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Oxidation of Ferrous Iron and Elemental Sulfur by *Thiobacillus ferrooxidans*

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The oxidation of ferrous iron and elemental sulfur by *Thiobacillus ferrooxidans* that was absorbed and unabsorbed onto the surface of sulfur prills was studied. Unadsorbed sulfur-grown cells oxidized ferrous iron at a rate that was 3 to 7 times slower than that of ferrous iron-grown cells, but sulfur-grown cells were able to reach the oxidation rate of the ferrous iron-adapted cells after only 1.5 generations in a medium containing ferrous iron. Bacteria that were adsorbed to sulfur prills oxidized ferrous iron at a rate similar to that of unadsorbed sulfur-grown bacteria. They also showed the enhancement of ferrous iron oxidation activity in the presence of ferrous iron, even though sulfur continued to be available to the bacteria in this case. An increase in the level of rusticyanin together with the enhancement of the ferrous iron oxidation rate were observed in both sulfur-adsorbed and unadsorbed cells. On the other hand, sulfur oxidation by the adsorbed bacteria was not affected by the presence of ferrous iron in the medium. When bacteria that were adsorbed to sulfur prills were grown at a higher pH (ca. 2.5) in the presence of ferrous iron, they rapidly lost both ferrous iron and sulfur oxidation capacities and became inactive, apparently because of the deposition of a jarosite-like precipitate onto the surface to which they were attached.

In the leaching process of sulfide materials in the presence of *Thiobacillus ferrooxidans*, the bacteria increase the overall leaching rate by at least two different mechanisms: indirect and direct leaching effects. Indirect leaching entails oxidation of the minerals by the ferric iron produced by bacterial oxidation of the soluble ferrous iron that is present in the leaching solution, while direct leaching proceeds through the interaction of the bacteria with the surface of the sulfide mineral or with elemental sulfur and other sulfur-containing compounds that are formed on the surface of the mineral particles during its oxidation with ferric iron (see, for example, Hutchins et al. [6]). The regulation of the iron and sulfur oxidation systems may then be important for understanding the relative importance of both direct and indirect leaching by *T. ferrooxidans* and might help to obtain regimens which favor one or another bacterial action under industrial process conditions.

Contradictory results have been published on the inducible or constitutive nature of the iron and sulfur oxidation systems of *T. ferrooxidans*. Margalith and co-workers (13), using *T. ferrooxidans* ATCC 23270, and Sugio et al. (15), using *T. ferrooxidans* AP19-3, have reported that cells grown on sulfur oxidize ferrous iron almost as rapidly as cells grown on ferrous iron. On the contrary, Landesman et al. (11) and Kulpa et al. (7), using strains ATCC 19859 and ATCC 13661, respectively, reported that sulfur-grown cells oxidized ferrous iron at least 50 times slower than ferrous iron-grown cells did. The sulfur-oxidizing system, however, was found to be constitutive in these studies, although Lundgren et al. (12) have reported that the rate of sulfur oxidation by iron-grown cells is about half that by sulfur-grown cells. The behavior of *T. ferrooxidans* when both ferrous iron and sulfur are supplied together is also unclear. Landesman et al. (11) concluded that iron and sulfur can be oxidized simultaneously, because they obtained a respiration rate in ferrous iron plus sulfur substrate that was equal to the sum of the maximum rates observed when each substrate was used individually. However, Unz and Lundgren (17) and Beck (1) observed independently that when both ferrous iron and elemental sulfur are provided, *T. ferrooxidans* preferentially oxidizes iron.

The contradictory conclusions of those reports can be attributed to the fact that the dynamics of bacterial growth in sulfur were not considered in detail. In a culture containing sulfur, there were two different populations of bacteria: the metabolically active and replicating cells that adsorbed to the insoluble substrate and the free, possibly starving, unadsorbed cells in the liquid phase that did not replicate (3). Therefore, free cells derived either from a sulfur- or a ferrous iron-containing culture must have first adsorbed onto the insoluble substrate before they could oxidize sulfur. On the other hand, adsorbed and unadsorbed bacteria in a sulfur-containing culture could have behaved differently in the presence of ferrous iron; with unadsorbed bacteria the sulfur was not actually available to the bacteria, and hence, the response of a growing culture to the presence of both substrates simultaneously could only be observed in sulfur-adsorbed bacteria.

Here we report the rates of ferrous iron oxidation by both adsorbed and unadsorbed *T. ferrooxidans* in a sulfur-containing culture and compare the results with those obtained with cells from a ferrous iron-containing culture.

MATERIALS AND METHODS

T. ferrooxidans ATCC 19859 was purified after colony plating and was transferred 5 or more times in MS9b medium (3) that contained either elemental sulfur or ferrous iron as the energy source. This organism was used throughout these experiments. Spherical prills of elemental sulfur (diameter, 0.15 to 0.17 or 0.28 to 0.34 cm) were prepared as described

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previously (10) and added to the MS9b salt solution at a concentration of 0.04 or 0.07 g/ml, respectively, to attain an approximate sulfur surface area of 1 cm^2/ml. MS9b salt solution adjusted to pH 2.5 with H_2SO_4 was used in every experiment except when indicated otherwise. The prills were sterilized in MS9b salt solution by heating them in a small autoclave at 105°C (approximately 2.5 lb/in^2) for 0.5 h on 2 successive days. Media were inoculated with the supernatant that was obtained after the sulfur prills were allowed to sediment at $1 \times g$ in a culture containing 1×10^8 to 4×10^8 bacteria per ml. A total of 1 ml of this supernatant was added to each 100 ml of medium. The incubation conditions and the determination of bacterial numbers were performed as described elsewhere (3).

To determine ferrous iron and sulfur oxidation rates, 100 small prills or 25 large prills were washed 3 times with MS-HCl which is a salt solution that is similar to MS9b but that is low in sulfate and which contained the following salts, in grams per liter; NH_4Cl, 0.1; $K_2HPO_4 \cdot 3H_2O$, 0.04; and $MgSO_4 \cdot 7H_2O$, 0.1. The pH was adjusted to 2.5 with HCl except when indicated otherwise. Unadsorbed bacteria were washed in the same solution by centrifugation at $12,000 \times g$ for 15 min at 4°C. Prills or sedimented bacteria were then suspended in 5 ml of MS-HCl and, after the addition of $FeSO_4$ to a final concentration of 1.25 mM, were incubated in 25-ml Erlenmeyer flasks in a gyratory incubator at 120 rpm and 30°C. Samples of 0.1 ml were taken every 10 to 30 min according to the expected oxidation rate. The amount of remaining ferrous iron was determined by adding 0.4 ml of *o*-phenanthroline and 2 ml of water, as described previously (14). The amount of sulfate produced was measured by a turbidometric procedure based on excess barium chloride precipitation as follows. Five successive samples of 0.4 ml each taken every hour were added to 0.4 ml of a solution of 0.1 N HCl and 0.4 ml of 0.05 M $BaCl_2$; and the A_{550} of the $BaSO_4$ precipitate, which was suspended by strong shaking in a vortex shaker, was measured. To calculate the total amount of both ferrous iron and sulfate produced per sulfur prill, the concentrations found in the samples were corrected for the decrease in volume of liquid in the flask containing the prills caused by the samples that were withdrawn earlier.

Transfer experiments to study the effect of ferrous iron on the oxidation system of *T. ferrooxidans* that adsorbed the sulfur prills were performed as follows. For the experiment performed at pH 1.6, prills of 0.28 to 0.34 cm in diameter were inoculated with bacteria as indicated above and incubated in M59b salt medium for 8 weeks; the salt medium was changed every week. After this time the prills were washed once with MS9b salt medium; and half of these prills were subsequently incubated in MS9b salt medium (pH 1.6) containing 12.5 mM ferrous iron, while the other half were maintained in MS9b medium without ferrous iron. The medium was changed every day in both culture flasks to avoid depletion of the ferrous iron in the medium containing iron and to maintain the control culture without iron under equivalent conditions. Ferrous iron and sulfur oxidations were performed as described above, except that the MS-HCl was adjusted to pH 1.6. The experiment performed at pH 2.5 was done in a similar way; but in this case the prills used were 0.15 to 0.17 cm in diameter, the pH of the MS9b salt medium for incubation with and without ferrous iron was 2.5, and the pH of the MS-HCl assay solution was also 2.5.

Polyacrylamide gel electrophoresis was performed as described by Laemmli (9) by using 15% (wt/vol) acrylamide and 0.4% (wt/vol) bisacrylamide gels. The bacteria that were either adsorbed to the prills or sedimented from the liquid phase after three successive washes with MS-HCl were disrupted with 100 μl of the mixture described by Laemmli (9) for each 5×10^8 bacteria or 50 prills and heated in boiling water for 2 min. After heating, the tubes were centrifuged for 5 s in a microfuge, and subsequently, 50 μl of supernatant was loaded into the gel wells.

RESULTS

T. ferrooxidans was grown on elemental sulfur prills of defined size to allow the appropriate control of both the amount of adsorbed bacteria and the amount of substrate actually available to the microorganism, as described previously (4). To study the regulation of the iron and sulfur oxidation systems, a suitable medium was defined that supported bacterial oxidation of both iron and sulfur but that contained minute amounts of sulfate so that the sulfate produced by sulfur oxidation could be determined. Among the different media tested, MS9b that was acidified with HCl instead of H_2SO_4 was found to be suitable for the planned experiments. MS-HCl which contained only 1.5 μmol of sulfate per ml under the assay conditions, supported ferrous iron oxidation by both free and adsorbed bacteria at efficiencies similar to those obtained in the MS9b growth medium (Fig. 1). The MS-HCl salt solution was then chosen for the assay of both the oxidation of ferrous iron and the oxidation of elementary sulfur. Unadsorbed bacteria oxidized Fe(II) at a rate of 1.3×10^{-10} and 1.9×10^{-10} μmol per bacteria min in the MS9b and MS-HCl salt solutions, respectively. This rate was 3.3×10^{-4} μmol per prill min when adsorbed bacteria were assayed. If it is assumed that there were 1.25×10^7 bacteria per cm^2 of sulfur surface, as determined previously by ^{32}P incorporation (3), in the prills of the size used in these experiments (diameter, 0.15 to 0.17 cm), there were 10^6 bacteria per prill. An oxidation rate of 3.3×10^{-10} μmol per bacteria min was then estimated for the adsorbed bacteria. It is likely, however, that unadsorbed and adsorbed bacteria oxidized Fe(II) at the same rate and that

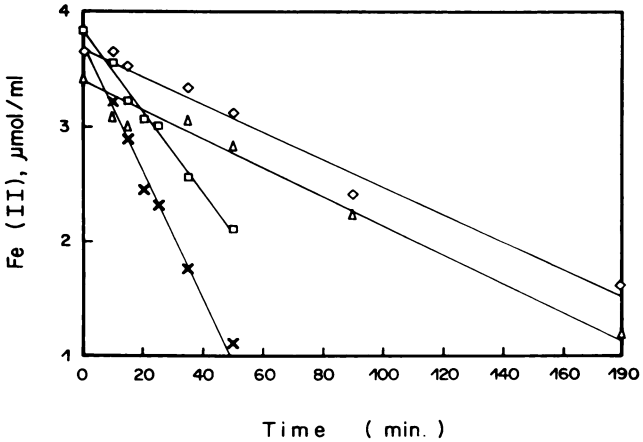

FIG. 1. Oxidation of ferrous iron in medium acidified with H_2SO_4 or HCl. The decrease of ferrous iron in medium acidified either with H_2SO_4 (MS9b) or HCl (MS-HCl) and containing either unadsorbed or prill-adsorbed bacteria was measured at different incubation times. Symbols: □, unadsorbed bacteria suspended in MS9b; ×, unadsorbed bacteria suspended in MS-HCl; ◇, sulfur prills in MS9b; △, sulfur prills in MS-HCl. The concentration of bacteria and prills in the assay medium were 3×10^8 bacteria per ml and 30 prills per ml, respectively.

the observed difference in the rates was actually due to the uncertainty in the estimation of the number of adsorbed bacteria per prill. As discussed elsewhere (3), this value apparently increases with incubation time, as well as with the number and frequency of salt medium changes.

The rate of ferrous iron oxidation observed in samples of both prills and the liquid phase that were obtained at different times from a batch culture (expressed per prill and per amount of liquid containing one prill, respectively) is given in Fig. 2. Figure 2 shows that at the beginning of growth, the oxidation rate of Fe(II) in the liquid phase was proportional to the number of bacteria present. The rate observed in the prills was close to that expected for prills that became saturated with bacteria (3). If it is assumed that there are 3×10^6 bacteria in these prills, with a surface 3 times larger than those used in the experiment for which the results are shown in Fig. 1, it can be concluded that both the unadsorbed and adsorbed bacteria obtained from a sulfur prill culture oxidized ferrous iron at the same rate (about 1×10^{-10} to 2×10^{-10} μmol per bacteria min). At later times, when the stationary phase of the growth curve was reached, the rate of oxidation in the liquid decreased, probably because of the inactivation of the bacteria caused by the low pH reached at this time (below 1.5; data not shown), as indicated by the number of CFU found in solution. The oxidation capacity of bacteria that attached to the prills also decreased at later times; however, it was rapidly recovered when the prills were transferred to fresh medium (the medium was changed on day 19) (Fig. 2). The unadsorbed bacteria obtained during exponential growth from a sulfur prill culture consistently oxidized ferrous iron at a rate of $2.0 \times 10^{-10} \pm 0.3 \times 10^{-10}$ μmol per bacteria min; which was 3 to 7 times slower than the rate for bacteria adapted to growth in ferrous iron, which did it at a rate of $6.4 \times 10^{-10} \pm 1.9 \times 10^{-10}$ μmol per bacteria min. However, unadsorbed sulfur-adapted bacteria were able to reach this last value after only 1.5 generations in MS9b medium containing ferrous iron as the sole energy source. In a typical experiment, the oxidation rate of Fe(II) by sulfur-adapted cells, which was 1.5×10^{-10} μmol per cell min, increased to 5.7×10^{-10}, 5.0×10^{-10}, and 5.5×10^{-10} μmol per cell min after transfer and growth for 1.5, 10, and 100 generations, respectively, in medium containing only Fe(II) as the energy source. These differences in the rate of Fe(II) oxidation correlated well with the apparent amount of putative rusticyanin observed after gel electrophoresis of the proteins from these cells (Fig. 3).

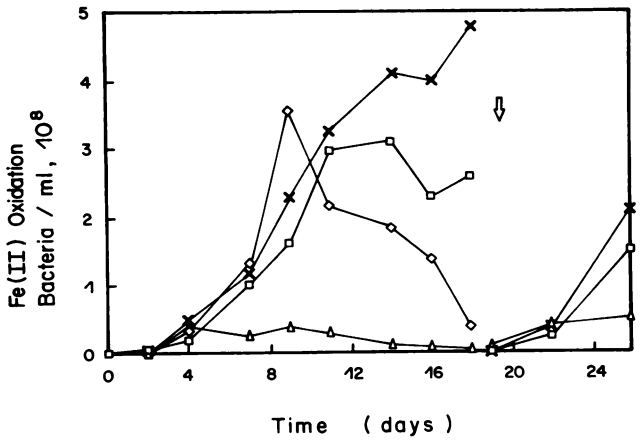

FIG. 2. Growth curve of *T. ferrooxidans* in elemental sulfur prills and Fe(II) oxidation capacity of *T. ferrooxidans*. Fe(II) oxidation by bacteria in the liquid and solid (prills) phases from a batch culture was measured after different incubation times. The culture contained three prills (diameters, 2.8 to 3.4 mm) per ml of salt medium solution. The arrow indicates the times when prills were transferred to fresh medium. Symbols: ◇, micromoles of Fe(II) oxidized for each 0.33 ml of salt medium solution (the volume of salt medium solution per prill; 10^3); △, micromoles of Fe(II) oxidized per prill (10^4); □, CFU/ml of medium; ×, number of bacteria per ml of medium counted under a microscope.

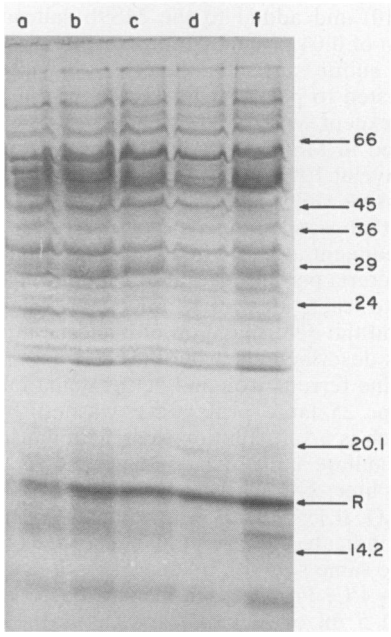

FIG. 3. Polyacrylamide gel electrophoresis of *T. ferrooxidans* grown with either elemental sulfur (unadsorbed bacteria only) or ferrous iron as the sole energy source. The wells contained *T. ferrooxidans*, which was grown as follows: more than 100 generations with elemental sulfur (lane a); sulfur-adapted bacteria grown for 1.5, 3.5, and 10 generations with ferrous iron only (lanes b to d, respectively); and more than 100 generations with ferrous iron as the sole energy source (lane e). The arrows indicate the position of the markers and their molecular sizes, in kilodaltons (lane f); R indicates the position of rusticyanin.

To study the regulation of ferrous iron and sulfur oxidation when both substrates were actually available to the bacteria, prills with adsorbed bacteria were transferred to medium containing 12.5 mM ferrous iron. The oxidation capacity of the adsorbed bacteria was then measured for each substrate after different times of incubation with ferrous iron. This was compared with the oxidation capacity of the bacteria that absorbed onto the surface of the prills that were maintained in medium containing only traces of the ferrous iron that is normally present in the sulfur medium. In these experiments the medium was changed every day, to avoid depletion of the ferrous iron by bacterial oxidation. The salt medium solution of the control flask containing no additional ferrous iron was also changed, to keep the control under the same incubation conditions. The results obtained when this experiment was performed in medium at pH 1.6 are given in Fig. 4. At this pH an activation of Fe(II) oxidation that was similar in nature to that which occurred when sulfur-adapted unadsorbed bacteria were transferred to medium containing ferrous iron was observed. However, the oxidation rate of elemental sulfur was unaffected by the presence of ferrous

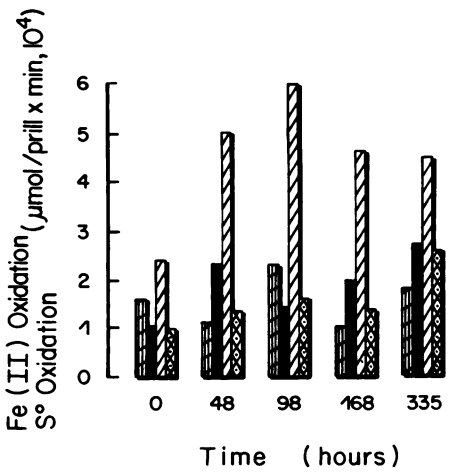

FIG. 4. Oxidation of ferrous iron and elemental sulfur by prill-adsorbed bacteria at different times after transfer to medium containing ferrous iron (pH 1.6). The first and second bars of the four bars shown for each culture time correspond, respectively, to the Fe(II) and sulfur oxidation rates for prills shifted to new medium containing only traces of Fe(II). The third and fourth bars correspond, respectively, to the Fe(II) and sulfur oxidation rates for prills shifted to medium containing 12.5 mM Fe(II) (prills were 0.15 to 0.16 cm in diameter).

iron in the medium. In this experiment the oxidation rates were determined at pH 1.6, which rendered a significantly lower oxidation rate for ferrous iron but not for sulfur (data not shown). Assuming that there were about 3.5×10^6 bacteria per sulfur prill (diameters, 0.28 to 0.34), the oxidation rates of sulfur-adapted *T. ferrooxidans* at this pH were about 0.3×10^{-10} μmol per bacteria min for both ferrous iron and elemental sulfur. The rate for ferrous iron oxidation was much lower than that observed when it was determined at pH 2.5. In other experiments (data not shown) it was observed that the ferrous iron oxidation rate decreased drastically when the pH of the MS-HCl salt solution was below 2.0. Like with unadsorbed bacteria, an increase of rusticyanin was also observed when the adsorbed bacteria were shifted to medium with ferrous iron and were disrupted and subjected to polyacrylamide gel electrophoresis (Fig. 5). This increase, however, was apparently smaller than that observed with unadsorbed bacteria (see below for a discussion). The results obtained when the same experiment shown in Fig. 4 was performed in medium at pH 2.5 are shown in Fig. 6. No relative differences were observed in the oxidation rates between the bacteria maintained in the absence of ferrous iron and those maintained in the presence of ferrous iron until 72 h of incubation. Later, ferrous iron and sulfur oxidation rates for the bacteria that were adsorbed to prills in medium with ferrous iron decreased steadily, becoming undetectable after about 300 h of incubation. This inhibition of bacterial activity was apparently due to the deposition of a ferric iron precipitate, possibly jarosite (16), onto the prills, which could be observed with the naked eye after 300 h of incubation in medium with ferrous iron. At this time the prills presented a dark brown color that was clearly distinguishable from the yellow color observed at the beginning of the experiments or in the cultures that lacked ferrous iron. In this experiment the oxidation rates were determined at pH 2.5. Assuming that the same number of bacteria adsorbed per square centimeter, the oxidation rates of adsorbed *T. ferrooxidans* at this pH were about 3×10^{-10} and 0.5×10^{-10} μmol per bacteria min for ferrous iron and elemental sulfur, respectively.

DISCUSSION

The oxidation of Fe(II) and elemental sulfur by *T. ferrooxidans* ATCC 19859 was studied by considering the two types of bacteria that were present in a sulfur-containing culture. The metabolically active bacteria adsorbed onto the insoluble substrate and the unadsorbed bacteria that were present in the liquid phase, which lacked an external energy source. When bacteria from the liquid phase, which were probably starving, were suspended in medium with ferrous iron, they initially oxidized this ion at about one third of the rate found for ferrous iron-adapted *T. ferrooxidans*. However, they were able to reach the rate of ferrous iron-adapted cells after growth for at least 1.5 generations in medium containing ferrous iron as the only energy source. Hence, the ferrous iron oxidation system is regulated, but the nature of this regulation is not clear, because repression in the sulfur-adapted bacteria was very low. It is unlikely that ferrous iron traces in the sulfur medium, by themselves, were responsible for this low repression. Ferrous iron traces were required to obtain reproducible and efficient growth of *T. ferrooxidans* in sulfur-containing medium. Otherwise, as is generally accepted, growth is dependent on the iron that is present in the other salts contained in the medium. The 0.0125 mM ferrous iron in the medium used was, however, too low to act as an inducer of the ferrous iron system, since Braddock et al. (2) showed that the apparent threshold ferrous iron concentration for iron oxidation is 0.25 mM for *T. ferrooxidans* AK1. Sugio et al. (15) have recently reported

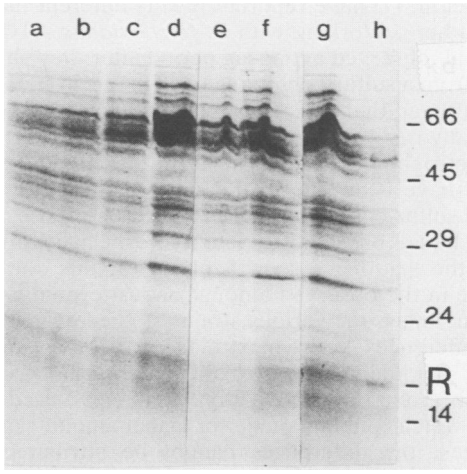

FIG. 5. Polyacrylamide gel electrophoresis of *T. ferrooxidans* adsorbed to sulfur prills incubated in the presence or absence of Fe(II). Sulfur prills inoculated with *T. ferrooxidans* were incubated and subsequently transferred to MS9b salt solution at pH 1.6 with either traces of Fe(II) or 12.5 mM of Fe(II). The bacteria in the same prills that were used for the determination of ferrous iron and sulfur oxidation shown in Fig. 4 were analyzed by gel electrophoresis. Lanes a, b, c, d, and g, adsorbed bacteria after 0, 48, 96, 168, and 336 h of incubation with ferrous iron, respectively; lanes e and f, adsorbed bacteria after 168 and 336 h of incubation with traces of Fe(II), respectively; lane h, bacteria grown for more than 100 generations (more than 10 subcultures) with ferrous iron as the sole energy source. The positions of the markers and their molecular sizes, in kilodaltons, are indicated on the right; R indicates the position of rusticyanin.

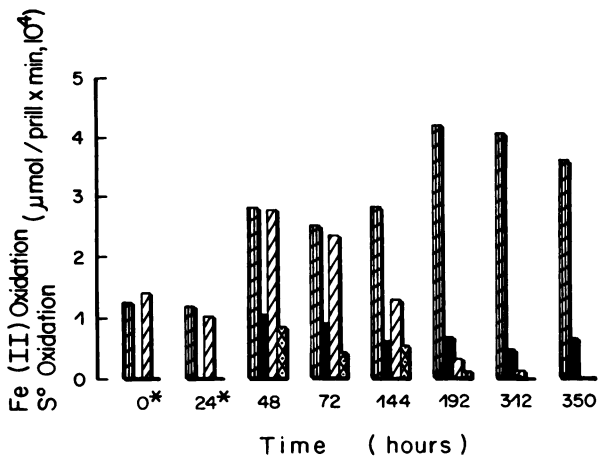

FIG. 6. Oxidation of ferrous iron and elemental sulfur by prill-adsorbed bacteria at different times after transfer to a medium containing ferrous iron at pH 2.5. The first and second bars of the four bars shown for each time correspond, respectively, to the Fe(II) and sulfur oxidation rates for prills shifted to the new medium containing only traces of Fe(II). The third and fourth bars correspond, respectively, to the Fe(II) and sulfur oxidation rates for prills shifted to medium containing 12.5 mM Fe(II) (prills were 0.28 to 0.34 cm in diameter). At the times indicated with asterisks, the amount of sulfur oxidation was not determined.

that for *T. ferrooxidans* AP19-3, the sulfur oxidation route under aerobic conditions makes up both the ferric ion-reducing and the iron-oxidizing systems. This observation could explain the maintenance of an appreciable ferrous iron oxidation activity in the bacteria grown on sulfur-containing medium for many generations.

Kulpa et al. (7) have reported results different than these described here. Working with *T. ferrooxidans* ATCC 3661, Kulpa et al. observed a long lag period after they shifted the bacteria from a sulfur-containing to a ferrous iron-containing medium (7) and that cells grown on sulfur did not appear to produce any rusticyanin when analyzed by an activity assay or gel electrophoresis (7, 8). The lag observed by Kulpa et al. (7) could have been due to the state of the cells obtained from the sulfur-containing culture, because those released into the liquid phase lost viability, especially after they reached the stationary growth phase (3; this study). The difference in the observed amount of rusticyanin is difficult to explain, unless the various strains studied behaved differently. Differences between strains of *T. ferrooxidans* is, however, not unlikely due to the heterogeneity observed within this group (5). Since Kulpa et al. (7, 8) used Starkey medium, which contains similar or higher amounts of ferrous iron traces, the differences cannot be attributed to the presence of higher amounts of Fe(II) in our medium.

An understanding of the regulation of the Fe(II) and elemental sulfur oxidation systems when both substrates are available to *T. ferrooxidans* is necessary in order to visualize the role of bacteria in the bioleaching process of sulfide minerals. The results reported here indicate that bacteria that adsorb to sulfur and that actively metabolize this substrate can also oxidize ferrous iron, which is in agreement with the interpretation of the report of Landesman et al. (11). The ferrous iron oxidation rate by sulfur-adsorbed bacteria was estimated to be equal to that by unadsorbed bacteria, and it increased in an amount similar to that observed with unadsorbed bacteria when ferrous iron was supplied to the sulfur-containing medium. On the other hand, the sulfur oxidation rate was not affected by the presence of ferrous iron at concentrations required for gross oxidation, in disagreement with results of previous studies (2, 17). These observations indicate that the presence of sulfur, which is actively metabolized by *T. ferrooxidans*, does not completely inhibit ferrous iron oxidation. Vice versa, the presence of ferrous iron does not inhibit sulfur oxidation. These results indicate that the determination of the overall ferrous iron oxidation rate in an ore that is leached under controlled conditions, as was reported previously (4), is indeed a fair indication of the total *T. ferrooxidans* activity in industrial applications.

The low increase in the relative amount of rusticyanin observed after we shifted the prills to medium containing ferrous iron deserves further comment, because this increase was apparently lower than that observed when we shifted unadsorbed bacteria. It seems likely that this difference was due to the presence in these prills of a significant proportion of bacteria that were inactive metabolically and that did not react to the presence of Fe(II). The presence of metabolically inactive bacteria that adsorbed to the prills is suggested by the fact that there was an increase with time in the amount of protein per prill (Fig. 5); this was not observable in the oxidation rate per prill (Fig. 4). It is unlikely that the differences observed were due to different efficiencies of the protein extraction procedure. By this procedure we extracted more than 90% of the incorporated ^{32}P when prills with bacteria grown in radioactive medium were used (data not shown). In a previously reported experiment (3), it was found that about 10% of the adsorbed bacteria apparently did not replicate when prills incubated for 20 days were tested. It is likely that the proportion of inactive, adsorbed bacteria increases considerably with prolonged incubations.

ACKNOWLEDGMENTS

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