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Aerobic Heterotrophic Bacteria Indigenous to *p*H 2.8 Acid Mine Water: Predominant Slime-Producing Bacteria in Acid Streamers

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Five gram-negative bacteria, two gram-positive bacteria, and one yeast were isolated from "acid streamers" taken from acid mine water. One gram-positive rod which has been tentatively identified as a *Bacillus* species appeared to be the predominant organism in the streamers. This isolate produced copious amounts of extracellular polymer at 10 C in the laboratory and was considered to be the primary source of polymer in the "acid streamer" slime matrix. The organism grew slowly at *p*H 2.8 in mine water media, but the optimal *p*H was approximately 7.0.

In a previous communication, we reported that "acid streamers" which are produced in mine water at *p*H 2.8 are of microbial origin (2). The streamers appeared to consist of a fibrillar polymer network produced by aerobic bacteria. The bacterial cells and inorganic precipitates could be observed within the fibrillar network. At least two different types of cells were observed within the streamer matrix, one of which produced microcolonies that could be recognized by fluorescent dye accumulation.

The purpose of this report is to describe the predominant organism which has been isolated from the acid streamers and to establish that the streamers are produced by heterotrophic bacteria.

MATERIALS AND METHODS

Enumeration of chemoautotrophic bacteria. Iron- and sulfur-oxidizing chemoautotrophic bacteria were enumerated by the dilution method in the 9K medium of Silverman and Lundgren (5) and in 9K medium which had 0.1 g of sulfur substituted for ferrous sulfate (7). Inocula (1 ml) consisting of mine water or dilutions made in sterile 9-ml acid blanks (H_2SO_4, *p*H 3.1) were used. Cultures and uninoculated controls were incubated in slanted racks at ambient temperature for 21 days. After incubation of the cultures containing sulfur, 0.4% aqueous thymol blue was used to indicate *p*H change by comparison to the uninoculated control. Most-probable-number (MPN) values were determined by using five-tube MPN tables (1). Results were expressed as MPN per 100 ml of the original sample.

The enrichment medium of Kucera and Wolfe (4) was employed, although not routinely, to test for the presence of *Gallionella ferruginia*.

Isolation and cultivation of heterotrophic bacteria. Primary isolation of bacteria and fungi routinely included six different media. Medium A was Plate Count Agar (Difco), medium B was Sabouraud Dextrose Agar (Difco), and media C, D, E, and F contained the same basal nutrients but differed in solidifying agent and in the presence or absence of "soil extract." Agarose (Sea-Kem, distributed by Bausch & Lomb, Inc., Rochester, N.Y.) and Ionagar No. 2 (Consolidated Laboratories, Inc., Chicago Heights, Ill.) were employed as solidifying agents. The "soil extract" (SE) was prepared from precipitate and leaves from the acid stream bed. A mixture of approximately two parts distilled water to one part "soil" (v/v) plus 3 g of sodium hydroxide per liter of water was steamed for 2 hr in an autoclave. The extract was then filtered through Whatman no. 1 filter paper to yield a metallic grey liquid at *p*H 2.25. Basal nutrients for the media (TGBYE) consisted of: tryptone (Difco), 0.5% (w/v); D-glucose (Difco), 0.1% (w/v); beef extract (Difco), 0.3% (w/v); and yeast extract (Difco), 0.1% (w/v).

The composition of media C, D, E, and F was as follows: (C) TGBYE-Ionagar [basal nutrients; Ionagar No. 2, 0.85% (w/v); final *p*H, 6.9]; (D) TGBYE-Ionagar-SE [basal nutrients; Ionagar No. 2, 0.85% (w/v); soil extract, 1% (v/v); final *p*H, 6.5]; (E) TGBYE-agarose [basal nutrients; agarose, 0.6% (w/v); final *p*H, 6.9]; and (F) TGBYE-agarose-SE [basal nutrients; agarose, 0.6% (w/v); soil extract, 1% (v/v); final *p*H, 6.5].

The enumeration and isolation of aerobic heterotrophic bacteria from the mine water was accomplished by plating on each of the solid media described above. The water sample was mixed by repeated inversion of the sample bottle. Triplicate plates were inoculated with 0.5 ml of undiluted mine water and were spread with an alcohol-flamed glass

spreader bar. All plates were sealed with masking tape and incubated for periods as long as 21 days at 10 and at 20 C. All media for biochemical determinations were prepared by standard methods and techniques (6).

Freeze-etching. Cultures were taken from 96-hr TGBYE slants, pre-frozen, and freeze-etched as previously described (3).

Phase-contrast and ultraviolet microscopy. Cells were stained and photographed as previously described (2).

RESULTS

The MPN of iron-oxidizing autotrophic bacteria in the mine water at the site of acid streamer formation was 9.2×10^3 per ml. No *Gallionella* could be detected by the methods employed. Although a high autotroph count was consistently obtained from the water, the observed streamers did not appear typical of autotrophic bacterial growth.

Use of media D and F gave a threefold increase in total colony counts (5,000/ml) as compared to counts obtained on medium A (1,600/ml) and a 20% increase over counts obtained on media C and E (4,000/ml). This indicates that Plate Count Agar is slightly inhibitory to some of the heterotrophic cells present and that growth factors were being supplied by the soil extract which were not present in TGBYE. Since most of the colonies were clear pinpoint types which are difficult to observe without magnification, it was not determined whether the soil extract stimulated all of the species present or whether the increase in numbers recovered represented only certain species. Eight different cultures were isolated from streamers when cultivated on media D and F which contained soil extract in addition to purified agar as the solidifying agent.

The eight isolates were transferred to media identical to those from which they were isolated and to media with the following variations: medium D-F to which acid mine water was added in place of distilled water (final *p*H 3.5), liquid medium D-F (solidifying agents omitted), and liquid medium D-F with acid mine water.

Table 1 is a summary of some characteristics of the eight isolates. Two isolates were gram-positive rods and differed only with reaction to nitrate, five were gram-negative rods, and 1 was a yeast. Three of the organisms did not grow on Plate Count Agar. Two of the gram-negative bacteria failed to grow in the absence of soil extract (medium C–E). None of the organisms fermented any of the carbon sources examined. The gram-negative isolates (no. 2, 3, 5, 6, and 7) could not be subcultured for more than three transfers on any medium examined but could be repeatedly re-isolated from acid streamers and acid water in the vicinity of acid streamers. These organisms have not been studied in detail.

None of the isolates grew in 9K medium or in 9K which had sulfur substituted for iron. Isolate no. 1 appeared to be the predominant organism in the streamer as judged by the number of colonies present on isolation media. It is an aerobic sporeforming rod which could be transferred routinely and which produced slime on TGBYE plates at 10 and 20 C. It also produced

TABLE 1. *Heterotrophs from pH 2.8 mine water*[a]

Determination	Isolate no.							
	1	2	3	4	5	6	7	8
Gram reaction	+	−	−	+	−	−	−	Yeast
Growth on								
Medium D-F	+	+	+	+	+	+	+	+
Medium C-E	+	+	NG	+	NG	+	+	+
Medium A	+	+	NG	+	NG	+	+	NG
Fermentation of								
Glucose	−	−	LG	−	LG	LG	−	LG
Sucrose	−	−	−	−	−	−	−	−
Lactose	−	−	−	−	−	−	−	−
Mannitol	−	−	−	−	−	−	−	−
Gelatin	−	−	NG	−	NG	NG	+	NG
Nitrate	−	−	Gas	+	−	Gas	−	NG
Citrate	NG	NG	NG	NG	NG	NG	NG	NG
Pigment	−	−	−	−	−	−	−	−

[a] Symbols: +, growth; −, growth with negative reaction; LG, very little growth; NG, no growth.

slime in liquid acid mine water medium at 10 and 20 C. The optimal temperature, as judged by growth rate, appeared to be 20 to 24 C, although greater amounts of slime were produced at 10 than at 20 C. The optimal *p*H for growth appeared to be 7.0 although growth occurred at *p*H 2.8.

Figures 1 to 4 are freeze-etch electron micrographs of isolate no. 1. Figure 1 shows a chain of cells within a fibrillar polymer network which corresponds to a capsule, slime layer, or matrix when observed with a light microscope. The fibrils (F) originate as an outer layer of the cell wall (CW). Another view of isolate no. 1 is shown in Fig. 2. Structures corresponding to cytoplasm (Cy), cell membrane (CM), cell wall (CW), and slime layer are present. The fibrillar array is made up of elementary fibrils and larger fibrils which may be an artifact of the freeze-etching procedure as previously discussed (3). Vesicular structures can be seen within vegetative cells, and structures resembling spores can also be seen. Figure 3 shows a chain of cells which contains a spore (Sp). Cross walls (W) are evident as well as cytoplasm (Cy) and fibrils (F). Unidentified particulate structures (P) are also present. The particles (P) can also be observed in Fig. 4, attached both to the outer layer of the cell and to fibrils (F) away from the cells. The possibility exists that the particles have either enzyme functions and are involved in polymer synthesis since they seem to be associated with polymer fibrils, or they are viral in nature, which might explain the crenated appearance of some cells (Cr). These aspects are being further investigated.

When isolate no. 1 was stained with Paper White BP and examined under phase-contrast and fluorescence microscopy, the cells reacted to the dye. Figure 5 is a phase-contrast photograph of a wet mount of the slimy culture. The phase-dark cells could not be brought into focus because of light diffraction by the slime. Figure 6 is a fluorescence photograph (ultraviolet illumination only) of the identical field shown in Fig. 5 and can be superimposed on Fig. 5. The phase-dark cells are seen to possess greater fluorescence and hence have a higher concen-

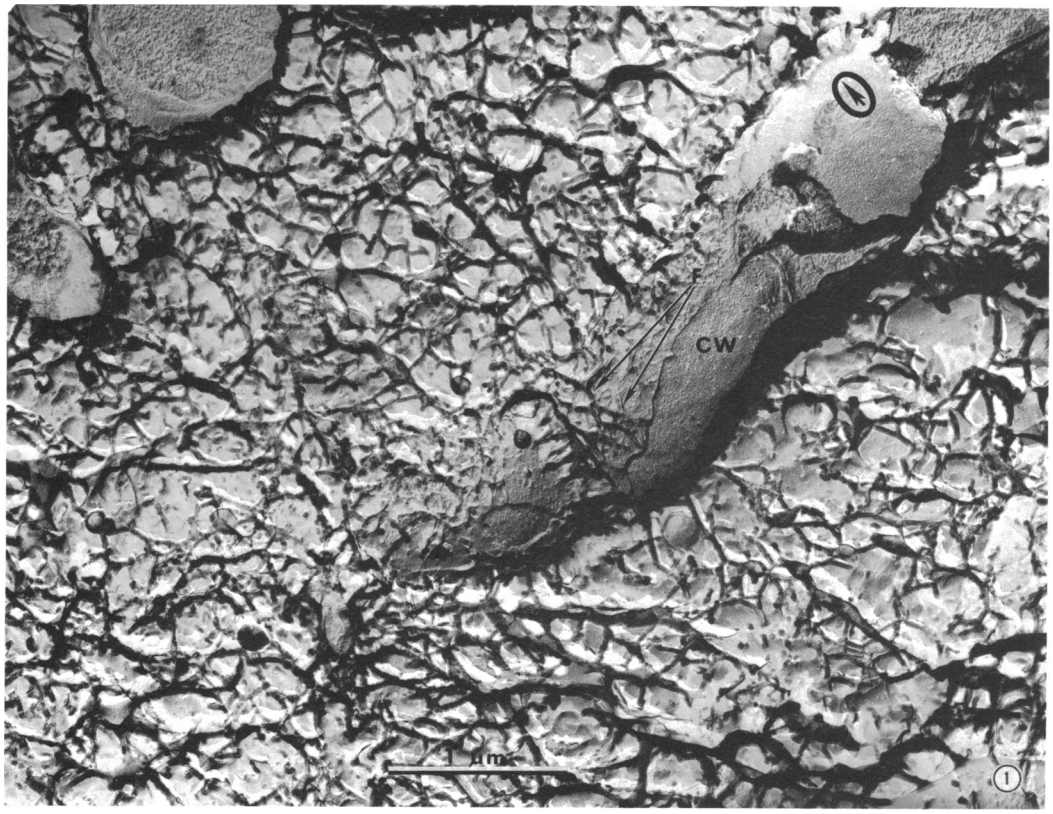

FIG. 1. *Freeze-etch preparation of isolate no. 1 showing cells enmeshed within a fibrillar polymer network. The fibrils (F) originate as an outerlayer of the cell wall (CW).*

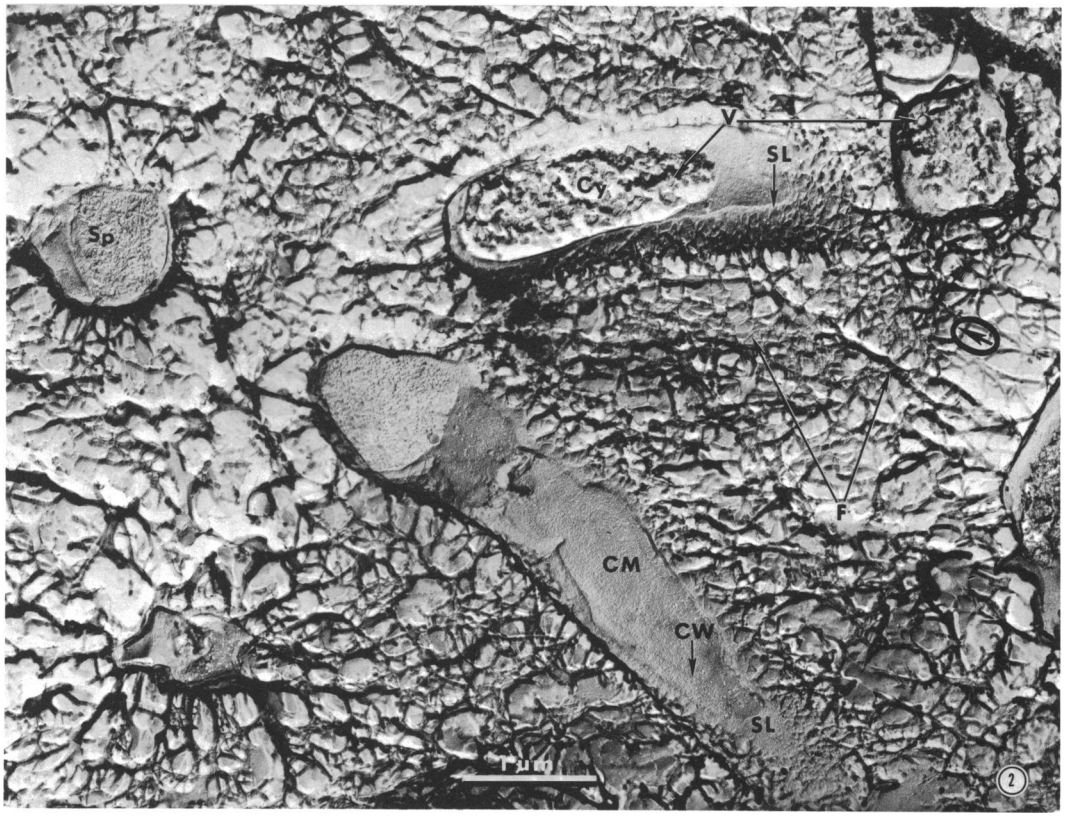

FIG. 2. *Freeze-etch preparation of isolate no. 1 showing cytoplasm (Cy), cell membrane (CM), cell wall (CW), and a slime layer or capsule (SL). Unidentified vesicular structures (V) are present in the cytoplasm of a vegetative cell, and other cells resembling spores (Sp) are present.*

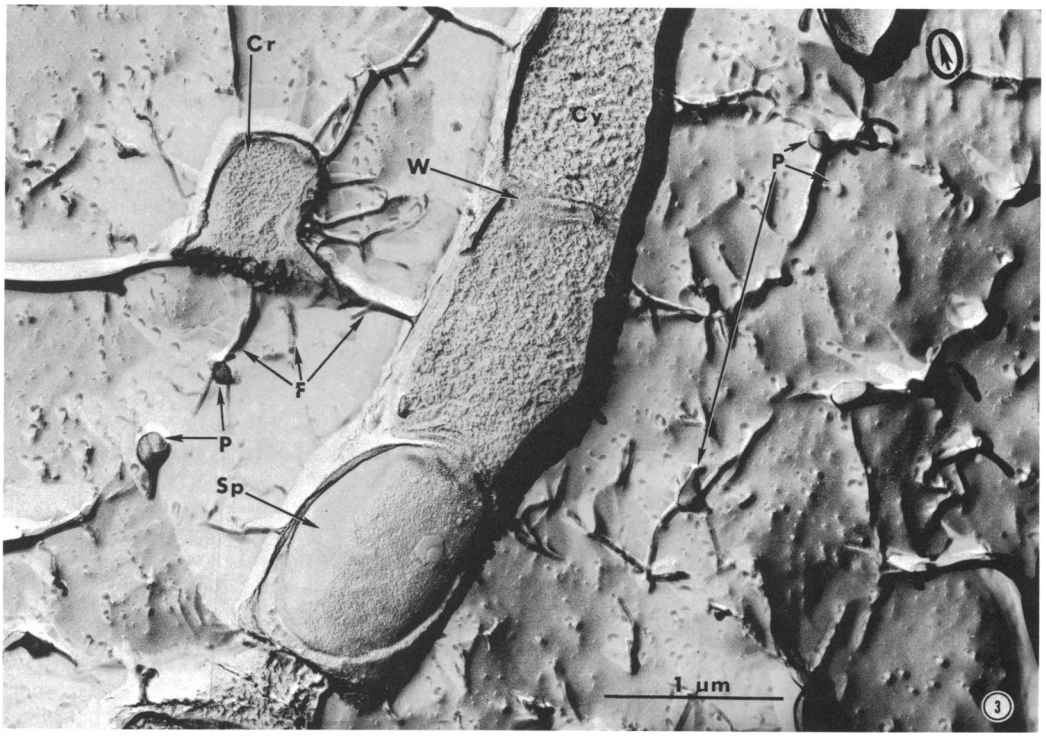

FIG. 3. *Freeze-etch preparation of a chain of cells which contains a spore (Sp). Cross walls (W), cytoplasm (Cy), and extracellular fibrils (F) can be seen. Unidentified particulate structures (P) are present outside the cells which may be the vesicles shown in Fig. 2. These particles resemble phage in many respects.*

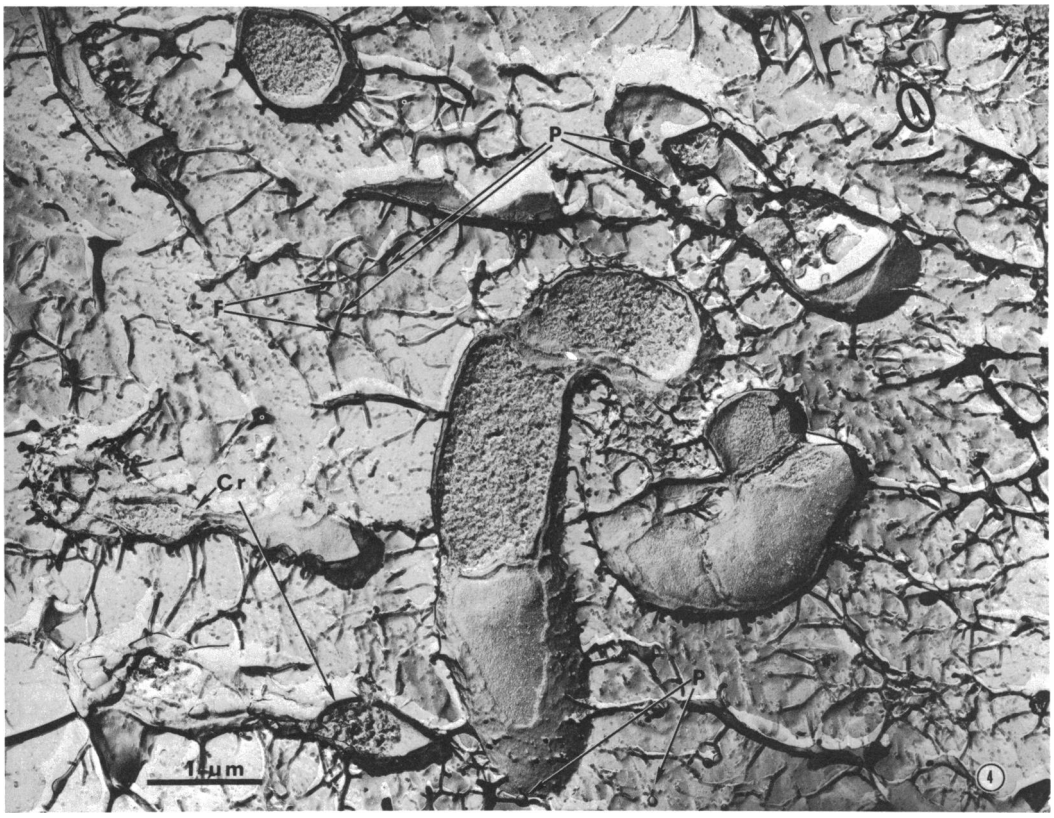

FIG. 4. *Freeze-etch preparation of the same cells shown in Fig. 1 to 3. Particles (P) are attached to the outer layer of the cell and to fibrils (F) away from the cells. Crenated (Cr) cells are also present which contain vesicles and may be partially lysed.*

tration of polysaccharide around the cells. These cell are presumed to be viable cells. Cell outlines having greater fluorescence at the ends of cells can be seen in the background of Fig. 6. These are not visible under phase contrast in Fig. 5 and represent the polysaccharide slime matrix. Individual fibrils cannot be observed at this magnification but are presumed to interconnect the cell outlines or ghosts (G). These ghost cells may be the fibrous masses of sheath-like structures which led Leathen to believe that acid streamers are devoid of cells (2).

DISCUSSION

Seven different aerobic heterotrophic bacteria have been isolated from acid streamers. Most of the isolates have not been subcultured for more than two transfers and have not been adequately studied. One isolate (no. 1) is a gram-positive sporeforming rod identified as a *Bacillus* species and grows well on laboratory media. This isolate produced copious amounts of slime under the same temperature and *p*H conditions found in its environment and is regarded as the organism primarily responsible for acid streamer formation.

The optimal temperature and *p*H for growth of isolate no. 1 are higher in the laboratory than in its habitat, although more slime is produced at 10 C than at 20 C when the *p*H is 6.9 in the laboratory. The organism grows much more slowly at *p*H 2.8 in the laboratory than at 6.9, which made quantitative estimates of slime polymer production at *p*H 2.8 versus temperature rather difficult; thus, no conclusions were drawn in this regard. The slime polymer is more soluble in alkali than in acid, suggesting that these streamers may be formed only in highly acid water. If the organism were growing and producing polymer in neutral or alkaline water, the polymer would be somewhat soluble and no streamer would form.

It is suggested that the *Bacillus* isolate is the same organism described as a gram-positive polymer fibril producer in our previous report (2) on the intact streamer, although no direct proof is available. The sculptured outer layer is not evident in the freeze-etched preparations

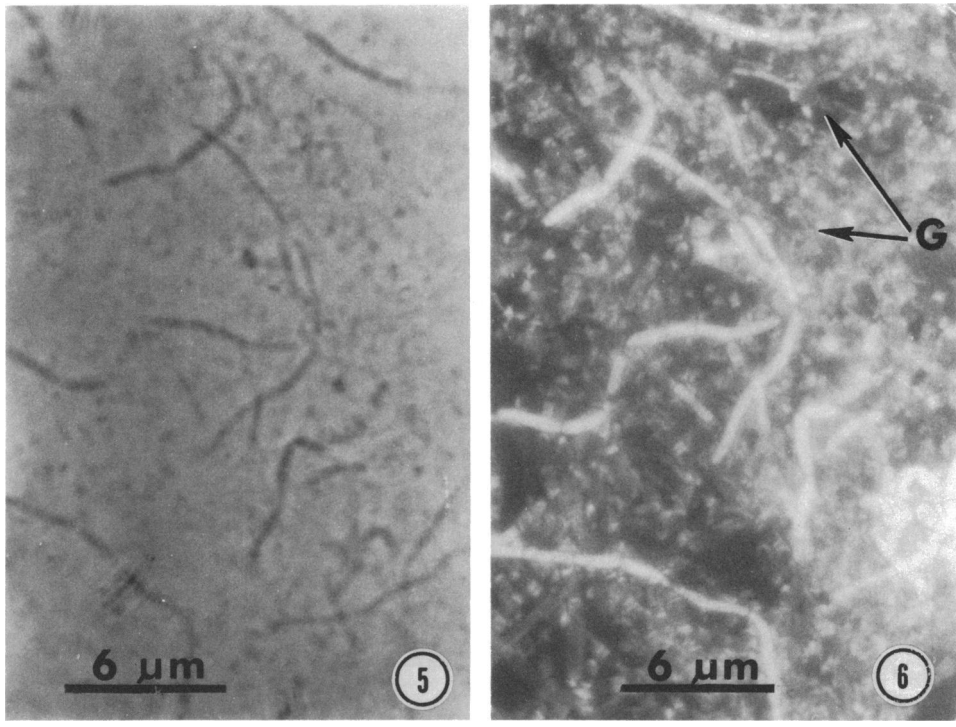

FIG. 5. *Phase-contrast photomicrograph of a wet mount of isolate no. 1 cells stained with a water-clear solution of Paper White BP. Light diffraction by slime diminishes contrast.*

FIG. 6. *Photomicrograph of the identical field shown in Fig. 5 but photographed under ultraviolet illumination. Cells having greatest fluorescence correspond to the phase-dark cells in Fig. 5, which are presumed to be viable. Cell ghosts (G) are also present which could not be observed under phase contrast (Fig. 5). The cell ghosts have greater fluorescence at the ends, which corresponds to greater polysaccharide accumulation at the ends of the cells. These cells are presumed to be nonviable.*

of the isolate cultivated in the laboratory (Fig. 1 to 4). The sculptured outer layer may have been produced in the acid environment in response to the high ionic content or it may be present but covered with fibrils in the laboratory culture.

There is insufficient information concerning nutrients available for growth and polymer synthesis in the acid environment or with respect to the hydrogen ion barrier of the cells. Metabolic by-products from acidophilic autotrophic bacteria represent a possible source of nutrients for heterotrophic growth.

When cells lyse, large amounts of cytoplasmic material are released into the surrounding medium. Some of this material is vesicular and can be seen attached to cells or polymer fibrils (Fig. 1 to 4). A small number of phase-dark (viable) cells as well as phase-dark debris can be seen in the background of Fig. 5. Use of the stain Paper White BP has allowed observation of detail (Fig. 6) and shows that the background debris consists primarily of empty cell ghosts. There appear to be areas at the terminal ends of cells which retain stain after lysis. The preponderance of cell ghosts in Fig. 6 is consistent with the observation of crenated cell forms in freeze-etched preparations (Fig. 1 to 4). The extent to which lysis could be detected in all preparations may be attributable to lytic vesicles, sporulation, or phage in the natural environment. The unidentified particulate structures (P, Fig. 3 and 4) could be phage or the vesicles which are in the cytoplasm shown in Fig. 2.

Growth of heterotrophic bacteria, which have an optimal *p*H near neutrality, in highly acid water suggests that streams would support a microbial flora which would aid in rapid recovery of the stream to a normal condition once acid discharge from the mine subsided.

It is evident that capsules, slime layers, zoogloeal matrices, etc. are composed of extracellular polymer fibrils that form networks of strands. The physical and chemical properties of the strands are, of course, related to the chemical composition and structural configuration of the polymers (e.g., the extent to which polymers bind water, ions, and other chemicals).

Some capsules will therefore appear more viscous than others and have greater integrity. The relationship of *p*H, ionic strength of solvent, and any other variable which influences the physical properties of polymers should therefore be considered in regard to slime layers, capsules, and zoogloea matrices of microorganisms.

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