Selective Adhesion of *Thiobacillus ferrooxidans* to Pyrite

NAOYA OHMURA,* KEIKO KITAMURA, AND HIROSHI SAIKI

Department of Biotechnology, Abiko Research Laboratory, Central Research Institute of Electric Power Industry, 1646 Abiko, Abiko-shi, Chiba 270-11, Japan

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Bacterial adhesion to mineral surfaces plays an important role not only in bacterial survival in natural ecosystems, but also in mining industry applications. Selective adhesion was investigated with *Thiobacillus ferrooxidans* by using four minerals, pyrite, quartz, chalcopyrite, and galena. *Escherichia coli* was used as a control bacterium. Contact angles were used as indicators of hydrophobicity, which was an important factor in the interaction between minerals and bacteria. The contact angle of *E. coli* in a 0.5% sodium chloride solution was 31°, and the contact angle of *T. ferrooxidans* in a pH 2.0 sulfuric acid solution was 23°. *E. coli* tended to adhere to more hydrophobic minerals by hydrophobic interaction, while *T. ferrooxidans* selectively adhered to iron-containing minerals, such as pyrite and chalcopyrite. Ferrous ion inhibited the selective adhesion of *T. ferrooxidans* to pyrite competitively, while ferric ion scarcely inhibited such adhesion. When selective adhesion was quenched by ferrous ion completely, adhesion of *T. ferrooxidans* was controlled by hydrophilic interactions. Adhesion of *E. coli* to pyrite exhibited a liner relationship on langmuir isotherm plots, but adhesion of *T. ferrooxidans* did not. *T. ferrooxidans* recognized the reduced iron in minerals and selectively adhered to pyrite and chalcopyrite by a strong interaction other than the physical interaction.

**MATERIALS AND METHODS**

Microorganisms, medium, and conditions of cultivation. The iron-oxidizing bacterium *T. ferrooxidans* ATCC 23270 and *Escherichia coli* HB101 were used in this study. *T. ferrooxidans* was cultured in 9K medium (23), which contained (per liter of distilled water) 3.0 g of (NH₄)₂SO₄, 0.5 g of MgSO₄ · 7H₂O, 0.1 g of KCl, 0.5 g of K₂HPO₄, 0.01 g of Ca(NO₃)₂, and 42.2 g of FeSO₄ · 7H₂O; the pH was adjusted to 2.5 with 6 N H₂SO₄. Large-scale cultivation of *T. ferrooxidans* was carried out by using 7 liters of 9K medium in a 10-liter culture flask; this culture was aerated and was incubated at 30°C for 3 days. A preculture of *T. ferrooxidans* (350 ml) which had been grown for 1 week was used as the inoculum for the large-scale culture.

*E. coli* was cultured in medium containing (per liter of distilled water) 1.6 g of trypsin, 1 g of yeast extract, and 5 g of sodium chloride. The bacterium was cultured at 37°C in 100 ml of medium in a Sakaguchi flask for 2 days on a rotary shaker.

Minerals. Pyrite, chalcopyrite, quartz, and galena were used for bacterial adhesion experiments. The pyrite used was produced by the Yanahara mineral mine in Japan; the chalcopyrite used was produced by the Copper Queen mine in the United States; the galena used was produced by the Gerais mine in Brazil; and the quartz used was produced by the Honkko mine in Japan. The pyrite was supplied by Dowa Co. Ltd., Tokyo, Japan, and the other minerals were supplied by Nihon Chikagaku Co. Ltd., Kyoto, Japan.

Single-crystal minerals were used to measure contact angles. The pyrite used was produced by the Navajun mine in Spain; the chalcopyrite used was produced by the Copper Queen mine in the United States; used was produced by the galena Honko mine in Japan; and the quartz used was produced by the Gerais mine in Brazil. These single-crystal minerals were supplied as plates by Nihon Chikagaku Co. Ltd., Kyoto, Japan. The levels of purity of all of the minerals used in this study were more than 95%.

Granular distribution. Pyrite, chalcopyrite, quartz, and galena were crushed into fine particles with a mortar and
adjusted to pass through 200- but not 300-mesh sieves. The resulting mineral preparations were washed with 5 ml of pH 2.0 sulfuric acid or a 0.5% (wt/vol) sodium chloride solution several times until the supernatant was clear. This procedure removed ultrafine particles which attached to the particles. The washed minerals were suspended in a 1% sodium chloride solution. The distribution of granule sizes for each mineral preparation was determined with an ELZONE particle counter (model 8XY; Particle Data Co. Ltd., Elmhurst, Ill.). As shown in Fig. 1, the means of the granule sizes ranged from 61.7 to 64.6 μm, and for each preparation the variance was ±5%. The distributions of granule sizes for the different preparations were also almost the same. Therefore, identical volumes of the different mineral preparations had the same surface area.

Adhesion experiments. The culture medium used for *T. ferrooxidans* ATCC 23270 was filtered through filter paper (no. 2; Toyo Co. Ltd., Tokyo, Japan) to remove the precipitated ferric compounds. The filtrate was centrifuged at 15,000 × g for 15 min to collect the cells. The cells were washed three times with a pH 2.0 sulfuric acid solution and then resuspended in a pH 2.0 sulfuric acid solution.

The *E. coli* culture was centrifuged at 8,000 × g for 15 min. The sedimented cells were washed three times with a 0.5% sodium chloride solution and then resuspended in a 0.5% sodium chloride solution. The suspensions of *T. ferrooxidans* and *E. coli* cells were used for adhesion experiments.

Mineral preparations for which distributions of granule sizes were determined as above were used for bacterial adhesion experiments. Pyrite (0.13 g), chalcopyrite (0.15 g), quartz (0.067 g), or galena (0.2 g) was added to each vessel (the same volume was added to each tube; the volumes were calculated from the specific gravities). Then, 10 ml of a cell suspension containing *T. ferrooxidans* or *E. coli* was added to each tube; various cell densities were used. The contents of each tube were stirred with a vortex mixer for 1 min and then kept stationary for 5 min. Then, the supernatant was removed by decantation. After the supernatant was diluted, its cell density was measured visually by counting the cells, using a bacterial counting chamber (Kayagaki Irika Kogyo Co. Ltd., Tokyo, Japan) and a phase-contrast microscope (magnification, ×600). The number of cells that adhered to the mineral was determined by subtracting the number of cells in the supernatant from the number of cells added. The level of adhesion to the vessel wall was also determined by performing experiments without minerals to reduce the experimental error.

In order to investigate the effect of ferric or ferrous ions on bacterial adhesion, various concentrations of ferric or ferrous sulfate in a pH 2.0 sulfuric acid solution were used as suspending solutions for *T. ferrooxidans*. The cell suspensions kept stationary for 5 min after cells were added, and then the preparations were used for adhesion experiments.

A pH 2.0 sulfuric acid solution containing 20% n-propanol was used to determine the adhesion of *T. ferrooxidans* to pyrite and chalcopyrite in a hydrophobic solution by the method described above.

**Contact angle.** The contact angles of minerals were measured with mineral plates (5 by 40 by 10 mm). The plates were polished with 6-, 1-, and 0.25-μm diamond pastes (Buehler Co. Ltd., Lake Bluff, Ill.) to produce mirror surfaces before the measurements.

Cell suspensions of *T. ferrooxidans* and *E. coli* were concentrated as much as possible by centrifugation. These suspensions were spread on glass and acrylic plates, and then the plates were dried at room temperature for 4 to 5 h. The dried cells formed thin layers on the plates. A pH 2.0 sulfuric acid or 0.5% sodium chloride solution was dropped on the bacterial layer on each plate and on mineral plates (15, 18, 20, 21). The contact angles were measured with a contact angle meter (type CA-A; Kyowa Surface Science Co. Ltd., Tokyo, Japan). The contact angles were measured by using photographs which were taken immediately after dropping.

**Hydrophobicity tests in a biphasic system.** The biphasic system described previously (10, 21, 29) was used to measure cell hydrophobicity.

A 0.1- to 0.5-ml portion of *n*-hexadecane was added to each test tube containing 3 ml of a *T. ferrooxidans* cell suspension in pH 2.0 sulfuric acid or an *E. coli* cell suspension in 0.5% sodium chloride. Each mixture was stirred with a vortex mixer for 1 min and then was kept stationary 5 min for to separate the aqueous phase from *n*-hexadecane. The optical density at 660 nm of the aqueous phase in each tube was measured with a Spectronic 21 spectrophotometer (Milton Roy Co. Ltd., Rochester, N.Y.) to determine cell density.

**Zeta potential.** Zeta potentials of *T. ferrooxidans* and pyrite were determined in a pH 2.0 sulfuric acid solution by using a particle electrophoresis apparatus (Lazer Zee model 501; Pen Kem Co. Ltd., Betfort Hills, N.Y.).

**Coverage of the pyrite surface by bacteria.** According to Bergey's Manual of Systematic Bacteriology (13), each *T. ferrooxidans* cell can be regarded as a rod that is 0.5 by 1.0 μm. Each cell of *E. coli* was regarded as a rod that was 1.0 by 2.5 μm (mean dimensions of 100 cells as determined by phase-contrast microscopy). We assumed that the average cells of *T. ferrooxidans* and *E. coli* covered 0.5 and 2.5 μm² of pyrite surface, respectively. The total surface areas covered by the cells were estimated by multiplying the number of adhered cells by the area covered by a single cell. The total surface area of pyrite particles was determined by a calculation based on the assumption that each pyrite particle is a cube; the average dimension of the cube was determined from the distribution of granule sizes (Fig. 1).
RESULTS AND DISCUSSION

Hydrophobicity of minerals and bacteria. Contact angles have been used previously to express surface hydrophobicity (15, 18, 20, 21). In this study, contact angles were also used as indicators of the hydrophobicity of bacteria and minerals. The contact angles of quartz, pyrite, chalcopyrite, and galena with pH 2.0 sulfuric acid and 0.5% sodium chloride solutions were measured. The contact angles of the four minerals with the pH 2.0 sulfuric acid solution became smaller in the following order: chalcopyrite, galena, pyrite, and quartz. The hydrophobic strengths of the minerals decreased in the same order, since the contact angles with hydrophilic solutions became larger for more hydrophobic minerals. When the 0.5% sodium chloride solution was used, the largest contact angle was obtained with galena, followed by chalcopyrite, pyrite, and quartz (Table 1). The contact angles of the four minerals were greater with the 0.5% sodium chloride solution than with the pH 2.0 sulfuric acid solution.

The contact angles of T. ferrooxidans and E. coli were measured on glass and acrylic plates. The glass and acrylic plates had different hydrophilicities and were used for bacterial contact angle measurements to check the effect of plates. The contact angles of E. coli were 30.8° on glass plates and 31.6° on acrylic plates; the contact angles of T. ferrooxidans were 22.7° and 24.0°, respectively (Table 2). In both cases, the contact angles of the dried bacteria on the two types of plates were similar despite the fact that the glass and acrylic had different hydrophilicities. Therefore, the values obtained represent the correct contact angles for the dry bacteria.

The previously reported values for the contact angles of some E. coli strains range from 15.7 to 32.0° (1, 7, 29). The range of reported values might have been due to differences in experimental conditions. For example, the growth phase of the bacterium influences the surface hydrophobicity (14), and the nutrient composition of the culture medium used for bacterial growth also influences it (24). Our value for E. coli in this study was around 31° (Table 2), which agreed well with the value of 32.0° obtained by Dickson and Koohmarai (7). On the other hand, the contact angle for T. ferrooxidans has not been determined previously, and our value is the first value reported for this parameter.

In a water–n-hexadecane biphasic system (10, 21, 29) a pH 2.0 sulfuric acid solution was used as the water phase for T. ferrooxidans and a 0.5% sodium chloride solution was used as the water phase for E. coli. If the cell surface was more hydrophobic, more cells were transferred to n-hexadecane from the water phase. When the amount of n-hexadecane was increased, more E. coli cells were transferred to the n-hexadecane phase. The numbers of E. coli cells in the water phase decreased to 40% of the number of cells added when 0.5 ml of n-hexadecane was added, while 75% of the T. ferrooxidans cells remained in the water phase after 0.5 ml of n-hexadecane was added (Fig. 2). These results showed that T. ferrooxidans was more hydrophilic than E. coli and were consistent with the contact angles shown in Table 2.

Bacterial adhesion to minerals. The adhesion of T. ferrooxidans and E. coli was investigated with four minerals which had different hydrophilicities. The numbers of adhered E. coli cells became greater in the following order: galena, chalcopyrite, pyrite, and quartz. However, a lot of T. ferrooxidans cells adhered to iron-containing minerals, such as pyrite or chalcopyrite, and only a few T. ferrooxidans cells adhered to galena and quartz (Table 3).

As T. ferrooxidans showed a tendency to adhere to iron-containing minerals, the influence of reduced iron was investigated. Experiments to investigate adhesion to pyrite were carried out in the presence of ferrous ion. The adhesion of T. ferrooxidans to pyrite decreased when the concentra-

<table>
<thead>
<tr>
<th>Solution</th>
<th>Contact angle(°)</th>
<th>Quartz (SiO₂)</th>
<th>Pyrite (FeS₂)</th>
<th>Chalcopyrite (CuFeS₂)</th>
<th>Galena (PbS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 2.0 sulfuric acid</td>
<td></td>
<td>28.4 ± 4.3</td>
<td>68.9 ± 2.1</td>
<td>83.4 ± 4.5</td>
<td>80.9 ± 2.5</td>
</tr>
<tr>
<td>0.5% sodium chloride</td>
<td></td>
<td>32.5 ± 2.7</td>
<td>76.3 ± 3.5</td>
<td>88.6 ± 3.0</td>
<td>94.3 ± 2.1</td>
</tr>
</tbody>
</table>

TABLE 2. Contact angles of T. ferrooxidans and E. coli

<table>
<thead>
<tr>
<th>Plate</th>
<th>Bacterium</th>
<th>Contact angle(°)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5% Sodium chloride solution</td>
</tr>
<tr>
<td>Glass</td>
<td>T. ferrooxidans</td>
<td>13.6 ± 2.2</td>
</tr>
<tr>
<td>Acrylic</td>
<td>T. ferrooxidans</td>
<td>53.3 ± 5.1</td>
</tr>
<tr>
<td>Glass</td>
<td>E. coli</td>
<td>22.7 ± 1.9</td>
</tr>
<tr>
<td>Acrylic</td>
<td>E. coli</td>
<td>30.8 ± 1.0</td>
</tr>
</tbody>
</table>

* The contact angles of T. ferrooxidans and E. coli were determined with pH 2.0 sulfuric acid and 0.5% sodium chloride solutions, respectively.
tion of ferrous ion increased. A liner relationship was observed between the number of adhered cells and the concentration of ferrous ion in the range from zero to 20 mM (Fig. 3A). The relationship within this range showed that the adhesion of *T. ferroxidans* to pyrite was inhibited competitively by ferrous ion. In the presence of more than 50 mM ferrous ion, the linear relationship was no longer observed, and a curved line was obtained (Fig. 3B). The quenching effect of ferrous ion on adhesion was almost complete at a concentration around 200 mM, and the level of adhesion decreased from 26 to 3%. Similarly, the level of adhesion to chalcopyrite decreased from 14 to 2% when 200 mM ferrous ion was added.

On the other hand, the adhesion of *T. ferroxidans* was only slightly affected in the presence of oxidized iron. Adhesion to pyrite decreased from 24.1 × 10⁶ cells (iron free) to 18.0 × 10⁶ cells (74.7% of the iron was free) when 160 mM ferric ion was added, while adhesion decreased from 24.1 × 10⁶ cells (iron free) to 3.9 × 10⁶ cells (16.2% of the iron was free) when 160 mM ferrous ion was added (Fig. 3B). As the ion strength in the ferric ion solution was greater than the ion strength in the ferrous ion solution under the experimental conditions used, the quenching effect was due to the ferrous ion itself rather than to the ion strength in the solution.

These results suggested that there is a special interaction between *T. ferroxidans* and the reduced iron in the minerals. The surface proteins in outer membranes or fimbriae of other gram-negative bacteria were considered to be related to adhesion (11, 21, 26, 32). However, no components which mediate the interaction have been identified.

**Selective adhesion and physical adhesion.** The data in Table 1 and Table 3 were replotted in Fig. 4A. *E. coli* adhered to more hydrophobic minerals. A clear relationship was observed between the number of adhered cells and the hydrophobic strength of the minerals, as shown by the solid curve in Fig. 4A. This relationship showed that there was a hydrophobic interaction between minerals and *E. coli*, which agreed with the results of previous reports (1, 21, 25, 28). In the case of *T. ferroxidans*, high levels of adhesion to pyrite and chalcopyrite were observed. The adhesive tendency of *T. ferroxidans* is not related to the hydrophobic strength of minerals.

When the special interaction of *T. ferroxidans* with pyrite and chalcopyrite was quenched by ferrous ion, a clear relationship appeared (Fig. 4A, dashed line). In this relationship, lower levels of adhesion were observed with more hydrophobic minerals. This relationship showed that there was a hydrophilic interaction between the minerals and the bacterium. The relationship was confirmed by the results of experiments performed with a sulfuric acid solution containing 20% n-propanol (Fig. 4B). The addition of n-propanol increased the hydrophobicity of the suspending solution (6). Increased hydrophobicity of the solution should have resulted in enhancement of the hydrophilic interaction between *T. ferroxidans* and the minerals (1). In the presence of 20% n-propanol, *T. ferroxidans* also tended to adhere to more hydrophilic minerals (Fig. 4B, solid line), and the number of adhered cells became greater. The curve obtained clearly showed that there was a hydrophilic interaction.

The difference in adhesive tendencies between *E. coli* and *T. ferroxidans* is much greater than we expected from the contact angles. The contact angle of dried bacteria might not reflect the surface properties of living cells correctly, since the drying process might alter the membrane structure and its hydrophobicity. However, at the present time there is no way to measure the absolute hydrophobicity of living cells. On the basis of a comparison of the adhesion tendencies of *T. ferroxidans* and *E. coli*, living *T. ferroxidans* cells might have more hydrophilic surfaces than we estimated.

Electrostatic interactions between minerals and bacteria have been shown to affect bacterial adhesion (5, 7, 12, 21).

### Table 3. Comparison of levels of adhesion of *E. coli* and *T. ferroxidans* cells to four minerals

<table>
<thead>
<tr>
<th>Organism*</th>
<th>Cells</th>
<th>10⁶ Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control*a</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Added</td>
<td>97 ± 5.4 (100)</td>
</tr>
<tr>
<td></td>
<td>Adhered</td>
<td>7.5 ± 2.3 (7.7)</td>
</tr>
<tr>
<td><em>T. ferroxidans</em></td>
<td>Added</td>
<td>100 ± 2.3 (100)</td>
</tr>
<tr>
<td></td>
<td>Adhered</td>
<td>0 ± 0.4 (0)</td>
</tr>
</tbody>
</table>

*a The adhesion experiments were performed with a pH 2.0 sulfuric acid solution for *T. ferroxidans* and with a 0.5% sodium chloride solution for *E. coli*.

*b The levels of adhesion of cells to vessel walls were determined in the control experiment.

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**FIG. 3.** Effect of ferrous ion on the adhesion of *T. ferroxidans* to pyrite. (A) The concentrations of ferrous ion in the cell suspensions ranged from 0 to 16 mM. (B) The concentrations of ferrous ion ranged from 0 to 200 mM.
and have been expressed as zeta potentials in solutions (27, 30). The zeta potentials of pyrite and *T. ferroxidans* in the pH 2.0 sulfuric acid solution were −7.0 and −4.6 mV, respectively. As the charges were both negative, these entities repelled each other in the solution. However, *T. ferroxidans* could adhere to pyrite despite the electrostatic repulsion.

These findings clearly showed that *T. ferroxidans* could selectively adhere to pyrite. This selective adhesion was due to a stronger interaction than the hydrophobic and electrostatic interactions.

*T. ferroxidans* can selectively adhere to pyrite and chalcopyrite among various minerals. The selectivity of this organism supports its growth on mineral surfaces under environmental conditions. This selectivity might have evolved spontaneously. Our findings may clarify the ecological niche of *T. ferroxidans* under environmental conditions. On the other hand, the selective adhesion of *T. ferroxidans* may contribute to a new separation method. *T. ferroxidans* has been used in microbial flotation for coal desulfurization (2, 9, 19). In microbial flotation the bacteria can act as a selective surfactant for changing the surface properties of pyrite. The selective surfactant could be applied as an ore dressing. This is a novel use for bacteria.

**FIG. 4.** Selective adhesion of *T. ferroxidans*. (A) Values obtained from Tables 1 and 3. Symbols: O, adhesion of *E. coli* in 0.5% sodium chloride; ∆, adhesion of *T. ferroxidans* in an iron-free sulfuric acid solution; ▲, adhesion of *T. ferroxidans* in a sulfuric acid solution supplemented with 200 mM ferrous ion. (B) Experiments carried out in a pH 2.0 sulfuric acid solution containing 20% n-propanol. Symbols: ○, adhesion of *T. ferroxidans* in an iron-free sulfuric acid solution containing 20% n-propanol (□); ▲, adhesion of *T. ferroxidans* in a solution containing 20% n-propanol supplemented with 200 mM ferrous ion. The dashed line is the same line shown in panel A. The arrows indicate the quenching effect of ferrous ion on the adhesion of *T. ferroxidans*.

**FIG. 5.** Langmuir isotherm plots for the adhesion of *T. ferroxidans* and *E. coli* to pyrite. (A) Relationship between the concentration of added cells and number of cells that adhered to pyrite. (B) Reciprocals of the data in panel A replotted as langmuir isotherm plots. Symbols: ●, *T. ferroxidans*; ▲, and *E. coli*.

**Adhesion of *E. coli* and *T. ferroxidans* to pyrite.** The adhesion of *E. coli* and *T. ferroxidans* to pyrite was examined at various cell densities. For both bacteria, the number of adhered cells increased when the number of cells of added increased (Fig. 5A). The adhesion to pyrite became saturated at an *E. coli* density of 9.0 × 10⁸ cells per ml and a *T. ferroxidans* density of 10.0 × 10⁶ cells per ml. Under these conditions, the total numbers of *T. ferroxidans* and *E. coli* cells that adhered to pyrite were 2.3 × 10⁹ and 1.2 × 10⁹ cells, respectively. The total surface area of pyrite particles was 2.58 × 10⁹ µm², based on the calculation in which approximate granular size was used. On the basis of these values, we calculated that the levels of coverage of the pyrite surface (coverage ratios) for *E. coli* and *T. ferroxidans* were 120 and 44.6%, respectively.

The high coverage ratio value for *E. coli* at the saturated density showed that the cells adhered not in a single layer but multiple layers. The multiple layers might be explained by the aggregation of *E. coli* cells since hydrophobic cells were in a hydrophobic solution. However, the coverage ratio was close to 100%. The influence of aggregation on adhesion should have been small, and the aggregation effect should have been negligible under these experimental conditions. On the other hand, the low coverage ratio value for *T. ferroxidans* indicated that its cells were distributed in a single layer. As *T. ferroxidans* cells had hydrophilic and negatively charged surfaces, they repelled and disturbed each other, even though the interaction of *T. ferroxidans*...
with pyrite was stronger than the hydrophobic interaction. Therefore, the coverage ratio should have been less than 100%. If *T. ferrooxidans* cells adhere to pyrite when the cells disturb their adhesion to each other, the minimum coverage ratio should be 32%, as shown in Fig. 6. The measured value was larger than but close to 32%.

The values in Fig. 5A were replotted as a langmuir isotherm in Fig. 5B. The plots for *E. coli* were consistent with the langmuir isotherm, and a linear relationship was observed between the reciprocal of the number of adhered cells and the reciprocal of the number of added cells. On the other hand, a linear relationship was not observed with *T. ferrooxidans* cells.

The linear relationship means that the adhesion of *E. coli* to pyrite is isothermal adhesion. This adhesion is due to the hydrophobic interaction; this means that isothermal adhesion of *E. coli* is based on the hydrophobic interaction. On the other hand, *T. ferrooxidans* cells interact strongly with pyrite. This strong interaction is responsible for much adhesion of *T. ferrooxidans* cells to pyrite at a low cell density. As a result, the plots did not reveal a linear relationship with the langmuir isotherm.

REFERENCES