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ABSTRACT

The Richmond mine in Iron Mountain, California, provides an unusual ecosystem suitable for the growth of microbial biofilms which produce many unique proteins. Through iron oxidation, these proteins facilitate acid mine drainage (AMD). Because this habitat is extremely acidic, survival is an extraordinary feat and the process of environmental selection is rare. In order to understand the mechanisms by which these organisms oxidize iron and gain electrons for energy, biochemical studies were applied. More specifically, column chromatography, spectrophotometry, and gel electrophoresis were used to determine the proteins present in different biofilms. Two specific locations of the mine researched were the AB drift and Ultraback C (UBC), which were both found to contain at least five different types of protein and a large amount of heme-bound cytochromes. Another application of these methods was to investigate proteins playing a major role within the community; one protein selected was cytochrome 579 (Cyt_{579}) due to its abundance in the biofilm, iron oxidizing potential, and signature absorbance of 579nm. The structure and function of Cyt_{579} could be characterized by the isolation of its heme, which was completed using column chromatography; however, one of the challenges has been liberating the heme from the column. Further research, including acid-base and temperature profiling of Cyt_{579} should help elucidate its structural changes within alternate environments and metabolism within the community.

INTRODUCTION

Acid mine drainage is a current environmental problem which exists throughout the world. Water flowing through coal or metal mines contributes toxic metals and acidity to downstream aquifers, which in turn pollute water supplies. Biogeochemical studies implicate micro-organisms as a key component in AMD formation. Studies of microbial communities that thrive in these acidic and metal-rich environments may not only lead to remediation strategies which would prevent AMD, but also illuminate the function of these unique, extermophilic organisms. Furthermore, these organisms could potentially be used for industrial purposes, such as in bio-leaching of valuable metals, or in the biotechnological use of highly stable proteins.

Our work focuses on an AMD site in Iron Mountain, California, where a biofilm forms along the surface of the acidic water (pH<1). This biofilm consists of self-sufficient extremophiles that accelerate the dissolution of the pyrite (FeS_2) bed in the mine. The biofilm gains energy by Fe (II) oxidation and grows autotrophically by fixing CO_2 and N_2 from the air [1]. The acidic mine water also has high concentrations of copper, zinc and arsenic [2].

To understand how the biofilm functions, metagenomic and proteomic studies were conducted. Greater than 12,000 genes within the community were identified by genomic sequencing, and two genomes were then reconstructed for the dominant species, *Leptospirillum* group II and *Ferroplasma* type II [1]. Shotgun proteomics, utilizing two-dimensional mass spectrometry, was used to identify proteins expressed within the biofilm [3]. Proteins from the extracellular and periplasmic regions were studied because exterior proteins not only play a vital role in the metabolism of the organism and interactions within the community, but are also in contact with the extreme environment. The majority of the proteins in the extracellular fraction were hypothetical, meaning they have low sequence similarity to proteins of known function.

Of particular interest was an abundant protein, Cyt_{579}, which has a sequence that corresponds to a hypothetical gene of the source organism, *Leptospirillum* group II. Although Cyt_{579} contains a cytochrome c like amino acid sequence motif (CysXaaXaaCysHis), it has little sequence similarity to cytochrome c [4]. This important protein was isolated from environmental biofilm samples and then highly enriched by column chromatography. The visible spectra of Cyt_{579} is indicative of an unusual heme co-factor and reaffirms a cytochrome structure. To study the heme and the specific mechanisms of electron transport from Fe(II) to *Leptospirillum* bacteria, methods were developed to isolate this unusual heme using the model substrate cytochrome c and then the uncharacterized Cyt_{579}.

MATERIALS AND METHODS

Heme Extraction from Cytochrome C

To form a solution of free heme and cytochrome c, 48 mg of cytochrome c was first dissolved in 1 ml of water. Then, this was added to a solution of 80 mg of Ag_2So_4 dissolved in 9 ml of water and 0.8 ml of acetic acid. This mixture was incubated in the dark for 4 hours at 40˚C and then centrifuged to remove heme aggregates and silver precipitate. See reference [5] for heme extraction method.

The supernatant from the above procedure, containing cytochrome c (protein-bound heme), apo-cytochrome c and free heme, was then separated by SDS-polyacrylamide gel electrophoresis [6] in order to test for efficiency of heme extraction. Four samples were loaded, including two of the previously silver-treated cytochrome c samples and two non-treated, cytochrome c controls (1 mg/mL). One of each was loaded on opposite sides of the gel; the left side was treated with o-dianisidine to stain specifically for heme [7], and the right was stained with Coomassie blue dye for protein.

2 mL of the treated cytochrome c solution was also applied to a SP-sepharose column in order to separate the free heme from the apo-cytochrome c and heme-bound protein and also to collect a sample

of heme. Three buffers were successively passed through the column: 2 M NaCl in 100 mL $NaC_2H_3O_2$ pH 5, 100 mM Na_2CO_3 pH 10, and 0.1 - 0.2 M NaOH.

Furthermore, 1 mL of cytochrome c was also applied to a G-25 Sephadex column in order to obtain a sample of free heme by elution with 0.1 - 0.2 M NaOH.

Acid-Base Profiling of Cytochrome C

For testing structural changes in cytochrome c at different pH values, thirteen buffers were composed (see Table 1) [8]. Each cytochrome c sample was diluted by adding 800 μL of the specific pH buffer to 200 μL of the 1 mg/mL cytochrome c solution. Then, 25 μL of each diluted cytochrome sample was added to 975 μL of pH 7 buffer to make 1 mL samples for spectrophotometry.

Processing the Biofilm: Isolation of Extracellular Fraction

The Ultraback C (UBC) biofilm (collected 6/16/06) was fractioned into extracellular and cellular membrane samples in order to separate the different types of proteins. First, a 50 mL sample of biofilm was thawed by adding a small amount (~10 mL) of 0.2 M H_2SO_4 pH 1.1 into the tube and placing it in a beaker of warm water. Once thawed and transferred into a 300 mL glass beaker, it was suspended in 150 mL of 0.2 M H_2SO_4 pH 1.1 and stirred for 10 minutes at room temperature. The solution was homogenized, stirred for 1-1.5 hours at 4 ˚C, homogenized again, and then centrifuged for 12 minutes at 17500 rpm/rcf and 20 ˚C in the SS-34 rotor. The supernatant was decanted and the pellet (cellular fraction) was stored in the freezer for further analysis.

25 grams of $(NH_4)_2SO_4$ was added per 100 mL of supernatant and this solution was stirred for 1 hour at 4 ˚C, centrifuged for 15 minutes at 17.5 K rpm/rcf, and decanted (supernatant for 95% fraction). The precipitate from this step made up the 45% precipitate fraction. The pellet was dissolved in 3-5 mL of 20 mM H_2SO_4/100 mM ammonium sulfate pH 2.2 (buffer A), centrifuged, decanted, and then stored.

Next, 28 grams of $(NH_4)_2SO_4$ was added to each 100 mL of supernatant from the 45% precipitate fraction. This mixture was stirred for 2 hours at 4 ˚C, centrifuged for 20 minutes at 17.5 K rpm/rcf, and decanted. The pellet was dissolved in 3-5 mL of buffer A, which made the 95% precipitate fraction. Then, it was dialyzed overnight in a 500 mL of buffer A. Protein concentrations were estimated by the Bradford protein assay, using bovine serum albumin as a standard [9].

Enrichment of Cyt_{579} and Heme Extraction from Cyt_{579}

Cation-exchange chromatography was used in order to enrich Cyt_{579}. About 5 mL of the 45% precipitate UBC extracellular fraction was loaded onto an SP-sepharose fast flow column equilibrated with 6 column volumes of buffer A. The absorbed sample was washed with ~3 columns of buffer A and the cytochrome fraction eluted with 100 mM NaOAc pH 5.0. Remaining protein was eluted with a 0-2M NaCl gradient in pH 5.0 buffer.

A G-25 sephadex column was loaded with about 1 mL of treated Cyt_{579} and washed with 100 mL of 100 mM NaOAc pH 5.0 buffer and 100 mL of 1 M NaCl.

RESULTS and DISCUSSION

Heme Extraction Using Cytochrome C as a Model

Cytochrome c is an analog for Cyt_{579} because both have the heme-binding sequence motif, implying that heme is covalently bound through disulfide bonds to the two Cysteines. Because samples of Cyt_{579} were limited, biochemical tests were first performed on cytochrome c. Previous attempts to extract heme from Cyt_{579} were not successful, but a silver sulfate treatment [5] appeared promising. Gel electrophoresis was initially used to verify that the silver sulfate treatment was effective in heme extraction. Following separation of silver-treated and untreated cytochrome c proteins, staining the polyacrylamide gel with Coomassie-blue dye revealed protein in both samples. However, staining the gel with o-dianisidine resulted in the sole appearance of free heme at a position of < 4 kDa, while the non-treated protein appeared at ~16 kDa (**Figure 1**).

The SP-sepharose column eluted apo-cytochrome c protein with the pH 10 buffer, and heme-bound protein and some free heme with 0.1 M NaOH. The majority of free heme remained attached to the column, even after an hour exposure with 0.2M NaOH. The G-25 sephadex column eluted apo-cytochrome c protein immediately after washing pH 5 buffer through the column. Then, 3 heme-bound 3 mL fractions were eluted using 0.1 M NaOH. However, similar to the SP-sepharose column, most of the free heme remained spread out and bound tightly along the column. This is further discussed below in "Heme Extraction from Cyt_{579} and Cytochrome C".

Acid Base Profiling of Cytochrome C

As an indicator of structure and stability of cytochrome c, visible spectra from 350 to 800 nm were taken after 24 hours incubation in different pH buffers. These spectra indicated that the heme group of cytochrome c was reduced at both pH 9 and 10, whereas no other pH significantly affected the protein. In all cases, the visible spectra indicated the cytochrome remained intact after 24 hours.

Cytochrome c was used again as a model for Cyt_{579}, which will soon be tested when more biofilm samples are processed.

Enrichment of Cyt_{579} and Heme Extraction

In the UBC biofilm sample, much more protein from the extracellular fraction was detected in the 45% ammonium sulfate precipitate sample than in the 95% precipitate. In contrast, most of the extracellular protein of previously studied AMD biofilms was recovered from the 95% precipitate fraction. This observation is interesting in that the UBC biofilm compared to previously characterized biofilms perhaps has a different structure, contains different amounts of metals, or that Cyt_{579} carries a different charge. Cation-exchange chromatography of the UBC 45% fraction eluted fractions which were red due to Cyt_{579}, yielding 1.5 mg protein from the biofilm sample.

Following silver treatment of Cyt_{579}, heme aggregates were seen and the solution turned slightly red probably due to liberated heme (similar to cytochrome c). After gel electrophoresis of the treated sample, heme-bound protein was detected through heme staining (**Figure 2**). However, free heme did not appear to be present. Perhaps this occurred because the heme migrated too far in the gel or the free heme was not concentrated enough for detection. Another gel will be run with Cyt_{579} for a shorter period in order to eliminate one of these possibilities.

The G-25 sephadex column eluted a protein fraction with the pH 5.0 buffer; however, the heme remained bound to the column, even after passing 1M NaCl through the column. Cyt_{579} has not yet been tested in column experiments with NaOH.

Comparison of Cyt_{579} and Cytochrome C Heme Groups

The results from the SP-Sepharose and Sephadex-25 columns demonstrated that the free heme bound tightly to both columns. The dark, red band that remained at the top of these columns could

contain precipitated cytochrome or aggregated heme derived from the silver sulfate treatment. Although the heme structure of Cyt_{579} is probably similar to the common porphyrin ring structure of most heme compounds that are soluble in alkaline solutions, it appears to be unique due to its specific visible (red-shifted) spectral characteristics. One possible explanation of the strong binding of heme to these columns is that it may be structurally altered by the addition of a formyl group at one of its vinyl side chains. This heme alteration theory has been reported for a recombinant cytochrome purified from *E. coli* [10] and confers a higher reduction potential that would make it more efficient in iron oxidation.

CONCLUSIONS

In the process of isolating heme from cytochrome c, the heme-bound protein and some free heme could be recovered using either Sephadex G-25 or SP-sepharose columns with 0.1 M NaOH buffer. Some free heme was also removed from Cyt_{579} using a Sephadex G-25 column, however the yield was low. Other methods for eluting greater quantities of free heme from cytochrome c will be developed prior to isolating the heme from Cyt_{579}. Cytochrome c was shown to be stable over a wide range of pH and reduced spontaneously at pH 9 and 10, but not at lower or higher pH values. The potential results of acid-base profiling of Cyt_{579} may exhibit similar or distinct results compared to those of cytochrome c and will allude to the structure and stability of Cyt_{579}. Ammonium sulfate treatment of the UBC and AB Drift extracellular fractions resulted in enriched samples of Cyt_{579}. Through purification and heme characterization studies, the properties of this unique cytochrome appear to be distinct in different biofilms. This could indicate that the structure of Cyt_{579} is selected for its optimum redox function by the influence of environmental conditions on the microbial community.

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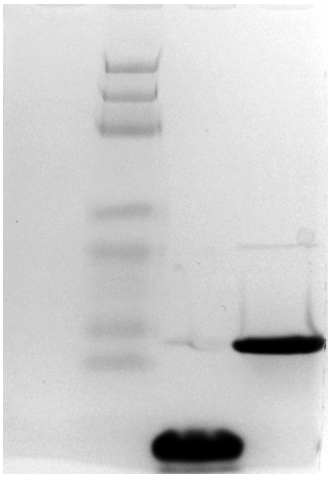

Figure 1. Heme stain of pure Cytochrome C

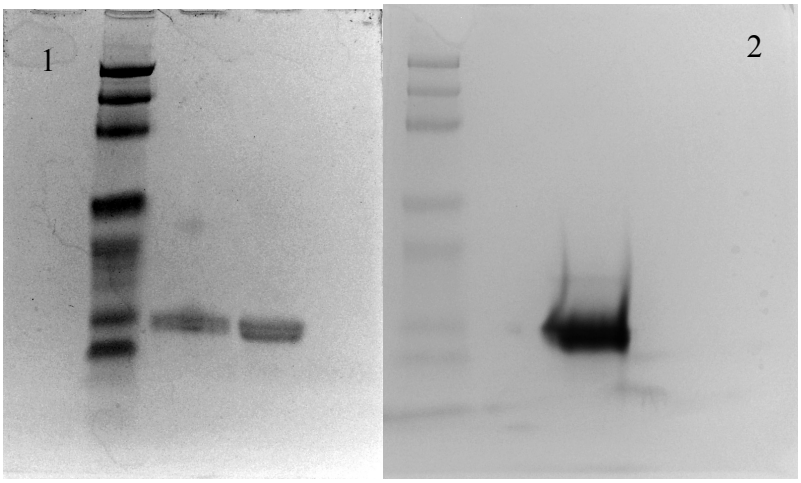

Figure 2. Protein stain (1) and heme stain (2) of enriched Cyt_{579}

Table 1- Buffers

Buffer Type	Components
pH 1 KCl/HCl	2.5 mL 0.2 M KCl 6.7 mL 0.2 M HCl 0.8 mL H_2O
pH 2 KCl/HCl	2.5 mL 0.2 M KCl 6.5 mL 0.2 M HCl 1.0 mL H_2O
pH 3 Glycine-HCl	2.5 mL 0.2 M glycine 0.57 mL 0.2 M HCl 6.93 mL H_2O
pH 4 $NaC_2H_3O_2$	1.8 mL 0.2 M NaOAc 8.2 mL 0.2 M HOAc
pH 5 $NaC_2H_3O_2$	7.0 mL 0.2 M NaOAc 3.0 mL 0.2 M HOAc
pH 6 MES-NaOH	2.5 mL 0.1 M MES 0.8 mL 0.1 M NaOH 1.7 mL H_2O
pH 7 KH_2PO_4-NaOH	5 mL 0.1 M KH_2PO_4 2.91 mL 0.1 M NaOH 2.09 mL H_2O
pH 8 KH_2PO_4-NaOH	5 mL 0.1 M KH_2PO_4 4.61 mL 0.1 M NaOH 0.39 mL H_2O
pH 9 Glycine-NaOH	2.5 mL 0.2 M glycine 0.44 mL 0.2 M NaOH 7.06 mL H_2O
pH 10 Glycine-NaOH	2.5 mL 0.2 M glycine 1.6 mL 0.2 M NaOH 5.9 mL H_2O
pH 11 Phosphate	5 mL 0.005 Na_2HPO_4 0.41 mL 0.1 M NaOH 4.59 mL H_2O
pH 12 Hydroxide-chloride	2.5 mL 0.2 M KCl 0.6 mL 0.2 M NaOH 6.9 mL H_2O
pH 13 Hydroxide-chloride	2.5 mL 0.2 M KCl 6.6 mL 0.2 M NaOH 0.9 mL H_2O