Enumeration of Thiobacilli within pH-Neutral and Acidic Mine Tailings and Their Role in the Development of Secondary Mineral Soil

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The Lemoine tailings of Chibougamau, Quebec, Canada, were deposited as a pH-neutral mineral conglomerate consisting of aluminum-silicates, iron-aluminum-silicates, pyrite, chalcopryrite, and sphalerite. These tailings are colonized by an active population of *Thiobacillus ferrooxidans* which is localized to an acid zone occupying 40% of the tailings' surface. This population peaked at $7 \times 10^{6}$ most probable number per gram of tailings during July and August 1990 and extended to a depth of 40 cm from the surface. Examination of samples over this depth profile by transmission electron microscopy and electron dispersive spectroscopy revealed a microbially mediated mineral transition from sulfides (below 40 cm) to chlorides and phosphates (at the surface). Silicate minerals were unaltered by microbial action. Transmission electron microscopy showed a tight association between *Thiobacillus* species and the sulfide minerals, which helps account for their prominence in tailings environments. Accurate enumeration of *T. ferrooxidans* from tailings required the disruption of their bonding to the mineral interface. Vortexing of a 10% aqueous suspension of the tailings material prior to most-probable-number analysis best facilitated this release. Even though heavy metals were highly mobile under acidic conditions at the Lemoine tailings, it was evident by transmission electron microscopy and electron dispersive spectroscopy that they were being immobilized as bona fide fine-grain minerals containing iron, copper, chlorine, phosphorus, and oxygen on bacterial surfaces and exopolymers. This biomineralization increased with increasing bacterial numbers and was most evident in the upper 3 cm of the acid zone.

The acidification of pyritic mine tailings and the concomitant release of heavy metals in leachates is facilitated by *Thiobacillus* spp. As a group, these organisms oxidize reduced sulfur compounds and ferrous iron to produce sulfuric acid and ferric iron (as by-products of their metabolism) (12). Carbon nutrition is provided through carbon dioxide fixation (16). Most thiobacilli are capable of growth on a variety of pyritic minerals (as sources of reducing power) (12), and most are easily grown on synthetic laboratory media (22).

Most environmental impact studies on pyritic mine tailings concentrate on the acidification of toxic heavy metals and their release to the environment surrounding the tailings. In these environments, biomineralization or biopromoted concentration of heavy metals by bacteria (4, 9), fungi (10), algae (17), and higher plants (11) have been observed and could be used as a natural bioremediation control to heavy metal pollution. In addition to tailings environments, biomineralization is also known to occur in a diverse variety of more natural environments such as downstream from pyritic soils (14, 25), around hydrothermal vents (8, 13), and in bog iron deposits (5).

Biomineralization by thiobacilli within tailings has received little attention, since the acid environment extracts and mobilizes metals from the existing mineral phase. Yet, since acidic holding ponds downstream from tailings are recognized for their microbium-induced precipitation of leachates (9), biomineralization can occur at a low pH. Indeed, in laboratory culture, growth of *Thiobacillus ferrooxidans* in a ferrous iron-based medium can be measured by the presence of jarosite precipitates (ferric hydroxy sulfates) associated with the thiobacilli and the surfaces of the culture vessel (18, 20).

The goals of this study were to characterize the microbiologically active zone in the Lemoine tailings and to evaluate the microbiota's effect on mineral dissolution and biomineralization within a natural tailings environment.

**MATERIALS AND METHODS**

**Tailings location and sampling procedure.** The Lemoine tailings are derived from a copper mine located 40 km west of Chibougamau, Quebec, Canada. They were first established by the mine in the early 1980s and have been subjected to seasonal freeze-thaw conditions since that time. The tailings were considered to be pH neutral on the basis of pH analysis done at a single site within the tailings (SEDAC, Inc., Chicoutimi, Quebec, Canada).

A grid pattern was established on the surface of the tailings (Fig. 1) and was used to obtain samples for microbiological and pH analyses over the entire area in order to determine active sites of microbial growth and leaching. Tailings were collected in sterile scintillation vials with a spatula cleaned with 70% (vol/vol) ethanol$_{aq}$. A site located in a moist surface depression within the acid zone on the tailings surface was typical (Fig. 1) and was selected for further microbiological analysis.

At this site, selective colonization of mineral surfaces was evaluated by measuring colonization of thiobacilli on inert plastic (Epon 812; Marivac, Halifax, Nova Scotia, Canada) surfaces (1 cm$^2$) which were inserted into and buried within the tailings. The advantage of this technique is that these
were examined for the presence of bacterial cells with a Phillips EM300 electron microscope.

**Tailings wash experiments.** Aliquots (2 g) of Lemoine tailings were washed (10 times) by gravity with 10-mL volumes of dH₂O, pH 3.0 9K buffer, and pH 7.0 9K buffer. Thiobacilli were enumerated from all wash samples as previously described. Tailings samples were also enumerated before and after the washing procedure.

Used in conjunction with vortexing (1 min) and to determine the chemical nature of the bonding forces adhering the bacteria to the minerals, tailings samples (1 g) were treated by sonication (1 min), detergents (0.1% [wt/vol] sodium dodecyl sulfate [SDS], 0.1% [vol/vol] Tween 20, 0.1% [wt/vol] CHAPS [3-[3-cholamidopropyl]-dimethyl-ammonio]-1-propanesulphonate]), ionic conditions (100 mM NaCl and 100 mM NaNO₃), or 10% oxalic acid (wt/vol) in pH 3.0 9K buffer and in pH 7.0 9K buffer for 1 min and 10 min prior to performing limiting dilutions for the recovery of *T. ferrooxidans*.

**RESULTS**

The pH determinations of the grid samples (Fig. 1) demonstrated that approximately 40% of the Lemoine tailings surface possessed a pH of less than 3, 23% was between pH 3 and 6, and the remainder was pH 6 to 7. Since 10% (wt/vol) suspensions of very acid soils are known to overestimate the pH of these acid soils (7), the actual pH values for the tailings samples are likely lower than the measured pH values (by 0.4 pH units [22]). MPN analysis at all sites consistently showed *T. ferrooxidans* to be present. The numbers of thiobacilli increased with decreasing pH and those regions at a pH of <3 are areas of intense metabolic activity by acidophiles and, consequently, of active mineral leaching. Regions of pH greater than 6 had very little microbial activity, and no leaching was detected.

At the site selected for detailed microbiological analysis (Fig. 1), the tailings possessed extensive microbial activity during the months of July and August (MPN, approximately 10⁷/g) but this was retarded by cooler temperatures in June (MPN, approximately 10⁵/g) and September (MPN, approximately 10⁷/g) (Fig. 2). Because of freezing conditions, the tailings are probably even more inactive during the remaining months of the year (1).

The depth profile at this site revealed microbial colonization down to approximately 40 cm below the tailings surface (Fig. 3). At 50 cm, few bacterial cells could be recovered by our procedures (Fig. 3) and none were observed in thin section (Fig. 4). By using EDS analysis, this 50-cm sample contained mostly silicates (Fig. 4B and C) and chalcopyrite (Fig. 5B). The latter was small grained and could only be identified by TEM at high magnification which revealed fractures throughout the mineral in thin section (Fig. 5A).

This 50-cm sample served as a control for the microbiologically altered upper regions.

Interaction between *T. ferrooxidans* and pyritic mineral surfaces was observed in samples above the 50-cm depth and increased towards the surface. A sample from the 23-cm depth observed by TEM is representative of the intermediate depths (Fig. 5C). Few bacterial cells were observed in this sample, and this may be due to the limited sample volume represented in thin section. What is evident in this micrograph is the close association between the bacteria and the pyrite (identified by EDS).

Thin-section analysis of the surface material (upper 3 cm) collected from the tailings (Fig. 6A) contained more bacteria
and their abundant exopolymers (cf. Fig. 4A and 6A) and demonstrated a transition of mineral forms from sulfides (similar to those seen in Fig. 5A and B) to chlorides (Fig. 6B) and phosphates (Fig. 6C). A diffuse precipitate originating from and connecting all cells was spread throughout (Fig. 6A and 7A) and was shown by EDS analysis to be an iron chloride (Fig. 6B); our experience with other systems suggest that these iron precipitates have congealed on microbial exopolymers. Since chlorine is not usually indigenous to the chemical composition of tailings, it must come from an external source (likely from pulp and paper industry emissions). Another iron-mineralized component occurred as dense spherical grains (Fig. 7B) which, when analyzed, were found to consist of iron with low levels of copper, phosphorus (likely as biologically derived phosphate), sulfur, chlorine, and oxygen (Fig. 6C). These micrographs highlight the striking effect of bacterial action on the geochemical processes occurring in a tailings environment. Not only are the bacteria contributing to the acid leaching of pyritic minerals, they are also mediating, through cell wall and exopolymer interaction, the development of distinct mineral phases not normally indigenous to the original tailing. We call this secondary mineral development.

The disruption of bacteria-mineral bonding while maintaining cell viability was a difficult goal to achieve. Washing acidic tailings samples with dH2O, pH 3.0 buffer, or pH 7.0 buffer likely removed only a small proportion of the bacterial population present (Fig. 8). Although viable cell counts were low, wetting the sample by washing increased the MPN per gram tailings determined at the end of the washing trial compared with before the washings. Further attempts to wash viable thiobacilli from tailings by using physical (vortexing and sonication) and chemical treatments (i.e., detergents [SDS, Tween 20, CHAPS], ionic interactions (NaCl, NaNO3), and an iron solubilizing agent (oxalic acid) at both pH 3.0 and pH 7.0 tested several methods for enhanced bacterial recovery from tailings. Two criteria were used to evaluate the efficiency of the washing procedures: first, the number of viable bacteria enumerated by MPN analysis and, second, the consistency of the bacterial recovery within each category (treatment). On the basis of these criteria, vortexing alone is a suitable method for the recovery of viable thiobacilli from tailings material (Fig. 9).

**DISCUSSION**

The pattern of colonization of *T. ferrooxidans* in the Lemoine tailings (Fig. 1) relates to the distribution of pyritic minerals as they were laid down during tailings deposition and to the hydrogeological flow within the tailings itself. We have analyzed the tailings for only one season and can, therefore, only speculate about the rate of acidification during the 8 years since the tailings were decommissioned. Certainly there are now localized acidic zones in this pH-neutral tailings and we believe these will increase in size (width and depth) through time because of the increased dissemination of *T. ferrooxidans*. Their presence will speed

**FIG. 2.** Microbial analyses of the Lemoine tailings comparing bacterial (*T. ferrooxidans*) associations with plastic (biofilm per square centimeter) and mineral surfaces (per gram). Heterotroph populations (per gram) were also determined.

**FIG. 3.** Bacterial (*T. ferrooxidans*) and pH profiles (0 to 50 cm) down from the surface of the tailings at the site characterized in Fig. 1.

**FIG. 4.** (A) Low magnification thin section of the Lemoine tailings from a depth of 50 cm (see Fig. 3). No bacterial cells were observed in this specimen. Positions B and C correspond to the EDS analyses seen in panels B and C, respectively. Bar, 5.0 µm. (B) EDS analysis of a nonfractured grain from position B in panel A. The mineral is an aluminum silicate with traces of iron and copper. EDS controls were performed for all samples since they were analyzed on aluminum grids. (C) EDS analysis of particulate material from position C in panel A possessing linear crystalline lattices. The mineral is a complicated iron-copper silicate with traces of potassium, titanium, and zinc.
FIG. 5. (A) High-magnification thin section of the Lemoine tailings from a depth of 50 cm. The large, highly fractured grain in this micrograph is chalcopyrite. Position B on this micrograph corresponds to the EDS analysis seen in panel B. Bar, 0.5 μm. (B) EDS analysis of a large fractured grain similar to that indicated by position B in panel A. (C) Thin section of the fraction collected at a depth of 23 cm from the tailings (see Fig. 3). Note the close juncture of pyrite to the bacterial surfaces of the *Thiobacillus* spp. (arrows). Bar, 0.5 μm.
FIG. 6. (A) Low-magnification thin section of the surface material collected from the tailings (see Fig. 3). Extensive biomineralization, resulting in new mineral phases, is evident. Positions B and C on this micrograph correspond to the EDS analyses seen in panels B and C, respectively. Bar, 5.0 μm. (B) EDS analysis of the diffuse precipitate at position B in panel A. Iron and chlorine predominate, with a trace of copper. (C) EDS analysis of a single spherical precipitate observed at position C in panel A. This is a complicated aggregate of iron, chlorine, phosphorus, and oxygen.
the release of acid effluents and toxic heavy metals to surface and ground waters as the tailings mature. It is unclear how initial acidification occurs in such tailings dumps, and some researchers have implicated Gallionella spp. (14) and Leptothrix spp. (5). None of these sheathed organisms were seen during our isolations or in situ sightings. Although the optimum conditions for growth of Thiobacillus spp. is a pH of <3 (2, 25), viable thiobacilli were cultured from tailings possessing environmental pHs of >4. The implication that T. ferrooxidans can colonize a neutral tailings and develop an
acid environment (pH < 3) by themselves lends a new insight to tailings ecology.

The mechanism for the colonization of neutral sulfide minerals involves very close interaction between the surfaces of the minerals and the bacteria (Fig. 5C). It is possible that this close juncture would make possible acidic interfaces between mineral face and bacterium that could more easily lead to sustained chemolithotrophy, eventual multiplication of the bacterium, and growing acidification within the tailings as a whole. Bennet and Tributsch (3) demonstrated that 

*T. ferrooxidans* chose to colonize fracture lines and dislocations, and these may be preferred bacterial microenvironments on pyritic mineral surfaces. The recognition of sulfide minerals by *T. ferrooxidans* is unknown (6). The molecular interaction responsible for this close bacteria-mineral bonding may also be responsible for mineral selective leaching.

Although corrosion products (iron hydroxides) are commonly observed during growth of *T. ferrooxidans* in synthetic laboratory media (20, 23), their identification within tailings has received little attention. A laboratory-scale leaching study carried out by McCready and Gould (18) demonstrated that *T. ferrooxidans* was capable of precipitating both ferric oxyhydroxide and ferric hydroxysulfate. Biomineralization of thiobacilli was observed in the upper 3 cm of the Lemoine tailings (Fig. 6A). In this region, secondary mineral formation resulted from the transition of mineral sulfides to iron chlorides (Fig. 6B) and iron phosphates (Fig. 6C). The mineralized bacteria (*T. ferrooxidans* and possibly, to a lesser degree, indigenous heterotrophs) encased within the secondary mineral aggregates make up an organic fraction of approx. 0.03% (wt/wt) dry weight. This organic fraction was estimated (19) according to the maximum viable population of *T. ferrooxidans* determined in the Lemoine tailings (Fig. 3).

Although natural drying at the tailings surface (depth, <1 cm) decreased numbers of viable thiobacilli it did not reduce acidity (Fig. 3). The maintenance of acid conditions within this drying zone may be the result of upward movement of sulfuric acid by capillary action through the fine-grain tailings. Their acidity (pH < 3) coupled with the presence of the toxic heavy metals reduces the chance of higher plants developing.

The development of secondary minerals from the primary pyritic phase with the concomitant production of organic matter through microbial growth and decay represent the earliest phase in the formation of a fertile soil over the tailings. We do not know the time required for the natural development of a productive soil from pyritic tailings, but it will depend on tailings depth and the amount and availability of the sulfur within the pyrite. Presumably, leaching of all available sulfide minerals is required prior to the development of a stable neutral top soil and, for this reason, long times should be necessary. Certain pyritic soils have caused major problems for the agricultural industry for decades because of acidification (14, 25).

The accurate enumeration of *T. ferrooxidans* and the identification of different subspecies are essential for the study of population growth in the natural tailings environments. Several methods exist for studying this growth, but not all can be used for natural samples. Protein analysis is unsuitable for the study of population growth because it also measures dead cells and because it does not allow for the characterization of bacterial subspecies. Ferrous iron oxidation (20, 24) is a good technique to study metabolic activity, but it does not account for metabolic variability among subspecies nor for the identification of the subspecies themselves. MPN analysis, in addition to being a quantitative measure of viable thiobacilli, provides a sample of cells which can be later used to identify subspecies. Enumeration of *T. ferrooxidans* by MPN analysis eliminates the problem of low plating efficiency and makes it the best available technique to estimate the population of *T. ferrooxidans* in tailings environments even though it provides underestimates of the actual population (Fig. 8 and 9). Because of the tight bonding between *T. ferrooxidans* and mineral surfaces, 100% accuracy in determining viable cell counts in natural samples is a difficult problem and, indeed, may not be possible. It is not known whether this interaction is mediated by the bacterial cell surface (e.g., lipopolysaccharide [21]) or by extracellular, surface active substances (15).

**FIG. 9.** MPN of *T. ferrooxidans* per gram of Lemoine tailings treated by vortexing in dH_2O (vortex), vortexing and sonication in dH_2O (sonicate), vortexing in 0.1% Tween 20 (Tween), vortexing in 0.1% SDS, vortexing in 0.1% CHAPS, vortexing in 100 mM NaCl, vortexing in 100 mM NaNO_3_, and vortexing in 10% oxalic acid.

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is achieved, can instigate the development of newly immobilized mineral phases which contribute to secondary soil formation.

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REFERENCES