RESEARCH ARTICLE

Metagenomic analysis reveals adaptations to a cold-adapted lifestyle in a low-temperature acid mine drainage stream

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One sentence summary: This study identifies the dominant microorganisms present in a low temperature acid mine drainage microbial community, describes potential adaptations to the low temperature, and reconstructs the function of community members.

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ABSTRACT

An acid mine drainage (pH 2.5–2.7) stream biofilm situated 250 m below ground in the low-temperature (6–10°C) Kristineberg mine, northern Sweden, contained a microbial community equipped for growth at low temperature and acidic pH. Metagenomic sequencing of the biofilm and planktonic fractions identified the most abundant microorganism to be similar to the psychrotolerant acidophile, Acidithiobacillus ferrivorans. In addition, metagenome contigs were most similar to other Acidithiobacillus species, an Acidobacteria-like species, and a Gallionellaceae-like species. Analyses of the metagenomes indicated functional characteristics previously characterized as related to growth at low temperature including cold-shock proteins, several pathways for the production of compatible solutes and an anti-freeze protein. In addition, genes were predicted to encode functions related to pH homeostasis and metal resistance related to growth in the acidic metal-containing mine water. Metagenome analyses identified microorganisms capable of nitrogen fixation and exhibiting a primarily autotrophic lifestyle driven by the oxidation of the ferrous iron and inorganic sulfur compounds contained in the sulfidic mine waters. The study identified a low diversity of abundant microorganisms adapted to a low-temperature acidic environment as well as identifying some of the strategies the microorganisms employ to grow in this extreme environment.

Keywords: metagenome; acid mine drainage; psychrotolerant; Acidithiobacillus ferrivorans; low temperature
INTRODUCTION

Sulfidic mine sites are characterized by high concentrations of iron and sulfur that are utilized as both electron donors and acceptors by acidophilic microorganisms (Bonnefoy and Holmes 2012; Dopson and Johnson 2012). The final product of S\(^8\) and inorganic sulfur compound (ISC) oxidation is sulfuric acid, which generates the low pH in which acidophilic microorganisms optimally grow. The oxidation of Fe\(^{2+}\) and ISCs by acidophiles is exploited in the biotechnological process of ‘biomining’, whereby the microorganisms catalyze sulfide mineral dissolution and release soluble metals (Vera, Schippers and Sand 2013). However, uncontrolled discharge of acidic, metal-laden solutions from sulfide mineral mines, termed acid mine drainage (AMD), can cause catastrophic environmental damage. Although several studies address AMD generation mediated by mesophilic microorganisms (e.g. Tyson et al., 2004), knowledge of the microbial community that catalyzes AMD generation in the cold is lacking.

The majority of the earth’s surface is cold and these milieus include the deep oceans, polar ice, the mesosphere and stratosphere, and permafrost. Psychrophiles are defined as having a temperature optimum for growth <-15°C (maximum growth temperature of 20°C) while microorganisms able to grow at <0°C but having a temperature optimum of 20-25°C are defined as psychrotolerant. Psychrotolerant species are more likely to be isolated from land environments that are prone to seasonal changes in temperature (Helmkne and Weyland 2004). Cold-adapted microorganisms are found in all three domains of life and they are able to grow by utilizing a range of metabolic strategies in diverse environments. The ability to adapt to low temperature requires the microorganisms to sense a decrease in temperature that induces upregulation of cold-associated genes (Shivaji and Prakash 2010). Major prokaryotic processes that are either affected by the cold or cold induced include: (i) transcription and translation (Noon et al., 2003); (ii) adaptation of proteins to allow them more flexibility (Grzymski et al., 2006); (iii) increased membrane fluidity (Chintalapati, Kiran and Shivaji 2004); (iv) cryoprotectants such as compatible solutes and anti-freeze proteins to protect against freezing (Gilbert et al., 2004); (v) production of stress proteins (Weber and Marahiel 2002); (vi) mechanisms to reduce reactive oxygen species damage caused by increased solubility of gases at low temperature (Methe et al., 2005) and (vii) production of large amounts of exopolymeric substances in aquatic environments (Krembs and Deming 2008). In addition to the cold, low-temperature habitats can have multiple stresses including limited water availability, oligotrophy, high salinity, UV irradiation and high pressure (Tehei and Zaccai 2005). For example, microorganisms in the stratosphere and mesosphere are challenged by temperatures as low as −100°C, UV radiation, desiccation and poor nutrient availability (reviewed in Margesin and Miteva 2013). Acidophile responses to abiotic stresses related to growth in an AMD environment, such as production of oxygen radicals by Fe\(^{2+}\) and high osmolarity due to ISC oxidation to sulfate may also protect against the cold.

Only a few acidophiles that are metabolically active at low temperature have been described. These include Acidithiobacillus (At.) ferrivorans (Hallberg, Gonzalez-Toril and Johnson 2010), Ferrovum (F.) myxofaciens (Johnson, Hallberg and Hedrich 2014), and an Acidiphilium-like bacteria (Berthelot, Leduc and Ferrari 1993, 1994). An ∼8.5°C underground AMD community in Caec Coch, Wales is dominated in various parts of the mine by At. ferrivorans and F. myxofaciens (Kimura et al., 2011; Kay et al., 2013; Johnson, Hallberg and Hedrich 2014). Acidithiobacillus ferrivorans oxidizes Fe\(^{2+}\) and ISCs by acidophiles is ex-}

2004 (Johnson, Hallberg and Hedrich 1994 F. myxofaciens alone and yeast extract (Johnson and Hallberg 2006 and high osmolarity due to ISC oxidation to sulfate may 2011a and N2 (Kay et al., 2013). Metagenomics is the isolation, sequencing and character- ection of microbial community DNA and has allowed elucidation of the phylogeny and metabolic potential of mixed populations (Sharon and Banfield 2013). The technique has been applied to acidic environments such as Iron Mountain, California that is characterized by extremely low pH and a temperature of 35-45°C (Tyson et al., 2004; Dick et al., 1999; Goltsman et al., 2009); an arsenic-rich ecosystem in Carnoulés, France that has a temperature in May of 15.1°C (Bertin et al., 2011); and a snottite biofilm fed by Fe\(^{3+}\)S in the Frassassi cave system, Italy that has a constant temperature of ∼13°C (Jones et al., 2012). Despite the relatively low temperature of the arsenic-rich ecosystem in Carnoulés and snottite biofilm, none of the cited acid pH environment metagenome studies focused on temperature adaptations.

In this study, community DNA was analyzed from a low temperature (6–10°C) underground AMD stream (pH 2.5–2.7) situated at a depth of 250 m below the surface in the Kristineberg mine, northern Sweden. The Kristineberg multimetal sulfide mine has been in operation since 1940 and is worked at a maximum depth of ∼1300 m below the surface. The analyses provide insights into how acidophiles are able to grow at low temperatures.

MATERIALS AND METHODS

Mine site, sampling and colony isolation

Kristineberg mine is a multimetal sulfide mineral mine located in northern Sweden (65.064325N, 18.565183E) from which Zn, Cu, Pb, Au and Ag are extracted (File 1, Supplementary Information). Measurements for annual temperature fluctuations in the mine were based upon multiple readings at two distinct sampling times, in April 2010 and November 2011 when the temperature was 6°C and 10°C, respectively. In April 2010, a total of 50 L of mixed AMD liquid and biofilm was removed from a stream flowing along a tunnel at 250 m below the surface. The mixed liquid and biofilm were stored in sterile containers and transported to the laboratory where they were immediately processed as described below (processing took place ∼3 h after sampling). From the same sample, liquid from the stream was inoculated onto plates containing 5 mM tetrathionate and tetrathionate plus 0.02% yeast extract at pH 2.5 and incubated at 4°C until colonies were observed. A further sampling was carried out in November 2011 to carry out a second isolation of pure cultures from both the planktonic and biofilm fractions, this time incubated at a temperature of 11°C and colonies were observed. A further sampling was carried out in November 2011 to carry out a second isolation of pure cultures from both the planktonic and biofilm fractions, this time incubated at a temperature of 11°C and colonies were observed. The incubation temperatures were chosen to be close to the temperature in the mine stream at time of the first sampling (4°C), while the second sampling for overlay plates experiments were incubated at a temperature similar to the stream temperature at time of sampling (i.e. 11°C) and −22°C to encourage potential psychrotolerant microorganisms present in the mine water to grow at a higher rate. Other measured parameters were redox potential (Pt electrode against an AgCl; 3.5 M KCl), soluble Fe\(^{2+}\) by titration with ceric sulfate, and soluble total iron and copper by atomic adsorption spectroscopy.
(Dopson and Lindström 1999). Metal concentrations were analyzed from two water samples with technical replicates making a total of five replicates.

**DNA preparation and metagenome sequencing**

The biofilm and planktonic cell fractions were separated by removing the solid biofilm before pelleting the planktonic cells by centrifugation (10 000 g for 10 min). The biofilm fraction was resuspended in mineral salts medium (Dopson and Lindström 1999) pH 2.5 and homogenized by a two-step bead-beating method: the biofilm was roughly dispersed using 5 mm beads for 10 min and then divided into smaller aliquots and further homogenized for 2 min using 1 mm glass beads. Cells from both the biofilm and planktonic fractions were resuspended in lysis buffer (100 mM Tris-HCl and 1 mM EDTA, pH 8) and treated with 20 mg mL$^{-1}$ lysozyme (Sigma) for 30 min and 400 μg mL$^{-1}$ proteinase K (Sigma) for 20 min. SDS (2% wt vol$^{-1}$; final concentration) was added to enhance cell lysis. DNA was extracted using phenol/chloroform/isoamyl alcohol (ratio 25:24:1), washed once with 70% ethanol, and precipitated with 100% ethanol and 3 M sodium acetate pH 5.2. To remove small DNA fragments from the biofilm preparation, high molecular weight DNA (∼40 kDa) was extracted from agarose gels with GELase™ agarose gel-digesting system (Epicentre Technologies Corporation) according to the manufacturer’s instructions. DNA was sent to Matís ohf, Reykjavík, Iceland for sequencing. First, a DNA library consisting of 800–1000 bp single-stranded fragments was constructed using the GS FLX Titanium General Library Preparation Kit (Roche, 454 Life Sciences). Shotgun sequencing of the DNA library was carried out using the GS FLX Titanium reagents as described by the manufacturer (Roche, 454 Life Sciences). This involved hybridization of the purified single-stranded DNA fragments onto DNA capture beads and amplification of separate fragments by emulsion PCR. Beads containing amplified DNA were deposited onto a 75 × 75 mm Titanium PicoTiterPlate equipped with a two-lane gasket and the pyrosequencing was performed in a single run. A single titanium plate was equally split between the biofilm and planktonic DNA samples. Generated sequence data were assembled using the GS De Novo Assembler software, version 2.6, from Roche, 454 Life Sciences with the default stringency settings.

**Metagenome bioinformatic analysis**

The biofilm and planktonic reads were assembled separately using the Newbler Assembler 2.3 (454 Life Science) using the default parameters. 16S rRNA genes were identified by comparing both metagenomes against the GREENGENES (DeSantis et al., 2006), RDP (Cole et al., 2009) and SILVA (Pruesse et al., 2007) databases using an E-value threshold of 1×$^{-10}$. Contig sequences (>500 bp) from the two separate planktonic and biofilm metagenomes were binned to taxonomic groups using a number of algorithms including: MEGAN v4.60.1 (Mitra, Stark and Huson 2011) using a bit score threshold of 35; RAlphy (Nalbantoglu et al., 2011) using a custom-built database containing complete and draft genomes from acidophilic representatives (File 2, Supplementary Information) and the default RAlphy database; WEBCARMA (Gerlach and Stoye 2011) and SOrt-ITEMS (Monzoorul et al., 2009). The results from these different techniques were similar and MEGAN results were chosen as representative for further analysis. In addition, TBLASTX (Altschul et al., 1997) searches were performed against all the RefSeq genomes (Fruit et al., 2012) for all contigs with lengths ≥180 nucleotides. Custom-made Perl scripts were used to align each contig nucleotide sequence against RefSeq database genomes using TBLASTX the results were ordered by E-value first, then query coverage (percentage of the contig participating in the alignment) and finally, alignment similarity and the best 10 genomes were retained. The top hit with the best statistics was then assigned to the contig as ‘Best Species’. Contigs associated with more than one taxonomic group were classified as unassigned.

In addition, the two metagenomes were assembled as a single metagenome. Combined contigs were annotated using Glimmer (Kelley et al., 2012) in default mode and Prodigal (Hyatt et al., 2010) in metagenomic mode, using a minimum length of 100 nucleotides. After manually comparing the performance of both tools, Prodigal results were selected for further analysis because they predicted more open reading frames (ORFs). MEGAN was used to identify the operational taxonomic units present in the reassembly of both metagenomes.

Predicted coding sequences (>60 amino acids) were selected to perform Blastp searches against the NCBI-NR and SWISSPROT protein databases, while domain searches were also performed against the Conserved Domain Database (Marchler-Bauer et al., 2013) using RPS-Blast, which includes COG (Tatusov et al., 2001), Pfam (Punta et al., 2012), PRK (Klimke et al., 2009) and TIGRfam (Haft et al., 2013); for all searches, an E-value cut-off of 1×$^{-10}$ was used. The NCBI accession number of the sequence data is AOMP0000000-AOMQ0000000.

**De-novo assembly of metagenome samples**

For metabolic reconstruction and curation of pathways, the two Kristineberg metagenome samples were pooled together and re-assembled as a new, single assembly using the CLC Genomics Workbench (version 6.5.2; http://www.clcbio.com). ORFs were predicted and annotated as described for the individual planktonic and biofilm metagenomes. Metabolic reconstruction and curation of specific pathways was performed on using the PRIAM (Claudel-Renard et al., 2003) and Ptools (Karp, Paley and Romero 2002) software.

**Comparison of metagenomes in this and other studies**

The planktonic and biofilms metagenomes (this study) were compared to the Iron Mountain (Tyson et al., 2004; Dick et al., 2009; Goltsman et al., 2009; Yelton et al., 2013), Carnoulès (Bertin et al., 2011) and Frasassi cave system (Jones et al., 2012) metagenomes. First, a cold-resistance Blast database was made with amino acid sequences of all the trehalose pathway genes (Avonce et al., 2006). Then, amino acid sequences of all metagenome annotated genes were compared with the database using Blastp with an E-value cut-off of 1×$^{-5}$, and the best database hit for each gene was registered. Second, and complementary to the first approach, a word search was also done using a word list crafted for that purpose that included cold-resistance gene acronyms, gene descriptions and terms related to cold resistance.

**Molecular phylogenetic analysis of isolates grown on overlay plates**

16S rRNA gene copies from colonies isolated on the overlay plates were PCR amplified using illustra PuReTaq Ready-To-Go PCR Beads and the 16S rRNA gene primers GMSF and 907R (Morales et al., 2005). PCR products were purified using a PCR Clean-up Kit (Qiagen), quantified and submitted to
Macrogen (Netherlands) for capillary Sanger sequencing. Phylogenetic analysis was carried out by removing primer sequences and aligning the isolate 16S rRNA gene sequences with representative acidophiles (File 3, Supplementary Information) using Muscle (Edgar 2004). The maximum number of iterations was set to 100 and poorly aligned and divergent regions were eliminated using Gblocks (Castresana 2000). The maximum number of contiguous non-conserved positions was set to 8 and intermediate gap positions were allowed. This resulted in 676 positions in the alignment that were used to generate maximum-likelihood trees using PhyML (Guindon and Gascuel 2003). The phylogenetic tree was assessed using 1000 bootstrap replicates.

RESULTS AND DISCUSSION

Environmental conditions and sampling

The AMD stream contained large streamer-type biofilms consisting of chains of cells twisted together (File 1, Supplementary Information). When the samples for the metagenomes were taken in April 2010 the stream had in situ characteristics of: 6°C, pH 2.7, an oxidation reduction potential value below the standard hydrogen potential of 640 mV and 16.8 mM soluble Fe²⁺. During a subsequent visit in November 2011, the stream was 11°C, pH 2.5 and had soluble iron (both Fe²⁺ and Fe³⁺) and copper concentrations of 39 ± 0.9 and 5 ± 0.1 mM, respectively (number of replicates = 5). This suggests that while the pH did not significantly change, small variations in temperature occurred potentially due to infiltration of surface waters.

Overview of the metagenomic data set

Metagenome sequencing generated 14 279 423 (19 051 contigs) and 8 006 447 (8115 contigs) nucleotides in the planktonic and biofilm libraries, respectively (File 4, Supplementary Information). A total of 20 606 and 10 694 ORFs from planktonic and biofilm datasets were predicted, respectively, from which 16 271 (78%) and 8230 (77%) had significant similarity to sequences available in public databases. A normalized comparison of the COG classifications (against the total number of coding sequences) from both communities (File 5, Supplementary Information) demonstrated essentially similar percentages of protein-encoding sequences assigned to the COG classifications. The total reads for COG classifications ‘RNA processing and modification (A)’ and ‘chromatin structure and dynamics (B)’ were <0.05% in both metagenomes indicating either a low incidence of eukaryotic sequences or a bias in the method for DNA preparation that preferentially lyzed prokaryotes. However, of the coding sequences matching COGs A and B, nearly 2-fold normalized sequences were present in the biofilm fraction. This suggested that eukaryotes, such as the identified acidophilic yeast (see below), were more prevalent in the biofilm where a higher concentration of organic carbon was likely available compared to typically oligotrophic AMD waters.

Molecular phylogeny of the biofilm and planktonic species

A phylogenetic tree was constructed using 16S rRNA gene sequences from isolated colonies and the Kristineberg mine metagenome (File 6, Supplementary Information). All 27 of the 16S rRNA gene sequences derived from the overlay plates incubated at ~22 and 12°C (13 and 14 colonies, respectively) aligned with the psychrotolerant acidophile, At. ferrivorans (for clarity, only two representative sequences are presented). The presence of an At. ferrivorans-like strain on the enrichment plates agreed with previous data showing a low-temperature bioreactor for ISC removal inoculated with Kristineberg AMD water contained At. ferrivorans (Liljevist et al., 2011a). In addition, gene sequences identified from the metagenomes included: two 16S rRNA gene copies phylogenetically most related to At. ferrivorans; three 16S rRNA gene copies most related to Gram-positive, Fe³⁺ oxidizing acidophilic heterotrophs of the genera Acidimicrobium and Ferrimicrobium (Wisotzkey et al., 1992; Johnson et al., 2009); two 16S rRNA gene copies most related to Alicyclobacillus acidocaldarius (Wisotzkey et al., 1992); one 16S rRNA gene copy most related to the Fe³⁺ oxidizing neutrophile Gallionella (G.) capsiferriformans (Emerson et al., 2007); and two 16S rRNA gene copies most related to the low-temperature species Terriglobus (Te.) saanensis (Männistö et al., 2011) and Acidobacterium (Ac.) capsulatum (Kishimoto, Kosako and Tano 1991). Two colonies from plates incubated at 4°C were found to be yeast species related to Cryptooccus sp. (data not shown). Acidophilic yeasts from AMD environments have been previously reported (Gadanho and Sampaio 2006) and likely metabolize organic carbon produced by the autotrophic acidophiles. However, the lack of 18S rRNA genes and other eukaryotic related species in the metagenome suggests that they constituted a minor portion of the mixed population.

Species identification and binning of the metagenomic sequences

Binning of the metagenome sequences was carried out with MEGAN (Fig. 1 & File 7, Supplementary Information) and other methods (File 8, Supplementary Information). The binning methods consistently demonstrated that species from the Acidithiobacillus genus and, in particular, an At. ferrivorans-like species dominated both the biofilm and planktonic communities.

Based upon the number of reads against the RefSeq genomes, the dominant species in both the planktonic (307 185 of assigned 639 459 reads; 48.0%) and biofilm (430 290 of assigned 672 719 reads; 64.0%) fractions was At. ferrivorans-like (File 8, Supplementary Information). The number of planktonic and biofilm contigs assigned to the At. ferrivorans-like species were 5574 (constituting 90% of the genome) and 2631 (also constituting 90% of the genome), respectively. The next most abundant strains were binned as a Fe³⁺ and ISC oxidizing At. ferrooxidans-like strain (both ATCC 23270 and ATCC 53993) with a total of 9.9% and 8.7% in the planktonic and biofilm metagenomes, respectively followed by an ISC oxidizing At. caldus-like strain (3.6% and 2.7%, respectively). In addition, a Gallionella-like sp. (G. capsiferriformans; 1.0% and 0.2%, respectively) that is a neutrophilic ferrrous oxidizer capable of growth at 6°C but not 30°C (Emerson and Moyer 1997) and an Acidobacterium-like sp. (Ac. capsulatum; 1.0% and 0.1%, respectively) that is a Gram-negative, organotrophic acidophile that grows at 25°C (Kishimoto, Kosako and Tano 1991) were also identified. The presence of contigs attributed to relatives of mesophilic and moderately thermophilic species is most likely explained by these being the only sequenced genomes available to which metagenome contigs can be matched. A low species diversity dominated by just two (Tyson et al., 2004), seven (Bertin et al., 2011) and one (Jones et al., 2012) strain(s) appears to be a typical trait of acidic environments (Hallberg et al., 2006), where primary producers able to fix carbon constitute the majority of the sampled microbial population.

Small differences in binning were observed between the two metagenomes with a higher percentage of contigs assigned
to the Acidithiobacillus genus in the biofilm fraction (25.5%) compared to the planktonic fraction (19.2%); At. ferrioxidans-like species (24.5% and 14.6%, respectively); At. ferrooxidans-like species (7.8% and 6.3%, respectively); and At. caldus-like species (2.8% and 2.6%, respectively) (File 8, Supplementary Information). In contrast, more contigs were assigned to the Acidobacteria-like species in the planktonic fraction (5.4%) compared to the biofilm (0.9%) and the Gallionellaceae-like species (3.9% and 0.2%, respectively) (File 8, Supplementary Information).

Although the environment was dominated by a few species, there was also a significant ‘tail’ of low-abundance species. These included contigs that were assigned by sequence similarity to: (i) an Acidimicrobium-like sp. (Acidimicrobium ferrooxidans), a Gram-positive moderately thermophilic ferrous iron oxidizer (Clark and Norris 1996); (ii) a Te. saanensis-like sp. which is an organotrophic Acidobacterium capable of growth at pH 4.5–7.5 and 4–30°C (Männistö et al., 2011); (iii) a Sideroxydans lithotrophicus-like sp., an acid-tolerant ferrous oxidizer (Ludecke et al., 2010); (iv) a Rhodofex xerireducens-like sp., a psychrotolerant iron reducer that grows at 4–30°C (Finneran, Johnsen and Lovley 2003) and (v) a Leptospirillum ferrodiazotrophum-like sp., an acidophilic obligate ferrous oxidizer (Goitsman et al., 2009).

### Cold-shock and cold acclimation strategies

Microorganisms undergo a cold shock when their external temperature drops below its optimal range. The response to cold shock in the psychrophilic Psychrobacter arcticus included down-regulation of genes associated with energy metabolism and substrate incorporation and upregulation of genes for membrane maintenance and a DEAD-box RNA helicase protein A (Kuhn 2012). In contrast, genes for RNA and protein chaperones were not upregulated under the tested conditions (Kuhn 2012). The response to cold shock between, for example, mesophilic and psychrophilic microorganisms can vary as a cold-shock gene in a mesophile can act as a cold acclimation protein in a psychrophile. For example, CspA was the most abundant protein in Escherichia coli upon downshift from 37 to 10°C (Goldstein, Pollitt and Inouye 1990) while it acted as a cold acclimation protein in the psychrotolerant species, Arthrobacter globiformis (Berger et al., 1996). It is unclear if the genes identified in this study were cold-shock or acclimation proteins.

An increase in membrane rigidity as a response to cold shock is sensed by a membrane-associated two-component system in Bacillus subtilis (Aguilar et al., 2001). Also, methyl-accepting chemotaxis proteins (Maeda and Imae 1979) and 3-oxoacyl-(acyl carrier protein) synthase II (Garwin and Cronan 1980) play an important regulatory role in sensing temperature changes in E. coli. A total of 34 membrane-associated two-component systems, 26 methyl-accepting chemotaxis systems and 6 copies of 3-oxoacyl-(acyl carrier protein) synthase II were found in the metagenomes. These proteins could be used to sense a wide variety of environmental signals and a potential role in sensing varying temperatures remains to be investigated.

Increased membrane fluidity can be achieved by changes in fatty acid isomerization, changing the composition of sterol-like compounds, and adjusting the levels of saturated and unsaturated fatty acids (Russell 2008). Fatty acid desaturases not only modulate the fluidity of the membrane but also protect against increased concentrations of reactive oxygen species. Oxidative stress proteins are especially important at low temperature due to the increased solubility of gases. Two types of desaturases can be found in the metagenome, fatty acid desaturase and stearoyl-CoA 9-desaturase. In addition, several species in the metagenome contained genes for catalase, superoxide dismutase, peroxiredoxin and alkyl hydroperoxide reductase as well as several dioxygenases of various functions that protect against reactive oxygen species. Oxidative stress defense strategies are common in AMD environments due to the presence of Fe⁴⁺. For example, peroxiredoxins are upregulated in Ferroplasma acidarmanus in response to the presence of Fe⁴⁺ (Dopson, Baker-Austin and Bond 2005). A requirement for these systems at low temperature may be increased due to the higher solubility of gases contributing to increased oxidative stress.

Cold sensing occurs via DNA super coiling at low temperature and by the ability of some RNA molecules to alter their secondary structure as a result of a temperature shift (reviewed in Shivaji and Prakash 2010). The most studied response is generally referred to as the ‘cold-shock response’. The cold-shock
response is characterized by the induction of small DNA/RNA-binding cold-shock proteins (CSPs), cold-shock RNA helicases and several proteins involved in replication, transcription and translation. The majority of cold-induced RNA helicases contain the DEAD/H-box motif (reviewed in Owttrim 2006). The biofilm and planktonic metagenomes contained 5 and 6 CSPs and 11 and 26 DEAD/H-box helicases, respectively. However, some of the CSPs have significant similarity to CspD that is upregulated in stationary phase and under nutrient starvation, rather than being associated with cold shock (Yamanaka and Inouye 1997).

Many microorganisms accumulate compatible solutes such as trehalose, glycine-betaine, choline and sucrose that along with other functions are involved in the cold-stress response (Kawahara 2008). Trehalose appears to be common in AMD environments (Yelton et al., 2013) and its other functions include mitigating osmotic stress, carbon storage and heat shock (Martins et al., 1997). The metagenome contains genes encoding five pathways for microbial trehalose production (Avonce et al., 2006; Fig. 2 and File 9, Supplementary Information). Functional redundancy for trehalose biosynthesis has been suggested to be due to the need to accumulate trehalose under changing environmental conditions (Reina-Bueno et al., 2012) and the presence of multiple pathways may reflect its relative importance in this milieu. Binning of the sequences demonstrated that the At. ferrivorans-like species had complete trehalose synthase (TS), TreYZ and TreT pathways as well as the gene-encoding trehalose-6-phosphate synthase (TPS) from the TPS/trehalose-6-phosphate phosphatase (TPP) pathway; the Acidobacteria-like species had the TS and TreP pathways; and the At. ferrooxidans-like species the TreT pathway as well as a gene-encoding TreY. It was interesting that contigs assigned to the psychrotolerant At. ferrivorans-like species encoded three separate trehalose synthesis pathways and that the At. ferrooxidans-like species had an additional partial trehalose synthesis pathway compared to the two sequenced At. ferrooxidans genomes. The multiple synthesis pathways suggest the importance of trehalose as a potential response to low temperature in this environment.

In addition, the biofilm metagenome contained predictions for genes involved in the production of other compatible solutes and cryoprotectants. As for trehalose, the compatible solutes glycine betaine and sucrose also have other functions and their role in cold adaptation has not been confirmed. Genes associated with cryoprotection included glycine betaine synthesis including the betaine transcriptional regulator (betI) and two copies of the gene-encoding choline dehydrogenase (betA), although the second enzyme in the pathway, betaine aldehyde dehydrogenase (betB), was not detected. The genes encoding sucrose synthase and sucrose-phosphate synthase involved in sucrose synthesis from UDP-D-glucose were also predicted in both fractions. Anti-freeze proteins are ice-binding proteins that induce thermal hysteresis and thereby, inhibit the growth of ice crystals (Kawahara 2008). The planktonic fraction contained a gene predicted to encode a Type II anti-freeze protein previously identified in Te. saanensis (Rawat, Mannisto and Starovoytov 2012). A final investigated signature for low-temperature adaptation was for the presence of de novo adenosine 5'-monophosphate synthetic enzymes (also involved in purine biosynthesis) that are enriched in low-temperature-adapted microorganisms compared to mesophiles (Parry and Shain 2011). Four and five gene copies encoding adenylosuccinate synthetase (purA) were identified in the biofilm and planktonic metagenomes while five and two copies of adenylosuccinate lyase (purB) were found, respectively.

Figure 2. The various pathways for trehalose production in bacterial acidophiles. Figure adapted from Liljeqvist (2012).
Biofilm formation

Genes were predicted for all stages in biofilm formation in the At. ferrivorans-like DNA sequences (File 10, Supplementary Information). Genes predicted to encode pili formation and a suite of genes for exopolysaccharide formation and export was also present in the At. ferrooxidans-like DNA sequences but they lacked any evidence for genes involved in the Lux quorum sensing system, flagella formation, the signaling pathway quorum sensing genes and the diguanylate cyclase signaling system. The At. caldus-like strain contained predicted genes for chemotaxis, pilus formation and flagella formation. This attribution of predicted genes and functions to specific reconstructed microbial species in the metagenome sequences is consistent with their respective individual genomes (Valdes et al., 2008; Valdes et al., 2009; Liljeqvist et al., 2011b) and additional bioinformatic evidence (Cardenas et al., 2010). Evidence was found for the ability to form a biofilm by the Gallionellaceae-like (principally Gallionella-like and Sideroxydans-like) and Acidobacteria-like metagenome bins. This includes pili formation in both species as well as genes coding for EPS biosynthesis in the Acidimicrobiium-like strain.

Substrate oxidation and energy conservation

Of the acidophiles, At. ferrooxidans has the best characterized Fe\(^{2+}\) oxidation system (Quatrini et al., 2010). All components of this system were detected in the metagenomes (File 11, Supplementary Information) with orthologs found in both the At. ferrivorans-like and At. ferrooxidans-like contigs. This suggests that these species may oxidize Fe\(^{2+}\), although expression of the genes has not been confirmed. Acidophile sulfur oxidation models have been suggested for At. ferrooxidans (Quatrini et al., 2009) and At. caldus (Mangold et al., 2011; Chen et al., 2012). Although a model was not generated, genes encoded by the At. ferrivorans genome have also been assigned to ISC oxidation (Liljeqvist, Rzhepishevskaya and Dospn, 2013). Based upon the earlier studies, an ISC oxidation pathway is proposed for the At. ferrivorans-like species (File 12, Supplementary Information). Other identified genes annotated as involved in ISC oxidation included components of heterodisulfide reductase from At. ferrooxidans-like, At. caldus-like, Ac. ferrooxidans-like and an Acidobacteria-like species.

Genes encoding electron transport components were identified in the At. ferrivorans-like species. These included cytochrome b terminal oxidase (cyoABCD), an aa\(_2\)-type cytochrome c oxidase (coxABC) and subunits of a cytochrome c oxidase. Additionally, two ccb\(_2\) genes (ccoN & ccoG) and a cytochrome bd ubiquinol oxidase gene (cydA) were also identified. All subunits from a respiratory nitrate reductase (narGHI) were annotated to the At. ferrivorans-like strain suggesting that it may utilize nitrate as a terminal electron acceptor.

Carbon dioxide and nitrogen fixation

Genes predicted to encode the two unique enzymes involved in carbon assimilation via the Calvin–Benson–Bassham cycle, namely ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) and phosphoribulokinase (CbbP), were found in At. ferrivorans-like and At. ferrooxidans-like contigs (File 13, Supplementary Information). This is in agreement with published data that these microorganisms fix carbon (Esparza et al., 2010;

| Table 1. Genes associated with a low-temperature lifestyle identified in the Kristineberg metagenome (this study) compared to Iron Mountain, California (Tyson et al., 2004; Dick et al., 2009; Goltzman et al., 2009; Velton et al., 2013); Carnoules, France (Bertin et al., 2011) and the Frasassi cave system, Italy (Jones et al., 2012) |
|----------------|----------------|----------------|----------------|
| Temperature     | 6–11°C         | 35–45°C        | 15.1°C         | ~13°C         |
| Response to low temperature | Fatty acid desaturase; SCD | SCD | SCD; DesA |
| Membrane fluidity\(^a\) | TS; TPS; TPP; TreY; TreZ; TreT; TreP | TS; TPS; TPP; TreY; TreZ; TreT; TreP | TS; TPS; TPP; TreY; TreZ; TreT; TreP | TPS; TreZ |
| Compatible solutes and antifreeze proteins | BetA; BetI | BetB | BetA; BetB; Gbu | BetB |
| Glycine betaine\(^c\) | Type II | Type II | Type II | Type II |
| Anti-freeze proteins | | | | |
| Oxidative stress and low-temperature response | | | | |
| Cold-shock response\(^d\) | CSP; CsBD; GyRA; DeaD; RecA; PpiC; SurA; RbfA; Pnpase; Pnp; NusA; ClpB | GyRA; DeaD; RecA; RbfA; Pnp; NusA | CsBD; GyRA; DeaD; RecA; PpiC; RbfA; Pnpase; Pnp; NusA; ClpB | Gpx; Ahp; SOD; Prx; CcpA; Cat |
| Oxidative stress\(^e\) | Gpx; Prx; Ahp | Gpx; Prx; Ahp | Gpx; Ahp; SOD; Prx (Bcp) | Gpx; Ahp; SOD; Prx (Bcp) |

\(^a\) SCD, Stearoyl-CoA 9-desaturase; DesA, Δ12 acyl-lipid desaturase.
\(^b\) Description of genes given in File 9 (Supplementary Information).
\(^c\) BetaA, choline dehydrogenase; BetI, betaine aldehyde dehydrogenase; BetI, BetII DNA-binding transcriptional repressor; Gbu, glycine betaine ABC transporter.
\(^d\) CSP, DNA binding cold-shock protein; CsBD, cold-induced stress protein; GyRA, DNA gyrase; DeaD, DEAD-box RNA helicase; PpiC, peptidyl-prolyl cis-trans isomerase; SurA, survival protein; RecA, DNA strand exchange and recombination protein; RbfA, ribosome binding factor; Pnpase, polynucleotide phosphorylase/polynucleosidase; Pnp, polyribonucleotide nucleotidyltransferase; NusA, transcription termination factor; ClpB, ATP-dependent chaperone.
\(^e\) Gpx, glutaredoxin; GrxB, reduced glutaredoxin 2; Ahp, alkyl hydroperoxide reductase; SOD, superoxide dismutase; Prx, peroxiredoxin; CcpA, cytochrome c peroxidase; Cat, catalase; BtuE, glutathione peroxidase.
Figure 3. Model of the Kristineberg low-temperature AMD microbial community showing the major functions of the identified Acidithiobacillus-like spp., an unidentified Acidobacteria-like species and an unidentified Gallionellaceae-like species. The figure was adapted from Valdes et al. (2008).
Hallberg, Gonzalez-Toril and Johnson (2010). There was no genetic evidence for CO₂ fixation by other known pathways. Evidence of heterotrophic lifestyles included candidate genes encoding licheninase, neopullulanase, dextranase and other proteins involved in sugar biodegradation and transport. Due to the typically low availability of organic carbon in AMD environments, primary producers are likely dominant and the relatively low number of heterotrophs in the biofilm was expected. The likely role of the heterotrophs is to metabolize organic carbon that can be toxic to autotrophic acidophiles (Nancucheo and Johnson 2010).

Genes annotated for ammonium and nitrate uptake systems in the *At. ferrivorans*-like species were present together with nitrate and nitrite reductases, suggesting that nitrogen was assimilated from different sources (File 14, Supplementary Information). Nitrate is a potential nitrogen source from explosives used for mining in an otherwise oligotrophic environment. In addition, nitrogen fixation genes were identified in the *Gallionellaceae*-like species. There was no genetic evidence for ammonia or ammonium oxidation, nitrification or denitrification (nitric oxide reductases and nitrous oxide reductases catalyzing the last two steps of denitrification were not found).

A cold-adapted metagenome

A search of the metagenome sequences identified unique genes assigned to the *At. ferrivorans*-like strain (but not to the other characterized *Acidithiobacillus* spp.) suggesting it was adapted to low temperature (File 15, Supplementary Information). These genes included a trehalose synthase, stressing its potential importance for adaptation to low temperature; a multisensor hybrid histidine kinase that acts as a cold sensor (Phadtare 2004); helicase containing proteins; phosphoadenosine phosphosulfate reductase previously shown to be upregulated at low temperature (Topanurak et al., 2005); the 30S ribosomal protein S21 involved in adaptation to low temperature (Sato et al., 1997); oxidative stress proteins; CsbD involved in survival at low temperature (Raengpradub, Wiedmann and Boor 2008) and genes encoding for biofilm formation. The presence of these genes supports that the *At. ferrivorans*-like strain is adapted to the low temperature. However, without isolation of the other species detected it is impossible to determine if they are psychrophilic, psychrotolerant or mesophilic.

A comparison of the genes identified in this study to the Iron Mountain (Tyson et al., 2004; Dick et al., 2009; Goltsman et al., 2009; Yelton et al., 2013), Carnoulès (Bertin et al., 2011) and Frasassi cave system (Jones et al., 2012) metagenomes also suggested adaptations to a low-temperature lifestyle (Table 1). This comparison must be taken with care as it will be affected by the number of species in the community, with a less diverse community likely to have a smaller ‘pan genome’ as well as the depth of sequencing. However, a higher incidence of genes suggested to contribute to life in the cold was evident in the Kristineberg metagenome followed by the Carnoulès AMD stream that had a temperature in May of 15.1 °C. These differences were particularly evident for genes assigned as encoding for compatible solutes and antifreeze proteins as well as genes associated with oxidative stress associated due to the higher solubility of gases at low temperature (Table 1). We hypothesize that the higher degree of similarity between the Kristineberg and Carnoulès AMD stream metagenomes was likely due to adaptations to the temperature rather than the presence of similar species. This is supported by more genes suggesting adaptations to a low temperature in the Carnoulès AMD stream despite that the Frasassi cave system contained a similar microbial population to the Kristineberg community.

Model of the Kristineberg low-temperature AMD microbial community

The metagenome binning was summarized to provide the attributes of the dominating *Acidithiobacillus*-like spp., *Acidobacteria*-like sp. and *Gallionellaceae*-like sp. (File 16, Supplementary Information). These attributes were used to create a model of the Kristineberg low-temperature AMD community (Fig. 3). All analyses suggested a predominantly chemolithotrophic lifestyle with the *At. ferrivorans*-like species fixing carbon dioxide. The other identified species are suggested to play a lesser role such as via metabolizing organic carbon that is toxic to chemolithoautotrophic acidophiles and potentially contributing to biofilm formation.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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