A FERROUS-ION-OXIDIZING BACTERIUM

I. Isolation and Some General Physiological Characteristics

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Microbial oxidation of iron pyrite by microorganisms in acid leach water at Bingham Canyon, Utah, was demonstrated by Bryner *et al.* (1954). Further studies showed that the leaching water contains a ferrous-iron-oxidizing, strongly acidophilic bacterium (King, 1956; Bryner and Jameson, 1958) which is similar to the organisms described by Colmer *et al.* (1950) and by Leathen *et al.* (1956). The purpose of this paper is to report results of experiments on the isolation and characterization of the iron-oxidizing bacterium found in acidic leaching water.

MATERIALS AND METHODS

All chemicals used were those commercially available and were used without purification. Qualitative tests on ordinary "flowers of sulfur" indicated no reduced iron was present.

Media. The liquid medium (M) used for enrichment cultures and for maintenance of pure cultures was prepared as follows:

Solution A contained $(NH_4)_2SO_4$, 0.1 per cent (0.0076 M); KH_2PO_4, 0.05 per cent (0.0037 M); and $MgSO_4 \cdot 7H_2O$, 0.02 per cent (0.008 M).

Solution B contained $FeSO_4 \cdot 7H_2O$, 10 per cent (0.35 M), and H_2SO_4(1 N), 1 volume per cent.

Solutions A and B, autoclaved separately, were mixed in the ratio of 4 parts A to 1 part B. The pH of the mixture is 2.6 to 2.8. Some iron precipitates while solution B is being autoclaved but the amount is small. A slight turbidity results when solutions A and B are mixed.

Solid media were prepared as follows:

Agar medium contained 2 parts 3 per cent sterile agar (Difco, Noble), 1 part solution A (sterile), and 1 part solution B (sterile). After mixing, this medium was either tubed for slants or poured into sterile petri plates.

Silica gel medium (Kingsbury and Barghoorn, 1954) contained 150 ml Ludox, 10 ml solution A, and 40 ml solution B. After mixing, plates were poured and placed in autoclave for 10 min at 120 C which sterilized and solidified the medium.

Cultures maintained in the liquid medium were transferred at approximately weekly intervals by placing a loopful (about 0.005 ml) of culture into fresh medium. Inocula for experiments were usually taken from 5 to 6-day cultures which were probably in early stationary phase.

Bacteria for cell suspension experiments. Bacteria were grown in 2-L Erlenmeyer flasks containing 300 ml of liquid medium. The flasks were inoculated with 1 ml of culture and incubated at room temperature (25 to 28 C) for 8 days. The heavy ferric precipitate which formed was removed by sedimentation in tall cylinders, and the supernatant was centrifuged at 13,000 × G for 5 min. The supernatants from the centrifuge tubes were discarded; the residues were suspended in about 40 ml solution B, and centrifuged at about 100 × G for 5 min. The residue was discarded. The cells in the supernatant which showed little or no presence of ferric iron, were collected by centrifugation, at 13,000 × G and suspended in 5 or 10 ml H_2O. Cell suspensions thus prepared, free of suspended ferric precipitate, retain about 50 per cent of their ferrous-ion-oxidizing activity after 90 days at 4 to 5 C; whereas in the presence of ferric precipitate about 60 per cent of original activity was found after storage for 180 days at 4 to 5 C.

Bacterial growth. Growth was measured by formation of ferric ion in liquid media. This will be shown later to have high correlation with carbon dioxide fixation. Cell counts were made by noting highest dilution of inoculum at which growth would occur in the liquid medium.

Carbon dioxide fixation. Fixation by cell suspensions or by growing cultures has been determined by adding radioactive carbon dioxide of known specific activity to the reaction vessel. At the end of the experiment samples of the entire contents were removed to small tubes treated

with concentrated HCl and aspirated with $C^{12}O_2$ for 15 to 30 min. After filtration through coarse filter paper, samples were plated in stainless steel planchets and counted with the usual β-ray detection equipment. Correction for self-absorption was necessary in most experiments.

Usual laboratory procedures were employed for manometric, photo- and spectrophotometric determinations. Nitrogen was determined by the micro-Kjeldahl method and carbon by the method described by Sakami (1955). Ferric iron estimations were made as follows: To 3 ml of 0.3 M acetic acid in a 10-ml volumetric tube were added the sample containing ferric ion (0.2 to 3.0 μmoles Fe^{+3}), 0.25 ml 0.1 M Tiron (disodium-1,2-dihydroxybenzene-3,5-disulfonate), and water to make 10 ml. After mixing and allowing to stand 10 min, the optical density of the solution (maximal absorption at 680 mμ) was measured against a control containing no ferric iron. Ferric ion can be accurately determined in presence of excess ferrous ion, which does not oxidize under these conditions. A standard curve showing the relationship between ferric ion concentration and optical density was prepared (Fisher Electrophotometer, filter A, 650 mμ) and used for all iron determinations.

RESULTS

Isolation of bacterium. Enrichment cultures of the iron-oxidizing bacteria were obtained by inoculating 25-ml portions of sterile liquid medium in 125-ml conical flasks with 1-ml portions of the acidic leach water, which was sampled after passage through the waste ore and just before its entry into the copper recovery plant. All flasks showed extensive oxidation of iron after incubation for several days at 30 C. Transfer of 1 ml into fresh sterile medium yielded noticeable oxidation in 1 to 2 days and transfer of a loopful of culture produced apparent activity in 4 to 5 days. After 6 days for the former and 8 days for the latter the ferrous iron was completely oxidized and additional enrichment media were inoculated. After several transfers enrichment cultures were used as inocula for streak plates. Silica gel and washed agar plates proved equally useful, yielding well-isolated colonies after 10 to 14 days of incubation at 30 C.

Colonies on agar were typically larger and showed less tendency to be entirely darkened than those on silica gel plates. The latter were identical to those shown by Bryner and Jameson (1958). Isolated colonies were examined microscopically and found to be uniform in appearance and composition. Repeated transfers from single, well-isolated, colonies to fresh sterile solid media (silica gel or agar) showed no deviation from the characteristic colonial type. Cultures obtained from single colonies were considered to be pure cultures. Three strains of those isolated have been maintained and used in most of the work reported here. These have been designated B_1, B_2, and 9 and although isolated at different times, seem to be identical in appearance, colonial habit, and physiological characteristics. A strain used in some early work (*Beck and Elsden, 1958*), and subsequently lost, appeared identical to those used more recently. The bacterium is a small ovoid or short rod which occurs singly or more frequently in pairs (figure 1). Single cells range in size from 0.5 to 0.7 μ in diameter and 0.8 to 1.0 μ in length; the length of pairs is about double that of single cells. The organism is gram-negative and weakly motile. In a dried smear from the culture medium the cells stain very poorly with basic stains but strongly with acidic stains. After suspending in solutions of about pH 7.0 basic stains are more readily taken up. Except for considerably reduced motility, the iron-oxidizing bacterium is morphologically similar to *Thiobacillus thiooxidans.*

Growth experiments. Cell counts were made by noting the highest dilution of inoculum at which growth would occur in medium M. Although this method is not highly reliable unless large numbers of tubes are used, it was found to be more convenient and to yield higher values than direct cell counts, which, because of inability to prepare plates for counting or to use turbidometric cell density estimations, is the only other method applicable to these cultures. The cell count in the acidic leach water has been found on two separate occasions to be between 10^4 and 10^5 cells per ml. Stationary phase cultures (medium M) have consistently shown cell counts of between 10^7 and 10^8 cells per ml. The medium during growth becomes more acidic; the pH changes from about 2.8 to about 2.5.

Numerous attempts to culture this organism in other media, lacking ferrous iron, or sulfur, have been unsuccessful. Nutrient broth at normal and at high acidity, yeast extract broth, and glucose broth have been tried without success. Peptone and yeast extract (Difco) added to

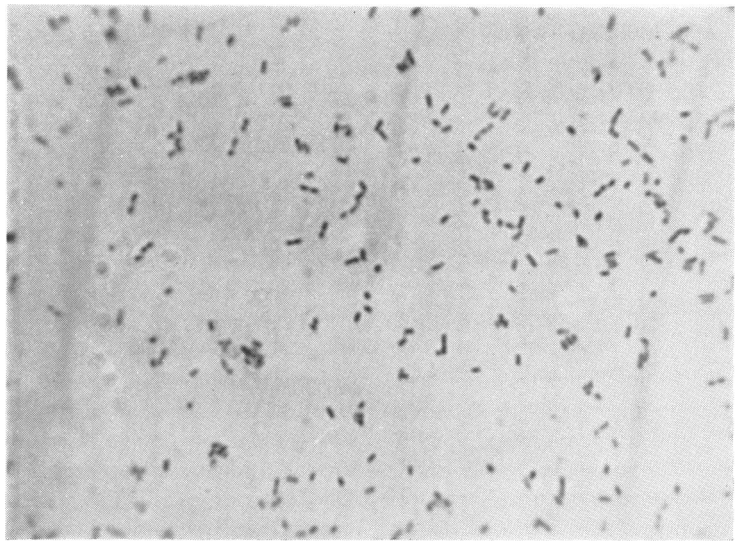

Figure 1. Iron-oxidizing bacterium isolated from acidic leach water in Bingham Canyon, Utah. Stationary phase culture. Crystal violet. 1300×.

medium M at a concentration of 0.05 per cent proved inhibitory to the iron bacteria. Slow growth on sulfur-containing media has been consistently observed. Substitution of powdered sulfur for ferrous iron in medium M produces a medium on which all strains grow slowly, producing noticeable turbidity after about 10 days. Upon inoculation of medium M with cultures of the iron-oxidizing bacterium which had been grown on sulfur, growth was initiated and continued at the same rate as if inoculated with cultures grown on ferrous iron. The cells in these cultures are morphologically unchanged from those in medium M. Thiosulfate media have not been observed to support the growth of the iron-oxidizing organism.

The formation of ferric iron in medium M during growth of the iron-oxidizing bacterium is shown in figure 2. In this experiment 100 ml of medium M were placed in a 1-L flask which was sealed subsequent to sterilization with a thin rubber stopper. Using a syringe, the medium was inoculated through the stopper with 15 ml of a stationary phase culture. Also, using a syringe, 1.0 ml of a 0.125 M solution of $NaHCO_3$ (C^{14} = 1,280,000 cpm) was introduced into the flask. This, with the normal CO_2 in the fluid and in the air, was calculated to yield 140 μmoles CO_2, specific activity = 9 × 10^3 cpm/μmole. The flask was incubated at 30 C and samples taken by syringe at intervals as shown. Each

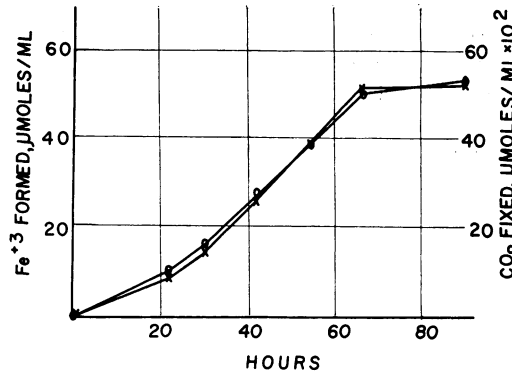

Figure 2. Correlation of ferrous ion oxidation and carbon dioxide fixation during growth of the iron-oxidizing bacterium. Medium M (125 ml) was inoculated with 15 ml of a stationary culture. $NaHC^{14}O_3$ was added. Incubation was at 30 C. × = μmoles ferric ion formed per ml; ○ = μmoles CO_2 fixed per ml × 10^2.

sample was acidified with HCl and flushed with $C^{12}O_2$ for 30 min, after which samples were taken for ferric iron and C^{14} determination. The plotted data show good correlation between CO_2-fixed and ferric iron formed, also complete cessation of CO_2 fixation upon exhaustion of the ferrous iron. Fixation of 1 mole of CO_2 occurred, in this experiment, as a result of oxidation of about 100 moles of ferrous iron. The data plotted in figure 2 may be used to calculate the generation time

of this bacterium, which is seen to be about 10 to 15 hr.

At the conclusion of the experiment the cells were obtained and analyzed for C^{14} content by oxidation and collection of CO_2. The specific activity of cellular carbon (corrected for the initial cellular carbon) was found to be 7600 cpm/μmole as contrasted with a calculated value of about 9000 cpm/μmole. Two other experiments showed similar low specific activity of carbon recovered from cells as compared to its initial activity. This may be due in part to a higher than 0.03 per cent CO_2 in laboratory atmosphere which would lower the calculated initial specific activity of the CO_2 in the growth vessel. Nevertheless, at least 85 per cent of the cellular carbon had its origin in CO_2 and the cells are truly chemolithoautotrophic bacteria. Attempts to grow these bacteria in contact with an atmosphere free of CO_2 (presence of NaOH) have been unsuccessful.

Cell-suspension experiments. The oxidation of ferrous iron by cell suspensions of the iron-oxidizing bacteria has been studied using Warburg-type manometric equipment. Data obtained in an experiment in which a cell suspension was allowed to act on ferrous sulfate as substrate are shown in figure 3. The uptake of oxygen is stoichiometric according to the following equation:

$$2FeSO_4 + H_2SO_4 + \tfrac{1}{2}O_2 \rightarrow Fe_2(SO_4)_3 + H_2O$$

Note also that the rate of oxidation is independent of Fe^{+2} concentration over a wide range. No endogenous activity of cell suspensions could be detected here or in other experiments with or without alkali in the center well. Boiled cells showed no oxygen uptake, nor did a ferrous iron control after 3 hr under conditions of this experiment.

The rate of oxygen uptake by cell suspensions is high, and with high cell concentrations failure to obtain oxygen equilibrium between the fluid and gaseous phase may result. This is shown in table 1 by the decreased $Q_{O_2}(N)$ values at cell concentrations above 0.129 mg cellular N per Warburg vessel. Note also the loss of activity caused by storage of cell suspensions. The data for table 1 were obtained from several experiments including the one described in figure 3 which shows further the stoichiometry of the iron oxidation reaction.

Cell suspensions are able to oxidize sulfur as is evidenced by the oxygen uptake data shown in figure 4. There is noted a uniform rate of oxy-

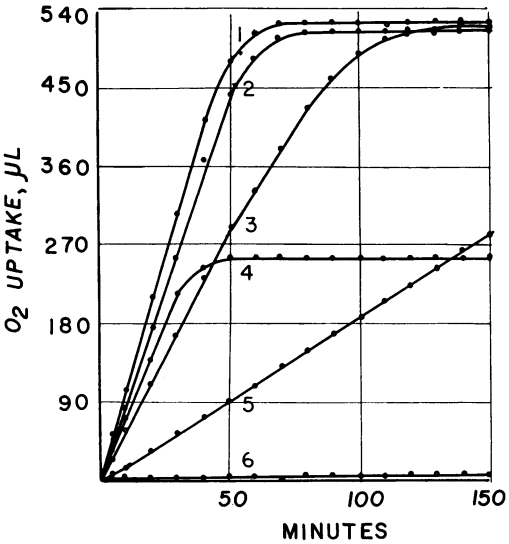

Figure 3. Oxidation of ferrous ion by washed cell suspensions of the iron-oxidizing bacterium. Each Warburg vessel contained 100 μmoles Fe^{+2}, water, and cell suspension to make 2 ml, except flask 4 which contained 50 μmoles Fe^{+2}. A cell suspension containing 0.43 mg cellular N per ml was added to the flasks as follows; flask *1*, 1 ml; flask *2*, 0.7 ml; flask *4*, 0.5 ml; flask *3*, 0.3 ml; flask *5*, 0.1 ml; and flask *6*, none. Endogenous activity with or without KOH in the center well was nil. Atmosphere, air; temperature, 30 C; pH 2.8 (with H_2SO_4).

TABLE 1

Rate of oxygen uptake by the iron-oxidizing bacterium

Storage[a]	Cellular N	Oxygen Uptake	$Q_{O_2}(N)$
days	*mg*	*$\mu LO_2/hr$*	
51	0.00[b]	0	
51	0.043[b]	120	2800
51	0.129[b]	350	2710
51	0.215[b]	484	2250
51	0.301[b]	588	1950
51	0.43[b]	642	1500
77	0.035		2770
63	0.115		2800
36	0.075		5100
36	0.105		4400

[a] Thoroughly washed cell suspensions in distilled water were stored in refrigerator at 4 to 6 C.

[b] Data from figure 3.

gen absorption which is very roughly proportional to the sulfur content of the flasks. The dependence of the rate of oxidation of sulfur on the initial sulfur content of the flasks may be more directly proportional than the data of figure 4 indicate because sulfur tends to clump and accumulates above the fluid level or in the side arm of the Warburg flasks. Therefore, the proportion of the added sulfur in active contact with cells may vary widely in different flasks.

Note that the rate of oxidation of sulfur in curve 1, containing 50 mg S, is somewhat greater than in either flasks 3 or 4, both containing over 50 mg S. A higher rate of oxygen uptake in flasks, containing both iron and sulfur and after ferrous iron depletion, than in flasks containing sulfur only has been observed several times. A marked increase is shown in table 2, experiment 5. The $Q_{O_2}(N)$ value for the flask with sulfur only was noted to be 160, whereas for the sulfur portion of the flask containing both iron and sulfur the value was found to be 320.

Somewhat higher $Q_{O_2}(N)$ values were obtained by use of cells grown in presence of both iron and sulfur. It was thought that the sulfur-oxidizing ability of cell suspension may have been due to growth of a contaminant sulfur-oxidizing bacterium whose density in the culture would be greatly increased by the presence of sulfur in the growth medium. The increased $Q_{O_2}(N)$ value of only about 25 per cent, less than that observed between other supposedly identical suspensions grown on iron only, does not support the idea that sulfur oxidation is due to a contaminant in the iron-oxidizing culture. Qualitative tests showed that sulfate is produced during sulfur oxidation.

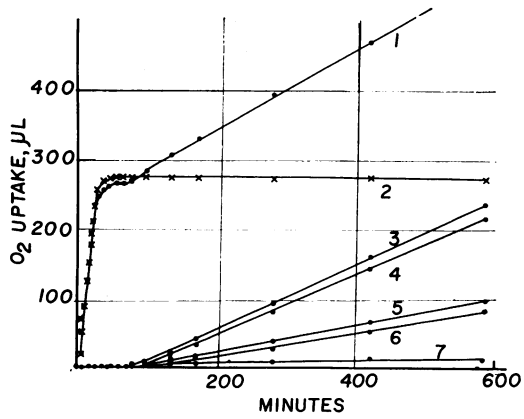

Figure 4. Oxidation of powdered sulfur by cell suspensions of the iron-oxidizing bacterium. Each Warburg vessel contained 0.5 ml of a cell suspension (0.37 mg cellular N per ml) of a 7-day culture grown on medium M with added sulfur and 0.01 N H_2SO_4 added to make total volume 2 ml. Sulfur or iron were added as follows; flask *1*, 50 µmoles Fe^{+2} and 50 mg S; flask *2*, 50 µmoles Fe^{+2}; flask *3*, 100 mg S; flask *4*, 57 mg S; flask *5*, 32 mg S; flask *6*, 16 mg S; and flask *7*, 4 mg S. Control flask with sulfur, but no cells, and flasks for endogenous activity showed no oxygen absorption in the 10-hr period. Atmosphere, air; temperature, 30 C; pH 2.8 (with H_2SO_4).

The uniform rate of oxygen uptake in the presence of sulfur always follows a lag period of no observable activity. The lag is slightly shortened by increased sulfur content but in no case has it been found to be less than 40 min, even using so-called sulfur-adapted cells grown in the presence of ferrous iron and sulfur. The data plotted on curve 1, figure 4, show that the lag period is not affected by the presence of ferrous iron in the Warburg flask, because following its depletion 10 min elapsed before oxygen uptake due to sulfur oxidation started. The latter coincided with onset of sulfur oxidation in flasks containing no ferrous iron. Under the conditions of these experiments sulfur oxidation by cell suspensions of *T. thiooxidans* also showed a lag period of 5 to 10 min followed by a gradually increasing rate, becoming constant after about 1 hr.

Cell suspensions of the iron-oxidizing bacterium

TABLE 2

Rate of oxygen uptake by cell suspensions of the iron-oxidizing bacterium and Thiobacillus thiooxidans

Organism	Ferrous-iron-oxidizing bacterium			*T. thiooxidans*		
Mg cellular N/ml	0.35			1.23		
Substrate	Fe^{+2}	S	$Na_2S_2O_3$	Fe^{+2}	S	$Na_2S_2O_3$
$Q_{O_2}(N)$	3800	250	35	0	270	14

Each Warburg vessel contained 100 µmoles Fe^{+2}, 100 mg sulfur or 10 µmole $Na_2S_2O_3$, cell suspension, and 0.01 N H_2SO_4 to 2 ml total volume; pH = 2.8.

$Q_{O_2}(N)$ values are the constant rate values following the lag period for sulfur and the high rate period for thiosulfate.

slowly oxidize low concentrations of $Na_2S_2O_3$. The oxygen uptake pattern on $Na_2S_2O_3$ was found to be almost identical for the iron oxidizer and for *T. thiooxidans*. There is an initial lag period of 5 to 10 min followed by a moderatly high rate of oxygen uptake to 0.2 to 0.3 μmole O_2 per μmole $Na_2S_2O_3$ which is followed by a prolonged period of constant slow rate. Table 2 summarizes the activity of cell suspension of the iron oxidizer and *T. thiooxidans* on ferrous iron, sulfur, and thiosulfate. Sulfur is oxidized by both organisms more rapidly than is thiosulfate.

Higher levels of $Na_2S_2O_3$ concentration inhibit the oxygen uptake by cell suspensions using as substrate $Na_2S_2O_3$ only or $Na_2S_2O_3$ plus ferrous ion. Thus at 0.005 M $Na_2S_2O_3$ the oxygen uptake in 110 min was 56 μL, whereas at 0.025 M the observed uptake was 5 μL. Also the latter concentration reduced the oxygen uptake with Fe^{+2} as substrate from 215 to 10 μL at 40 min. Vishniac (1952) showed a similar inhibition of *T. thioparus*.

The extent of CO_2 fixation has been measured in numerous experiments by introducing a solution of labeled $NaHC^{14}O_3$ or $Na_2C^{14}O_3$ into reaction mixtures containing ferrous iron or sulfur or both. The results of several typical experiments are shown in table 3. In every case CO_2 fixation has fallen in the range of 0.015 to 0.030 μmole CO_2 fixed per μmole O_2 absorbed, except, as noted above, for certain suspensions in the presence of both ferrous iron and sulfur. In the latter, higher fixation has been noted in several cases. The higher value has always been attributed to the sulfur portion of the oxidation. However, this may not be correct; the presence of sulfur may in some manner increase the efficiency of CO_2 fixation by the cells during Fe^{+2} oxidation. In cases of increased efficiency of CO_2 fixation

TABLE 3

Oxygen absorption and carbon dioxide fixation by cell suspensions of the iron-oxidizing bacterium

Expt. No.	Substrate		Oxygen Uptake			Carbon Dioxide Fixation		
	F^{+2}	S	Total	Fe^{+2} $Q_{O_2}(N)$	S $Q_{O_2}(N)$	C^{14} in cells	μMoles CO_2/μMole O_2[a]	
							Fe^{+2}	S
	μmoles	*mg*	*μL*			*c/m*		
1	20	0	91	2400		249	0.027	
	50	0	272	2500		746	0.026	
	100	0	504	2500		1804	0.029	
2	80	0	441	2600		500	0.026	
3	100	60	726	2400	145	2150	0.030[b]	0.015
4	80	50	630	3200	105	710	0.029[b]	0.026
5	100	0	518	4200		2100	0.021	
	100	100	700	4200	320	5400	0.021[b]	0.081
	0	100	104		160	570		0.025
	100	0	506	4000		75[c]	0.0005	
6	0	50	286		155	1200		0.021
7[d]	0	100	726		270	7280		0.047

[a] Specific activity of $C^{14}O_2$ used was 2500 c/m/μmole for experiments 1 and 3; 900 c/m/μmole for experiments 2 and 4; and 4700 c/m μmole for experiments 5, 6, and 7.

[b] Values are those of control flask with no sulfur.

[c] KOH in center well.

[d] *Thiobacillus thiooxidans* instead of the iron-oxidizing bacterium.

there has always been noted an increased rate of O_2 uptake with sulfur as the substrate.

The increased CO_2 fixation efficiency of cells in contact with both iron and sulfur is an interesting phenomenon which cannot be explained at this time. The rather wide range of CO_2 fixation efficiency may be due in part to experimental error, but seems to be more dependent on the variability of individual cell suspensions. It has been difficult to obtain sufficient cells to carry out several experiments with one preparation.

Adding KOH to the center well which presumably reduced the CO_2 level in the Warburg flask to very low levels had no effect on the rate or final level of iron oxidation. In one experiment (table 2, experiment 5) $NaHC^{14}O_3$ was added to a flask in which KOH was present in the center well. At the conclusion of the experiment cellular C^{14} was found to be 0.015 μmole compared with 0.45 μmole in cells in a control flask with no KOH in the center well. This shows conclusively that fixation of CO_2 is not associated in an obligatory manner with ferrous iron oxidation.

DISCUSSION

Although 2 species of iron-oxidizing bacteria recently have been added to *Bergey's Manual of Determinative Bacteriology* (Breed *et al.*, 1957), the bacterium described here has physiological properties differing significantly from those ascribed to either *Ferrobacillus ferrooxidans* or *Thiobacillus ferrooxidans*. The former, described by Leathen *et al.* (1956), grows well on ferrous ion, but does not oxidize thiosulfate. Leathen, in a personal communication, has stated that it does oxidize sulfur. *Thiobacillus ferrooxidans* is described by Temple and Colmer (1951) as being an iron-oxidizing bacterium with little or no ability to grow on or utilize sulfur, but with ability to grow using thiosulfate as the sole energy source.

The organism isolated from Bingham Canyon, Utah, grows well on ferrous iron or on sulfur. Its growth on acid thiosulfate media is very slow and perhaps nil; this point is difficult to decide because of turbidity due to formation of colloidal sulfur. Cell suspensions at pH 2.8 rapidly oxidize ferrous salts and oxidize sulfur, after an initial lag period, at a more rapid rate than thiosulfate.

Growing cultures and cell suspensions of the iron-oxidizing bacterium fix CO_2 into cellular material during the oxidation of both iron and sulfur. The efficiency of this process as measured by the ratio of μmoles CO_2 fixed to μmole oxygen uptake is rather constant and is in general agreement with values reported for other chemoautotrophic bacteria (Baalsrud, 1954). The range of efficiency during iron oxidation, which may be more accurately measured than that during sulfur oxidation, was found to be from 0.021 to 0.030 μmole CO_2 per μmole O_2. The concentration of cells used, the time of storage of the suspensions, and age of cultures at time of harvest varied considerably. The rather narrow range of efficiency is therefore quite surprising. The observed range of efficiency of CO_2 fixation during oxidation of sulfur is shown to be 0.015 to 0.025. On mixed iron-sulfur substrate the calculated efficiency frequently is higher rising to 0.081 μmole CO_2 per μmole O_2. The reasons for greater efficiency on a mixed substrate are not known.

Oxidation of ferrous ion or sulfur yields about the same carbon dioxide fixation. This indicates a single site of entry of electrons into the electron transport system of the bacterium regardless of the electron potential in the oxidizable substrate. This suggests the possibility of interesting studies on the electron transport system and on the yield of high energy compounds during oxidation of iron or sulfur by this bacterium.

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SUMMARY

An iron-oxidizing bacterium found in the acidic leaching water in Bingham Canyon, Utah, has been isolated by conventional methods. It grows on completely inorganic media at pH 2.0 to 3.0 obtaining its carbon from carbon dioxide and its energy by oxidation of either ferrous to ferric ion or sulfur to sulfate. Its utilization of thiosulfate, although detectable in manometric experiments, is slow compared to its action on sul-

fur. Morphologically it seems identical to other previously described autotrophic iron-oxidizing Pseudomonadales.

Cell suspensions of the bacterium oxidize ferrous ion rapidly with stoichiometric oxygen uptake. The $Q_{O_2}(N)$ values range from 2400 to 4200, becoming considerably less with increased cell density. The $Q_{O_2}(N)$ values for sulfur oxidation range from 120 to 320, being dependent on the amount of sulfur used and, interestingly, are usually increased by use of mixed ferrous ion and sulfur substrate. Endogenous activity as measured by oxygen uptake in air or CO_2-free atmosphere at 30 C in Warburg manometers was not detectable even after 10 hr, nor was fixation of CO_2 by cell suspensions in the absence of oxidizable substrate as measured by uptake of $C^{14}O_2$.

Carbon dioxide fixation by growing cultures or cell suspensions was determined by measuring cellular radioactivity after contact with $C^{14}O_2$. During growth about 1 μmole of CO_2 was fixed for each 100 μmoles of ferrous ion oxidized; whereas cell suspensions fixed about 1 μmole CO_2 for each 40 μmoles oxygen absorbed while oxidizing either ferrous iron or sulfur. With mixed ferrous iron-sulfur substrate the efficiency of carbon dioxide fixation was greatly increased to values as high as 1 μmole CO_2 for each 13 μmoles oxygen uptake.

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