Examination of Lipopolysaccharide (O-Antigen) Populations of *Thiobacillus ferrooxidans* from Two Mine Tailings

G. SOUTHAM* and T. J. BEVERIDGE

Department of Microbiology, College of Biological Science, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Received 21 January 1993/Accepted 28 February 1993

Net acid-generating capacities of 39.74 kg of H$_2$SO$_4$ per ton (ca. 0.05 kg/kg) (pH 2.68) for the Lemoine copper mine tailings (closed ca. 8 years ago; located 40 km west of Chibougamau, Quebec, Canada) and 16.07 kg of H$_2$SO$_4$ per ton (ca. 0.02 kg/kg) (pH 3.01) for the Copper Rand tailings (in current use and 50 km distant [east] from those of Lemoine) demonstrate that these sulfide tailings can support populations of acidophilic thiobacilli. Oxidized regions in both tailings environments were readily visible, were extremely acidic (Lemoine, pH 2.36; Copper Rand, pH 3.07), and provided natural isolates for our study. A 10% (wt/vol) oxalic acid treatment, which solubilizes both ferric sulfate and ferric hydroxide precipitates (B. Ramsay, J. Ramsay, M. deTremblay, and C. Chavarie, Geomicrobiol. J. 6:171–177, 1988), enabled the recovery of intact bacterial cells from the tailings material and from liquid synthetic medium for lipopolysaccharide analysis. No viable cells could be cultured after this oxalic acid treatment. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic profiles of lipopolysaccharides extracted from the Lemoine tailings were complex, indicating a heterogeneous population of *Thiobacillus ferrooxidans*. Six *T. ferrooxidans* subspecies as identified by lipopolysaccharide analysis (i.e., lipopolysaccharide chemotypes) were eventually isolated from a total of 112 cultures from the Lemoine tailings. Using the same isolate and lipopolysaccharide typing techniques, we identified only a single lipopolysaccharide chemotype from 20 cultures of *T. ferrooxidans* isolated from the Copper Rand tailings. This homogeneity of lipopolysaccharide chemotype was much different from what was found for the older Lemoine tailings and may reflect a progressive lipopolysaccharide heterogeneity of *Thiobacillus* isolates as tailings leach and age.

*Thiobacillus* species are typically responsible for the solubilization and leaching of heavy metals from pyritic tailings. For their growth, these microorganisms utilize atmospheric CO$_2$ as a source of carbon and reduced iron and sulfur as sources of electrons. Ferric iron and sulfuric acid are oxidative by-products of their metabolism (12). Leaching of toxic heavy metals from tailings therefore is a chemical process which is enhanced by biocatalysis (1, 23).

Growth on minerals is facilitated by close bacterium-mineral interaction (2), and it is usually assumed that these bacteria are tightly associated with the mineral surface (Fig. 5 of reference 24; 25). The details of this bacterium-mineral interaction are ill defined, although a combination of ionic forces and perhaps hydrophobicity may play a role in mineral colonization (24). It is clear, however, that the overriding factor in the establishment of active, growing *Thiobacillus* populations in tailings and the subsequent acidification and leaching of resident toxic metals lies in the association between the bacterium and the mineral. Whether there is a physical adherence of the bacterium to the mineral faces is debatable, but certainly the juncture must be close enough for iron-sulfur utilization and transfer of constituent electrons (29). The enzymes responsible for growth are not released extracellularly but are associated with the *Thiobacillus* cell envelope (7).

The bacterial surface component most likely responsible for bacterium-mineral interaction in this gram-negative bacterium is lipopolysaccharide (LPS), since its side chains extend above the outer face of the outer membrane and since capsular material has not been described for *Thiobacillus* spp. (10, 19, 21, 27, 28, 30). The goals of this study were to develop a method to study LPS chemotypes of thiobacilli grown in tailings and in synthetic media, and to determine whether LPS heterogeneity exists among *Thiobacillus* spp. within natural tailings environments.

**MATERIALS AND METHODS**

**Tailings location and sampling procedure.** The Lemoine and Copper Rand tailings were derived from copper mines located 40 km west and 10 km east of Chibougamau, Quebec, Canada, respectively. Mining operations at the Lemoine site were discontinued in 1984, and because of subsequent biological and chemical weathering, the tailings possess an extensive acid zone encompassing 63% of the tailings surface (24). A sample of Lemoine tailings (approximately 1 kg) was taken from the central region of this acid zone, placed in a heavy plastic bag, and stored at 4°C upon return to the laboratory. Tailings were collected with a common garden trowel that had been rinsed with 70% (vol/vol) ethanol(aq). The Copper Rand mine is still in operation, and most of the tailings were saturated with water and unoxidized. However, several discrete oxidized regions (ca. 1-m$^2$ surface area) existed on a raised plateau immediately downstream from a previous tailings discharge point. One of these oxidized zones was sampled as the Lemoine tailings were. Nonacidic regions of both tailings adjacent to these acid zones were also sampled for use in the net acid-generating (NAG) procedure (see below). Unless otherwise indicated, chemicals were purchased from Fisher Scientific (Fairlawn, N.J.).

**Determination of pH of tailings material.** Samples (1 g) of tailings were resuspended into 1 ml of NAPure water...
(dH₂O; Barnstead; 18 MΩ/cm), vortexed several times over 30 min, and allowed to settle by gravity (1 h) prior to pH determination.

**Total Sulfur.** Oxidized tailings samples were assayed in duplicate for total sulfur with a Leco analyzer (model SC-232).

**NAG Capacity.** Oxidized tailings material (2.5 g) was weighted into a 500-ml flask. The reaction to determine NAG (a chemical oxidation) was initiated (time zero) by the addition of 250 ml of 15% (vol/vol) H₂O₂ (aq) (9, 15, 26). The temperature of the reaction vessel was monitored while the mixture was stirred, and the reaction was allowed to proceed for 24 h. The fluid phase was then collected by filtration (0.22-µm-pore-size filter), boiled for 1 h to remove residual H₂O₂, and cooled to room temperature. The pH was determined and monitored during a back titration with 2 N NaOH.

The NAG capacity was calculated as kilograms of H₂SO₄ per ton (1 ton = ca. 900 kg) on the basis of the back titration.

**Isolation and Growth of T. ferrooxidans.** Tailings samples were serially diluted in 9K buffer (22) containing 33 g of FeSO₄·7H₂O per liter and were also plated on 9K plus iron solidified with 2% (wt/vol) agarose. *T. ferrooxidans* strains were identified in end point dilutions from liquid culture enrichment and from the solidified medium as single colonies. The individual *T. ferrooxidans* strains were identified by LPS analysis according to their LPS chemotypes.

**LPS (O-antigen) Preparation.** *Thiobacillus* spp. grown in tailings and synthetic medium were treated with dH₂O, 1% (wt/vol) oxalic acid, or 10% (wt/vol) oxalic acid to release the bacterial cells from the precipitated metals (18). The two oxalic acid concentrations were used in an attempt to determine the toxicity of the treatment (see below). LPS was then extracted for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (13) by the method of Hitchcock and Brown (11), which was modified as a single incubation at 37°C in the presence of 1 µg of proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml. The gels were silver stained by the protocol described by Morrissey (16).

**Toxicity of Oxalic Acid.** Tailings samples (1 g) were treated with dH₂O, 1% (wt/vol) oxalic acid, or 10% (wt/vol) oxalic acid in 9K buffer at the 10⁻¹ dilution for 15 min with repeated vortexing. These pretreated mixtures were then serially diluted in 9K buffer alone, and the *T. ferrooxidans* strains were enumerated by most-probable-number analysis in 9K buffer containing 33 g of FeSO₄ per liter. The tubes for most-probable-number analysis were incubated at room temperature for 6 weeks to ensure that the growth end point had been reached. Total bacterial counts (expressed as an average of 10 replicates), determined with a Coulter Counter (model ZM; Coulter Electronics Ltd., Luton, England), were also obtained for the three tailings treatments at the 10⁻¹ dilution.

**LM.** Samples of tailings (1 g) were washed with 9 ml of dH₂O to suspend the fines from the coarse tailings (gravity sedimentation). These fines were collected from the upper phase of the water wash, and 1-ml aliquots of the suspension of fines were collected by centrifugation (14,000 × g for 1 min). For the control experiment and the oxalic acid treatment, samples of fines were resuspended into either 1 ml of dH₂O or 1 ml of 10% (wt/vol) oxalic acid, respectively, for 1 h. The samples were then centrifuged as before and resuspended into 1 ml of dH₂O for phase-contrast light microscopy (LM) analysis. For LM, 10-µl aliquots of each sample were dried onto a microscope slide, stained for 1 min with Gram's crystal violet, washed under running water, and examined with a Zeiss photomicroscope. For comparison, *T. ferrooxidans* strains grown in synthetic medium were also examined by simple staining for LM both with and without the oxalic acid treatment.

**RESULTS**

The tailings from both the Lemoine and Copper Rand mines possess the ability to generate acid mine drainage. Chemical (H₂O₂) oxidation of the sulfide minerals in samples (2.5 g) of tailings demonstrated that final pH values of 2.68 and 3.01 for the Lemoine and Copper Rand tailings, respectively, could be achieved after successful colonization by *Thiobacillus* spp. (Table 1). The temperature isotherms for the NAG process on the tailings samples (Fig. 1) are consistent with those of reactive (acid-generating) tailings (15) in that the NAG is inversely proportional to the time of reactivity. Only *T. ferrooxidans* could be enumerated from these acid zones in both tailings as outlined by Southam and Beveridge (24).

Water and 1 and 10% (wt/vol) oxalic acid wash procedures were evaluated for their abilities to elute thiobacilli from iron hydroxides, thereby rendering them free from precipitates so that LPS purification from "clean" cells could be done. Since these experiments eluted the cells in bulk from the tailings and since there was no attempt to separate the cells into distinct phylcogenic groups, these populations could contain a variety of bacteria of various LPS types. In this experiment, both oxalic acid pretreatments enhanced the recovery of LPS from *T. ferrooxidans* cells (the dominant bacterial population [24]) grown in tailings material; 10% (wt/vol) oxalic acid provided better recovery of LPS (Fig. 2). The oxalic acid treatments were found to be toxic to *T. ferrooxidans*, as *T. ferrooxidans* could not be recultured after 10% (wt/vol) acid treatment (Fig. 3). SDS-PAGE profiles of LPSs extracted from the Lemoine tailings were complex, with some dominant bands intermingled with a ladder-like pattern which indicated a heterogeneous population of *T. ferrooxidans* (Fig. 2). Conversely, only a ladder-like LPS profile was seen for the Copper Rand tailings, and this was indicative of a dominant or pure culture of *T. ferrooxidans* (Fig. 2).

**TABLE 1. Determination of NAG capacity of tailings material collected from the pH-neutral regions of the Lemoine and Copper Rand mines**

<table>
<thead>
<tr>
<th>Mine</th>
<th>Initial pH</th>
<th>% S</th>
<th>Theoretical acid production (kg of H₂SO₄/ton)</th>
<th>Measured pH from biooxidized tailings</th>
<th>pH after NAG procedure</th>
<th>Expnl acid production (kg of H₂SO₄/ton)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lemoine</td>
<td>7.04</td>
<td>7.79</td>
<td>214.73</td>
<td>2.36</td>
<td>2.68</td>
<td>39.74</td>
</tr>
<tr>
<td>Copper Rand</td>
<td>8.32</td>
<td>3.63</td>
<td>100.62</td>
<td>3.07</td>
<td>3.01</td>
<td>16.07</td>
</tr>
</tbody>
</table>

* From percent S. One ton = ca. 900 kg.
  * Determined by back titration with NaOH after the NAG procedure.
We had variable success in our attempts to isolate LPS from dried tailings samples from various acidic regions of the tailings (data not shown). These unsuccessful LPS preparations were likely due to low bacterial numbers in the samples and perhaps due to adsorptive forces between the bacteria and mineral surfaces, which may have increased upon drying. Successful extraction of LPS from tailings material was routinely achieved in samples which had been kept moist. Since this would allow for continued growth of bacteria after sampling until oxalic acid elution, the success or failure of LPS preparations from tailings material may be a reflection of the vitality and size of the *Thiobacillus* population as well as of adhesive forces which could be increased by drying. With LM, a comparison of the water wash with the 10% (wt/vol) oxalic acid wash in tailings (cf. Fig. 4A and B) and in synthetic medium (cf. Fig. 4C and D) confirmed that the oxalic acid treatment was an excellent technique (compared with the dH₂O wash) for releasing thiobacilli from ferric oxy-hydroxides in either culture system. Enhanced recovery of LPS from *T. ferrooxidans* grown in tailings (Fig. 4B) and in synthetic iron-based medium (Fig. 4D) was due to the release of entrapped thiobacilli from the iron precipitates indigenous to these culture systems (3) (Fig. 4A and C). SDS-PAGE analysis of LPS extracted from pure cultures of *T. ferrooxidans* from the Lemoine tailings demonstrated the presence of six O-antigen LPS chemotypes (Fig. 5). By analyzing 112 isolates of *T. ferrooxidans* from the Lemoine tailings, we also determined the dominant chemotypes in this heterogeneous population (Table 2). These six LPS chemotypes are presented from the highest (chemotype 1) to lowest (chemotype 6) frequency in which they were recovered from the natural environment (Fig. 5). *T. ferrooxidans* isolates from the Copper Rand tailings all possessed the same LPS profile, which was also seen in the LPS extracted from tailings (Fig. 2 and 5).

**DISCUSSION**

In the natural environment, pH values in the Lemoine tailings have been measured to be below 2 (24). These low pH values, in comparison with the theoretical acid production (pH 2.68) (Table 1), presumably result from variable mineralogies throughout the fabric of the tailings (e.g., high

---

**FIG. 1.** Temperature isotherms of 1% (wt/vol) suspensions of Lemoine and Copper Rand tailings collected from nonacidic zones and reacted with 15% (vol/vol) H₂O₂(aq). Compare the time required to reach the temperature maximum with the experimental acid production (Table 1).

**FIG. 2.** Silver-stained SDS-PAGE gel (15% polyacrylamide) of LPS profiles of *Thiobacillus* spp. growing in the Lemoine (lanes 1 through 3) and Copper Rand (lanes 4 through 6) tailings and washed with dH₂O (lanes 1 and 4), 1% (wt/vol) oxalic acid (lanes 2 and 5), or 10% (wt/vol) oxalic acid (lanes 3 and 6) prior to LPS extraction.

**FIG. 3.** Amount (most probable number per gram) of *Thiobacillus* spp. from the Lemoine tailings treated with increasing concentrations of oxalic acid (aq). No viable cells were recovered after the 10% (wt/vol) oxalic acid treatment.
Pseudomonas require specific plastic concentrations as cells medium of different plastic petitive in the decline method ling of the oxidation surface.

This is also pH dependent (4); therefore, neutral versus acidic environmental pHs would also affect the ability of a T. ferrooxidans strain to colonize tailings.

Suzuki et al. (25) observed that ore-grown cells are difficult to dissociate from the ore particles, suggesting that a tight association occurs between Thiobacillus spp. and mineral surfaces. Although LPS may initially be responsible for this tight association, it is more likely that in later stages the bacterial cells are cemented to the mineral surfaces by ferric oxy-hydroxide precipitates (24), since the solubilization of these precipitates (cf. Fig. 4A and B) resulted in the release of bacteria from the mineral surfaces (Fig. 3). Strong adherence of T. ferrooxidans to minerals via iron precipitates (24) may play an important ecological role by reducing the diffusion of metabolic products (e.g., Fe³⁺ and sulfuric acid) away from the cell-mineral interface. This would help maintain an acidic microenvironment at the mineral surface and thereby promote growth of T. ferrooxidans (23, 29).

T. ferrooxidans is known to adapt to various sources (types) of sulfide ores prior to the initiation of active microbial leaching (25). This adaptation mechanism is not well understood. It is possible that binding of T. ferrooxidans to mineral surfaces and the development of an oxidized “crust” (17) is related to this adaptation process. Although phenotypic switching has recently been demonstrated in T. ferrooxidans, it has not been related to LPS chemistry (20). Early chemical analysis of LPS from T. ferrooxidans strains grown on iron (Fe-LPS) and sulfur (S-LPS) has produced controversial ideas on the nutritional modulation of LPS chemistry. Fe-LPS and S-LPS were found to be chemically different (10, 27) or identical (30) for different T. ferrooxidans strains. This discrepancy in LPS chemistry may be related to the presence of acidicophilic heterotrophs, which have been found in many T. ferrooxidans cultures (8). Our data from the Copper Rand tailings suggest that LPS modulation does not occur in this T. ferrooxidans subspecies grown on

**TABLE 2. Population distribution of T. ferrooxidans strains from LPS (O-antigen) chemotype**

<table>
<thead>
<tr>
<th>LPS profile*</th>
<th>No. of isolates</th>
<th>% of population</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>47</td>
<td>42.0</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>16.1</td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>15.2</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>13.4</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>8.0</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>5.4</td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* Profiles 1 through 6 were for T. ferrooxidans strains isolated from the Lemoine tailings; profile 7 was for a T. ferrooxidans strain isolated from the Copper Rand tailings.

correspond to different LPS chemistries. Differences in LPS chemistry (i.e., O-antigen type) may confer a different cell surface charge character and/or hydrophobicity, which could affect the strain’s ability to colonize mineral surfaces. Charge character is also pH dependent (4); therefore, neutral versus acidic environmental pHs would also affect the ability of a T. ferrooxidans strain to colonize tailings.

In a previous study of the Lemoine tailings (24), we examined the colonization of T. ferrooxidans on inert competitive plastic surfaces. In all cases, the mineral-associated counts were at least 10²-fold higher than counts on the plastic surfaces. This supports the concept that thiobacilli require specific interfaces, such as crystal lattice defects of sulfide mineral surfaces (2), for growth to occur. Since Dispirito et al. (6) found that extraction of LPS resulted in a decline in the rate and total amount of cell adsorption to pyrite, it is probable that LPS is responsible for the initial bacterium-mineral interaction of T. ferrooxidans. For Pseudomonas aeruginosa, a phylogenetically related gram-negative eubacterium (14), LPS heterogeneity is a reflection of different chemistries and chain lengths (5). If this analogy holds true for T. ferrooxidans, the different LPS profiles determined for our T. ferrooxidans isolates (Fig. 5) would

**FIG. 5.** LPS profiles of T. ferrooxidans subspecies isolated from the Lemoine and Copper Rand tailings in pure culture and grown in synthetic medium. A 10% (wt/vol) oxalic acid wash was used prior to LPS extraction. Lane numbers correspond to LPS chemotypes 1 through 6 from the Lemoine tailings and LPS chemotype 7 from the Copper Rand tailings.

sulfide concentrations will decrease pH, whereas high carbonate concentrations will increase it) and perhaps, to some extent, from the evaporative concentration of acid at the tailings surface. Biological (pH 3.07) and chemical (pH 3.01) oxidation of the Copper Rand tailings resulted in comparable acid generation as determined by pH measurement. Modeling acid mine drainage by chemical oxidation is an effective method to perform environmental impact assessments of tailings.

In a previous study of the Lemoine tailings (24), we examined the colonization of T. ferrooxidans on inert competitive plastic surfaces. In all cases, the mineral-associated counts were at least 10²-fold higher than counts on the plastic surfaces. This supports the concept that thiobacilli require specific interfaces, such as crystal lattice defects of sulfide mineral surfaces (2), for growth to occur. Since Dispirito et al. (6) found that extraction of LPS resulted in a decline in the rate and total amount of cell adsorption to pyrite, it is probable that LPS is responsible for the initial bacterium-mineral interaction of T. ferrooxidans. For Pseudomonas aeruginosa, a phylogenetically related gram-negative eubacterium (14), LPS heterogeneity is a reflection of different chemistries and chain lengths (5). If this analogy holds true for T. ferrooxidans, the different LPS profiles determined for our T. ferrooxidans isolates (Fig. 5) would

**FIG. 4.** Simple-stain (Gram's crystal violet) LM. (A) Fine particulate material washed from a sample of Lemoine tailings with dH₂O. Note the ferric (oxy-)hydroxide-bacterium precipitates. (B) Tailings material seen in panel A, treated with 10% (wt/vol) oxalic acid and resuspended into dH₂O. (C) Thiobacillus spp. grown in a ferrous iron-based medium without oxalic acid treatment. (D) Thiobacillus spp. grown in a ferrous iron-based medium and then treated with 10% (wt/vol) oxalic acid and resuspended into dH₂O. The increase in individual bacterial cells (B and D) resulted from solubilization of the iron (oxy-hydroxides). Note that all of the LMs have been underfocused to highlight the bacterial cells as spherical halos. This differentiates the cells from inorganic particulate material (iron oxy-hydroxide fines), which are also of bacterial dimensions. Bars, 10 μm.
mineral-based (primarily pyrite and chalcopyrite) and in ferrous iron-based (synthetic) media.

The Copper Rand tailings, which represent young or newly colonized tailings, contained several oxidized zones, one of which was colonized and acidified by a single strain of *Thiobacillus ferrooxidans* (Fig. 5, lane 7). Therefore, it is theoretically possible for sulfide tailings occurring in the natural environment to be acidified by a single strain of *Thiobacillus ferrooxidans*. In practical terms, however, older tailings are probably colonized by several strains of *Thiobacillus ferrooxidans*, since it is almost impossible to maintain pure cultures in the natural environment. The Lemoine tailings are an example of relatively older tailings which have been colonized and acidified by *Thiobacillus ferrooxidans* (24). In the site selected for microbiological analysis, one *T. ferrooxidans* strain (Fig. 5, lane 1) was dominant (42.0%) (Table 2), and five other strains (Fig. 5, lanes 2 through 6) existed as smaller populations (16.1 to 5.4%) (Table 2). This range in population density probably resulted from overgrowth of individual oxidized zones (strains), similar to what was observed in the Copper Rand tailings. To conclude, LPS chemotyping by SDS-PAGE is an effective method for the identification of individual strains of *T. ferrooxidans* in the laboratory and in the natural environment.

ACKNOWLEDGMENTS

We thank J. Loral and R. MacFarlane of Westminer Canada Ltd. for the funding of this project. We also thank R. Woodall and P. MacGeehan of the Western Mining Corp., Australia for scientific discussions and for their help in obtaining funding. The total sulfur determination was provided by the Copper Rand mine, Chibougamau, Quebec, Canada. Some aspects of this project were funded through an NSERC operating grant to T.J.B.

Special thanks to C. MacKenzie for word processing.

REFERENCES