Analysis of differential protein expression during growth states of *Ferroplasma* strains and insights into electron transport for iron oxidation

Mark Dopson,^{1†} Craig Baker-Austin¹ and Philip L. Bond^{1,2}

School of Biological Sciences¹ and Centre for Ecology, Evolution and Conservation² University of East Anglia, Norwich NR4 7TJ, UK

Correspondence

Philip Bond
phil.bond@uea.ac.uk

To investigate the metabolic biochemistry of iron-oxidizing extreme acidophiles, a proteomic analysis of chemomixotrophic and chemo-organotrophic growth, as well as protein expression in the absence of organic carbon, was carried out in *Ferroplasma* species. Electron transport chain inhibitor studies, spectrophotometric analysis and proteomic results suggest that oxidation of ferrous iron may be mediated by the blue copper-haem protein sulfocyanin and the derived electron passes to a *cbb*₃ terminal electron acceptor. Despite previous suggestions of a putative carbon dioxide fixation pathway, no up-regulation of proteins typically associated with carbon dioxide fixation was evident during incubation in the absence of organic carbon. Although a lack of known carbon dioxide fixation proteins does not constitute proof, the results suggest that these strains are not autotrophic. Proteins putatively involved in central metabolic pathways, a probable sugar permease and flavoproteins were up-regulated during chemo-organotrophic growth in comparison to the protein complement during chemomixotrophic growth. These results reflect a higher energy demand to be derived from the organic carbon during chemo-organotrophic growth. Proteins with suggested function as central metabolic enzymes were expressed at higher levels during chemomixotrophic growth by *Ferroplasma acidiphilum* Y^T compared to '*Ferroplasma acidarmanus*' Fer1. This study addresses some of the biochemical and bioenergetic questions fundamental for survival of these organisms in extreme acid-leaching environments.

Received 21 July 2005

Revised 7 September 2005

Accepted 9 September 2005

INTRODUCTION

Acidophilic metal-mobilizing micro-organisms have been isolated from acidic environments and their biodiversity and ecology have been reviewed (Hallberg & Johnson, 2001). In environments containing mineral sulfides, metals are released via oxidation of the metal sulfide bond by ferric iron and the process is catalysed by the regeneration of ferric iron by ferrous-iron-oxidizing micro-organisms (Singer & Stumm, 1970). As a consequence of the pivotal role that acidophilic micro-organisms play in the generation of acid mine drainage (AMD), acid rock drainage (ARD) and biologically catalysed mobilization and extraction of metals (termed bioleaching), these micro-organisms have attracted significant attention.

[†]Present address: Molecular Biology, Umeå University, S-90187 Umeå, Sweden.

Abbreviations: AMD, acid mine drainage; ARD, acid rock drainage; 1D-PAGE, one-dimensional polyacrylamide gel electrophoresis; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

Supplementary tables with details of the proteins induced under various conditions are available with the online version of this paper.

Ferroplasma spp. are of particular interest as they have been isolated from the most acidic and metal-rich natural environment reported to date (Edwards *et al.*, 2000; Nordstrom & Alpers, 1999), as well as having been detected from a number of other AMD sites and commercial bioleaching plants (Dopson *et al.*, 2004b; Golyshina *et al.*, 2000; Gonzalez-Toril *et al.*, 2003; Kinnunen & Puhakka, 2004; Okibe *et al.*, 2003). The genus includes '*Ferroplasma acidarmanus*' Fer1 isolated from Iron Mountain, CA, USA, where typically the drainage water pH is between 0.5 and 1.0 and metal ion concentrations are in the range of tens of grams per litre (Edwards *et al.*, 2000). The Fer1 genome has been sequenced (97 % complete) and draft results are available at <http://genome.ornl.gov/microbial/faci/>. Other *Ferroplasma* strains include *Ferroplasma acidiphilum* Y^T, Y-2, MT17 and DR1, all isolated from bioreactors for metal removal (Dopson *et al.*, 2004b; Golyshina *et al.*, 2000; Okibe *et al.*, 2003; Pivovarova *et al.*, 2002). A further *Ferroplasma* lineage is detected from an Iron Mountain biofilm via DNA sequencing directly from the biofilm, termed *Ferroplasma* Type II (Tyson *et al.*, 2004). Finally, a *Ferroplasma*-like isolate, which grows optimally at 55 °C, has recently been identified from a chalcocite heap leach operation in Perth, Western Australia (Hawkes *et al.*, 2005).

Ferroplasma spp. are capable of chemo-organotrophic growth on yeast extract (Baumler *et al.*, 2005; Dopson *et al.*, 2004b). They also gain energy via oxidation of Fe^{2+} (Dopson *et al.*, 2004b; Golyshina *et al.*, 2000). However, with the exception of *Acidithiobacillus ferrooxidans* (Brasseur *et al.*, 2004; Elbehti *et al.*, 1999; Yarzabal *et al.*, 2003, 2004), little is known regarding the biochemistry of oxidation of Fe^{2+} in acidophiles. But, from a biotechnological viewpoint and in conditions of very low pH AMD, Fe^{2+} oxidation is probably more important in *Ferroplasma* spp., as they are the predominant iron oxidizers present at low pH and they have been found in many industrial bioleaching operations (Edwards *et al.*, 2000). Furthermore, preliminary investigations of acidophile Fe^{2+} oxidation indicate that mechanisms of other acidophiles differ from that of *A. ferrooxidans* (Barr *et al.*, 1990; Blake *et al.*, 1993; Hart *et al.*, 1991). A putative electron transport chain for *Ferroplasma* Type II is suggested to contain components involved in oxidation of both organic carbon and Fe^{2+} (Tyson *et al.*, 2004).

Availability of organic carbon is important to the microbial biochemistry at AMD-producing sites. Organic carbon inhibits the growth of many extreme acidophiles, a result of their reversed membrane potential and accumulation of organic acids that results in acidification of the cytoplasm (Alexander *et al.*, 1987). However, *Ferroplasma* spp. grow chemo-organotrophically by aerobic and anaerobic respiration at pH 1.0 (Baumler *et al.*, 2005; Dopson *et al.*, 2004b). It is likely that acid-producing sites such as Iron Mountain receive little fixed carbon from external sources. Presently there is some incongruity concerning the autotrophic capabilities of *Ferroplasma* spp. From environmental genomic information some acidophiles detected at Iron Mountain are considered to be autotrophic. Genes for CO_2 fixation attributed to *Leptospirillum* strains have been detected, and a mechanism for CO_2 fixation is suggested for *Ferroplasma* Type II (Tyson *et al.*, 2004). However, both *Ferroplasma* strains MT17 and Fer1 are described as organotrophic iron oxidizers (Okibe & Johnson, 2004). In contrast, *Ferroplasma* Y^T and Y-2 are described as able to fix CO_2 for organic carbon and only require yeast extract as an essential growth factor, and these strains are classified as autotrophs (Golyshina *et al.*, 2000; Pivovarova *et al.*, 2002). Our recent characterization failed to conclusively prove CO_2 fixation by either strain Y^T or strain Fer1 (Dopson *et al.*, 2004b). *Ferroplasma* spp. in general and the issue of CO_2 fixation in particular has been recently reviewed (Golyshina & Timmis, 2005). It is of critical importance to elucidate the exact biochemical mechanisms of CO_2 fixation/carbon assimilation in these organisms because of their importance as intermediates in key geochemical cycling, such as the mobilization of heavy metals (Dopson *et al.*, 2003), and their role in catalysing AMD and bioleaching.

The *Ferroplasmaceae* represent a particularly important group of organisms from both an environmental (AMD genesis) and biotechnological perspective (bioleaching capability and a possible source of acid-tolerant proteins

for industrial applications). In this study we combine proteomic analyses of different growth states with the characterization of electron transport. This is believed to constitute the first detailed proteomic examination of growth in an acidophilic archaeon, and insight has been gained into metabolic and physiological details of *Ferroplasma* strains.

METHODS

Strains and batch growth conditions. The *Ferroplasma* strains used were '*F. acidarmanus*' Fer1 (Dopson *et al.*, 2004b; Edwards *et al.*, 2000), *F. acidiphilum* Y^T (DSM 12658; Golyshina *et al.*, 2000), *F. acidiphilum* MT17 (Okibe *et al.*, 2003) and *F. acidiphilum* DR1 (Dopson *et al.*, 2004b). All strains were initially grown in shake flasks (100 ml) in mineral salts medium (MSM) containing trace elements (Dopson & Lindström, 1999), 20 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ l^{-1} and 0.02 % (w/v) yeast extract. The basal salts were adjusted to pH 1.2 with H_2SO_4 and autoclaved. Sterile yeast extract, trace elements, Fe^{2+} and inhibitors were added to the MSM as required. Growth experiments were inoculated with cells equivalent to 10 µg protein per 100 ml and measured as protein concentration (Bradford assay, Sigma) after 63 h incubation at 37 °C (Dopson *et al.*, 2004b). Unless stated, all experiments were carried out in triplicate and results presented as means ± SD. To test if growth results were significantly different, a Student's *t*-test was carried out.

Growth in continuous culture. For comparison of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) protein expression profiles, cells were grown to steady state in glass air-lift continuous-culture vessels (Dopson *et al.*, 2004b). All vessels were sparged with ≥ 800 ml air min^{-1} ; the dissolved oxygen concentration was measured for chemomixotrophic growth and found to be 96.1 % of the oxygen concentration in air at 8.6 mg protein l^{-1} . Continuous flow of the medium was as stated in Table 1 and the vessels were regularly sampled and protein concentration measured (as described above). Flow rates for the chemomixotrophic and chemo-organotrophic continuous cultures were based on maximum rates obtained for batch growth under the respective conditions [4.2 and 9.6 h for chemomixotrophic and chemo-organotrophic conditions, respectively (Dopson *et al.*, 2004b)].

Protein preparation and 2D-PAGE. 2D-PAGE was carried out according to Dopson *et al.* (2004a). Proteins were stained using EZBlue Coomassie brilliant blue (Sigma). When required, gels were further stained using silver nitrate (Blum *et al.*, 1987) or mass spectrometry compatible silver staining using the Plus-One silver staining kit (Amersham Biosciences) with the following amendments to the manufacturer's instructions: fixation was performed twice in 40 % ethanol/10 % acetic acid, glutaraldehyde was omitted from the sensitizing solution and formaldehyde was omitted from the silver nitrate solution. Gel images were analysed with ProteomWeaver version 1.3 (Definiens) and composite gels produced from replicates [number of replicates (*n*) = 2–4]. Protein spot matching, spot presence or absence, and statistics were determined using ProteomWeaver. This software normalizes protein spot expression data according to the entire protein complement expressed on the gels, and therefore intensities are adjusted on the basis of expression of every protein on replicate gels, alleviating the need for internal protein standards and adjusting for slight differences in protein development in individual gels. Proteins were only considered for excision and analysis if they were present in ≥ 50 % of the replicate gels for each condition.

Protein excision and mass spectrometry. A portion of the protein spots of interest were punched out of EZBlue or mass spectrometry compatible silver-stained gels with a sterile micropipette tip. Samples were treated by trypsin digestion and analysed using

Table 1. Continuous culture growth conditions and steady state protein concentrations

Data for incubation without added organic carbon were taken from Dopson *et al.* (2004b).

Strain	Mode of growth	<i>D</i> (h ⁻¹)	Culture volumes*	Steady-state concn (mg protein l ⁻¹)
Fer1	Chemomixotrophic†	0.020	53.3	7.5 ± 2.4 (<i>n</i> = 58)
	Chemo-organotrophic‡	0.008	5.3	5.2 ± 1.2 (<i>n</i> = 12)
	No added organic carbon§	0.002	3.2	0.4 ± 0.4 (<i>n</i> = 1)
MT17	Chemomixotrophic	0.015	17.3	9.1 ± 2.7 (<i>n</i> = 27)
	No added organic carbon	0.004	3.0	1.6 ± 1.3 (<i>n</i> = 9)
DR1	Chemomixotrophic	0.010	15.8	8.8 ± 2.3 (<i>n</i> = 27)
	No added organic carbon	0.003	3.5	2.1 ± 1.4 (<i>n</i> = 9)
Y ^T	Chemomixotrophic	0.015	15.8	4.8 ± 1.3 (<i>n</i> = 9)
	No added organic carbon	0.003	3.0	2.4 ± 0.7 (<i>n</i> = 8)

*Culture volume refers to the number of culture-vessel volumes of media passed through the culture-vessel for that mode of growth.

†20 g Fe²⁺ l⁻¹ + 0.02 % (w/v) yeast extract.

‡0.02 % (w/v) yeast extract.

§20 g Fe²⁺ l⁻¹.

||No steady state was observed.

matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Bruker Reflex III; Hesketh *et al.*, 2002). MALDI-TOF peptide mass fingerprint data were matched directly against the '*F. acidarmanus*' Fer1 genome sequence data (<http://genome.ornl.gov/microbial/faci/>) using MASCOT (<http://www.matrixscience.com>). Results were only included when peptide matching was statistically significant (MOWSE score greater than 75). Putative protein functions were inferred from the analyses contained in the annotated genome site (BLAST, KEGG hit, COGS Comparison, PFam model Comparison, and InterPro). Inferences regarding gene operons were drawn from STRING (<http://www.bork.embl-heidelberg.de/STRING/>).

Extraction and analysis of membrane proteins. '*F. acidarmanus*' Fer1 cells were harvested by centrifugation at 10 000 *g* for 20 min and washed twice in MSM pH 1.2. Pellets were resuspended in 15 ml 50 mM Tris pH 8 + 250 mM sucrose + 1 mM EDTA + Complete protease inhibitor cocktail (Roche). The cells were disrupted by passing twice through a French press at 1100 p.s.i. (7.6 MPa) and centrifuged at 5000 *g* for 10 min to remove whole cells and large debris. Membranes were pelleted by centrifugation at 120 000 *g* for 30 min. The resulting membrane pellet was resuspended in 500 µl 10 mM Tris + 1 mM EDTA (TE) + 2 % dodecyl maltoside and proteins were dissolved by rocking gently overnight at 4 °C. Undissolved proteins were removed by centrifugation at 120 000 *g* for 30 min and the remaining solubilized protein concentration was quantified using Bio-Rad protein assay reagent (as above). Protein was separated via one-dimensional polyacrylamide gel electrophoresis (1D-PAGE; Sambrook *et al.*, 1989) and stained with either Coomassie or haem stain (Thomas *et al.*, 1976). Differentially expressed protein bands in the 1D-PAGE gel were detected and quantified using Quantity One (Bio-Rad) and chosen bands excised and identified by mass spectrometry (as described above). UV-visible spectra of membrane proteins were obtained using a Hitachi U4001 UV-visible spectrophotometer and membrane fractions were analysed as prepared (oxidized) and after addition of sodium dithionite (reduced). Fe(CN)₆ (2 mM) was added to ensure that the membrane redox proteins were fully oxidized, whilst 2 and 1.5 mM dithionite for the chemomixotrophic and chemo-organotrophic membranes, respectively, was added to reduce the proteins.

Measurement of Fe²⁺ oxidation in the presence and absence of inhibitors. '*F. acidarmanus*' Fer1 cells were grown in continuous culture vessels (as described above) and harvested by centrifugation at 10 000 *g* for 10 min. The cells were washed in MSM and then Fe²⁺ oxidation was detected in the presence and absence of inhibitors in a thermostatted, stirred reaction chamber at 37 °C. The reaction mixture (1 ml total volume) contained MSM (without trace elements), inhibitors (as indicated), and cell mass equivalent to 10 µg protein. The cells were allowed to equilibrate at 37 °C for 3 min in the thermostatted chamber before the reaction was initiated by the addition of 50 µM Fe²⁺. Fer1 resting cells were stored ≤2 h prior to use. Samples (100 µl) were removed at the indicated time points, diluted in a Tris buffer (100 mM Tris, pH 7), and the Fe²⁺ concentration analysed utilizing ferrozine (Dawson & Lyle, 1990).

RESULTS

It was previously determined that all four *Ferroplasma* strains grow chemo-organotrophically on yeast extract and chemomixotrophically on yeast extract and Fe²⁺ (Dopson *et al.*, 2004b). Also, *F. acidiphilum* strains Y^T, MT17 and DR1 grow in the presence of only a very small concentration of organic carbon, defined as 'extreme oligotrophic conditions' (Dopson *et al.*, 2004b). To further characterize the growth of *Ferroplasma* strains, proteomic analysis of protein expression under the different growth conditions was analysed.

Growth of *Ferroplasma* strains in continuous culture

For the purpose of proteomic analyses, all four *Ferroplasma* strains were grown in continuous culture to steady state under substrate-limiting conditions (Table 1). Increases in yeast extract resulted in increased steady-state protein concentrations, e.g. with 0.02 and 0.04 % (w/v) yeast extract

the '*F. acidarmanus*' Fer1 steady-state protein concentration was 7.5 ± 2.4 and 16.5 ± 2.4 mg protein l^{-1} , respectively ($n=7$). The Fe^{2+} concentration inside a subsequent chemomixotrophic continuous-culture vessel incubated under the same conditions was found to be 0.5 ± 0.2 mM Fe^{2+} ($n=11$ over a 17 day period). '*F. acidarmanus*' Fer1 steady-state protein concentration under chemo-organotrophic conditions was lower than that observed for chemomixotrophic growth on Fe^{2+} and yeast extract, probably reflecting the extra energy the organism obtains while oxidizing Fe^{2+} as opposed to growing on organic carbon alone (Table 1). For protein expression comparisons, cells were harvested from the continuous-culture vessel during steady-state growth (except for incubations with no organic carbon addition), thus avoiding pitfalls in comparison of the vastly uneven growth conditions and states that can occur in batch culture.

Comparative protein expression of '*F. acidarmanus*' Fer1 grown under chemomixotrophic and chemo-organotrophic conditions

When '*F. acidarmanus*' Fer1 was grown under chemomixotrophic (Fig. 1a) and chemo-organotrophic (Fig. 1b) conditions, many of the proteins were common between the sets of 2D-PAGE separations. However, 52 % of the proteins were more than twofold differentially expressed when comparing the two conditions.

When '*F. acidarmanus*' Fer1 was grown under chemomixotrophic conditions, 10 proteins with high similarity to ribosomal subunits were up-regulated, possibly reflecting its increased growth rate (see Supplementary Table S1, available with the online version of this paper). The genes for the ribosomal proteins S19E and L31E, and L2, L4 and L22 are expressed as an operon in a number of acidophilic archaea, including *Sulfolobus solfataricus* and *Sulfolobus tokodaii*. It is possible that up-regulation of proteins with homology to chaperones HSP70 and DnaK is a response to increased protein production. Two electron transport proteins with similarity to ferredoxins were also up-regulated. Two subunits of a protein similar to alkyl hydroperoxide reductase, which is the primary scavenger for hydrogen peroxide

in *Escherichia coli* (Seaver & Imlay, 2001), were also up-regulated. During Fer1 chemomixotrophic growth, increased hydrogen peroxide may be caused by the presence of Fe^{2+} , or as a byproduct of a higher growth rate. Many proteins with homology to biosynthetic enzymes were up-regulated, including a protein involved in riboflavin, FMN and FAD biosynthesis, and four proteins involved in the biosynthesis of methionine possibly relating to increased protein production rates.

Under chemo-organotrophic conditions, '*F. acidarmanus*' Fer1 up-regulated 15 proteins with high homology to proteins having a suggested function in central metabolic pathways (see Supplementary Table S1). These included proteins similar to a transketolase that is a link between glycolysis and the pentose phosphate pathway; glyceraldehyde-3-phosphate dehydrogenase, which plays an important role in glycolysis and gluconeogenesis; a flavoprotein involved in central fermentation pathways; a further flavoprotein that has been implicated in the transfer of electrons from a dehydrogenase to a ubiquinone oxidoreductase; and glucose dehydrogenase. Four enzymes with similarity to those involved in the tricarboxylic acid (TCA) cycle were up-regulated; these include two subunits (expressed in a single operon in *Thermoplasma acidophilum* and *Thermoplasma volcanium*) of the pyruvate/2-oxoglutarate dehydrogenase complex that produces acetyl-CoA feeding into the TCA cycle (Patel & Roche, 1990), aconitate hydratase, succinyl-CoA synthetase β subunit, and succinate dehydrogenase. Proteins with homology to 3-hydroxyacyl-CoA dehydrogenase, glutamate/leucine dehydrogenase and acyl-CoA dehydrogenase involved in fatty acid metabolism (another central metabolic process) were also up-regulated. Other proteins up-regulated had homology to the thermosome β subunit and the 20S proteasome. In archaea these stress proteins protect intracellular proteins from aggregation as well as aiding protein translocation across the membrane (Klumpp & Baumeister, 1998). A protein similar to a member of the major facilitator permease family was also up-regulated. This probable sugar permease may be up-regulated to provide substrate for central metabolic pathways, in contrast to chemomixotrophic growth, where energy would be obtained from Fe^{2+} oxidation.

Fig. 1. Representative 2D-PAGE gels of whole-cell protein from chemomixotrophically [$20 \text{ g } Fe^{2+} \text{ l}^{-1} + 0.02 \%$ (w/v) yeast extract; a] and chemo-organotrophically grown '*F. acidarmanus*' Fer1 [0.02% (w/v) yeast extract; b]. Inset (c) shows a portion of Fer1 under chemomixotrophic conditions (left portion of panel) compared to chemo-organotrophic growth (right portion). Inset (d) is Fer1 chemo-organotrophic growth (left portion) compared to chemomixotrophic conditions (right portion; for (a)–(d) all spot numbers correspond to Supplementary Table S1). Panel (e) represents protein expression from chemomixotrophically grown *F. acidiphilum* Y^T (spot labels for e to g correspond to Supplementary Table S2) with spots chosen upon comparison with incubation in the absence of added organic carbon ($20 \text{ g } Fe^{2+} \text{ l}^{-1}$; 2D-PAGE gel not shown). Inset (f) shows a portion of Y^T protein expression under chemomixotrophic conditions (left) compared to incubation without addition of organic carbon (right). Inset (g) is a portion of Y^T incubated in the absence of added organic carbon (left) showing up-regulation of spots in comparison to chemomixotrophically grown cells (right). Finally, inset (h) shows spots (Supplementary Table S3) up-regulated in chemomixotrophically grown *F. acidiphilum* Y^T (left) compared to Fer1 (right). Apparent spot-intensity differences in the figure may not precisely match those listed in the tables (stimulation), as the latter were derived by comparison of composite images (not shown) prepared from replicate 2D-PAGE gels ($n=2-4$).

F. acidiphilum Y^T proteins expressed in the presence and absence of yeast extract

Differences of protein expression from *F. acidiphilum* Y^T were detected during continuous culture on Fe²⁺ in the presence and absence of organic carbon (Fig. 1e–g). While steady-state growth was not achieved in the absence of yeast extract addition, cells were harvested for protein extraction following continuous culture incubation for three reactor volumes (28 days). The proteome of these cells would be indicative of extremely oligotrophic conditions. During incubation of *F. acidiphilum* Y^T on Fe²⁺ in the absence of organic carbon, four proteins with homology to anti-oxidants and related proteins were up-regulated (see Supplementary Table S2). These were superoxide dismutase, which converts toxic superoxides to the less toxic hydrogen peroxide (Cannio *et al.*, 2000); two proteins similar to a peroxiredoxin subunit (an alkyl hydroperoxide reductase), which is the primary scavenger for hydrogen peroxide in *E. coli* (Seaver & Imlay, 2001); and finally, a protein with homology to superoxide-inducible cysteine synthase. Other proteins up-regulated during incubation of *F. acidiphilum* Y^T in the absence of organic carbon were similar to thermosome α and β subunits (chaperone HSP60 family group II) and a component of the H⁺ ATP synthase. Furthermore, two proteins with significant DNA sequence homology to methionine synthase II were expressed. This appears to be a gene duplication event, and although the sequences had different percentage identities, the same gene arrangement is apparent in the genomes of *Thermoplasma* spp. and *Pyrococcus* spp.

A *F. acidiphilum* Y^T protein with significant gene sequence homology to an anti-oxidant hydroperoxide reductase (Supplementary Table S2) was up-regulated during chemomixotrophic growth compared to incubation without added organic carbon. Three proteins with homology to transcription and translation components were up-regulated; these were a ribosomal protein L7A, an eIF-2B initiation factor, and a DNA primase, possibly reflecting the increased growth rate. In addition, proteins similar to a number of electron transport and metabolic proteins possibly involved in growth on organic substrates were up-regulated. Among others, these included pyruvate/ferredoxin oxidoreductase, which forms acetyl-CoA, and an uncharacterized NAD-(FAD)-dependent dehydrogenase, which possibly feeds electrons into the electron transport chain.

Comparative protein expression of chemomixotrophic growth between *F. acidiphilum* Y^T and '*F. acidarmanus*' Fer1

To investigate differences between *Ferroplasma* spp., protein expression profiles during chemomixotrophic growth were compared (Fig. 1a, e, h). Of the proteins detected, 21 and 31 % were uniquely expressed by '*F. acidarmanus*' Fer1 and *F. acidiphilum* Y^T, respectively (data not shown). Many of the proteins up-regulated in *F. acidiphilum* Y^T had high homology to proteins involved in central energy production

from organic carbon (see Supplementary Table S3). These included malate dehydrogenase in the TCA cycle, fructose 1,6-bisphosphatase and phosphoglycerate mutase involved in gluconeogenesis and both subunits of an aerobic carbon monoxide dehydrogenase involved in acetyl-CoA metabolism. Finally, proteins with high similarity to electron transfer proteins that may be involved in energy conservation were also up-regulated (contig 166, gene 1240; contig 166, gene 1220; and contig 168, gene 1522; see Supplementary Table S3).

Identification of respiratory complexes

Spectrophotometric scans of chemomixotrophically and chemo-organotrophically grown '*F. acidarmanus*' Fer1 membranes were successfully obtained with high signal-to-noise ratio (Fig. 2). Importantly, the membranes from the two growth conditions have quite distinct spectral characteristics, indicating differential expression of redox proteins. Each of the spectra has a number of different features that suggest contributions from a range of different protein-bound cofactors, including haems and iron–sulfur clusters. From the chemomixotrophically grown cells the broad oxidized peak from approximately 415 to 455 nm is due to the Soret band and may be made up of a combination of cytochromes *b* and *c* from a terminal oxidase (Brasseur *et al.*, 2004; Komorowski *et al.*, 2002). The shift of the 423 nm peak to 437 nm is indicative of the reduction of a partially oxidized haem protein (423 nm) to the reduced

Fig. 2. Spectrophotometric analysis of air-oxidized (solid line) and dithionite-reduced (dotted line) chemomixotrophic (a) and chemo-organotrophic (b) grown '*F. acidarmanus*' Fer1 membranes. Insets are dithionite-reduced minus air-oxidized difference spectra (the scale bar gives a difference in optical density of 0.1).

state (437 nm). Characteristics of the observed spectra reflect differential protein expression. For example, the broad absorbance at 460–510 nm is typical of Fe–S centres and this correlates with proteins identified as up-regulated in chemomixotrophic growth (contig 168, gene 1454 and contig 157, gene 545). The peak at 583 nm suggests the presence of cytochrome a_{583} found in *Sulfolobus solfataricus* strain 7 that has been tentatively suggested to have a cytochrome c -like function in terminal oxidases (Schafer *et al.*, 1996). The peak at 596 nm is possibly a blue copper-haem sulfocyanin, although it has been shown that the absorbance associated with a purified, truncated sulfocyanin is lost upon reduction (Komorowski & Schafer, 2001). Such a protein may have an important role mediating Fe^{2+} oxidation similar to rusticyanin (as in *A. ferrooxidans*) or SoxE (as in *Sulfolobus acidocaldarius*), replacing the function of cytochrome c (Komorowski & Schafer, 2001). An alternative explanation is that the peak is due to a cytochrome aa_3 oxidase (peak at 597 nm) that has been identified in *A. ferrooxidans* when grown on Fe^{2+} (Brasseur *et al.*, 2004), although no genes encoding a cytochrome aa_3 oxidase have been identified on the 97% complete Fer1 genome. In addition, a protein containing c -type haem was detected in '*F. acidarmanus*' Fer1 grown under chemomixotrophic, but not chemo-organotrophic conditions (by haem-stained 1D-PAGE gel; Fig. 3).

A different pattern is observed in the chemo-organotrophic spectra, with a peak at 385 nm indicating the presence of flavoproteins that feed electrons from central metabolic pathways into the electron transport chain. Possibly the flavoprotein peak correlates with the up-regulation of two flavoproteins (contig 157, gene 557 and contig 166, gene 1219) identified by our proteomic analysis. The broad peak in the reduced minus oxidized spectrum at 425 nm with a shoulder at 428 nm is probably due to a combination of cytochromes b and c from a terminal oxidase as seen in the chemomixotrophic-grown membranes, but the ratio between the oxidized and reduced forms is different between the two growth states. A lack of peak shift between the oxidized and reduced spectra suggests that this putative cytochrome has a high midpoint potential. Absence of

absorption at 418 nm in the Soret band indicates a lack of a cytochrome c , supporting the haem-stain data. Other notable observations include the small peaks at 531 and 562 nm typical of cytochrome b haems (Komorowski *et al.*, 2002), and the peak for the putative blue copper-haem sulfocyanin or cytochrome aa_3 oxidase is absent (or much reduced) in the spectra of chemo-organotrophic-grown '*F. acidarmanus*' Fer1.

'*F. acidarmanus*' Fer1 growth and ferrous iron oxidation in the presence of electron transport inhibitors

'*F. acidarmanus*' Fer1 was grown in the presence of electron transport inhibitors (Table 2) and their effect on Fe^{2+} oxidation in '*F. acidarmanus*' Fer1 resting cells was measured (Fig. 4). Rotenone (a competitive inhibitor of NADH-ubiquinone reductase) did not have a statistically significant effect on chemomixotrophically grown '*F. acidarmanus*' Fer1, whereas it did inhibit chemo-organotrophic growth (Table 2). Quinacrine (a competitive inhibitor of succinate dehydrogenase) inhibited 51% of chemomixotrophic growth, but had no statistically significant effect on chemo-organotrophic growth (Table 2). Antimycin A non-competitively reduces electron flow through the cytochrome b and iron-sulfur protein in a bc_1 complex or its analogue, whilst 2-heptyl-4-hydroxyquinoline N -oxide (HQNO) inhibits most quinol oxidases and reductases. The point of cytochrome b inhibition by HQNO is at the site of ubiquinone reduction. Both antimycin A and HQNO strongly reduced chemomixotrophic and chemo-organotrophic growth (Table 2), despite no cytochrome bc_1 complex genes having been identified on the genome (Tyson *et al.*, 2004). It is possible that a cytochrome b and a Fe–S protein, detected in the membrane spectra, may act as a bc_1 complex [similar to *S. acidocaldarius* (Komorowski *et al.*,

Fig. 3. Coomassie-stained (A, B) and haem-stained (C, D) 1D-PAGE gel of membrane preparations from chemomixotrophic- (A, C) and chemo-organotrophic- (B, D) grown '*F. acidarmanus*' Fer1. Molecular mass markers are given in kDa.

Table 2. Growth of '*F. acidarmanus*' Fer1 in the absence and presence of electron transport chain inhibitors

Inhibitor	Chemomixotrophic growth (mg protein l ⁻¹)*	Chemo-organotrophic growth (mg protein l ⁻¹)†
None	4.1 ± 0.5‡	1.1 ± 0.1
Rotenone (40 µM)	3.7 ± 1.2	0.6 ± 0.3
Quinacrine (1 mM)	2.0 ± 0.9	2.2 ± 1.3
Antimycin A (0.5 µM)	0.0 ± 0.1	0.6 ± 0.1
HQNO (40 µM)	0.3 ± 0.8	0.7 ± 0.2
Azide (1 mM)	0.1 ± 0.6	0.5 ± 0.7

*20 g Fe^{2+} l⁻¹ + 0.02% (w/v) yeast extract.

†0.02% (w/v) yeast extract.

‡Growth was measured as protein concentration after 63 h at 37 °C. Values are means minus the no substrate control ± SD calculated as the square root of the sum of the squares of the no substrate control and test (both $n=3$).

Fig. 4. Oxidation of Fe^{2+} by '*F. acidarmanus*' Fer1 in the absence (■) and presence of 2 (●), 20 (▲) and 100 μM (▼) HQNO (a), 2 (●), 5 (▲) and 10 (▼) μM antimycin A (b), and 1 (●), 10 (▲) and 100 μM (▼) azide (c). Values are means of three replicate samples $\pm$ SD.

2002)] and inhibition of electron flow in this portion of the complex may result in the lowered growth. Although both antimycin A and HQNO inhibited growth, they do not significantly affect Fe^{2+} oxidation by '*F. acidarmanus*' Fer1 resting cells, even at high concentrations (Fig. 4a, b). This suggests that electrons from the oxidation of Fe^{2+} bypass a bc_1 complex or its analogue. In contrast, azide (a terminal oxidase inhibitor) completely inhibited Fe^{2+} oxidation at 100 μM , and only allowed 40 % of the available Fe^{2+} to be oxidized after 20 min at 1 μM (Fig. 4c). The degree of inhibition by azide on chemomixotrophic and chemo-organotrophic growth was not statistically different.

DISCUSSION

Oxidation of Fe^{2+} by acidophiles is a very important biochemical process as Fe^{3+} serves as the primary oxidant in the dissolution of sulfide minerals in AMD and bioleaching. It has been proposed that *Ferroplasma* Type II mediates Fe^{2+} oxidation via a terminal oxidase supercomplex similar to SoxM in *S. acidocaldarius* (Tyson *et al.*, 2004). However, this hypothesis is not wholly supported by our analyses of protein expression profiles and membrane spectra data. It is

unlikely that a complete SoxM supercomplex is utilized in '*F. acidarmanus*' Fer1 or *Ferroplasma* Type II, as gene homologues for components of the supercomplex are missing from the completed genome sequence of *Ferroplasma* Type II and the 97 % complete Fer1 genome. Also, genes encoding an alternative oxidase have been detected on the '*F. acidarmanus*' Fer1 genome and our membrane spectra data lack some expected absorbance maxima for the presence of a SoxM supercomplex (e.g. a ba_3 -type haem copper SoxM terminal oxidase would absorb at 605 nm).

To facilitate discussion of our proteomic, genomic, membrane spectra, and inhibition studies, a very speculative model for electron transport in *Ferroplasma* spp. is provided (Fig. 5), with the suggested points of inhibition for each of the electron transport inhibitors given. It is possible that Fe^{2+} oxidation is mediated by a blue copper-haem sulfo-cyanin that is suggested to have a function similar to *S. acidocaldarius* SoxE and *A. ferrooxidans* rusticyanin (Tyson *et al.*, 2004). Following that, we suggest that transfer of the electron from Fe^{2+} oxidation to the cbb_3 terminal oxidase occurs, without passing higher up the electron transport chain (Fig. 5). This hypothesis is supported by the membrane scans, which indicated that a blue copper-haem

Fig. 5. A speculative model for electron transport for chemo-organotrophic and chemo-mixotrophic growth by '*F. acidarmanus*' Fer1 (updated from Tyson *et al.*, 2004). Proteins identified by up-regulation, visible spectra or inhibition have been underlined, and points of electron transport inhibition are given by curvilinear arrows. NDH-I, NADH ubiquinone oxidoreductase; SDH, succinate dehydrogenase.

sulfocyanin at 596 nm was present during chemomixotrophic growth but greatly reduced or absent during chemo-organotrophic growth. Our suggested model is also supported by the biochemical data, whereby HQNO and antimycin A did not inhibit Fe^{2+} oxidation, whereas the terminal electron acceptor inhibitor azide inhibited virtually 100 % of this activity at 10 μM . Only a few redox proteins were identified from 2D-PAGE to support this hypothesis. It is possible that Fe^{2+} -oxidizing proteins are constitutively expressed, although detection of redox proteins from membrane scans suggests that some regulation is occurring. More likely, the lack of proteomic data is due to the accepted difficulty of resolving membrane proteins on 2D-PAGE gels (Smith, 2000).

Placement of some components in the electron transport chain model (Fig. 5) is highly speculative. Evidence for attachment between the quinol and terminal oxidase elements is based on inhibition of anaerobic growth and Fe^{3+} reduction by '*F. acidarmanus*' Fer1 resting cells by the terminal oxidase inhibitor azide (M. Dopson and others, unpublished). The proposed pattern of cytochrome usage contrasts with Fe^{2+} oxidation in *A. ferrooxidans*, which involves the passage of electrons from Fe^{2+} via an outer-membrane cytochrome *c* (Cyc2) to rusticyanin, then a membrane-bound cytochrome *c*₄, and finally the terminal aa₃ oxidase (Appia-Ayme *et al.*, 1999; Brasseur *et al.*, 2004; Yarzabal *et al.*, 2002). Inhibition of chemo-organotrophic growth by the complex I respiratory chain inhibitor suggests that under these conditions the majority of the electrons pass through the NADH-ubiquinone reductase, rather than the succinate dehydrogenase, which was much less affected by quinacrine. The electrons then probably pass through the quinol-oxidizing element and into the terminal oxidase. This passage of electrons is supported by inhibition by HQNO, antimycin A and azide.

A key issue remaining for characterization of the genus *Ferroplasma* is the question of chemolithoautotrophic growth. Previously we maintained a viable culture of *F. acidiphilum* in a continuous-culture reactor for more than three culture volumes in the absence of organic carbon, but the protein level was very close to the concentration calculated from the theoretical washout rate (Dopson *et al.*, 2004b). However, '*F. acidarmanus*' Fer1 and *Ferroplasma* Type II contain candidate genes for CO_2 fixation via the reductive acetyl-CoA pathway (Tyson *et al.*, 2004). The pathway works by reduction of CO_2 to form acetyl-CoA and then pyruvate and is found in anaerobic prokaryotes. A key enzyme in this pathway is a Ni-Fe carbon monoxide dehydrogenase, which is unlikely to be functional in the aerobic iron-oxidizing conditions of *Ferroplasma* spp. In contrast, we have demonstrated the expression of proteins with similarity to two subunits (CutM and CutL) of an aerobic Mo-Fe-flavin carbon monoxide dehydrogenase. One possibility is that this dehydrogenase may be utilized to fix CO_2 via the reductive pentose phosphate pathway (Ferry, 1995). However, this also seems unlikely, as key genes for this

process have not been identified in *Ferroplasma* spp. and we found these proteins to be up-regulated in chemomixotrophic growth compared to incubation in the absence of added organic carbon (Supplementary Table S2). Tyson *et al.* (2004) suggested that due to the large numbers of sugar and amino acid transporters identified, *Ferroplasma* Type II may predominantly grow chemo-organotrophically, and *F. acidiphilum* MT17 has been described as an obligate chemo-organotroph (Okibe & Johnson, 2004). Nothing in our results contradicts these hypotheses that *Ferroplasma* spp. are chemo-organotrophs. We also find no evidence indicating that these strains can fix CO_2 , although lack of evidence does not preclude that it occurs.

During incubation in the absence of organic carbon, a complete pathway of anti-oxidant proteins was expressed in *F. acidiphilum* Y^T, possibly due to a general lack of intracellular reducing power and to oxidative damage caused by interaction of Fe^{2+} with superoxide and hydrogen peroxide to form highly toxic hydroxyl radicals (Andrews *et al.*, 2003). During chemo-organotrophic growth, enzymes associated with central metabolic pathways were up-regulated. These include components of gluconeogenesis, glycolysis, the TCA cycle, and fatty acid metabolism. None of these proteins were up-regulated during any of the other comparisons between growth states. Along with the probable sugar transporter identified during chemo-organotrophic growth, this was almost certainly due to the increased energy requirement from organic carbon during growth in the absence of Fe^{2+} . The identification of flavoproteins in the chemo-organotrophic membrane spectra could be one of the up-regulated proteins identified from the proteomic analysis, for example succinate dehydrogenase and an electron transfer flavoprotein (Supplementary Table S1).

Differences in the protein profile between chemomixotrophically grown *F. acidiphilum* Y^T and '*F. acidarmanus*' Fer1 revealed that a number of central metabolic proteins were up-regulated in *F. acidiphilum* Y^T. These proteins were similar to those up-regulated during chemo-organotrophic growth of '*F. acidarmanus*' Fer1 compared to chemomixotrophic growth. For instance, components of the TCA cycle, gluconeogenesis, and electron transfer proteins, which feed electrons into the electron transport chain at a more negative redox potential than Fe^{2+} oxidation, were up-regulated under both conditions. These results may indicate a more versatile physiology of *F. acidiphilum* Y^T compared to '*F. acidarmanus*' Fer1 in response to changes in organic carbon in its environment.

This study is believed to constitute the first in-depth proteomic analysis of growth states in acidophilic archaea. This study also addresses key biochemical and bioenergetic issues from the characterization of the genus. The results do not support the utilization of any known chemolithoautotrophic CO_2 fixation pathway in *Ferroplasma* spp., suggesting that these organisms are chemo-organotrophs. Also, membrane scans and inhibitor experiments suggest that Fe^{2+} oxidation and organization of the redox components

are different from the SoxM terminal oxidase supercomplex from *S. acidocaldarius* (Fig. 5). Protein expression during chemo-organotrophic growth suggests that many of the central metabolic pathways are up-regulated to compensate for the lack of energy derived from Fe^{2+} oxidation. Finally, key intermediates in central metabolic pathways are also up-regulated in chemomixotrophically grown *F. acidiphilum* Y^T compared to '*F. acidarmanus*' Fer1.

ACKNOWLEDGEMENTS

We would like to acknowledge Drs J. F. Banfield and K. J. Edwards for provision of '*F. acidarmanus*' Fer1 and helpful discussions. Strains MT17 and DR1 were kindly supplied by Dr D. B. Johnson and Dr D. E. Rawlings, respectively. Dr F. Mulholland kindly provided proteomic scanning equipment and many helpful suggestions. We would also like to thank Dr D. J. Richardson for assistance in interpreting redox spectra and critical comments on the manuscript. Trypsin digests and MALDI-TOF analysis was carried out at the Joint Proteomics Facility (Institute of Food Research/John Innes Centre, Norwich). We would also like to thank the technical assistance of Martina Maidment, Lynda Flegg, and Ann Reilly. This work was funded by a BBSRC Research grant.

REFERENCES

- Alexander, B., Leach, S. & Ingledew, W. J. (1987). The relationship between chemiosmotic parameters and sensitivity to anions and organic acids in the acidophile *Thiobacillus ferrooxidans*. *J Gen Microbiol* **133**, 1171–1179.
- Andrews, S. C., Robinson, A. K. & Rodriguez-Quinones, F. (2003). Bacterial iron homeostasis. *FEMS Microbiol Rev* **27**, 215–237.
- Appia-Ayme, C., Guilian, N., Ratouchniak, J. & Bonnefoy, V. (1999). Characterization of an operon encoding two *c*-type cytochromes, an *aa₃*-type cytochrome oxidase, and rusticyanin in *Thiobacillus ferrooxidans* ATCC 33020. *Appl Environ Microbiol* **65**, 4781–4787.
- Barr, D. W., Ingledew, W. J. & Norris, P. R. (1990). Respiratory-chain components of iron-oxidizing, acidophilic bacteria. *FEMS Microbiol Lett* **70**, 85–89.
- Baumler, D. J., Jeong, K.-W., Fox, B. G., Banfield, J. F. & Kaspar, C. W. (2005). Sulfate requirement for heterotrophic growth of '*Ferroplasma acidarmanus*' strain fer1. *Res Microbiol* **156**, 492–498.
- Blake, R. C., Shute, E. A., Greenwood, M. M., Spencer, G. H. & Ingledew, W. J. (1993). Enzymes of aerobic respiration on iron. *FEMS Microbiol Rev* **11**, 9–18.
- Blum, H., Beier, H. & Gross, H. J. (1987). Improved silver staining of plant-proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**, 93–99.
- Brasseur, G., Levican, G., Bonnefoy, V., Holmes, D., Jedlicki, E. & Lemesle-Meunier, D. (2004). Apparent redundancy of electron transfer pathways via *bc₁* complexes and terminal oxidases in the extremophilic chemolithoautotrophic *Acidithiobacillus ferrooxidans*. *Biochim Biophys Acta* **1656**, 114–126.
- Cannio, R., Fiorentino, G., Morana, A., Rossi, M. & Bartolucci, S. (2000). Oxygen: friend or foe? Archaeal superoxide dismutases in the protection of intra- and extracellular oxidative stress. *Front Biosci* **5**, 768–779.
- Dawson, M. V. & Lyle, S. J. (1990). Spectrophotometric determination of iron and cobalt with ferrozine and dithizone. *Talanta* **37**, 1189–1191.
- Dopson, M. & Lindström, E. B. (1999). Potential role of *Thiobacillus caldus* in arsenopyrite bioleaching. *Appl Environ Microbiol* **65**, 36–40.
- Dopson, M., Baker-Austin, C., Koppineedi, P. R. & Bond, P. L. (2003). Growth in sulfidic mineral environments: metal resistance mechanisms in acidophilic micro-organisms. *Microbiology* **149**, 1959–1970.
- Dopson, M., Baker-Austin, C. & Bond, P. L. (2004a). First use of 2-dimensional polyacrylamide gel electrophoresis to determine phylogenetic relationships. *J Microbiol Methods* **58**, 297–302.
- Dopson, M., Baker-Austin, C., Hind, A., Bowman, J. P. & Bond, P. L. (2004b). Characterization of *Ferroplasma* isolates and *Ferroplasma acidarmanus* sp. nov., extreme acidophiles from acid mine drainage and industrial bioleaching environments. *Appl Environ Microbiol* **70**, 2079–2088.
- Edwards, K. J., Bond, P. L., Gihring, T. M. & Banfield, J. F. (2000). An archaeal iron-oxidizing extreme acidophile important in acid mine drainage. *Science* **287**, 1796–1799.
- Elbehti, A., Nitschke, W., Tron, P., Michel, C. & Lemesle-Meunier, D. (1999). Redox components of cytochrome *bc*-type enzymes in acidophilic prokaryotes. I. Characterization of the cytochrome *bc₁*-type complex of the acidophilic ferrous ion-oxidizing bacterium *Thiobacillus ferrooxidans*. *J Biol Chem* **274**, 16760–16765.
- Ferry, J. G. (1995). CO dehydrogenase. *Annu Rev Microbiol* **49**, 305–333.
- Golyshina, O. V. & Timmis, K. N. (2005). *Ferroplasma* and relatives, recently discovered cell wall-lacking archaea making a living in extremely acid, heavy metal-rich environments. *Environ Microbiol* **7**, 1277–1288.
- Golyshina, O. V., Pivovarova, T. A., Karavaiko, G. I. & 7 other authors (2000). *Ferroplasma acidiphilum* gen. nov., sp nov., an acidophilic, autotrophic, ferrous-iron-oxidizing, cell-wall-lacking, mesophilic member of the *Ferroplasmaceae* fam. nov., comprising a distinct lineage of the Archaea. *Int J Syst Evol Microbiol* **50**, 997–1006.
- Gonzalez-Toril, E., Llobet-Brossa, E., Casamayor, E. O., Amann, R. & Amils, R. (2003). Microbial ecology of an extreme acidic environment, the Tinto River. *Appl Environ Microbiol* **69**, 4853–4865.
- Hallberg, K. B. & Johnson, D. B. (2001). Biodiversity of acidophilic prokaryotes. *Adv Appl Microbiol* **49**, 37–84.
- Hart, A., Murrell, J. C., Poole, R. K. & Norris, P. R. (1991). An acid-stable cytochrome in iron-oxidizing *Leptospirillum-ferrooxidans*. *FEMS Microbiol Lett* **81**, 89–94.
- Hawkes, R. B., Franzman, P. D. & Plumb, J. J. (2005). Microbiology of an industrial-scale chalcocite heap bioleaching operation. In *Bac-Min Conference*, pp. 11–17. Bendigo, Victoria, Australia: AusIMM.
- Hesketh, A., Fink, D., Gust, B., Rexer, H. U., Scheel, B., Chater, K., Wohlleben, W. & Engels, A. (2002). The GlnD and GlnK homologues of *Streptomyces coelicolor* A3(2) are functionally dissimilar to their nitrogen regulatory system counterparts from enteric bacteria. *Mol Microbiol* **46**, 319–330.
- Kinnunen, P. H. M. & Puhakka, J. A. (2004). High-rate ferric sulfate generation by a *Leptospirillum ferrophilum*-dominated biofilm and the role of jarosite in biomass retainment in a fluidized-bed reactor. *Biotechnol Bioeng* **85**, 697–705.
- Klumpp, M. & Baumeister, W. (1998). The thermosome: archetype of group II chaperonins. *FEBS Lett* **430**, 73–77.
- Komorowski, L. & Schafer, G. (2001). Sulfocyanin and subunit II, two copper proteins with novel features, provide new insight into the archaeal SoxM oxidase supercomplex. *FEBS Lett* **487**, 351–355.
- Komorowski, L., Verheyen, W. & Schafer, G. (2002). The archaeal respiratory supercomplex SoxM from *S. acidocaldarius* combines features of quinone and cytochrome *c* oxidases. *Biol Chem* **383**, 1791–1799.

- Nordstrom, D. K. & Alpers, C. N. (1999).** Negative pH, efflorescent mineralogy, and consequences for environmental restoration at the iron mountain superfund site, California. *Proc Natl Acad Sci U S A* **96**, 3455–3462.
- Okibe, N. & Johnson, D. B. (2004).** Biooxidation of pyrite by defined mixed cultures of moderately thermophilic acidophiles in pH-controlled bioreactors: significance of microbial interactions. *Biotechnol Bioeng* **87**, 574–583.
- Okibe, N., Gericke, M., Hallberg, K. B. & Johnson, D. B. (2003).** Enumeration and characterization of acidophilic microorganisms isolated from a pilot plant stirred-tank bioleaching operation. *Appl Environ Microbiol* **69**, 1936–1943.
- Patel, M. S. & Roche, T. E. (1990).** Molecular biology and biochemistry of pyruvate dehydrogenase complexes. *FASEB J* **4**, 3224–3233.
- Pivovarova, T. A., Kondrat'eva, T. F., Batrakov, S. G., Esipov, S. E., Sheichenko, V. I., Bykova, S. A., Lysenko, A. M. & Karavaiko, G. I. (2002).** Phenotypic features of *Ferroplasma acidiphilum* strains Y-T and Y-2. *Microbiology* **71**, 698–706.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor.
- Schafer, G., Purschke, W. & Schmidt, C. L. (1996).** On the origin of respiration: electron transport proteins from archaea to man. *FEMS Microbiol Rev* **18**, 173–188.
- Seaver, L. C. & Imlay, J. A. (2001).** Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. *J Bacteriol* **183**, 7173–7181.
- Singer, P. C. & Stumm, W. (1970).** Acid mine drainage: the rate-determining step. *Science* **167**, 1121–1123.
- Smith, R. D. (2000).** Probing proteomes – seeing the whole picture? *Nat Biotechnol* **18**, 1041–1042.
- Thomas, P. E., Ryan, D. & Levin, W. (1976).** An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. *Anal Biochem* **75**, 168–176.
- Tyson, G. W., Chapman, J., Hugenholtz, P. & 7 other authors (2004).** Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* **428**, 37–43.
- Yarzabal, A., Brasseur, G., Ratouchniak, J., Lund, K., Lemesle-Meunier, D., DeMoss, J. A. & Bonnefoy, V. (2002).** The high-molecular-weight cytochrome *c* *cyc2* of *Acidithiobacillus ferrooxidans* is an outer membrane protein. *J Bacteriol* **184**, 313–317.
- Yarzabal, A., Duquesne, K. & Bonnefoy, V. (2003).** Rusticyanin gene expression of *Acidithiobacillus ferrooxidans* ATCC 33020 in sulfur- and in ferrous iron media. *Hydrometallurgy* **71**, 107–114.
- Yarzabal, A., Appia-Ayme, C., Ratouchniak, J. & Bonnefoy, V. (2004).** Regulation of the expression of the *Acidithiobacillus ferrooxidans* *rus* operon encoding two cytochromes *c₁* a cytochrome oxidase and rusticyanin. *Microbiology* **150**, 2113–2123.