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ORIGINAL ARTICLE

Cytochrome 572 is a conspicuous membrane protein with iron oxidation activity purified directly from a natural acidophilic microbial community

Chris Jeans[1], Steven W Singer[1], Clara S Chan[2], Nathan C VerBerkmoes[3], Manesh Shah[4], Robert L Hettich[3], Jillian F Banfield[2] and Michael P Thelen[1]

[1]*Chemistry Directorate, Lawrence Livermore National Laboratory, Livermore, CA, USA;* [2]*Department of Earth and Planetary Sciences, University of California, Berkeley, CA, USA;* [3]*Chemical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN, USA and* [4]*Life Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN, USA*

Recently, there has been intense interest in the role of electron transfer by microbial communities in biogeochemical systems. We examined the process of iron oxidation by microbial biofilms in one of the most extreme environments on earth, where the inhabited water is pH 0.5–1.2 and laden with toxic metals. To approach the mechanism of Fe(II) oxidation as a means of cellular energy acquisition, we isolated proteins from natural samples and found a conspicuous and novel cytochrome, Cyt_{572}, which is unlike any known cytochrome. Both the character of its covalently bound prosthetic heme group and protein sequence are unusual. Extraction of proteins directly from environmental biofilm samples followed by membrane fractionation, detergent solubilization and gel filtration chromatography resulted in the purification of an abundant yellow-red protein. The purified protein has a cytochrome *c*-type heme binding motif, CxxCH, but a unique spectral signature at 572 nm, and thus is called Cyt_{572}. It readily oxidizes Fe^{2+} in the physiologically relevant acidic regime, from pH 0.95–3.4. Other physical characteristics are indicative of a membrane-bound multimeric protein. Circular dichroism spectroscopy indicates that the protein is largely beta-stranded, and 2D Blue-Native polyacrylamide gel electrophoresis and chemical crosslinking independently point to a multi-subunit structure for Cyt_{572}. By analyzing environmental genomic information from biofilms in several distinctly different mine locations, we found multiple genetic variants of Cyt_{572}. MS proteomics of extracts from these biofilms substantiated the prevalence of these variants in the ecosystem. Due to its abundance, cellular location and Fe^{2+} oxidation activity at very low pH, we propose that Cyt_{572} provides a critical function for fitness within the ecological niche of these acidophilic microbial communities.

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Introduction

Acid mine drainage (AMD) is a global environmental problem that occurs when metal sulfide ore deposits, dominated by FeS_2 (pyrite) and containing a range of other metal sulfide phases, are exposed to air and water (Baker and Banfield, 2003). Pyrite undergoes oxidative dissolution through several reactions, depicted in Equation (1), forming solutions of low pH that generate high levels of toxic metals.

$$FeS_2 + 3.5\,O_2 + H_2O \rightarrow Fe^{2+} + 2\,SO_4^{2-} + 2\,H^+ \quad (1)$$

Experiments have shown that microbial ferrous iron [Fe(II)] oxidation can accelerate Equation (1) over the inorganic rate by up to 10^6 (Singer and Stumm, 1970). Based on such experiments, it has been inferred that the microbial catalyzed reaction is the rate-limiting step for AMD generation. Consequently, the study of enzymatic mechanisms of microbial iron oxidation is central to understanding the biology and environmental impact of AMD (Druschel *et al.*, 2004).

Correspondence: MP Thelen, Chemistry Directorate, Lawrence Livermore National Laboratory, L-452, PO Box 808, Livermore, CA 94550, USA.
E-mail: mthelen@llnl.gov
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Previous approaches to understanding biological iron oxidation under acidophilic conditions have concentrated on isolating organisms from acidic, metal rich environments and culturing them in the laboratory to obtain proteins that oxidize Fe(II) (Blake *et al.*, 1993). An alternative strategy is to isolate proteins directly from natural microbial consortia and interrogate their functions by biochemical techniques (Kruger *et al.*, 2003). This enables the identification of important proteins in their natural environment produced by organisms that are difficult to culture under laboratory conditions. For this reason, we have chosen to study low-diversity microbial communities that establish floating biofilms in extremely acidic water (pH 0.5–1.0) at high temperatures (30–50 °C) in the Richmond Mine at Iron Mountain, California (Bond *et al.*, 2000). The mine water in this environment contains high levels of metals, including 0.2–0.4 M Fe(II) and millimolar levels of As, Zn and Cu. Genomic analyses of a biofilm isolated from the Richmond Mine at two different sites (Supplementary Figure S1, for map) reveals that *Leptospirillum* group II (*Lepto*II) dominates these communities, with *Leptospirillum* group III (*Lepto*III) and several archaeal members present in lower abundance (Tyson *et al.*, 2004; Lo *et al.*, 2007).

Proteomic mass spectrometry (MS) analyses of a related biofilm collected at the AB end site identified 2033 proteins, of which >500 were expressed hypothetical proteins (Ram *et al.*, 2005); a subset of these were among the most abundant proteins. We anticipate that many of the proteins of unknown function are central to processes that are essential for survival in the AMD habitat, including the oxidation of Fe(II) to drive cellular metabolism. Outer membrane-bound *c*-type cytochromes have been frequently implicated as electron transfer proteins that interact directly with metals in the environment (Newman and Banfield, 2002; Marshall *et al.*, 2006; Weber *et al.*, 2006). For acidophilic Fe(II) oxidation by *Acidithiobacillus ferrooxidans*, a bacterium often observed in less acidic (pH 2–3) AMD environments, biochemical and gene expression analyses have implicated an outer-membrane *c*-type cytochrome (Cyc2) as the iron oxidase (Yarzabal *et al.*, 2002, 2004).

Here, we report the identification and purification of a conspicuous cytochrome belonging to *Lepto*II from biofilms obtained in the Richmond Mine. Biochemical studies of this protein indicate that it is membrane bound, contains a unique heme group, is not homologous to known *c*-type cytochromes and oxidizes iron at low pH, a possible link between the microbial community and the generation of AMD. This work illustrates that a classical biochemical approach combined with new proteomics and genomics methods can be used to identify proteins of interest from a natural, heterogeneous microbial community.

Materials and methods

Sample collection

Biofilm samples were collected from the C-drift region (AMD dam, March 2005, and 15 m beyond the AMD dam into the C-drift, November 2005; Supplementary Figure S1) of Richmond Mine near Redding CA, USA (Ram *et al.*, 2005). Purified Cyt_{572} from both samples had identical spectral and redox properties over a range of pH from 0.95 to 5.0. Samples were frozen in 50 ml aliquots in dry ice at the site, and later moved to −80 °C for storage.

Protein purification

A sample of frozen biofilm (50 ml) was slowly thawed and mine water removed by centrifugation at 5000 *g* at 4 °C for 10 min. Biofilm was resuspended in 4 volumes of H_2SO_4 (pH 1.1) using a glass Dounce homogenizer. Cells were collected at 12 000 *g* at 4 °C for 10 min, and similarly resuspended in 50 mM MES-NaOH, pH 5 (MES (2-(*N*-morpholino) ethanesulfonic acid) buffer), to a volume of 50 ml. Cells were kept on ice and broken by sonication (Misonix, Farmingdale, NY, USA; 50% intensity, 20 cycles of 30 s on, 1 min off). After centrifugation at 12 000 *g* at 4 °C for 10 min, membranes in the opaque yellow supernatant were sedimented by centrifugation at 100 000 *g* at 4 °C for 1 h, resulting in a translucent reddish pellet. This was resuspended again in MES buffer to 50 ml by passing through a narrow bore needle several times to obtain a homogeneous suspension, then pelleted again and resuspended in 20 mM Tris-HCl pH 7, 10 mM EDTA (TE buffer) to a volume of 3 ml. This membrane suspension was loaded onto a discontinuous sucrose gradient and centrifuged in a Beckman SW41 Ti swinging bucket rotor at 39 000 r.p.m. at 4 °C for 18 h. Sucrose concentrations (w/w in TE buffer) and volumes per tube were: 60% (0.4 ml); 55% (0.9 ml); 50% (2 ml); 45% (2 ml); 40% (2 ml); 35% (2 ml) and 30% (2 ml). Yellow colored bands of membrane were removed from the gradient and diluted into 50 ml TE buffer. The membranes were then pelleted at 100 000 *g* for 1 h and washed three times in TE buffer to remove sucrose. The membranes were resuspended to a final concentration of 1 mg ml^{-1} in TE buffer.

Proteins were extracted from membranes with n-dodecyl-β-D-maltoside (DM, ULTROL grade Calbiochem, Gibbstown, NJ, USA). DM was chosen as the solubilizing agent as it is a mild detergent that has been shown to retain activity in a number of isolated integral membrane complexes (Seddon *et al.*, 2004), and because in preliminary experiments it was better at extracting proteins than *n*-octyl-β-d-glucopyranoside, amidosulfobetaine-14 or Triton X-100. DM was added to the membrane sample to a final concentration of 1%, and the suspension was incubated with gentle mixing at 4 °C for 3 h. Insoluble material was pelleted by centrifugation at 8000 *g* for 20 min, the supernatant was

recovered and concentrated in Centricon spin concentrators (Millipore, Billerica, MA, USA) with a 3 kDa molecular weight cutoff. Samples were typically concentrated to 5 mg ml^{-1}, as determined by a detergent compatible protein assay (DC protein assay, BioRad, Hercules, CA, USA). Approximately 1.3 mg protein in the detergent extract was run on a 1 × 30 cm Superdex 200HR column in TE buffer containing 0.05% DM. Each peak fraction was pooled, and where necessary, concentrated in Centricon spin concentrators to 5–10 mg ml^{-1} (for example, for spectroscopic assays requiring dilution into acidic buffers).

Protein electrophoresis and staining

For routine analysis of membrane proteins, samples were treated to remove excess detergent and lipid (PAGEprep Advance, Pierce, Rockford, IL, USA) prior to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. Staining for heme binding proteins following SDS-PAGE was carried out using *o*-dianisidine (Francis and Becker, 1984). Prior to MS analysis, individual protein bands were excised from colloidal-Coomassie stained polyacrylamide gels, reduced, alkylated and digested with trypsin.

Spectral analysis of Cyt$_{572}$

Spectrophotometric analysis was carried out on a Pharmacia Ultraspec 3000Pro. Protein samples were diluted to approximately 0.1 mg ml^{-1} in buffer at the required pH. Four buffers were used: H_2SO_4 (pH 0.95–1.1), 50 mM KCl-HCl (pH 1 to 2), 50 mM glycine-HCl (pH 2.2–3.4) and 50 mM MES-NaOH (pH 5). For pH values from 0.95 to 3.4, 1 ml of Cyt$_{572}$ solution was oxidized by addition of 10 µl of 0.1 g per 1 ml Fe(III)$SO_4 \cdot H_2O$ (23% Fe) and reduced by addition of 10 µl of 0.7 M Fe(II)$SO_4 \cdot 7H_2O$ (7 mM final concentration). Because Fe(III) is only soluble in acidic buffers, reaction of iron with Cyt$_{572}$ was not carried out above pH 3.5. Instead, at pH 5, Cyt$_{572}$ was oxidized by addition of 10 µl of 0.2 M ammonium cerium (IV) nitrate (2 mM final concentration) and reduced with 10 µl of 0.5 M sodium dithionite (5 mM final concentration). Under these conditions, redox activity and spectra are similar at both pH 3.4 and 5.

2D Blue-Native electrophoresis

For analysis of membrane protein complexes, 2D Blue-Native PAGE was used (Stenberg *et al*., 2005).

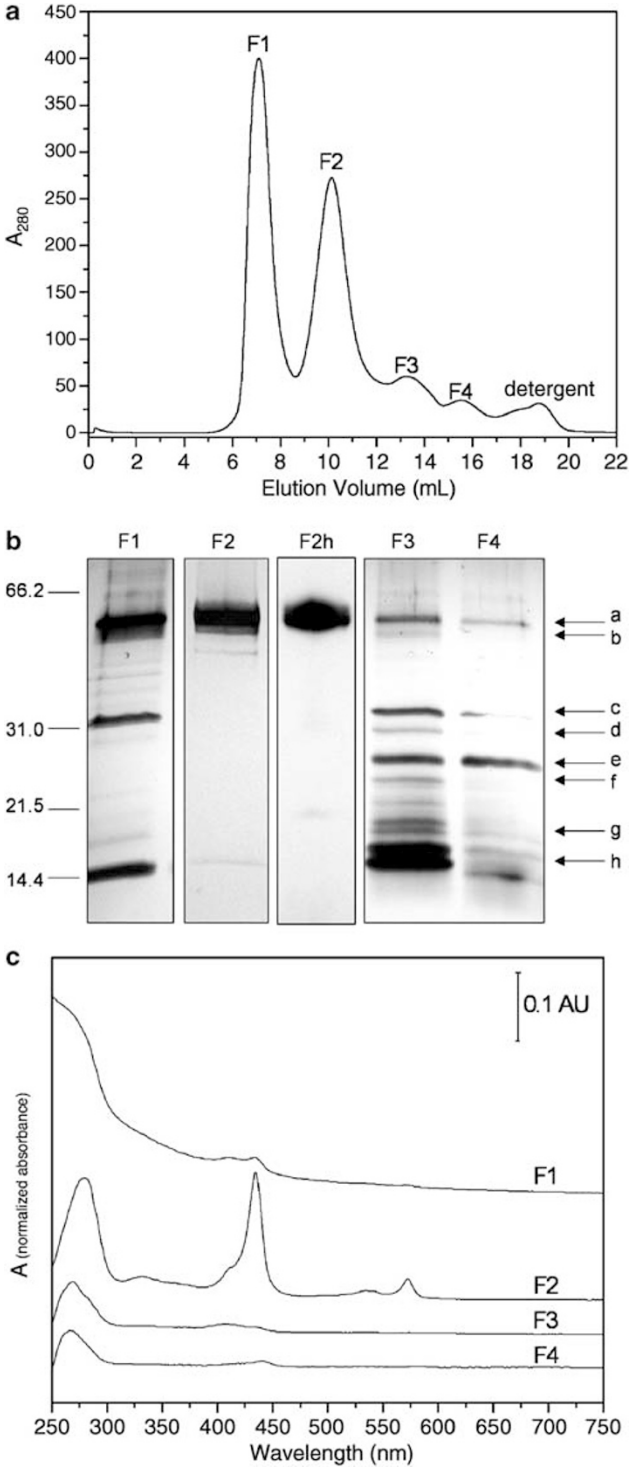


Figure 1 Purification of Cyt$_{572}$. (**a**) Size exclusion chromatogram of DM (n-dodecyl-β-D-maltoside) extracted membrane proteins. Protein was monitored by absorbance at 280 nm, and each peak fraction (F1–F4) was pooled and analyzed in (b and c). (**b**) Analysis of column fractions after separation by SDS-PAGE (SDS-polyacrylamide gel electrophoresis). Samples (2 µg total protein in each lane) were stained with silver, and lane F2h (10 µg) was excised and stained separately for the presence of heme (Supplementary Methods for further details). The mass values of standard protein markers are indicated (left). Positions of protein bands a–h identified in gel slices by MS (mass spectrometry; right): (a) Cyt$_{572}$, identified in community genome data set as gene 630-6 from *Lepto*II; (b) hypothetical protein (*Lepto*II, 32-36); (c) OmpA; (d) FliC, flagellar protein; (e) Pal, peptidoglycan-associated lipoprotein; (f) ATP synthase β-subunit; (g) hypothetical (*Lepto*II, 141-2); (h) hypothetical (*Lepto*II, 21-20). (**c**) Absorption spectra of chromatographic fractions. Peaks in the absorption spectrum of F2 are at 280, 435 and 572 nm.

Briefly, 10–20 μg protein in TE buffer with 1% DM (w/w) was mixed with Coomassie blue G250 to a final dye concentration of 0.25% (w/w), loaded onto a 5–20% acrylamide gradient gel, and separated by electrophoresis in a Mini-Protean II system at 15 mA constant current. After electrophoresis in the first dimension, gel strips were excised, laid on a glass plate and soaked in 2% SDS in 250 mM Tris-HCl pH 6.8 for 30 min. The second glass plate was placed on top, and a 15% acrylamide gel containing 0.1% SDS was poured. A 5% acrylamide stacking gel was poured around the first dimension gel slice, and the denaturing gel was run according to standard protocols and then stained with silver.

Protein identification by mass spectrometry
All protein samples were denatured, reduced and digested with sequencing grade trypsin (Promega, Madison, WI, USA) or pepsin (Sigma, St Louis, MO, USA), and analyzed by MS as described previously (Lo *et al.*, 2007). For detailed LC-MS methods, see Supplementary Methods.

Results

Identification and purification of a novel membrane-bound cytochrome
While examining extracts of microbial biofilms taken from the toxic drainage water of a pyrite mine, we searched for heme containing proteins potentially involved in Fe(II) oxidation. One of the membrane proteins most highly detected by MS proteomic analyses was identified as a hypothetical gene product from our biofilm community genomics data set, corresponding to scaffold 630-gene 6 (630-6) from *Lepto*II (Ram *et al.*, 2005). This protein has been detected with relatively high spectral counts in membrane fractions from five distinct biofilms (Supplementary Table S1A). The encoded 570 amino-acid (61.3 kDa) sequence has no significant homology to any known proteins, but does contain a single heme binding motif, CxxCH, found in *c*-type cytochromes. To purify this putative cytochrome, biofilm samples obtained at the C-drift site (Supplementary Figure S1) were disrupted by sonication and membranes were isolated by sucrose density gradient centrifugation to reduce protein heterogeneity. The major density band was analyzed by MS and found to contain several prominent proteins, including flagellar proteins and porins indicative of outer membranes (Figure 1B). This fraction also contained proteins localized to the cytoplasmic membrane, most likely due to mixing by the sonication process; however, the majority of the proteins could be assigned to the outer membrane. Similar results have been obtained from protein purified from membranes without using a sucrose gradient (data not shown). The protein detected with the highest number of mass spectral counts was the 630-6 gene product, the dominant protein stained by both Coomassie blue and silver staining following separation by SDS-PAGE. DM was used to release the cytochrome from membranes for purification. DM consistently extracted approximately 75% of the protein from isolated and washed membranes.

Proteins in the DM extract were separated by size exclusion chromatography into four major fractions (Figure 1A). The second peak (Fraction 2), eluting at an apparent molecular weight of 400 kDa, had a distinct yellow color. Analysis of fractions following SDS-PAGE indicated that Fraction 2 contained a 57 kDa heme binding protein of 97% relative purity (Figure 1B). This represents a 30% yield of protein from the crude membrane preparation, which, along with spectral counts from MS analysis, indicates the abundance of this cytochrome. Moreover, absorption spectra of the column fractions confirm a heme containing cytochrome in Fraction 2 (Figure 1C). The absorbance of the α-band at 572 nm is unique among known cytochromes and is the basis for the name Cytochrome 572 (Cyt_{572}).

To further characterize the heme group of purified Cyt_{572}, an alkaline pyridine hemochrome spectrum of purified Cyt_{572} reduced with sodium dithionite was taken (Figure 2). The α-band of Cyt_{572} in the pyridine hemochrome spectrum was observed at 568 nm, instead of 550 nm for known *c*-type cytochromes (Berry and Trumpower, 1987), thus indicating novel properties of the Cyt_{572} heme.

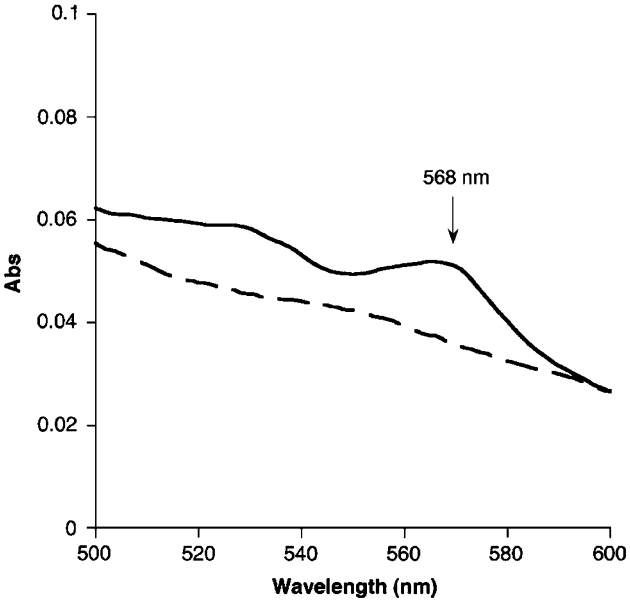
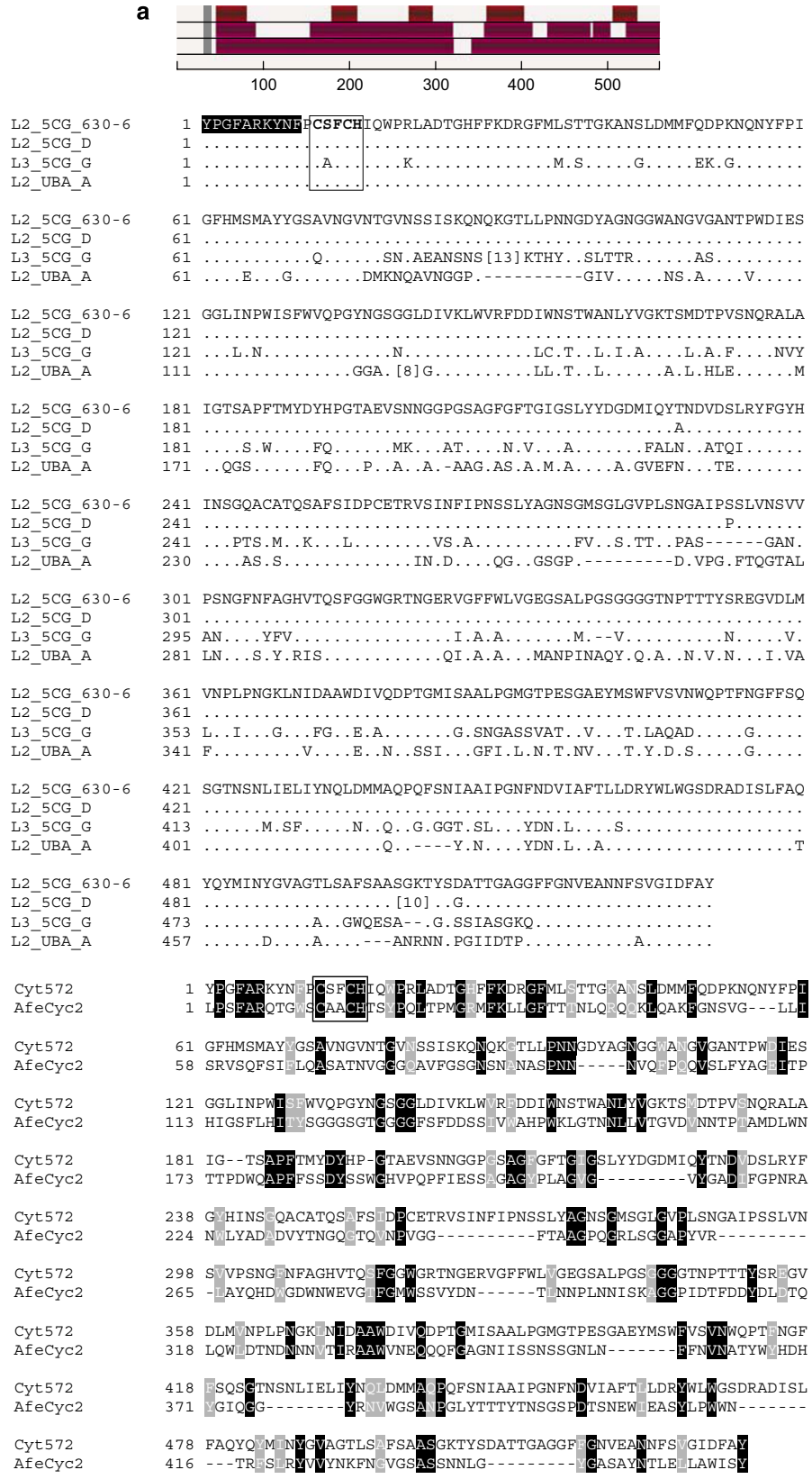


Figure 2 Alkaline pyridine spectra of Cyt_{572}. Purified Cyt_{572} (100 μl of 0.2 mg ml^{-1}) was diluted into 400 μl of 0.2 M NaOH, and 500 μl of pyridine was added. The pyridine hemichrome (oxidized) spectrum was obtained in the presence of 0.1 mM $K_3Fe(CN)_6$ and the pyridine hemochrome (reduced) spectrum was obtained in the presence of 2 mM sodium dithionite. In the latter spectrum, the arrow indicates the maximum absorbance at 568 nm.

Edman degradation of purified Cyt$_{572}$ determined the N-terminal 10-amino-acid sequence, YPGFARKYNF, which matches exactly to the translated 630-6 gene. This sequence is preceded by a signal peptide, as predicted by the SignalP program (Bendtsen *et al.*, 2004), with a signal peptidase I



cleavage site between Ala and Tyr in the sequence AANA/YPGF. The identity of the purified protein was confirmed using LC-MS/MS analysis after treatment with either trypsin or pepsin (Figure 3a; see Supplementary Tables S2A and S2B for sequence coverage and detailed mass spectral data, respectively). In the Cyt_{572} sequence there are five long stretches of amino acids that lack either R or K residues, so trypsin digestion resulted in large peptides that are more difficult to detect by standard LC-MS/MS methods; nevertheless, tryptic peptides were detected over 30% of the sequence, giving a positive identification of the 630-6 gene. A higher number of peptides were detected following the pepsin digest, with 72% of the sequence covered (Figure 3a). Using a deeper 2D LC-MS/MS analysis of the pepsin digest, 92% of the sequence was detected; moreover, Cyt_{572} was the only protein significantly identified. The 22 amino-acid stretch from the observed N-terminus containing the single heme binding site and flanking residues was not detected by MS, most likely due to the interference by the covalently bound heme.

Analysis of the detailed reconstruction of the *Lepto*II genome from environmental sequence data (Tyson *et al.*, 2004) revealed five strain variants from the 5-way site, each at $\geqslant$99% amino-acid sequence identity with 630-6; subsequent analyses indicated that gene 630-6 is a composite of these sequences. A second environmental genomic data set was obtained from a biofilm isolated at a different site in the UBA location within the Richmond mine (Supplementary Figure S1), in which three additional *Lepto*II Cyt_{572} homologs were identified (Lo *et al.*, 2007). To determine if Cyt_{572} is also present in the less abundant bacterium, *Lepto*III, we examined these sequences and found nine different homologs. Alignment of the composite 630-6 sequence with one representative from each of these three sets of sequence variants indicates greater divergence of UBA *Lepto*II and 5-way *Lepto*III from the 5-way *Lepto*II strain variants (Figure 3b). The protein purified is most likely one or a mixture of 5-way *Lepto*II variants, with nearly all residues in peptides detected by MS in these particular sequences. The relationship between the 17 Cyt_{572} variant sequences from both bacterial types is especially strong in the region surrounding the CxxCH heme binding site, implicating an N-terminal cytochrome *c*-like structural domain of approximately 80 amino acids (Supplementary Figure S2). Alignment of Cyt_{572} with the Cyc2, the putative iron oxidase from *A. ferrooxidans*, illustrates that despite significant sequence divergence (15% identity), the position of the heme binding site and some surrounding residues are conserved (Yarzabal *et al.*, 2002) (Figure 3c).

Spectroscopic and redox properties of Cyt_{572}

The absorption spectrum of the purified cytochrome contains a Soret band at 434 nm and α-band at 572 nm (Figure 1C), both red-shifted compared with the spectra of known *c*-type cytochromes. Reduced Cyt_{572} was stable to oxidation for prolonged periods under ambient O_2 levels (months at 4 °C in pH 7 buffer). However, when diluted into buffers with pH values below 3, multiple spectral changes occurred (Figure 4a). The α-band at 572 nm was replaced by a split α-band with maxima at 575 and 585 nm. The ratio of the two sets of α-bands was pH dependent, with the split α-band predominating at low pH. At pH$\leqslant$1.8, partial oxidation of Cyt_{572} occurred, as observed by the appearance of a new Soret band at 419 nm. Although partial oxidation of the cytochrome occurs at pH 2.6, addition of excess Fe(II) caused full reduction of the cytochrome and the appearance of the symmetric α-band at 572 nm (data not shown).

Addition of Fe(III) to fully reduced Cyt_{572} caused complete oxidation of the heme, reflected in loss of the 572 nm peak and a shift of the 435 nm Soret band to 419 nm (Figure 4b). The oxidized state of Cyt_{572} was unstable, and removal of excess iron from the protein by dialysis or desalting resulted in partial reduction of Cyt_{572}. To study the reactions of oxidized Cyt_{572} with Fe(II), it was necessary to minimize the quantity of Fe(III) used to generate oxidized Cyt_{572}, then add a molar excess of reductant. Based on the reappearance of the symmetric 572 nm peak, Cyt_{572} was re-reduced by the addition of excess Fe(II). Reduction of oxidized Cyt_{572} occurred with equal efficiency between pH 0.95 and 3.4; however, at pH$\leqslant$1.4, the split α-band at 575

Figure 3 Identification of Cyt_{572} sequence. (**a**) Schematic of MS (mass spectrometry) detection of peptides across the Cyt_{572} sequence, with the observed N-terminal 10 amino acids depicted in red, and positions of one or more detected peptides represented as colored bars. 1D LC-MS trypsin digest (top), 1D LC-MS pepsin digest (middle) and 2D LC-MS/MS pepsin digest (bottom). (**b**) Multiple sequence alignment of Cyt_{572} representative environmental sequences with the 630-6 sequence. Corresponding genes are from the *Lepto*II 5-way community genomic data (L2.5CG_D, gene 630-6 variant D), *Lepto*II UBA community genomic data (L2.UBA_A, gene 8241-0149c) and *Lepto*III 5-way data set (L3.5CG_G, gene 9350). The 630-6 sequence is 99% identical to L2.5CG_D, 70% to L2.UBA_A and 67% to L3.5CG_G. The theoretical isoelectric point and molecular weight of these cytochromes calculated after removal of predicted signal peptides indicate that the proteins are physically distinct: 5CG_D is 4.7/57.5 kDa (pI/MW); UBA_A, 4.4/55 kDa and L3.5CG_G, 5.0/58 kDa. Key to alignment: sequenced N-terminal peptide, black highlight; heme binding site, boxed bold residues; residues identical to 630-6, '…'; gaps in aligned sequence, '—'; short insertions are indicated as the number of residues, (*n*), removed to maintain alignment. Further details of sequence variants are given in Supplementary Figure S2. (**c**) Alignment of 630-6 sequence with Cyc2 from *Acidithiobacillus ferrooxidans* ATCC 23720. N-terminus of each sequence is derived from Edman degradation of Cyt_{572} (this work) and Cyc2 (Yarzabal *et al.*, 2002). Box indicates heme-binding motif of *c*-type cytochromes.

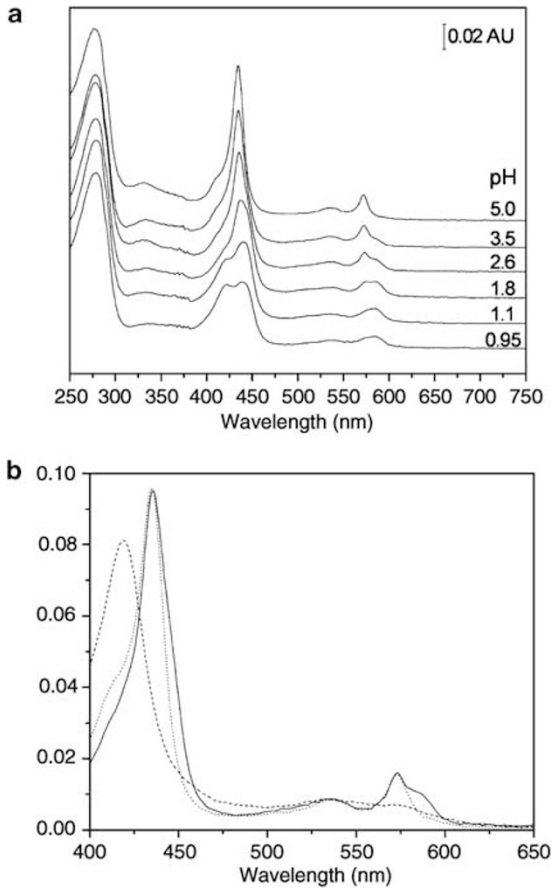


Figure 4 Redox properties of Cyt$_{572}$. (**a**) Absorption spectra of purified, reduced Cyt$_{572}$ at different pH values. Cyt$_{572}$ was diluted to 0.1 mg ml^{-1} into an appropriate buffer (Supplementary Methods). (**b**) Oxidation and reduction of Cyt$_{572}$ by Fe(III) and Fe(II). Absorption spectra of purified, reduced Cyt$_{572}$, 0.1 mg ml^{-1} in 50 mM glycine-HCl, pH 2.6, were taken immediately after addition of iron solutions. Starting sample, solid line; after oxidation by Fe(III), dashed line; after re-reduction by Fe(II), dotted line.

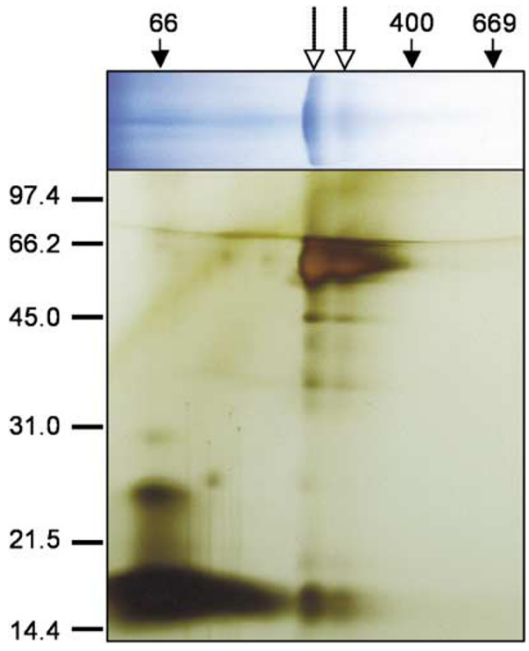


Figure 5 Structural characterization of Cyt$_{572}$. 2D Blue-Native/SDS-PAGE of an enriched preparation of Cyt$_{572}$. The dominant protein species resolved in the first (nondenaturing) dimension are 210 and 260 kDa, although the latter is a less well-defined band of substantially weaker staining. The 210 kDa band was present in all membrane extracts tested, whereas the 260 kDa band varied with sample preparation. Black arrows indicate positions and mass of protein standards, and white arrows mark the positions of the 210 and 260 kDa proteins. This gel was rotated 90° (electrophoresis direction is from right to left) and overlaid as shown at the top of the second (denaturing) dimension gel, which was stained with silver. A protein complex migrating at 66 kDa in the first dimension is resolved into subunits of 23 kDa, and was identified as a peptidoglycan-associated lipoprotein (Pal) corresponding to Figure 1B, band 'e'. A broad smear stained across the bottom of the second dimension gel likely represents lipids or nucleic acids, as it is not stained by Coomassie dyes (data not shown). Standard markers of protein mass for the second dimension are indicated on the left. SDS-PAGE, SDS polyacrylamide gel electrophoresis.

and 585 nm was observed instead of the 572 nm band for reduced Cyt$_{572}$ (data not shown). Buffer composition and presence of additional DM did not influence these results: at the same pH in different buffers, spectral changes upon oxidation and reduction of Cyt$_{572}$ were the same.

Structural properties of Cyt$_{572}$

Chromatographic experiments indicated that Cyt$_{572}$ is a multimer or complexed with other proteins under the solution conditions used (Figure 1A). To investigate the nature of the Cyt$_{572}$ complex, we used 2D Blue-Native PAGE, an electrophoresis technique specifically designed for identifying membrane protein complexes and their subunits (Schagger *et al.*, 1994). Protein complexes bind to Coomassie dye, which is negatively charged, and migrate in a predictable relationship according to their native molecular weights. The first dimension, non-denaturing gel was calibrated with protein standards (Supplementary Figure S3). This was used to estimate the molecular weight of the Cyt$_{572}$ complex run under the same conditions (Figure 5).

The second (denaturing) dimension indicated that the two apparent complex protein species were composed primarily of Cyt$_{572}$, identified by the prominent (saturated) silver-staining at approximately 60 kDa. *In situ* tryptic digests and MS analysis of the first dimension blue-stained bands at 210 and 260 kDa confirmed this assignment. The relative intensities of Coomassie-stained bands in both Blue-Native and denaturing gels indicated that the 260 kDa band represents a minor species, possibly due to a distribution of oligomeric forms, and that $\geqslant$90% of the Cyt$_{572}$ in the sample was found in the 210 kDa band. A protein identified as peptidoglycan-associated lipoprotein is often found with Cyt$_{572}$ complexes by this analysis.

In standard SDS-PAGE, the purified Cyt$_{572}$ protein contains minor protein species of higher molecular weight, estimated at 114, 170 and 226 kDa (data not

shown). These mass values are almost exactly what would be expected for dimeric, trimeric and tetrameric Cyt_{572}, suggesting that some oligomeric species persist under denaturing conditions. We tested this further with chemical crosslinking, and found that the dimeric and tetrameric species were specifically enhanced (Supplementary Figure S4). Higher oligomeric forms are possible, which is one possible explanation for the different mass values calculated for Cyt_{572} in 2D Blue-Native PAGE and size exclusion chromatography.

The requirement of detergent to solubilize Cyt_{572} indicates that it is an integral membrane protein. Based on examination of the 630-6 protein sequence by the PRED-TMBB program (Bagos *et al.*, 2004), a mostly β-strand structure is predicted with up to 20 transmembrane strands, a prediction that is consistent with an outer membrane localization. To experimentally test these folding characteristics, we used circular dichroism spectroscopy to analyze purified Cyt_{572} (Supplementary Figure S5). These results revealed a dominance of β-strand structure (43%), similar to that predicted by PRED-TMBB (38%), and a low proportion of α-helical structure (7%).

Discussion

We found an abundant and unusual cytochrome by extracting and purifying proteins from natural, low-diversity microbial biofilm samples. The protein, Cyt_{572}, was purified as a homo-multimeric complex from membranes and identified by MS as a gene product of *Lepto*II, matching a protein of unknown function documented in our previous proteogenomic data (Ram *et al.*, 2005). The prosthetic heme group of this cytochrome is novel, ascertained by both the visible absorbance spectrum and pyridine hemochrome spectrum of purified Cyt_{572}. We speculate that the observed spectral red shift of the α-absorption band in Cyt_{572} relative to conventional *c*-type cytochromes may be caused by oxidation of the organic portion of the heme, which would raise the cytochrome midpoint potential (Zhuang *et al.*, 2006). An elevated potential for Cyt_{572} may be essential to efficiently oxidize Fe(II) in the low pH regime of AMD. Cyt_{572} also displays unusual pH-dependent spectral properties. At higher pH (>2.6) the 572 nm absorbance peak predominates in the spectrum of reduced Cyt_{572}, but at low pH, a split band replaces this band with maxima at 575 nm and 585 nm. Similar split α-bands have been observed for *c*-type cytochromes, especially at low temperature (77 K), and have been attributed to changes in the heme binding pocket (Reddy *et al.*, 1996). This observation, along with alteration of this pH-dependence in the presence of excess Fe(II), suggests that the heme binding site is sensitive to pH and may have important implications for the biological activity of Cyt_{572}. Current studies are focused on obtaining sufficient Cyt_{572} from the acidophilic biofilms for further biophysical characterization.

Although no archaeal homologs of Cyt_{572} were found in the database searches, an absorption band at 572/573 nm has been observed as a feature of the visible spectra of cell extracts from the acidophilic archaea *Sulfolobus metallicus*, *Metallosphaera sedula* and *Acidanus brierlyi* when grown on soluble Fe(II) or pyrite (Blake *et al.*, 1993; Kappler *et al.*, 2005; Bathe and Norris, 2007). These observations suggest that the heme found in Cyt_{572} may be a specialized prosthetic group in microbes that catalyze Fe(II) oxidation under acidic conditions.

Despite minimal sequence similarity, Cyt_{572} shares many properties with Cyc2, the putative iron oxidase localized to the outer membrane of *A. ferrooxidans.* Both proteins have structures that are primarily composed of β-strands, are monoheme cytochromes with heme binding sequences that begin 12 amino acids from the N-terminus of the mature protein and oxidize Fe(II) readily at low pH. Cyc2 undergoes a partial digestion upon treatment of whole cells of *A. ferrooxidans* with proteinase K, indicating that domains of this protein are exposed to the exterior of the cell (Yarzabal *et al.*, 2002). Experiments are currently underway to determine whether portions of Cyt_{572} are exposed to the exterior of *Lepto*II cells in Richmond Mine biofilms.

A small soluble cytochrome with unusual absorbance characteristics similar to Cyt_{572}, Cytochrome 579 (Cyt_{579}), has also been purified directly from Richmond Mine biofilms (Singer *et al.*, manuscript in preparation). Biochemical studies of Cyt_{579} are most consistent with it serving as a periplasmic electron transfer protein that shuttles electrons derived from Fe(II) oxidation to protein complexes localized on the cytoplasmic membrane. Future biochemical studies will focus on determining if Cyt_{572} oxidizes Fe(II) at the surface of *Lepto*II cells and donates electrons to Cyt_{579} as part of an effort to reconstruct the Fe(II)-dependent respiration pathway of *Lepto*II in the Richmond Mine biofilms.

Environmental genomic data obtained from the 5-way site of the Richmond Mine contained five variant sequences that result in the composite 630-6 gene of the original data set (Tyson *et al.*, 2004), and an additional 12 variants of Cyt_{572} were identified in the reconstructed genomes of UBA *Lepto*II (Lo *et al.*, 2007) and 5-way *Lepto*III. The high level of variation in genes encoding Cyt_{572} is striking because of the protein abundance and its iron oxidation activity. The extent of sequence diversity and the effect of this variation on the biochemical characteristics of Cyt_{572} will be determined by examining multiple environmental samples. The high peptide coverage of the Cyt_{572} by the combination of pepsin digestion and 2D LC-MS/MS (Figure 3a) will enable us to distinguish the presence of these variants. Of particular interest is whether strain variation affects the midpoint potential of Cyt_{572}, which we are

currently investigating in an ecological context. We postulate that a high level of recombination in a relevant genomic region (Lo *et al.*, 2007) leads to sequence variation and duplication of Cyt_{572} genes. If Cyt_{572} is confirmed as an iron oxidase in *Lepto*II, this will be a notable example of geochemical forces shaping the fitness of a complex community by refining the mechanisms required to exploit these conditions.

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