AN IRON-OXIDIZING BACTERIUM FROM THE ACID DRAINAGE OF SOME BITUMINOUS COAL MINES

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As late as 1945 Starkey called attention to the incompleteness of the information concerning the true autotrophic nature of iron bacteria, and to the confusion existing as to whether any bacteria in iron-bearing waters are concerned directly or indirectly with the precipitation of iron from the waters. The recent critical summary of researches on the iron bacteria by Pringsheim (1949) indicates a continuing interest in this member group of the *Chlamydobacteriales*, and, as Pringsheim states, "The general interest in iron bacteria is apparently greater than that bestowed upon other biological groups of comparable standing. This may partly be due to the fact that deposits of iron bacteria are readily perceived by the naked eye."

Carpenter and Herndon (1933) investigated the drainage produced by bituminous coal mines on different coal seams. They reported some waters were alkaline or near neutrality, whereas others were very acid, and suggested that the reaction responsible for the production of the acid waters was:

$$2FeS_2 + 7O_2 + 2H_2O \rightarrow 2FeSO_4 + 2H_2SO_4$$

The high sulfate and iron content, together with high acidity, makes these acid waters of major import as an industrial waste. The clear or light tan acid waters containing $FeSO_4$ soon become amber colored as the iron oxidizes, and subsequently hydrated iron oxides coat the stream bed with their rusty-appearing precipitate. Carpenter and Herndon (1933) suggested the following reactions to explain the transformations in the $FeSO_4$:

$$4FeSO_4 + 2H_2SO_4 + O_2 \rightarrow 2Fe_2(SO_4)_3 + 2H_2O$$
$$Fe_2(SO_4)_3 + 6H_2O \rightarrow 2Fe(OH)_3 + 3H_2SO_4$$

Colmer and Hinkle (1947) reported a bacterium in acid mine drainage whose growth in this type of water would cause the oxidation of the ferrous iron to ferric iron with the subsequent precipitation of hydrated ferric oxides. Samples of the same water sterilized by Seitz filtration did not become amber nor form a rusty precipitate even after months of storage.

Harder (1919), Starkey and Halvorson (1927), and Halvorson (1931) have shown that the precipitation of iron may be effected by some heterotrophic bacteria and, indeed, that iron may be precipitated from its solutions without microbial involvement.

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Since acid mine drainage is an unusual environment for bacteria and since the interest in iron organisms continues high, it was thought worth while to report findings on the organism involved in the iron transformations in this industrial waste.

MATERIALS AND METHODS

Liquid media used: Natural mine waters. The acid waters used in this study came from mines operating on the Pittsburgh, the Sewickley, and the Upper Freeport coal seams. In most instances waters entering bituminous coal mines as roof drips are near neutrality or alkaline. The alkaline roof drips used were secured as the water came into a mine working the Pittsburgh seam.

Artificial mine waters. Three artificial mine waters were made based on the pH (2.3 to 2.6) and iron content (1,500 to 2,000 ppm) of many natural acid mine waters. Water number 1 consisted of distilled water, 2,000 ppm Fe as $FeSO_4 \cdot 7H_2O$, and 100 ppm N as $(NH_4)_2SO_4$. The pH was adjusted to 2.5 with concentrated H_2SO_4. Water number 2 consisted of alkaline roof drips similarly treated to have approximately 2,000 ppm Fe, 100 ppm N, and a pH of 2.5. Water number 3 had as its base 100 ml of completely oxidized natural acid mine water containing only ferric iron to which was added 7 ml of a 1 per cent solution of acidified Na_2SO_3 at pH 2.9. Reagent grade chemicals were used throughout.

These three artificial mine waters were passed through sterile Seitz filters previously washed with 200 ml of distilled water adjusted to pH 2.5 with H_2SO_4. The acid washing prevents undesirable changes in the medium.

Sodium thiosulfate medium. A liquid medium was made using

$Na_2S_2O_3 \cdot 5H_2O$	5	g
KH_2PO_4	3	g
$(NH_4)_2SO_4$	0.2	g
$MgSO_4 \cdot 7H_2O$	0.1	g
$CaCl_2$	0.2	g
distilled H_2O	1,000	ml

The medium was autoclaved at 17 pounds pressure for 15 minutes. The final pH was 4.7 to 5.0. A 1 per cent $Na_2S_2O_3$ liquid medium, number two of Starkey (1934), that has an increased buffer content and a pH of 6.5 was used in one test.

Solid media used: Natural mine water with agar. Agar plates containing acid mine water were made by adding Seitz-filtered water to sterile 3 per cent agar at 45 to 50 C. The medium was made only as needed since reheating the solidified agar would change its properties.

Sodium thiosulfate agar. The liquid medium containing 0.5 per cent $Na_2S_2O_3$ was solidified by the use of 1.5 per cent agar.

Isolation of organism. Acid mine drainage from the Pittsburgh seam served as the source of the iron-oxidizing bacterium, although Upper Freeport and Sewickley waters were plated and the colonial characteristics of the organism noted. Approximately 50 ml of the chosen acid drainage were placed in a sterile 250-ml Erlenmyer flask; the flask was closed with a cotton stopper and incubated at room temperature, approximately 30 C. After the water had become turbid and

there was a precipitate on the sides of the flask, 1 ml of it was taken to serve as the inoculum for another 50 ml of acid drainage in a similar flask. Successive transfers were made as soon as the characteristic precipitate was formed. Streak plates of acid mine water agar were made from one of the successively enriched flasks.

The agar plates were examined under a stereoscopic wide-field microscope, and well-isolated colonies surrounded by an amber precipitate were removed with a microneedle to test tubes of sterile acid mine drainage. To ensure the purity of the culture, acid mine water agar plates were streaked from the growth of the test tube and, after colonies were formed, reisolations were made. Although the fresh acid drainage might be plated directly to gain pure cultures, the successive transfer method proved to be the easier technique.

Procedures used for study of characteristics. The morphological and cultural characters of the iron-oxidizing bacterium were determined on the natural mine water media and on the 0.5 per cent $Na_2S_2O_3$ media. These media, together with the artificial mine waters, were used to study the organism's physiology.

The inocula used were from either the natural acid mine water or the $Na_2S_2O_3$ broth. Since the phosphate content of the latter medium and the iron of the mine water or agar brought about a murky precipitate of iron phosphate, inoculations were made usually from iron medium to iron medium or from $Na_2S_2O_3$ medium to $Na_2S_2O_3$ medium.

The series of tests made with the artificial mine waters numbers 1 and 2 were inoculated with a pure culture of the iron-oxidizing organism. One ml of a 3-day-old culture grown in a natural acid mine water was placed in about 125 ml of the artificial mine water and mixed thoroughly. A sample was then taken for the determinations of pH, redox potential, total acidity, and ferrous and ferric iron. Two 50-ml portions of the inoculated water were placed in sterile 250-ml Erlenmyer flasks; to one portion was added 1 ml of toluol, and the flask was then closed with a rubber stopper. The other portion was cotton-stoppered as usual.

The artificial acid mine water number 3 was divided into two portions and placed in sterile 250-ml Erlenmyer flasks. One flask was left as the uninoculated control; the other flask received 5 drops of a pure culture of the iron-oxidizing bacterium grown in a natural acid mine water medium. At intervals samples were removed aseptically for testing.

To test the possibility that compounds in the original inoculum might influence the oxidation of the iron of the artificial mine waters, transfers were made successively from an oxidized portion of the artificial mine waters to sterile unoxidized portions of these artificial mine waters. All incubations were made at room temperature.

The Beckman pH meter model G was used for all pH and millivolt readings. The millivolt values secured with the saturated calomel electrode were corrected to the normal hydrogen electrode by adding 243 millivolts. The readings were further corrected to pH 2.5 by adding or subtracting 60 millivolts for each pH unit above or below 2.5.

The method of Goodman (1947), utilizing *ortho*-phenanthroline, was used for

the iron determinations. Any necessary dilutions were made with water acidified with H_2SO_4 to a pH of 2.6. A reagent blank and a known iron standard solution were run concurrently with the test samples. All samples were filtered through Whatman no. 42 filter paper prior to testing.

Total acidity was determined by titrating an aliquot of the mine water at boiling temperature with N/50 NaOH to the phenolphthalein end point. The results are expressed as parts per million of $CaCO_3$.

The determinations of sodium thiosulfate utilization were made by titrating 5- or 10-ml paper-filtered aliquots of the broth with N/100 iodine solution; starch solution was used to determine the end point.

EXPERIMENTAL RESULTS

Morphological characteristics. The iron-oxidizing bacterium is a short, motile gram-negative rod occurring singly or, occasionally, in pairs. In natural acid mine water it ranges in size from 0.4 μ by 0.8 to 1.0 μ, but in $Na_2S_2O_3$ broth it appears to be larger. It is nonsporeforming.

Cultural characteristics: Sodium thiosulfate media. Growth in $Na_2S_2O_3$ broth is turbid. A wet mount made after several days of growth shows globules of sulfur and organisms. Frequently, upon undisturbed incubation of 2 to 3 weeks, a delicate, fragile pellicle of cells is formed. Handling of the culture causes the pellicle to flake and settle to the bottom. Crystals of undetermined composition are formed in the medium after several weeks' incubation.

The iron-oxidizing organism does not give the copious, slimy, creamy growth characteristic of *Thiobacillus thioparus* in $Na_2S_2O_3$ broth. Streak plates of the iron-oxidizing bacterium on $Na_2S_2O_3$ agar frequently show colonies with a covering of white, frosty material. This material is needlelike and does not resemble the whitish sulfur of *T. thioparus* growing on $Na_2S_2O_3$ agar. If portions of the pellicle from $Na_2S_2O_3$ broth or if organisms from a colony on the $Na_2S_2O_3$ agar are mixed in a drop of sterile mine water on the surface of acid mine water agar and then streaked out, the typical amber color is produced about the growing colonies.

Acid mine drainage. Sterile acid mine water inoculated with the iron-oxidizing bacterium becomes murky in 3 to 4 days; an amber-colored coating forms across the surface of the liquid and on the sides of the tube. A wet mount of the surface crust shows flakes of the precipitated iron compound with entrapped cells of the organism.

The change from the clear, nearly colorless, uninoculated acid mine water to the murky, amber-colored water, which results only after growth of the organisms, is enhanced if the water is placed in a flask rather than in a test tube. The increased surface exposure speeds up the oxidation of the iron. As the incubation time is increased, the liquid loses its murkiness and again becomes clear although its amber color persists.

Iron-oxidizing organisms have been detected in mine water after bottles stored for a year in a filled, sealed condition were opened and exposed to the air. The rapidity of change-over was much slower in these stored waters than in the fresh mine water.

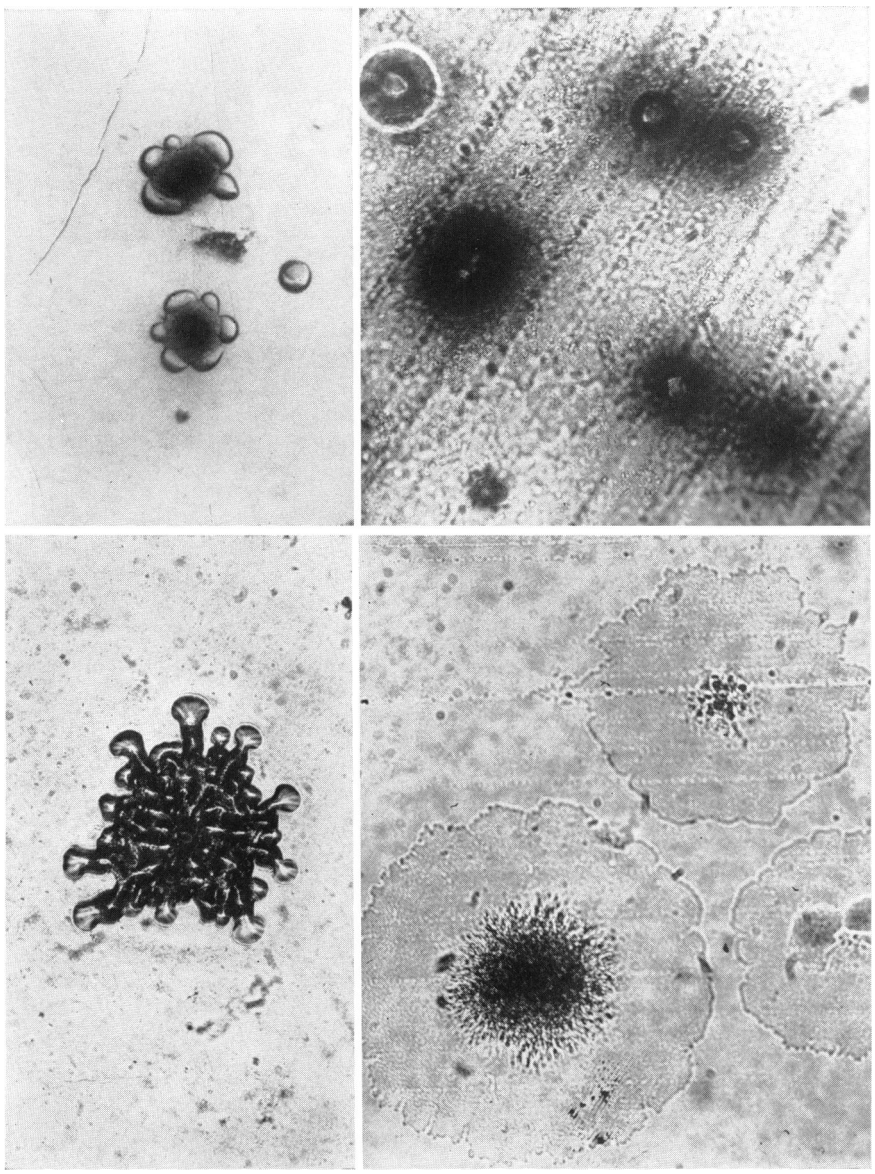

Figure 1. Colonial appearance of iron-oxidizing bacterium. *Top left*: Young colonies on acid mine water agar with early lobed growth. *Top right:* Young colonies showing iron precipitation. *Bottom left:* Old colony with marked lobe formation and iron precipitation within colony. *Bottom right:* Colonies on sodium thiosulfate agar showing different stages in the formation of frosty-appearing material in the colony.

The colony appearance on acid mine water agar is striking. After about 6 or 8 days of incubation very small glycerollike colonies can be seen. If these colonies are crowded, the amber precipitate appears first in that area. With well-isolated

colonies the amber precipitate appears later about the growth. The illustrations show the tendency toward lobe formation. Frequently the iron precipitation occurs only at the center of the colony and is produced out from the center as the lobed growth ages. Many bizarre colonial forms have been seen as a result of the growth of the lobes coming out from the original point of growth. Old colonies are encrusted with the precipitated iron compound.

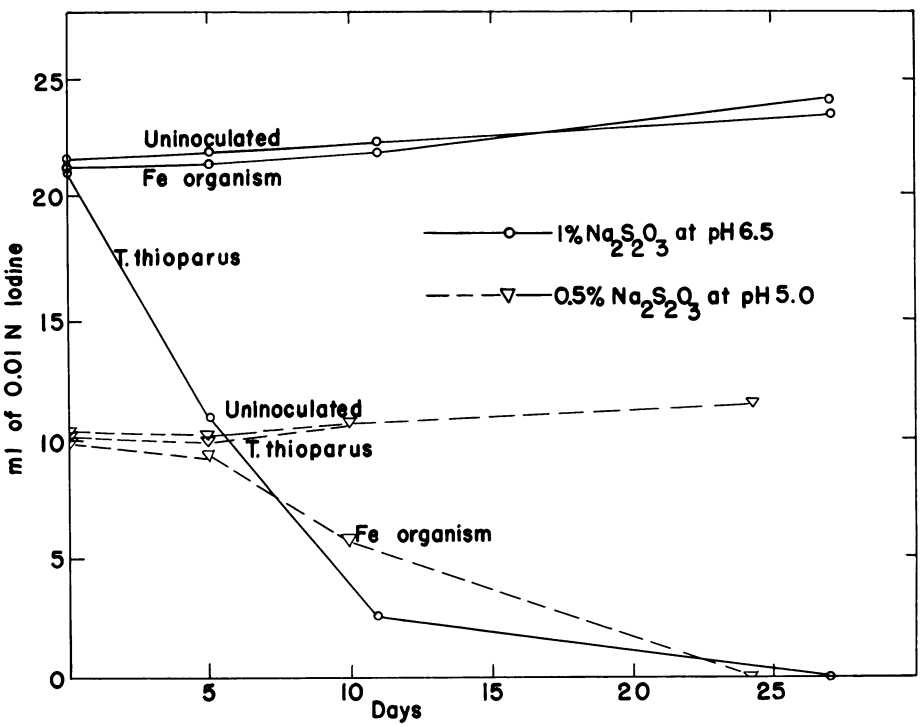

Figure 2. Sodium thiosulfate utilization (titration with 0.01 N I_2) and pH change by iron-oxidizing bacterium and *T. thioparus* in 0.5 per cent $Na_2S_2O_3$ medium at initial pH 5.0 and in 1 per cent $Na_2S_2O_3$ medium at initial pH 6.5.

The organism used in these experiments is definitely not heterotrophic but has every indication of being an obligate autotroph. It can get its energy from the oxidation of $Na_2S_2O_3$, and the CO_2 of the air can serve as the sole source of carbon. Attempts to grow the organism on nutrient agar or broth, on acidified glucose peptone agar or broth, or in acidified solutions in which the iron was in the form of the citrate, butyrate, or lactate have failed. At present only the $Na_2S_2O_3$ media or the natural and artificial mine waters have served for its growth.

Physiological characteristics: Sodium thiosulfate broths. Figure 2 shows the comparative action of the iron-oxidizing bacterium and *T. thioparus* on two $Na_2S_2O_3$ media. In the 0.5 per cent thiosulfate, pH 5.0, medium, the iron-oxidizing bac-

terium decomposed the energy source with acid production, but the action of *T. thioparus* was negligible. With the 1 per cent thiosulfate, pH 6.5, medium, the reversal was found to hold. *T. thioparus* grew well but the iron-oxidizing bacterium did not. The failure of the iron-oxidizing bacterium to grow here may be due to the H^+ concentration. It was found in other work that a 1 per cent, pH 4.5, sodium thiosulfate medium that did not support good growth of *T. thioparus* did become adequate when the pH was raised to 6.5.

Natural and artificial acid mine waters. Figure 3 shows the rapid oxidation of ferrous iron in a Pittsburgh seam water. The oxidation was particularly rapid in

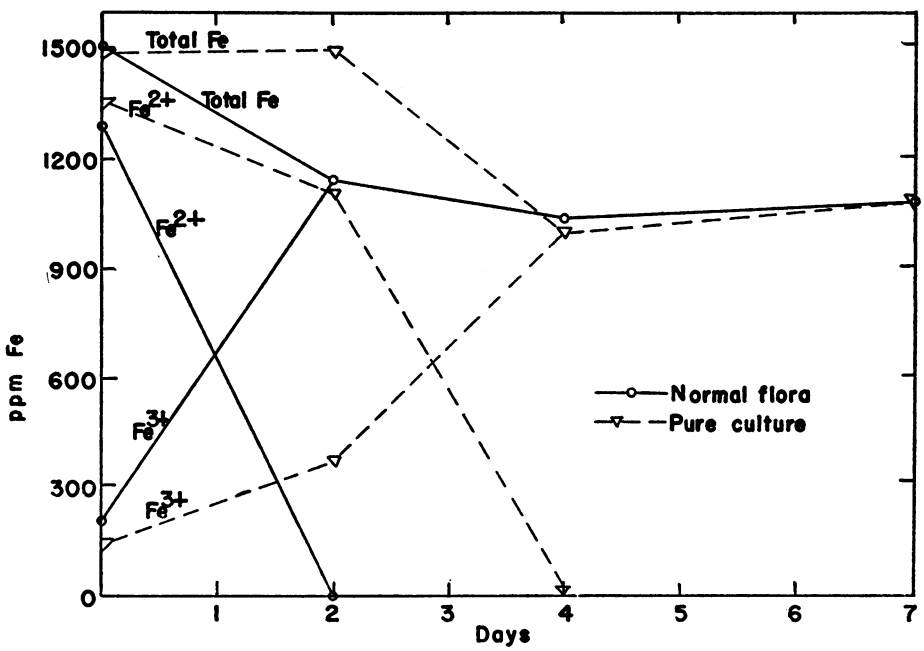

Figure 3. Pittsburgh seam drainage showing iron transformations with the normal flora and a pure culture of the iron-oxidizing bacterium.

the water containing the normal mixed flora; when another portion of the same drainage was sterilized and inoculated with the iron-oxidizing bacterium, the over-all changes in the iron were the same.

Table 1 shows that the total acidity, pH, and the redox potential of these waters also are modified as the environment changes due to the action of the microflora present.

Figure 4 shows the iron changes in the distilled water base medium. It is interesting to note the efficiency of toluol in preventing the activity of the iron-oxidizing bacterium and to note the absence of any agent from the bacterium, in the absence of its growth, which could bring about marked alterations in the iron content of the water.

The growth of the iron-oxidizing bacterium in the distilled water base medium was slower than the growth of the organism in either the natural acid mine water or in the altered roof drips water, but the changes in the iron content of the distilled water medium were due to the growth of the organism and not to other agents in the original inoculum. Serial transfers in the distilled water medium always gave the characteristic precipitate. Table 1 shows the changes in total

TABLE 1

Changes in total acidity, pH, and E_h of natural and artificial acid mine waters undergoing iron transformations

DAYS OF INCUBATION	TOTAL ACIDITY AS PPM $CaCO_3$		pH		E_h* + mv	
	t	nt	t	nt	t	nt
Normal mixed flora, Pittsburgh seam water						
0	—	6,000	—	2.60	—	619
2	—	5,750	—	2.40	—	812
4	—	5,500	—	2.30	—	831
7	—	5,750	—	2.28	—	820
Pure culture iron-oxidizing bacterium, Pittsburgh seam water						
0	5,550	5,550	2.65	2.65	614	614
2	5,750	6,200	2.70	2.72	617	646
4	5,500	5,850	2.70	2.35	620	814
7	5,600	5,750	2.70	2.30	620	811
Pure culture iron-oxidizing bacterium, distilled water base medium						
0	5,750	5,750	2.00	2.00	†	†
2	†	6,170	2.25	2.10	†	†
5	5,720	6,620	2.05	2.15	571	777
Pure culture iron-oxidizing bacterium, roof drips base medium						
0	4,950	4,950	2.70	2.70	607	607
3	5,200	5,100	2.72	2.72	611	628
5	4,900	4,800	2.70	2.50	615	648
8	5,050	4,900	2.62	2.28	610	740

* Corrected to the normal hydrogen electrode at pH 2.5.

† Not determined; t = toluol present; nt = no toluol present.

acidity, pH, and redox potential accompanying the changes in the iron content of the artificial acid mine waters. It can be seen from table 1 that with a pH alteration and an increased ferrous iron content the iron-oxidizing bacterium found roof drips acid mine water an adequate medium.

Table 2 indicates that if the ferrous iron content of a completely oxidized natural acid mine water is restored by a reducing agent, such as Na_2SO_3, the iron-oxidizing bacterium can again oxidize the iron.

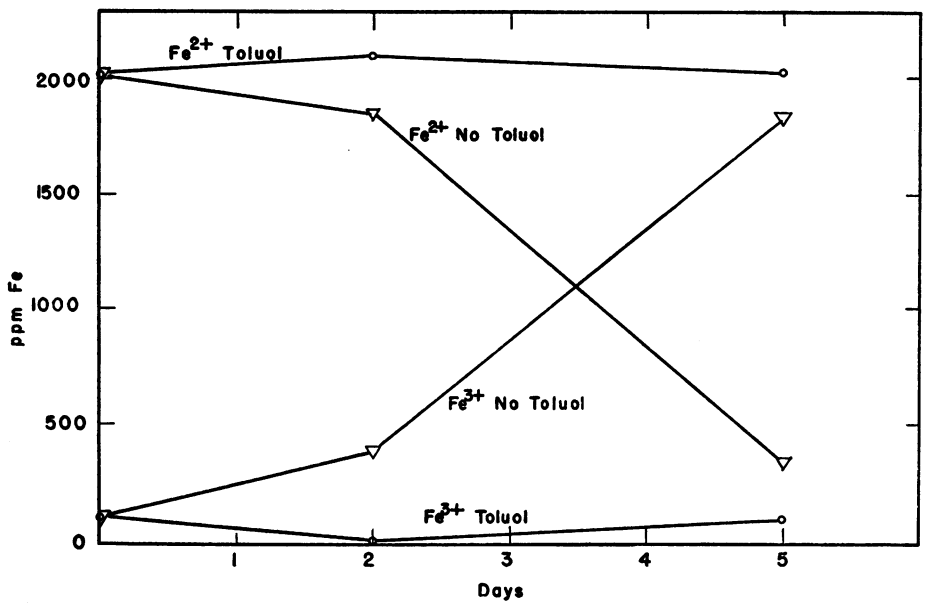

Figure 4. Iron transformations in artificial acid mine water, distilled water base, inoculated with a pure culture of iron-oxidizing bacterium.

TABLE 2

Changes in pH and E_h in Na_2SO_3-reduced natural mine water

	pH			E_h*		
				+mv		
Original oxidized H_2O	2.35			789		
Original oxidized H_2O reduced with Na_2SO_3	2.15			618		
	DAYS OF INCUBATION					
	4	12	15	4	12	15
Reduced H_2O uninoculated	2.20	†	2.10	625	625	619
Reduced H_2O inoculated	2.25	2.30	2.30	658	786	806

* Corrected to the normal hydrogen electrode at pH 2.5.

† Not determined.

DISCUSSION

Colmer and Hinkle (1947) reported that two microorganisms were isolated consistently from acid mine drainage. One was an organism indistinguishable in its morphological, cultural, and physiological properties from *Thiobacillus thiooxidans*. It was suggested that this organism might be involved in the oxidation of pyrite and marcasite, FeS_2, which are common in some seams of coal as concretions, lenses, bands, etc., to $FeSO_4$ and H_2SO_4. This possibility is at present being explored. The second organism was involved in the oxidation of the $FeSO_4$

to $Fe_2(SO_4)_3$, and it was shown that, if the growth of the organism was prevented, the oxidation was prevented or would proceed at a markedly slower rate.

The colonial appearance of the iron-oxidizing bacterium on $Na_2S_2O_3$ agar is different from that of *T. thiooxidans* or *T. thioparus*. Its growth in $Na_2S_2O_3$ broth is different from that of *T. thioparus*, even though amorphous sulfur is found in the menstruum. It resembles *T. thiooxidans* in that the medium becomes turbid with no slimy growth and the energy source is oxidized to H_2SO_4. The low pH, as shown in figure 2, indicates this acid production. The presence of the amorphous sulfur may be similar to that found by Starkey (1925), who attributed sulfur formation to the decomposition of the thiosulfate by acid. There is no iron in either of the $Na_2S_2O_3$ media except that which might be a contaminant of the distilled water or the reagent grade chemicals used. In other words, the organism grows in an environment without iron and reacts, in one instance, similarly to *T. thiooxidans*. All attempts to induce *T. thiooxidans* or *T. thioparus* to bring about the characteristic iron precipitation have failed.

In view of the unsatisfactory status of the so-called iron bacteria it is difficult to assign a place for this new organism. It bears no relation to the heterotrophic iron-precipitating bacteria of Mumford (1913) or Starkey and Halvorson (1927) or to the heterotrophic thiosulfate oxidizers of Sijderius (1946). The mine water organism belongs to the *Eubacteriales* and appears to live autotrophically on $Na_2S_2O_3$. It has nothing in common with the iron bacteria previously reported and critically summarized by Pringsheim (1949). There is no sheath or band or stalk formation. There is no colonial manifestation as demonstrated by *Siderocapsa*. However, as the crust of iron is formed on the water, organisms are entrapped in it.

Acid mine water has little in common with natural waters of other iron bacteria reported by Halvorson (1931) and Harder (1919). The pH is exceedingly low, the salt concentration high, and the iron is in much greater concentration and occurs as the sulfate instead of the carbonate. Acid mine water is the first natural source to provide a stable ferrous solution not associated with organic compounds, a failure of other waters deplored by Pringsheim (1949) as a principal deterrent to studies of autotrophy.

Iron oxidation in very acid waters was rapid, and in the natural waters the change was faster than with the pure cultures. Since no iron organism counts were made, it is impossible to ascribe the difference in speed to the number of organisms. However, from the appearance of the many streak plates made of natural waters it might well be that this rapid change was influenced by the diversity of the types of organisms growing in the water as well as by the change induced only by the iron organisms.

The mechanism of the precipitation of the iron in the acid mine waters is not known as yet. One might postulate the presence of a substance in these complex mineral solutions that is acted upon by the iron-oxidizing bacterium so that its decomposition affects the stability of the iron. This premise is difficult to hold when it is known that ferrous sulfate solutions, at the pH levels in natural acid mine waters, are stable, and when these solutions occur in the artificial distilled

water acid mine water made from reagent grade chemicals in which such a substance is not likely to be present, precipitation takes place upon growth of the bacterium. Nor can the iron precipitation be due to the decomposition of an organic iron complex, since the artificial distilled water acid mine water had none and it is doubtful if such complexes exist in large amounts in the natural acid mine water used.

If the Winogradsky concept of iron bacteria is to be accepted, one should expect to find these bacteria securing their energy from the oxidation of ferrous iron. In the exploratory tests made thus far with the synthetic medium, it is premature to draw any conclusions concerning the possibility that oxidation of the ferrous iron is the energy source for the organism.

A consideration of the known physiological characteristics of the iron-oxidizing bacterium shows that it does not correspond to the description of any species heretofore described. If the growth in iron media and the oxidation of ferrous iron are ignored, an apparent classification in the genus *Thiobacillus* seems reasonable. This is the only logical place for a small gram-negative rod living autotrophically on thiosulfate and reducing the pH, by H_2SO_4 production, to 2 to 3 pH units. The extreme acid tolerance and sulfate production resemble *T. thiooxidans*; the failure to grow on elemental sulfur constitutes a difference. The action on ferrous solutions and the lobed colony growth are anomalous properties for a *Thiobacillus*. Until the mechanism of iron oxidation is better understood and the biochemistry further investigated, it would be well to reserve classification of this bacterium.

SUMMARY

A bacterium concerned with iron transformations has been isolated repeatedly from the acid mine drainage of mines on the Pittsburgh, the Sewickley, and the Upper Freeport bituminous coal seams. The ferrous iron of such drainage is rapidly oxidized to the ferric state in the presence of the bacterium. If the drainage is made sterile as, e.g., by Seitz filtration, the ferrous iron is stable for long periods of time.

The bacterium can bring about comparable changes in the iron content of Seitz-filtered artificial acid mine drainage composed of distilled water, sulfuric acid, ferrous sulfate, and ammonium sulfate. The mechanism responsible for the oxidation of the ferrous iron in the natural and artificial acid mine waters has not yet been found.

The morphological characters of the bacterium, its autotrophic nature on thiosulfate medium, and its oxidative action on thiosulfate with the production of sulfuric acid to give a low pH indicate properties that most closely resemble those displayed in the genus *Thiobacillus*.

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