FERROBACILLUS FERROOXIDANS: A CHEMOSYNTHETIC AUTOTROPHIC BACTERIUM

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Investigations into the cause and control of the production of sulfuric acid in effluents of coal mines, and the effects of such effluents upon streams were begun in 1946.[1] Working and abandoned mines of Western Pennsylvania alone contribute an estimated million tons of sulfuric acid per year to the drainage area of the Ohio River. A field survey of mine drainage and receiving streams was inaugurated, and the waters were examined by chemical and bacteriological procedures. The sulfuric acid enters the drainage as ferrous sulfate, $FeSO_4$, which is derived from finely dispersed pyrite, FeS_2, in the coal and associated rock strata. By inference, sulfur-oxidizing bacteria were considered as participating in the oxidation of such pyritic materials, but this was proved to be an erroneous supposition (Leathen, Braley, and McIntyre, 1953). However, in an examination of the drainage from the Bradenville Mine in 1947, the change from ferrous to ferric sulfate was observed to increase more rapidly than could be accounted for by absorption of atmospheric oxygen. From such rapidly oxidizing effluents, it became evident that chemosynthetic autotrophic bacteria were associated with bituminous coal mines. One of these microorganisms, described in 1954 (Leathen and Braley), was definitely shown to oxidize ferrous to ferric sulfate and was given the specific name, *Ferrobacillus ferrooxidans*. Physiological studies of this bacterium have been continued, and it is the purpose of this report to describe further this autotroph, which is indigenous in all bituminous coal regions. Such ferruginous waters, being highly acidic, restrict the autotrophic population and usually kill the heterotrophs which normally inhabit streams.

To account for the high acidities of some bituminous coal mine drainage, Powell and Parr (1919) suggested that bacteria, or some catalytic agent, hastened the oxidation of pyritic and marcasitic sulfur of coal. Such oxidations were attributed by Carpenter and Herndon (1933) to *Thiobacillus thiooxidans*, or a very closely related species. The presence of two microorganisms in acid mine water was observed by Colmer and Hinkle (1947). One bacterium was believed to be similar to *Thiobacillus thiooxidans*, and the other was stated to be involved in the oxidation of ferrous iron to the ferric condition.

In 1949 Leathen and Madison reported the isolation of pure cultures of typical strains of *Thiobacillus thiooxidans* from all of the acid mine effluents examined during a 2-year study. In addition, a bacterium capable of rapidly oxidizing ferrous iron to the ferric state was grown in a synthetic medium (Leathen, McIntyre, and Braley, 1951) composed of ferrous sulfate and other simple inorganic salts.

Gleen (1950) demonstrated the biological oxidation of ferrous iron in acid soils by the use of a perfusion technique, but did not isolate the etiologic agent. A microorganism which oxidized ferrous iron to the ferric state and also oxidized thiosulfate to sulfuric acid was reported by Colmer, Temple, and Hinkle (1950). It was assigned to the genus *Thiobacillus*, and the specific name *Thiobacillus ferrooxidans*, n. sp., was suggested (Temple and Colmer, 1951). The results of our studies have not confirmed Dr. Temple's observation of both ferrous iron and thiosulfate oxidation under acid conditions by a single bacterial species (Leathen, Braley, McIntyre, 1953). In the acid drainage from Bingham Canyon, Utah, both iron- and sulfur-oxidizing bacteria were observed by Bryner, Beck, Davis, and Wilson (1954). The organisms were reported not to be in pure culture. An organism similar to *Thiobacillus ferrooxidans* was isolated by Ashmead (1955) from acid mine waters in Scotland.

The role of chemosynthetic autotrophic

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bacteria in the formation of acid from certain sulfuritic constituents associated with bituminous coal was studied by Leathen and Braley (1950, 1951) and Leathen, Braley, and McIntyre (1953) who reported that the amount of acid produced from such sulfuritic materials was increased threefold by the action of iron-oxidizing bacteria. *Thiobacillus thiooxidans* failed to cause an appreciable increase in acidity, except when marcasite was used as the source of oxidizable sulfur. These observations were confirmed and somewhat extended by Temple and Delchamps (1953) and Temple and Koehler (1954).

The iron-oxidizing bacterium, used throughout our studies, was given the generic name *Ferrobacillus* to indicate the transfer of iron from the ferro to the ferric state, and the specific designation, *Ferrobacillus ferrooxidans*, was proposed (Leathen and Braley, 1954).

MATERIALS AND METHODS

The iron-oxidizing bacteria found in acid bituminous coal mine effluents are strict autotrophs, and grow well in a medium of the following composition: $(NH_4)_2SO_4$, 0.15 g; KCl, 0.05 g; $MgSO_4 \cdot 7\ H_2O$, 0.50 g; K_2HPO_4, 0.05 g; $Ca(NO_3)_2$, 0.01 g; in 1,000 ml of distilled water. This solution of inorganic salts is dispensed in 100-ml quantities in 250-ml Erlenmeyer flasks and sterilized by autoclaving for 15 min at 121 C. A stock solution consisting of 10 per cent $FeSO_4 \cdot 7\ H_2O$ in distilled water is sterilized by filtration, and 1.0 ml is added aseptically to each flask containing the inorganic salt solution. The resultant medium is opalescent and has a pH of 3.5. Appreciable oxidation does not occur as long as the medium is refrigerated.

A solid medium having essentially the same composition may be prepared with silicic acid (Leathen, Kinsel, and Braley, 1955). The inorganic solution for such a solid medium is composed of the following salts: $(NH_4)_2SO_4$, 6.0 g; KCl, 0.05 g; $MgSO_4 \cdot 7\ H_2O$, 0.50 g; $Ca(NO_3)_2$, 0.01 g; in 250 ml of distilled water. This solution is dispensed in 25-ml aliquots in 125-ml Erlenmeyer flasks and sterilized for 15 min at 121 C. A buffer solution consisting of 13.5 g K_2HPO_4 in 100 ml of distilled water is autoclaved separately. The ferrous sulfate solution is prepared as previously described.

The silicic acid was prepared by passing sodium silicate through an ion exchange column. This column was constructed of pyrex glass tubing 2 in in diameter and 24 in long, with a side-arm 2 in from the top and a 3-way stopcock at the bottom. The stopcock served as the outlet for the silicic acid and as the inlet for the distilled water used in the back-washing procedure. The side-arm at the top served as the outlet for the distilled water. The column was packed with 500 g of analytical grade "amberlite" IR-120 (H). A solution containing approximately 7 per cent SiO_2 was prepared by dissolving 15 g of anhydrous *meta* sodium silicate in 100 ml of distilled water. This solution was run through the resin at the rate of 20–30 ml per min. The acid collected at the bottom of the column had a pH of 2.0. The silicic acid was distributed in 75-ml quantities in 300-ml Erlenmeyer flasks and sterilized for 15 min at 121 C.

The operating efficiency of the column was followed by the use of wide range pH indicator paper. Until a pH of 2.0 was reached, all fractions were discarded. Exhaustion of the resin was indicated by a rapid rise in pH to that of the sodium silicate solution. Passage of 1,500 ml of 10 per cent HCl through the resin completely regenerated the column. The Amberlite was freed of chloride ions by thoroughly back-washing with distilled water.

To complete the preparation of the solid medium, 1 ml of the ferrous sulfate solution was added aseptically to 75 ml of sterile silicic acid, and 1 ml of the buffer solution was added to 25 ml of the sterile solution of inorganic salts. These two solutions were combined to form the completed medium. This medium was poured into petri dishes and set aside until a suitable gel was formed, which usually occurred in 24 hr.

The resultant solid medium is opaque and has a pH of 3.5. Except for the additional ammonium sulfate and buffer added to produce a satisfactory gel within a reasonable time, the final concentration of inorganic salts is the same as that of the liquid medium.

In addition to the two iron media described above, the acidic thiosulfate medium of Colmer, *et al.* (1950) was used over a wide pH range to test for oxidation of thiosulfate. The oxidation of elemental sulfur was determined by attempting to grow the microorganism in Waksman's medium (1922).

Morphological studies were made by negative staining and by the use of both bright and dark phase contrast microscopy.

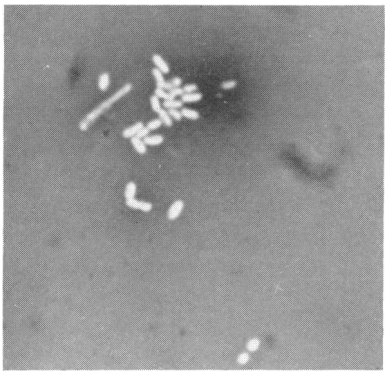

Figure 1. Cells of *Ferrobacillus ferrooxidans* negatively stained with Congo red. 1455 ×.

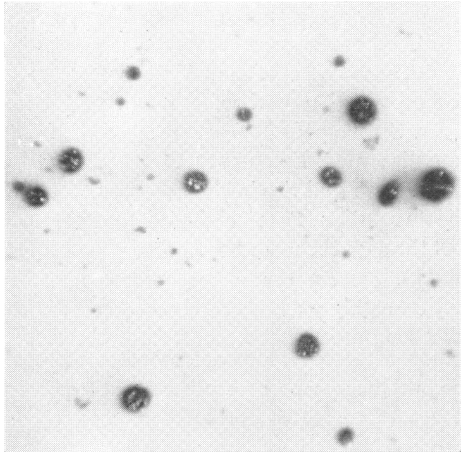

Figure 2. Colonies of *Ferrobacillus ferrooxidans* on silica gel-ferrous iron media after 7 days' incubation at 20–25 C. 50 ×.

RESULTS AND DISCUSSION

The chemosynthetic autotroph, isolated from bituminous coal mine effluents and designated as *Ferrobacillus ferrooxidans*, does not grow in any of the usual bacteriological media. The presence of carbohydrate or peptone inhibited the oxidizing ability of the microorganism. Even the inclusion of agar, as the solidifying agent of the liquid ferrous iron medium, retarded growth to the extent that only a few serial transfers were possible.

The microorganism oxidizes ferrous iron to the ferric state within the pH range of 2.0 to 4.5. Except for a slight atmospheric oxidation, chemical solutions of similar reaction are stable, and oxidation of the ferrous iron of the medium may be attributed wholly to the action of this microorganism.

The optimum reaction for growth of *Ferrobacillus ferrooxidans* is pH 3.5 and the optimum temperature is 20–25 C. The ammonium ion, in a concentration of 41 ppm, is the essential source of nitrogen. Nitrates do not appear necessary for growth. The only carbon source was the CO_2 of the atmosphere.

Young, actively growing cultures oxidize the ferrous iron of the liquid media (200 ppm) to the ferric state in 3 days, while atmospheric oxidation of a similar concentration of sterile ferrous iron required more than 2 years to go to completion. Analogous cultures failed to oxidize acid thiosulfate, though such media were tested under a wide variety of conditions and with a score of stock cultures of *F. ferrooxidans*. The elemental sulfur of Waksman's medium was not oxidized by any of the cultures tested. The latter reactions separate this chemosynthetic autotroph from the genus *Thiobacillus*.

The cells are rod shaped, measuring from 0.6 to 1.0 μ in width and from 1.0 to 1.6 μ in length (figure 1). They are actively motile, probably by means of a single polar flagellum. The cells often become attached to the cover slip and move with a whiplike motion, before becoming detached and darting across the microscopic field.

The cells stain only with the greatest difficulty. When they do take a gram stain, they are negative. The cells can best be demonstrated by using Congo red in a negative staining procedure.

Colonies of the bacteria, often embedded in the ferrous iron-silica gel medium, are small and raised, with irregular margins (figure 2). Young colonies are cream to glistening tan, but become granular and brown as they grow older. Often, there is a tan to brown area of oxidized iron underneath and around the colony.

In nature, *F. ferrooxidans* is indigenous in bituminous coal regions (Leathen, 1953). The effluents of such mines are often highly acidic and contain large quantities of ferrous iron. Such effluents rapidly deplete streams of dissolved oxygen, which inhibits aquatic life. By rapidly oxidizing the ferrous iron to the ferric state, this microorganism permits the recovery of the stream, as far as dissolved oxygen is concerned, within the shortest possible distance. In this respect, *F. ferrooxidans* is of distinct economic importance.

In addition, the role of this autotroph in the increase of stream acidity may be postulated as follows: amorphous, or microcrystalline, pyrite found in coal measures is oxidized on exposure to the atmosphere without bacterial intervention, producing ferrous sulfate and sulfuric acid. Steam, or dry-heat, sterilized sulfuritic material is just as susceptible to such oxidation as that exposed in a mine. Water seeping into a mine, or from a broken underground water course, leaches the soluble oxidation products from the exposed surfaces. Accelerated by *F. ferrooxidans*, ferrous sulfate is rapidly oxidized to the ferric sulfate. The ferric sulfate in solution, contacting other sulfuritic material, accelerates its oxidation to ferrous sulfate and sulfuric acid and is itself reduced to ferrous sulfate, making an increased quantity of ferrous sulfate available for bacterial, or air, oxidation. The extent to which this cycle enters is dependent upon the volume and speed of flow from the mine.

Eventually, in or out of the mine, all of the ferrous sulfate oxidizes to ferric sulfate and basic ferric sulfates. Ferric iron being weakly basic, dilution of the mine effluent shortly permits hydrolysis of the soluble ferric sulfates to insoluble basic ferric sulfate, ferric hydroxide, and sulfuric acid. Such iron compounds deposit as a brownish yellow sludge in the streams ("yellow boy") while the stream itself remains acid.

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SUMMARY

The chemosynthetic autotroph, *Ferrobacillus ferrooxidans*, has been further characterized. It oxidizes ferro to ferric iron, as indicated by the generic term, *Ferrobacillus*. Neither sulfur nor thiosulfate are oxidized under favorable environmental conditions. This fact distinguishes the organism from the genus *Thiobacillus*.

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