6. *C*-type cytochrome, soluble and membrane protein analysis of *Rhodobacter* sp SW2 and *Rhodopseudomonas palustris* TIE-1

ABSTRACT

The ability to grown on Fe(II) is thought to be a primitive metabolism and of the bacteria able to use Fe(II) as a source of energy for growth, it is believed that the anoxygenic phototrophs are the most ancient. Substantiation of this hypothesis requires phylogenetic investigations of the enzymes involved in this metabolism, particularly the enzyme that catalyzes the oxidation of Fe(II); however, the identity of this enzyme remains unknown. The high reduction potentials of Class I *c*-type cytochromes and existing precedent for the involvement of *c*-type cytochromes in Fe(II) oxidation by *Acidithiobacillus ferrooxidans* make a protein of this type a strong candidate for the role of Fe(II) oxidase in Fe(II)-oxidizing phototrophs. To identify components involved in photoautotrophic Fe(II) oxidation, and potentially the Fe(II) oxidase, we characterized the soluble, membrane and *c*-type cytochrome protein profiles of the Fe(II)-oxidizing phototrophs, *Rhodobacter* sp SW2 and *Rhodopseudomonas palustris* TIE-1, grown on different electron donors, and in particular on Fe(II). *C*-type cytochromes and other proteins unique or more highly expressed under Fe(II) growth conditions in *Rhodobacter* sp SW2 and *Rhodopseudomonas palustris*

TIE-1 were observed. Whether these proteins are involved in phototrophic Fe(II)-oxidation by these strains is under current investigation.

INTRODUCTION

The photosynthetic electron transport chain of purple non-sulfur bacteria of the *Rhodospirillaceae* family contains two multi-subunit transmembrane proteins: the reaction center and the cytochrome bc_1 complex. While exceptions exist, in these types of bacteria, the cyclic electron flow between these two complexes that results in ATP formation is mediated by the membrane soluble quinone pool in the cytoplasmic membrane and cytochrome c_2 (Cyt c_2), located in the periplasmic space [119]. Electrons from inorganic substrates such as H_2 or S^{2-}, enter the cyclic electron transport chain via these soluble carriers in a reaction that is catalyzed by enzymes specific to growth on the respective substrate [64, 178].

In a bicarbonate containing system the relevant Fe couple, $Fe(OH)_3$ + $HCO_3^-/FeCO_3$, has a high redox potential of +0.2 V [52]. Thus, in purple non-sulfur, anoxygenic phototrophs able to use Fe(II) as an electron donor for photosynthesis, a carrier(s) that mediates electron transfer between Fe(II) and the photosynthetic electron transport chain must have a redox potential higher than +0.2 V. Because the reduction potential of the ubiquinone pool (+0.113 V) is higher than that of the Fe(II) couple, it is unlikely that electrons from Fe(II) enter the chain at this point. It has been proposed that electrons from Fe(II) enter the electron transport chain of these organisms via Cyt c_2 directly [52].

However, it is also possible that entry of these electrons into the cyclic electron flow is mediated by an enzyme unique to growth on Fe(II) (*i.e.*, an Fe(II) oxidase), similar to the case of H_2 and S^{2-}.

Class I *c*-type cytochromes, of which bacterial Cyt c_2 is a representative [16], have reduction potentials that vary from +0.2 - +0.35 V [9], making them able to accept electrons from Fe(II). Given this, and the fact that *Rhodobacter capsulatus* has at least 12 *c*-type cytochromes and *Rhodopseudomonas palustris* and *Rhodobacter sphaeroides* each have at least 21 (some of which have no known function) [118], a *c*-type cytochrome is a likely candidate for the role of Fe(II) oxidase. In addition, a number of *c*-type cytochromes have been implicated in Fe(II) respiratory chain of *Acidithiobacillus ferrooxidans* [189], an obligately autotrophic and acidophilic bacterium capable of aerobic respiration on Fe(II) and reduced forms of sulfur (H_2S, S^o, $S_2O_3^{2-}$) [53, 139]. Further, it has been proposed that one of them, the product of the *cyc2* gene, Cyc2, is the primary acceptor for electrons from Fe(II) [7].

Another protein implicated in the Fe(II) respiratory chain of *Acidithiobacillus ferrooxidans* that is also postulated to be the primary electron acceptor in some strains is the high potential iron-sulfur protein (HiPIP), encoded by the *iro* gene [63, 104]. HiPIPs, have redox potentials in the range of +0.05 to +0.45 V and are also commonly found in purple photosynthetic bacteria [120]. These soluble ferredoxins are found primarily in purple sulfur bacteria of the *Chomatiaceae and Ectothiorhodospiraceae* families, but are also found in some *Rhodospirillaceae* [118]. In these bacteria, it is thought that these HiPIPs can

serve the same purpose as Cyt c_2, that is, to mediate electron flow between the reaction center and the cytochrome bc_1 complex [118]. Thus, another potential candidate for the role of Fe(II) oxidase may be a HiPIP.

Because *c*-type cytochromes can be easily detected through specific staining on polyacrylamide gels [61], we characterized the *c*-type cytochrome contents of the Fe(II) oxidizing phototrophs, *Rhodobacter* sp SW2 and *Rhodopseudomonas palustris* TIE-1, grown on different electron donors and in particular, on Fe(II). We have also begun investigations of the membrane and soluble proteins of these two bacteria to identify proteins expressed exclusively under Fe(II) growth conditions. Protocols with which to identify proteins with Fe(II) oxidation activity in polyacrylamide gels exist [46] and it is our goal to identify a protein(s) with such activity.

EXPERIMENTAL PROCEDURES

Organisms and cultivation

Cultures of *Rhodopseudomonas palustris* TIE-1 (TIE-1) and *Rhodobacter* sp. SW2 (SW2) were maintained in a previously described anoxic minimal salts medium for freshwater cultures [52] and were incubated 20 to 30 cm from a 34 W tungsten, incandescent light source at 30°C for TIE-1 and 16°C for SW2. *Rhodobacter capsulatus* SB1003 (1003) grown photoheterotrophically on RCV [180] and incubated at 30°C. Electron donors for photosynthetic growth were added to the basal medium as follows: thiosulfate was added from an anoxic filter sterilized stock to a final concentration of 10 mM; acetate was added from a 1 M,

filter sterilized, anoxic solution at pH 7 to a final concentration of 10 mM, and H_2 was provided as a headspace of 80% H_2: 20% CO_2. For growth on Fe(II), 4 mls of a filter sterilized, anoxic 1 M Fe(II)$Cl_2 \cdot H_2O$ stock solution was added per 1 liter (L) of anaerobic, basal medium (final concentration ~4 mM). To avoid the precipitation of ferrous Fe minerals that results upon addition of Fe(II)$Cl_2 \cdot H_2O$ to the bicarbonate buffered basal medium and the precipitation of ferric Fe minerals that form during the growth of these bacteria on Fe, the metal chelator, nitrilotriacetic acid (NTA, disodium salt from Sigma), was supplied from a 1 M filter sterilized stock solution to a final concentration of 10 mM. This NTA addition greatly facilitated the harvesting of cells, free of Fe minerals, from Fe(II) grown cultures.

Soluble and membrane protein extraction

To extract soluble and membrane protein fractions from SW2, 1 L cultures of this strain grown phototrophically on H_2, acetate and Fe(II) were harvested in exponential phase by centrifugation (10,000 rpm in a Beckman JLA 10.5 rotor for 20 min). The pellets were resuspended in 3 mls of 10 mM HEPES buffer at pH 7 and sonicated on ice for a total of 5 minutes using a 10 second on/10 second off program. 18 µl of a 0.2 M PMSF stock (0.0348 g in 1 ml 100% EtOH) and 1 µl of a 50 mg/ml DNAse stock were added and the lysate was incubated 30 min on ice. After incubation, cell debris was removed by centrifugation (6000 rpm on a Beckman JLA 10.5 rotor for 30 min at 4ºC). The supernatant from this centrifugation was subjected to ultracentrifugation at 200,000 x g for 90 min at

4ºC. The resultant supernatant represented the soluble protein fraction. The resultant pellet was resuspended in 50 µl buffer to give the membrane protein fraction. Soluble and membrane protein fractions from *Rhodobacter capsulatus* SB1003 grown phototrophically on RCV, a condition under which *c*-type cytochromes are known to be expressed in a similar manner to that described above.

To extract soluble and membrane protein fractions from TIE-1, ~10 L of cells grown phototrophically on H_2, thiosulfate and ~ 50 L of cells grown phototrophically on Fe(II) were harvested in exponential phase by centrifugation (10,000 rpm on a Beckman JLA 10.5 rotor for 20 min). The pellets were washed and resuspended in 20 mls of buffer (50 mM HEPES, 20 mM NaCl, pH 7), DNAase and protease inhibitors were added and the suspension was subjected to 3 passages through a French pressure cell at 18,000 psi. Cell debris was removed by low speed centrifugation (10,000 x g for 20 minutes) and soluble and membrane protein fractions were isolated as described above.

Protein concentrations were measured using the Bio-Rad protein assay (Hercules, CA). For SW2 sample storage, glycerol was added to a final concentration of 10% before freezing the sample at -20ºC. TIE-1 samples were frozen with liquid N_2 and stored at -80 C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and gel staining

SDS-PAGE was performed by standard procedures according to the Laemmli method [1], using dithiothreitol (DTT) as the reducing agent in the Laemmli sample buffer. For SW2, 15 µg protein samples of the soluble and membrane protein fractions from the different cultures were incubated with sample buffer for 5 min at 25°C. These samples were then separated on a 12% polyacrylamide gel at a current of 20 mA. For TIE-1, 100 µg samples of crude cell extract and soluble fraction and 60 µg of the membrane fraction from the different cultures were prepared as described above. Here, the samples proteins were separated on a 4-20% Tris·HCl mini-gradient pre-cast gel from Biorad at a current of 25 mA.

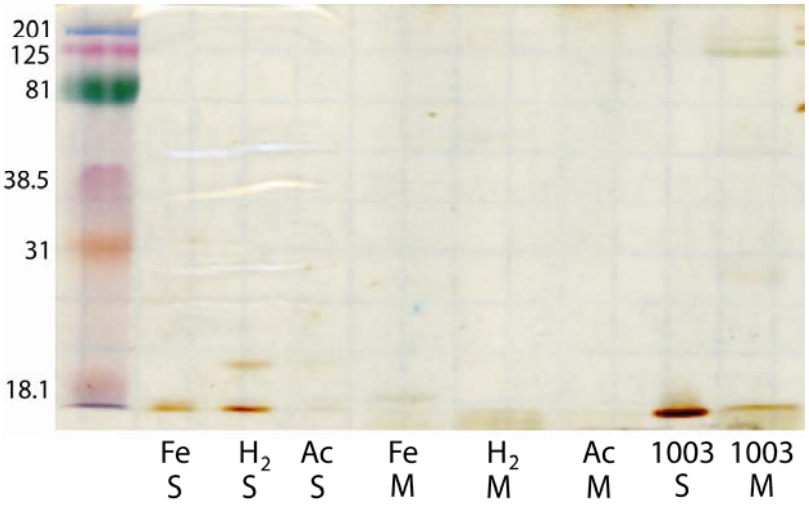
Gels were stained for protein with the Bio-Safe Coomassie Stain from Bio-Rad. Gels were stained for heme-containing proteins according to the in-gel peroxidase activity assay of Francis and Becker [61]. Here, the gel was first incubated in 12.5% trichloroacetic acid for 30 minutes and then washed with dH_2O for 30 minutes. After these incubations, the gel was transferred to a solution containing 20 mls of 0.5 M Na-citrate buffer (pH 4.4), 0.4 mls of 30% H_2O_2 and180 mls of a freshly prepared solution of *o*-dianisidine (200 mg *o*-dianisidine (Sigma), 180 mls dH_2O). The staining reaction was allowed to proceed from 2 hours to overnight.

RESULTS

C-type cytochromes and other proteins unique to Fe(II) growth in SW2

Heme and protein stains of soluble and membrane proteins of SW2 grown on Fe(II), H_2, and acetate, separated by SDS-PAGE, are shown in Figure 6-1A and 6-1B, respectively. Here, a *c*-type cytochrome of approximately 15 kDa (kiloDaltons) that appears unique to the membrane fraction of Fe(II)-grown cells was observed (Figure 6-1A). This cytochrome was not present in 1003, a strain that is unable to grow photoautotrophically on Fe(II). High molecular weight non-*c*-type cytochrome proteins that appear to be unique to the membrane fraction of Fe(II)-grown SW2 cells were also observed (Figure 6-1B). It is important to note, however, that the concentration of protein loaded here was low (15 µg). For example, the *c*-type cytochromes (likely cyc_1 or cyc_y [81]), present in 1003 of approximately 30 kDa, are very faint. Thus, it is possible that the cytochrome present under Fe(II) growth conditions also exists in the cells grown on H_2 and acetate, but its concentration in our sample is below the detection limit of the peroxidase activity assay. This caveat stands for the unique proteins identified in the gel stained for protein as well.

A.


201
125
81
38.5
31
18.1
Fe S
H2 S
Ac S
Fe M
H2 M
Ac M
1003 S
1003 M

B.

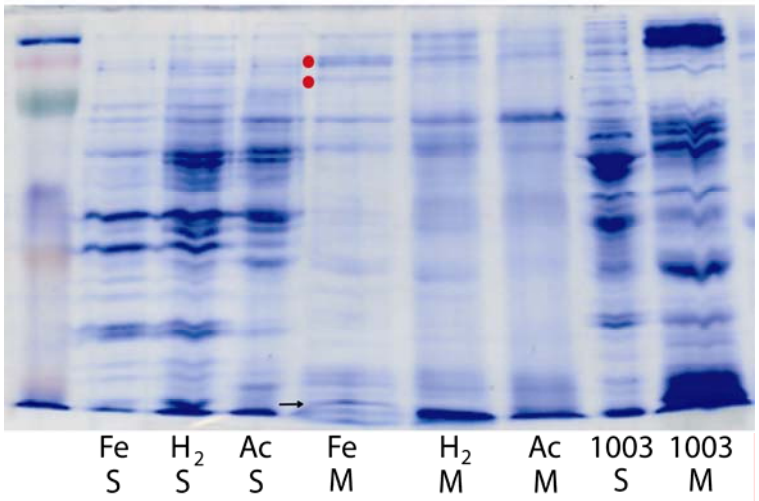
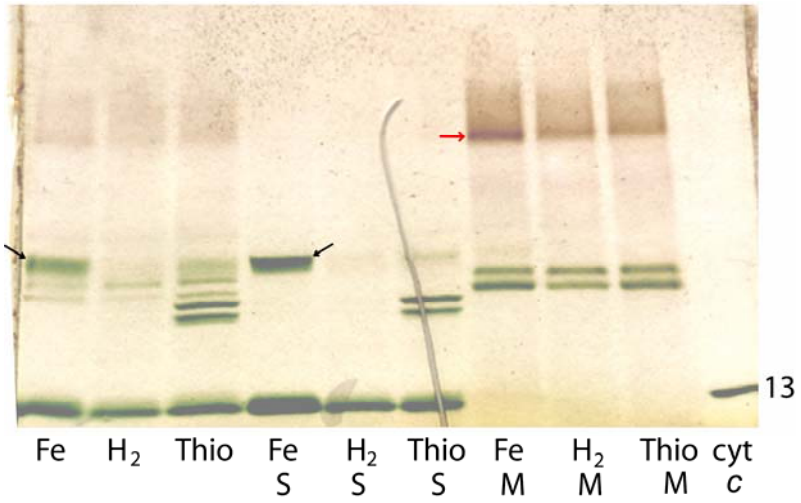

Fe S
H2 S
Ac S
Fe M
H2 M
Ac M
1003 S
1003 M

Figure 6-1: A. Heme stain of soluble (S) and membrane (M) proteins of SW2 cells grown phototrophically on Fe(II), H_2, and acetate, and *Rhodobacter capsulatus* SB1003 grown photoheterotrophically on RCV. The arrow highlights a *c*-type cytochrome of approximately 15 kDa that appears unique to the membrane fraction of Fe(II)-grown SW2 cells. B. Total soluble and membrane protein profiles of SW2 grown on Fe(II), H_2, and acetate. The red dots highlight proteins that appear unique to the membrane fraction of Fe(II)-grown cells and the arrow identifies the protein that corresponds to the heme in part A.

C-type cytochromes upregulated under Fe(II) growth conditions and other proteins unique to Fe(II) growth conditions in TIE-1

Heme and protein stains of soluble and membrane proteins of TIE-1 cells grown on Fe(II), H_2, and thiosulfate, separated by SDS-PAGE, are shown in Figure 6-2A and 6-2B, respectively. In the heme stain, we observed *c*-type cytochromes of approximately 35 kDa that were much more highly expressed in the crude and soluble fractions of Fe(II)-grown TIE-1 cells (Figure 6-2A). In addition, it seems an approximately 90 kDa *c*-type cytochrome associated with the membrane fraction that is more highly expressed under Fe(II) growth conditions (Figure 6-2A). On a gel stained for protein, an approximately 23 kDa, non-*c*-type cytochrome protein that appears unique to Fe(II) grown cells is present in soluble protein fraction (Figure 6-2B).

A.


13
Fe
H2
Thio
Fe S
H2 S
Thio S
Fe M
H2 M
Thio M
cyt c

B.

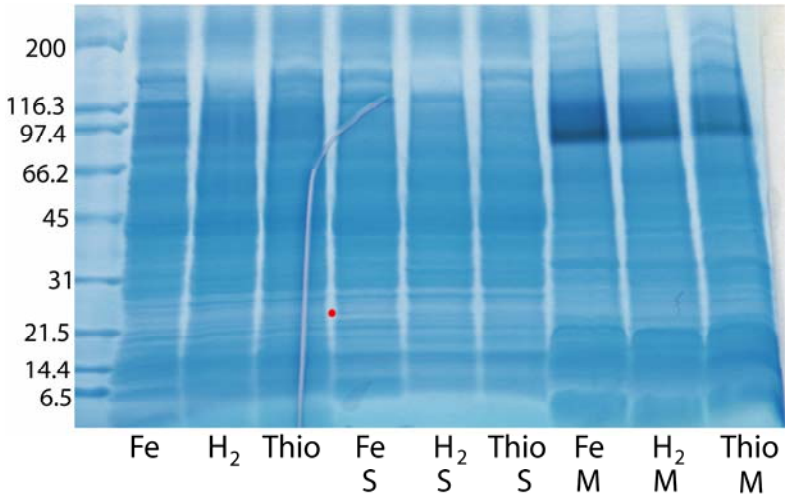

200
116.3
97.4
66.2
45
31
21.5
14.4
6.5
Fe
H2
Thio
Fe S
H2 S
Thio S
Fe M
H2 M
Thio M

Figure 6-2: A. Heme stain of soluble (S) and membrane (M) proteins of TIE-1 cells grown on Fe(II), H_2, and thiosulfate, separated by SDS-PAGE. The black arrows highlight *c*-type cytochromes of approximately 35 kDa that are much more highly expressed in the crude and soluble fractions of Fe(II)-grown cells. In addition, there is a *c*-type cytochrome of approximately 90 kDa that is more highly expressed in the membrane fraction (indicted by the red arrow). B. Total soluble and membrane protein profiles of TIE-1 grown on Fe(II), H_2, and acetate. The red dots highlight proteins that appear unique to the soluble fraction of Fe(II)-grown cells.

DISCUSSION AND FUTURE WORK

Preliminary work presented here provides evidence that *c*-type cytochromes and other proteins unique or more highly expressed under Fe(II) growth conditions are present in *Rhodobacter* sp. SW2 and *Rhodopseudomonas palustris* TIE-1. Whether these proteins are involved in phototrophic Fe(II)-oxidation by these strains remains to be investigated, however precedent for *c*-type cytochromes being involved in Fe(II) respiratory processes exists [6, 38, 174, 177, 189]. Further, the redox potentials of *c*-type cytochromes are consistent with the hypothesis that the enzyme that shuttles electrons from Fe(II) to the photosynthetic electron transport chain is a *c*-type cytochrome.

Interestingly, the product of the *cyc2* gene of *Acidithiobacillus ferrooxidans* is a high molecular weight *c*-type cytochrome (46 kDa) that is localized to the outer membrane and this protein is proposed to catalyze the first step in the

transfer of electrons from Fe(II) to O_2 by this organism [6]. While the 35 kDa *c*-type cytochrome we observed in TIE-1 appears to be soluble, the similarity here is encouraging. In addition, a gene that is predicted to encode a putative deca-heme *c*-type cytochrome with similarity at the C-terminal to MtrA (a cytochrome involved in Fe(III) respiration in *Shewanella oneidensis* MR-1), is found in the genome of *Rhodopseudomonas palustris* CGA009 (CGA009), a strain unable to growth photosynthetically on Fe(II), and can be amplified by PCR from TIE-1 (Figure 6-3, [82]). The predicted product of this gene has a multi-copper oxidase copper binding motif at the N-terminal. Proteins with such motifs have been implicated in divalent metal oxidation coupled to growth in *Acidithiobacillus ferrooxidans*, *Leptothrix discophora*, *Pseudomonas putida, Bacillus SG-1* and some eukaryotic organisms [26].

A gene with similarity to the *iro* gene of *Acidithiobacillus ferrooxidans* (predicted to encode an Fe oxidase in some strains of this organism) is also found in the genome of CGA009, and is detected in TIE-1 (Figure 6-3). A gene that is predicted to encode a cytochrome of 90 kDa, however, is not found in the genome of CGA009. Thus, it is possible that this cytochrome is unique to *Rhodopseudomonas palustris* strains able to growth on Fe(II). Finally, a gene predicted to encode an outer membrane protein, homologous to MtrA, an outer membrane protein involved in Fe(III) respiration in *Shewanella oneidensis*, is found in the same cluster as the two genes described above (Figure 6-3).

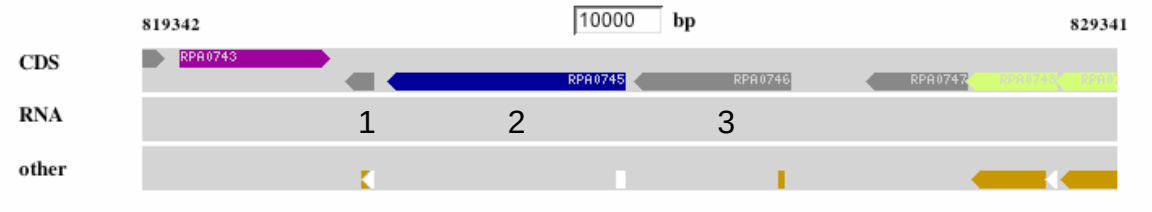


Figure 6-3: Gene cluster in *Rhodopseudomonas palustris* GCA009 with homologs in TIE-1 containing genes encoding proteins with possible function in photoautotrophic Fe(II) oxidation. 1: A gene predicted to encode a high redox potential Fe-S protein that is homologous to the *iro* (possible iron oxidase) in *Acidithiobacillus ferrooxidans*. 2: A gene predicted to encode an outer membrane protein, homologous to MtrA, (an outer membrane protein involved in Fe(III) respiration in *Shewanella oneidensis*. 3: The predicted product of this gene has a multi-copper oxidase copper binding motif at the N-terminal and a C-terminal sequence is homologous to a deca-heme cytochrome *c* in *Shewanella*.

Current efforts are underway to construct mutants of these genes in TIE-1 and test their Fe(II) oxidation capabilities. Finally, we are also working to obtain N-terminal sequence for the observed *c*-type cytochromes of TIE-1 and SW2 and to develop an in-gel assay to test if these cytochromes or the other proteins unique to Fe(II) growth conditions in these strains have Fe(II)-oxidation activity.

Once components of photoautotrophic growth on Fe(II) are identified, we can begin to uncover the degree to which electron transfer from Fe(II) is conserved among phototrophs and other bacteria able to oxidize Fe(II). In addition, knowledge of the components involved in this form of metabolism will direct our efforts to identify traces of Fe(II) oxidation in the rock record [41].