Research in Microbiology 167 (2016) 555–567

www.elsevier.com/locate/resmic

Detection, identification and typing of *Acidithiobacillus* species and strains: a review

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Received 29 February 2016; accepted 23 May 2016
Available online 8 June 2016

Abstract

The genus *Acidithiobacillus* comprises several species of Gram-negative acidophilic bacteria that thrive in natural and man-made low pH environments in a variety of geo-climatic contexts. Beyond their fundamental interest as model extreme acidophiles, these bacteria are involved in the processing of minerals and the desulfurization of coal and natural gas, and are also sources of environmental pollution due to their generation of acid mine drainage and corrosion of cement and concrete structures. *Acidithiobacillus* spp. are therefore considered a biotechnologically relevant group of bacteria, and their identification and screening in natural and industrial environments is of great concern.

Several molecular typing methodologies have been instrumental in improving knowledge of the inherent diversity of acidithiobacilli by providing information on the genetic subtypes sampled in public and private culture collections; more recently, they have provided specific insight into the diversity of acidithiobacilli present in industrial and natural environments. The aim of this review is to provide an overview of techniques used in molecular detection, identification and typing of *Acidithiobacillus* spp. These methods will be discussed in the context of their contribution to the general and specific understanding of the role of the acidithiobacilli in microbial ecology and industrial biotechnology. Emerging opportunities for industrial and environmental surveillance of acidithiobacilli using next-generation molecular typing methodologies are also reviewed.

Keywords: *Acidithiobacillus*; Diversity; Typing; Fingerprinting; Acidophile; Bioleaching

1. Introduction

The genus *Acidithiobacillus* consists of a group of obligatory acidophilic, Gram-negative, rod-shaped bacteria that derive energy from oxidation of elemental sulfur and reduced sulfur compounds to support autotrophic growth. They thrive in both natural and man-made low pH environments in a variety of geo-climatic contexts, including acidic ponds, lakes and rivers, sulfur springs, acid mine/rock drainage waters and mining areas around the world. While all *Acidithiobacillus* spp. oxidize reduced sulfur, some can also catalyze the dissimilatory oxidation of ferrous iron and/or hydrogen. *Acidithiobacillus* spp. include those that are moderately thermophilic, mesophilic or psychro-tolerant. Because of their metabolic capabilities, these bacteria are relevant in the processing of minerals and electronic wastes and desulfurization of coal and natural gas. They are also responsible for environmental pollution due to their generation of acid mine

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http://dx.doi.org/10.1016/j.resmic.2016.05.006
0923-2508/

Table 1
Molecular markers and typing techniques used in the study of the acidithiobacilli and their communities.

	Technique	Marker	Advantages	Limitations	Discriminatory power[a]	Development/1st use in *Acidithiobacilli*	Strains/Communities	Refs.
Restriction based fingerprinting	AFLP	gDNA, rDNA	High reproducibility	Complex, difficulty interpretation	High-Strain level	1995/No date	–	–
	RFLP	gDNA (HKG)	Rapid, low cost, high reproducibility	Laborious set-up	Mid-Species level	1974/2001	10 *Thiobacillus ferrooxidans*	[19]
	T-RFLP	rDNA (HKG)	Rapid, high sensitivity and reproducibility, HT.	PCR-derived biases	Mid-Species level	1997/2009	Microbial community in AMD from pyrite mine	[23]
	ARDRA	rDNA	Rapid, low cost, high reproducibility	Limited resolution	Mid-Species level	1991/1998	5 *Thiobacillus* spp.	[21]
Anonymous fingerprinting	RAPD	gDNA (random targets)	Rapid, low cost, no previous sequence knowledge required, HT	Limited reproducibility, not universal	High-Strain level	1990/1996	8 *Thiobacillus ferrooxidans*	[32]
	AP-PCR	gDNA (random targets)			High-Strain level	1990/2008	32 *Acidithiobacillus* spp.	[22]
	ERIC-PCR	gDNA (ERIC-type repeats)			High-Strain level	1991/2001	19 strains of *A. ferrooxidans* and *A. thiooxidans*	[33]
	BOX-PCR	gDNA (BOX-type repeats)			High-Strain level	1996/2001	19 strains of *A. ferrooxidans* and *A. thiooxidans*	[33]
	REP-PCR	gDNA (Palindromic repeats)			High-Strain level	1991/1998	5 *Thiobacillus* spp.	[21]
Conformational polymorphism fingerprinting	SSCP	rDNA, ssDNA	Rapid, semi-quantitative	Complex, low reproducibility, laborious phylogenetic identification	Mid-Species level	1989/2002	Microbial community inside a pyrite bio-reactor	[39]
	DGGE	rDNA, dsDNA	Rapid, semi-quantitative.		Mid-Species level	1979/1996	3 *T. ferrooxidans* and *T. thiooxidans*	[40]
	TGGE	rDNA, dsDNA			Mid-Species level	1987/No date	–	–
Hybridization dependent	DDH	gDNA	Universal standard. Genome level comparison	Complex implementation. No central database. High experimental error.	Mid-Species level	1961/1980	10 *Thiobacillus* spp.	[3]
	FISH	rRNA (HKG)	Phylogenetic identification, semi-quantitative	Dependent on probe sequences, unable to identify unknown species	Mid-Species level	1980/1998	Microbial community in AMD from pyrite mine	[51]
	Microarray	rRNA (Multiple genes)	Phylogenetic identification, semi-quantitative, rapid	Cross hybridization, PCR-derived biases, low abundance species are difficult to detect	Mid-Species level	1995/2007	Microbial community in AMD from copper mine	[64]
Real time PCR dependent	Q-PCR	rDNA, gDNA, rRNA, mRNA	Quantitative, high sensitivity and reproducibility, Rapid,	Genetic information required for probe design, unable to identify unknown species, cost	High-Strain level	1992/2006	Microbial community in AMD from pyrite mine	[69]
Sequence dependent	Sequencing	rDNA	Phylogenetic identification, quantitative, rapid. Discovery of unknown bacteria	Cost, HGT that may distort relationships	Mid-Species level	1977/1985	11 *Thiobacillus* spp.	[101]
	MLST	gDNA (HKG)	High reproducibility	Genetic information required for selection of informative genes, cost, laborious	High-Strain level	1998/2011	21 *Acidithiobacillus* spp.	[9]
	NGS	rDNA, gDNA, mgDNA	High sensitivity, rapid	Cost, laborious data management	Mid to High-Species to Strain level	2005/2009–2011	Microbial community in pyrite mine. Biofilm community.	[23,91]

Abbreviations: HT = High-throughput; LT = Low-throughput; HGT, Horizontal gene transfer; mgDNA: Metagenomic DNA.
[a] Levels of discriminatory power. They are based on the tools capacities to discriminate between Genus (low), Species (mid) and Strains (high).

drainage and corrosion of cement and concrete structures. For these reasons, *Acidithiobacillus* spp. are considered a biotechnologically and environmentally important group of bacteria, beyond their fundamental interest as model extreme acidophiles.

Detection, enumeration and screening of particular strains and species of *Acidithiobacillus* in natural and industrial environments are important in monitoring and controlling sources of environmental pollution and for tracking bioleaching progress. Both classic and modern molecular typing technologies, accessed from bacterial systematics, have been used for identification in a wealth of biomining environments (Table 1). Several of these molecular typing methodologies have been instrumental in improving knowledge of the inherent genetic diversity of the acidithiobacilli, and have contributed to recent phylogenetic revision of the genus. In addition, several (semi-) quantitative molecular techniques have helped to improve understanding of the structure and dynamics of acidophilic communities and provide insight into the distinct roles played by the acidithiobacilli within them.

Typing of a bacterium of interest, i.e. identifying and assigning it to a particular species, entails an operational definition of a microbial species. The currently accepted definition of a species in microbiology requires that the strains concerned share >70% global DNA similarity (from DNA–DNA hybridization (DDH) analysis) and have <5% difference at their genomic DNA melting temperatures [1]. Typically, this level of hybridization correlates to 16S rRNA gene sequence identity greater than 97% and to an average nucleotide identity at the whole genome scale above 94% [2].

Using these gold-standard criteria and physiological and morphological distinctive traits, the genus *Acidithiobacillus* has been shown to consist of at least seven species, four of which oxidize iron as well as sulfur (*Acidithiobacillus ferrooxidans*, *Acidithiobacillus ferridurans*, *Acidithiobacillus ferrivorans* and *Acidithiobacillus ferriphilus*), while three do not (*Acidithiobacillus thiooxidans*, *Acidithiobacillus caldus* and *Acidithiobacillus albertensis*). Using DNA–DNA hybridization, Harrison and colleagues differentiated *Thiobacillus thiooxidans* strains from the *Thiobacillus ferrooxidans* genomovar, with DDH values between 9 and 12% [3,4]. In 1983, Bryant and colleagues described a new autotrophic sulfur oxidizer, *Thiobacillus albertensis*, which was later confirmed to belong to the acidithiobacilli during revision of the genus [5,6]. A decade later, Hallberg and Lindstrom differentiated two *A. caldus* strains from *A. thiooxidans* and *A. ferrooxidans*, with DDH values of 12–16% and 2–6% respectively [7]. Harrison, later followed by Karavaiko and several other researchers, was the first to highlight that genomovars of the *A. ferrooxidans* group had sufficient phylogenetic heterogeneity to be classified as separate species [4,8,9]. These findings lay the foundations of the more recent reassignment of the iron-oxidizing acidithiobacilli strains into the four currently recognized species [10–12].

This review examines some of the most common molecular DNA-based techniques used for bacterial typing in general and

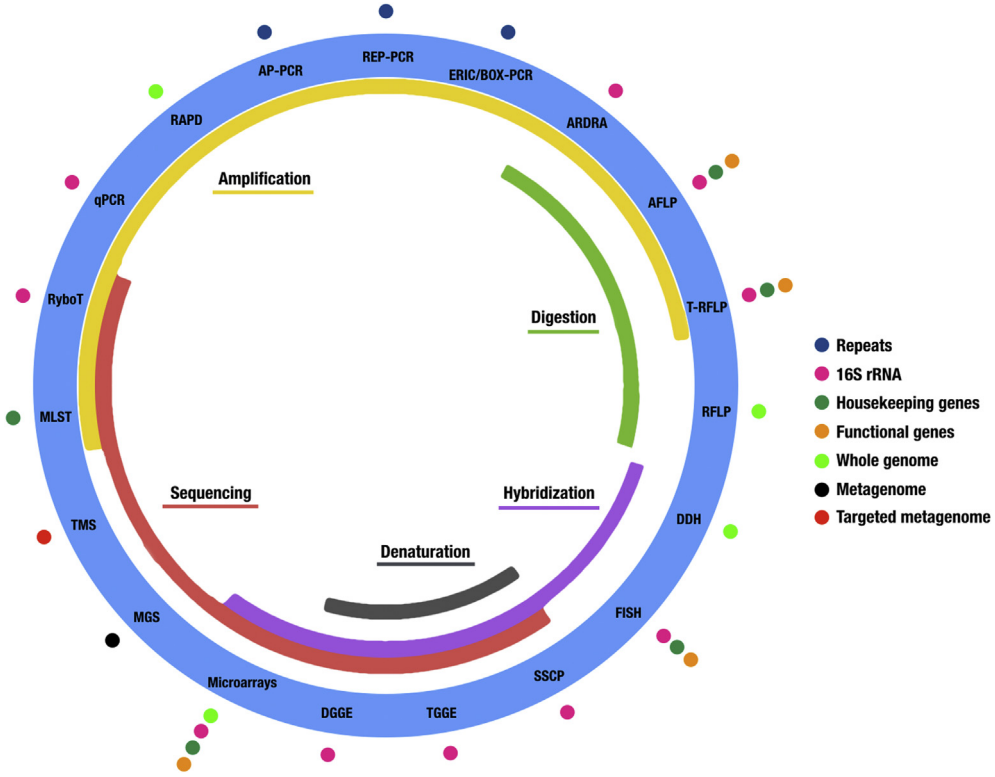

Fig. 1. Molecular markers and typing techniques used in the study of the acidithiobacilli and their communities. The techniques are shown in the blue circle. The technology or technologies associated with each of the techniques are indicated as colored hemi-circles inside the blue circle. Molecular markers used in each case are represented by colored dots outside the blue circle.

applied to the detection, identification and study of the different members of the genus *Acidithiobacillus* in particular, and their contribution to our understanding of the ecological role of the acidithiobacilli in natural and industrial systems.

2. Molecular markers used in molecular detection, identification and typing

As a consequence of technological progress, but primarily because DNA reassociation kinetic parameters are not readily evaluated and defined, there is an ever growing tendency to search for more accessible molecular markers and techniques to identify and classify bacterial strains. Different types of DNA sequences are currently used to mark a particular genotype provided that they are stably inherited, informative and readily detectable. A molecular marker may well be a single (e.g. the 16S rRNA gene) or a group of genes (e.g. a number of housekeeping genes), a set of sequence repeats or restriction sites, or even a single nucleotide that exists in several polymorphic states in different microorganisms (Fig. 1). Since all taxonomic information about a bacterium is contained in its nucleotide sequence, the marker may even be the whole genome sequence or the signatures that emerge from it (e.g. di-, tri- and tetranucleotides) [13,14]. Regardless of the marker chosen, it is preferable that it reflect as closely as possible the natural relationship between bacteria.

The molecular marker par excellence in microbial phylogenetic reconstruction, modern taxonomy and typing is the 16S ribosomal RNA gene. These ancient molecules occur universally in all bacteria, and are highly conserved in sequence and structure [15]. Since protein synthesis is essential to all growing cells and since ribosomes and rRNA are found at high copy numbers in every cell, these markers are easy to isolate and detect. Due to their small size (~1500 bp), these genes are relatively easy to sequence, yet they provide a huge amount of information [16]. Furthermore, there are plenty of 16S rRNA gene sequences in public databases such as NCBI and secondary dedicated databases (e.g. SILVA [17]), which greatly facilitate comparisons. However, the resolution possible from comparative analysis of 16S rRNA gene sequences is, at best, to the species level, providing in many cases the phylogenetic neighborhood and only tentative identification. Therefore, several other markers have been put forward as additional or alternative approaches during the past fifty years (Fig. 1).

Coupled with a number of well-established molecular technologies such as digestion using restriction enzymes, sequence amplification using (end-point or real time) polymerase chain reaction (PCR/qPCR), hybridization and sequencing (Fig. 1), the different types of markers give rise to an extensive collection of techniques for detection, identification and typing of bacterial strains and isolates, as well as for diversity studies. Most of these techniques have been applied in taxonomic analyses of virtually all bacteria (e.g. RFLP, DDH), including the acidithiobacilli (Table 1). In the present review, these methods have been categorized into five principal categories according to the technology they rely on to resolve or detect inherent differences between target molecular markers: restriction-based, anonymous and conformational polymorphism fingerprinting techniques, and hybridization-real time PCR- and sequence-dependent approaches.

3. Restriction-based fingerprinting methods

Restriction-based fingerprinting methods produce molecular fingerprints resulting from the variation in length of defined DNA fragments produced by digestion of a reference and/or a sample DNA, using restriction endonucleases. These patterns are resolved using agarose gel electrophoresis, frequently in combination with Southern blotting, and more recently, also through capillary-based electrophoresis. Similarities or differences in the restriction patterns obtained uncover sequence variations (insertions, deletions, rearrangements, or nucleotide differences) and are thus useful for typing and diversity analysis. While restriction fragment length polymorphism (RFLP) analysis efficiently maps changes in the DNA sequence of total genomic DNA or other DNA fragments, the derived methodology, PCR-RFLP, amplifies a specific gene or DNA region using specific primers before applying the RFLP

Table 2
Restriction enzymes used in restriction-based fingerprinting of the acidithiobacilli.

Enzyme	Restriction site	Species assayed/detected[a]	Method	Refs.
*Alu*I	AGCT	AFE – AFV – ATH – ACA	ARDRA, T-RFLP	[18–20,22,24]
*Ava*II	GGWCC	AFE – ATH	ARDRA	[21]
*Cfo*I	GCGC	AFE – AFV – ATH – ACA	ARDRA	[18,21,22,24]
*Dde*I	CTNAG	AFE – ATH	ARDRA	[20,21]
*Hah*I	GCGC	AFE – ATH	T-RFLP	[19,23]
*Hae*III	GGCC	AFE – ATH – ACA	ARDRA, T-RFLP	[19–23]
*Hap*II	CCGG	AFE	ARDRA	[19]
*Hinf*I	GANTC	AFE – ATH – ACA	ARDRA	[20]
*Msp*I	CCGG	AFE – AFV – ATH – ACA	ARDRA, T-RFLP	[19–24]
*Nde*II	NGATCN	AFE – ATH	ARDRA	[21]
*Rsa*I	GTAC	AFE – ATH – ACA	ARDRA	[19,21,22]
*Taq*I	TCGA	AFE – ATH – ACA	ARDRA	[22]

[a] Abbreviations: AFE: *A. ferrooxidans*; AFV: *A. ferrivorans*; ATH: *A. thiooxidans*; ACA: *A. caldus*.

assay. This combination of techniques greatly increases the reproducibility and resolution of the assay and has given rise to a number of variant techniques, namely: amplification of ribosomal DNA restriction analysis (ARDRA), amplified fragment length polymorphism (AFLP) and terminal-RFLP (T-RFLP).

Enzymes used in restriction-based fingerprinting in the acidithiobacilli are listed in Table 2. The first applications of these techniques to the study of the acidithiobacilli were focused on the investigation of inter- and intraspecific variability of culture collection strains and environmental isolates [18–20]. In 1998, Selenska-Pobell and colleagues used ARDRA to analyze the variability of a number of culture collection strains of the genus [21]. They used the 16S rRNA gene, the central region of the 23S rRNA gene and the 16S–23S internal transcribed spacer sequence (ITS) as molecular markers, in order to include both conservative and variable regions of the ribosomal RNA operon in the analysis. Using this approach, they found that the acidithiobacilli possessed species-specific ARDRA patterns and hinted for the first time at genomic differences between the *A. ferrooxidans* type strain (ATCC 23270) and strain ATCC 33020, which was later classified as the type strain of *A. ferridurans* [12].

The first reports on record to apply PCR-RFLP also aimed to explore genetic variability of uncultured sequence clones assigned to the acidithiobacilli [19,20]. Restriction patterns obtained from digestion of amplified 16S rRNA genes and intergenic 16S–23S rRNA sequence amplicons recovered from industrial or environmental DNA samples were generated and compared. Analyses were performed with restriction endonucleases AluI, DdeI, HaeIII, HapII, HinfI, HhaI and MspI, showing discriminatory power between the three recognized species of the genus at that time (*A. ferrooxidans*, *A. thiooxidans* and *A. caldus*), with some degree of intraspecific discrimination for both *A. ferrooxidans* and *A. thiooxidans* [20]. Later, Ni and colleagues explored the intraspecific genetic variability of 32 environmental isolates of *Acidithiobacillus* spp. using RFLP of similar PCR-amplified rDNA regions and a total of six different restriction enzymes [22]. In that study, the authors found 15 different RFLP genotypes within the *Acidithiobacillus* strains evaluated, and uncovered a high degree of genomic and phenotypic variation among *A. ferrooxidans* strains isolated in China. The existence of one or two novel species within the genus was thus suggested, anticipating later findings in the field. Although PCR-RFLP results for *A. thiooxidans* strains revealed far more genetic heterogeneity than *A. ferrooxidans*-like strains, other methods used in the study contradicted these results and were thus disregarded [22]. At present, genetic variability of the *A. thiooxidans* group remains largely unexplored.

These techniques have also been applied to the exploration of spatial and temporal variations in prokaryotic communities from different environments. Using T-RFLP of 16S rRNA gene sequences, Tan and colleagues showed that the microbial population inside an acidic stream draining across a pyrite mine in China varied spatially and seasonally, exhibiting a strong correlation with geochemical and physical parameters [23]. Within this community, *A. ferrooxidans*-affiliated microorganisms increased along the stream as less acidic conditions were encountered (pH 2.2–3.8), peaking in the storage pond, and remained common over the year. Based on these findings, iron-oxidizing *Acidithiobacillus* species were attributed a key role in the generation of ferric iron and its precipitates under the less acidic conditions.

The composition and structure of the microbial community associated with the Cae Coch abandoned pyrite mine in Wales were also explored through the use of T-RFLP and other techniques. Although water flowing through the mine chamber and out of the system was dominated by "*Ferrovum myxofaciens*" and *Gallionella* spp., water droplets dripping from the under-surface mine roof were found to be dominated by acidithiobacilli, according to FISH data [24]. T-RFLP fingerprints identified *A. ferrivorans* as the single acidithiobacilli in the droplets, representing up to 94% of the active bacteria present. Since it is a psychrotolerant species, the authors proposed that the dominance of *A. ferrivorans* could be related to its ability to oxidize sulfur as well as ferrous iron at the stably low temperature (8–9 °C) of this deep underground cave. This work is in agreement with previous evidence obtained at an abandoned cold Norwegian copper mine [25], and was among the first to establish the relevance of *A. ferrivorans* as a major player in the generation of acid mine drainage (AMD), typically associated with *Leptospirillum*-dominated communities.

Performance and composition of a microbial consortium used in column bioleaching assays of low-grade uranium-porphyry ore was also investigated using restriction-based typing methods [26]. 16S rRNA gene clone libraries derived from planktonic cells in the feed solution and leach liquor, and attached cells on a residual mineral surface, were analyzed by ARDRA. Seven different ARDRA patterns were detected, revealing a difference in the composition of the microbial communities associated with the three samples analyzed, with a clear dominance of *A. ferrooxidans*-like bacteria in the feed solution and leach liquor, while the residual mineral was enriched in *Leptospirillum* species. *A. albertensis* was also found in the residual mineral in small relative abundance.

4. Anonymous fingerprinting techniques

A number of PCR-based fingerprinting techniques, relying on the use of short oligonucleotide primers (8–12 bases in length) to hybridize randomly with chromosomal DNA, and produce distinct genomic fingerprints, emerged in the early 1990s [27,28]. Either arbitrary, low selectivity or repeat-specific oligonucleotide primers are used to initiate amplification of multiple target sites in the genome. If the primer binding sites are in the correct orientation and at appropriate distances for amplification, amplicons of various length that are resolvable by agarose or acrylamide gel electrophoresis are generated. The main advantages of anonymous fingerprinting over restriction-based techniques and those further described below are their ease of use and lower costs. These techniques encompass several variant methods including randomly

Table 3
PCR primers used in anonymous fingerprinting of the acidithiobacilli.

PCR	Primer	Sequence (5′ – 3′)	Tested species[a]	Band size detection range	Refs.
RAPD	AP18	CACACGCACACGGAAGAA	AFE – ATH	Not reported	[21]
	AP19	CAGGCACACGCAGACAGA			
	OPA primers kit 1 to 20	Available from Eurofins Genomics	AFE – ATH - ACA	200 bp to 4 kb	[32,34]
	OPB primers kit 1 to 20	Available from Eurofins Genomics			
	OPC primers kit 1 to 20	Available from Eurofins Genomics			
AP	M13	TTATGTAAAACGACGGCCAGT	AFE – ATH - ACA	200 bp to 4 kb	[22,34]
	PJ108	GCTTATTCTTGACATCCA			
	PJ118	TGTTCGTGCTGTTTCTG			
ERIC	ERIC1R	ATGTAAGCTCCTGGGGATTCAC	AFE – ATH	350 bp to 5 kb	[21,33,34]
	ERIC2	AAGTAAGTGACTGGGGTGAGCG			
BOX	BOX-A1R	CTACGGCAAGGCGACGCTGACG	AFE – ATH	300 bp to 5 kb	[21,33,34]

[a] Abbreviations: AFE: *A. ferrooxidans*; ATH: *A. thiooxidans*; ACA: *A. caldus*.

amplified polymorphic DNA analysis (RAPD), arbitrary primed-PCR (AP-PCR), enterobacterial repetitive intergenic consensus sequence (ERIC-PCR), BOX-PCR, and repetitive extragenic palindromic–PCR (REP-PCR) [27,29,30]. RAPD-PCR and AP-PCR are both based on arbitrary priming under low specificity conditions, but the two protocols differ in that RAPD-PCR makes use of a collection of short random primers and AP-PCR of a single 10-bp-long arbitrary primer. In turn, ERIC-PCR, BOX-PCR and REP-PCR use, as target sites, naturally occurring repetitive DNA sequences that are partially conserved and dispersed in multiple copies throughout the genome of many bacteria [31].

Primers used in anonymous fingerprinting of the acidithiobacilli are summarized in Table 3. The application of anonymous fingerprinting techniques to type *Acidithiobacillus* spp. was first reported in 1996 by Novo and colleagues [32]. Using RAPD fingerprints they assessed the genomic variability of eight *A. ferrooxidans* isolates from different sources, mostly mine sites, establishing the presence of two groups with a high degree of genomic diversity and supporting the usefulness of RAPD as a quick and reliable alternative for identifying and differentiating *A. ferrooxidans* strains [32]. A broader analysis was performed by Selenska-Pobell and colleagues in 1998, comparing results from RAPD and REP-PCR to those obtained by the restriction-based method, ARDRA. These authors found RAPD and REP-PCR to be adequate tools in the discrimination of species and closely-related strains of *Acidithiobacillus*, being more informative than ARDRA [21]. Consistency between RAPD fingerprints clustering and phylogenetic analysis of the 16S rRNA gene and ITS sequences markers, obtained from a set of 35 *Acidithiobacillus* strains, was also reported by Ni and colleagues [22]. Similar results were obtained using 19 *Acidithiobacillus* strains isolated from coal, copper, gold and uranium mine wastes in Brazil, when using BOX- and ERIC-PCR [33]. In turn, work from Wu and colleagues, using 23 different *Acidithiobacillus* strains isolated from Chinese sites and anonymous fingerprinting techniques, uncovered a much higher degree of genetic variability using these methods rather than 16S rDNA sequence phylogenetic analysis [34]. While the former clustered the strains into four or five major groups, 16S rRNA gene-based analysis distinguished only two groups. None of these studies has been able to correlate the genetic relatedness of the strains to either the geographic location or the type of source environment/sample [22,32,33].

Both the number and origin of the strains used in fingerprinting analysis can have a strong influence in the results obtained. Also, due to the promiscuous nature of anonymous fingerprinting techniques, the set-up of the assays suffers from reproducibility issues. Yet all studies agree on the fact that acidithiobacilli strains encompass a higher degree of genetic variability than acknowledged by existing taxonomical classifications. These results have motivated different instances of revision of the genus and promoted specific reassignment of *A. ferrooxidans* strains into three new species, as outlined in the preceding sections.

5. Conformational polymorphism fingerprinting techniques

Conformational polymorphism fingerprinting results from the differential electrophoretic mobility of defined DNA fragments, generally produced by PCR amplification, due to differences in their secondary structure. Changes in the conformation of the amplicons reflect differences in their nucleotide composition [35]. These methods detect shifts in the migration of double- or single-stranded DNA fragments during electrophoresis, either in the presence or absence of a denaturing agent. The denaturant can be a chemical (e.g. formamide), as in the case of denaturing gradient gel electrophoresis (DGGE), or a physical factor (e.g. temperature) for temperature gradient gel electrophoresis (TGGE). In the case of both DGGE and TGGE, the shift in the migration pattern occurs in double-stranded DNA fragments over polyacrylamide gels containing a linear gradient of the denaturant [36]. In turn, single-strand conformation polymorphism (SSCP) analysis works on single-stranded DNA molecules which have slightly different folded conformations and migrate differentially on a non-denaturing gel [37]. Using these techniques, DNA fragments of similar length but with different sequences can be resolved on a gel as band patterns of varying complexity. Due to their resolution, these

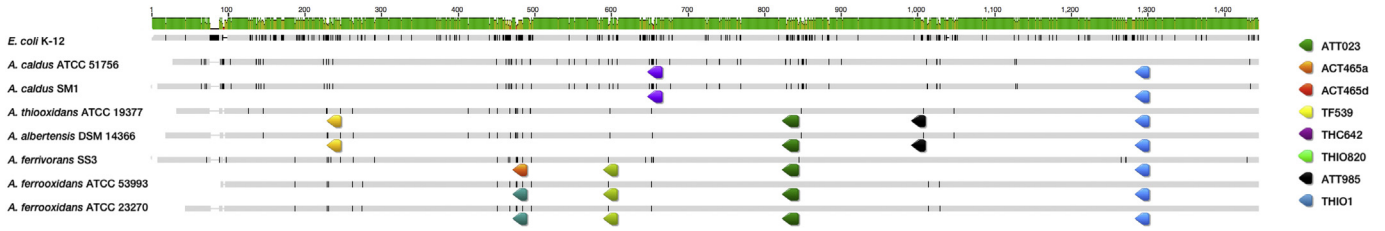

Fig. 2. Fish probes used in the study of the acidithiobacilli. Alignment of the 16S rRNA gene sequences of seven acidithiobacilli with respect to *Escherichia coli* K-12 is shown above. Conserved regions are represented in green and variable regions in yellow and red. Probes are color-coded as indicated. Hybridization specificity of the probes is shown by the presence of a colored arrows in the lane representing each species and/or strain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

techniques are very useful in the analysis and comparison of whole microbial communities and their spatial and/or temporal variations, enabling the correlation of shifts in the genetic structure with environmental properties or events [38]. Detection and identification of species of interest can be achieved, either by hybridization of specific probes or by sequencing of excised gel bands [35,36].

Among conformational polymorphism fingerprinting techniques, DGGE is by far the most utilized in the characterization of microbial populations from acidic environments where *Acidithiobacillus* spp. thrive, perhaps with the exception of one study using SSCP to follow the evolution of a bacterial consortium inside cobaltiferous pyrite bioreactors [39]. The first practical demonstration of the utility of the DGGE method in the characterization of mixed microbial populations from acidic mining environments comes from Stoner and colleagues in 1996 [40]. They evaluated the performance of DGGE in the identification of 15 known acidophiles using the 5S rDNA as target, and showed that DGGE had powerful resolution at the species level, being able to discriminate neatly between *A. ferrooxidans* (ATCC 19859) and *A. thiooxidans* (ATCC 8085). Moreover, the patterns of 5S rDNA migration from both *Acidithiobacillus* strains were clearly distinguishable from other bacteria commonly isolated from acidic environments, such as *Acidiphilium* and *Leptospirillum*.

The following DGGE-based studies have focused on the analysis of the composition of acidophilic communities in different natural and industrial biotopes, and their shifts in time or space. Work led by Amils and colleagues has used DGGE as a key tool in the in-depth characterization of the microbial diversity and composition of the Tinto River ecosystem (Spain), a current model for the study of natural AMD environments. Correlation of diversity and compositional patterns unveiled through the use of DGGE, with physicochemical parameters collected along the river, enabled Amils and colleagues to propose that *Acidithiobacillus* species play crucial ecological roles in the iron cycle of this river, as they are catalysts of ferrous iron oxidation under aerobic conditions and ferric iron reduction in anaerobiosis [41,42].

DGGE analysis of PCR-amplified 16S rRNA gene fragments has also been used to evaluate changes in industrial microbial consortia compositions during heap bioleaching of low-grade copper sulfides in northern Chile and bioleaching of chalcopyrite in shaken flask assays [43–45]. *A. ferrooxidans* was identified as the most abundant bacterium during the beginning of the leaching cycle inside the heap, while other iron-oxidizers, *Leptospirillum ferriphilum* and *Ferroplasma acidiphilum*, increased with time. A high relative abundance of the sulfur-oxidizer *A. thiooxidans* was also found throughout the leaching cycle in all sampling sites. Based on these results, and associated metadata, a model was proposed by the authors in which *A. ferrooxidans* colonizes the mineral at early stages due to the higher pH values (>2) and lower iron content, and is followed by other iron-oxidizers, as more extreme pH conditions and higher ionic strength develop. In turn, *A. thiooxidans* was proposed to play an important role in the sulfur oxidation cycle throughout the leaching cycle.

6. Hybridization-dependent approaches

Nucleic acid hybridization can be used to detect a particular microbial species or to assess the degree of relatedness between two microorganisms based upon the level of identity of marker genes or even the entire genome. These methods entail one or multiple probes to hybridize with the test sample and some kind of fluorescent label for visualization using epifluorescence or confocal laser scanning microscopy flow cytometry or other specialized scanning devices (e.g. microarray scanners).

FISH and its technically improved assay variants, like catalyzed reporter deposition-FISH (CARD-FISH), can be applied to detect, identify and quantify microorganisms of interest and also to follow the dynamics of specific bacterial populations that are metabolically active, in time and/or space [46]. Genus- and species-specific probes suitable for FISH analysis of *Acidithiobacillus* bacteria have been designed over the years and are summarized in Fig. 2 and Table S1 [43,47–49]. The first known application of FISH for the study of the acidithiobacilli dates back to the late 1990s. Using conventional FISH and species-specific probes targeting the 16S rRNA gene from *A. ferrooxidans* and *Leptospirillum ferrooxidans*, Banfield and colleagues analysed the underground AMD system of the Richmond mine in California [50,51]. They established that the acid-generating areas of this abandoned mine were populated by species other than *A. ferrooxidans*. *A. ferrooxidans* was abundant only in cooler

(20–30 °C) and more moderate-pH (1.5–2.3) waters peripheral to the ore body of the mine, and virtually absent from the sites that were in contact with the ore body. Because of this distribution, *A. ferrooxidans* was considered to be involved in the oxidization of dissolved ferrous iron to ferric iron, rather than in the direct attack of the sulfide minerals, and thus to contribute through the precipitation of ferric iron oxides to metal decontamination of the mine effluents. Other studies using FISH or CARD-FISH to explore the composition of microbial communities inhabiting different natural and man-made acidic econiches, and the presence and role of the acidithiobacilli, can be found in the literature [42,46,52–56].

The presence, relative abundance and activity of *Acidithiobacillus* spp. have been evaluated using this technology in laboratory and industrial setups with a fair degree of success [57,58]. Okibe and colleagues followed the changes in relative abundance of *A. caldus* in mixed cultures with a strain of *L. ferriphilum* in pH-controlled bioreactor assays to assess the role of this bacterium in the bioleaching of pyrite [57]. That study concluded that production of acidity by *A. caldus*, in mixed cultures with iron oxidizers, may be more important in limiting formation of jarosite than in removing elemental sulfur from passivation layers, as originally suggested [59]. Harrison and colleagues also used FISH to assay the composition of the microbial population in two different industrial bioleaching settings. Quantification of the community was achieved using species- and genus-specific probes from a temperature-controlled mesophilic heap at the Agnes Gold mine and high temperature test columns at SGS Lakefield Research (South Africa), detecting *A. ferrooxidans* only in the mesophilic temperature samples [60]. Kock and Schippers also quantitatively assessed the presence of *Acidithiobacillus* spp. in three different mine tailing dumps using a combination of FISH, CARD-FISH and Q-PCR. They demonstrated that the abundance of iron- and sulfur-oxidizing acidithiobacilli varies greatly between dumps, but were unable to define which physical, chemical and mineralogical parameters drove the qualitative and quantitative changes in species distributions [58].

Genome-level typing of a collection of strains, or parallel detection and identification of diverse microorganisms within a community, can also be achieved using DNA microarrays. Depending on the type of microarray (phylochips, functional gene arrays and whole-genome coverage arrays), a few taxonomically or metabolically informative marker genes, or even the whole gene collection of a microorganism, can be utilized [61,62]. In the mid-2000s, a number of dedicated microarrays were designed for the screening of acidophiles in industrial biotopes [63,64]. Yin and colleagues designed an array consisting of close to 1000 probes, half of which corresponded to 16S rRNA genes [63]. The other half were functional genes, including those involved in iron and sulfur oxidation by typical acidophiles. This array was used to monitor the composition and structure of the microbial communities in three samples from mining environments in the Jiangxi Province, China. Results obtained showed clear differences in the richness of *Leptospirillum* spp. and *A. ferrooxidans* in the three samples analyzed, correlating to changes in the available substrates (iron and sulfur) and the pH values of each sample. A second array constructed by Garrido and colleagues is better described as a phylochip, being an oligonucleotide microarray containing close to 60 probes targeting the 16S rRNA gene of a variety of acidophilic bacteria and archaea [64]. This array was validated against artificial mixtures of genomic DNA from diverse acidophiles and three water samples from the Tinto River, producing results that were comparable, and in some cases more sensitive, than the ones generated using alternative techniques, such as DGGE and FISH. This array was successfully used to describe the dynamics of active bioleaching communities in an industrial copper-sulfide heap in northern Chile [44]. A few other multi-specific microarrays have been designed for use in the bioleaching industry, which are protected by patents (BioSigma Patent 2009-0101085 and 2010-2006241345 B2) or undergoing patenting processes (InnovaChile Patent application 2012-02111). Also, a customized whole-genome microarray from *A. ferrooxidans* ATCC 23270^T has been used in the analysis of the genomic diversity of isolates of this species collected from different environments in China, identifying nearly 40% strain specific genes and further supporting the high genetic variability within this group of bacteria [65].

7. Real-time PCR-dependent approaches

Because of its quantitative nature, medium-throughput, simplicity and costs, real-time PCR (qPCR) is being increasingly used to detect and monitor microorganisms in the environment. Developed in the 90's, qPCR uses fluorescent dyes such as SYBR green, oligonucleotide hybridization probes or hydrolysis probes, such as in TaqMan assays, to specifically detect and monitor PCR products in real time [66]. In this field of application, qPCR has been used to evaluate the presence and assess the roles of different acidophiles in a wide variety of sample types [67], stirred tank and airlift reactors [68,69], mine tailings [58], bioleaching heaps [44] and AMDs [70], querying either total DNA or RNA extracted from these samples. For this technique also, the 16S rDNA gene has been most frequently targeted. However, suitability of this marker in enumeration procedures has been called into question due to frequent occurrence of multiple exact and occasionally variant copies/genome of this gene, which can lead to over- and sub-estimation of the true abundance of the target microbes [71]. This potential problem may not apply to the acidithiobacilli, which seem to invariantly possess two identical copies of the 16S rRNA gene. In addition, qPCR approaches are highly sensitive to nucleic acids quality and inhibitory in/organic species that are frequently co-extracted with the nucleic acids in mineral and metal-rich samples. Despite this caveat, the setup of adequate nucleic acid extraction methods that remove impurities and minimize sample-to-sample variations in biomining samples have been reported in the literature [67,71,72]. Proof of this, for example, is the successful application of qPCR to assess the dynamics of

microorganisms inhabiting an industrial heap in northern Chile [44]. Quantitative analysis using this technique has revealed that iron-oxidizing *Acidithiobacillus* strains are the numerically dominant (80–98%) active microorganisms at early stages of the bioleaching cycle (30 days) in industrial heaps, which reached their highest abundances at pH values over 2, redox potentials around 680 mV and ferric and total iron concentrations below 1 and 1.2 g l−1, respectively.

8. Sequence-dependent approaches

Traditional and next-generation nucleic acids sequencing are also molecular genotyping techniques used to assess the presence and the degree of relatedness between microorganisms, based upon the level of identity of one or a few marker genes. Traditional Sanger-based sequencing of clone libraries of the 16S rRNA gene has been used for many years in the phylogenetic analysis of bacteria, and has laid the basis for modern microbial taxonomy. However, this marker has limited resolution when it comes to distinguishing strains or even closely related bacteria from certain taxa [73,74]. Multilocus sequence typing (MLST) has emerged as an alternative method due to its greater discriminatory power. It entails sequencing of internal gene fragments (~500 nt long) of five to seven single copies, conserved, yet informative loci, and indexing of the variant positions with respect to reference sequences [74]. Relatedness between target microorganisms is then inferred from the comparison of the allelic profiles generated for each individual marker or a concatenate of these. Bacterial housekeeping genes (HKGs) meet all the above criteria and are frequently used in strain typing, taxonomy assignment and phylogenetic analysis. Although MLST only typically examines a small sample of the core genome (seven genes) of a species, clonal assignments inferred by MLST data have been confirmed by analysis of complete genome sequences.

Few studies to date have used this kind of approach in the detection and typing of acidithiobacilli from industrial or natural environments, though they have been central to the reclassification of species within this group. Amouric and colleagues used a concatenate of three HKGs (*recA*, *atpD* and *nifH*) to investigate the phylogenetic relationship of 21 iron-oxidizing strains of *Acidithiobacillus* [8]. Information content within these three markers was sufficient to support the division of the iron-oxidizing acidithiobacilli into four robust groups with defined phenotypic traits, matching currently recognized species [10–12]. These new divisions explained past discrepancies in the distribution among isolates of key

Table 4
Targeted and conventional metagenomes where the presence of acidithiobacilli has been demonstrated.

Site	Sample type	Origin	Sequencing method	Detected Acidithiobacillus species	Approach	Nº samples	Ore	References
Yunfu Mine	AMD	Guangdong, China	Clone library	AFE	Targeted Metagenome	7	Pyrite	[23]
Xitieshan Mine	Bioreactor	Qinghai, China	Clone library	ACA, ATH, ALB	Targeted Metagenome	10	Pyrite	[81]
Several mining areas	AMD	Several provinces, Southeast, China	Pyrosequencing	AFE, ACA	Targeted Metagenome	59	Polymetalic	[77]
The Mushroom Farm	AMD	Ohio, USA	Pyrosequencing	AFV	Targeted Metagenome	10	Coal	[78]
Shuimuchong	Tailings	Anhui, China	Pyrosequencing	AFE, ACA	Targeted Metagenome	90	Copper	[82]
Los Rueldos	AMD/Biofilm	Asturias, Spain	Clone library	AFV	Targeted Metagenome	3	Pyrite	[79]
Ala Moana Park	Concrete pipes	Honolulu, Hawaii	Pyrosequencing	ATH, *Acidithiobacillus* sp.	Targeted Metagenome	3	–	[83]
Carnoulès Mine	AMD	Gard, France	Pyrosequencing	AFE	Targeted Metagenome	6	Pb/Zn	[80]
Frasassi cave	Biofilm	Ancona, Italy	Pyrosequencing	ATH	Metagenome	1	Limestone	[90]
Carnoulès Mine	AMD/Biofilm	Gard, France	Pyrosequencing	*Acidithiobacillus* sp.	Metagenome	1	Pb/Zn	[86]
Colombian Andes	Acid Hot Spring	Andes, Colombia	Pyrosequencing	ACA	Metagenome	1	–	[89]
Dabaoshan Mine	AMD	Guangdong, China	Pyrosequencing	AFV, *Acidithiobacillus* spp.	Metagenome	1	Polymetalic	[85]
Fankou Mine	AMD	Guangdong, China	Pyrosequencing	AFV, *Acidithiobacillus* spp.	Metagenome	1	Pb/Zn	[85]
Yunfu Mine	AMD	Guangdong, China	Pyrosequencing	AFV, *Acidithiobacillus* spp.	Metagenome	2	Pyrite	[85]
Dexing Copper Mine	AMD	Jiangxi, China	Pyrosequencing	AFE, AFV	Metagenome	1	Copper	[102]
Dexing Copper Mine	Ore/PLS	Jiangxi, China	Pyrosequencing	AFE, ACA, ATH, AFV	Metagenome	10	Copper	[87]
Fankou Mine	AMD	Guangdong, China	Illumina	AFE, ATH	Metagenome	1	Polymetalic	[91]
Kristineberg Mine	AMD/Biofilm	Malå, Sweden	Pyrosequencing	AFV	Metagenome	2	Polymetalic	[86]
Dexing Copper Mine	Heap	Jiangxi, China	Illumina	AFE, AFV	Metagenome	1	Copper	[88]

*Abbreviations: AFE: A. ferrooxidans; ATH: A. thiooxidans; ACA: A. caldus; ALB: A. albertensis.

genes related to the iron oxidation pathway [8]. Nuñez and colleagues designed a MLST scheme for the sulfur oxidizer *A. caldus* based on seven protein-encoding housekeeping genes (*pilC*, *lnt*, *alt*, *dhaL*, *era*, *htrA* and *gdp*) and applied it to investigate genetic diversity within this group, using a collection of thirteen *A. caldus* strains from diverse geographical origins and ore mining industrial settings [75]. The allelic profiles detected grouped together strains that are known to share certain genotypic and/or phenotypic traits, such as patterns of occurrence of specific mobile genetic elements, serotypes and/or trans-alternating field electrophoresis migration profiles. However, genetic diversity within *A. caldus* proved to be lower than that reported for the iron-oxidizing acidithiobacilli. Using available genomic information for seven strains encompassing four *Acidithiobacillus* species and a variation of the MLST approach which concatenates more than 50 ribosomal proteins, Williams and colleagues reassigned the genus to a new proteobacterial class, the *Acidithiobacillia* [76].

The massification of high-throughput sequencing methods has fundamentally changed our capacity to assess the taxonomic and functional profiles of microbial communities, and thus our understanding of the biology and ecology of an ever increasing number of ecosystems. One approach, referred to by some authors as targeted metagenomics, combines PCR-based amplification of the 16S rRNA taxonomic marker or other specific functional markers with next-generation sequencing (NGS) and powerful bioinformatic analysis techniques, in order to generate exhaustive community profiles. This approach has been applied to the study of microbial ecology within acidophilic communities in recent years, and taxonomic profiles for a handful of AMD waters [22,77–80], a bioreactor [81], a tailing deposit in China [82] and corroding concrete pipes in Hawaii [83] have been reported (Table 4). Similarly, following the seminal work by Banfield and colleagues [84], an increasing number of metagenomic analyses of acidophilic communities from AMD [85,86], bioleaching heaps [87,88], acidic hot springs [89] and sulfide-rich caves [90] have been performed, enabling the establishment of relevant links between the occurrence of certain microbes, their activities and the geochemistry of cognate sites (Table 4). Due to the extreme sensitivity of both approaches, members of the *Acidithiobacillus* genus have been found to be ubiquitous and to occur in most acidic econiches studied to date, in varying abundances and diversity. In a number of sites, such as the low temperature underground AMD stream below the Kristineberg multi-metal sulfide mine in Sweden and the AMD waters originating in the Fankou Pb/Zn mine in Guangdong, China, a high relative abundance of *A. ferrivorans*-like, *A. ferrooxidans*-like and *A. thiooxidans*-like uncultured representatives has enabled the reconstruction of populational genomes [91] and variability analyses [92]. All these studies agree on the fact that the relative abundance of iron-oxidizing *Acidithiobacillus* species varies consistently with changing ferric ion and copper concentrations [82], being the dominant primary producers of the communities populating these systems at lower ferric to ferrous iron concentrations and pHs above 3. In turn, sulfur-oxidizing *Acidithiobacillus* are dominant members of the biofilm communities populating walls and ceilings of caves, where sulfide-rich springs de-gas H_2S into the cave air [90] or in sewer pipes during the final acidic stage of microbiologically induced concrete corrosion, when H_2S gas concentrations in the sewer effluent increase [83]. In both cases, sulfide and highly acidic pH seem to be driving the dominance patterns. Emerging correlations between microbial diversity and composition patterns and variations in specific geo- and physicochemical factors are providing a unique opportunity to disentangle the ecology and evolution of extreme acidic microbial assemblages.

9. Outlook

Regardless of the method preferred, all genotyping approaches have emerged to assess the genetic similarity between bacteria as a proxy for the whole genome sequence (WGS). With the advent of NGS technologies, WGS data are rapidly becoming more widely available and WGS-based analyses for characterization of a larger number of strains and isolates are now not only feasible but possible. It is foreseeable that the study of *Acidithiobacillus* will experience a shift from traditional typing to genomic-based typing in the near future and adopt one or more possible genomic indexes, such as in silico genome-to-genome hybridization similarity (GGDH), average nucleotide identity (ANI), average amino acid identity (AAI), the Karlin genomic signature or supertrees [93,94]. This will be accompanied by development of analytical workflows for high-resolution genomic schemes that relate fine-grain biological information derived to ecological and industrial parameters of performance of the microorganisms under study. A handful of *Acidithiobacillus* genomes are currently available, including the type strain of *A. ferrooxidans*, *A. caldus*, *A. thiooxidans* and the SS3 strain of *A. ferrivorans* [95–98]. However, only one or two genomes per species have been sequenced thus far, and no genome, complete or draft, has yet been reported for the three other recognized species of the genus, *A. ferridurans*, *A. ferriphilus* and *A. albertensis*. These are absolute requirements if genome-based typing is to overtake identification and characterization of bacterial isolates within the genus. To date, only two studies have reported ANI indexes for comparison of sequenced *Acidithiobacillus* strains [99,100], and no study to date has truly assessed the taxonomy and phylogeny of the genus from a genomic standpoint. For the moment, this kind of approach can only be equated by 'whole-organism' fingerprints achievable through Raman spectroscopy, high-throughput MALDI-TOF mass spectrometry or infrared spectra analysis of intact microbial cells to produce specific functional profiles so as to differentiate, classify, and identify *Acidithiobacillus* species and strains, and ultimately understand how functional potential dictates functional activity in these microorganisms.

Conflict of interest

None declared.

Acknowledgments

This work was supported by the Fondo Nacional de Desarrollo Científico y Tecnológico FONDECYT (http://www.conicyt.cl/fondecyt/) [3130376 and 1140048], and the Programa de Financiamiento Basal de la Comisión Nacional de Investigación Científica y Tecnológica CONICYT (http://www.conicyt.cl/pia/category/lineas-del-programa/creacion-consolidacion-centros/) [PFB16]. P.C.C. and F.I. are recipients of graduate study fellowships from CONICYT.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2016.05.006.

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