Journal of Industrial Microbiology & Biotechnology (1999) 22, 88–92

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The role of exopolymers in the bioleaching of a non-ferrous metal sulphide

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Exocellular polysaccharides were extracted from *Thiobacillus ferrooxidans* cells grown in the presence of iron. Cells without these compounds could not adhere to covellite. The loss of the layer of exocellular polysaccharides also affected the direct mechanism of bioleaching of covellite in a negative way. This ability to attach to and leach covellite was restored within a few hours when exopolymeric material was produced again. The addition of exocellular compounds to cells stripped of exocellular polymers also restored their ability to the same level as that of untreated cells. *Thiobacillus thiooxidans* was not able to attach to and leach covellite even when exocellular compounds from *Thiobacillus ferrooxidans* were added.

Keywords: exopolymers; *Thiobacillus ferrooxidans*; covellite; bioleaching

Introduction

The ability of acidophilic bacteria to assist in the recovery of metals by the dissolution of sulphide minerals is well known but the mechanism is not fully understood. Although a variety of bacteria are involved in the leaching process, *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* are the most effective microorganisms [2,8].

Two different mechanisms have been proposed to explain bacterial attack by *T. ferrooxidans*: a direct one and an indirect one. The direct mechanism is based on catalytic sulphide oxidation, while the indirect one implies sulphide oxidation by ferric ions. Both products, sulphur and ferrous ion are oxidised by the microorganisms allowing the iron redox cycle to be repeated. *T. ferrooxidans* is also able to use other terminal electron acceptors under anaerobic conditions [6].

T. thiooxidans oxidises elemental sulphur but not ferrous ions and is therefore unable to degrade sulphide minerals by the indirect mechanism described for *T. ferrooxidans*. Moreover, the ability of *T. thiooxidans* to oxidise sulphides has been demonstrated only for more soluble sulphides [12,17,18].

Because of the insolubility of metal sulphides, direct bacterial attack must be initiated by adhesion of cells to the substrate surface. Physical evidence of the adsorption of *T. ferrooxidans* onto mineral surfaces has been described in several papers [3,14,16,23]. The surface properties of microorganisms influence the attachment of bacteria to ores [4]. Thus the partial loss of surface lipopolysaccharides affects the adhesion of *T. ferrooxidans* [1,9].

Recently, Gehrke *et al* [10] demonstrated that *T. ferrooxidans* possesses an extracellular layer of polysaccharides, proteins and lipids (exopolymeric substances, EPS). They also showed that EPS was necessary for attachment and subsequent leaching of pyrite. Furthermore, the attachment ability was restored by addition of ferric ions to cells stripped of EPS (EPS-deficient cells).

These studies were carried out using pyrite, which contains iron, allowing both mechanisms of bacterial action. Thus it is difficult to determine how the addition of ferric ion initiates the dissolution of pyrite. Gehrke *et al* [10] assumed that they stimulated the rapid re-establishment of an EPS layer by EPS-deficient cells. They also proposed that ferric ions were entrapped in the EPS layer allowing bacterial attachment to and leaching of pyrite. However, the addition of ferric ion could produce an initial chemical oxidation of pyrite generating a series of intermediate compounds (ferrous ion, polythionates) which can be used by *T. ferrooxidans* [20]. The production of ferric ion would continue the dissolution of pyrite.

In the present work, we have studied the role of exocellular polysaccharides on the attachment and the dissolution of non-ferrous sulphide (synthetic covellite, CuS) by *T. ferrooxidans*. We also investigated whether *T. thiooxidans* was able to attach to and leach covellite when EPS from *T. ferrooxidans* was added to the culture.

Materials and methods

Bacteria

A *T. ferrooxidans* strain from Santa Rosa de Arequipa (DSM 11477) was used. Bacteria were grown in 9 K medium [21] at pH 1.8 and harvested during the late logarithmic growth phase (85–90% of ferrous iron oxidised). After removal of jarosite by filtration through blue ribbon filter paper, the culture suspension was filtered through a 0.22-μm pore size filter (Nuclepore, Osmonics Lab, Minnesota, USA) and washed several times with acidified water (pH 1.5) to eliminate soluble ferric iron. Finally, the bacterial pellet was suspended in iron-free 9 K medium and used as an inoculum (containing approximately 3×10^8 cells ml^{-1}).

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Received 2 June 1998; accepted 8 January 1999

A *T. thiooxidans* strain from Minais Gerais (DSM 11478) was also used. The organism was propagated in iron-free 9 K medium at an initial pH of 2.0 with powdered sulphur (10 g L^{-1}) as the energy source. After removal of sulphur by filtration through blue ribbon filter paper, bacteria were harvested at a pH of about 1.0, suspended in medium and used as inoculum. In the same way, *T. ferrooxidans* inocula were obtained from cultures using sulphur as the energy source. In both cases the bacterial population in the inoculum was approximately 3×10^8 cells ml^{-1}.

T. ferrooxidans cells growing on iron(II) or elemental sulphur were treated by centrifugation ($12\,000 \times g$, for 25 min), a treatment known to strip EPS from the cells [10,18,19], and re-suspended in medium at a density of 3×10^8 cells ml^{-1}. The resultant supernatants containing EPS, were dialysed against acidified distilled water (pH 1.5) for 24 h (Spectra/por dialyser tubing; cut-off 12 000 Da) [10,22]. Cells collected by this procedure were defined as 'treated' or 'EPS-deficient' cells. Cells collected by filtration without further centrifugation (thus retaining EPS) were designated as 'untreated' cells.

Attachment

Iron-free 9 K medium (2.5 ml) and 10 mg of synthetic covellite (99% pure, particle size $<74\ \mu m$, from Strem Chemicals Inc, Newburyport, USA) were added to 2.5 ml of bacterial suspension containing either *T. ferrooxidans* (untreated or treated) or *T. thiooxidans*. Similar experiments were carried out with 10 mg of sulphur (powder, 100 mesh from Carlo Erba) instead of covellite. The test tubes were incubated at 30°C and 200 rpm for 15 min. The mixtures were then filtered through black ribbon filter-paper and the number of cells remaining in suspension was determined by direct microscopic counting with a Petroff-Hausser chamber. All experiments were carried out at least twice and the standard deviations were equal to or less than 7.5%.

In some experiments, 2.5 ml of treated *T. ferrooxidans* was exposed to an equal volume of extracted EPS for 2 min before addition of covellite. In other experiments, covellite was previously exposed to 2.5 ml of extracted EPS for 2 min before addition of 2.5 ml of treated cells. These cells were subsequently incubated at 30°C and 200 rpm for 15 min, as before.

Media (pH 1.8) used in the experiments were sterilised by filtration through a 0.22-μm pore size bacterial filter.

The number of cells that adhered to the solids was calculated by subtracting the cells remaining in the supernatant from the total number of cells added. Cell removal by the glass walls of the test tubes was determined in control tests in the absence of solids, and was taken into consideration when calculating the percentage of attachment to solids.

Leaching

Leaching was carried out in 250-ml flasks with 95 ml of iron-free 9 K medium at pH 1.8. Medium was previously sterilised by filtration (through a 0.22-μm pore size bacterial filter). Synthetic covellite (200 mg; 0.2% pulp density) was added to each flask. Flasks were inoculated at 5% v/v with *T. ferrooxidans* (untreated or treated cells) or *T. thiooxidans* to give an initial bacterial population equal to 1.5×10^7 cells ml^{-1}. In some cases inocula of EPS-deficient cells or *T. thiooxidans* were exposed to EPS suspension (from a similar bacterial population) and then added to the cultures with covellite. Flasks were incubated in an orbital shaker at 180 rpm and 30°C.

Sterile controls were done with inocula replaced by the same volume of medium (or EPS); except for this, conditions were identical. All treatments were carried out at least in duplicate.

Analytical methods

Dissolved copper was analysed by atomic absorption spectrophotometry. Total polysaccharides were estimated by the phenolsulphuric method [7] using glucose as standard.

Results

The extent of cell adhesion to covellite is shown in Figure 1. A decrease of attachment of *T. ferrooxidans* to covellite was produced by the loss of EPS. This property could be

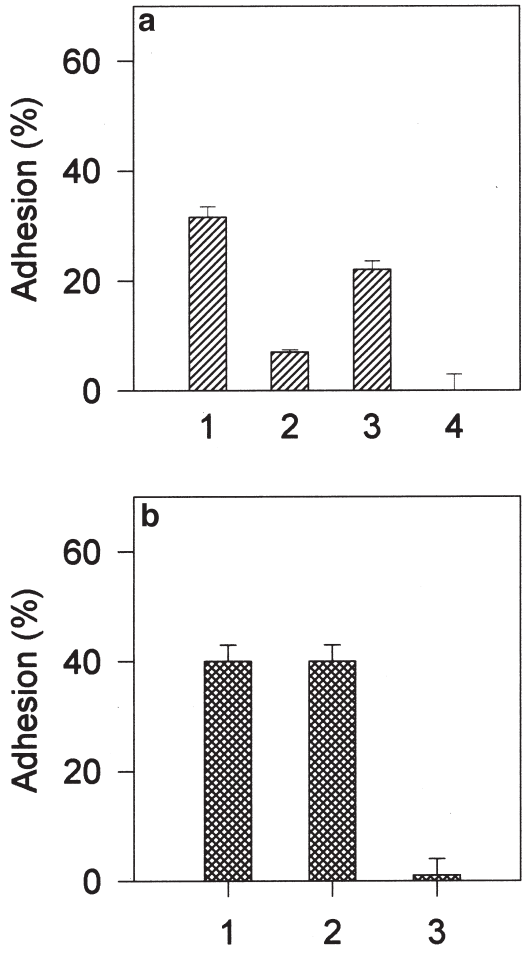

Figure 1 (a) Attachment of untreated and EPS-deficient cells of iron-grown *T. ferrooxidans* to covellite. 1: Untreated cells. 2: EPS-deficient cells. 3: EPS-deficient cells pre-exposed to EPS. 4: EPS-deficient cells (covellite pre-exposed to EPS). (b) Attachment of *T. thiooxidans* to covellite. Influence of the addition of EPS from iron-grown *T. ferrooxidans*. 1: Untreated cells. 2: Untreated cells pre-exposed to EPS. 3: Untreated cells (covellite pre-exposed to EPS). EPS was obtained from iron-grown *T. ferrooxidans*.

partially recovered by pre-exposure of EPS-deficient cells to EPS. Prior contact between covellite and EPS fully repressed the adhesion of EPS-deficient *T. ferrooxidans*. The decrease in *T. ferrooxidans* attachment to covellite, previously in contact with EPS, suggests that EPS attached to covellite prevents the adhesion of cells. This was also observed when the attachment of *T. thiooxidans* was studied. However the extent of adhesion of untreated *T. thiooxidans* was not affected by later addition of EPS from *T. ferrooxidans*.

Leaching of copper from covellite in the presence of *T. thiooxidans* was not significantly different from that observed in sterile controls (Figure 2), confirming previous reports [5,15] of its inability to leach insoluble sulphides. Pre-incubation of *T. thiooxidans* with EPS obtained from *T. ferrooxidans* failed to promote improved attachment to covellite (Figure 1b) or significant bioleaching (Figure 2).

Untreated cells of *T. ferrooxidans* promoted significant leaching of copper from covellite (Figure 2). The initial rate of dissolution was depressed when EPS-deficient cells were used. EPS-deficient cells which had been pre-incubated with EPS produced an initial rate of dissolution which was not significantly different from that observed for untreated cells. Similarity between these results and those of cell adhesion to covellite suggests a close relation between attachment and bioleaching by *T. ferrooxidans*. Rates of dissolution by untreated and treated cells were similar after 24 h incubation, possibly due to regeneration of EPS by the treated cells.

Changes in the number of unattached bacteria during incubation in the presence of covellite are described in Table 1. There was no decrease in the number of unattached bacteria after inoculation of treated *T. ferrooxidans* cells, as expected from the attachment experiments. Other inoculated flasks showed a decrease in the population of unattached bacteria due to partial attachment.

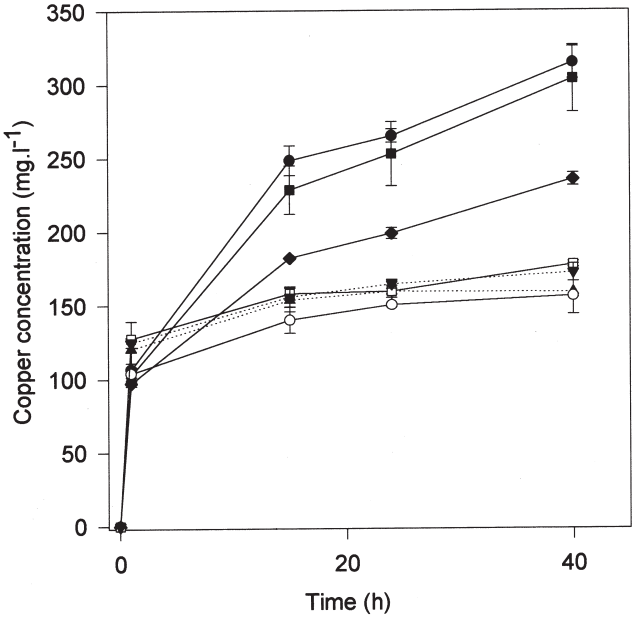

Figure 2 Copper dissolution during bioleaching of covellite by EPS-deficient and untreated cells of iron-grown *T. ferrooxidans*. EPS was extracted from iron-grown *T. ferrooxidans*. ● *T. ferrooxidans* (untreated); ◆ *T. ferrooxidans* (EPS-deficient cells); ■ *T. ferrooxidans* (EPS-deficient cells) + EPS; ○ *T. thiooxidans*; □ *T. thiooxidans* + EPS; ▲ Sterile; ▼ Sterile + EPS.

Table 1 The numbers of unattached bacteria during the bioleaching of covellite by iron-grown *T. ferrooxidans*

Time (h)	Unattached bacteria ($\times 10^6$ ml^{-1} ± standard deviation)		
	Untreated cells[a]	Treated cells[b]	Treated cells + EPS[c]
0	9.5 ± 1.7	14.0 ± 0.3	11.3 ± 0.9
24	25.7 ± 3.7	8.6 ± 1.6	17.9 ± 0.9
40	37.0 ± 4.3	27.4 ± 2.0	30.0 ± 4.8

[a]Cells including EPS.
[b]EPS-deficient cells.
[c]EPS-deficient cells + EPS suspension.

After 20 h incubation, the number of unattached bacteria increased, indicating bacterial growth using covellite as an energy source. This was not observed for EPS-deficient cells whose population decreased. This could indicate that EPS-deficient cells had produced EPS and were then able to attach to covellite. Subsequently, covellite oxidation and bacterial growth continued at the same rates as in other inoculated flasks.

In order to determine whether *T. ferrooxidans* had lost its viability after EPS removal, both untreated and treated bacteria at the same concentration were incubated in medium with 9 g L^{-1} of ferrous ion. There were no differences in growth, confirming that the treatment did not change the bacterial capability to oxidise a soluble substrate (ferrous ion).

To investigate the effect of the bacterial energy source on EPS, we extracted EPS from *T. ferrooxidans* grown on sulphur. The amount of EPS (expressed as glucose) was slightly higher than that found for iron-grown cells (60 mg from 10^{12} sulphur-grown cells and 53 mg from 10^{12} iron-grown cells).

Figure 3a shows the attachment of untreated and treated sulphur-grown *T. ferrooxidans* to covellite. Treated sulphur-grown cells attached better than untreated ones, but much worse than untreated iron-grown cells.

To investigate whether the extraction of EPS from sulphur-grown cells had decreased their hydrophobicity, we tested the attachment of sulphur-grown untreated and treated *T. ferrooxidans* to sulphur, which is more hydrophobic than covellite. The results are shown in Figure 3b. Nearly 40% of the untreated cells were adsorbed on sulphur. When the layer of EPS was removed, there was negligible attachment but addition of EPS restored cell capacity to attach to sulphur to the same level as that found for untreated cells.

Discussion

Our results suggest that the presence of EPS on the cell surface of *T. ferrooxidans* is an important factor in its ability to attach to and leach covellite. These results are in agreement with those obtained by Gehrke and collaborators [10] with respect to pyrite.

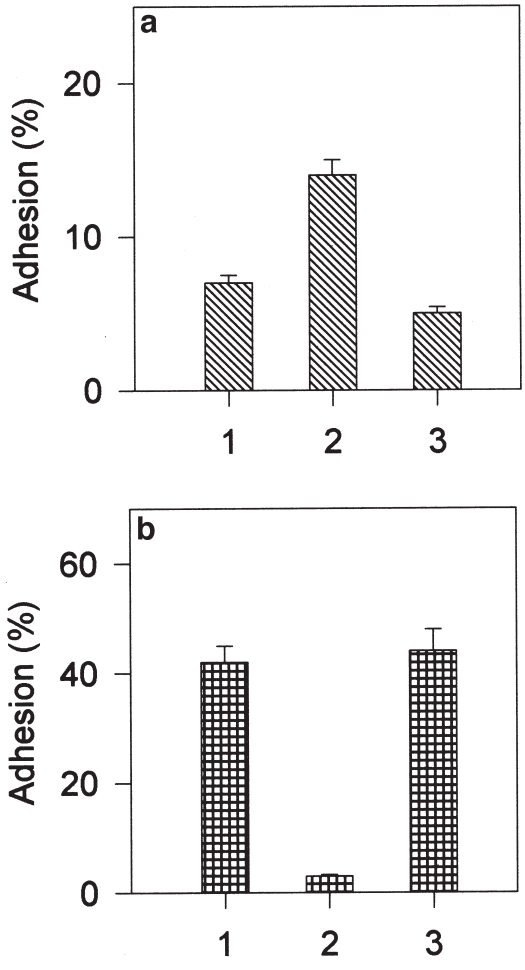

Figure 3 (a) Attachment of untreated and EPS-deficient cells of sulphur-grown *T. ferrooxidans* to covellite. 1: Untreated cells. 2: EPS-deficient cells. 3: EPS-deficient cells pre-exposed to EPS. (b) Attachment of untreated and EPS-deficient cells of sulphur-grown *T. ferrooxidans* to sulphur. 1: Untreated cells. 2: EPS-deficient cells. 3: EPS-deficient cells pre-exposed to EPS. EPS was obtained from sulphur-grown *T. ferrooxidans*.

When EPS-deficient cells were pre-exposed to EPS-suspension, their capacity for attachment to covellite was restored. In contrast, there was negligible adherence of EPS-deficient cells to covellite previously in contact with EPS. These facts are similar to the results of previous studies about LPS [9].

After about 24 h EPS-deficient cells regained their ability to attach to and leach covellite, even in the absence of iron. Ferric ions were not necessary for EPS restoration.

Gehrke *et al* [10] proposed that ferric ions were entrapped in the EPS layer. A higher rate of leaching by cells with Fe^{3+}-containing EPS would then be expected due to chemical oxidation. However, our results suggest that ferric ions were not present in the EPS since copper dissolution in sterile controls with and without EPS was similar (Figure 2).

Hydrophobicity and electrostatic interactions are the most important factors governing the mechanism of bacterial adhesion. It has been proposed that hydrophobicity is dominant except for more hydrophilic cell surfaces when electrokinetic potential (a measurement of the electrostatic state of a bacterium) becomes more influential [24]. However, other studies have found the opposite [22].

Copper sulphides have a negative surface charge [22] and the isoelectric points are at pH 2.0 and pH 4.0 for iron-grown and sulphur-grown cells, respectively [4,11]. This implies that the cells were positively charged in our attachment experiments (pH 1.8). Accordingly, should electrostatic interactions play the main role in initial adhesion, it would be expected that the attachment of sulphur-grown cells to covellite would be higher than, or at least similar to that of iron-grown cells. On the other hand, previous studies in our laboratory [15] showed that sulphur-grown cells are more hydrophobic than iron-grown cells, but in the latter case greater attachment to covellite was found.

According to our results, initial attachment of iron-grown cells and sulphur-grown cells to covellite cannot be explained by the same mechanism. Moreover, our results suggest that initial attachment of more hydrophilic (grown on iron) cells may be due to electrostatic forces rather than hydrophobic interactions. On the other hand, the contribution of electrostatic forces should be lower for sulphur-grown cells due to their higher hydrophobicity.

We propose that EPS from sulphur-grown cells is more hydrophobic allowing EPS-deficient cells to attach better to covellite than untreated ones, since covellite is less hydrophobic than sulphur. Moreover, EPS-deficient cells grown on sulphur showed a lesser degree of attachment to sulphur than did untreated cells, indicating that cells without EPS became less hydrophobic.

In contrast, the cell surface of iron-grown *T. ferrooxidans* is essentially hydrophilic [13]. Thus, our results suggest that in this case cells without EPS lose their charge and their capacity to attach to covellite.

Both sulphur-grown and iron-grown cells attached to covellite to a similar extent when they were stripped of their EPS. Thus, it may be concluded that the characteristics of the bacterial surface under the layer of EPS did not vary significantly between cells grown on ferrous iron or sulphur.

Finally, we conclude that the direct mechanism of bioleaching of covellite cannot occur when iron-grown *T. ferrooxidans* cells have been stripped of their layer of EPS because these compounds are necessary for attachment to a substrate, whether iron is present or not. Such cells generated EPS again even in the absence of iron, and were subsequently able to bioleach covellite. Besides, after analysing the addition of EPS to *T. thiooxidans* cultures, we conclude that the exopolymers do not directly participate in bioleaching of covellite.

Acknowledgements

E Donati is research member of CONICET (Argentine Research Council). Financial support from Agencia Nacional de Promoción Cientifica y Tecnológica (research grant No. 13-00000-00059) as well as from the Foundations Andes-Antorchas is acknowledged.

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