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# Partial Removal of Lipopolysaccharide from *Thiobacillus ferrooxidans* Affects Its Adhesion to Solids

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**Conditions for the partial removal of lipopolysaccharide (LPS) from *Thiobacillus ferrooxidans* are described. Raising the pH of the solution containing the cells from pH 1.5 to pH 6.8 to 8.0 releases about 50% of the LPS without cell lysis. The release of LPS begins at pH 3.5, and it was not affected by EDTA concentration. Partial removal of LPS exposed higher amounts of a 40-kDa outer membrane protein in the bacteria, as detected by a dot immunoassay employing an antiserum against the *T. ferrooxidans* surface protein. This higher protein exposure and the reduced LPS content increased the hydrophobicity of the cell surface, as determined by an increased adhesion (50%) to hydrophobic sulfur prills and $^{14}$C-dodecanoic acid binding (2.5-fold) compared with control cells. In addition, adhesion of radioactively labeled microorganisms to a sulfide mineral was inhibited (40%) in the presence of previously added LPS. Our results suggest that not only LPS but also surface proteins probably play important roles in *T. ferrooxidans* adhesion to solid surfaces.**

*Thiobacillus ferrooxidans* is a gram-negative chemolithotrophic bacterium active in the bioleaching of minerals (7). Direct and indirect mechanisms have been proposed for the bacterial dissolution of sulfide minerals (39). By the direct mechanism, bacteria would attack the mineral enzymatically; therefore, intimate contact with and adhesion of the microorganism to the solid are required. Bacterial adhesion to these minerals is an initial step in oxidation (7). *T. ferrooxidans* has been shown to adhere to the surfaces of various solid substrates such as sulfur (13, 32), mineral sulfides (11, 27), and coal (5). In general, a nonspecific binding to these substrates has been explained in terms of hydrophobicity and the electrical charge of the bacterial cell surface (40). Very recently, both hydrophilic (28) and hydrophobic (11, 36) interactions between *T. ferrooxidans* and minerals have been shown.

The main components of the cell surface of gram-negative bacteria that may contribute to the adhesion properties of the microorganism are lipopolysaccharide (LPS) and outer membrane proteins (24). In this regard, it has been shown that LPS polymers may play a role in the interaction of some microbial cells with solid surfaces (10, 21, 33). LPS has been characterized in *T. ferrooxidans* (15, 38, 41, 42), and it was found that treatment of the microorganism with 0.5 M sucrose decreased its rate of adhesion and the density of cells that adhered to several surfaces tested (12). Sucrose treatment removes the LPS from the cell wall of marine pseudomonad B-16 (14). However, that the LPS was actually removed from *T. ferrooxidans* by this treatment was not demonstrated (12).

In other microorganisms, surface proteins have been implicated in adhesion of the bacteria to solids (1, 8, 6, 26, 30). In *T. ferrooxidans*, some outer membrane proteins have been partially characterized (19, 30). A 40-kDa major outer membrane protein (Omp40) has been described (19, 35). The expression of this poplypeptide is regulated by environmental conditions (19), and the protein is able to form low-conductance ion channels in planar lipid bilayers (35). However, the possible role of this surface protein in the bacterial attachment to minerals has not been analyzed. Very recently, however, it has been suggested that a proteinaceous new cell surface component is synthesized in mineral-grown *T. ferrooxidans* cells, bringing about adhesion to the solid substrates (11).

In the present report, we have studied the conditions for the partial removal of LPS from *T. ferrooxidans* and the effect of this treatment on exposure of outer membrane proteins and adherence of the microorganisms to solid substrates.

## MATERIALS AND METHODS

**Bacterial strain and growth conditions.** *T. ferrooxidans* R2 (30) was used in these studies. It was grown in modified 9K medium containing ferrous iron, as described before (2, 4, 18), or in sulfur prills (13). Cells were labeled in the presence of $Na_2{}^{14}CO_3$ as described before (2).

**Immunological procedures.** A dot immunoassay to estimate the total amount of outer membrane protein Omp40 present in *T. ferrooxidans* cells (19, 35) was similar to our previously reported dot immunoassay (4), except that an antiserum against Omp40 from *T. ferrooxidans* R2 (kindly supplied by M. Rodríguez, Universidad Católica de Chile) was used as the primary antibody (1:3,000 dilution) and immunoglobulin conjugated with peroxidase was used as the secondary antibody. Densitometric tracings of the dots and quantification of the immunological reaction were done by using a Hewlett-Packard Scan Jet Plus device and the GelPerfect image analysis program (kindly provided by S. Bozzo, Universidad de Chile).

**SDS-PAGE and autoradiography.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) used 7.5 to 15% or 11.5 to 20% polyacrylamide gradients in the absence of urea followed by autoradiography, as described previously (2, 4).

**Extraction of LPS.** Radioactively labeled or unlabeled *T. ferrooxidans* cells ($10^{10}$ cells per ml) were incubated in the presence of 50 mM Tris-HCl (pH 8.0) for 2 h at 30°C. After this treatment, the cells were centrifuged at 12,000 × *g* for 10 min, and the supernatant containing the LPS was used directly or after previous concentration by freeze-drying for SDS-PAGE analysis or adhesion and competition studies.

**Effect of EDTA and pH on LPS removal.** To study the effect of EDTA concentration on LPS removal from *T. ferrooxidans*, radioactively labeled cells were incubated for 1 h at 30°C in the absence or in the presence of increasing EDTA concentrations

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ranging from 10 to 100 mM in 50 mM Tris-HCl buffer at pH 8.0. After these incubations, each sample was centrifuged to pellet the cells. The supernatants were previously subjected to proteinase K digestion or were directly analyzed by SDS-PAGE (7.5 to 15%) and autoradiography. The effect of different pHs on LPS removal from *T. ferrooxidans* was studied by incubating radioactively labeled cells for 1 h at 30°C at different pH values ranging from 1.5 to 8.0. After these incubations, each sample was centrifuged to pellet the cells. Some of the supernatants were subjected to proteinase K digestion and were then analyzed by SDS-PAGE and autoradiography. To determine the extent of LPS removal by the pH 6.8 extraction, radioactively labeled cells were incubated for 1 h at 30°C and pH 6.8. After this incubation, cells were pelleted by centrifugation. The cell pellet and the supernatant were then subjected to proteinase K digestion and analyzed as described above.

**Adhesion studies.** A sulfide ore from El Teniente copper mine, Chile (particle size, 0.104 to 0.417 mm), was employed; it was kindly supplied by Tomás Vargas (Universidad de Chile). It contained 1.0% (wt/wt) Cu and 5.8% (wt/wt) Fe, determined by chemical analysis. Mineralogical analysis by light reflection in an optical microscope indicated that copper was mainly present as chalcopyrite, with much smaller amounts of chalcocite and covellite. Pyrite was the main iron-containing species. Radioactively labeled cells ($10^8$) grown in ferrous iron medium were incubated in a 15-ml borosilicate glass scintillation vial with 100 mg of the sulfide ore in 3 ml of sulfuric acid at pH 1.5 for 2 h at 30°C. After incubation, the fluid was decanted from the mineral, and the remaining solid particles at the bottom of the vial, which contained the radiolabelled adherent cells, were subjected to four 3-ml washings by hand stirring in sulfuric acid at pH 1.5 and room temperature. The radioactivity associated with the particles was determined by scintillation counting. For this, 5 ml of a toluene-Triton X-100 scintillation fluid containing 2,5-diphenyloxazole-1,4-bis(2-[5-phenyloxazolyl])benzene was directly added to the vial containing the ore with the attached bacteria. There was no significant quenching by the mineral particles under the conditions employed. The percentage of cell adhesion to the ore was calculated from curves correlating the number of cells with their radioactivity.

For determination of the kinetics of adhesion of *T. ferrooxidans* to elementary sulfur, we employed spherical sulfur particles (sulfur prills) according to the procedure of Espejo and Romero (13), except that the prills were adjusted to pass through an appropriate sieve to have a uniform diameter of 2 mm. Nonradioactive control or pH 6.8-extracted cells ($10^8$) grown in elemental sulfur were added to 20 prills in 3 ml of 9K medium salts without ferrous iron at pH 2.5 and were incubated for 1 h at 30°C with gentle rotary shaking (50 rpm). To determine the degree of adhesion to the borosilicate glass container, control vials without added solids were also employed. The numbers of cells remaining in suspension were estimated at different times by using a Petroff-Hausser counting chamber. To estimate the number of cells that adhered per surface unit, we determined the total surface area of the prills by a calculation based on the assumption that they were perfect spheres. For the glass container, the surface area in contact with the cell suspension was estimated from its geometrical shape.

To measure the binding of radioactive LPS to the solids or for competition experiments, the same procedure was employed, except that the cells were replaced by LPS. The amount of LPS used was estimated by measuring its radioactivity.

**Proteinase K digestion.** Proteinase K digestion of whole cells or of the supernatant containing the extracted LPS was carried out as described before (18) and essentially as described by Hitchcock and Brown (17). The cells were resuspended in sample buffer (0.0625 M Tris-HCl [pH 6.8], 10% [vol/vol] glycerol, 2% [wt/vol] SDS, 0.05% [vol/vol] 2-mercaptoethanol, 0.00125% [wt/vol] bromophenol blue), and the suspension was heated at 100°C for 5 min. Twenty-five micrograms of proteinase K was added to 100 μg of protein (type XI protease adjusted to 2.5 mg/ml in sample buffer; Sigma), and the mixture was heated at 60°C for 2 h.

**Binding of $^{14}$C-dodecanoic acid to *T. ferrooxidans* cells.** To estimate the relative hydrophobicity of *T. ferrooxidans* cells, we employed the method described by Kjelleberg et al. (20), except that a nitrocellulose membrane was used as a solid support for the bacterial cells. Between $10^6$ and $10^8$ control or pH 6.8-extracted cells were applied to the membrane by means of a commercial Bio-Dot apparatus (Bio-Rad Laboratories), and after drying at room temperature to fix the cells, the membrane was incubated in a Parafilm-sealed petri dish containing 10 ml of a salt solution at pH 2.5 and containing 0.1 g of $(NH_4)_2SO_4$, 0.4 g of $MgSO_4 \cdot 6H_2O$, and 0.052 g of $K_2HPO_4 \cdot 3H_2O$ per liter and about 134,000 cpm of 1-$^{14}$C-dodecanoic acid (58 Ci/mmol; Amersham International, S.A.) added in 5 μl of methanol. The membrane was incubated at 30°C for 12 h with gentle rotary shaking. This incubation was followed by three washes of the membrane in a petri dish containing 10 ml of salts, with shaking for 10 min each time. Finally, each of the dots (included in the center of 1 cm$^2$ of membrane) was cut out and subjected to scintillation counting. The radioactivity bound to equivalent pieces of membrane without attached cells was subtracted from each point. The number of cells released from the nitrocellulose membranes during the assay was similar in control and pH 6.8-extracted cells and was determined by using radiolabeled cells in the same assay conditions, except that 1-$^{14}$C-dodecanoic acid was omitted.

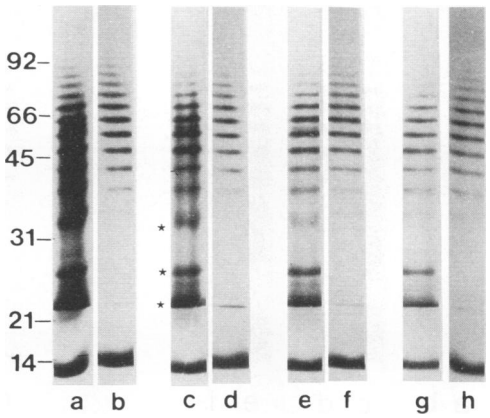


FIG. 1. Effect of EDTA concentration on LPS removal from *T. ferrooxidans*. Radioactively labeled *T. ferrooxidans* cells were incubated for 1 h at 30°C in the absence (lanes a and b) or in the presence of 10 (lanes c and d), 50 (lanes e and f), or 100 (lanes g and h) mM EDTA in 50 mM Tris-HCl buffer, pH 8.0. After the cells were pelleted, the supernatants were subjected to proteinase K digestion (lanes b, d, f, and h) or were directly (lanes a, c, e, and g) analyzed by SDS-PAGE (7.5 to 15%) and autoradiography. Numbers to the left are molecular mass standards for proteins, in kilodaltons. Asterisks indicate some proteins degraded by proteinase K.

## RESULTS

**Effect of EDTA on removal of LPS (O antigen) from *T. ferrooxidans*.** Figure 1 shows that incubation of *T. ferrooxidans* cells with increasing concentrations of EDTA removed LPS, as judged by the electrophoretic migration of the typical ladder-like pattern of this compound (18, 42). In addition, some proteins were also extracted by this treatment. The proteins were degraded by proteinase K (for example, compare bands indicated by asterisks in lanes c and d, Fig. 1). It is possible that the release of protein could be due to lysis of some of the bacterial cells. However, only very few protein bands were present before the proteinase K treatment, suggesting that lysis of the bacteria upon EDTA treatment was negligible. When increasing concentrations of EDTA were employed, the same result was obtained. Furthermore, in the absence of EDTA, the same components were removed (lanes a and b, Fig. 1), indicating that the LPS and proteins were probably removed by the pH change during incubation with the Tris-HCl buffer and not due to the presence of EDTA.

**Effect of pH on removal of LPS from *T. ferrooxidans*.** Cells were incubated in 0.01 N sulfuric acid (pH 1.5) or 50 mM Tris-HCl (pH 6.8 and 8.0), and the release of LPS was analyzed by gel electrophoresis. The results of this experiment are shown in Fig. 2A. At pH 1.5, there was no release of radioactive material from *T. ferrooxidans* (lanes a and b). However, at pH 6.8 (lanes c and d) and 8.0 (lanes e and f), there was a considerable release of LPS. This release accounts for up to 50% of the total radioactivity initially present in the cells as LPS when they were incubated at pH 6.8 (Fig. 2B, lanes g and h). Cells extracted at pH 6.8 grew upon subculture on ferrous iron, although at a highly reduced rate (not shown), indicating that some damage was produced in the cells. This was also observed during treatment of *T. ferrooxidans* with sucrose (12). *T. ferrooxidans* normally grows at between pH 1.5 and 4.0. Therefore, it was of interest to determine the pH at which the LPS start to be released. Figure 3 shows that at a pH as low as 3.5 there was release of LPS.

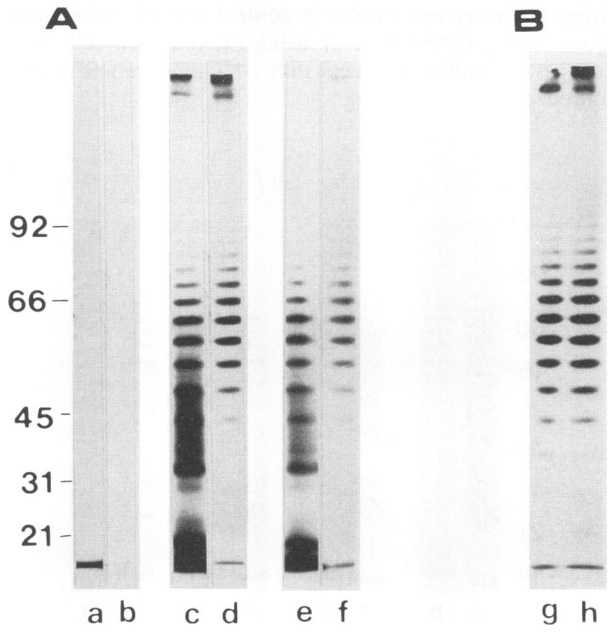


FIG. 2. Effect of different pHs on LPS removal from *T. ferrooxidans*. (A) Radioactively labeled cells were incubated at pH 1.5 (lanes a and b), 6.8 (lanes c and d), or 8.0 (lanes e and f). After the cells were pelleted, the supernatants were left untreated (lanes a, c, and e) or were subjected to proteinase K digestion (lanes b, d, and f) and were then analyzed by SDS-PAGE and autoradiography. (B) Determination of the extent of LPS removal by the pH 6.8 extraction. Radioactively labeled cells were incubated for 1 h at 30°C and pH 6.8. The cell pellet (lane g) and the supernatant (lane h) were then subjected to proteinase K digestion and analyzed as described in the legend to Fig. 1.

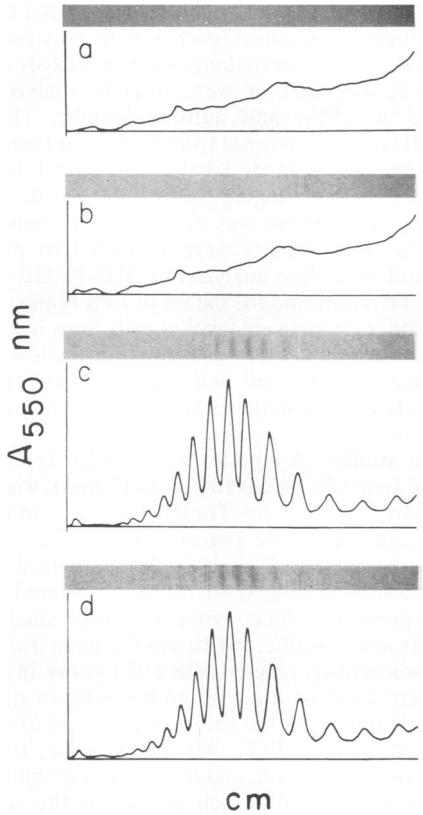


FIG. 3. Release of LPS from *T. ferrooxidans* in the acid pH range. Radioactively labeled cells were incubated for 1 h at 30°C at pH 1.5 (a), 2.5 (b), 3.5 (c), and 5.0 (d). After these incubations, each sample was centrifuged to pellet the cells. The supernatants were subsequently subjected to proteinase K digestion, and the final products were analyzed by SDS-PAGE and autoradiography. The X-ray films and their corresponding densitometric tracings are shown.

**Effect of pH extraction on reactivity with anti-Omp40 antiserum.** The removal of LPS from the cell surface could expose some of the outer membrane proteins, changing the surface characteristics of the cells. To analyze the degree of exposure of an outer membrane protein from *T. ferrooxidans* on the surfaces of control and pH 6.8-extracted *T. ferrooxidans* cells, we measured Omp40 by employing a dot immunoassay with a polyclonal antibody against Omp40 from *T. ferrooxidans* (19, 35). We can see (Fig. 4) that the cells extracted at pH 6.8 under the conditions that remove part of their LPS exhibited a more intense reaction (30 to 40%) with the antiserum against *T. ferrooxidans* Omp40 compared with the control cells (for example, compare the relative absorbance in dots b and e, Fig. 4).

**Kinetics of attachment of treated *T. ferrooxidans* to sulfur prills.** To establish if the observed removal of LPS and the increased exposure of outer membrane protein affect the ability of *T. ferrooxidans* to adhere to a solid substrate, we measured the sorption rates of *T. ferrooxidans* to the hydro-

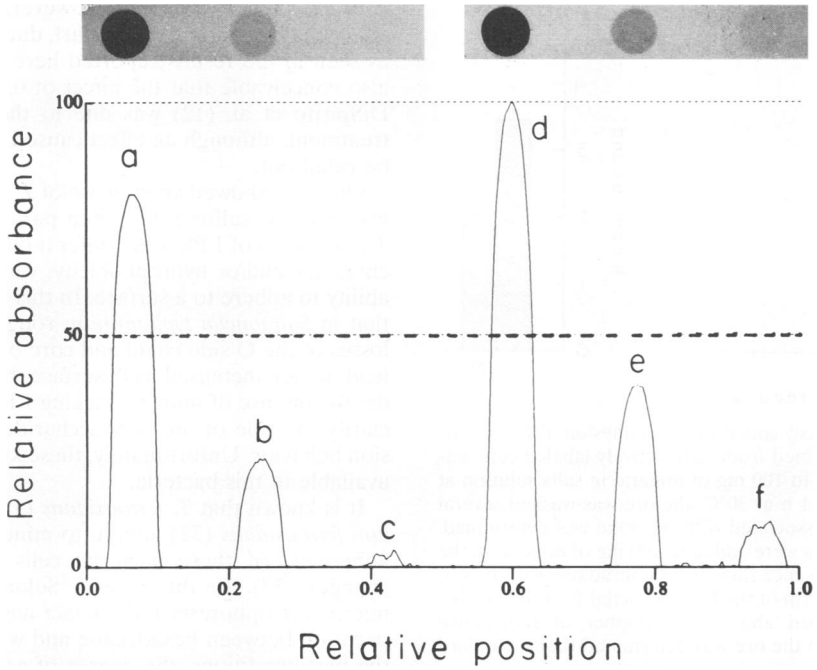


FIG. 4. Dot immunobinding assay of Omp40 in pH 6.8-extracted and control *T. ferrooxidans* cells. Control (dots a, b, and c) and pH 6.8-extracted (dots d, e, and f) cells were applied to a nitrocellulose membrane and reacted and developed with anti-Omp40 serum as described in Materials and Methods. The number of cells applied were as follows: dots a and d, $6 \times 10^6$; dots b and e, $6 \times 10^5$; dots c and f, $10^5$. The densitometric tracings of the dots were obtained as described in Materials and Methods.

phobic solid, elemental sulfur. The pH 6.8-extracted microorganisms showed higher rates of sorption to the sulfur prills than control cells, resulting in 50% more adhesion to sulfur at 60 min of incubation (Fig. 5). On the other hand, the levels of adhesion to the hydrophilic borosilicate glass container were similar in both kinds of microorganisms. This result indicates that the cells that have been subjected to alkaline pH extraction and have lost part of their surface components have an increased capacity to adhere to hydrophobic sulfur.

**Effect of externally added LPS on attachment of *T. ferrooxidans* to an ore.** To find out if the LPS released from the cells by the pH 6.8 extraction competes with whole cells for their binding to an ore, we first measured the interaction between the released LPS and the mineral. As seen in Fig. 6A, the LPS itself is capable of binding to the ore. In addition, when the extracted LPS was first incubated with the ore, and then the bacteria were added, there was an inhibition of cell attachment to the particles (up to 40%) (Fig. 6B).

**Binding of hydrophobic dodecanoic acid by *T. ferrooxidans* cells.** Since the pH 6.8-extracted cells appeared to have a higher hydrophobicity, as shown by their increased adhesion to sulfur prills, we measured the capacity of *T. ferrooxidans* to bind $^{14}$C-dodecanoic acid. As seen in Fig. 7, control cells bound lauric acid, in agreement with their slightly hydrophobic character at low pH (34). On the other hand, the cells with partially removed LPS bound about 2.5-fold more dodecanoic acid than control cells, supporting the notion that the hydrophobicity of pH 6.8-extracted cells was increased.

## DISCUSSION

It has been reported that treatment of *Escherichia coli* with EDTA results in loss of LPS (30 to 50%) and of minor amounts of proteins and phospholipids (22, 23) without cell

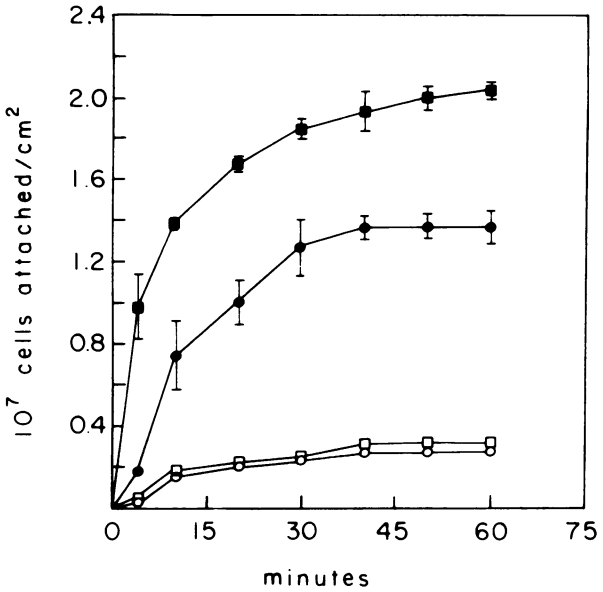


FIG. 5. Kinetics of adhesion of *T. ferrooxidans* to elementary sulfur. Control cells (○, ●) and pH 6.8-extracted bacteria (■, □) were incubated at pH 2.5 in the presence (●, ■) or in the absence (○, □) of sulfur prills, and the numbers of cells remaining in the solution were determined by direct microscopic counting. A total cell number of $10^8$ was employed in each case, and the total percentages of cells adhering at 60 min of incubation were 32 and 50% for the control and pH 6.8-extracted cells, respectively (bars, standard deviation; $n = 5$).

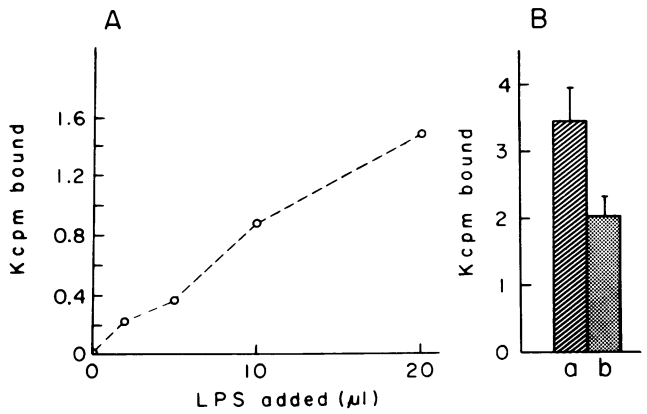


FIG. 6. Effect of exogenously added LPS on adhesion of *T. ferrooxidans* to an ore. (A) LPS obtained from radioactively labeled cells was added in increasing amounts to 100 mg of mineral in salts solution at pH 3.5. After incubation for 1 h at 30°C, the ore was washed several times, and the radioactivity associated with the solid was determined. (B) Radioactively labeled cells were added to 100 mg of mineral in the absence (lane a) or in the presence (lane b) of nonradioactive LPS (an amount equivalent to one-fourth of the LPS extracted from $10^{10}$ cells). After incubation as described above, the number of radioactive bacterial cells that adhered to the ore was determined (bars, standard deviation; $n = 3$).

lysis (25). Also, the antigens extracted by EDTA treatment of *Legionella pneumophila* are LPS (29). However, our results show that EDTA has no effect on LPS removal from *T. ferrooxidans*, and it is the pH increase that causes its release. Nonetheless, the pH 6.8 extraction we describe appears to be a good and simple alternative method to prepare LPS (O antigen) from *T. ferrooxidans*.

Cell components such as LPS or other extracellular polymers may mediate bacterial adhesion to a solid surface (11, 31). If LPS are involved in the adhesion of *T. ferrooxidans* to solid substrates, removal of part of this polymer should decrease sorption rates, as suggested previously by the results of DiSpirito et al. (12). Our preliminary results of inhibition of cell adhesion to a mineral by previously added LPS would also indicate that the LPS may play a role in *T. ferrooxidans* attachment to a solid.

Since charge character is pH dependent, neutral versus acidic environmental pHs would affect the ability of *T. ferrooxidans* to adhere to a surface. However, it is also possible that pH changes affect adherence, in part, due to partial release of LPS, as seen in the results reported here. On the other hand, it is also conceivable that the effect of 0.5 M sucrose observed by DiSpirito et al. (12) was due to the pH change during the treatment, although an effect caused by the sugar itself cannot be ruled out.

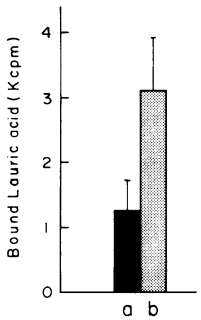


FIG. 7. Binding of $^{14}$C-dodecanoic acid to *T. ferrooxidans* cells. A total of $10^8$ control (a) or pH 6.8-extracted (b) cells were applied to a nitrocellulose membrane, and the membrane was incubated in the presence of radioactive dodecanoic acid (bars, standard deviation; $n = 3$).

Our data showed an increase of *T. ferrooxidans* adherence to hydrophobic sulfur prills when part of its LPS was removed. The removal of LPS may confer a different cell surface charge character and/or hydrophobicity, which could affect the cell's ability to adhere to a surface. In this regard, it has been shown that in *Salmonella typhimurium* rough mutants the sequential losses of the O side chain and core oligosaccharide monomers lead to an increased cell surface hydrophobicity (15). Evidently, the use of mutants lacking LPS should be employed to clarify the role of the polysaccharide in *T. ferrooxidans* adhesion behavior. Unfortunately, these kind of mutants are not yet available in this bacteria.

It is known that *T. ferrooxidans* (11, 13, 27) and *Leptospirillum ferrooxidans* (32) adhere to mineral surfaces and that the adherence of these bacterial cells can be affected by pH changes (37). In this regard, Solari et al. (36) showed by microelectrophoresis and contact angle and measurements of partition between hexadecane and water on day 2 that, under the best conditions, the degree of adhesion of *T. ferrooxidans* per unit area was larger to hydrophobic surfaces (mineral sulfides) than to hydrophilic quartz and that *T. ferrooxidans* cells themselves are slightly hydrophobic. The hydrophobicity of *T. ferrooxidans* has also been implicated in cell adhesion to minerals (11, 36).

The higher sorption rates that we observed for the pH 6.8-extracted *T. ferrooxidans* cells could be due to a higher exposure of surface proteins favoring hydrophobic or other interactions. In this regard, surface proteins have been implicated in adhesion of some microorganisms to solids (1, 6, 8, 9, 26, 30).

We have previously found that phosphate-starved *T. ferrooxidans* cells have greatly induced levels of outer membrane proteins of various sizes in their surface (19, 34), and these cells also showed a higher adhesion to sulfur prills and sulfides, apparently due to an increased hydrophobicity (3). This was supported by our measurements of $^{14}$C-dodecanoic acid binding to *T. ferrooxidans* cells, by adapting the assay of Kjelleberg et al. (20) previously employed to determine the hydrophobicity of bacterial cells. The binding of the hydrophobic fatty acid was done at pH 2.5. Therefore, the carboxyl group should be almost entirely protonated, giving a good measure of hydrophobic interaction under our conditions of measurement.

Hydrophobic interactions are not the only factor in determining the degree of adhesion of microorganisms, since they are themselves charged to varying degrees (6, 28). Further analysis of *T. ferrooxidans* surface components will be required to understand better the mechanism of adhesion of the bacteria to ores, since this probably involves the participation of other components (31, 38) in addition to LPS and surface proteins. Adhesion of *T. ferrooxidans* to ores is important for their bioleaching activity (11, 28, 38, 39), and our studies indicate that growth conditions affect their surface components and their adhesion abilities. Therefore, it may be important to pay attention to growth conditions in bioleaching operations in order to maximize bioleaching capabilities.

## ACKNOWLEDGMENTS

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