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Surface Chemistry of *Thiobacillus ferrooxidans* Relevant to Adhesion on Mineral Surfaces

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Thiobacillus ferrooxidans **cells grown on sulfur, pyrite, and chalcopyrite exhibit greater hydrophobicity than ferrous ion-grown cells. The isoelectric points of sulfur-, pyrite-, and chalcopyrite-grown cells were observed to be at a pH higher than that for ferrous ion-grown cells. Microbe-mineral interactions result in change in the surface chemistry of the organism as well as that of the minerals with which it has interacted. Sulfur, pyrite, and chalcopyrite after interaction with *T. ferrooxidans* exhibited a significant shift in their isoelectric points from the initial values exhibited by uninteracted minerals. With antibodies raised against sulfur-grown *T. ferrooxidans*, pyrite- and chalcopyrite-grown cells showed immunoreactivity, whereas ferrous ion-grown cells failed to do so. Fourier transform infrared spectroscopy of sulfur-grown cells suggested that a proteinaceous new cell surface appendage synthesized in mineral-grown cells brings about adhesion to the solid mineral substrates. Such an appendage was found to be absent in ferrous ion-grown cells as it is not required during growth in liquid substrates.**

Thiobacillus ferrooxidans is a gram-negative chemoautotrophic acidophile which obtains its energy by the oxidation of ferrous, sulfur, and reduced-sulfur compounds (6). *T. ferrooxidans* is commercially used in the extraction of copper and uranium (2) and in the pretreatment of gold-bearing refractory sulfides for liberation of encapsulated gold before cyanidization (8). It can oxidize a number of sulfide minerals such as pyrite, chalcopyrite, galena, sphalerite, and pentlandite. Two mechanisms are known to be responsible for the biodissolution of sulfidic ores, namely, indirect and direct mechanisms. The indirect mechanism operates by the chemical action of acidic ferric sulfate produced by the bacterial metabolism. In this mechanism, adhesion of the bacteria to the mineral surfaces is not required. The direct mechanism, on the other hand, involves the enzymatic attack of the mineral by the bacteria, for which intimate contact and hence adhesion are required. Previous studies in our laboratory have shown that direct attack plays an important role in the bioleaching of pyrite and chalcopyrite (13, 17). Adhesion of *T. ferrooxidans* to sulfur and mineral surfaces has been reported by several workers (4, 5, 24). Scanning electron micrographs of ore particles during bioleaching also show adhesion of *T. ferrooxidans* (12). It has also been shown that conditioning minerals with *T. ferrooxidans*, which results in cell adhesion, could induce either hydrophilicity or hydrophobicity, making the minerals either floatable or nonfloatable (7, 10, 25). Even though it has been known that bacterial cells play an important role in biooxidation, the actual mechanism of adhesion of *T. ferrooxidans* and its role in altering the surface chemistry of the mineral have not been well understood.

Bacterial adhesion is dependent not only on the biochemical properties of the organism but also on the interfacial properties of the various interfaces existing in a bioleaching system. The surface properties of bacteria that have been found to affect adhesion are cell surface hydrophobicity and electrokinetic potential (22, 23). Ferrous ion provides a soluble substrate for growth of *T. ferrooxidans*, whereas minerals such as sulfur, pyrite (FeS_2), and chalcopyrite ($CuFeS_2$) provide insoluble substrates. Adhesion is a necessary event for bacterial growth on sulfur, pyrite, and chalcopyrite (solid substrates), in contrast to growth in a ferrous ion medium in which iron is dissolved (soluble substrate). Thus, growth of *T. ferrooxidans* in the presence of mineral is a model system for investigating the mechanism of adhesion.

This work was undertaken with the objective of studying the role of surface chemistry in the adhesion of *T. ferrooxidans* to sulfur, pyrite, and chalcopyrite.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *T. ferrooxidans* MAL 4-1 used in this study was isolated from the Malanjkhand copper mines (Malanjkhand, India) and maintained on 9K medium (18), in which ferrous ion is the energy source, at pH 2.3. Ferrous ion-grown cells were obtained by growth on 9K medium for 2 days on a rotary shaker at 240 rpm and 30°C. The culture was filtered through Whatman no. 1 filter paper and centrifuged at 27,000 × *g* for 15 min. The pellet was washed and resuspended in distilled water adjusted to pH 2.0 with sulfuric acid. Sulfur-grown cells were obtained by growth on $9K^-$ mineral salts medium (without ferrous sulfate) containing 10 g of sulfur powder per 100 ml, adjusted to pH 2.3, and incubated at 30°C and 240 rpm for 10 days. For growth of *T. ferrooxidans* on sulfur, it was necessary to supplement the medium with trace amounts of iron (10 mg of ferric chloride per liter). The culture was filtered through Whatman no. 1 filter paper and centrifuged. Pyrite-grown and chalcopyrite-grown cells were obtained from $9K^-$ medium supplemented with 4 g of the mineral per 100 ml of the solution. The cells were harvested as described above.

Minerals. Hand-picked pure samples of pyrite and chalcopyrite and analytical grade sulfur particles were used in all the experiments. The purity of the mineral was determined by mineralogical analysis, X-ray diffraction, and chemical

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TABLE 1. Hydrophobic interaction chromatography of *T. ferrooxidans* grown on different substrates

Substrate	% Cells bound to octyl-Sepharose
Ferrous ion	17.7
Sulfur	52
Pyrite	51.2
Chalcopyrite	50.4

analysis. The minerals were ground with an agate mortar and pestle to a particle size of less than 10 μm for electrophoretic mobility studies.

Hydrophobicity measurements. The hydrophobicity of cell suspensions was measured by liquid-liquid partition in aqueous and organic phase as described previously (15) by using *n*-hexadecane. Hydrophobic interaction chromatography was carried out (19) with octyl-Sepharose (Pharmacia).

Electrophoretic mobility. The electrophoretic mobilities of the bacteria and mineral particles were determined by using Zeta-Meter 3.0 as described previously (20). Electrokinetic measurements were conducted on bacterial suspensions of 1.0×10^7 cells per ml. Minerals were suspended at a concentration of 10 mg/100 ml and conditioned for 1 h. For microbe-mineral interaction studies, sulfur-grown cells (10^7 cells per ml) and the respective mineral (10 mg/100 ml) were conditioned in 10^{-3} M KCl at the required pH for 1 h unless stated otherwise. Such conditioning of the mineral particles in the presence of the bacteria results in modification of their surfaces by adhesion of cells.

Raising of antibodies and ELISA. Antibodies against the cell surface of sulfur-grown cells were raised in rabbits by injecting them with whole cells. Cross-reacting antibodies were removed by incubating ferrous ion-grown cells (10^9) with 0.5 ml of serum for 3 h at 37°C. The cells were removed by spinning in a Microfuge. This was repeated three times. Whole-cell enzyme-linked immunosorbent assay (ELISA) was carried out as described by Bartlett and Noelle (1).

Fourier transform infrared spectroscopy. Infrared absorption spectra were recorded on a Fourier transform infrared (FTIR) spectrometer, Bio-Rad SPC 3200 (21), from ferrous ion- and sulfur-grown cells. The difference spectrum was obtained by subtracting the spectrum of ferrous ion-grown cells from that of sulfur-grown cells by using a program available for the instrument.

RESULTS AND DISCUSSION

The results of hydrophobic interaction chromatography and phase partitioning with *T. ferrooxidans* are illustrated in Table 1 and Fig. 1. In Table 1 the percentage of cells bound to octyl-Sepharose after growth on different substrates is shown. As can be seen, over 50% of the cells grown on mineral substrates were bound to octyl-Sepharose, whereas only about 18% of the cells grown in a liquid ferrous ion medium were so bound. It can also be seen from Fig. 1 that while about 20% of *T. ferrooxidans* cells grown on mineral substrates are transferred to the hexadecane organic phase, only negligible numbers of ferrous ion-grown cells appeared in this phase. It should be noted that the populations of cells obtained by growth on sulfur, pyrite, and chalcopyrite may not be homogeneous. In sulfur oxidation, partially oxidized species of sulfur that freely suspended *T. ferrooxidans* cells oxidize appear in solution (16). Similarly, in some phases of pyrite and chalcopyrite oxidation, ferrous ion that freely suspended *T. ferrooxidans* cells may oxidize is released into solution. The mineral-grown cells were more hydrophobic than the ferrous ion-grown cells. The greater hydrophobicity may help in adhesion.

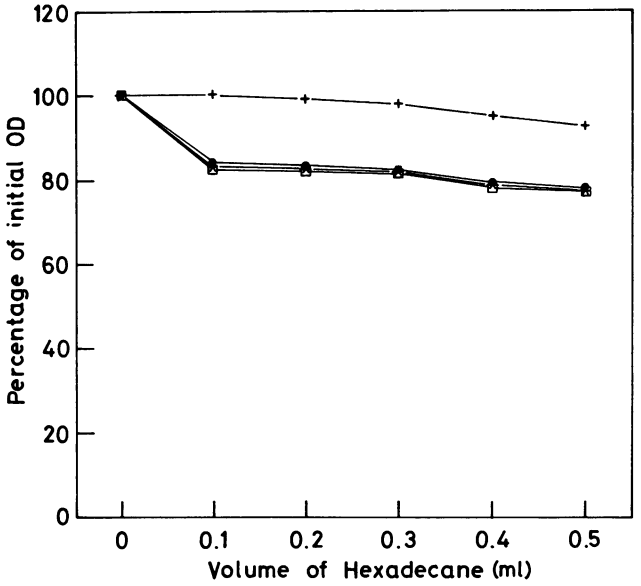

FIG. 1. Hydrophobicity of *T. ferrooxidans* grown on ferrous ion (+), sulfur (●), pyrite (×), and chalcopyrite (□) by partitioning in *n*-hexadecane and aqueous phase. OD, optical density.

Figure 2 illustrates the different electrophoretic mobilities of *T. ferrooxidans* cells grown on different substrates as a function of pH. It was observed that *T. ferrooxidans* grown on ferrous ion exhibited an isoelectric point (IEP) at about pH 2. This implies that the bacterial cells are positively charged below pH 2.0, the cells becoming increasingly negative as the pH is increased. When the electrokinetic behavior of the iron-grown cells is compared with that of those grown on the mineral substrates, we observed a significant difference. The cells which were grown on sulfur, pyrite, and chalcopyrite exhibited IEPs corresponding to a pH of about 3.8, implying that the cells are positively charged below pH 3.8. Thus, we conclude that the surface

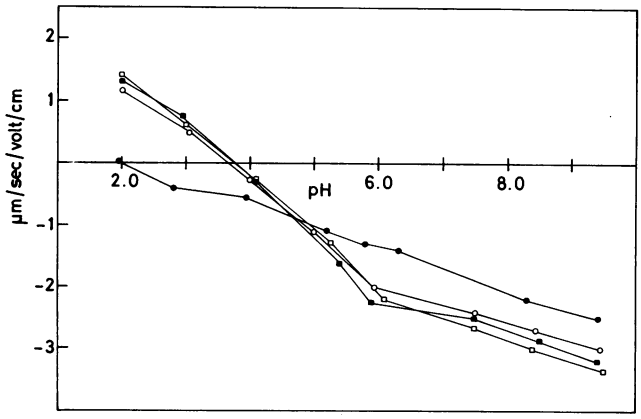

FIG. 2. Electrophoretic mobilities of *T. ferrooxidans* cells grown on ferrous ion (●), sulfur (■), pyrite (○), and chalcopyrite (□).

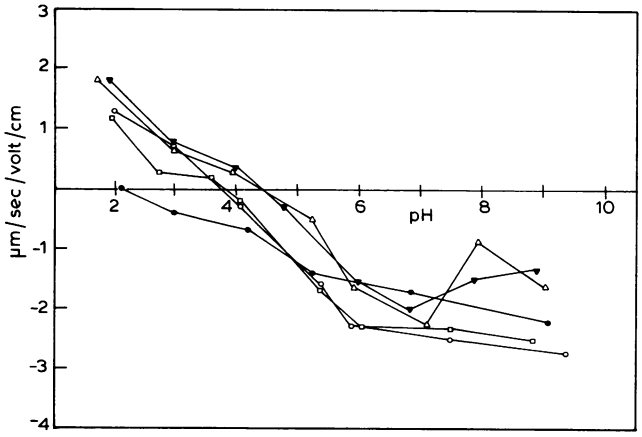

FIG. 3. Electrophoretic mobilities of sulfur before interaction with *T. ferrooxidans* (●) and after interaction with *T. ferrooxidans* for 1 h (○), 24 h (□), 120 h (△), and 240 h (▼).

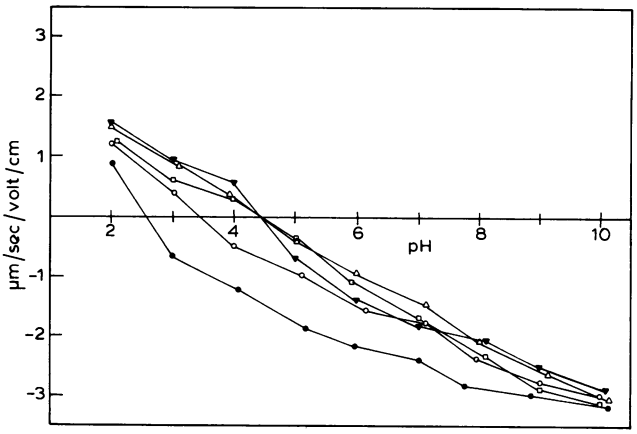

FIG. 5. Electrophoretic mobilities of chalcopyrite before interaction with *T. ferrooxidans* (●) and after interaction with *T. ferrooxidans* for 1 h (○), 24 h (□), 120 h (△), and 240 h (▼).

chemical (electrokinetic) natures of the ferrous ion- and mineral-grown bacteria are significantly different. It is noteworthy that all the mineral-grown bacteria show similar surface chemical properties. The positive charge of mineral-grown cells at acidic pH may indicate the presence of a $-NH_3$ group on the surface (9). This is of significance with respect to microbe-mineral interactions in bioleaching and bioflotation. The optimum pH used in many sulfide-leaching processes is between 2.0 and 2.5. In beneficiation of sulfide minerals by flotation, conditioning with *T. ferrooxidans* should be in an acidic range between pH 2 and 4.

Mozes and Rouxhet (11) have studied the interplay of hydrophobic and electrostatic interactions in influencing cell adhesion. They report that although hydrophobic interactions are predominant in adhesion of hydrophobic cells, electrostatic interactions are often present and influence the process. The coverage of any solid substrate was most dense slightly above the IEP of the cells.

We examined the electrokinetic behavior of minerals such as sulfur, pyrite, and chalcopyrite before and after interaction with *T. ferrooxidans*, as illustrated in Fig. 3, 4, and 5. As can be seen from Fig. 3, the IEP of sulfur (at about pH 2.0 in the absence of interaction with bacteria) was shifted to higher pH values between 3.8 and 4.5 after interaction with the bacteria over a period of time ranging from 1 to 240 h. A similar trend in the electrokinetic behavior of the other minerals, namely, pyrite and chalcopyrite, could also be observed. A fresh sample of pyrite has an IEP at pH 2.9 (Fig. 4) which shifts to pH 4.7 after interaction with the bacteria. Freshly ground chalcopyrite has an IEP at pH 2.6 (Fig. 5) which shifts to pH 4.4 after interaction with the bacteria. The above observations indicate that the interaction of *T. ferrooxidans* with a mineral changes not only the electrokinetic behavior of the bacterial cell but also that of the mineral with which it has interacted.

Common immunoreactivity was seen between sulfur-, pyrite-, and chalcopyrite-grown cells with antisera raised against the surface of sulfur-grown cells. This is shown in the ELISA readings given in Table 2. Ferrous ion-grown cells showed no immunoreactivity to the same antisera. This indicates that a new surface component was produced on mineral-grown cells which is absent in ferrous ion-grown cells and is presumably involved in aiding adhesion to mineral surfaces. The data from hydrophobicity measurements and electrokinetic studies also support such a conclusion, as the changes in their values may be due to the presence of a new surface component. It is also known that hydrophobicity and electrostatic interactions play important roles in initial adhesion, with irreversible adhesion being brought about by polymeric bridging (23). The surface appendage postulated to be present on mineral-grown cells may be involved in bringing about polymeric bridging for firm adhesion. Other workers (14) have previously shown by electron spectroscopic and X-ray fluorescence studies that a

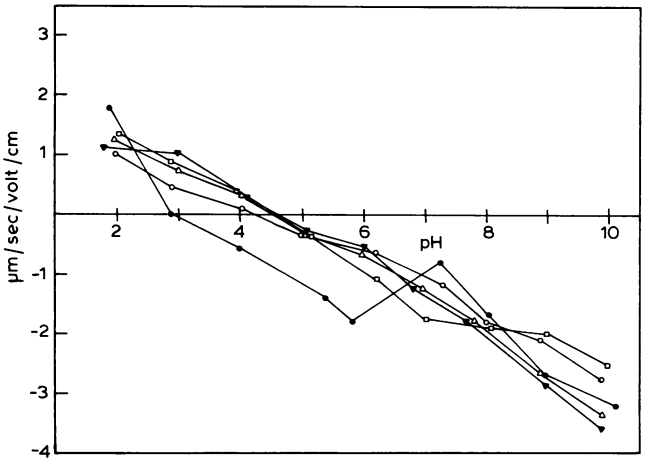

FIG. 4. Electrophoretic mobilities of pyrite before interaction with *T. ferrooxidans* (●) and after interaction with *T. ferrooxidans* for 1 h (○), 24 h (□), 120 h (△), and 240 h (▼).

TABLE 2. ELISA readings for *T. ferrooxidans* grown on different substrates

Substrate	OD_{490}[a]
Ferrous ion	0
Sulfur	0.324
Pyrite	0.312
Chalcopyrite	0.292

[a] Optical density of cells at 490 nm.

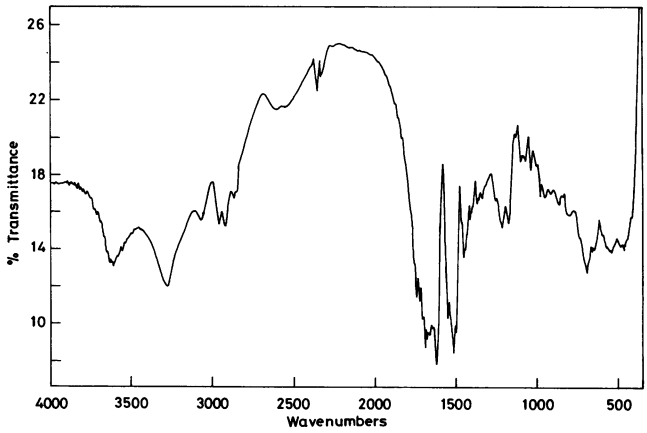

FIG. 6. FTIR difference spectra obtained by subtracting the spectra of ferrous ion-grown *T. ferrooxidans* cells from those of sulfur-grown cells.

layer of organic substance is secreted by *T. ferrooxidans* when grown on pyrite crystals.

To understand the nature of the new cell surface component present on mineral-grown cells but absent on ferrous ion-grown cells, FTIR difference spectra obtained by subtracting the spectra of ferrous ion-grown cells from those of sulfur-grown cells were obtained. While FTIR spectroscopy yields cell-surface-sensitive information (21), depending upon the concentrations of the adhesion-specific and nonspecific components, the spectrum may reflect the traits of both components. A typical difference spectrum is shown in Fig. 6. There is a broad, strong band near 3,300 cm^{-1} and a weaker one at 3,100 cm^{-1} assignable to asymmetric and symmetric stretching of the NH_2 group. The spectrum also suggests the presence of NH, since the band at 3,277 cm^{-1} is asymmetric and NH stretching occurs near 3,200 cm^{-1}. The group of bands between 3,000 and 2,800 cm^{-1} attributable to C—H stretching modes indicate the presence of alkyl groups (CH_3, CH_2, CH). Very intense bands between 1,740 and 1,620 cm^{-1} indicate the presence of $-C{=}O$ groups. The intense broad band at 1,650 cm^{-1} indicates the presence of an amide group (amide I band). The sharp strong band near 1,700 cm^{-1} is assignable to the $-CO$ stretching frequency of the aliphatic carboxylic group. The amide content appears to be high, judging from the intensity of the amide I band. The 1,622-cm^{-1} band is assignable to NH_2 bending of the primary amide group or $-NH_3$ of the amino acid (zwitterion form). The band at 1,550 to 1,515 cm^{-1} is assignable to NH bending of the secondary amide group $-CONH$ (amide II band). The bands at 1,450 and 1,400 cm^{-1} are assignable to bending of $-CH_3$ and $-CH_2$ groups. The $-CH$ bending vibrations occur in the 1,350-cm^{-1} region where weak absorptions are seen. The bands at 1,220 and 1,176 cm^{-1} are assignable to $-CH_3$ wagging and $-CH_2$ twisting modes, respectively. The band at 1,095 cm^{-1} is attributable to $-CH_3$ rocking and $-CH_2$ wagging modes. The band at 694 cm^{-1} is assignable to $C{=}O$ bending (amide IV band) or COO^- bending of the carboxylic groups (3). FTIR spectra obtained thus show the presence of NH_3, NH_2, NH, CONH, CO, CH_3, CH_2, CH, and COOH groups on the surface of sulfur-grown cells. This indicates that the new surface component may be proteinaceous in nature.

The study strongly suggests a correlation between hydrophobicity and electrokinetic properties with adhesion of *T. ferrooxidans* to minerals. It provides evidence for production of a new surface component with proteinaceous properties by cells grown in the presence of a solid energy source, a component which may play a role in surface adhesion and may account for the change in hydrophobicity and electrokinetics.

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