Characterization of Cytochrome 579, an Unusual Cytochrome Isolated from an Iron-Oxidizing Microbial Community

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A novel, soluble cytochrome with an unusual visible spectral signature at 579 nm (Cyt579) has been characterized after isolation from several different microbial biofilms collected in an extremely acidic ecosystem. Previous proteogenomic studies of an Fe(II)-oxidizing community indicated that this abundant red cytochrome could be extracted from the biofilms with dilute sulfuric acid. Here, we found that the Fe(II)-dependent reduction of Cyt579 was thermodynamically favorable at a pH of >3, raising the possibility that Cyt579 acts as an accessory protein for electron transfer. The results of transmission electron microscopy of a biofilm identified that Cyt579 is localized near the bacterial cell surface, consistent with periplasmic localization. The results of further-protein analysis corroborated the posttranslational modifications of these forms and identified a genomically characterized Cyt579 variant. Homology modeling was used to predict the overall cytochrome structure and heme binding site; the positions of nine amino acid substitutions found in three Cyt579 variants all map to the surface of the protein and away from the heme group. Based on this detailed characterization of Cyt579, we propose that Cyt579 acts as an electron transfer protein, shuttling electrons derived from Fe(II) oxidation to support critical metabolic functions in the acidophilic microbial community.

Biological oxidation of Fe(II) by acidophilic microbial communities found in mines with exposed pyrite ore accelerates the dissolution of FeS₂ and acidification of the mine water, resulting in acid mine drainage (AMD), a global environmental problem (8). One of the most-intensively studied AMD sites is the Richmond Mine at Iron Mountain, CA, where copious biofilm communities are found in extremely low-pH (0.5 to 1.0) solutions (2). Most of these communities are pink biofilms dominated by Leptospirillum group II bacteria, with several archaeal species (4). A Leptospirillum group II bacterium-dominated biofilm was collected at the “5-way” site at the Richmond Mine (11) and analyzed by metagenomic sequencing (5-way community genomics data set [24]). Proteomic characterization by mass spectrometry (MS) of a similar biofilm isolated from the “AB end” site of the Richmond Mine identified an abundant extracellular protein from Leptospirillum group II bacteria, encoded by gene 20 on sequencing scaffold 20 (gene 14-20), that has a CXXCH heme binding motif common to c-type cytochromes but otherwise insignificant sequence similarity to known proteins (17). The results of gel electrophoresis and N-terminal sequencing confirmed that this protein contained heme and was abundant in the extracellular fraction. The first 40 amino acids deduced from the environmental genomic sequence were nearly identical to the N-terminal sequence deduced for the Fe(II)-oxidizing cytochrome 579 (Cyt579) purified from an isolate of Leptospirillum ferriphilum. The reduction potential of L. ferriphilum Cyt579 was estimated to be 660 mV, and the cytochrome was fully reduced in the presence of excess Fe(II) at pH 2.0 (17). A cytochrome with very similar spectral and pH-dependent-redox properties had also been isolated from Leptospirillum ferrooxidans (10). The ability of L. ferriphilum and L. ferrooxidans Cyt579 to oxidize Fe(II) at low pH led to the hypothesis that this novel cytochrome identified in the biofilm acted as the primary Fe(II) oxidant for Leptospirillum group II bacteria.

Here we report the purification and characterization of Cyt579 from a Leptospirillum group II bacterium-dominated biofilm collected at Richmond Mine. The results of detailed biochemical and MS studies of Cyt579 from the biofilm suggest that it functions as a periplasmic electron transfer protein.

MATERIALS AND METHODS

Isolation of extracellular proteins. Richmond Mine biofilm samples were collected in 50-ml conical Falcon tubes (BD Biosciences, San Jose, CA), frozen at the site on dry ice, and later stored at −80°C. Biofilm samples were collected from the AB end site (near the junction of the “A drift” and B drift) in January 2004; from the C drift site (15 m beyond the AMD dam) in November 2005; and from the “UBA” site (in the A drift) in November 2005. A map describing the field site can be found in online supplementary information of reference 11. To obtain the extracellular fraction, the biofilm was thawed, suspended in 110 ml of 0.2 M H₂SO₄ (pH 1.1), and homogenized in a glass tube by using several vigorous strokes of a tight-fitting, round, glass pestle. The resulting homogeneous cell suspension was stirred for 2 h at 4°C and then centrifuged at 24,000 × g for 12 strokes of a tight-fitting, round, glass pestle. The resulting homogeneous cell suspension was stirred for 2 h at 4°C and then centrifuged at 24,000 × g for 12
min. The supernatant is the extracellular fraction used for cytochrome purification. For proteomic analysis, proteins from a 10-ml sample of the extracellular fraction of the biofilm from the C drift were precipitated with 10% trichloroacetic acid and the precipitate was collected by centrifugation, rinsed twice with cold methanol, and air dried.

**Purification of Cyt579.** Proteins in the extracellular fraction (150 ml) were precipitated with (NH₄)₂SO₄ and redissolved in ~5 ml sample buffer (SB) containing 20 mM H₂SO₄ and 100 mM NH₄(SO₄)₂ at pH 2.2. A light red precipitate at 45% NH₄(SO₄)₂ saturation was gelatinous, indicating the presence of exopolysaccharides. A deeper red precipitate at 95% NH₄(SO₄)₂ contained 75% of the protein found in the extracellular fraction. This precipitate was dialyzed for 16 h at 4°C against 1 liter of 25 mM-l-histidine–HCl, pH 6.2. The remaining protein was removed with a 0 to 2 M NaCl gradient (30 ml) in pH 5.0 buffer. Between 1.2 M and 2.0 M NaCl, light yellow fractions (3 ml each; 2 mg total protein) eluted that had visible spectra consistent with the presence of c-type cytochromes (α-band at 552 nm for reduced samples).

**Immunogold labeling of Cyt579 and transmission electron microscopy (TEM) of biofilms.** Polyclonal antibodies were produced in rabbits (Covance, Denver, PA) by using the cation-exchange fraction of Cyt579 as the antigen. Prior to immunization, the antigen was concentrated by using MicroCon spin filters (10-kDa-molecular-mass cutoff; Millipore, Billerica, MA) and resuspended in phosphate-buffered saline. Immunoblotting of a biofilm lysate indicated a high specificity of the antibody preparation for Cyt579 (data not shown). Antibodies were purified by using a Melon gel antibody purification kit (Pierce, Rockville, IL).

A biofilm sample was frozen under high pressure (Bal-tec HPM 010) and freeze substituted in 0.2% glutaraldehyde and 0.1% uranyl acetate in acetone. The resulting fragment ions were measured at high resolution in the FTICR analyzer with infrared laser irradiation (30% maximum laser power for 1.5 s), and the ion was isolated by ejecting all other ions from the analyzer cell and dissociated with ion-molecule dissociation (IRMPD) with a Synrad carbon dioxide laser (75-W maximum power and 10.2-psec pulses). Ion dissociation was accomplished by infrared multiple photon dissociation (IRMPD) with a Synrad carbon dioxide laser (75-W maximum power and 10.2-psec pulses) and mass resolutions of 50,000 to 160,000 Da (full width at half maximum), as previously described (7). Ion dissociation was accomplished by infrared multiphoton dissociation (IRMPD) with a Synrad carbon dioxide laser (75-W maximum power and 10.2-psec wavelength). For this experiment, the desired parent ion was isolated by ejecting all other ions from the analyzer cell and dissociated with infrared laser irradiation (30% maximum laser power for 1.5 s), and the resulting fragment ions were measured at high resolution in the FTICR analyzer cell.

To verify amino acid differences in Cyt579 variants, purified samples were denatured, reduced, and digested with trypsin (sequencing grade; Promega, Madison, WI). Peptides were analyzed by using one-dimensional liquid chromatography-tandem MS (LC–MS–MS) on a Thermo Fisher linear-trapping quadrupole instrument. All MS-MS spectra were searched with DDBJ (22) against a database of all proteins predicted by genomic sequencing of biofilm samples (15, 24), as well as all potential amino acid variants of Cyt579. The output data files were then filtered and sorted with the DTA Select algorithm (21) using the following parameters: fully tryptic peptides only; delta correlation value of at least 0.01; cross-correlation scores of at least 25 (+1 ions), 30 (+2 ions), and 45 (+3 ions); and at least two unique peptides per protein.

Amino acid variants were also verified from crude extracellular fractions of biofilms from the A bend, C drift, and UBA sites. Extracellular proteins were denatured, reduced, trypsin digested, and analyzed by using two-dimensional LC–MS–MS on a linear-trapping quadrupole instrument as previously described.
Environmental genomic data indicate three distinct Cyt_{579} genes. In addition to the previous metagenomics data for the 5-way site, we examined a second genomic data set obtained from a biofilm collected at the UBA site, which was dominated by a *Leptospirillum* group II species closely related to the characterized species from the 5-way site (15). Two homologs of Cyt_{579} were identified. One is encoded by gene 8062-147, with an amino acid sequence 99% identical to the amino acid sequence encoded by a paralog of this gene, 8062-147, from the AB end site; the other is an amino acid sequence 99% identical to the amino acid sequence encoded by gene 14-20 from the AB end site; the distances between all identified templates and preliminary structural alignments were built as loops. These regions were modeled using LGA (28) by “grafting” in suitable fragments from related structures in PDB. Finally, SCWRL (5) was used to add coordinates for missing side chain atoms.

**RESULTS**

The visible spectrum of purified Cyt_{579} oxidized with Fe(III) at pH 2.0 exhibited a Soret band at 427 nm. In addition, a weak absorption band at 695 nm characteristic of an axial methionine ligand was observed in concentrated solutions (>0.2 mM) of oxidized Cyt_{579} (data not shown). Upon reduction of isolated Cyt_{579} with 50 μM sodium ascorbate, the Soret band shifted to 441 nm and β (539 nm) and α (579 nm) bands were observed (Fig. 2A). The Soret band of the reduced spectrum also had a distinct shoulder at 419 nm, a feature absent in the spectrum of reduced Cyt_{579} isolated from *L. ferriphilum* (17).

The alkaline pyridine hemochrome spectrum had a Soret band at 443 nm and an α band at 587 nm (Fig. 2B). The results of SDS-PAGE of this fraction revealed two closely spaced protein bands at ~16 kDa (Fig. 3). Since MS proteomics of this frac-
tion digested with trypsin indicated that >98% of the peptides were from Cyt579. We concluded that two protein species represented different forms of Cyt579 (data not shown). The results of Edman degradation identified two N-terminal sequences of Cyt579 from the C drift biofilm (AELDILKPRV and ILKPRVPAD) that corresponded to the predicted amino acid sequence for all the Cyt579 variants. Identical N-terminal sequences were obtained for a Cyt579 preparation from the AB end site, the original proteomic sample (data not shown). The predicted N-terminal cleavage site to give the N-terminal sequence AELDILKPRV of signal peptidase I is between residues 23 and 24 for the variant sequences of Cyt579. The Cyt579 fraction eluted as a single band at an apparent molecular mass of 20 kDa from a Superdex 75 gel filtration column, consistent with the assignment of Cyt579 as a monomer.

Cyt579 was localized in Leptospirillum group II cells by TEM imaging of a thin section of the C drift biofilm that had been treated with polyclonal antibodies raised against Cyt579 and a secondary gold-labeled antibody. Visualization of the antibody-treated thin section by TEM indicated that Cyt579 was localized on the exterior of the Leptospirillum group II cells and was not distributed throughout the biofilm (Fig. 4). Since Cyt579 contains a signal peptide and has no other hydrophobic regions in its amino acid sequence, we hypothesize that it is located in the periplasm of Leptospirillum group II cells.

**Multiple forms of Cyt579 separated by chromatofocusing.** As mentioned above, the results of SDS-PAGE indicated that multiple forms of Cyt579 were present in the purified fraction. The forms were too close in molecular weight to separate successfully by gel filtration. However, the forms of Cyt579 were separated by using a preparative chromatofocusing column. Two red bands were eluted at pH 5.5 (C1) and pH 5.1 (C2) in a pH gradient of 6.2 to 5.0. The red fraction remaining on the column was eluted with pH 5.0 1 M NaCl buffer (C3). All three red fractions had nearly identical visible spectra; however, C1 had a Soret band for the oxidized Cyt579 that was shifted to 425 nm, compared to 428 nm for C2 and C3. The results of SDS-PAGE of the separated Cyt579 fractions confirmed that the pH 5.5 and pH 5.1 fractions represented the higher band in the crude Cyt579 fraction, while the pH 5.0 1 M NaCl fraction represented the lower band (Fig. 3). N-terminal sequencing of the individual bands revealed different start sites for each (Table 1). Cyt579-specific polyclonal antibodies detected all three forms of the protein.

**Mass spectrometry of separated Cyt579 forms.** To determine the accurate molecular masses and fragmentation products for the individual forms of C drift biofilm Cyt579, the separated proteins were examined by FTICR-MS. The measured average molecular masses of the peaks in each Cyt579 fraction are given in Table 1.

The amino acid sequences of each of these proteins were examined by MS-based fragmentation techniques. Isolation and IRMPD fragmentation of the (M + 13H)$^{13+}$ ion for the 16,060-Da species revealed a variety of fragment ions, including a sequence tag, MVWVVSNGS, which is representative of the 8062-147-encoded sequence (Fig. 5, upper panel).

![FIG. 3. Separation of different forms of Cyt579. Chromatofocusing was used to fractionate a Cyt579 sample, and proteins were analyzed on a 10 to 20% acrylamide gel using SDS-PAGE. First lane, C drift biofilm Cyt579 fraction; second lane, C1 fraction; third lane, C2 fraction; and fourth lane, C3 fraction.](image)

![FIG. 4. TEM images of immunogold-labeled biofilm. Ultrathin section of biofilm showing Cyt579 distribution on the edges of cells, possibly in the periplasm, and along the exterior of cells. Two representative fields are shown. Black arrows show gold particles; scale bars show 500 nm.](image)

<table>
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<tr>
<th>Cyt579 fraction</th>
<th>N-terminal sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Avg molecular mass (Da) of species</th>
<th>Sequence start and end</th>
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<tr>
<td>C1</td>
<td>AELDILKPRV</td>
<td>16,058.97 16,045.60</td>
<td>AELD... LKPE</td>
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<tr>
<td>C2</td>
<td>ILKPRVPAD</td>
<td>15,691.10 15,705.87</td>
<td>ILKP... LKPE</td>
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<tr>
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<td>AKAMPPFV</td>
<td>14,316.57 14,332.22</td>
<td>AKAM... LKPE</td>
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<tr>
<td></td>
<td></td>
<td>14,571.63</td>
<td>LAAK ... LKPE</td>
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</table>

<sup>a</sup> N-terminal sequences determined in each fraction by Edman degradation. Overlap in sequences is indicated in bold type. 
<sup>b</sup> --, species not detected by MS.
larger $b$-type fragment ions verified the presence of a truncated N terminus, supporting the experimentally determined N terminus, AELDILKPRV, and provided sequence information for the first 110 amino acids of the mature protein. Interestingly, some of the smaller $y$-type fragment ions revealed truncation of the C terminus, indicating that this form of Cyt579 corresponds to the sequence AELD...LKPE of the product of gene 8062-147 lacking the C-terminal eight amino acids. The
observed mass is also consistent with removal of the heme group from the protein. However, the predicted average molecular mass of this species at 16,075.26 Da is 16 Da heavier than the measured value stated above. Further studies will determine if the discrepancy between the observed and calculated molecular masses of C1 is due to posttranslational modification or is an artifact of purification and mass spectrometry analysis.

The 15,691 Da (C2) and 14,317 Da (C3) species most closely corresponded to the 8062-372-encoded sequences ILKPR . . . . . . LKPE and AKAMP . . . . . . LKPE, respectively, based on the observed N-terminal sequences (see Table 1). The additional satellite peak at 14,572 Da in C3 was assigned to the sequence LAAAK . . . . . . LKPE, although this N-terminal sequence was not observed by Edman degradation. Based on the measured molecular masses, the C-terminal truncations are identical in the C1 to C3 samples. In each of these cases, the predicted average molecular mass based on the predicted sequence of 8062-372 was 32 Da heavier than the observed mass. To determine if an amino acid variation could account for this difference, the relevant ions from these species were isolated and fragmented by IRMPD as described above. In both C2 and C3, the fragmentation revealed a sequence tag corresponding to the amino acid sequence MFWVV GVSAGQITDK. O. Amino acid substitutions are indicated in bold type.

<table>
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<tr>
<th>Cyt579 gene</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Spectral count&lt;sup&gt;b&lt;/sup&gt; of sample from:</th>
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<tr>
<td>8062-147 (14-20)</td>
<td>MWVVVSN</td>
<td>28</td>
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<tr>
<td>8062-372</td>
<td>MFWVVSNS</td>
<td>85</td>
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<tr>
<td>8062-372 C drift (S112A)</td>
<td>MFWVVAN</td>
<td>73</td>
</tr>
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<sup>a</sup> Spectral counts are derived from peptide RTAGEMXWVVXNPSLQPM VGFVSAGQITDK. O. Amino acid substitutions are indicated in bold type.

<sup>b</sup> Spectral counts refer to the total number of MS-MS spectra taken for the peptide as an indicator of overall abundance. Each count is the average of the results for three technical replicates.

Fe(II) oxidation by Cyt579 forms. Previous work on Cyt579 purified from *L. ferriphilum* and *L. ferrooxidans* demonstrated that the oxidized form was fully reduced with excess Fe(II) at pH 2.0 (10, 17). When subjected to the same conditions as *L. ferriphilum* Cyt579 (30 mM FeSO₄, 0.2 M total SO₄²⁻), the C drift Cyt579 fraction before separation by chromatofocusing was ~30% reduced at pH 2.0, as determined by measuring the amplitude of the 579-nm band, in comparison to reduction with sodium ascorbate (data not shown). Studies of the pH dependence of Fe(II) oxidation by Cyt579 indicated that minimal oxidation occurred at pH 1 to 2, but the equilibrium shifted to reduced Cyt579 at a pH of >3, and Cyt579 was almost fully reduced in the presence of 30 mM Fe(II) at pH 4 (Fig. 6). A nearly identical pH dependence of Fe(II) oxidation was observed for the crude Cyt579 fraction obtained from the AB end biofilm, as well as the separated Cyt579 forms (C1 to C3) obtained by chromatofocusing (data not shown).

**Structural model of Cyt579.** Although no significant homology to Cyt579 was found in protein database searches, over 100 candidate structural templates for modeling Cyt579 were detected, ranging from 7% to 25% sequence identity. Secondary-structure predictions, along with high levels of structural similarities observed between the analyzed templates, narrowed the candidates to 25. An initial 3D model was constructed based on an alignment of the Cyt579 sequence with that of cytochrome c₅₅₃, 1cjjA (Fig. 7A).

Based on calculated alignments to several structural templates (including RCSB Protein Data Bank accession no. 1cjj, 2dge, 1w5c, 1ls9, 1h1o, 1jdl, 1kv9, and 1nir), the final 3D model was created, including the position of a c-type heme group from cytochrome c₅₅₃ (13). The heme in Cyt579 is likely to be different, as discussed above, due to the unique spectral character of the cytochrome (see also Discussion). This model was

![FIG. 6. pH-dependent Fe(II) oxidation by Cyt579. The results of redox experiments are shown as follows: pH 1.2 (red), pH 2.0 (gray), pH 3.0 (green), and pH 4.0 (blue). Abs, absorbance.](http://aem.asm.org/)

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compared by sequence to structure alignments and in 3D plots with selected structural templates (Fig. 7B and C). The heme orientation and structural elements were compared with the cytochrome c₆ structure, 1cyj_A (13) (Fig. 8A). Models for the two major genetic variants of Cyt579 were then superimposed to indicate the positions of all nine side chain substitutions, thioether linkages between heme and Cys68 and Cys71, and heme-Fe complex with axial ligands His72 and Met121 (Fig. 8B).

DISCUSSION

In this study, we have purified the abundant, novel bacterial cytochrome first identified by proteogenomic studies in the acidic-wash fraction of biofilms collected at the Richmond Mine in Iron Mountain, CA. We have confirmed the prediction that this protein is Cyt579, a modified c-type cytochrome that has been implicated as the Fe(II) oxidase in biochemical and physiological studies of Leptospirillum isolates (17). In the initial genomic data set obtained from a biofilm at the Richmond Mine, only one gene was sequenced that coded for Cyt579; however, two paralogs of Cyt579 were sequenced in a genomic data set from a second biofilm (15, 24). The amino acid substitutions observed in these genetic variants can be predicted in a 3D rendering of the protein structure based on homology modeling (Fig. 8B). It is noteworthy that the predicted variant residues are all located on the surface of the protein and in contact with solvent and thus do not appear to impose any perturbance to structural elements or to the putative interactions with heme.

Modeling also predicts a His-Met axial ligation for Cyt579 that is consistent with the observation of an absorption band at 695 nm and a mostly helical protein structure that is corroborated by CD spectroscopy.

Detailed biochemical studies of Cyt579 isolated from the biofilms have revealed some unexpected features of Cyt579. The alkaline pyridine hemochrome spectrum closely resem-
bles the spectrum of heme A (Soret band, 430 nm, and α band, 587 nm), suggesting that the heme in Cyt579 may contain a formyl group (3). The spectrum is also consistent with the removal of the heme from the protein, since the α band is red shifted from 579 nm to 587 nm. The presence of a CXXCH amino acid motif and the periplasmic localization of Cyt579 are evidence that this unusual heme is covalently bound to the protein, so its removal under alkaline pyridine conditions is unexpected. One interpretation of this result is that the covalent thioether linkages of the modified heme in Cyt579 are more sensitive to alkaline pH than those of conventional c-type cytochromes.

The second unexpected feature of Cyt579 was the isolation of three forms of the protein, truncated at different sites on the N terminus. One of these forms, with a detected N-terminal sequence of AELDILKPRV, was consistent with removal of the predicted signal peptide; however, the other two forms may result from additional proteolysis. Cyt579 from L. ferrispiillum was isolated in one form, corresponding to an N terminus of AELDILKPRV, that is identical to the highest-molecular-weight form of Cyt579 from the biofilm (17). These truncations may be due to proteolytic activity during the preparation of Cyt579; however, identical N-terminal sequences were observed for Cyt579 preparations from the AB end and C drift biofilms, suggesting that the cleavages are not random and are posttranslational modifications that occur in vivo. N-terminal cleavage sites of Cyt579 have been correlated with the different stages of the biofilm life cycle, establishing their ecological relevance (S. W. Singer and M. P. Thelen, unpublished results).

Accurate molecular-mass values for each of the forms of Cyt579 were determined by intact-protein analysis using MS. This confirmed the N-terminal cleavage sites observed by Edman degradation and revealed a C-terminal cleavage site. A particularly significant finding was that a sequence variant of Cyt579 in the C drift sample was not observed in environmental genomic sequences obtained from Richmond Mine biofilms. The sequence was identified by fragmenting the intact protein and isolating a sequence tag that contained an Ala to Ser variation. The presence of the sequence variant was verified by MS-MS analysis of tryptic peptides. High-resolution intact-protein MS will be invaluable in discriminating between variants of the protein isolated from the environment, allowing the correlation of protein variation with changes in environmental conditions.

FIG. 8. Structural comparison and variants of Cyt579. (A) Structure of cytochrome c6, 1cylA (13), compared with the final model of Cyt579, predicting heme orientation, covalent binding with two Cys residues, and iron coordination complex with axial His and Met residues. (B) Amino acid substitutions are depicted for the two major variants of the Cyt579 gene, CG14-20 (red) and 8062-372 (blue) (see Fig. 1 for sequence alignment).
The third unexpected feature of Cyt579 was that Fe(II) oxidation was not favored thermodynamically at a pH of <3. This result is inconsistent with the results of previous studies with *Leptospirillum* isolates, where complete reduction of Cytochromes in the presence of 30 mM Fe(II) was observed at pH 2, and casts doubt on the proposed role of Cyt579 as the Fe(II) oxidase for *Leptospirillum* group II bacteria (10, 17).

The properties of Cyt579 from *Leptospirillum* group II bacteria are analogous to those of rusticyanin, a periplasmic Cu-containing protein expressed by *Acidithiobacillus ferrooxidans*, an acidophilic Fe(II)-oxidizing bacterium found in environments similar to those where members of *Leptospirillum* group II are found. Biochemical and transcriptomic evidence has implicated rusticyanin as the initial electron acceptor for an Fe(II) oxidase for *A. ferrooxidans* (25–27). In support of this analogy, we have recently purified a novel membrane cytochrome, Cyt572, that is expressed by *Leptospirillum* group II in the Richmond biofilms (11). In contrast to Cyt579, Cyt572 oxidizes Fe(II) at low pH and may donate electrons to Cyt579. Efforts to reconstruct the Fe(II)-dependent electron transfer pathway in *Leptospirillum* group II bacteria and clarify the role of Cyt579 in this pathway are currently under way.

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