Heterotrophic Bacterial Population in Acid Coal Mine Water: *Flavobacterium acidurans* sp. n.¹

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Acid water from a closed deep coal mine was examined for the presence of heterotrophic bacteria. This water, which is routinely pumped to a surface stream for runoff, did not contain "acid streamers" or slime-producing, sporeforming bacteria. However, aerobic, nonmotile, yellow, nonfermentative, gram-negative rods were found by using the most-probable-number technique with a dilute tryptone-yeast extract medium. The isolates displayed binary fission as the mode of reproduction although abortive division, as evidenced by mini-cell formation, occurred at a low frequency. Based on morphological and other characteristics, including deoxyribonucleic acid base composition, a new species, *Flavobacterium acidurans*, is proposed for these bacteria. The type strain of this organism is ATCC 27383. Periodic sampling of mine effluent over a 1-year period disclosed that this organism comprises a major microbial component of the acid environment.

Drainage of acid waters from coal mines is a serious pollution problem in Appalachia due to the high levels of iron, sulfate, and hydrogen ions in these waters. These components are derived, in part, from oxidations of reduced iron and sulfur compounds, such as pyrite and marcasite, by members of the genera *Thiobacillus* and *Ferrobacillus* (4). Such iron-oxidizing bacteria possess a chemolithotrophic nutrition. They grow in the complete absence of preformed organic compounds if ferrous iron, carbon dioxide, and mineral salts are supplied. One consequence of such growth, however, is the release of organic compounds into the surrounding medium. For example, Schnaitman and Lundgren (25) found that 9.6% of the carbon dioxide fixed by *Ferrobacillus ferrooxidans* was released extracellularly.

Because organic matter is liberated by *F. ferrooxidans* during growth, the possibility exists that acid mine water may support the growth of heterotrophic bacteria. Dugan et al. (7) investigated this possibility with pH 2.8 water from an Ohio coal mine that displayed "acid streamers." From such streamers five gram-negative bacteria, two gram-positive bacteria, and one yeast were isolated (8). One of the isolates, a *Bacillus* species, was shown to be

responsible for production of the acid streamers. The gram-negative isolates, however, could not be maintained for more than three transfers in the laboratory.

The purpose of the present study was to determine whether acid coal mine water that lacked streamers also possessed an indigenous heterotrophic bacterial microflora.

MATERIALS AND METHODS

Mine water. The acid mine water examined was the effluent from a pump, which removes water from the Pursglove no. 15 Mine. This mine, located about 5 miles (about 8 km) north of Morgantown, W. Va., and owned by the Christopher Coal Co., has been closed for approximately 20 years. Over the past 2 years the pump, operating semicontinuously, has eliminated an average of 800,000 gallons (about 3,028,000 liters) of water each day. The water has a pH of 2.6 and, because it is drawn from a depth of 292 feet (about 89 meters), has a constant temperature of 20 C.

Sampling procedure. Water samples were collected in sterile Erlenmeyer flasks directly from the mouth of the pump to minimize chances of introducing microorganisms not indigenous to the mine water. The flasks, filled to capacity to prevent oxidation during transportation to the laboratory, were examined bacteriologically and chemically within 2 h after collection.

Chemical determinations. Total dissolved iron levels in mine water samples were determined using the 1,10-phenanthroline method described (catalog no.

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10, 1969, Hach Chemical Company, Ames, Iowa) for a Hach DR Colorimeter. Sulfate ion concentrations were assayed by the barium sulfate turbidimetric method, also according to the Hach procedure.

Isolation and enumeration of bacteria. All bacterial populations in acid mine water were enumerated by eight-tube, most-probable-number (MPN) series after incubation for 21 days at 20 C (19). For iron-oxidizing chemolithotrophic bacteria, 9K medium was employed (27), and for the sulfur-oxidizing chemolithotrophic bacteria, 9K basal salts medium containing elemental sulfur was used (31).

Initial attempts at isolating heterotrophic bacteria from the mine pump included five- or eight-tube MPN series with the following media: filter-sterilized acid mine water containing 0.1% yeast extract, pH 3.0 and 7.0; medium no. 3 of Postgate (21) for sulfate-reducing bacteria, pH 4.5 and 7.6; soil extract broth (14), pH 3.3 and 7.3; thioglycolate medium, pH 7.0; tryptic soy broth, pH 3.3, 4.8, and 6.6; and a broth composed of 0.1% each tryptone and yeast extract, pH 3.4, 5.2, and 7.0. In these trials heterotrophic bacteria were isolated only with the tryptone-yeast extract broth at pH values of 5.2 and 7.0. It was subsequently determined that the greatest number of heterotrophic bacteria could be isolated from the pump samples using a medium (TYE) of the following composition: tryptone, 0.1%; yeast extract, 0.1%; Na_2SO_4 , 0.1%; pH 6.0 with sulfuric acid. Heterotrophic isolates were obtained by streaking organisms from those positive MPN tubes at the highest dilutions onto TYE solidified with 2.0% agar. For characterization purposes, 15 isolates were selected from seven different pump samples collected over a period of six months (Table 1). After initial isolation, all heterotrophic cultures were grown in TYE at 25 C and stored at 4 C. Working cultures were transferred at 3-week intervals.

Growth of heterotrophs in acid mine water. A modified replacement culture technique was employed to demonstrate growth of the heterotrophic bacteria in pH 2.6 acid mine water. This approach was adopted since the pump samples contained insufficient organic carbon (8.2 mg/liter, D. H. Rothermel and W. N. Millar, unpublished data) to support significant heterotrophic growth in batch culture. Flasks containing distilled water, 9K medium, and filter-sterilized acid mine water, each at pH 2.6, were inoculated with washed cells (isolate number 1-51-22) and incubated for 13 days at 25 C on a reciprocal shaker. At four different times during incubation, 20% of the contents of each flask was removed and replaced with sterile medium of similar composition. This resulted in a net physical removal of approximately 60% of the original flask contents. MPN determinations in TYE were performed before and after the incubation period to determine population levels. Small numerical changes or a numerical gain during incubation in the flasks would indicate heterotrophic growth and multiplication. In contrast, numerical losses of 60% or more of the initial population would indicate that the heterotrophs were unable to grow.

Biochemical tests. Final interpretations of all growth and biochemical determinations were made after incubation for 21 days at 25 C. Catalase

TABLE 1. Strain designations of isolated acid mine water heterotrophs

Strain	Water sampling date
1-39-26	July 24, 1970
1-39-29	July 24, 1970
1-41-14	September 1, 1970
1-41-15	September 1, 1970
1-42-12	September 27, 1970
1-42-13	September 27, 1970
1-47-11	October 14, 1970
1-47-24	October 30, 1970
1-47-27	October 30, 1970
1-51-7	November 16, 1970
1-51-10	November 16, 1970
1-51-22	December 2, 1970
1-51-23	December 2, 1970
1-51-31	December 2, 1970
1-51-32	December 2, 1970

determinations were made by adding 3% H_2O_2 to cells grown on TYE agar. The oxidase test (16), interpreted according to the criteria of Steel (29), and the benzidine test for iron porphyrins (5) were performed to determine the presence of a cytochrome system. The phosphatase test was performed on TYE agar containing 0.01% phenolphthalein diphosphoric acid (2).

The hydrolysis of gelatin and starch and the utilization of tyrosine and xanthine were examined with TYE agar containing the appropriate substrate. Lipolytic activity was evaluated by adding 1.0% Tween 60 and 0.01% $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ to TYE agar. Cellulose hydrolysis was examined by placing a filter paper strip into tubes of TYE.

Hydrogen sulfide production was examined in SIM Medium (Difco, Detroit, Mich.) initially adjusted to pH 6.0. Indole production and nitrate reduction were determined in TYE supplemented, respectively, with 1.0% tryptone and 0.1% KNO_3 . Brain Heart Infusion, Casamino Acids, heart infusion broth, Koser citrate medium, litmus milk, MR-VP medium, neopeptone, nutrient broth, proteose peptone, soytone, thioglycolate medium, tryptic soy broth, tryptone, tryptose broth, urea broth, and yeast extract were Difco products.

Tests for autotrophic growth of the acid mine water heterotrophs were made in the salts solution of Starkey (28) containing either thiosulfate (0.5%) or sulfur (1.0%) at pH 6.0. In addition, the thiosulfate-containing media of Charles and Suzuki (3), Santer et al. (23), and Trautwein (30) were used, as were the media described above, for the enumeration of iron-oxidizing and sulfur-oxidizing chemolithotrophs. To determine a possible mixotrophic utilization of sulfur and thiosulfate, tubes of Starkey thiosulfate and sulfur media containing 0.1% each of tryptone and yeast extract were employed.

The utilization of single organic compounds as sole sources of carbon was investigated using a mineral salts medium (MS) of the following composition: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 g; Na_2SO_4 , 1.0 g; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.04 g; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.04 g;

KCl, 0.1 g; NH_4NO_3 , 1.0 g; H_2O , 1,000 ml; pH 6.0. The organic compounds, sterilized by filtration, were added to the medium at a final concentration of 0.2%. The utilization of nitrogen-containing organic compounds as sole sources of carbon and nitrogen was investigated using MS medium without the NH_4NO_3 . The nitrogen-containing organic compounds were either membrane filtered or autoclave sterilized and added to give a final concentration of 0.2%.

Acid production from carbohydrates and polyalcohols (13) was determined using basal media of TYE and one-tenth strength TYE with 0.002% bromocresol purple as an indicator. All carbohydrates and polyalcohols were membrane filter sterilized as 10% (wt/vol) solutions and added to the basal media to give final concentrations of 0.5%.

Susceptibility to antibiotics was determined using Sensi-Discs (BBL) on heavily seeded TYE agar plates. Sensitivity to the vibriostatic compound 0/129 (2,4-diamino-6,7-diisopropylpteridine) was assayed by applying the crystals to the surface of spread plates.

Deoxyribonucleic acid base composition. Duplicate analyses of the guanine plus cytosine contents of the deoxyribonucleic acid (DNA) of representative isolates growing on TYE agar were performed by M. Mandel by means of the CsCl density gradient method (24).

Electron microscopy. Whole-cell observations were made on cells mounted on carbon-supported Formvar grids and stained with 1% uranyl acetate. For sectioning, cells grown in TYE were centrifuged and fixed overnight (16 h) in 1% OsO_4 (15). The fixed preparations were dehydrated in a graded ethanol series, embedded in Epon 812 (17), and sectioned with a glass knife. The sections were post-stained with 2% uranyl acetate and lead citrate (22) and observed in an RCA EMU-36 electron microscope at 100 kV.

RESULTS

Occurrence. Pump effluent was sampled over a 1-year period for counts of heterotrophic and chemolithotrophic bacteria as well as for concentrations of iron and sulfate ions. Fig. 1 shows that major fluctuations of these parameters did not occur over the sampling period. The fact that all bacterial populations were able to maintain stable population levels despite dilution by influx of ground waters and elimination by pump action suggests that they are able to multiply in this environment. The pH (2.6) and temperature (20 C) of the effluent did not vary during the sampling period.

Cell and colony morphology. All isolates were gram-negative, procaryotic rods, 0.3 to 0.6 by 1.0 to 3.0 μm (Fig. 2). Spores, branching, and motility were not detected in any of the strains under the cultural conditions used. The division process was constrictive in nature with no initial formation of a transverse septum. Occasionally multiple constrictions occurred, resulting in the formation of very small cells (Fig. 3). Such small structures were evident on

1 to 5% of the cells during growth in TYE and were observed in both polar and nonpolar configurations. Because thin sections through these small structures revealed the absence of DNA, it is believed that they are "mini-cells" (1). Mini-cells are small, anucleate bodies resulting from the asymmetric segregation of cytoplasmic material during division. Culture filtrates passed through 0.45- μm membrane filters were unable to initiate growth in fresh TYE.

All isolates were slow growing. Growth was rarely noticeable to the unaided eye after incubation for 1 week, 4 weeks being required for the isolates to attain a maximum colony diameter of 1 mm on TYE agar. Such colonies were characterized by a pale, yet distinct, yellow, water-insoluble pigment.

Growth conditions. Because initial attempts to isolate heterotrophic bacteria from acid mine water using several heterotrophic media were unsuccessful, the ability of isolated heterotrophs to grow in a number of conventional nutritive media was investigated (Table 2). In trial I, where the initial pH values of the media were not altered, few of the media examined were able to support growth of all the isolates. In trial II, similar media were adjusted initially to pH 6.0 by the addition of sulfuric acid. In this latter case most of the media examined supported growth of the heterotrophs. Tryptic soy broth and Brain heart infusion were unable to support growth of the heterotrophs even at pH 6.0.

Because none of the media examined provided better growth of the isolates than TYE, this medium was used to determine temperature and pH ranges for growth. As recorded in Table 3, the heterotrophs were mesophilic with none able to grow at 45 C. Growth of all isolates also occurred between pH values of 4.5 and 6.5. The lack of turbidity at pH values of 3.5 and below was unexpected since the heterotrophs were apparently indigenous to waters at pH 2.6. This apparent anomaly was investigated by a replacement culture technique using acid mine water at pH 2.6 as the growth medium. Table 4 shows that the heterotrophic populations in distilled water and 9K medium (both at pH 2.6) decreased in excess of the expected 60%. These losses are probably due to starvation and to the effects of hydrogen and iron ions. In the case of the filter-sterilized acid mine water medium, no such loss of viable cells occurred. Such a result is explained only by the growth of the heterotrophic bacteria at pH 2.6 under the low nutrient conditions of acid mine water.

Biochemical characteristics. The isolates were

FIG. 1. Analysis of Brewer Pump effluent over a 1-year period. A, Ionic concentrations: ■, sulfate ion; ●, total iron. B, Bacterial populations: ■, iron-oxidizing chemolithotrophs; ●, heterotrophs; ▲, sulfur-oxidizing chemolithotrophs.

aerobic, unable to grow under a Vaspar seal, and catalase positive. The oxidase reaction was slow in development (i.e., greater than 10 s), and the cultures were judged to be oxidase negative. The presence of a cytochrome system is indicated, however, by positive benzidine tests and sensitivities to sodium azide and sodium cyanide. Results of additional biochemical characteristics are presented in Table 5.

DNA base composition. Based on four representative strains, the average DNA base composition of the acid mine water heterotrophs was found to be 66.4% guanine plus

cytosine (Table 6). The small standard deviation suggests that the isolates are members of the same population.

Taxonomy. Because of their morphology, nutrition, yellow colonial pigmentation, and nonfermentative nature, the heterotrophic bacteria isolated from acid mine water are believed to be members of the genus *Flavobacterium*. The isolates, however, do not conform to descriptions of other flavobacteria. Because of their ability to withstand a pH value of 2.6, a new species, *Flavobacterium acidurans*, is thus proposed for these organisms.

Flavobacterium acidurans sp. n. (M. L. neut.

FIG. 2. Section of acid mine water heterotroph grown in TYE. Bar represents 0.5 μ m.

FIG. 3. Uranyl acetate-stained acid mine water heterotroph displaying multiple constrictions. Note mini-cell (M). Bar represents 0.5 μ m.

TABLE 2. Growth of acid mine water heterotrophs in various media

Medium	Trial I		Trial II	
	Initial pH	Positive strains ^a	Initial pH	Positive strains ^a
TYE	6.0	15	6.0	15
Casamino Acids, 1.0%	6.4	14	6.0	15
Tryptone, 1.0%	7.3	0	6.0	15
Soytone, 1.0%	7.1	0	6.0	15
Proteose peptone, 1.0%	6.4	2	6.0	15
Tryptose broth, 1.0%	7.2	1	6.0	15
Nutrient broth	6.8	8	6.0	15
Heart infusion broth	6.8	1	6.0	14
Tryptic soy broth	7.2	1	6.0	0
Brain Heart Infusion	6.9	0	6.0	1

^a Number of isolates displaying growth out of 15 examined.

n. *acidum*, acid; L. part. adj. *durans*, resisting; M. L. adj. *acidurans*, acid resistant.).

Cells rod shaped, 0.3 to 0.6 by 1.0 to 3.0 μm , occurring singly or in pairs. Produce an asymmetric segregation of cytoplasmic material (mini-cells). Cell replication is by binary fission. Cells are aerobic, catalase positive, nonmotile, gram-negative, and nonsporeforming. Cells are oxidase negative, benzidine positive, and sensitive to sodium azide and potassium cyanide. Cells are slow growing, with turbidity or colonies evident after approximately 1 week of incubation. Mature colonies develop within 4 weeks with a diameter of 1 mm and a pale yellow, water-insoluble pigment. Growth occurs between 20 to 30 C; poor (seven strains) or no growth at 37 C. In laboratory media, growth occurs at pH 4.5 to 6.5; growth occurs in acid mine water at pH 2.6. Cells maintain their viability when incubated at 25 C in non-nutritive solutions at pH 2.6 for periods as long as 1 month.

Tween 60 is hydrolyzed. Nitrate not reduced to nitrite. Xanthine and tyrosine are not decomposed. Indole and hydrogen sulfide are not produced. No growth in the presence of 3.0% NaCl. No acid from carbohydrates and polyalcohols. α -Ketoglutarate is utilized as a sole carbon source in a mineral salts solution; there is no growth with pyruvate, acetate, oxalacetate, or benzoate and variable utilization of oxalate (2 strains), citrate (1 strain), malate (14 strains), and succinate (14 strains).

Ornithine, proline, and lysine are utilized as sole sources of carbon and nitrogen in mineral salts solutions; there is variable utilization of aspartate (6 strains), glutamate (11 strains), and arginine (5 strains), but no growth with other amino acids, purines, pyrimidines, or ethylamine. Sensitive to neomycin, polymyxin B, penicillin, terramycin, and vibriostatic agent 0/129; variable sensitivity to bacitracin (11 strains). The DNA base composition is 66.4 mol

TABLE 3. Temperature and pH ranges for growth of acid mine water heterotrophs in TYE

Condition	Positive strains ^a
Temperature (C) ^b	
10	10
20	15
30	15
37	7
45	0
pH ^c	
3.5	0
4.0	11
4.5	15
6.5	15
7.0	11
7.5	0

^a Number of isolates displaying growth out of 15 examined.

^b Medium at initial pH 6.0.

^c Incubation at 20 C.

% guanine plus cytosine. Habitat is acidic water from a West Virginia coal mine.

Type strain. The type strain, number 1-51-22, has been deposited with the American Type Culture Collection under the number 27383. Cells of this strain are rod shaped, 0.3 to 0.6 by 1.0 to 3.0 μm , aerobic, nonmotile, gram-negative, and nonsporeforming. The DNA base composition is 66.3 mol % guanine plus cytosine. Cell division is constrictive with the occasional formation of mini-cells. Growth occurs in acid mine water at pH 2.6, and viability is maintained for at least 1 month in non-nutritive solutions at pH 2.6.

Pale yellow colonies develop on solid media (pH 6.0) within 4 weeks with a diameter of 1 mm. Growth at 20 C and 30 C; no growth at 37 C. Cultures are catalase positive, oxidase negative, sensitive to sodium azide and potassium cyanide, and display a positive benzidine test.

TABLE 4. Growth of acid mine water isolate 1-51-22 under replacement culture conditions at pH 2.6^a

Medium ^b	Day 0 ^c	Day 13 ^c	Population change ^d
Distilled water	2.3×10^6	3.3×10^4	-98.6
9K	1.3×10^6	1.3×10^3	-99.9
Acid mine water	3.3×10^6	7.0×10^6	+112.1

^a At four intervals during incubation, 20% of the contents of each flask was removed and replaced with sterile medium of similar composition. Thus, 60% of the original contents of each flask was ultimately discarded.

^b Each medium was initially adjusted to pH 2.6 with sulfuric acid.

^c Cells per milliliter as determined by five-tube MPN series in TYE.

^d Expressed as a percent of the initial population.

TABLE 5. *Biochemical characteristics of Flavobacterium acidurans*

Test	Positive strains ^a	Type strain (ATCC 27383) ^b	Test	Positive strains ^a	Type strain (ATCC 27383) ^b
Phosphatase	15	+	Acid from:		
Nitrate reduction	0	—	Glycerol	0	—
Decomposition of:			Arabinose	0	—
Tyrosine	0	—	Ribose	0	—
Xanthine	0	—	Xylose	0	—
Growth on: ^c			Fructose	0	—
Koser citrate medium	0	—	Galactose	0	—
Litmus milk	0	—	Glucose	0	—
Urea broth	0	—	Mannose	0	—
Thioglycolate medium	0	—	Dulcitol	0	—
TYE containing:			Inositol	0	—
Sodium chloride (1.0%)	15	+	Mannitol	0	—
Sodium chloride (3.0%)	0	—	Sorbitol	0	—
Methyl red	0	—	Cellobiose	0	—
Voges-Proskauer	0	—	Sucrose	0	—
Production of:			Lactose	0	—
Indole	0	—	Maltose	0	—
Hydrogen sulfide	0	—	Melibiose	0	—
Hydrolysis of:			Raffinose	0	—
Cellulose strip	0	—	Utilization of: ^e		
Gelatin (3.0%)	0	—	Alanine	0	—
Starch (0.2%)	0	—	Glycine	0	—
Tween 60 (1.0%)	15	+	Serine	0	—
Utilization of: ^d			Threonine	0	—
Oxalate	2	—	Leucine	0	—
Citrate	1	—	Isoleucine	0	—
Pyruvate	0	—	Methionine	0	—
Acetate	0	—	Aspartate	6	—
Malate	14	+	Glutamate	11	+
Succinate	14	+	Ornithine	15	+
α -Ketoglutarate	15	+	Proline	15	+
Oxalacetate	0	—	Lysine	15	+
Benzoate	0	—	Arginine	5	—
Sensitivity to:			Tryptophane	0	—
Bacitracin, 10 units	11	+	Phenylalanine	0	—
Neomycin, 30 μ g	15	+	Histidine	0	—
Polymyxin B, 300 units	15	+	Asparagine	0	—
0/129 Crystals	15	+	Adenine	0	—
Penicillin, 10 units	15	+	Thymine	0	—
Terramycin, 50 μ g	15	+	Uracil	0	—
Autotrophic growth on:			Cytosine	0	—
Sulfur	0	—	Ethylamine	0	—
Thiosulfate	0	—			
Ferrous iron	0	—			

^a Number of isolates displaying a positive result out of 15 examined.^b +, positive reaction; —, negative reaction.^c Media adjusted initially to pH 6.0^d Utilization as sole carbon source.^e Utilization as sole source of carbon and nitrogen.

Additional biochemical characteristics of the type strain are presented in Table 5.

DISCUSSION

In this study, a population of gram-negative, heterotrophic bacteria was demonstrated in acid mine water. Isolates from this population

were sensitive to a range of antimicrobial compounds. Of interest was the inhibition of growth by 0/129, a vibriostatic agent. In this connection, certain marine *Flavobacterium* and *Cytophaga* species are reported to be sensitive to the compound (10); members of the genus *Pseudomonas* are considered resistant (26).

Based on their Gram reaction, yellow water-

TABLE 6. Deoxyribonucleic acid base composition of acid mine water heterotrophs

Strain no.	CsCl density (g/cm ³)	GC content ^a (mol %)
1-39-26	1.725	65.8
	1.724	
1-42-12	1.726	67.3
	1.726	
1-47-11	1.725	66.3
	1.725	
1-51-22	1.725	66.3
	1.725	
Mean	1.7251	66.4
Standard deviation	± 0.0006	± 0.6

^a GC content, guanine plus cytosine.

insoluble pigmentation, nonfermentative nature, and lack of motility, the isolates have been placed within the genus *Flavobacterium*. This generic designation is further supported by a guanine plus cytosine content of 66.4 mol %, a value which correlates well with the results for four *Flavobacterium* species as reported by Marmur et al. (18). However, in this context, there is considerable variation within the genus (11). For example, DeLey and van Muylem (6) reported guanine plus cytosine values of marine *Flavobacterium* isolates from 35.6 to 40.6 mol %. Further, the reisolated type species, *F. aquatile*, displays characteristics typical of *Cytophaga* species (20, 32). This latter genus has been eliminated from consideration in the present study since gliding motility was never observed with the acid-water isolates. On the basis of other morphological, physiological, and ecological considerations, a new species, *F. acidurans*, is proposed for the acid mine water isolates.

The formation of mini-cells by *F. acidurans* was of interest. Such structures have been noticed by other workers in *Escherichia coli* (1) and in a strain of *Erwinia amylovora* which had been induced to grow filamentously (12). While all bacteria now known to display mini-cells fall within the order *Eubacteriales*, little taxonomic value has been attached to them in the present work. It is conceivable, for example, that their presence might merely reflect a cellular response to suboptimal growth conditions.

The occurrence of heterotrophic microorganisms in acid mine water has been reported previously. Ehrlich (9) and Dugan et al. (8) isolated several heterotrophic microorganisms from the acidic effluents of copper and coal mines, respectively. In the latter work, a *Bacillus* species considered to be the primary

source of "acid streamers" was described. Neither streamers nor gram-positive bacilli were found in the acid-water samples, however. In that same report five gram-negative, heterotrophic, bacterial isolates were also described. Although these isolates were unable to be subcultured for more than three transfers, they nonetheless resembled *F. acidurans* by their inability to ferment carbon compounds.

F. acidurans was enumerated in samples collected over a 1-year period at a level exceeding 10³ cells per ml. They thus constituted the second most dominant population after the iron-oxidizing thiobacilli in this acid ecosystem. The relatively stable numerical level observed suggests that they were either being continually leached into the water serviced by the pump or that they were multiplying in the acid water at a rate that was balanced by the outflow of the pumped water. As a result of their ability to grow in acid mine water in the laboratory, and because of the constant temperature, ion concentrations, and flow rates of the pump effluent, the latter possibility seems more attractive at present. Although the source of carbon for the heterotrophs in acid mine water is unknown, organic compounds leached from the soil above or organic matter released as waste material by the iron-oxidizing thiobacilli are two possibilities. That such cross-feeding might occur has been suggested but never demonstrated (8).

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REPRINT REQUESTS

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