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# Protein Profile of *Acidithiobacillus ferrooxidans* Strains Exhibiting Different Levels of Tolerance to Metal Sulfates

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**Abstract.** Strains of *Acidithiobacillus ferrooxidans* exhibited differences in the inhibition of $Fe^{2+}$ oxidation in the presence of 250 mM of cadmium, zinc, and manganese sulfates in respirometric assays. Strains LR and I35 were practically not inhibited, whereas strains SSP and V3 showed significant inhibition (30–70%). Analysis by SDS-PAGE of total proteins from cells grown in the absence of metal sulfates showed different profiles between the more tolerant strains (LR and I35) and the more susceptible ones (SSP and V3). Total proteins of strains LR and V3 were also resolved by two-dimensional polyacrylamide gel electrophoresis (2-DE). A set of major proteins (40, 32, 22, and 20 kDa) could be identified only in the more tolerant strain LR. Our results show that protein profiles analysis could differentiate *A. ferrooxidans* strains that considerably differ in the tolerance to metal sulfates and present low genomic similarity as revealed by Random Amplified Polymorphic DNA (RAPD) data obtained previously in our laboratory.

*Acidithiobacillus ferrooxidans* is a Gram-negative, acidophilic, and chemolithotrophic bacterium that oxidizes ferrous ion, sulfur, and reduced-sulfur compounds as energy sources for its growth. This bacterium also oxidizes several sulfide minerals in natural environments, producing acid and releasing metals as acid mine drainage [10, 11]. This metabolic ability has been used industrially in the recovery of copper and uranium from dump or heap leaching operations [3]. Controlled processes in stirred tank bioreactors have also been utilized for pretreatment of gold bearing arsenopyrite concentrates [17]. In general, *A. ferrooxidans* is resistant to several toxic metals. However, strain-specific differences in the level of tolerance have been reported for *A. ferrooxidans* isolated from various mine sites [7, 13]. Several studies have demonstrated genetic variability among strains of this bacterium [16, 20] and elucidated genetic systems involved in tolerance to mercury [21] and arsenic [4]. However, relationships between metal tolerance, genomic variability, and protein synthesis are poorly understood in *A. ferrooxidans* strains. In the present work, the tolerance of some *A. ferrooxidans* strains to cadmium, zinc, and manganese sulfates was characterized by measurement of $O_2$ consumption coupled with $Fe^{2+}$ oxidation through respirometric experiments. These data were compared to total protein profiles in SDS-PAGE and 2-DE and previously obtained Random Amplified Polymorphic DNA (RAPD) results [16].

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## Materials and Methods

***A. ferrooxidans* strains and growth conditions.** Eight *A. ferrooxidans* strains (Table 1) were used in this work. They were grown for two days with shaking (250 rpm) at 30°C in 200-mL of T and K medium [22] at pH 1.8. The cells were then harvested by membrane filtration (pore size, 0.45 μm) followed by three washes with $H_2SO_4$ solution at pH 1.8 and suspension in 8 mL of $H_2SO_4$ solution.

**Respirometric experiments.** Cell suspensions were standardized by total protein determination [2]. The experiments were carried out with 10 μg of total cell protein in a Warburg respirometer, as described previously [15]. The cadmium, manganese, and zinc sulfates were used

Table 1. *Acidithiobacillus ferrooxidans* strains and sources

| Strain | Source |
|---|---|
| AMF* | Acid drainage of coal mine from Figueira, State of Paraná, Brazil |
| CMV | Effluent of gold mine, State of Minas Gerais, Brazil (supplied by R. Liberato, Morro Velho Company) |
| FG-460* | Acid drainage of uranium mine from Figueira, State of Paraná, Brazil |
| I35 | Supplied by O.H. Tuovinen (Ohio State University, USA) |
| LR* | Effluent of column leaching of uranium ore from Lagoa Real, State of Bahia, Brazil |
| S* | Effluent of column leaching of copper ore from Surubim mine (Caraiba), State of Bahia, Brazil |
| SSP* | Acid drainage of coal mine from Sideropolis, State of Santa Catarina, Brazil |
| V3 | Supplied by S. Groudev (University of Sofia, Bulgaria) |

* Isolated by Garcia [6].

in concentrations (mmol/$L^{-1}$) of 100, 200, 250, 300, 400, and 500 and the oxidizable substrate ($Fe^{2+}$) at 100 mmol/$L^{-1}$. All the assays were carried out in duplicate. The rate of $O_2$ consumption by cell suspensions was calculated for each experiment from the linear part of the respective curve. Two types of controls were carried out in parallel to the experiments: active and dead cell suspensions of each strain in the absence of the metal sulfates tested. The rates of oxygen consumption in the presence and absence of the metal sulfates were utilized to calculate the relative inhibition by the metal sulfates on $Fe^{2+}$ oxidation by each *A. ferrooxidans* strain.

**Total protein extraction and electrophoresis.** Total protein was extracted from the cells grown in the absence of the tested metal sulfates (~200-mL cultures), essentially as described by De Mot and Vanderleyden [5], and the protein concentration was determined by the method described by Bradford [2]. Approximately 80 μg of proteins were separated by electrophoresis on 12% polyacrylamide gel containing SDS [12].

Total protein (~60 μg) from strains LR and V3 were also separated by two-dimensional polyacrylamide gel electrophoresis (2-DE) [5, 15]. Second-dimension gels (15% polyacrylamide) [12] were stained with Coomassie Brilliant Blue R-250.

## Results and Discussion

Figure 1 shows the effect of 250 mmol/$L^{-1}$ of cadmium, zinc, and manganese sulfates on the oxygen consumption, coupled with $Fe^{2+}$ oxidation, of four strains of *A. ferrooxidans.* The concentration of 250 mmol/$L^{-1}$ of each metal sulfate was choose to differentiate the strains since, in general, it showed more significant differences. LR and I35 were the most tolerant strains to all three metal sulfates, and SSP and V3 were the most sensitive. The other strains tested, AMF, CMV, FG-460 and S, showed intermediate responses (results not shown).

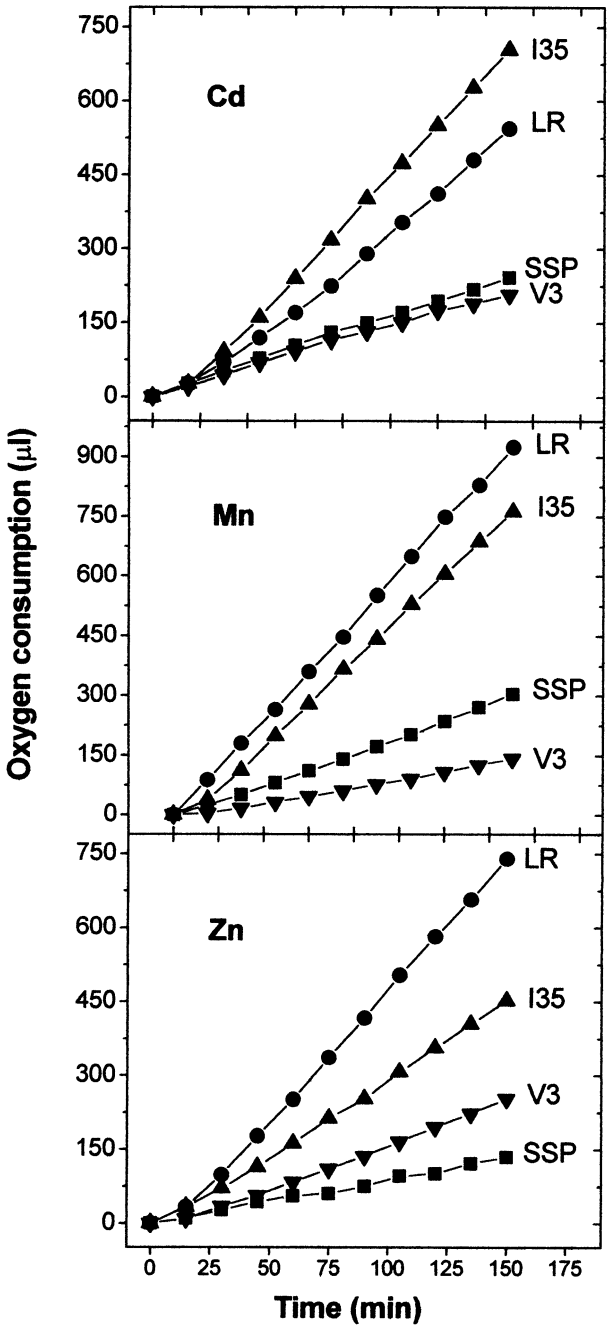


Fig. 1. Oxygen uptake coupled with $Fe^{2+}$ oxidation by *A. ferrooxidans* strains in the presence of cadmium, zinc, or manganese sulfates (250 mmol/$L^{-1}$).

As shown in Table 2, the relative inhibition on the initial rate of $Fe^{2+}$ oxidation in the most tolerant strains (LR and I35) ranged from no inhibition, for $MnSO_4$, to 11%, for $ZnSO_4$. The strains (SSP and V3) showed more significant inhibition, varying from 33 to 70% for $MnSO_4$; for $CdSO_4$ and $ZnSO_4$, the inhibition ranged

Table 2. Relative inhibition by metal sulfates (250 mmol/L) of the initial rate of $Fe^{2+}$ oxidation in respirometric experiments

| Strain | Inhibition (%)[a] | | |
|---|---|---|---|
| | Cd | Mn | Zn |
| LR | 9 | 0 | 6 |
| I35 | 4 | 0 | 11 |
| SSP | 41 | 33 | 49 |
| V3 | 64 | 70 | 33 |

[a] Compared to initial rates in absence of metal sulfates.

from 33 to 64%. Similar levels of inhibition for these strains have been obtained using 200 mmol/$L^{-1}$ $CuSO_4$ [15]. Strain LR is also more tolerant to $Co^{2+}$, $Ag^{+}$, and $Hg^{2+}$ than some *A. ferrooxidans* strains tested [7]. Thus, *A. ferrooxidans* strains have different levels of susceptibility to metal sulfates and this trait is an intrinsic cellular characteristic of each strain.

To investigate a possible correlation between metal sulfates tolerance levels and protein profile of the *A. ferrooxidans* strains, total proteins were extracted and analyzed by SDS-PAGE (Fig. 2). Strains I35 and LR showed very similar protein profiles (pattern I), whereas a distinctly different profile was observed for strains SSP and V3 (pattern II). For the strains which presented intermediate levels of inhibition (AMF, CMV, FG-460, and S) the protein profiles showed no detectable differences by SDS-PAGE analysis, resulting in a profile (pattern I) similar to those of LR and I35 strains. Proteins with apparent molecular weight of about 22 kDa and 40 kDa were only observed in the pattern I, whereas the proteins of approximately 47 and 43 kDa were specific for the pattern II (Fig. 2).

To investigate the 22-kDa and 40-kDa proteins present in pattern I and absent in the pattern II, 2-DE was performed for one of the most sensitive (V3) and one of the most tolerant strains (LR). Figure 3 shows the 2-DE profiles of total proteins from V3 and LR. Although common protein spots were observed in the two profiles, remarkable differences were also detected. A major 45-kDa protein was detected only in V3 (spot 1, Fig. 3A), probably corresponding to 47–43-kDa bands detected by SDS-PAGE (pattern II, Fig. 2). The apparent molecular masses of the major proteins detected only in strain LR were 40- (spot 2, Fig. 3B), 32- (spot 3, Fig. 3B), 20-, and 22-kDa (spots 4 and 5, Fig. 3B). Other differences between the 2-DE profiles of strains LR and V3 were also observed, but they were not as evident as the ones mentioned above.

The 40-kDa protein detected only in the more tolerant strain, LR, was acidic (Fig. 3B). In a previous work, the major acidic 40-kDa protein observed for LR strain was not significantly induced or repressed when the cells were grown in presence of 600 mmol/L of copper, zinc, nickel, or cadmium sulfates [15].

Outer membrane porins have been characterized as acidic proteins with a molecular mass in the range of 30–50 kDa [1, 14]. Jerez et al. [9] described major acidic outer membrane proteins of about 36 and 40 kDa (Omp40) in *A. ferrooxidans,* which apparently responded to external pH and phosphate starvation. Molecular characterization suggests that Omp40 is a porin [8]. Rodriguez et al. [19] also described a porin-like protein (~40 kDa) in the outer membrane of *A. ferrooxidans.*

RAPD analysis of the *A. ferrooxidans* strains that have previously been done in our laboratory [16] showed a low degree of similarity between the most and the least

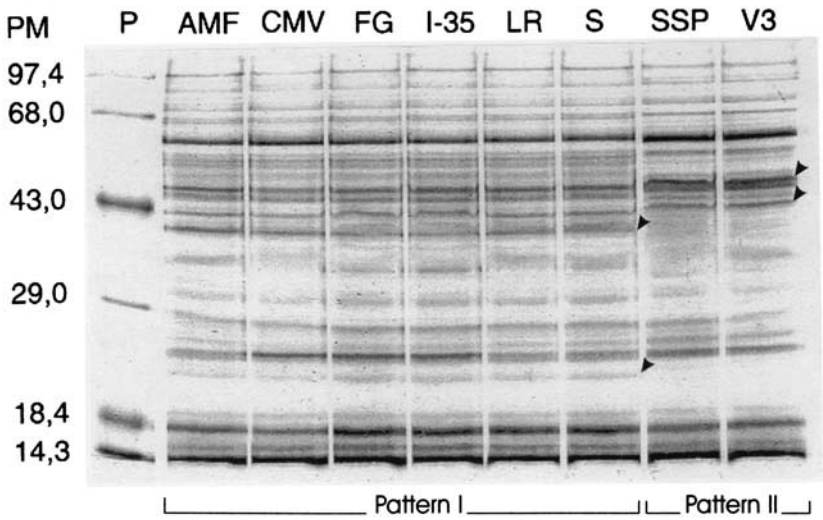


Fig. 2. SDS-PAGE analysis of total proteins from *A. ferrooxidans* strains in 12% polyacrylamide gel. Arrowheads indicate the major differential bands detected in the Pattern I (22 and 40 kDa) and in the Pattern II (47 and 43 kDa). The molecular mass (kDa) of standard proteins are indicated in the left side of the gel.

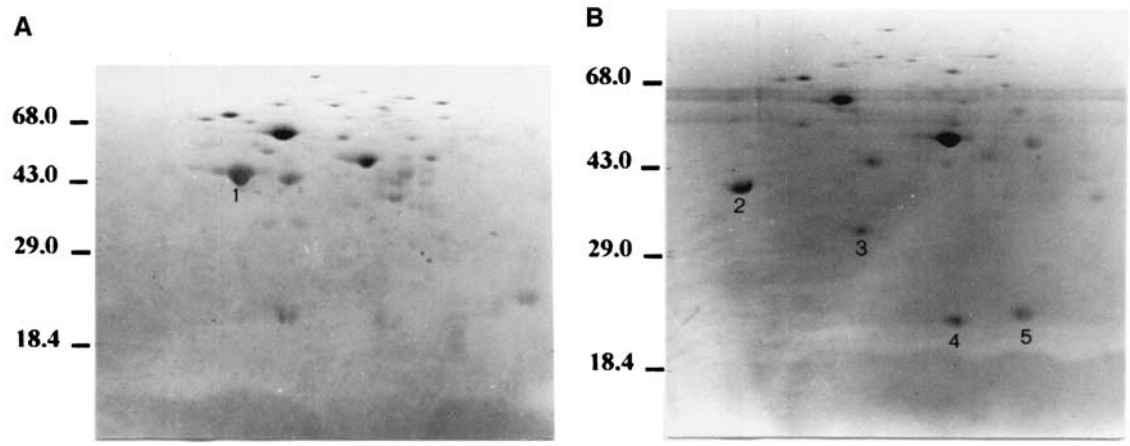


Fig. 3. Two-dimensional PAGE analysis of total proteins from *A. ferrooxidans* strain V3 (**A**) and LR (**B**). Numbers refer to the major differential bands mentioned in the text. The pH 5 and pH 8 side of the isoelectric focusing gel is on the left and the right side of the second dimension gel, respectively. The molecular mass (kDa) of standard proteins (Gibco BRL) are indicated in the left side of the gels.

Table 3. Relative inhibition by metal sulfates, protein profiles, and genomic similarity for some *A. ferrooxidans* strains used in this work

| Strain | Relative inhibition by metal sulfates[a] (%) | Protein profile[b] | | Genomic similarity (%) with[c] | | | |
|---|---|---|---|---|---|---|---|
| | | SDS-PAGE | 2-DE (40 kDa acidic protein) | LR | I35 | SSP | V3 |
| LR | <9 | I | + | 100 | 96.2 | 1.6 | 1.1 |
| I35 | <11 | I | na[d] | 96.2 | 100 | 1.6 | 1.1 |
| SSP | 33–49 | II | na | 1.6 | 1.6 | 100 | 97.6 |
| V3 | 33–70 | II | — | 1.1 | 1.1 | 97.6 | 100 |

[a] Respirometric experiments in presence of 250 mmol/L cadmium, zinc, or manganese sulfates.
[b] Total protein profiles of the strains grown in the absence of the metals. SDS-PAGE: patterns I and II from Fig. 2. 2-DE: the presence (+) or absence (—) of the ~40-kDa acidic protein discussed in the text is shown.
[c] RAPD data [16].
[d] Data not available.

tolerant strains. Strains LR and I35, the more tolerant strains, presented only 1.1–1.6% of genomic similarity with strains SSP and V3, the most sensitive ones. On the other hand, strains I35 and LR have high genomic similarity to each other (94.4–98.1%), as do the two sensitive strains, SSP and V3 (97.6%).

Despite such observed correlations, unexpected results were also observed. For example, strains AMF and S are very distinct from strains CMV, FG-460, I35, and LR by RAPD data—0.5–2% genomic similarity [16]—but they have similar protein profiles as detected by SDS-PAGE (Fig. 2). Besides, although strains AMF, CMV, FG-460, and S have protein profiles very similar to LR and I35 strains (Fig. 2), their tolerance levels are not the same.

It should be mentioned that the strain SSP has been recently analyzed by DNA–DNA hybridization [18] and the results obtained by these authors showed that this strain was not clearly related with *A. ferrooxidans.* The authors suggested that this strain could represent a new species of *Acidithiobacillus* with phenotypic properties similar to *A. ferrooxidans.*

Table 3 presents a summary of some strains comparisons. These data suggest an association between metal tolerance and the presence of an acidic 40-kDa protein. However, further investigations should be done in order to determine if this differential protein is related with the tolerance to metal sulfates in the LR strain, or if its presence/absence in *A. ferrooxidans* is only related with the high genomic diversity found in the species. The biochemical and genetic basis of tolerance to transition metals in acidithiobacilli is practically unknown, and future work in this area is warranted.

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