven organic acids tested; group 2 strains utilized at least four of seven organic acids tested and included a subgroup 2a which liberated sulfide from cysteine.
+c, Growth visible after 30 days; otherwise no growth was evident.

Exposure to a variety of metabolic products during growth in enrichment cultures may explain the increased versatility for organic acid utilization by enrichment culture isolates over strains recovered directly from the environment.

No strains required supplementary iron for growth. Despite the inability of a minor number of bacteria to utilize the compound, succinic acid rather than citric acid was employed in iron tolerance studies because the latter organic acid formed interfering precipitates in the presence of ferrous iron. A high ferrous iron concentration, typical of that present in culture media used to grow T. ferrooxidans, was tolerated by 23 of 27 strains capable of growth on succinic acid. No group 1 strain capable of growth on succinic acid was tolerant to high ferrous iron levels, whereas only subgroup 2a strain NNW was unable to develop in the presence of extensive ferrous iron.

With the exception of strains EEEC and EEB, which were unable to utilize glucose and glycerol, respectively, all group 2 and subgroup 2a bacteria grew on these carbon sources. Only two group 1 strains (CCP and HHY) grew on glucose, although a majority of group 1 bacteria (PMA, NW, FP, JPB, TOR, CCP, HW, and ZZZ) utilized glycerol. Glycerol and glucose utilization...
were accompanied by a decrease in pH from 3.0 to mean values of 2.76 (standard deviation = 0.072) and 2.71 (standard deviation = 0.045), respectively.

All strains, except strain HW, utilized aspartic acid as a sole carbon source. Glutamic acid supported growth of only three group 1 strains (QPB, PLP, and ZZP), although all but five group 2 strains (MW, MA, SW, EEB, SSO) and one subgroup 2, strain (NNW) utilized the amino acid. Growth of strains of amino acids produced an increase in the pH of culture fluids from 3.0 to mean values of 5.83 (standard deviation = 0.15) for glutamic acid and 5.2 (standard deviation = 0.21) for aspartic acid.

Only three strains failed to grow in complex nitrogenous media, namely, strain SW (Casanino Acids, peptone, and tryptone); strain HW (Casitone and tryptone); and strain PLP (tryptone). No strains active on tryptone formed indole. Yeast autolysate supported growth of all group 2 and subgroup 2, organisms, except strain EEEC. Only group 1 strains HHY and ZZP grew on yeast autolysate. No strains were found to be halotolerant when NaCl was included in the yeast autolysate medium.

Tween 80, but not starch, appeared to stimulate growth of strains based on a comparison of the size of colonies on plates containing and not containing substrates (mineral salts only). No hydrolysis of starch was detectable with iodine. The calcium soaps, normally formed around colonies active on Tweens, were not evident, possibly due to the low pH of the culture medium.

Justification for the separation of isolates into groups was strengthened by the finding that none of the group 1 bacteria hydrolyzed urea, whereas only two group 2 strains (AWB and BBW) did not. Inorganic nitrogen sources, nitrate and ammonia, were used by all bacteria. Nitrate did not serve as an ultimate electron acceptor.

**Natural acid mine drainage bacteria populations.** Citric acid supported greater numbers of the bacteria present in natural mine water samples than did malic acid, although the differences were not appreciable (Table 3). The largest numbers of organisms and diverse colony types were found in connection with the plating of *Euglena* sp. biomass, in particular, specimens taken from the Proctor no. 2 stream bed.

**DISCUSSION**

All bacteria obtained directly or indirectly from mine water samples were obligately acidophilic (capable of growth at pH 2.6 but not pH 6.0) and gram negative. No relationships could be drawn between specific characteristics of the bacteria and the sources from which they were isolated, except that it appeared that strains recovered from enrichment cultures were active on more carbon sources than were bacteria directly isolated from the environment. The data obtained support the contention that acidophilic, heterotrophic bacteria ubiquitously exist in the acidic, mine water environment and may be expected to encounter limited competition from allochthonous organisms, e.g., coliform bacteria (10).

<table>
<thead>
<tr>
<th>Table 3. Viable, heterotrophic bacteria densities and acidophilic strains isolated from acidic mine water environments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Source</strong></td>
</tr>
<tr>
<td>Proctor 2 mine water</td>
</tr>
<tr>
<td>Proctor 2 <em>Euglena</em> sp. biomass</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Borehole 1 mine water</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Borehole 1 ferric hydrates</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Borehole 1 <em>Euglena</em> sp. biomass</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Borehole 3 mine water</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

*a Viable counts obtained on a mineral salts medium containing the following (g/liter): carbon source, 0.15; ferrous iron as FeSO₄·7 H₂O, 0.325; and agarose, 10. Final pH was 3.0. Incubation was at 20°C for 28 days. Values reported are the mean of four determinations.

*b Values represent sum of colony types observed on the four countable plates used to estimate mean cell densities.

*c Strains isolated from among colonies present on countable plates.
Purification of enrichment culture bacteria and the isolation and enumeration of bacteria in natural samples were satisfactorily accomplished through conventional plating techniques only when agarose was the solidifying agent.

Previous workers have demonstrated the existence and nonexistence of gram-positive bacteria in acidic mine waters. Johnson et al. (14) isolated several gram-positive bacteria, including staphylococci, micrococci, corynebacteria, and sporeforming bacilli, from the drainage of an abandoned coal mine. Joseph (15) employed unspecified culture media to recover 14 strains of *Bacillus* sp., 20 strains of *Micrococcus* and *Sarcina* spp., and only two strains of *Escherichia* and *Enterobacter* (Aerobacter) from coal mine drainage-impregnated streams. Dugan et al. (7) used media solidified with agarose to isolate two gram-positive and five gram-negative bacteria from "streamers" present in an acidic (pH 2.8) mine drainage. A *Bacillus* sp., which produced exocellular polysaccharide and grew optimally at circumneutral pH, was identified as a major member of the slime population. These workers suggested that the predominant gram-positive bacteria of the streamers may exist in microzones, with pH values much closer to neutrality than those of the acidic bulk water.

Tuttle et al. (25) obtained eight gram-negative bacilli and no gram-positive bacteria from an acidic, coal pile drainage. The absence of gram-positive bacteria in the drainage was explained on the basis of a greater permeability of the gram-positive cell wall to toxic hydrogen ion. Guthrie et al. (9) noted reductions in the incidence of *Bacillus* and *Sarcina* species in coal ash drainage when the pH was lowered from 6.5 to 4.6 upon introduction of fly ash to the drainage basin. Harrison (11) described a microbial succession in artificial coal refuse which involved acid-tolerant heterotrophs. It is apparent that hydrogen ion alone may not be totally responsible for adverse effects on gram-positive bacteria in acidic environments since certain *Bacillus* spp., e.g., *B. coagulans* and *B. acidoaldrarius* require distinctly acid conditions for growth (2, 4).

Previous investigators have found a preponderance of gram-negative bacteria among organisms recovered on distinctly acid culture media. Manning (18) obtained 21 strains of gram-negative heterotrophic bacteria from acid mine drainage employing a mineral salts medium (pH 3.0) without organic supplements other than purified agar as a solidifying agent. The better characterized strains, designated *Pseudomonas acidophilia*, were motile, strictly aerobic, oxidase positive, indole and H$_2$S negative, and grew optimally at pH 3.5 and 37°C. Belly and Brock (3) observed yellow-pigmented, gram-negative bacilli in cultures developed at pH 2.5 from an inoculum of coal refuse. Millar (19) employed a tryptone-yeast extract (pH 6.0) medium to recover acid-tolerant heterotrophs, principally flavobacteria, in concentrations of 1,000 to 10,000 cells per ml of acidic deep mine drainage.

Little information is available on the numbers of acidophilic, heterotrophic bacteria present in coal mine environments. Tuttle et al. (25) found low numbers of microorganisms in acidic (pH 2.5 to 3) coal refuse drainage (mean aerobic, heterotrophic densities of 15 to 19 cells per ml of drainage, depending on culture medium composition), with the major populations represented by yeasts and fungi. It was suggested that the microorganisms were transient, acid tolerant, and nonproliferating in mine water. Our results indicate that a variety of bacteria, which require a distinctly acid pH for growth, exist in various locales of acidic mine streams, with largest densities found in association with ferric hydrates and stream vegetation. Acidophilic heterotrophs were within one log of the concentration of iron-oxidizing bacteria by comparison with data of Olem and Unz (21) collected on similar sample sites. The organic carbon content of mine water is low, with values of 1 to 5 μg of total organic carbon per ml reported for Proctor 2 drainage (21). Dugan (6) has proposed organic depot products of iron-oxidizing thiobacilli, excretions of algae, and coal leachates as possible nutrient sources for heterotrophic bacteria. Quantitative information on acidophilic heterotrophs in mine drainage must be interpreted with liberties since the choice of carbon source, pH of culture medium, and enumeration method will likely influence results obtained.

Harrison et al. (12) isolated acidophilic, glucose-utilizing heterotrophic bacteria from *T. ferrooxidans* cultures, and on the basis of deoxyribonucleic acid homology studies, concluded that the organisms were genetically unrelated to *T. ferrooxidans*. Furthermore, these workers questioned facultative autotrophy by *T. ferrooxidans*, as reported by others (22, 23). Guay and Silver (8) isolated from a *T. ferrooxidans* culture, a facultative autotroph (*T. acidophilus*) which utilized as energy sources elemental sulfur, several sugars, and citric, malic, aspartic, and glutamic acids. Tuovinen et al. (24) reported autotrophic development of *T. acidophilus* on ferrous iron.

The 37 strains described herein could not be induced to utilize inorganic energy sources even after long periods of exposure. Orange or rust-colored colonies, typical of *T. ferrooxidans*, never appeared on iron-containing solid media inoculated with heterotrophic bacteria. Moreover, all
strains in the collection were maintained through numerous subcultures on media lacking ferrous iron. However, we have demonstrated that strains PW1 and PW2 require approximately 200 μg of Fe(II)/ml for optimum development on succinic acid medium. Zavarzin (26) isolated from an acid (pH 4.0) peat bog inhabited by *T. ferrooxidans* an acidophilic heterotroph which grew best on citric acid supplied as a sole carbon source and required at least 3 μg of ferrous ammonium sulfate per ml for optimum development.

Insufficient knowledge on the behavior and characteristics of the newly isolated bacteria precludes assignment of binomial epithets. Presently, group 2 strains PW1 and PW2 are undergoing intensive scrutiny. It is evident from the results of our investigations and those of others that further research is needed to establish the taxonomic position of the acidophilic, heterotrophic bacteria of acidic mine drainage and to identify their role in the environment.

ACKNOWLEDGMENTS

The research was performed for the Institute for Research on Land and Water Resources at the Pennsylvania State University under grant 416 from the Pennsylvania Science and Engineering Foundation, Department of Commerce, Commonwealth of Pennsylvania.

LITERATURE CITED