APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1990, p. 1620–1626
Vol. 56, No. 6
0099-2240/90/061620-07$02.00/0
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Ferrous Iron and Sulfur Oxidation and Ferric Iron Reduction Activities of *Thiobacillus ferrooxidans* Are Affected by Growth on Ferrous Iron, Sulfur, or a Sulfide Ore

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Received 26 January 1990/Accepted 30 March 1990

Eight strains of *Thiobacillus ferrooxidans* (laboratory strains Tf-1 [= ATCC 13661] and Tf-2 [= ATCC 19859] and mine isolates SM-1, SM-2, SM-3, SM-4, SM-5, and SM-8) and three strains of *Thiobacillus thiooxidans* (laboratory strain Tt [= ATCC 8085] and mine isolates SM-6 and SM-7) were grown on ferrous iron (Fe^{2+}), elemental sulfur (S^0), or sulfide ore (Fe, Cu, and Zn). The cells were studied for their aerobic Fe^{2+}- and S^0-oxidizing activities (O_2 consumption) and anaerobic S^0-oxidizing activity with ferric iron (Fe^{3+}) (Fe^{2+} formation). Fe^{2+}-grown *T. ferrooxidans* cells oxidized S^0 aerobically at a rate of 2 to 4% of the Fe^{2+} oxidation rate. The rate of anaerobic S^0 oxidation with Fe^{3+} was equal to the aerobic oxidation rate in SM-1, SM-3, SM-4, and SM-5, but was only one-half or less that in Tf-1, Tf-2, SM-2, and SM-8. Transition from growth on Fe^{2+} to that on S^0 produced cells with relatively undiminished Fe^{2+} oxidation activities and increased S^0 oxidation (both aerobic and anaerobic) activities in Tf-2, SM-4, and SM-5, whereas it produced cells with dramatically reduced Fe^{2+} oxidation and anaerobic S^0 oxidation activities in Tf-1, SM-1, SM-2, SM-3, and SM-8. Growth on ore 1 of metal-leaching Fe^{2+}-grown strains and on ore 2 of all Fe^{2+}-grown strains resulted in very high yields of cells with high Fe^{2+} and S^0 oxidation (both aerobic and anaerobic) activities with similar ratios of various activities. Sulfur-grown Tf-2, SM-1, SM-4, SM-6, SM-7, and SM-8 cultures leached metals from ore 3, and Tf-2 and SM-4 cells recovered showed activity ratios similar to those of other ore-grown cells. It is concluded that all the *T. ferrooxidans* strains studied have the ability to produce cells with Fe^{2+} and S^0 oxidation and Fe^{3+} reduction activities, but their levels are influenced by growth substrates and strain differences.

Bacterial leaching of metals from sulfide ores by *Thiobacillus ferrooxidans* involves not only ferrous iron oxidation to ferric iron by the organism but also the oxidation of sulfide or the sulfur portion of sulfide minerals to sulfuric acid (6, 13, 16, 20, 36, 37). The latter reaction can be achieved also by *Thiobacillus thiooxidans*, a closely related organism. The metabolism of Fe^{2+} and sulfide or sulfur is interrelated through the activity of sulfur (sulfide):Fe^{3+} oxidoreductase (28, 30). Fe^{2+} is oxidized by *T. ferrooxidans* with molecular oxygen ($4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O$) by a mechanism involving Fe^{2+}-cytochrome *c* oxidoreductase, cytochrome *c*, rusticyanin, and cytochrome oxidase (3, 4, 8, 11, 12, 23). Sulfur is oxidized by *T. thiooxidans* and *T. ferrooxidans* with O_2 ($S^0 + 1\frac{1}{2}O_2 + H_2O \rightarrow H_2SO_4$). The enzymes involved for both *T. thiooxidans* and *T. ferrooxidans* are the sulfur-oxidizing enzyme ($S^0 + O_2 + H_2O \rightarrow H_2SO_3$, with reduced glutathione as cofactor) (25, 33, 34) and sulfite oxidase ($H_2SO_3 + \frac{1}{2}O_2 \rightarrow H_2SO_4$); for *T. ferrooxidans*, the enzymes are sulfur (sulfide):Fe^{3+} oxidoreductase ($S^0 + 4Fe^{3+} + 3H_2O \rightarrow H_2SO_3 + 4Fe^{2+} + 4H^+$) (28, 30) and sulfite: Fe^{3+} oxidoreductase ($H_2SO_3 + 2Fe^{3+} + H_2O \rightarrow H_2SO_4 + 2Fe^{2+} + 2H^+$) (29). In the latter organism, the Fe^{2+} formed is oxidized to Fe^{3+} with O_2, with the identical stoichiometry for the aerobic oxidation of sulfur as that for *T. thiooxidans*.

T. ferrooxidans is known for its ability to adapt to different environmental conditions, such as growth substrates and heavy metal concentrations. There are conflicting reports, however, concerning the effect of Fe^{2+} or S^0 as a growth substrate on the level of Fe^{2+} or S^0 oxidation activity of *T. ferrooxidans* cells (7, 14, 15, 21, 31). Recent genetic studies shed some light on possible explanations for these results. *T. ferrooxidans* has a remarkable ability to produce spontaneous phenotypic variants presumably by the transposition of mobile repeated DNA sequences (10, 24). These variants do not oxidize Fe^{2+} but revert at high frequency to the parental phenotype and regain the ability to oxidize Fe^{2+}. This type of mechanism could provide a foundation for understanding the process of adaptation and strain variations in *T. ferrooxidans* and possible selection of ideal strains for bacterial leaching.

We carried out a systematic study of the sulfur-oxidizing systems of various *T. ferrooxidans* and *T. thiooxidans* strains, both laboratory and recent mine isolates, to find out the activity levels of O_2-coupled and Fe^{3+}-coupled oxidation of sulfur as well as Fe^{2+} oxidation in cells grown on different substrates (S^0, Fe^{2+}, or sulfide ore). This is the first comprehensive study involving such a large number of strains, three different substrates, and three different activities, including the recently discovered Fe^{3+}-coupled oxidation of S^0 (27, 30). The aim was to see whether different strains respond to different substrates in quantitatively different manners, changing the respective activities during their adaptation. Our results indicate the existence of variability among different strains not only in their ability to grow on certain substrates but also in their response to different substrates by changing Fe^{2+} and S^0 oxidative activities. This variability in adaptation is in agreement with the possible mechanism described previously (10, 24).

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TABLE 1. Ferrous iron and sulfur oxidation activities of Fe^{2+}- and S^0-grown cells of the first group of *T. ferrooxidans* strains and of *T. thiooxidans* and SM-2 strains[a]

Strain (growth medium)	Growth substrate (no. of transfers)	Final pH of growth medium	Cell yield (mg of protein/liter)	Activity of cells					
				Aerobic oxidation (nmol of O_2/min per mg of protein)			Reduction of Fe^{3+} (nmol of Fe^{2+}/min per mg of protein) with S^0 (treatment IV)	Activity ratio	
				S^0 (treatment I)	S^0 (treatment II)	Fe^{2+} (treatment III)		Treatment III/treatment II	Treatment IV/treatment II
Tf-2 (Fe^{2+})	Fe^{2+}	ND	2.5	28	20	1,200	27	60	1.4
	S^0 (1)	ND	ND	280	45	1,919	ND	43	ND
	S^0 (2)	1.7	8.2	180	30	1,199	118	40	3.9
	S^0 (3)	1.5	10.2	14	25	55	9	2.2	0.4
	S^0 (4)	1.9	ND	142	29	1,280	166	44	5.7
SM-4 (Fe^{2+})	Fe^{2+}	2.5	2.0	29	28	1,251	122	45	4.4
	S^0 (1)	1.9	ND	248	63	1,308	214	21	3.4
	S^0 (2)	1.6	ND	72	36	720	52	20	1.4
	S^0 (4)	1.8	34.7	76	56	1,174	174	21	3.1
	S^0 (8)	1.9	23.4	119	54	373	132	6.9	2.4
	S^0 (11)	1.6	7.0	160	69	426	172	6.2	2.5
SM-4 (S^0, 11 transfers)	Fe^{2+} (1)	1.8	10.1	32	53	827	130	16	2.5
	Fe^{2+} (3)	2.3	3.9	40	71	1,118	210	16	3.0
	Fe^{2+} (5)	2.5	7.1	20	70	647	106	9.2	1.5
SM-5 (Fe^{2+})	Fe^{2+}	ND	10.8	27	32	1,514	176	47	5.5
	S^0 (1)	1.8	5.9	13	33	657	77	20	2.3
	S^0 (2)	1.7	12.9	38	35	152	33	4.3	0.9
	S^0 (3)	1.9	5.3	132	28	415	133	15	4.8
	S^0 (5)	2.0	5.0	163	52	888	233	17	4.5
Tt (S^0)	S^0 (2)	1.4	ND	184	36	0	0	0	0
SM-6 (S^0)	S^0 (2)	1.7	ND	119	87	0	0	0	0
SM-7 (S^0)	S^0 (2)	1.8	ND	139	70	0	0	0	0
SM-2 (Fe^{2+})	Fe^{2+}	ND	1.9	15	24	1,365	41	57	1.7

[a] Growth of the organism and activity determinations were as described in Materials and Methods. Strains were maintained on Fe^{2+} or S^0, as indicated. Growth substrate and the number of transfers were as shown, for example, SM-4 strain maintained on Fe^{2+} was grown once on Fe^{2+}, 11 times on S^0, and then 5 times on Fe^{2+} again. ND, Not determined.

MATERIALS AND METHODS

Organisms. Isolates from a sulfide ore mine site (16) and laboratory strains were used. Eight strains of *T. ferrooxidans* (laboratory stains Tf-1 [= ATCC 13661] and Tf-2 [= ATCC 19859] and mine isolates SM-1, SM-2, SM-3, SM-4, SM-5, and SM-8) and three strains of *T. thiooxidans* (laboratory strain Tt [= ATCC 8085] and mine isolates SM-6 and SM-7) were maintained either on Fe^{2+} (*T. ferrooxidans*) or elemental sulfur, S^0 (*T. ferrooxidans* and *T. thiooxidans*), as stock cultures. From time to time, cultures were plated out in solid media (9, 24) to check for purity. *T. ferrooxidans* strains used for S^0 stock cultures were initially grown on Fe^{2+} and were isolated as single colonies on solid Fe^{2+} medium (9). Each isolated culture was then adapted to growth on sulfur as described previously (35). SM-2 did not grow on S^0.

Media and growth of bacteria. The medium used for the maintenance of Fe^{2+}-grown strains was HP medium (amounts per liter) [0.4 g of $(NH_4)_2SO_4$, 0.1 g of K_2HPO_4, 0.4 g of $MgSO_4 \cdot 7H_2O$; adjusted to pH 2.3 with H_2SO_4] containing 33.3 g of $FeSO_4 \cdot 7H_2O$ (pH 2.3; filter sterilized) (22). Shake flask cultures (100 ml) with 10% inoculum (vol/vol) were grown in 250-ml Erlenmeyer flasks at 25°C on a rotary shaker at 150 rpm normally for 2 days.

The medium used for the maintenance of S^0-grown strains was Starkey medium (amounts per liter) [0.3 g of $(NH_4)_2SO_4$, 3.5 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.25 g of $CaCl_2$, 18 mg of $FeSO_4 \cdot 7H_2O$] and 50 g of elemental sulfur powder (sulfur precipitated; BDH Chemicals, Toronto, Ontario, Canada) spread evenly on the surface after inoculation (33). Stationary cultures of 1 liter in 2.8-liter Fernbach flasks or 200 ml in 500-ml Erlenmeyer flasks with 2.5% inoculum (vol/vol) were grown at 25°C without agitation.

The growth medium used for the activity experiments of Fe^{2+}- or S^0-grown cells was essentially the 9K medium (26) as modified by Sugio et al. (30, 32) (amounts per liter) [3 g of $(NH_4)_2SO_4$, 0.1 g of KCl, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 14 mg of $Ca(NO_3)_2 \cdot 4H_2O$] containing 33.3 g of $FeSO_4 \cdot 7H_2O$ (for Fe^{2+} growth) or 100 g of elemental sulfur powder (precipitated sulfur; J. T. Baker Chemical Co., Phillipsburg, N.J.) plus 0.2 g of $Fe_2(SO_4)_3$ (for S^0 growth) per liter. The pH of the medium was adjusted with H_2SO_4 to 2.3 for the Fe^{2+} medium or to 2.5 for the S^0 medium. The medium used for ore-leaching or ore growth

TABLE 2. Ferrous iron and sulfur oxidation activities of Fe^{2+}- and S^0-grown cells of the second group of *T. ferrooxidans* strains[a]

Strain (growth medium)	Growth substrate (no. of transfers)	Final pH of growth medium	Cell yield (mg of protein/ liter)	Activity of cells					
				Aerobic oxidation (nmol of O_2/min per mg of protein)			Reduction of Fe^{3+} (nmol of Fe^{2+}/min per mg of protein) with S^0 (treatment IV)	Activity ratio	
				S^0 (treatment I)	S^0 (treatment II)	Fe^{2+} (treatment III)		Treatment III/ treatment II	Treatment IV/ treatment II
Tf-2 (Fe^{2+})	Fe^{2+}	ND	7.6	39	51	1,380	50	27	1.0
	S^0 (1)	ND	ND	187	76	833	ND	11	ND
	S^0 (2)	1.7	12.4	16	30	23	5	0.8	0.2
	S^0 (3)	1.7	17.0	41	31	12	5	0.4	0.2
	S^0 (4)	1.9	ND	29	22	25	6	1.1	0.3
SM-1 (Fe^{2+})	Fe^{2+}	ND	7.6	17	45	1,890	135	42	3.0
	S^0 (1)	1.6	29.0	66	41	359	78	8.8	1.9
	S^0 (2)	1.5	22.4	65	34	124	34	3.6	1.0
	S^0 (3)	1.8	ND	47	35	83	20	2.4	0.6
SM-3 (Fe^{2+})	Fe^{2+}	ND	4.8	8	20	658	79	33	4.0
	S^0 (1)	1.8	15.9	59	24	171	10	7.1	0.4
	S^0 (2)	1.8	21.9	96	36	42	3	1.2	0.1
	S^0 (4)	1.7	24.1	38	42	31	3	0.7	0.1
	S^0 (6)	1.8	25.4	49	45	26	1	0.6	0.0
SM-8 (Fe^{2+})	Fe^{2+}	ND	1.4	40	30	1,056	56	35	1.9
	S^0 (1)	1.7	ND	152	55	103	47	1.9	0.9
	S^0 (2)	1.5	ND	65	23	41	14	1.8	0.6
	S^0 (4)	1.7	52.4	65	41	25	7	0.6	0.2
	S^0 (8)	1.8	29.0	51	43	40	0	0.9	0.0
	S^0 (11)	ND	30.4	42	38	20	1	0.5	0.0

[a] Conditions were the same as in Table 1. ND, Not determined.

experiments was HP medium at pH 2.3 without $FeSO_4$ but with 10 g of ore in 100 ml. Normally, shake flask cultures (100 ml) with 10% inoculum (vol/vol) were grown in 250-ml Erlenmeyer flasks at 25°C (28°C for S^0 growth) at 150 rpm.

Strains adapted to Cu or ore used in ore-leaching or growth experiments were grown and maintained in the presence of 50 mM $CuSO_4$ or 10 g of ore (instead of Fe^{2+} or S^0) per 100 ml after adaptation (35). They were grown without Cu or ore to obtain the inoculum for the experiments.

Collection of cells. All cultures were first filtered through Whatman no. 1 filter paper under suction before centrifugation at 12,000 × *g*. The cells were washed three times with 0.1 M β-alanine–H_2SO_4 (β-alanine buffer), pH 3.0, and were suspended in the same buffer at a concentration of 50 mg of wet cells per ml for the determination of activities.

Determination of activities. (Treatment I) Aerobic S^0 oxidation. The rate of S^0 oxidation was determined by measuring the rate of O_2 consumption polarographically in a Gilson Oxygraph with a Clark electrode and a magnetic stirrer at 25°C. The reaction mixture contained either 75 μl (Fe^{2+}- or ore-grown cells) or 300 μl (S^0-grown cells) of elemental sulfur suspension (320 mg of precipitated sulfur, low in Fe [BDH], per ml of 500-ppm [500-μg/ml] Tween 80), 50 μl of cell suspension, and β-alanine buffer in a total volume of 1.2 ml. The oxidation was completed after a short duration of less than 30 min because of the exhaustion of dissolved O_2 (0.26 mM).

(Treatment II) Aerobic S^0 oxidation. S^0 oxidation rate was determined in a Warburg apparatus (Braun, Germany) by using the standard manometric technique for the disappearance of O_2 (39) at 30°C. The reaction mixture in a total volume of 3.2 ml contained 0.2 ml of sulfur suspension, 0.167 ml of cell suspension, and β-alanine buffer. The reaction mixture was shaken reciprocally in an atmosphere of air so that it remained saturated with O_2 and the oxidation could be followed over a long period of time (2 to 5 h).

(Treatment III) Aerobic Fe^{2+} oxidation. The Fe^{2+} oxidation rate was determined by the O_2 consumption rate in a Gilson Oxygraph at 25°C. The reaction mixture in a total volume of 1.2 ml contained 50 μl of 0.1 M $FeSO_4 \cdot 7H_2O$ (pH 2.3), 10 μl of cell suspension, and β-alanine buffer.

(Treatment IV) Anaerobic reduction of Fe^{3+} with S^0. The anaerobic oxidation rate of S^0 with Fe^{3+} was determined by following the rate of Fe^{2+} production in Warburg flasks under O_2-free N_2 gas at 30°C by a modification of the method of Sugio et al. (27). The reaction mixture with a total volume of 3.33 ml contained 0.2 ml of sulfur suspension, 0.167 ml of 50 mM $Fe_2(SO_4)_3$, 0.167 ml of cell suspension, and β-alanine buffer. The reaction was started by tipping the cells from a side arm, and the flow of N_2 was maintained throughout the experiment. Samples (25 μl) were taken at time intervals with microsyringes through serum stoppers capped on the side arms of flasks and were injected into 1 ml of 0.1% *o*-phenanthroline. β-Alanine buffer (0.975 ml) and 3 ml of H_2O (pH 3.0 with H_2SO_4) were added, and after 10 min the red color due to Fe^{2+} (2) was measured in a Klett-Summerson photoelectric colorimeter with a green (no. 54) filter.

Determination of protein. Protein concentration in cell suspensions was determined by a modification of the method of Lowry et al. (19) by using bovine serum albumin as the standard. A sample (0.1 ml) was mixed with 0.1 M NaOH (0.9 ml) and was boiled for 10 min. After centrifugation at 12,000 × *g* for 10 min, 0.8 ml of the supernatant was mixed with 4 ml of reagent D (50 ml of 2% Na_2CO_3 with 1 ml of 0.5% $CuSO_4 \cdot 5H_2O$ in 1% potassium tartrate). After 10 min, 0.4 ml of phenol reagent (1 N) was added and mixed. The

TABLE 3. Ferrous iron and sulfur oxidation activities of ore 1-grown cells[a]

Strain (adaptation)	Final pH of growth medium	Cell yield (mg of protein/liter)	Activity of cells					
			Aerobic oxidation (nmol of O_2/min per mg of protein)			Reduction of Fe^{3+} (nmol of Fe^{2+}/min per mg of protein with S^0 [treatment IV])	Activity ratio	
			S^0 (treatment I)	S^0 (treatment II)	Fe^{2+} (treatment III)		Treatment III/ treatment II	Treatment IV/ treatment II
SM-1	2.5	41	130	49	841	167	17	3.4
SM-1 (Cu)	3.2	22	127	69	778	103	11	1.5
SM-1 (ore)	2.3	418	79	47	724	77	15	1.6
SM-2	2.2	42	40	29	134	ND	5	ND
SM-4	2.1	155	107	79	623	59	8	0.7
SM-4 (Cu)	1.9	4	185	ND	692	65	ND	ND
SM-5	2.9	114	26	25	214	32	9	1.3
SM-5 (Cu)	2.2	103	85	50	388	45	8	0.9
SM-5 (ore)	2.4	96	19	20	185	20	9	1.0
SM-8	2.2	258	68	52	449	74	9	1.4
SM-8 (Cu)	2.3	189	83	54	829	87	15	1.6
SM-8 (ore)	2.2	183	78	63	703	85	11	1.3

[a] Shake flask growth was on 10 g of ore 1 in 100 ml of HP medium for 21 days at 25°C with 10% inoculum of Fe^{2+}-grown bacterial strains. Adaptation to Cu or ore was carried out as described in Materials and Methods. ND, Not determined.

color was determined after 30 min in a Klett-Summerson photoelectric colorimeter with a no. 66 (red) filter.

Ore samples. Three sulfide ore samples (−200 mesh) were obtained from the Hudson Bay Mining and Smelting Co. Ltd., Flin Flon, Manitoba, Canada. Ore 1 (16) contained 4.9% Cu, 12.5% Zn, 30% Fe, and 37.5% S. Ore 2 contained 3.7% Cu, 10.2% Zn, 29% Fe, and 34.6% S. Ore 3 contained 3.1% Cu, 9.1% Zn, 33.4% Fe, and 37.8% S. Major minerals present in those samples were pyrite, chalcopyrite, and sphalerite, with small amounts of carbonates and pyrrhotite.

TABLE 4. Ferrous iron and sulfur oxidation activities of ore 2-grown cells[a]

Strain (adaptation)	Final pH of growth medium	Cell yield (mg of protein/ liter)	Activity of cells					
			Aerobic oxidation (nmol of O_2/min per mg of protein)			Reduction of Fe^{3+} (nmol of Fe^{2+}/min per mg of protein with S^0 [treatment IV])	Activity ratio	
			S^0 (treatment I)	S^0 (treatment II)	Fe^{2+} (treatment III)		Treatment III/ treatment II	Treatment IV/ treatment II
Tf-1 (Cu)	2.3	125	90	53	758	111	14	2.1
Tf-2	2.9	45	38	31	92	41	3	1.3
Tf-2 (Cu)	2.3	56	151	55	502	110	9	2.0
Tf-2 (Cu, ore)	2.3	87	84	51	474	86	9	1.7
SM-1	2.4	145	76	60	470	112	8	1.9
SM-1 (Cu)	2.4	49	125	78	924	87	12	1.1
SM-1 (ore)	2.4	272	62	45	553	46	12	1.0
SM-2	2.3	90	43	27	378	31	14	1.1
SM-3	2.7	47	88	49	703	131	14	2.7
SM-3 (ore)	2.4	234	76	ND	571	16	ND	ND
SM-4	2.4	212	72	42	595	48	14	1.1
SM-4 (Cu)	2.3	124	196	ND	933	104	ND	ND
SM-4 (ore)	2.2	207	88	79	989	110	13	1.4
SM-5	2.2	103	168	77	1,330	125	17	1.6
SM-5 (Cu)	2.3	133	93	50	825	84	17	1.7
SM-5 (ore)	2.5	185	38	43	357	62	8	1.4
SM-8	2.2	103	168	77	1,330	125	17	1.6
SM-8 (Cu)	2.4	189	63	ND	583	44	ND	ND
SM-8 (ore)	2.4	204	52	58	645	57	11	1.0

[a] Conditions were the same as in Table 3, except that ore 2 was used instead of ore 1. ND, Not determined.

TABLE 5. Shake flask leaching of ore 3 by sulfur-grown cells[a]

Strain	Adaptation	Final pH of growth medium	% Metal extraction		
			Cu	Zn	Fe
None		5.2	0	2	0
Tt		4.9[b]	0	2	0
Tf-1		4.8[b]	0	15	0
Tf-2		2.1	18	64	5
Tf-2	Ore	1.4	23	106	25
SM-1		4.8	0	22	0
SM-1	Ore	1.3	24	114	31
SM-3		4.4[b]	0	13	0
SM-4		4.7[b]	0	23	0
SM-4	Cu	2.1	6	25	3
SM-4	Cu, ore	1.8	24	79	6
SM-5		4.8[b]	3	21	0
SM-6		2.1	6	37	3
SM-6	Ore	1.3	24	114	31
SM-7		2.2	12	43	3
SM-7	Ore	1.3	25	120	3
SM-8		2.0	13	49	4
SM-8	Ore	1.3	23	120	32

[a] Shake flask leaching of 10 g of ore 3 (3.1% Cu, 9.1% Zn, 33.4% Fe) in 100 ml of HP medium was for 24 days at 25°C with 10% inoculum of sulfur-grown stationary cultures on Starkey no. 1 medium. Adaptation to Cu or ore was carried out as described in Materials and Methods.

[b] No adaptation to ore 3; cells were dead after the experiment (no growth on sulfur).

Shake flask leaching or growth experiments with 10 g of ore in 100 ml of HP medium required no H_2SO_4 addition with ore 1 but required 250 μl of 10 N H_2SO_4 initially and 25 μl after 1 day with ore 2 and 400 μl of 10 N H_2SO_4 initially with ore 3.

RESULTS

Fe^{2+}-grown cells. The Fe^{2+}-grown cells all showed very high Fe^{2+} oxidation activities (658 to 1,890 nmol of O_2/min per mg of protein) and relatively low sulfur oxidation activities (8 to 40 nmol of O_2/min per mg of protein [treatment I] and 20 to 51 nmol of O_2/min per mg of protein [treatment II]) (Tables 1 and 2). The sulfur oxidation activity (treatment II) was only 2 to 4% of the Fe^{2+} oxidation activity (treatment III). The anaerobic sulfur oxidation activity with Fe^{3+} (treatment IV) was close to the aerobic activity with O_2 (treatment II) in some strains (SM-1, SM-3, SM-4, and SM-5), whereas it was only one-half to one-fourth the aerobic activity in others (Tf-1, Tf-2, SM-2, and SM-8).

Transition from Fe^{2+} to sulfur growth. When the Fe^{2+}-grown cells were transferred to the sulfur medium, all of the *T. ferrooxidans* cultures except SM-2 grew and the cells collected showed characteristic changes in oxidation activities. The first group of organisms, Tf-2, SM-4, and SM-5, maintained Fe^{2+} oxidation activities at relatively high levels, even after growth in sulfur medium with several transfers, except when the pH was allowed to decrease below 1.7 (Table 1). A similar loss of Fe^{2+} oxidation and Fe^{3+} reduction activities at low pH values was reported by Sugio et al. (32). Sulfur oxidation activities increased upon transition from Fe^{2+} to sulfur, often dramatically, in treatments I and IV except at very low final pHs. The increase in treatment II was more moderate. Aerobic sulfur oxidation activities (treatments I and II) reached the levels of *T. thiooxidans* (Tt, SM-6, and SM-7) grown under the same conditions (Table 1). The activity ratio for Fe^{2+} to S^0 (treatment III to treatment II) remained high, and the ratio for anaerobic to aerobic activities (treatment IV to treatment II) approached the theoretical value of 4 in many cases. *T. thiooxidans* strains did not oxidize Fe^{2+} and showed no anaerobic reduction of Fe^{3+} to Fe^{2+} with S^0. The return to the Fe^{2+} medium from the S^0 medium (SM-4) tended to decrease S^0 (treatment I) and increase Fe^{2+} (treatment III) activities to the original levels of Fe^{2+}-grown cells.

Upon transfer to the sulfur medium from the Fe^{2+} medium, the second group of organisms (Tf-1, SM-1, SM-3, and SM-8) produced cells with dramatic decreases in the Fe^{2+} oxidation (treatment III) and anaerobic Fe^{3+}-coupled S^0 oxidation (treatment IV) activities (Table 2). The decreases were progressive with the number of transfers in the S^0 medium. Aerobic S^0 oxidation activities (treatments I and II) often increased, but the increases were less pronounced than in the first group. The activity ratio for Fe^{2+} to S^0 (treatment III to treatment II) decreased dramatically upon repeated transfers in the S^0 medium, and the ratio for anaerobic to aerobic activities (treatment IV to treatment II) showed a similar decrease. Thus, the second group of strains was quite distinct from the first group in its response to growth on sulfur, although both groups of organisms grew well on sulfur (except SM-2).

In both groups, the cell yields were higher with S^0 as the growth substrate than with Fe^{2+} (Tables 1 and 2), with the possible exception of SM-5, although the growth time was longer (4 to 6 days for S^0 growth and 1 to 2 days for Fe^{2+} growth). Thus, all the activities per liter of culture increased in the first group, while only some did in the second group.

Transition from Fe^{2+} to ore growth. As reported previously (16), the Fe^{2+}-grown cells of SM-1, SM-2, SM-4, and SM-5 leached Cu and Zn efficiently from the complex sulfide ore 1. These results were confirmed in the present study, and, in addition, the Fe^{2+}-grown SM-8 cells were found to be effective in metal leaching also. These cells were in fact growing on the ore as the growth substrate, and large quantities of cells were recovered from the culture filtrates. These cells often had the high sulfur oxidation activities

TABLE 6. Ferrous iron and sulfur oxidation activities of ore 3-grown cells

Strain	Final pH of growth medium	Cell yield (mg of protein/liter)	Activity of cells					
			Aerobic oxidation (nmol of O_2/min per mg of protein)			Reduction of Fe^{3+} (nmol of Fe^{2+}/min per mg of protein with S^0 [treatment IV])	Activity ratio	
			S^0 (treatment I)	S^0 (treatment II)	Fe^{2+} (treatment III)		Treatment III/ treatment II	Treatment IV/ treatment II
Tf-2	1.4	41	24	20	243	31	12	1.6
SM-4	1.5	89	39	28	308	63	11	2.3

[a] Both of the S^0-grown Tf-2 and SM-4 stock cultures were grown on ore six times and were regrown on S^0 as inoculum for the ore medium. The growth conditions were the same as in Table 5.

(treatments I and II) of sulfur-grown cells and Fe^{2+} oxidation activities only slightly lower than those of Fe^{2+}-grown cells (except SM-2) (Table 3). The cell yield was sometimes over 100 times that of Fe^{2+}-grown cultures, although the incubation period was much longer.

Since the supply of ore 1 was limited, we used ore 2 with a similar composition for further experiments. In a previous work (17), both samples behaved similarly in column leaching experiments. The powdered samples, however, responded differently in shake flask leaching experiments. Ore 2 required the addition of H_2SO_4 to maintain an acidic pH for the first day or so. The metal-leaching rates were very low and only marginally increased with Cu- or ore-adapted cultures. Thus, the rates were 0 to 2% Cu, 3 to 7% Zn, and 0 to 5% Fe above those of the uninoculated control after 21 days compared with 7 to 10% Cu, 59 to 67% Zn, and 10 to 22% Fe from ore 1 by effective strains in 18 days (16). Surprisingly, all strains grew well on ore 2, although some grew better after Cu or ore adaptation, while only the metal-leaching strains grew on ore 1. Table 4 shows a high growth yield of cells ranging from 45 to 272 mg of protein per liter with high sulfur- and Fe^{2+}-oxidizing activities. Thus, it appears that these cells grew on ore 2, oxidizing Fe^{2+} and sulfide or sulfur without solubilizing Cu, Zn, and Fe, while the metal-leaching strains grew on ore 1, oxidizing Fe^{2+} and sulfide or sulfur and solubilizing these metals.

Transition from sulfur to ore growth. Sulfur-grown SM-6 and SM-7 strains leached ore 1 effectively (16). The sulfur-grown *T. thiooxidans* and *T. ferrooxidans* strains were studied for their ability to leach metals from ore 3, which was very similar to the ore 1 sample except for the initial H_2SO_4 addition requirement. In addition to SM-6 and SM-7, Tf-2, SM-1 (after adaptation to ore), SM-4 (after adaptation to Cu), and SM-8 effectively leached metals from ore 3 (Table 5). Adaptation to ore improved metal extraction rates. These six strains obviously grew on ore 3 and were subcultured on the ore six times without losing leaching ability. The cells were, however, tightly bound to the ore particles and were difficult to obtain free of ore. Tf-2 and SM-4 cells successfully collected had reasonable levels of S^0 and Fe^{2+} oxidation activities considering the low pH attained (Table 6).

DISCUSSION

T. ferrooxidans is an extremely versatile organism capable of growth on ferrous iron, elemental sulfur, thiosulfate, and sulfide minerals by using carbon dioxide and inorganic compounds for the biosynthesis of cell materials (6, 13, 16, 20, 36–38). Transition from growth on one substrate to that on another is not always easy and often requires adaptation. The process may involve the mobile repeated DNA sequences as proposed by Holmes et al. (10, 24). Different strains adapt differently. Some strains grow on Fe^{2+} but not on sulfur (9). Among our isolates, SM-2 seems to be such an example. There has been a considerable amount of work on the levels of Fe^{2+} oxidation and S^0 oxidation activities when *T. ferrooxidans* is grown on Fe^{2+} or S^0, with conflicting results (7, 14, 15, 21, 31). Our data (Tables 1 and 2) clearly show that different strains can produce entirely different results not only in terms of aerobic oxidation activities with Fe^{2+} or S^0 but also anaerobic S^0 oxidation activities with Fe^{3+}. Thus, the first group of strains maintained Fe^{2+} oxidation activity (treatment III) when transferred from the Fe^{2+} to the S^0 growth medium, while the second group of strains lost it. Anaerobic S^0 oxidation activity with Fe^{3+} (treatment IV) increased in the first group but decreased in the second group. Thus, the physiological activities of the second group after growth on sulfur approached those of *T. thiooxidans*, which had none of these activities.

The anaerobic S^0 oxidation rate with Fe^{3+} as electron acceptor (treatment IV) was often equal to the aerobic rate (treatment II) in Fe^{2+}-grown *T. ferrooxidans* cells (treatment IV to treatment II = 4.0) in agreement with Corbett and Ingledew (5). Upon transition to growth on S^0, the first group of strains often maintained a high treatment-IV-to-treatment-II ratio, while the second group of strains lost anaerobic activity, with the treatment-IV-to-treatment-II ratio approaching zero with repeated transfers on the S^0 medium. According to data of Sugio et al. (31), the Fe^{3+} reduction rate with S^0 (aerobic, with 5 mM KCN)/the O_2 consumption rate with S^0 was 0.5 with the Fe^{2+}-grown cells and 0.4 with the S^0-grown cells.

Ore-grown cells were often difficult to dissociate from ore particles, requiring many centrifugation steps. Results were obtained with free cells not tightly adsorbed on ore particles; adsorbed cells might have shown different activities. Surprisingly, the cell yield was very high, and the activities were also generally high in all four categories in all strains which grew on a particular ore sample. Thus, all the activities per liter of culture were much higher than those found in Fe^{2+}- or S^0-grown cultures, although the growth period was much longer. Fe^{2+} oxidation activity (treatment III) was either equal to or only slightly below the level in the Fe^{2+}-grown cells, and aerobic S^0 oxidation activities (treatments I and II) were as high as those of S^0-grown cells. Anaerobic S^0 oxidation activities with Fe^{3+} were also as high as those of Fe^{2+}-grown cells. The activity ratio for Fe^{2+}-to-S^0 oxidation (treatment IV to treatment II) centered around 10, and the ratio anaerobic to aerobic S^0 oxidation (treatment IV to treatment II) was around 1 to 2 in general. Thus, the difference between the group one strains and group two strains observed in the transition from the Fe^{2+} to S^0 growth was not observed in the transition to ore growth. It is interesting that growth on an ore seems to dictate the level and ratio of these activities in *T. ferrooxidans*. This may be related to the fact that sulfide minerals are the major natural substrates for this organism, and growth on Fe^{2+} or S^0 in the laboratory, although convenient and necessary, places certain strains and restrictions on cell metabolism. In this regard, the ability of *T. ferrooxidans* resting cells to reduce Fe^{3+} with pyrite anaerobically (18) suggests a possible role of the sulfur (sulfide):Fe^{3+} oxidoreductase (treatment IV) in the solubilization of metals from sulfide ores, in agreement with the concept of sulfides or polysulfides being the substrate for oxidation by Fe^{3+} (1, 28).

ACKNOWLEDGMENTS

We thank the Natural Sciences and Engineering Research Council of Canada for grants and Canada Centre for Mineral & Energy Technology for contract 23440-6-9126.

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