Research in Microbiology 165 (2014) 705–712

www.elsevier.com/locate/resmic

Microorganisms in subterranean acidic waters within Europe's deepest metal mine

Catherine M. Kay [a], Anu Haanela [b], D. Barrie Johnson [a,*]

[a] College of Natural Sciences, Bangor University, Deiniol Road, Bangor LL57 2UW, UK
[b] Pyhäsalmi Mine Oy, P.O. Box 51, 86801 Pyhäsalmi, Finland

Received 2 April 2014; accepted 7 July 2014
Available online 22 July 2014

Abstract

The Pyhäsalmi mine, central Finland, has operated as a deep metal mine since 1967. It currently reaches a depth of almost 1500 m, making it the deepest mining operation in Europe. Around 900,000 m^3 of metal-rich, extremely acidic water are pumped out of the mine each year. The near constant air temperature of ~24 °C together with exposure of sulfidic rock surfaces to air and water, have created an environment that is highly suitable for colonization by acidophilic mineral-oxidizing microorganisms. Using a combined cultivation-dependent and molecular approach, indigenous bacteria in waters at two depths within the mine, and of an acid streamer sample were identified and isolated. Iron-oxidizing chemolithotrophs (*Acidithiobacillus* and *Leptospirillum* spp., and "*Ferrovum myxofaciens*" were the most abundant bacteria in mine water samples, whereas the acid streamer community contained a greater proportion of heterotrophic acidophiles (*Ferrimicrobium acidiphilum* and a gammaproteobacterium related to *Metallibacterium scheffleri*). The most abundant isolates obtained from both water and streamer samples were all strains of *Acidithiobacillus* Group IV, a proposed separate species of iron-oxidizing acidithiobacilli that has not yet been classified as such. Archaea were also detected in water and streamer samples using molecular methods, but most were not identified and no isolates were obtained.

Keywords: Acid mine drainage; *Acidithiobacillus*; Acidophiles; Biodiversity; Microbial communities; Pyhäsalmi

1. Introduction

Exposure of pyrite and other sulfide minerals to both air (oxygen) and water makes them susceptible to microbially-accelerated oxidative dissolution [1]. This process occurs in oxidised exposures of pyritic rock strata (gossans) though more frequently it is associated with mining of metals and coals [2,3]. While oxygen can act as the oxidant of sulfide minerals, in acidic liquors ferric iron assumes a more dominant role [1]. Iron is reduced (to ferrous) in this reaction and needs to be re-oxidized for the reaction to continue which, at ambient temperatures and low pH, is a relatively slow abiotic reaction. For this reason, ferrous iron-oxidizing acidophiles are considered to play a key role in generating acid mine drainage (AMD). The oxidation of elemental sulfur and reduced sulfur oxy-anions by sulfur-oxidizing prokaryotes generates acidity, which results in ferric iron and other metals (such as aluminium) being retained in solution, which both increases the toxicity of AMD to most forms of life and facilitates increased rates of mineral dissolution. Waters that drain abandoned mines and mine spoils (waste rock dumps and mineral tailings) are characteristically acidic, and enriched with the products of mineral dissolution, including sulfate, iron and other metals, and metalloids such as arsenic.

* Corresponding author.
E-mail addresses: c.kay@live.co.uk (C.M. Kay), Anu.Haanela@pyhasalmi.com (A. Haanela), d.b.johnson@bangor.ac.uk (D.B. Johnson).

http://dx.doi.org/10.1016/j.resmic.2014.07.007
0923-2508/

The diversity of acidophiles that are directly or indirectly involved in the genesis of AMD has been widely researched. Autotrophic bacteria that use ferrous iron (and also, in some cases, reduced sulfur) as electron donors have primary roles in generating AMD. The genus *Acidithiobacillus* (*At.*) currently includes three validated species of bacteria that oxidize both iron and sulfur: *Acidithiobacillus ferrooxidans* [4,5], *Acidithiobacillus ferrivorans* [6] and *Acidithiobacillus ferridurans* [7]. Other iron- and sulfur-oxidizing acidithiobacilli (*Acidithiobacillus* Group (IV)) have been identified as strains of a fourth species, though this group currently awaits formal description and validation as a novel species [8]. Other autotrophic iron-oxidizing acidophiles include species that do not oxidize reduced sulfur (*Leptospirillum* (*L.*) *ferrooxidans* [9], *Leptospirillum ferriphilum* [10] and "*Leptospirillum ferrodiazotrophum*" [11], and "*Ferrovum* (*Fv.*) *myxofaciens*" [12], while conversely some other autotrophic acidophiles (e.g. *Acidithiobacillus thiooxidans* [13] and *Acidithiobacillus caldus* [14] oxidize reduced sulfur but not ferrous iron. Obligately and facultatively heterotrophic acidophiles are also ubiquitous in mine waters (and other extremely acidic environments) where they interact with primary producers (chemolithotrophic prokaryotes and some species of micro-algae [15,16]. Some of these (such as the archaeon *Ferroplasma* spp., the actinobacterium *Ferrimicrobium* (*Fm.*) *acidiphilum* and the alphaproteobacterium *Acidicaldus organivorans*) can also accelerate the oxidative dissolution of sulfide minerals by oxidizing ferrous iron or reduced sulfur [17]), while others such as *Acidiphilium* (*A.*) and *Acidocella* (*Ac.*) spp. reduce ferric iron to ferrous in oxygen-limited environments [18]). The microbiology of mine drainage waters has been the subject of general review articles (e.g. [19–21]) as well as publications that have focused on a particular mine site (e.g. [22–25]).

The Pyhäsalmi Mine Oy. located in Oulu province in central Finland, was first worked as an opencast operation in 1962, but from 1967 has been operated as an underground mine. The ore is massive and coarse grained, and contains ~75% sulfide minerals (3% chalcopyrite, 6% sphalerite, 2% pyrrhotite, 66% pyrite, and minor amounts of galena and sulfosalts). Barite ($BaSO_4$) and carbonates are the main gangue minerals present. Pyhäsalmi produces copper and zinc concentrates (49,000 t and 40,000 t, respectively, in 2013), as well as pyrite (as a source of sulfur and sulfuric acid; 826,000 t in 2013). Some gold and silver are also produced from the ore at Pyhäsalmi. Currently the mine reaches to just under 1500 m below the land surface, making it the deepest mining operation in Europe. Large volumes of water (~900,000 m^3/annum) are pumped out of the mine to maintain suitable operational conditions. The depth-related warm temperatures (air temperature a near constant 24 °C) within the mine, together with exposure of sulfidic rock surfaces to air and water, have created an environment that is highly suitable for the colonization and activities of acidophilic mineral-oxidizing microorganisms. This study examined the diversity of the indigenous microflora in acidic waters that accumulate within Pyhäsalmi, and their role in AMD genesis in the mine.

2. Materials and methods

2.1. Sampling protocols and physico-chemical analysis

Samples were taken, in June 2012 and January 2013, at two depths (630 m and 970 m below the land surface) where water was collected and pumped out of the mine. Measurements of pH, redox potential, conductivity and temperature were taken *in situ*, and samples taken for laboratory analyses. Those used for chemical analysis were acidified with nitric and hydrochloric acids (to prevent metal precipitation); concentrations of transition metals, aluminium and arsenic in these were determined by ICP-OES, and those of sulfate using ion chromatography. Two samples were also taken at each site for microbiological analysis: one (non-acidified) water sample in sterile Falcon tubes, while the other involved collecting water filtrates on 0.2 μm pore-sized cellulose nitrate membranes. Between 500 mL and 1 L of mine water was filtered through each membrane to collect sufficient microbial biomass. A sample was also taken (in January 2013) of a small acid streamer growth found in mine water at 630 m. Samples used for microbiological analysis were stored at ~4 °C and processed as rapidly as possible, while those used for molecular analysis were stored at −20 °C.

2.2. Biomolecular analysis

DNA was extracted from membranes containing microorganisms filtered from Pyhäsalmi mine waters an intact acid streamer sample (~100 mg wet weight) using MO-BIO Ultraclean Soil DNA Isolation kits (MO-BIO Laboratories Inc. USA). Amplification of 16S rRNA genes was carried out in triplicate to minimise PCR bias, using Cy5-labelled forward primers, ahead of terminal restriction enzyme fragment length polymorphism (T-RFLP) analysis. This semi-quantitative PCR-based technique has been used on numerous occasions to determine microbial communities in other extremely acidic environments (e.g. [25]). The primer pairs used to amplify bacterial 16S rRNA genes were 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1387R (5′-GGGCGGWGTGTACAAGGC-3′). Amplification of archaeal 16S rRNA genes used 20F (5′–TCCGGTTGATCCYGCCRG-3′) and 915R (5′–GTGCTCCCCCGCCAATTCCT–3′) as the primer pair. Bacterial PCR conditions were: 95 °C (5 min), followed by 30 cycles at 95 °C (30 s), 55 °C (30 s) and 72 °C (1.5 min) and a final extension at 72 °C (10 min). Archaeal 16S rRNA gene PCR amplification conditions were 95 °C (5 min), followed by 30 cycles at 95 °C (30 s), 62 °C (30 s) and 72 °C (1.0 min), and a final extension at 72 °C (10 min). The combined PCR products were purified using SureClean reagent (Bioline Reagents Ltd., UK) according to the manufacturer's instructions and re-suspended in 10 μl sterile MilliQ water. Individual T-RFs were generated using restriction endonuclease digestion with the enzymes HaeIII, AluI, CfoI (Promega, UK). EcoO109I (New England Biolabs Inc., UK) was used to generate a determinative T-RF to distinguish members of *Acidithiobacillus* Group IV from other iron-oxidizing

acidithiobacilli (Groups I, II and III [8]) and the sulfur-oxidizers *At. caldus* and *At. thiooxidans*. The digested and labelled 16S rRNA genes were analysed via capillary electrophoresis, using a CEQ8000 genetic analysis system (Beckman–Coulter, UK). Individual terminal restriction enzyme fragments (T-RFs) were identified by reference to the acidophile T–RFLP database maintained at Bangor University.

2.3. Microbiological analysis

Preliminary data on the diversity of cultivatable acidophilic bacteria present in mine water samples from the Pyhäsalmi mine were obtained by streak-inoculating unfiltered mine water samples (from June, 2012) onto a range of solid media that have been designed to promote the growth of different physiological groups of these microorganisms [26]. These were: (i) iFeo medium (for autotrophic ferrous iron-oxidizers, such as *Leptospirillum* spp.); FeSo medium (for autotrophic and heterotrophic iron- and sulfur-oxidizers, such as *Acidithiobacillus* and *Ferrimicrobium* spp.); (iii) YE3o medium (for heterotrophic acidophiles, such as *Acidiphilium* spp.); (iv) FeTo medium (for mixotrophic sulphur-oxidizers, such as *Thiomonas* spp., and other moderate acidophiles) and non-overlaid FeTSB medium for heterotrophic iron-oxidizers. The same media were used to both enumerate and isolate acidophiles from mine water samples collected during January 2013. On this occasion the samples were serially-diluted and the plates spread-inoculated. To isolate bacteria from the acid streamer sample (from 630 m), a small amount of the material was suspended in 1 ml of sterile pH 2.5 basal salts solution and the biofilm disrupted by vortexing for 2 min, and samples of the cell suspension streak-inoculated onto the solid media listed above.

Bacterial isolates were purified by repeated single colony isolation and re-streaking onto fresh solid media on solid media. Isolates were then transferred to corresponding liquid media. Lysates were prepared either directly from colonies or from harvested biomass from liquid cultures. For sequencing purposes the 16S rRNA genes of isolates were amplified using the same protocols described above, except that un-labelled 27F primers were used. DNA concentrations of the 16S rRNA gene PCR products were measured using a Nanodrop 1000 system (Thermo Scientific, UK) and sequencing reactions were outsourced to Macrogen, Inc. (Korea). Electropherogram data were visualised using Chromas Lite and assembled contiguous sequences were compared to those in the Genbank database using BlastN on-line software [27].

2.4. Phylogenetic analysis

Sequences of isolates obtained from Pyhäsalmi mine waters were aligned with other selected sequences using ClustalX [28], followed by manual editing to remove gaps and positions of ambiguous nucleotides. Phylogenetic trees were generated by DNA parsimony, neighbour-joining and maximum-likelihood analyses. In all cases, general tree topology and clusters were stable, and reliability of the tree topologies was confirmed by bootstrap analysis using 1000 replicate alignments. As the topologies of trees generated by all three methods were nearly identical, only the neighbour-joining tree is presented.

3. Results

3.1. Mine water chemistry

The chemical compositions of water samples taken from 630 m to 970 m below the mine surface (June 2012 data) are shown in Table 1. These were typical of mine waters found within and draining metal mines in being extremely acidic (pH 2.6–2.7) and enriched with soluble sulfate, iron, aluminium and manganese. Concentrations of most of the solutes analysed (with the notable exception of iron) was greater in water sampled at 630 m depth than at 970 m. The elevated concentrations of zinc and copper in these mine waters reflected the fact that the ore body contains large amounts of sphalerite and chalcopyrite. In contrast, concentrations of arsenic in the mine waters were relatively low. Redox potentials (based on an E^0 value of ~ +720 to +740 mV for the ferrous/ferric couple in acidic sulfate-rich waters [29]) confirmed that most of the soluble iron in both water samples was present as ferric, and both samples were consequently highly orange-brown coloured.

3.2. Mine water bacteria: June 2012 samples

Profiles of T-RFs obtained of amplified 16S rRNA genes from DNA of mine water bacteria harvested in June 2012 showed that both samples contained a diverse range of microorganisms, though there were differences between water from the 630 m and 970 m collection points (Fig. 1, which shows profiles obtained of amplified genes digested with HaeIII). Where T-RFs are common to more than one acidophile species, their identities were confirmed in T-RFLP profiles obtained with CfoI and AluI digests. Most of the T-RFs identified in HaeIII digests of amplified genes from 630 m

Table 1
Chemical analysis of water samples from the Pyhäsalmi mine (June 2012). All concentrations shown are in mg/L.

Analyte	Mine depth	
	630 m	970 m
pH	2.70	2.60
E_H (mV)	+730	+720
E_C (mS/m)	913	774
T (°C)	20	21
Al	526	192
As	<0.04	<0.04
Cu	45	20
Fe	400	1100
Mn	40	20
SO_4–S	2660	1750
Zn	400	160

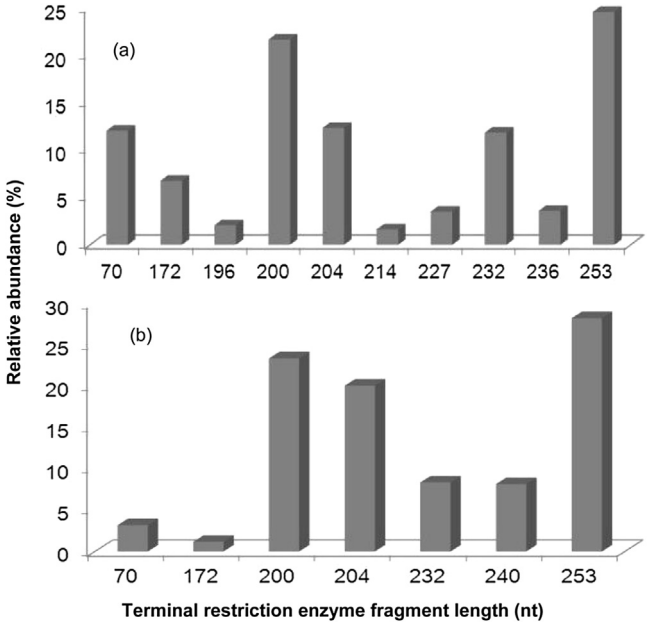

Fig. 1. Terminal restriction enzyme fragment length polymorphism (T-RFLP) profiles of bacterial 16S rRNA genes (digested with HaeIII) extracted from DNA from mine waters at (a) 630 m, and (b) 970 m depth at the Pyhäsalmi mine, sampled in June 2012. The T-RFs corresponded to: 70 nt, *At. ferrivorans*; 172, *A. rubrum*, 196 nt, *Gammaproteobacterium* WJ2; 200 nt, "*Fv. myxofaciens*"; 204 nt, *L. ferrooxidans*; 214 nt, *Acidimicrobium*; 227 nt, "*Acidithrix*"; 232 nt, *A. cryptum*; 236 nt, unclassified actinobacterium; 240 nt, "*Thiobacillus prosperus*"; 253 nt, *At. ferrooxidans/At. ferridurans* and *Acidithiobacillus* Group IV (shared T-RF).

corresponded to known species of iron-oxidizing autotrophic acidophiles (*Acidithiobacillus* spp., *Leptospirillum* spp. and "*Fv. myxofaciens*"), though two other peaks corresponded to iron-reducing *Acidiphilium* spp. (*Acidiphilium cryptum* and *A. rubrum*), and minor peaks to a heterotrophic iron-reducing acidophilic *Gammaproteobacterium* (strain WJ2; [30]), and to iron-oxidizing actinobacteria (*Acidimicrobium* and "*Acidithrix*" [17]). Peaks corresponding to actinobacteria and to strain WJ2 were not evident in the 970 m water sample, though peaks corresponding to autotrophic iron-oxidizers and *Acidiphilium* spp. were present, the latter in smaller relative abundance than in the 630 m water sample. Three ferric iron-encrusted colonies from the 630 m sample (PS1, PS2 and PS4) were all identified as strains of *Acidithiobacillus* Group IV (>99% gene identity with strain IFO 14246). An iron-oxidizing isolate (PS6) from the 970 m sample was also identified as a strain of *Acidithiobacillus* Group IV while the other (PS7) had 99.7% 16S rRNA gene identity with *L. ferriphilum*^{T} and 99.5% identity with *L. ferrooxidans*^{T}. Three out of four isolates (two from each mine water samples) that grew on acidified yeast extract-containing solid media (PS10, PS11 and PS12) were identified as *Acidiphilium* spp., while the other (PS13) had 99.2% 16S rRNA gene similarity to that of *Thiomonas arsenivorans* [31] (now reclassified as a strain of *Thiomonas delicata* [32]), a moderately acidophilic sulphur-oxidizing mixotrophic acidophile that was not identified in T-RFLP profiles.

3.3. Mine water bacteria: January 2013 samples

Spread plates were used to separately enumerate iron-oxidizing bacteria (ferric iron-encrusted colonies) and heterotrophic acidophiles (white/cream and pink-coloured colonies on yeast extract-containing media). Colony-forming units (CFUs) of iron-oxidizing bacteria were numerically dominant in both water samples, corresponding to 4.1×10^7 and 5.7×10^6 CFUs/mL in samples from 630 m to 970 m depths, while the corresponding numbers of heterotrophic CFUs were 2.2×10^6/mL and 4.0×10^4/mL, respectively.

T-RFLP profiles from water samples from 630 m to 970 m depths within the Pyhäsalmi mine taken in January 2013 showed some differences between each other and to the profiles obtained (at the same depths) from June 2012 samples (Fig. 2a and b). Five peaks were found in HaeIII digests of amplified genes from 630 m mine water, and four of these corresponded to autotrophic iron-oxidizing acidophiles (*At. ferrivorans* (70 nt), "*Fv. myxofaciens*" (200 nt), *L. ferrooxidans* (204 nt) and *At. ferrooxidans/At.ferridurans/Acidithiobacillus* Group IV (common T-RF of 253 nt)). No peaks corresponding to *Acidiphilium* spp. or acidophilic actinobacteria were apparent, and the sole T-RF (196 nt) corresponding to a heterotrophic acidophile was that of *Gammaproteobacterium* WJ2, which was also detected on this occasion in water from 970 m). *A. cryptum* was also detected in the 970 m water samples, though in smaller relative abundance than previously, but not *A. rubrum*. As was the case with both June 2012 samples, and in contrast to water from 670 m in January 2013, peaks corresponding to *At. ferrivorans* and "*Fv. myxofaciens*" were relatively small, and the dominant species indicated by T-RFLP analysis were iron-oxidizing acidithiobacilli (*At. ferrooxidans/At. ferridurans/Acidithiobacillus* Group IV).

Colonies of isolates obtained from Pyhäsalmi mine waters sampled in January 2013 are shown in Fig. S1. Seven colonies of iron-oxidizing isolates from 630 m depth were successfully sub-cultured and identified from their 16S rRNA gene sequences, four as *Acidithiobacillus* Group IV, two (PS101 and PS108) as *At. ferrooxidans* as one (PS105) as *L. ferriphilum*. One of the two heterotrophic isolates sequenced was confirmed as a strain of *A. cryptum* (109) while the other (PS110) was most closely related to *Acidisphaera rubrifaciens* [33]. However, the 16S rRNA gene similarity between PS110 and *As. rubrifaciens* was only 95.7% (96.1% and 96.4% to the unclassified *Acidisphaera* isolates MS-Y2 and nju-AMD-S1) indicating that PS110 is a strain of a novel species of *Acidisphaera*. Two colonies from 970 m mine water (representing the most abundant isolates) were successfully sub-cultured, and both identified as strains of *Acidithiobacillus* Group IV.

3.4. Acid streamer bacteria: January 2013 sample

The T-RFLP profile of digested amplified bacterial 16S rRNA genes from the acid streamer sample (Fig. 2c) showed some interesting differences to the 630 m water sample in which it was found. More T-RFs were apparent in HaeIII

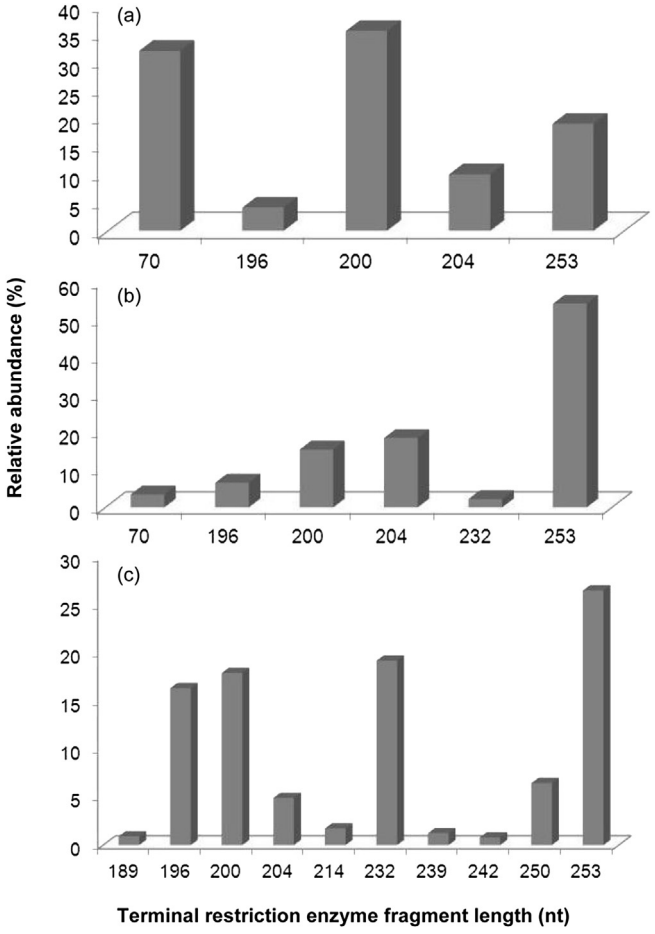

Fig. 2. Terminal restriction enzyme fragment length polymorphism (T-RFLP) profiles of bacterial 16S rRNA genes (digested with HaeIII) extracted from DNA from mine waters at (a) 630 m, (b) 970 m depth, and (c) an acid streamer sample (at 630 m) at the Pyhäsalmi mine, sampled in January 2013. The T-RFs corresponded to: 70 nt, *At. ferrivorans*; 189 nt, *L. ferriphilum*; 196 nt, *Gammaproteobacterium* WJ2; 200 nt, "*Fv. myxofaciens*"; 204 nt, *L. ferrooxidans*; 214 nt, *Acidimicrobium*; 232 nt, *A. cryptum*/isolate PS130; 250 nt, *Alicyclobacillus* sp.; 253 nt, *At. ferrooxidans/At. ferridurans* and *Acidithiobacillus* Group IV (shared T-RF).

digests, and of the four that had >15% relative abundance, only two corresponded to iron-oxidizing autotrophic acidophiles (*At. ferrooxidans/At. ferrivorans/Acidithiobacillus* Group IV, and "*Fv. myxofaciens*") while the other two corresponded to obligately heterotrophic acidophiles, one of which was confirmed in CfoI and AluI digests to be the iron-reducing *Gammaproteobacterium* WJ2. The 232 nt T-RF in HaeIII digests suggested the presence of *A. cryptum*, but unlike in the mine water samples, no corresponding T-RFs were found in CfoI and AluI digests. Both of these had peaks of ~20% relative abundance that corresponded to *Fm. acidiphilum* (366 nt in CfoI digests and 134 nt in AluI digests). A strain of *Fm. acidiphilum* isolated from the acid streamer (described below) was shown to have a 232 nt T-RF in HaeIII digests, confirming that this obligately heterotrophic iron-oxidizing actinobacterium was a major member of the acid streamer microbial community. *Leptospirillum* spp. (*Leptospirillum ferrooxidans*, and a smaller peak corresponding to *L. ferriphilum*) and a T-RF corresponding to *Alicyclobacillus* were also found in the T-RFLP profiles of the acid streamer, though none of these was isolated from this sample.

Relatively few colonies of isolates obtained from the acid streamer sample were sub-cultured and identified. Both iron-encrusted colonies from iFeo plates were confirmed as strains of *Acidithiobacillus* Group IV. Colonies obtained on non-overlay FeTSB medium which displayed "fried-egg"-like morphologies (e.g. PS130; Fig. S1), typical of heterotrophic iron-oxidizing acidophiles, were identified as being closely related (99.7% gene identity) to *Fm. acidiphilum*^{T}.

3.5. Differentiation of Group IV acidithiobacilli from other iron-oxidizing Acidithiobacillus spp.

One of the limitations of the three restriction enzymes used routinely in T-RFLP analysis was that two of them (CfoI and AluI) generated the same T-RFs for the three classified species iron-oxidizing acidithiobacilli (*At. ferrooxidans*, *At. ferridurans* and *At. ferrivorans*) and *Acidithiobacillus* Group IV, while the other (HaeIII) could distinguish between *At. ferrivorans* and other three Groups of iron-oxidizing acidithiobacilli only. In view of the evidence for cultivation-based analysis that *Acidithiobacillus* Group IV was the most abundant acidophile in Pyhäsalmi mine waters, it was necessary to devise an approach that could separate strains of this "species" from other iron-oxidizing acidithiobacilli. Database searches indicated that the restriction enzyme EcoO109I would generate a 444 nt T-RF from 16S rRNA genes of *Acidithiobacillus* Group IV strains, and T-RFs of 912 nt from the three classified species. This was confirmed in laboratory tests using pure cultures of *Acidithiobacillus* spp., though the 912 nt fragments were too large to be identified in the profiles obtained (maximum marker size 600 nt). EcoO109I digests of 16S rRNA genes of DNA extracted from January 2013 samples confirmed that *Acidithiobacillus* Group IV were abundant in both the Pyhäsalmi mine waters and the acid streamer sample (data not shown). The presence of *L. ferriphilum* in the acid streamer sample was also confirmed in EcoO109I and HaeIII digests of amplified 16S rRNA genes.

3.6. Phylogenetic relationships between acidophiles isolated from the Pyhäsalmi mine to known species of bacteria

Sequences of 16S rRNA genes of selected bacteria isolated from the Pyhäsalmi mine were used to generate a phylogenetic tree showing their relationships to known species of acidophiles (Fig. 3). These sequences have been deposited in GenBank, and have the accession numbers KC954526 to KC954532.

3.7. Archaea in Pyhäsalmi mine water and acid streamers

The T-RFLP profiles of digested amplified archaeal 16S rRNA genes were remarkably similar in mine waters from

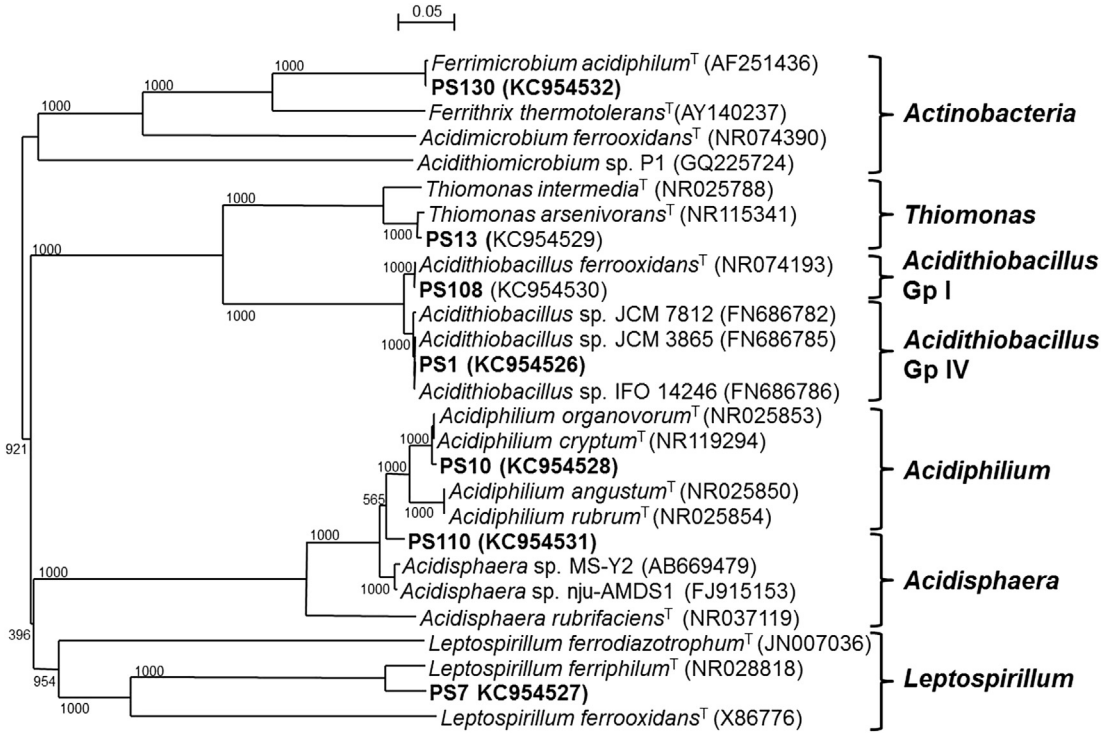

Fig. 3. Unrooted tree showing the phylogenetic relationship of bacteria isolated from the Pyhäsalmi mine acidic waste-water and acid streamers with known species of acidophilic bacteria by comparison of their 16S rRNA genes (988-1046 nucleotides). The scale bar represents 5% nucleotide sequence divergence. Numbers at the nodes indicate bootstrap values generated from 1000 trials. Pyhäsalmi isolates are indicated in bold text.

both depths and at both sampling occasions, with ~6 T-RFs evident on most HaeIII digests (Fig. 4). In contrast, only two T-RFs were found in HaeIII digests of genes amplified from the acid streamer samples, neither of which (a major 70 nt peak, and a minor 478 nt peak) were found in mine water profiles. Only two of the T-RFs in the archaeal profiles were identified, and both of these corresponded to euryarchaeotes (*Thermoplasma acidophilum* and to various *Thermoplasmatales* clones identified in AMD). No archaea were isolated from Pyhäsalmi mine waters or the acid streamer sample.

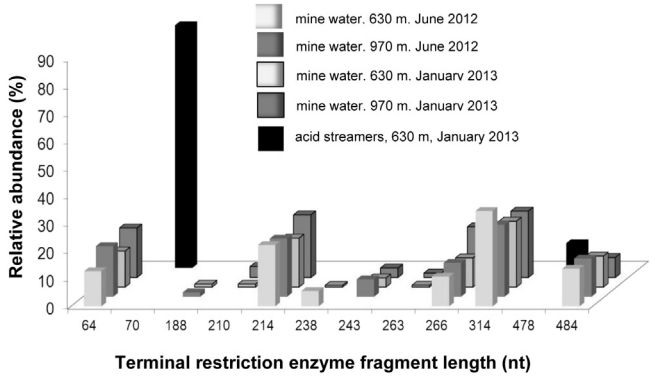

Fig. 4. Terminal restriction enzyme fragment length polymorphism (T-RFLP) profiles of archaeal 16S rRNA genes (digested with HaeIII) extracted from DNA from the Pyhäsalmi mine. The T-RFs corresponded to: 64 nt, *Thermoplasma acidophilum*; 214 nt, clones obtained from AMD at Mynydd Parys, Wales (*Thermoplasmatales*; Kay et al., 2013). Other T-RFs did not correspond to any entries in the Bangor University database.

4. Discussion

Relatively little is known about the abundance and biodiversity of microorganisms that inhabit subterranean acidic environments [34]. Waters in abandoned deep mines that are totally flooded have physico-chemical and microbiological characteristics that are more similar to those of stratified pit lakes (e.g. [35]) than sites that are more well drained and contain pools and streams of AMD (e.g. [36,37]). The Pyhäsalmi mine waters examined in the current work differ from most that have been previously described in that (i) the mine is currently fully operating and therefore needs to be continuously dewatered, and (ii) they were sourced from deep within the mine. Exposed faces of pyritic rock strata are contacted by groundwater and inflowing surface water and, combined with the immediate access of atmospheric air, this promotes the activities of chemolithotrophic mineral-oxidizing microorganisms. The latter is further enhanced by the warm temperatures maintained in the mine due to geothermal inputs, even though the mine itself is located in the northern latitudes. The mean temperature in Pyhäsalmi is, however, 10–20 °C less than that recorded within the abandoned Richmond mine at Iron Mountain, Ca. [36], where the elevated temperatures are due mostly to the rapid rate of pyrite oxidation (an exothermic reaction) rather than geothermal inputs. Active air circulation within the Pyhäsalmi mine maintains the lower and more uniform temperature within this mine, and consequently provides more suitable conditions for mesophilic acidophiles than, as is the case within Iron Mountain, more thermo-tolerant species.

The most abundant bacteria identified in the metal-rich, acidic waters within Pyhäsalmi were autotrophic iron- (*Leptospirillum* spp. and "*Fv. myxofaciens*") and iron/sulfur- (*Acidithiobacillus* spp.) oxidizers. *Leptospirillum* and *Acidithiobacillus* spp. are frequently encountered in mine waters [19–21], and clones closely related to "*Fv. myxofaciens*" have been obtained from pH 2–3 mine waters throughout the world, though few strains have been isolated [12]. For the first time, *Acidithiobacillus* Group IV iron-oxidizers were identified, from the combined cultivation-dependent and molecular approach used, as the most abundant mineral-oxidizers in a working or abandoned mine. One reason for this is that, until recently, these bacteria would have been classed as strains of *At. ferrooxidans*. Group IV iron-oxidizing *Acidithiobacillus* is the only one of four phylogenetic groups identified by Amouric et al. [8] that has not, as yet, been classified as a distinct species. The particular physiological traits of these bacteria that confer an advantage over other species in colonizing the Pyhäsalmi mine are unknown. Group IV iron-oxidizing acidithiobacilli, in contrast to *At. ferrooxidans* and *At. ferridurans* but in common with *At. ferrivorans*, tend to grow relatively slowly on sulfur and tetrathionate [8], and do not grow on hydrogen [38]. However, they differ from *At. ferrivorans* in not being psychro-tolerant, and being able to grow in extremely acidic (pH 1.6) media [8]. Other Group IV isolates have been obtained from an iron sulfide mine and mine waters in Japan, a coal mine in the USA, a copper–zinc mine in Romania (all listed in [8]) and the Rio Tinto, Spain (D.B. Johnson, unpublished).

As is invariably the case with AMD, heterotrophic acidophiles occupied the same environmental niches within the Pyhäsalmi mine as the chemolithotrophic primary producers. The fact that CFUs of heterotrophic bacteria were one or two orders of magnitude smaller than those of autotrophic iron/sulfur-oxidizers reflects the fact that, unlike in mine waters located at the land surface where extraneous organic carbon, such as leaf fall, can be present, the only significant carbon and energy source for heterotrophic acidophiles within the Pyhäsalmi mine is that originating from the chemolithotrophic primary producers [15,39]. Besides *Acidiphilium* spp., which are the most commonly-encountered species of heterotrophic bacteria in mine waters of pH 2 to 3, a mixotrophic *Thiomonas* sp. and a bacterium related to *As. rubrifaciens* were isolated from Pyhäsalmi mine waters, though they were not detected in T-RFLP profiles, implying low relative abundance. *As. rubrifaciens* is currently the only classified species of *Acidisphaera* [40], though strains of potentially novel species have been isolated from mine waters (e.g. [20]). *As. rubrifaciens* is a moderate acidophile (it grows between pH 3.5 and 6.0, with an optimum at ~pH 4.75 [40]) and does not oxidize or reduce iron [18]. The fact that *Acidisphaera* PS110 was isolated from pH 2.7 AMD and has been maintained subsequently in pH 3 liquid media suggests that the novel species represented by this isolate is significantly more acidophilic than *As. rubrifaciens* and warrants further investigation.

The biodiversity of the small acid streamer growth found at 630 m was significantly different to that of the mine water in which it grew, including the fact that the ratio of heterotrophic to autotrophic acidophiles was much greater in the macroscopic growth. "*Fv. myxofaciens*", which has also been identified as a major acidophile in much larger streamer growths [25,37], and *Acidithiobacillus* Group IV (one strain of which has been reported to form dense biofilms [41]) were found (T-RFLP analysis) to be the two most abundant chemolithotrophs present. *Fm. acidiphilum* (which was also isolated from the streamer sample) and gammaproteobacterium WJ2 were identified as the dominant heterotrophs present. A bacterium very closely related (99% 16S rRNA gene identity) to *Gammaproteobacterium* WJ2 was recently isolated from an acidic biofilm in a pyrite mine in Germany and classified as *Metallibacterium scheffleri* [42]. Both WJ2 and *M. schefferi* are moderate acidophiles (pH ~5) that reduce ferric iron.

Although archaea were present in both the mine water and the acid streamer samples, few were identified from the T-RFs (all as euryarchaeotes) and none was isolated. Knowledge of archaea that live in low pH environments that have low to moderate temperatures continues to lag well behind that of extremely acidic high temperature (>50 °C) sites, even though they appear to be ubiquitous in the former (e.g. [43]).

Conflict of interest

None declared.

Acknowledgements

The authors acknowledge the financial support given to this project by the European Commission under the Seventh Framework Programme for Research and Development. We are also grateful to Timo Maki and colleagues at Pyhäsalmi for their help in accessing samples within the mine. This work was carried out in the frame of ProMinE (European project contract NMP-2008-LARGE-2:# 228559).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2014.07.007.

References

[1] Vera M, Schippers A, Sand W. Progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation – part A. Appl Microbiol Biotechnol 2013;97:7529–41.
[2] Blowes DW, Ptacek CJ, Jambor JL, Weisener CG, Paktunc D, Gould WD, et al. The geochemistry of acid mine drainage. In: Holland HD, Turekian KK, editors. Treatise on geochemistry. 2nd ed., vol. 11. Oxford: Elsevier; 2014. pp. 131–90.
[3] Johnson DB. Chemical and microbiological characteristics of mineral spoils and drainage waters at abandoned coal and metal mines. Water Air Soil Poll 2003;3:47–66.
[4] Temple KL, Colmer AR. the autotrophic oxidation of iron by a new bacterium: *Thiobacillus ferrooxidans*. J Bacteriol 1951;62:605–11.
[5] Kelly DP, Wood AP. Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov.,

Halothiobacillus gen. nov., and *Thermothiobacillus* gen. nov. Int J Syst Evol Micr 2000;50:511–6.
[6] Hallberg KB, González-Toril E, Johnson DB. *Acidithiobacillus ferrivorans* sp. nov.; facultatively anaerobic, psychrotolerant, iron- and sulfur-oxidizing acidophiles isolated from metal mine-impacted environments. Extremophiles 2010;14:9–19.
[7] Hedrich S, Johnson D. *Acidithiobacillus ferridurans*, sp. nov.; an acidophilic iron-, sulfur- and hydrogen-metabolizing chemolithotrophic *Gammaproteobacterium*. Int J Syst Evol Micr 2013;63:4018–25.
[8] Amouric A, Brochier-Armanet C, Johnson DB, Bonnefoy V, Hallberg KB. Phylogenetic and genetic variation among Fe(II)-oxidizing acidithiobacilli supports the view that these comprise multiple species with different ferrous iron oxidation pathways. Microbiology 2011;157:111–22.
[9] Markosyan GE. A new iron-oxidizing bacterium, *Leptospirillum ferrooxidans* gen. et. sp. nov. Biol J Armen 1972;25:26–9 [in Russian].
[10] Coram NJ, Rawlings DE. Molecular relationship between two groups of the genus *Leptospirillum* and the finding that *Leptospirillum ferriphilum* sp. nov. dominates South African commercial biooxidation tanks that operate at 40 °C. Appl Environ Microbiol 2002;68:838–45.
[11] Tyson GW, Lo I, Baker BJ, Allen EE, Hugenholtz P, Banfield JF. Genome-directed isolation of the key nitrogen-fixer *Leptospirillum ferrodiazotrophum* sp. nov. from an acidophilic microbial community. Appl Environ Microbiol 2005;71:6319–24.
[12] Johnson DB, Hallberg KB, Hedrich S. Uncovering a microbial enigma: isolation and characterization of the streamer-generating, iron-oxidizing acidophilic bacterium, *"Ferrovum myxofaciens"*. Appl Environ Microbiol 2014;80:672–80.
[13] Waksman SA, Joffe JS. Microorganisms concerned in the oxidation of sulfur in the soil. II. *Thiobacillus thiooxidans*, a new sulfur-oxidizing organism. J Bacteriol 1922;7:239–56.
[14] Hallberg KB, Lindstrom EB. Characterization of *Thiobacillus caldus* sp. nov., a moderately thermophilic acidophile. Microbiology 1994;140:3451–6.
[15] Kermer R, Hedrich S, Taubert M, Baumann S, Schlömann M, Johnson DB, et al. Elucidation of carbon transfer in a mixed culture of *Acidiphilium cryptum* and *Acidithiobacillus ferrooxidans* using protein-based stable isotope probing. J Integr OMICS 2012;2:37–45.
[16] Ňancucheo I, Johnson DB. Acidophilic algae isolated from mine-impacted environments and their roles in sustaining heterotrophic acidophiles. Front Microbiol 2012;3:235.
[17] Johnson DB, Hallberg KB. Carbon, iron and sulfur metabolism in acidophilic micro-organisms. Adv Microb Physiol 2009;54:202–56.
[18] Coupland K, Johnson DB. Evidence that the potential for dissimilatory ferric iron reduction is widespread among acidophilic heterotrophic bacteria. FEMS Microbiol Lett 2008;279:30–5.
[19] Baker BJ, Banfield JF. Microbial communities in acid mine drainage. FEMS Microbiol Ecol 2003;44:139–52.
[20] Johnson DB, Hallberg KB. The microbiology of acidic mine waters. Res Microbiol 2003;154:466–73.
[21] Kuang JL, Huang LN, Chen LX, Hua ZS, Li SJ, Hu M, et al. Contemporary environmental variation determines microbial diversity patterns in acid mine drainage. ISME J 2013;7:1038–50.
[22] Bond PL, Smriga SP, Banfield JF. Phylogeny of microorganisms populating a thick, subaerial, predominantly lithotrophic biofilm at an extreme acid mine drainage site. Appl Environ Microbiol 2000;66:3842–9.
[23] González-Toril E, Llobet-Brossa E, Casamayor EO, Amman R, Amils R. Microbial ecology of an extreme acidic environment, the Tinto river. Appl Environ Microbiol 2003;69:4853–65.
[24] Bruneel O, Duran R, Casiot C, Elbaz-Poulichet F, Personne J-C. Diversity of microorganisms in Fe-As-rich acid mine drainage waters of Carnoulès, France. Appl Environ Microbiol 2006;72:551–6.
[25] Kay CM, Rowe OF, Rocchetti L, Coupland K, Hallberg KB, Johnson DB. Evolution of microbial "streamer" growths in an acidic, metal-contaminated stream draining an abandoned underground copper mine. Life 2013;3:189–210.
[26] Johnson DB, Hallberg KB. Techniques for detecting and identifying acidophilic mineral-oxidising microorganisms. In: Rawlings DE, Johnson DB, editors. Biomining. Heidelberg: Springer-Verlag; 2007. pp. 237–62.
[27] Altschulet SF, Madden TL, Schaffer AA, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389–402.
[28] Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and clustal X version 2.0. Bioinformatics 2007;23:2947–8.
[29] Johnson DB, Kanao T, Hedrich S. Redox transformations of iron at extremely low pH: fundamental and applied aspects. Front Microbiol 2012;3:96.
[30] Hallberg KB, Johnson DB. Novel acidophiles isolated from moderately acidic mine drainage waters. Hydrometallurgy 2003;71:139–48.
[31] Battaglia-Brunet F, Joulian C, Garrido F, Dictor M-C, Morin D, Coupland K, et al. Oxidation of arsenite by *Thiomonas* strains and characterization of *Thiomonas arsenivorans* sp. nov. A Van Leeuw 2006;89:99–108.
[32] Battaglia-Brunet F, El Achbouni H, Quemeneur M, Hallberg KB, Kelly DP, Joulian C. Proposal that the arsenite-oxidizing organisms *Thiomonas cuprina* and *'Thiomonas arsenivorans'* be reclassified as strains of *Thiomonas delicata*, and emended description of *Thiomonas delicata*. Int J Syst Evol Micr 2011;61:2816–21.
[33] Hiraishi A, Matsuzawa Y, Kanbe T, Wakao N. *Acidisphaera rubrifaciens* gen. nov., sp. nov., an aerobic bacteriochlorophyll-containing bacterium isolated from acidic environments. Int J Syst Evol Microbiol 2000;50:1539–46.
[34] Johnson DB. Geomicrobiology of extremely acidic subsurface environments. FEMS Microbiol Ecol 2012;81:2–12.
[35] Falagan C, Sanchez-Espana J, Johnson DB. New insights into the biogeochemistry of extremely acidic environments revealed by a combined cultivation-based and culture-independent study of two stratified pit lakes. FEMS Microbiol Ecol 2014;87:231–43.
[36] Edwards KJ, Goebel BM, Rodgers TM, Schrenk MO, Gihring TM, Cardona MM, et al. Geomicrobiology of pyrite (FeS_2) dissolution: case studies at Iron Mountain, California. Geomicrobiol J 1999;16:155–79.
[37] Kimura S, Bryan CG, Hallberg KB, Johnson DB. Biodiversity and geochemistry of an extremely acidic, low temperature subterranean environment sustained by chemolithotrophy. Environ Microbiol 2011;13:2092–104.
[38] Hedrich S, Johnson DB. Aerobic and anaerobic oxidation of hydrogen by acidophilic bacteria. FEMS Microbiol Lett 2013;349:40–5.
[39] Ňancucheo I, Johnson DB. Production of glycolic acid by chemolithotrophic iron- and sulfur-oxidizing bacteria and its role in delineating and sustaining acidophilic sulfide mineral-oxidizing consortia. Appl Environ Microbiol 2010;76:461–7.
[40] Hiraishi A, Matsuzawa Y, Kanbe T, Wakao N. *Acidisphaera rubrifaciens* gen. nov., sp. nov., an aerobic bacteriochlorophyll-containing bacterium isolated from acidic environments. Int J Syst Evol Micr 2010;50:1539–46.
[41] Harneit K, Göksel A, Klock J-H, Gehrke T, Sand W. Adhesion to metal sulfide surfaces by cells of *Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans*. Hydrometallurgy 2006;83:245–54.
[42] Ziegler S, Waldner B, Itoh T, Schumann P, Spring S, Gescher J. *Metallibacterium scheffleri* gen. nov., sp. nov., an alkalinizing gammaproteobacterium isolated from an acidic biofilm. J Syst Evol Microbiol 2013;63:1499–504.
[43] Justice NB, Pan C, Mueller R, Spaulding SE, Shah V, Sun CL, et al. Heterotrophic archaea contribute to carbon cycling in low-pH suboxic biofilm communities. Appl Environ Microbiol 2012;78:8321–30.