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Dynamic of active microorganisms inhabiting a bioleaching industrial heap of low-grade copper sulfide ore monitored by real-time PCR and oligonucleotide prokaryotic acidophile microarray

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Summary

The bioleaching of metal sulfide has developed into a very important industrial process and understanding the microbial dynamic is key to advancing commercial bioleaching operations. Here we report the first quantitative description of the dynamic of active communities in an industrial bioleaching heap. *Acidithiobacillus ferrooxidans* was the most abundant during the first part of the leaching cycle, while the abundance of *Leptospirillum ferriphilum* and *Ferroplasma acidiphilum* increased with age of the heap. *Acidithiobacillus thiooxidans* kept constant throughout the leaching cycle, and *Firmicutes* group showed a low and a patchy distribution in the heap. The *Acidiphilium*-like bacteria reached their highest abundance corresponding to the amount of autotrophs. The active microorganisms in the leaching system were determined using two RNA-based sensitive techniques. In most cases, the 16S rRNA copy numbers of *At. ferrooxidans*, *L. ferriphilum*, *At. thiooxidans* and *F. acidiphilum*, was concomitant with the DNA copy numbers, whereas *Acidiphilium*-like bacteria and some *Firmicutes* members did not show a clear correlation between 16S rRNA accumulation and DNA copy numbers. However, the prokaryotic acidophile microarray (PAM) analysis showed active members of *Alphaproteobacteria* in all samples and of *Sulfobacillus* genus in older ones. Also, new active groups such as *Actinobacteria* and *Acidobacterium* genus were detected by PAM. The results suggest that changes during the leaching cycle in chemical and physical conditions, such as pH and Fe^{3+}/Fe^{2+} ion rate, are primary factors shaping the microbial dynamic in the heap.

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Introduction

Heap bioleaching is currently the most successful technology for the extraction of base metals from low-grade sulfide ores (Watling, 2006), and during the last few decades this technology has become increasingly important due to the depletion of high-grade copper ores and the existence of huge natural reserves of copper in the form of secondary copper sulfides (Galleguillos *et al.*, 2008). In recent years scientific and commercial interest has emerged to study the microbial ecology of industrial bioleaching processes (Demergasso *et al.*, 2005; Hawkes *et al.*, 2006; Xie *et al.*, 2007; Wakeman *et al.*, 2008), because the understanding of the microbiological aspects would facilitate the design and operation of industrial heaps to improve this technology (Brierley, 2001; Rawlings, 2002; Watling, 2006; Yin *et al.*, 2007). In case of metal sulfide bioleaching, metal sulfides are oxidized to metal ions and sulfate by aerobic, acidophilic Fe(II) and/or sulfur-compound oxidizing *Bacteria* or *Archaea* (Schippers, 2007). These microorganisms are responsible for producing the ferric iron and sulfuric acid for the bioleaching reactions (Rawlings, 2007; Johnson and Hallberg, 2007). In addition to those acidophiles that have direct roles in accelerating mineral dissolution, other acidophiles, most of them heterotrophic, could have a positive impact on the overall process (Johnson and Hallberg, 2007).

Some recent studies have corroborated that *Acidithiobacillus ferrooxidans* and *Leptospirillum* species seem to be the most abundant microorganisms in heap leaching and mine waste environments (Diaby *et al.*, 2007; Remonsellez *et al.*, 2007; Xie *et al.*, 2007; Galleguillos *et al.*, 2008; He *et al.*, 2008; Kock and Schippers, 2008).

Several mesophilic and moderately thermophilic species of phylum *Firmicutes* have been identified and isolated from sulfide ore heaps, stirred tanks and mine waste, but many of them are not validly described yet (Diaby *et al.*, 2007; Schippers, 2007; Xie *et al.*, 2007; He *et al.*, 2008; Johnson *et al.*, 2008). Some acidophilic heterotrophic bacteria have been found in bioleaching operations and environments (Diaby *et al.*, 2007; Rowe *et al.*, 2007; Xie *et al.*, 2007; Kock and Schippers, 2008). *Ferroplasma* and *Thermoplasma* lineages have been regularly found in bioleaching operations as well (Hawkes *et al.*, 2006; Xie *et al.*, 2007; Galleguillos *et al.*, 2008; He *et al.*, 2008), and other *Archaea* microorganisms like *Sulfurisphaera* and *Sulfolobus* genera have been identified in a copper test-heap (Demergasso *et al.*, 2005).

A variety of molecular techniques have been widely used for the analyses of mineral-leaching populations (Johnson and Hallberg, 2007). After that, the use of PCR associated with fluorescence emission (q-PCR) has emerged as a new approach to quantitatively describe the community composition, and several works have reported the quantification of *Bacteria* and *Archaea* in water, soil and sediment samples, and even in mine waste tailing and leaching solutions (Fey *et al.*, 2004; Kock and Schippers, 2006; 2008; Liu *et al.*, 2006; Schippers and Neretin, 2006; Remonsellez *et al.*, 2007). Despite the available techniques, the studies of bioleaching heaps have described the microbial diversity by relative abundance of communities (Demergasso *et al.*, 2005; Wakeman *et al.*, 2008) and most of them in some specific samples from the processes (Rawlings and Johnson, 2007; Xie *et al.*, 2007; He *et al.*, 2008). Therefore, the predominance of certain members of the population at specific stages of the bioleaching heap process and the reasons of that dynamic is just being described (Demergasso *et al.*, 2005; Galleguillos *et al.*, 2008; Wakeman *et al.*, 2008).

The DNA approaches used in the analysis of acidophiles are based in the detection and amplification of one specific gene (mainly the 16S rRNA gene), indicating the presence of the microorganism containing that gene (Johnson and Hallberg, 2007). However, a large part of the microorganisms could be dormant or ever dead and yet retain stable DNA. Instead, experiences with pure cultures have shown that cells with significant ribosome content are living and metabolically active (Schippers *et al.*, 2005). To get information on the active microorganisms in a process, RNA-based analyses should be performed. The last years have brought rapid advances especially in tools used for molecular microbial ecology that merit to be explode (Johnson and Hallberg, 2007). One important advance is the use of oligonucleotide arrays to detect and to analyse microbial communities (Zhou, 2003). Oligonucleotide arrays have been used successfully in environmental studies in the last years (Wu *et al.*, 2001; Valinsky *et al.*, 2002; Brodie *et al.*, 2006; Gentry *et al.*, 2006). Recently, two oligonucleotide microarrays have been developed to monitor the diversity in extremely acidic environments (Yin *et al.*, 2007; Garrido *et al.*, 2008). One of them has been used to detect the most metabolically active microorganisms using labelled environmental RNA (Garrido *et al.*, 2008).

In order to get new insights and understanding the role that microorganisms play in mineral processing operations, we analysed the composition and the dynamic of the microbial communities in an industrial bioleaching heap in Chile (Fig. 1). To achieve this objective, we determined the active microorganisms during the leaching cycle in the industrial heap by means of a combination of two sensitive

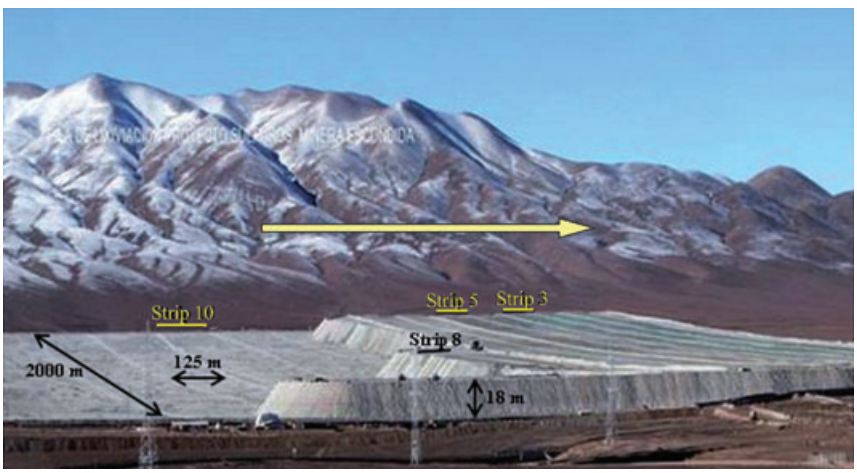

Fig. 1. Image of the industrial bioleaching heap in Escondida Mine in January 2007. Arrows indicate the dimension of strips, and lines indicate the main strips (S3, S5, S8 and S10) analysed in this study. The direction of the yellow arrow indicates the increase in days of operation of the strips.

Table 1. Summary of the microbial community composition of the industrial heap using DGGE and 16S rRNA gene clone libraries during year 2006.

	DGGE				Clone libraries			
	August		November		September		August	
Microorganism	S1	S3	S1	S3	Feed	S1	S3	S4
Bacteria								
At. ferrooxidans	2	2	2	2	57	18	87	96
At. thiooxidans	1	1	1	1	9	5	11	–
L. ferriphilum	1	1	1	1	8	13	–	2
Ab. disulfidooxidans	1	1	–	–	–	–	2	–
Uncultured *Alicyclobacillus*	1	–	1	1	–	–	–	1
Sulfobacillus spp.	–	–	1	–	–	–	–	–
Acidiphilum-like	–	–	–	–	–	–	–	1
Archaea								
F. acidiphilum	2	2	2	2	5	6	ND	ND

DGGE, number of sequenced bands. Clone libraries, number of 16S rRNA clones analysed.
ND, not done. –, not found.
S1, strip 1; S2, strip 2; S3, strip 3; Feed, irrigation solution.

molecular techniques, such as quantitative real-time PCR and a microarray for prokaryotic acidophiles.

Results

Composition of the microbial community in the industrial bioleaching system

Since the beginning of the bioleaching cycle in the heap, DGGE and 16S rRNA clone libraries were used to identify the microbial diversity in early strips in operation and in the feed solution (year 2006). Comparative sequence analysis of DGGE bands showed the presence of organisms related to *At. ferrooxidans*, *At. thiooxidans*, *Leptospirillum ferriphilum* and the archaeon *Ferroplasma acidiphilum* (Table 1) with percentages of similarity between 97% and 99% with-type strains. The rest of the bands showed sequence similarities between 80% and 85% to Gram-positive *Sulfobacillus* spp. (called *Sulfobacillus*-like bacteria), *Alicyclobacillus disulfidooxidans* and an uncultured *Alicyclobacillus*. The results showed in the Table 1 were comparable with DGGE bands analysed from other new strips in different times of the leaching cycle (data not shown).

Moreover, we constructed bacterial and archaeal 16S rRNA clone libraries to complement the data obtained by DGGE. The analysis of all different clones showed that the community is mainly composed by sequences related to *Acidithiobacillus* genus (Table 1). We found two different phylotypes of *At. ferrooxidans* (called D2 and DM) and around of 75% of analysed clones were *At. ferrooxidans* D2. *At. ferrooxidans* D2 is similar to the sequence of *At. ferrooxidans* strain D2 (AJ278723) and these sequences have just 99% of identity with *At. ferrooxidans* DM (Fig. S1). Moreover, we found two different phylotypes of *L. ferriphilum* with 98% of identity by using BLAST algorithm (Altschul *et al*., 1997), and one of them is similar to *L. ferriphilum* type Warwick (Fig. S2). Sequences $\geq$ 98% related with *At. thiooxidans*, $\geq$ 93% with *Ab. disulfidooxidans*, 94% with uncultured *Alicyclobacillus*, and one sequence 94% similar to *Acidiphilium* spp. (called *Acidiphilium*-like bacteria), were also identified. Sequences with high similarity ($\geq$ 99%) related to *F. acidiphilum* from the Archaea domain were also detected (Table 1).

These results showed that the identified microorganisms correspond to phylogenetic groups such as *Alphaproteobacteria*, *Gammaproteobacteria*, *Nitrospira*, *Firmicutes* and *Thermoplasmata*, normally found in heap- and tank-bioleaching processes (Espejo and Romero, 1997; Demergasso *et al*., 2005; Rawlings and Johnson, 2007) and acid mine waste (Johnson and Hallberg, 2003; Diaby *et al*., 2007).

Dynamic of the microbial communities in the heap

Sequences obtained by DGGE and clone libraries were used to design specific primers (Table 2) and, following standardization, real-time PCR was used to quantify the dynamic of microorganisms inhabiting the industrial heap.

The microorganisms inhabiting the heap throughout the leaching cycle are mainly bacterial species and reached total 16S rRNA gene copy numbers greater than 10^7 copies ml^{-1} and, in general, this value decreased around one order of magnitude in most of the strips when these aged more than 200 days (Fig. 2A and B; all other strips: data not shown). *At. ferrooxidans* phylotypes were the most abundant microorganisms during the first part of the leaching cycle (around 200 days of operation) and

Table 2. Oligonucleotides used in quantitative real-time PCR analysis.

Targeted group	Primer	Sequence (5′→3′)	Reference
Universal[a,b]	907R	CCGTCAATTCMTTTGAGTTT	Casamayor *et al.* (2002)
Bacteria[b]	UBactF	TCCTACGGGAGGCAGCAGT	Nadkarni *et al.* (2002)
Bacteria[b]	UBactR	GGACTACCAGGGTATCTAATCCTGTT	Nadkarni *et al.* (2002)
Archaea[b]	ARCH349F	GYGCASCAGKCGMGAAW	Takai and Horikoshi (2000)
Archaea[b]	ARCH806R	GGACTACVSGGGTATCTAAT	Takai and Horikoshi (2000)
Archaea[a,b]	ARCH-R	TGCTCCCCCGCCAATTCC	This study
At. ferrooxidans D2[b]	ATFD2-F	CGGGTCCTAATACGATCTGCT	This study
At. ferrooxidans DM[b]	ATFDM-F	TGGTTCCTAATACGAGCTACTG	This study
At. thiooxidans[b]	ATT-F	GGGTGCTAATANCGCCTGCT	This study
L. ferriphilum[b]	Lferri-F	CGTCAGAAIACGGCGCTTC	This study
L. ferriphilum Warwick[b]	LferriW-F	GATGTCAGAACACGGCATTT	This study
Sulfobacillus-like[b]	Sesc-F	GGAGACCGTGCCGTCG	This study
Ab. disulfidooxidans[b]	SG1-F	AGTGGCGAAGGCGCCTTGCTGG	This study
Uncultured *Alicyclobacillus*[a]	Adunc-F	CCTCTCCGACCCTCAAGTCT	This study
Uncultured *Alicyclobacillus*[b]	Adunc-R	AGGAGAGGGAATGCTTTTGG	This study
Acidiphilium-like[b]	Acesc-F	AGGCGGCTTRTACAGTCAGGC	This study
F. acidiphilum[b]	Fer-F	GAAGCTTAACTCCANAAAGTCTG	This study

a. cDNA synthesis for quantitative real-time PCR and real-time PCR amplification analysis.
b. Real-time PCR amplification analysis.

specifically *At. ferrooxidans* D2 reached around of 10^7 copies ml^{-1} during this period (Fig. 2A and B). These 16S rRNA gene copy numbers have been observed in all strips during the first month of operation (Remonsellez *et al.*, 2007; Galleguillos *et al.*, 2008; data not shown). *At. ferrooxidans* DM reached between 10^5 and 10^6 copies ml^{-1} in most of the strips (Fig. 2A and B and Table 3; all other strips: data not shown). In marked contrast, *L. ferriphilum* phylotypes were present with lower abundance (between 10^2 and 10^3 copies ml^{-1}) during the first month of operation in all strips, but copy numbers of both *Leptospirillum* phylotypes, increased with age reaching around of 10^5 copies ml^{-1} (Fig. 2A and B and Table 3; all other strips: data not shown). The sulfur-oxidizer *At. thiooxidans* maintained a constant copy numbers (10^5 copies ml^{-1}) throughout the leaching cycle in all strips (Fig. 2A and B and Table 3; most of other strips: data not shown). The last microorganism has been identified from other heap operations (Goebel and Stackebrandt, 1994; Espejo and Romero, 1997), but its dynamic had not been reported until this study.

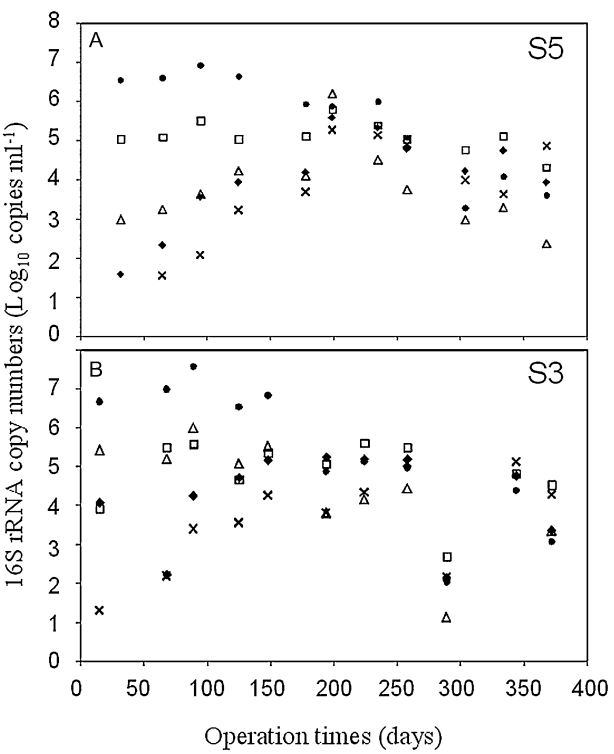

Fig. 2. Dynamic of main microorganisms present in the bioleaching industrial process. DNA copy numbers of 16S rRNA genes of main bacterial species during the leach cycle of strip S3 (A) and strip S5 (B) are shown. Strips S3 and S5 began its operations in May and August 2006 respectively. ●*At. ferrooxidans* D2; △*At. ferrooxidans* DM; □*At. thiooxidans*; ×*L. ferriphilum*; and ◆*L. ferriphilum* Warwick.

The *Firmicutes* group, such as *Ab. disulfidooxidans*, uncultured *Alicyclobacillus* and *Sulfobacillus*-like bacteria, generally were detected in low copy numbers in comparison with the other groups of *Bacteria*, but all of them reached number between 10^3 and 10^4 copies ml^{-1} and showed a patchy distribution in all strips (data not shown). The *Acidiphilium*-like bacteria presented copy numbers between 10^2 and 10^4 cells ml^{-1} in all strips, but reached its highest abundance when autotrophs microorganisms presented high copy numbers, generally between 150 and 200 days of operation in all strips (data not shown). The archaeon *F. acidiphilum* showed the same behaviour than *Leptospirillum* species but reached a low copy numbers around of 10^3 copies ml^{-1} (Table 3; all strips: data not shown). In all cases, the data from quantitative real-time PCR indicated that 16S rRNA gene copy numbers of *Bacteria* showed similar values to the sum of all species analysed, and 16S rRNA gene copy numbers of *Archaea*

Table 3. Copy numbers and accumulation levels of 16S rRNA of microorganisms present in the industrial heap (samples of August 2007) by real-time PCR analysis.

	Copy numbers (copies ml^{-1})			Transcripts levels (copies ml^{-1})		
	Strips			Strips		
Microorganism	S10	S8	S5	S10	S8	S5
Bacteria	6.7×10^6	9.0×10^5	3.2×10^5	ND	ND	ND
At. ferrooxidans D2	3.4×10^6	3.1×10^5	1.2×10^3	1.1×10^7	5.9×10^4	2.1×10^4
At. ferrooxidans DM	2.9×10^6	8.1×10^4	2.3×10^3	1.0×10^7	4.1×10^4	4.7×10^4
At. thiooxidans	1.3×10^5	8.9×10^4	3.4×10^4	6.9×10^5	7.5×10^4	2.7×10^5
L. ferriphilum	3.8×10^3	1.9×10^4	1.4×10^5	5.3×10^2	3.8×10^3	2.2×10^5
L. ferriphilum Warwick	2.2×10^3	4.2×10^5	5.6×10^4	1.5×10^3	4.0×10^4	2.8×10^4
Ab. disulfidooxidans	7.4×10^3	2.1×10^4	2.7×10^3	3.3×10^3	1.3×10^2	3.3×10^3
Sulfobacillus-like	8.9×10^2	6.0×10^2	BDL	BDL	1.9×10^2	1.3×10^3
Uncultured Alicyclobacillus	2.2×10^2	1.1×10^2	BDL	BDL	BDL	BDL
Acidiphilum-like	9.6×10^3	9.2×10^3	BDL	5.1×10^4	2.9×10^4	1.4×10^3
Archaea	BDL	2.0×10^2	2.0×10^3	ND	ND	ND
F. acidiphilum	BDL	1.4×10^2	2.1×10^3	4.1×10^2	2.2×10^3	1.3×10^4

BDL, below detection limit. ND, not done.

were almost identical to *F. acidiphilum* copy numbers (Table 3; data not shown).

The younger parts of the heap (strips) running since May 2008 (S7–S14) also showed remarkable differences in the microbial communities depending on the strip age. *At. ferrooxidans* phylotypes were found between 80% and 98% of abundance in the youngest strips (S13 and S14) with approximately 30 days of operation (Fig. 3). We observed one gap between the dominance of *At. ferrooxidans* and *L. ferriphilum* phylotypes in the strips S11 and S12 (150 and 120 days of operation respectively, Fig. 3), while *L. ferriphilum* phylotypes were found between 89% and 96% of relative abundance in the older strips (S7, S8, S9 and S10, over 250 days of operation, Fig. 3). The archaeon *F. acidiphilum* showed the same behaviour than *L. ferriphilum* but with lower abundance (4–9%). The rest of the bacteria such as *At. thiooxidans*, uncultured *Alicyclobacillus* and *Sulfobacillus*-like bacteria were the less abundant (between 4.5% and 0.4%), with the exception of the strip S11 where *Sulfobacillus*-like bacteria accounted for the 15% (Fig. 3). *Ab. disulfidooxidans* and *Acidiphilium*-like bacteria species were not represented in the Fig. 3 because they showed relative abundances below 0.5%.

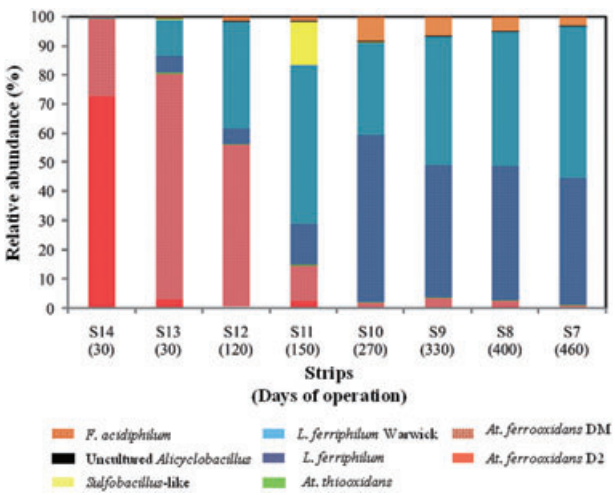

Fig. 3. Community structures based on 16S rRNA genes of *Bacteria* and *Archaea* species from different strips of the industrial heap. Relative abundances of different microorganisms were evaluated by quantitative real-time PCR of total DNA from different strips in operation until May 2008.

16S rRNA accumulation levels analysis

The 16S rRNA accumulation levels have been determined in different strips, as indicative of physiological activity (Parro *et al.*, 2007). In this work we show the results of the main phylogenetic groups that were detected throughout the leaching cycle from three strips (S5, S8 and S10) with different days of operations (Figs 2 and 3, Table 3). The ranges of the physicochemical parameters of the strips (S5, S8 and S10) are shown in Table 4. In correlation with the time of operation, the pH decreased with age strip;

Table 4. Physicochemical parameters governing three strips that represent the bioleaching cycle (samples of August 2007).

	Strips (days of operation)		
Parameters	S10 (30)	S8 (150)	S5 (330)
pH	2.3 ± 0.14	1.83 ± 0.12	1.67 ± 0.1
Total Fe (g l^{-1})	1.18 ± 0.19	1.9 ± 0.2	ND
Fe^{3+} (g l^{-1})	0.87 ± 0.19	1.86 ± 0.19	ND
Eh (mV)	682 ± 30.5	849 ± 11.5	837 ± 43.9
Cu (g l^{-1})	4.1 ± 0.25	2.55 ± 0.6	1.62 ± 0.49

ND, not done. Total Fe and Fe^{3+} of other strips (S8, S9, S10 and S11) with 300 days of operations reached average values of 1.9 ± 0.185 and 1.88 ± 0.183 g l^{-1} respectively.

total iron, Fe^{3+} and redox potential showed higher values in strips over 150 days of operation (S5 and S8), while the copper concentration showed a higher value in the strip S10 with less than 50 days of operation (Table 4).

The 16S rRNA accumulation levels indicate that *At. ferrooxidans* phylotypes were the most active microorganism in the strip S10 with a shorter operation time (Table 3). The same behaviour has been observed in other strips in the first months of operation (Galleguillos *et al.*, 2008). On the contrary, the results suggested that *L. ferriphilum* phylotypes were more actives in strips S5 and S8 with more than 150 days of operation (Table 3). The sulfur-oxidizer *At. thiooxidans* was active in all analysed strips (Table 3), indicating that this microorganism could have an important role throughout the leaching cycle. The archaeon *F. acidiphilum* also showed higher 16S rRNA accumulation in strips with a longer operation time (Table 3), and the same behaviour has been observed in other strips with more than 200 days of operation (Galleguillos *et al.*, 2008).

However, the rest of acidophilic microorganisms analysed did not show a clear correlation between DNA copy numbers and 16S rRNA accumulation. The *Acidiphilium*-like bacteria was active in all strips analysed, but was not detected in the strip S5 by DNA copy numbers analysis (Table 3). *Ab. disulfidooxidans* and *Sulfobacillus*-like bacteria showed a lower activity in all strips analysed, and a patchy activity of these *Firmicutes* members was observed. Finally, although the uncultured *Alicyclobacillus* showed low DNA copy numbers in strips S8 and S10, it was not active in the strips analysed (Table 3).

Time-course strip monitoring with total RNA and prokaryotic acidophile microarray

We used the prokaryotic acidophile microarray (PAM) developed and validated by Garrido and colleagues (2008), for a fast monitoring of the most active groups of microorganisms at the industrial heap (Figs S3 and 4). The same samples used for the 16S rRNA accumulation studies were subjected to PAM analysis. Total RNA of these samples was amplified following the method described by Moreno-Paz and Parro (2006), which was used for the amplification of total RNA from an acidophilic environment in transcriptome analysis (Parro *et al.*, 2007). The amplified RNAs from strips S5, S8 and S10 were labelled with Cy5 or Cy3, and hybridized with the PAM microarray in a classical simultaneous dual hybridization (S5 and S10; S8 and S10; S5 and S8) as shown in the Fig. 4. The relative proportion of each phylogenetic group in the sample was estimated by calculating the ratio between the fluorescent signals (Cy5/Cy3) for each probe on the microarray as described (Garrido *et al.*, 2008). The relative signal intensity for the analysed strips showed a dramatic change in the prokaryotic profile, from *Acidithiobacillus* spp. to *Nitrospira* group, *Thermoplasmata* class and *Actinobacteria*, from younger to older strips (Fig. 4A). Interestingly, strips closer in time of operation (strips S5 and S8, with more than 150 days of operation) showed similar preferential rRNA accumulation pattern (Fig. 4A) with some bias to *Actinobacteria*, *Archaea* and *At. thiooxidans* to the older one (strip S5). In agreement with this result, the patterns observed in older strips (strips S5 and S8) were compared with the youngest one (strip S10). The PAM results showed a sharp preferential distribution of some phylogenetic groups, with *Acidithiobacillus* spp., and *Acidobacterium* spp. dominating the first moments of the operation (strip S10, green colour in Fig. 4B), and *L. ferriphilum*, some *Actinobacteria*, *Sulfobacillus* spp. and members of the *Thermoplasmata* class dominating the older strips (strips S5 and S8, red colour in Fig. 4B). The total *Bacteria* showed a preferential expression in the strip S10, which correlated with data obtained from DNA copy numbers and 16 rRNA accumulation (Fig. 4A and Table 3). The *Alphaproteobacteria* and *At. thiooxidans* do not showed a preferential expression between the old and new strips (Fig. 4). Although members of the *Alphaproteobacteria* should be present in all the analysed samples, none of the specific probes for *Acidiphilium* spp. showed positive results, neither that for *At. ferrooxidans* group D, nor those for the *L. ferrooxidans* phylotypes (Fig. 4).

Discussion

Acidophilic microorganisms in the heap leaching solutions

The most prominent microorganisms making up these communities are *Gammaproteobacteria* (*At. ferrooxidans* and *At. thiooxidans*) and some members of phylum *Nitrospira* (*L. ferriphilum*). These microorganisms have been found in most studies in bioleaching heaps (Table S1). The community consists to a lesser extent of *Alphaproteobacteria*, *Firmicutes* and *Thermoplasmata* groups, which have also been identified in bioleaching heaps (Table S1). Our results also confirm previous observations that identified new groups that could participate in such process, like *Acidimicrobium* genus or some *Actinobacteria* (Rawlings and Johnson, 2007).

In this work we used quantitative real-time PCR to monitor the microbial diversity and speculate about the dynamic and physiological state of the microbial communities with respect to leaching cycle in industrial heaps. *At. ferrooxidans* phylotypes have always reached their highest abundance when pH values are over 2, and the Fe^{3+} ion and total iron concentrations are less than 1 and 1.2 g l^{-1} respectively (Tables 3 and 4). Moreover, we observed that the redox potential was around 680 mV when *At. ferrooxidans* reached higher abundance, and

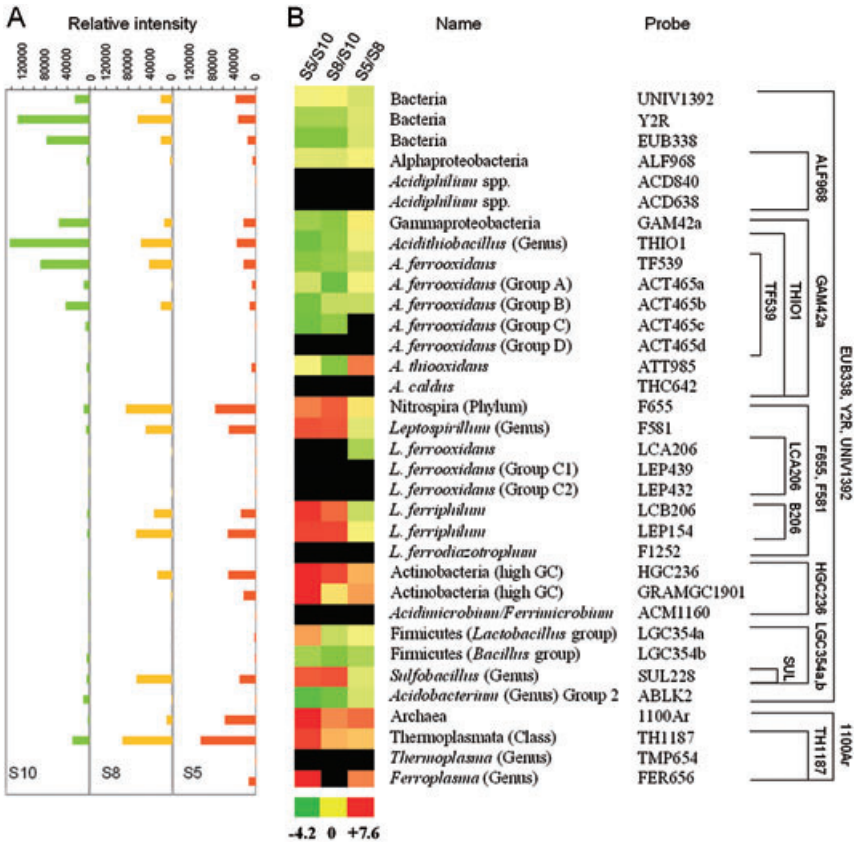

Fig. 4. A barcode for industrial biolixiviation process by assaying total industrial heap RNA with a prokaryotic acidophile microarray (PAM).
A. Histograms showing the average relative signal intensity of two different hybridizations for the different analysed strips, from younger to older ones (S10-S8-S5). Each bar of the histogram aligns horizontally with the corresponding strain name or phylogenetic group and probe (in B).
B. Relative proportion of the different metabolically active phylogenetic groups by using prokaryotic acidophile microarray (PAM). The PAM results were obtained after two-colour simultaneous hybridization with total fluorescent labelled environmental RNA from three samples of the industrial heap of August 2007 (S5, Cy5 labelled; S8, Cy5 and Cy3 labelled; S10, Cy3 labelled). After two-colour simultaneous hybridizations (S5/S10, S8/S10 and S5/S8), the Log_2 ratio between the signal intensity from each sample was calculated and clustered for comparison along sampling times (coloured figure). The log_2 ratio values ranged from –4.2 (the most green), through 0 (yellow), to +7.6 (the most red). Black colour indicates no signal detected with the corresponding probe. Representative phyla and species are indicated, as well as a phylogenetic tree showing the probe range (right scheme).

these results are consistent with the report of Boon and colleagues (1999) and Meruane and colleagues (2002), which showed that *At. ferrooxidans* are not capable of oxidation at higher redox potentials. Possibly in our case, the metabolism of *At. ferrooxidans* should change as the heap ages (Galleguillos *et al*., 2008) to sulfur oxidation coupled to oxygen like *At. thiooxidans* (Schippers and Sand, 1999) and/or to sulfur oxidation coupled to Fe^{3+} ion reduction (Sand, 1989). *Leptospirillum* species reached their highest abundance when the pH was below 2, high Fe^{3+} ion concentrations prevailed, and the concentration of copper was under 3 g l^{-1} (Tables 3 and 4). Their physiological particularities, such as tolerance to high redox potential and Fe^{3+} ion concentration (Rawlings *et al*., 1999; Bond *et al*., 2000), could be the reason for their dominance in these conditions as also described previously (Demergasso *et al*., 2005). The archaeon *F. acidiphilum*, as *Lepstospirillum* species, showed its highest abundance in old strips (Table 3 and Fig. 4), and possibly these microorganisms contribute to the mineral dissolution in environments with extremely low pH values (Bond *et al*., 2000). A recent study about the archaeal diversity in two bioleaching systems revealed that the presence of *Thermoplasma* and *Ferroplasma* was related to highest amounts of total iron and low pH values (Xiao *et al*., 2008).

One important finding in our work was the high abundance of the sulfur-oxidizer *At. thiooxidans* throughout the leaching cycle in all strips. In our case, the presence of acidic solution in the system could decompose the copper sulfide to elemental sulfur as previously described Schippers and Sand (1999), and we suggest that *At. thiooxidans* microorganism have high abundance in the process due to the constant production of sulfur and intermediate

reduced sulfur compounds by Fe^{3+} ion-mediated chemical sulfide oxidation.

The species belonging to *Firmicutes* occurred in low copy number and generally showed a patchy distribution in all strips. *Sulfobacillus* spp. has been described as chemolithotroph and show mixotrophic and autotrophic growth (Bond *et al*., 2000). But, despite that several species of *Firmicutes* group have been isolated from bioleaching systems or thermal springs, many of them have not been described and characterized yet (Schippers, 2007). Recently, some species of *Firmicutes* have been identified also from other bioleaching sites and sulfide columns-test (Xie *et al*., 2007; He *et al*., 2008; Wakeman *et al*., 2008). Some acidophilic heterotrophic *Bacteria* have been found usually in bioleaching operations (Hallberg and Johnson, 2001; Xie *et al*., 2007), but whether their capabilities contribute to the bioleaching efficiency of a microbial consortium in practice is still unclear (Johnson, 1998, Hallberg and Johnson, 2001). But whit respect to *Acidiphilum*-like bacteria, our data suggest that their abundance may vary significantly in the bioleaching cycle possibly depending of the autotrophic oxidizer dynamic.

Active microorganisms by using RNA from an industrial heap

Despite the results reviewed above, the question remains whether the 16S rRNA gene copy numbers in this study originate from livings cells or from cell debris. Therefore, using RNA one expects to detect the most active microorganisms in the system (Schippers *et al*., 2005). The 16S rRNA accumulation of *At. ferrooxidans*, *L. ferriphilum*, *At. thiooxidans* and *F. acidiphilum* were in agreement with the PAM analysis. Therefore, we suggest that *At. ferrooxidans* and *L. ferriphilum* would produce Fe^{3+} at different stages of the bioleaching cycle, *At. thiooxidans* would generate sulfuric acid to supply protons throughout the bioleaching cycle and, possibly under low Fe^{2+} concentrations (over 200 days of operation), *At. ferrooxidans* acts like *At. thiooxidans* by acid production.

Acidiphilium-like bacteria presented a high activity in all analysed strips (Table 3) and the PAM analysis showed active microorganisms belonging to *Alphaproteobacteria* with no preferential dominance between old and new strips (Fig. 4). We propose the hypothesis that the possible activity of heterotrophic bacteria could depend on the autotrophic oxidizer dynamics. Similarly, Schippers and colleagues (1995) described a positive correlation between cells count of acidophilic chemoorganotrophics microorganisms and those of *At. ferrooxidans* in two different uranium mine waste heaps.

With respect to the *Firmicutes* group, we did not find a clear activity depending on age strip (Table 3), possibly due to the fact that they could use sulfur and iron compounds as energy source (Bond *et al*., 2000). On the contrary, the *Sulfobacillus* genus was also detected by using PAM and was preferentially active in older strips (Fig. 4), as described previously (Demergasso *et al*., 2005) in one sulfide test-heap in Chile. Recently, some sequences related to *Firmicutes* group have been isolated from solutions with high iron concentration from Tong Shankou copper Mine in China (Xie *et al*., 2007).

Interestingly, the PAM detected new active groups of microorganisms such as *Actinobacteria* and *Acidobacterium* genus. Therefore, the use of RNA as target is expected to be more sensitive than DNA analysis (Galleguillos *et al*., 2008; Garrido *et al*., 2008). Members of *Actinobacteria* group have been identified in another industrial operation (Bruhn *et al*., 1999). Some microorganisms related with *Acidobacterium* species have been isolated from metal-rich mine waters with pH values between 2.4 and 2.7 (Hallberg *et al*., 2006; Rowe *et al*., 2007). Despite these studies, few microorganisms belonging to this phylogenetic group have been cultivated and detected in industrial bioleaching processes. The slight discrepancies between transcript levels by real-time PCR and PAM can be explained by the methodological differences, mainly in the RNA amplification step and the specificity of the probes used (Garrido *et al*., 2008). Finally, PAM microarray can proportionate a sort of 'barcode' or 'fingerprint' for characterizing certain industrial bioleaching processes.

Using pregnant leaching solution as an indicator of the microbial communities within an industrial bioheap

Based on the necessity of understanding the microbiology of heaps (Brierley, 2001), we monitored the composition and dynamic of the microbial communities from an industrial bioleaching sulfide heap.

Notwithstanding that the bioleaching reactions are carried out mainly by bacteria attached to the sulfide mineral inside the heap in order to provide Fe^{3+} ion and protons (Schippers, 2007), we decided to analyse the leach solutions for several reasons:

(i) The implications of taking ore samples during the bioleaching process at industrial level are enormous (Galleguillos *et al*., 2008), regarding the cost, the complexity of this process and the amount of samples needed because of the high heterogeneity inside the heap. In some previous studies, *At. ferrooxidans*, *L. ferrooxidans, L. ferriphilum* and *Sulfobacillus* spp. have been detected in sulfidic mine waste dumps (Diaby *et al*., 2007); in addition *At. thiooxidans*, *Acidithiobacillus* spp. and *Thiomonas* spp. have been

detected in metal sulfide mine wastes (tailings) (Wielinga *et al*., 1999). A work published recently by Kock and Schippers (2008), in which depth profiles have shown that the composition of the microbial communities varied between zones of oxidized and unoxidized tailings, and maximum cell numbers have been determined in the pyrite oxidation tailings zones, shows just an example of the factors that affect the microbial community associated to specific ore samples (oxygen levels, oxidation rates, sulfide–sulfur content). This is much more relevant because the system analysed in this work is composed of non-agglomerated run-of-mine ore, where heterogeneous niches must be common (Kock and Schippers, 2008; Wakeman *et al*., 2008).

(ii) The comparison between the microbial communities in associated mineral and solutions performed in industrial and laboratory samples have shown enough similarity to be considered as indicator of the community inside the heap. We have confirmed that the most abundant communities in ore samples of this heap correspond to those commonly found as predominant in leaching solutions. In the oldest strip S1 with more than 400 days of operation *At. ferrooxidans*, *At. thiooxidans* and *L. ferriphillum* are the most abundant microorganisms with 6×10^3 copies g^{-1}, 5×10^4 copies g^{-1} and 1×10^4 copies g^{-1} respectively. Also, we determined that *At. ferrooxidans* was the most abundant microorganism in one ore sample from strip S9, with 60 days of operation, reached 10^7 copies g^{-1} (F. Remonsellez, F. Galleguillos, A. Echeverria and C. Demergasso, unpublished data).

(iii) It is accepted for bioleaching that bacterial cells can affect the sulfide dissolution by 'contact' and 'non-contact' mechanisms (Rohwerder *et al*., 2003). The contact mechanism requires attachment of bacteria to the sulfide surface. Then, Fe^{2+} ions, elemental sulfur and other key intermediate sulfur compounds in oxidative sulfide degradation (Schippers *et al*., 1996; Boon *et al*., 1998; Sand *et al*., 2001), are biologically oxidized. This mechanism does not require the attachment of cells to the sulfide mineral (Sand *et al*., 2001).

Taking into account those arguments, we argue that the microorganisms detected in the leaching solutions represent an overview of what is happening in each strip. Therefore, we support the hypothesis that in large bioleaching operations the analysis of leaching solution could be a good alternative for monitoring these systems. Finally, we proposed that one periodic analysis of ore samples at different depths and locations to complement the data obtained from leach solutions could help to control the industrial bioleaching process from a microbial and a productive point of view.

Conclusion

The selection, control and monitoring of the microbial communities in biomining had been neither well studied nor fully understood. The question arise, therefore, which species are more abundant and effective during industrial bioleaching operations, and to answer this, more studies of microbial dynamics during different leaching stages containing different sulfide minerals are required. This is the first work to describe the dynamics of an active microbial community in an industrial heap using powerful RNA-based tools and, these techniques can rapidly evaluate the levels of acidophilic microorganisms present in these systems. Finally, we are proposing that the chemical and physical conditions (like pH and the Fe^{3+}/Fe^{2+} ratio) determine which bacteria are likely to dominate commercial bioleaching processes.

Experimental procedures

Industrial heap and samples

Escondida Mine is located 170 km South-East from Antofagasta, Chile. The heap was built 2 years ago with low-grade run-of-mine sulfide copper ore and air was supplied at the base of the heap through blowers. The ore was characterized as low-grade sulfide material averaging 0.60% total Cu consisting of chalcocite (40%), covellite (10%) and chalcopyrite (50%). The heap was designed to have one raffinate irrigation solution directed from the solvent-extraction plant that feeds 14 ore strips (S1–S14) at steady state. Each strip (125 m wide by 2000 m long) generates its own pregnant leaching solution (PLS) and the start of irrigation was approximately 1 month apart with the strip 1 being the oldest (Fig. 1). We defined that around of 400 days of operation correspond to the leaching cycle of each strip. Samples of Feed and PLS from each ore strip were collected every month during 2 years (from June 2006 to May 2008).

DNA and RNA extraction

DNA was extracted from cells collected by filtering 1 l of PLS through a 0.2 μm pore size membrane (Whatman) as described previously (Demergasso *et al*., 2005).

RNA was extracted from cells collected by filtering 4 l of PLS through a 0.2 μm pore size membrane (Whatman). To preserve the RNA the cells were recovered by scrapping with a sterile spatula in 2 ml of RNA*later* solution (Ambion). RNA was purified using RNeasy kit (QIAGEN). The yield of RNA was determined spectrophotometrically using a Nanodrop ND-1000 (NanoDrop technologies).

DGGE and 16S rRNA clone libraries analysis

DNA from industrial samples was directly used as template for PCR amplification of the 16S rRNA gene for DGGE and clone libraries. For DGGE analysis, PCR products were generated using the universal *Bacteria* and *Archaea* primer set

as previously described (Muyzer *et al*., 1993), and the DGGE analysis was performed as previously described (Casamayor *et al*., 2002; Demergasso *et al*., 2005). The excision of bands and reamplification was performed according to Casamayor and colleagues (2002). The bacterial clone libraries were constructed using the primer set EUB27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) and the archaeal clone libraries were constructed using the set ARQ21F (5′-TCCGGTTGATCCYGCCGG-3′) and 1492R. PCR products were cloned into the pGEM®T Easy Vector (Promega) and transformed into JM109 High Efficiency Competent Cells (Promega) according to the manufacturer's instructions. Sequences from DGGE and clone libraries were analysed using the BLAST algorithm (Altschul *et al*., 1997) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Quantitative real-time PCR analysis

Real-time PCR was performed with a Rotor-Gene™ 6200 (Corbett Research Pty Ltd) using SYBR Green PCR Master Mix (Biotools, Spain) to determine the copy numbers of the 16S rRNA gene with specific primers target to *Bacteria*, *Archaea*, two phylotypes of *At. ferrooxidans*, two phylotypes of *L. ferriphilum*, *At. thiooxidans*, *Sulfobacillus*-like bacteria, *Ab. disulfidooxidans*, uncultured *Alicyclobacillus*, *Acidiphilium*-like bacteria and *F. acidiphilum*. The real-time PCR analysis was used also to determine the transcription levels of 16S rRNA genes of the same analysed microorganisms. Table 2 shows the primer sequences used in this study.

Standard curves were generated extracting plasmid DNA from clones of 16S rRNA gene libraries containing sequences of bioleaching microorganisms inhabiting the industrial heap using a QIAprep Spin Miniprep Kit (QIAGEN). The DNA concentration in nanograms per μl was transformed to 16S rRNA gene copy number per μl as described previously (Remonsellez *et al*., 2007). Calibrations curves were generated by the RotorGene software 1.7 (Corbett Research), and for each standard the concentration was plotted against the cycle number, and the value at which the fluorescence signal increased above the threshold value was the Cycle threshold (Ct value).

To determine the transcript levels of 16S rRNA genes, the synthesis of cDNA from RNA samples was performed using the Sensiscript RT kit (QIAGEN), according to the manufacturer's instructions using 10 pmol of specific reverse primers (Table 2) and the reaction mixtures were incubated at 37°C for 30 min (Galleguillos *et al*., 2008).

The reaction mixture contained 10 μl of SYBR® Green PCR Master Mix (Biotools, Spain), 1 μl of DNA or cDNA, 1 μl of the corresponding oligonucleotide primers (Table 2), and nuclease-free H_2O added to a total of 20 μl. The amplification program was developed as described previously (Galleguillos *et al*., 2008). The melting curves were measured with a ramp raising the temperature from 50°C to 95°C by 1°C each 5 s.

RNA amplification, cDNA labelling and PAM

The RNA quality analysis, total RNA amplification and cDNA labelling were done as described elsewhere (Moreno-Paz and Parro, 2006). The oligonucleotide PAM construction, hybridization and analysis were done as described previously (Garrido *et al*., 2008).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Phylogenetic tree based on comparative analysis of 16S rRNA gene sequences representatives of *At. ferrooxidans* type strains. Sequences were aligned using the alignment tool of ARB program (Strunk and Ludwing, 1995). Phylogenetic tree was generated using the maximum-parsinomy algorithm in the ARB program. The bar indicates a 1% estimated sequence divergence. The sequences obtained in this study are indicated in bold.

Fig. S2. Phylogenetic tree based on comparative analysis of 16S rRNA gene sequences representatives of Leptospirillum strains. Sequences were aligned using the alignment tool of ARB program (Strunk and Ludwing, 1995). Phylogenetic tree was generated using the maximum-parsinomy algorithm in the ARB program. The bar indicates a 1% estimated sequence divergence. The sequences obtained in this study are indicated in bold.

Fig. S3. Assaying total industrial heap RNA with a Prokaryotic acidophile microarray (PAM).

A. Examples of two PAM images corresponding to hybridization with total RNA from strips S10 and S5. The yellow rectangles point out the universal probes UNI1392, Y2R and EU338 for a better orientation.

B. Names and the position of each probe which were described by Garrido *et al.* (2008). Different coloured rectangles indicate some relevant probes: universal (yellow), those showing higher intensity in earlier (S10, in green) or older (S5, red) stages of the industrial process.

C. Histograms showing the average relative signal intensity of two different hybridizations for the different analyzed strips, from younger to older ones (S10-S8-S5). BACT, *Bacteria*; Alpha, *Alphaproteobacteria*; HGC, probes for High GC containing bacteria; LGC, probes for Low GC containing bacteria, *Firmicutes*; Beta, *Betaproteobacteria*.

Table S1. Acidophilic microorganisms identified in heap processes.

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