Rapid and specific detection of *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* by PCR

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A B S T R A C T

Acidithiobacillus ferrooxidans and *Leptospirillum ferrooxidans* are two of the most important bacteria in heap bioleaching processes of copper sulphide at common operating temperatures (18–24 °C). In this paper, both microorganisms were detected in solutions and ores coming from processes in 2 days, using specific amplification of 16S rDNA sequences by PCR. The technique was first validated using template DNA from pure cultures of the microorganisms. Then it was applied to samples of solutions and ores from bioleaching processes. Results were confirmed using tRFLP (terminal Restriction Fragment Length Polymorphism) with universal primers and by identification of isolated bacteria by means of culture. This methodology is more rapid and specific than the identification by tRFLP or by culture, which require from 1 to three weeks to positively detect the bacteria. The detection limit of this technique is 10^5 cells per ml.

Keywords:
Bioleaching
A. ferrooxidans
L. ferrooxidans
PCR
16S rDNA

1. Introduction

Copper sulphide ore bioleaching processes are currently under full development in Chile and worldwide, for treatment of secondary sulphide in heaps (Brierley, 1999), low-grade ores in dumps or for bioleaching of concentrates in reactors at high temperatures (ACL Project, 2000). In these processes, microorganisms (bacteria and archaea) play a major role as catalysts of chemical reactions, which in their absence, would be carried out at low speed, making the process inefficient (Norris, 1991).

Amongst the microorganisms participating in these processes are iron-oxidising and sulphur-oxidising bacteria and archaea, heterotrophic bacteria, as well as other species that are active at different temperatures (Goebel and Stakebrandt, 1994; Yahya et al., 1999; Rawlings, 2001). Participation of these microorganisms in the dissolution of metal sulphides has been widely studied (Rohwerder et al., 2003). Today, it is accepted that iron-oxidising microorganisms are critical for keeping a high oxidising potential in solutions, which is a key condition for the dissolution of metal sulphides (Rawlings, 1998). Amongst mesophilic bacteria, the most important in bioleaching due to their oxidising capacity of Fe(II) to Fe(III) are *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* (Rawlings, 1998). *T. ferrooxidans* which was renamed *Acidithiobacillus ferrooxidans* by Kelly and Woods (2000), oxidises Fe(II) and reduced sulphur compounds; *L. ferrooxidans* oxidises only Fe(II). These microorganisms have been frequently described as participants in copper sulphide bioleaching processes in different parts of the world (Okibe et al., 2003; Nemati et al., 1998). Detection of these bacteria in pregnant leaching solutions (PLS) and in minerals from bioleaching processes has traditionally been made using culture dependent techniques, using liquid or solid media (Johnson, 1995). However, these methods do not always allow distinction between *A. ferrooxidans* and *L. ferrooxidans*. Due to their different growth kinetics it is often unfeasible to culture them simultaneously (Jerez, 1998). One additional disadvantage of this methodology is the slow pace of the detection process, which can take several weeks. In the last few years, several molecular techniques have been developed using PCR (polymerase chain reaction) for the identification of microorganisms, consisting to amplification of 16S rDNA sequences from isolated genomic DNA (Jensen et al., 1993). In the case of identification of microbial communities in bioleaching processes, the most widely used molecular techniques have been: 16S rDNA analysis by restriction enzymes (Rawlings, 1995); analysis of spacing regions from ribosomal operons (Pizarro et al., 1996); analysis of phylogenetic groups (De Wulf-Durand et al., 1997); DGGE (Denaturing Gradient Gel Electrophoresis) (Demergasso et al., 2005), and t-RFLP (terminal Restriction Fragment Length Polymorphism) (Bryan et al., 2005), amongst others. However, most of these techniques are slow, and in many cases, irrelevant microorganisms to the process are also detected in the samples. We present here a rapid and specific technique to detect *A. ferrooxidans* and *L. ferrooxidans* in solutions and mineral samples of bioleaching process, in order to monitor the growth dynamics of these organisms

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in response to varying environmental conditions. This technique can be incorporated specifically when extensive sampling is required to check continuously several points in the heap.

2. Materials and methods

2.1. Microorganisms

DNA from *A. ferrooxidans* ATCC 19859 and *L. ferrooxidans* ATCC 29047 were used as templates for PCR. Bacteria were cultured in basal medium (0.4 $(NH_4)_2SO_4$, 0.4 $MgSO_4 \times 7H_2O$ and 0.056 $K_2HPO_4 \times 3H_2O$ g/L) pH 1.6 (Escobar and Godoy, 1999) containing 3 g/L ferrous sulphate.

2.2. Ore and solution samples

DNA was extracted from bacteria contained in different solutions coming from a laboratory bioleaching column or PLS solution of a bioleaching heap process. A sample of agglomerated ore coming from a bioleaching heap process was also used in this study.

2.3. Genomic DNA extraction

For the extraction from cells coming from pure cultures, the initial volume was 100 mL; for bioleaching solutions, 4 or 5 L were used, due to the low concentration of bacteria in those solutions (about 1.0×10^6 bacteria/mL). Bacteria were concentrated by micro-filtration and washed twice with 1× PBS pH 1.2, and also with washing buffer (50% A2×, 25% Glycerol); cells were resuspended in 3 mL buffer A2× (200 mM Tris HCl pH 8.0, 50 mM EDTA, 200 mM NaCl, 2 mM sodium citrate and 10 mM $CaCl_2$) containing 3 mg/mL lysozyme and incubated at 37 °C for 1 h. After adding SDS at 4% and 10% CTAB (Hexadecyltrimethylammonium bromide) (De Wulf-Durand et al., 1997) in NaCl 0.7 M, samples were incubated at 65 °C for 1 h. Three cycles of freezing at −20 °C and thawing at 70 °C were carried out. DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1), washed with chloroform-isoamyl alcohol (24:1) and precipitated with absolute isopropanol in 0.3 M sodium acetate pH 5.2. The pellet was washed with 70% ethanol. DNA obtained was resuspended in 10 mM Tris–HCl pH 8 and 0.1 mM EDTA and stored at −20 °C.

For extraction of DNA in ores, 10 g of the ore was washed three times with 0.3 N sulphuric acid and the thickest particles were eliminated. Then the ore was washed in 50 mM Tris–HCl pH 7.6, 50 mM EDTA, 20% v/w sucrose. Lysis of the adhered cells was carried out by three successive incubations: 1 h at 37 °C in 15 mg/mL lysozyme; 1 h at 50 °C in 10 mg/mL proteinase K, SDS 4% w/v, 5 M NaCl and 1 h at 65 °C in 1% CTAB. After that, the same protocol used for DNA extraction from cultured or solutions cells was applied. DNA yield was checked by electrophoresis in 0.8% agarose gel (40 min at 100 V and 135 mA) with 0.01% ethidium bromide.

Table 1
Strains used for designing primers for *A. ferrooxidans* and *Leptospirillum ferrooxidans* detection

Microorganism	Strain	GenBank accession number (1)
A. ferrooxidans		
	DSM 9465	Y11595
	ATCC 23270	AF329205
	N-Fe3	X75268
	ATCC19859	AF362022
	N-Fe4	X75267
	N-Fe2	X75266
	TFI	AF46560
	ML1	AJ306698
	ML2	M79412
	S/N	M79402
	NASF-1	AB03982
	ATCC33020	AJ278719
	SS4	AJ27872
	OP4	X91222
	WJ13	AY495953
L. ferrooxidans		
	CF12	AF356834
	X72852	X72852
	BCT2	AF356833
	49879	AF356832
	P3a	AF356837
	Parys	AF356838
	Sy	AF356839
	L15	X86776
	Lf2	AF356835
	ATCC	M79442
	Z2	M79443
	Z2	M79441
	EPA15	X91228
	TzT-B1-K4	AJ295686
	WJ71	AY495960

(1) Sequences correspond to 16S rRNA gene.

Table 2
Primers used in this study for amplification of 16S rDNA from *A. ferrooxidans*, *Leptospirillum ferrooxidans* and universal primers to tRFLP analysis

Primer	Sequence (5′→ 3′)	Target site[a]
A. ferrooxidans		
F1_Thio (Sense)	ATGCGTAGGAATCTGTCTTT	120–139
R1_Thio (Antisense)	GGACTTAACCCAACATCTCA	1078–1097
L. ferrooxidans		
F1_Lepto (Sense)	GGGTGAGTAATACATGGGTG	111–130
R1_Lepto (Antisense)	AACTTGTCGCTGGCAGTC	1156–1173
Universal Primers		
341f (Sense)	CCTACGGGAGGCAGCAG	341–357
1100r (Antisense)	GGGTTGCGCTCGTTG	1100–1114

[a] *E. coli* numbering.

2.4. Primers design

The specific primers used in the PCR reactions for *A. ferrooxidans* and *L. ferrooxidans* were designed using the Ribosomal Database Project II (RDPII), available on the Internet (http://rdp.cme.msu.edu/index.jsp), and the Primer Premier v5 software (www.ncbi.nlm.nih.gov). By using the aforementioned database and software, specific primers to target *A. ferrooxidans* and *L. ferrooxidans* 16S rDNA were designed. Strains analysed are listed in Table 1, while selected primers are shown in Table 2. The sequences used and designed here are specific for each strain. At the moment we made this work, no specific sequences had been described in the literature for the 15 strain used in this work for *A. ferrooxidans* and *L. ferrooxidans.* For tRFLP analysis, different combinations of universal primers-enzyme were tested using the software Probe Match of RDPII, Enzyme Resolution and Virtual Digest available in MICA (http://mica.ibest.uidaho.edu). Universal primers 341f and 1100r, described in literature (Liu et al., 1997, Osborn et al., 2000), were validated using online databases. The enzyme RsaI was selected using as criteria the number of microorganims amplified, usually present in bioleaching processes and the best resolution of terminal fragments. Table 3 shows the tRFs lengths predicted for the most frequently found bacteria in bioleaching environments.

2.5. 16S rDNA amplification by PCR

PCR was carried out at a final volume of 25 μL, containing in each case 1 μM each *sense/antisense* primer, 0.2 mM each dNTP, buffer Taq 1× (Invitrogen), 1.5 mM $MgCl_2$, 2.5 U of Taq polymerase and 5 μL (10–100 ng) of the purified genomic DNA. The amplification programme consisted of 40 cycles of 1.5 min at 95 °C, 1.5 min at 58.5 °C for *A. ferrooxidans* primers and 53 °C for *L. ferrooxidans* primers; finally, extension was carried out at 72 °C. Reactions were carried out in an Eppendorf Thermocycler. PCR products were checked by 1.2% agarose gel electrophoresis stained with 0.01% ethidium bromide.

For tRFLP, universal bacterial primers were used. The sense primer was labelled fluorescently with FAM (6-carboxyfluorescein); the annealing temperature in the PCR was 56 °C. Resulting amplification products were purified using QIAquick PCR purification kit prior to digestion with the restriction enzyme. 18 ng/μL of purified PCR products were digested with 15 U endonuclease R*sa*I (Invitrogen) and 1× Buffer R*sa*I in a 20 μl reaction volume. Restriction digests were incubated at 37 °C for 3.5 h. Digested fragments were separated by capillary gel electrophoresis and detected in laser induction fluorescence Genetic Analyzer (ABI PRISM model 310, Applied Biosystems).

Table 3
Predicted tRF length for bioleaching bacteria using primer 341f and 1100r in combination with the restriction enzyme R*sa*I

Strain	tRF length (bp)
Acidiphilium sp	121
Acidithiobacillus caldus	551
Acidithiobacillus ferrooxidans	247
Acidithiobacillus thiooxidans	309
Leptospirillum ferriphilum	146
Leptospirillum ferrooxidans	146
Sulfobacillus sp	142

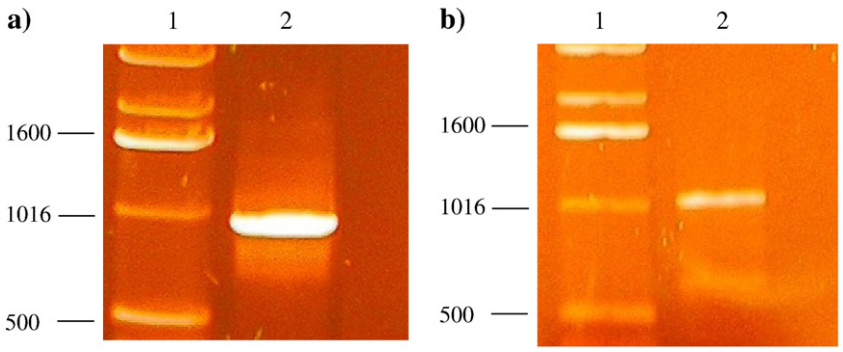

Fig. 1. 16S rDNA fragments amplified from the genomic DNA isolated from pure cultures of *A. ferrooxidans* ATCC 19859 and *L. ferrooxidans* ATCC 29047. Lane 1, 1Kb ladder (bp, Invitrogen), Lane 2, 16S rDNA amplified from *A. ferrooxidans* (a) and *L. ferrooxidans* genomic DNA (b).

2.6. Detection limit determination for this technique

The detection limit was determined using five cell suspension mixtures containing *A. ferrooxidans* and *Sulfolobus metallicus* cells, in which *A. ferrooxidans* cells were present in decreasing concentrations from 10^7 to 10^3 bacteria/mL. The DNA extracted from 100 mL of these mixtures containing 10^8 total cells/mL was used as template for PCR with the specific primers for *A. ferrooxidans*.

2.7. Isolation and detection of microorganisms from bioleaching solutions and mineral samples by culture

Concentrations of bacteria in bioleaching solutions are low. Therefore in order to isolate microorganisms from these solutions 1 L of PLS was concentrated by filtration through a membrane filter of 0.22 μm pore size. Bacteria on the filter were resuspended in 5 mL of basal medium pH 1.6, and added to basal medium containing 3 g/L of ferrous sulphate pH 1.6. The increase of the redox potential of this solution and production of ferric sulphate showed by the yellow to orange colour of the solution was indicative of the presence of ferrous oxidizing bacteria. To differentiate between both microorganisms, 5 mL of each culture were added to basal medium containing 0.01 M potassium tetrathionate pH 4.0; acidification of the medium to pH 1.5 demonstrated presence of *A. ferrooxidans* (a sulphur-oxidizing bacteria) (Escobar and Lazo, 2003). In the case of *L. ferrooxidans* there was not acidification of the culture medium. To isolate and identify bacteria present in agglomerated ore, 10 g of the ore were added to basal medium pH 1.6 containing 3 g/L of ferrous sulphate, after 2 weeks ferrous oxidizing bacteria were detected by the presence of ferric iron; the identification of bacteria was carried out according to the previously described methodology for bacteria coming from solutions.

3. Results and discussion

3.1. Amplification of the 16S rDNA sequence from pure cultures

Amplification conditions were optimised using genomic DNA from pure cultures of *A. ferrooxidans* ATCC 19859 and *L. ferrooxidans* ATCC 29047 as template. Electrophoretic analysis of the PCR products (Fig. 1) showed that the size of the fragments amplified from both *A. ferrooxidans* and *L. ferrooxidans* DNA matches the expected size of 985 and 1088 bp, respectively. Also, no other amplification bands were observed, which demonstrates the high specificity of each pair of primers for the tested strains. These results were controlled using the technique of tRFLP with primers 341f and 1100 r (*sense/antisense* respectively) in combination with the restriction enzyme RsaI. In both cases only one important peak was observed (Fig. 2), corresponding to tRF length of 247 and 143 bp. According to the data shown in Table 3, these values would correspond to *A. ferrooxidans* and *L. ferrooxidans*, respectively.

The detection limit using this technique was determined for *A. ferrooxidans*. As shown is Fig. 3, using this technique was possible to detect a concentration as low as 10^5 cells/mL of *A. ferrooxidans*, starting with a suspension of total population of 10^{10} cells. This value is ten times lower than the detection limit of direct count to microscope (10^6 cells/mL).

3.2. Identification of A. ferrooxidans and L. ferrooxidans in bioloeaching solutions and ores

In order to amplify the 16S rDNA of *A. ferrooxidans* and *L. ferrooxidans*, genomic DNA extracted from bacteria present in a PLS from a bioleaching plant was PCR-amplified using the specific primers described in Table 2. The same methodology was used with a sample of genomic DNA extracted from cells adhered to the ore, coming from a secondary sulphide bioleaching plant. As is shown in Fig. 4, amplification with both pairs of primers was very specific; in each case, size of the amplified DNA agrees to the amplified fragments from genomic DNA from pure cultures. tRFLP results of the same samples digested with RsaI (Fig. 5) showed, amongst others, restriction fragments of 146 and 247 bases, corresponding to the predicted length for *L. ferrooxidans* and *A. ferrooxidans*, respectively (Table 3). These results corroborated that both strains were present in the samples, although there were also other microorganisms, such as *A. thiooxidans* and probably *S. thermosulfidooxidans*, frequently found in PLS solutions and ores (Norris, 1983; Okibe et al., 2003; Coram-Uliana et al., 2005, Nemati et al., 1998; Rawlings, 1998). To confirm the results obtained with this technique, identification of the iron-oxidising bacteria in both samples was also made using culture techniques. In both cases, results showed the presence of both microorganisms in the samples. However, identification by culturing was possible after 15 days in the case of PLS solution and about 30 days in the case of ore, that is, much slower than detection by the PCR methodology. It can be observed that each pair of primers used in this technique does not give a positive amplification with the genomic DNA of the other specie, which allows us to conclude that this methodology is specific and effective for the identification of *A. ferrooxidans* and *L. ferrooxidans*, individually and also in a mixture.

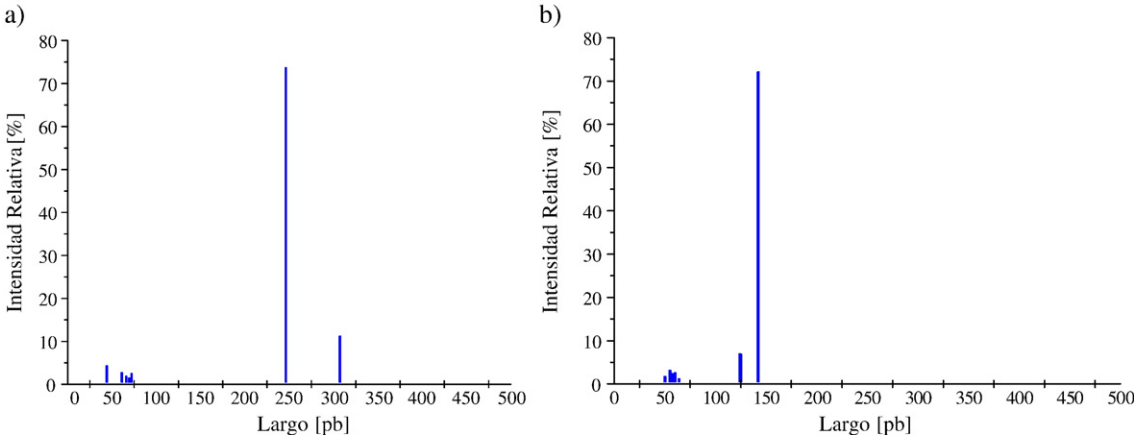

Fig. 2. Processed tRFLP results analysis for pure culture of *A. ferrooxidans* ATCC 19859 (a) and *L. ferrooxidans* ATCC 29047 (b).

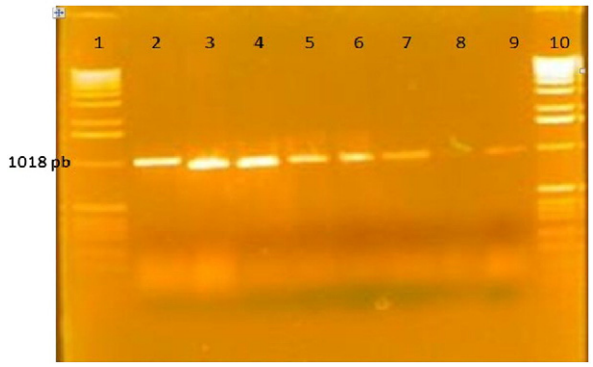

Fig. 3. Detection limit determination for *A. ferrooxidans*. Lane 2 and 3, 107 cells/mL; Lane 4 and 5, 106 cells/mL; Lane 6 and 7, 105 cells/mL, Lane 8 and 9, 104 cells/mL, Lane 1 and 10, 1 Kb DNA ladder (bp, Invitrogen).

Primers designed in this work are specific for each microorganism and, according to the computational analysis carried out for this work, they should efficiently amplify 15 different strains of both *A. ferrooxidans* and *L. ferrooxidans*, which is not possible using primers described in the literature (Schrenk et al., 1998; Pecchia et al., 2000).

This technique, like the FISH technique, uses specific primers to detect microorganisms in a determined environment, however, this technique is simpler and more rapid than FISH, because it only requires the use of a Thermocycler and an equipment for agarose gel electrophoresis. On the other hand, the FISH technique requires two more specific equipments, a hibridization oven and an epifluorescence microscope, which demonstrates that the technique presented in this work is more rapid and simpler to apply.

In principle in all bioleaching processes of metallic sulphides both microorganisms should be present. In case that they were not detected (both or one of them), what could be attributed to the negative effect on them of some solutions like high concentration of some ions (sulphate, aluminium, zinc, chlorine, etc), it is possible to introduce some modifications on the solutions in order to rapidly revert the problem.

For the implementation of this technique in this work (using specific primers), the starting material was 4×10^9 total bacteria. Although the technique is not quantitative, we observed that it is possible to detect microorganisms using 1 to 3 L of bioleaching solutions, with an initial concentration of 1×10^6 bacteria/mL. Since the concentration of bacteria in raffinate or PLS solutions is usually about 1×10^6 bacteria/mL, the methodology is perfectly applicable to this cellular density. Thus, this technique permits detection of microorganisms even at very low concentration in the solution (10^5 bacteria/mL). According to our experience the yield in the extraction processes of genomic DNA from leaching bacteria is extremely low. This also has been observed by other authors (Freslung et al., 2001). Furthermore, it is possible that the template DNA solution still retains some residual concentrations of ions and salts from culture medium, which are strong inhibitors of the PCR. Even so, we consider that the detection level obtained with this procedure is enough to detect bacteria in these environments. In the ores, if the concentration of attached bacteria is very low (less than 10^6 bacteria/g), it will be necessary to take at least 50 g of ore to have good results with the DNA extraction.

This technique can also be applied when the presence of *A. ferrooxidans* and *L. ferrooxidans* needs to be established in sulphide flotation tailings, which if colonised with these microorganisms, will generate acid water drainage (AMD) (Bond et al., 2000; Leveille et al., 2001; Lopez-Archilla et al., 2001; Gonzalez-Toril et al., 2003), with the well-known negative consequences (Dennison et al., 2001).

Although this technique has been implemented in this work only for these two microorganisms, it can also be applied to other microorganisms present in these processes, such as *A. thiooxidans*, *A. cryptum*, *S. thermosulfidooxidans*, etc., designing the specific primers in each case. In these cases response time should be the same, that is, 2 days. The technique described here is much faster than other molecular techniques, such as tRFLP or DGGE, which are laborious and time consuming (Liu et al., 2006). The tRFLP technique, on other hand, can be used to account for population changes during a bioleaching process because it allows only a partial identification of the microorganisms present in a sample.

In conclusion, the implemented molecular technique allows identification and differentiation in no more than 2 days, any microorganism provided that 16S rDNA sequences are available in RDPII to design specific primers for their detection.

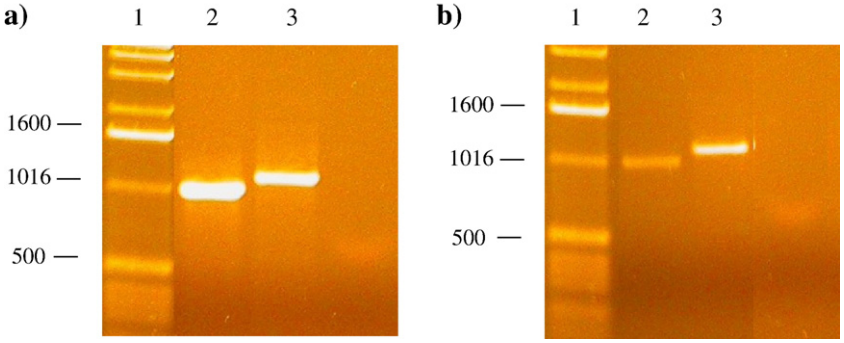

Fig. 4. Electrophoresis of the products amplified from genomic DNA extracted from bacteria in a raffinate solution (a) and ores (b) with specific primers against *A. ferrooxidans* (Lane 2) and *L. ferrooxidans* (Lane 3) 16S rDNA. Lane 1, 1 Kb DNA ladder (bp, Invitrogen).

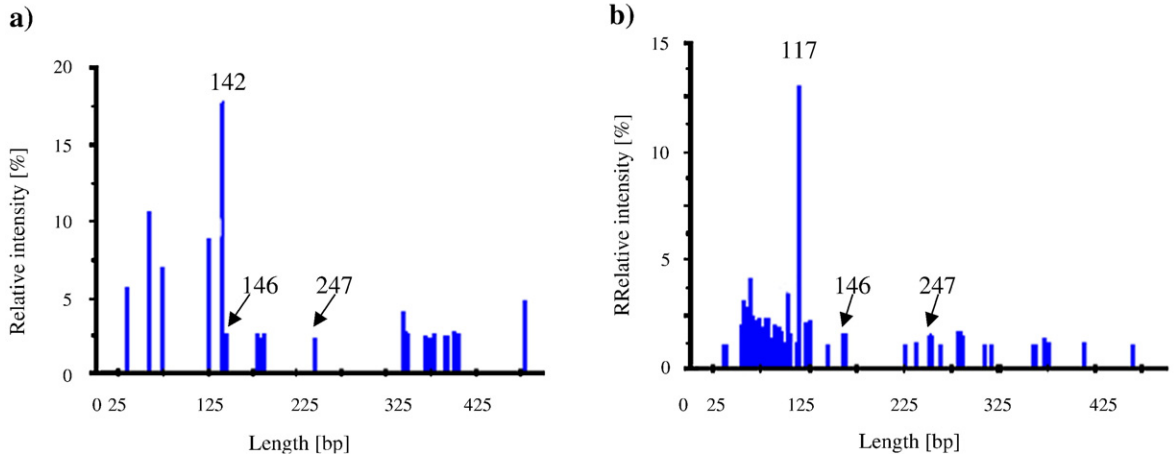

Fig. 5. Processed tRFLP results for DNA extracted from bacteria in an ore (a) and in a PLS solution (b). Numbers correspond to the length of restriction fragments for bioleaching bacteria shown in Table 3, predicted using the informatic tools available in MICA. 117 bp corresponds to *Bacillus* sp.

In this work the technique was applied at two of the most important and most frequent microorganisms present in bioleaching processes at environmental temperature: *A. ferrooxidans* and *L. ferrooxidans*. This PCR-based technique lies in the design of specific primers for 16S rDNA amplification, using genomic DNA directly extracted from bacteria in raffinate or PLS solutions and in ores.

Acknowledgments

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