PHYSIOLOGICAL AND MECHANISTIC STUDIES

OF PHOTOTROPHIC FE(II) OXIDATION IN

PURPLE NON-SULFUR BACTERIA

Thesis by

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Abstract

Phototrophic Fe(II)-oxidizing bacteria use electrons from ferrous iron [Fe(II)] and energy from light to drive reductive CO_2 fixation. This metabolism is thought to be ancient in origin, and plays an important role in environmental iron cycling. It has been implicated in the deposition of Banded Iron Formations, a class of ancient sedimentary iron deposits. Consistent with this hypothesis, we discovered that hydrogen gas, a thermodynamically favorable electron donor to Fe(II), in an Archean atmosphere would not have inhibited phototrophic Fe(II) oxidation. To understand this physiology and the connection to BIF formation at the molecular level, the mechanisms of phototrophic Fe(II) oxidation were examined in two purple non-sulfur bacteria, *Rhodopseudomonas palustris* TIE-1 and *Rhodobacter* sp. SW2.

Important advances were made in elucidating genes critical to phototrophic Fe(II) oxidation. In *R. palustris* TIE-1, the first genetically tractable phototrophic Fe(II) oxidizer isolated, transposon mutagenesis identified a putative integral membrane protein and a potential cobalamin (vitamin B_{12}) biosynthesis protein involved in Fe(II) oxidation. Increased expression of a putative decaheme *c*-type cytochrome, encoded by *pioA*, was observed when cells were grown under Fe(II)-oxidizing conditions. Two genes located immediately downstream of *pioA* in the same operon, *pioB* and *pioC*, encode a putative outer membrane beta-barrel protein and a putative high potential iron-sulfur protein, respectively. Deletion studies demonstrated that all three genes are involved in phototrophic Fe(II) oxidation.

In *Rhodobacter* sp. SW2, a three-gene operon, *foxEYZ,* was found to be involved in phototrophic Fe(II) oxidation through heterologous expression in a close relative,

Rhodobacter capsulatus SB1003. The first gene, *foxE*, encodes a novel *c*-type cytochrome located in the periplasm. Expression of *foxE* alone confers light-dependent Fe(II) oxidation activity to SB1003, but maximal activity is achieved when *foxE* is co-expressed with *foxY* and *foxZ*. FoxY appears to contain the redox cofactor pyrroloquinoline quinone and FoxZ a cytoplasmic membrane transporter. Recombinant PioC was overexpressed and partially purified from *Escherichia coli*.

This research presents a detailed study of the physiology and genetics of phototrophic Fe(II) oxidation in two purple non-sulfur bacteria, and provides our first insight into the molecular mechanisms of this metabolism.

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Chapter 1

Introduction

1.1   RESEARCH OBJECTIVES

The goal of this research is to examine the molecular mechanisms of Fe(II)

oxidation by anoxygenic bacteria. These bacteria grow photoautotrophically by coupling

electrons derived from Fe(II) oxidation with reductive CO_2 fixation powered by energy

from light. It has been hypothesized that these Fe(II)-oxidizing phototrophs played an

important role in the deposition of Banded Iron Formations (BIFs), a class of sedimentary

iron deposits formed during the early history of the Earth. The Fe(II) oxidation capability

of two strains of such bacteria was measured under the conditions relevant to the inferred

H_2 and CO_2 level of the ancient Earth. We found that phototrophic Fe(II) oxidation is not

significantly inhibited by the presence of H_2 gas, a thermodynamically favorable electron

donor to Fe(II), consistent with the hypothesis that these bacteria (or their ancient

counterparts) were involved in the deposition of ancient BIFs. However, in order to test

this hypothesis, we must be able to identify potential biomarkers preserved in ancient

rocks that are unique to this metabolism. Therefore, the elucidation of the molecular

components of the phototrophic Fe(II) oxidation pathway is critical (see section 2.1).

Accordingly, we have examined the mechanism of phototrophic Fe(II) oxidation

in two purple non-sulfur bacteria: *Rhodopseudomonas palustris* TIE-1 (TIE-1) and

Rhodobacter sp. SW2 (SW2). By combining physiology, genetics, and biochemistry

studies we have identified key molecular components essential for phototrophic Fe(II) oxidation in both systems. This research is the first mechanistic description of phototrophic Fe(II) oxidation and has established a basis for a detailed molecular understanding of this metabolism.

1.2   THESIS OUTLINE

I will first describe the motivation for this research and the geological relevance of phototrophic Fe(II) oxidation in the deposition of BIFs in chapter 2 (section 2.1). Further details concerning the search for biomarkers and why we think understanding the molecular basis of phototrophic Fe(II) oxidation is a key step toward this goal are highlighted. I provide additional background information relevant to my research that includes physiological studies about phototrophic Fe(II)-oxidizing bacteria and their environmental relevance (section 2.2), and biochemical studies on aerobic Fe(II) oxidation by the acidophilic Fe(II) oxidizing bacterium *Acidothiobacillus ferrooxidans* (section 2.3).

In chapter 3, I describe our studies on the effect of molecular hydrogen (H_2) on phototrophic Fe(II) oxidation with TIE-1 and SW2 to evaluate the role of phototrophic Fe(II)-oxidizing bacteria within a geological context. Both H_2 and Fe(II) can serve as electron donors for anoxygenic photosynthesis and are predicted to have been present in the atmosphere and ocean during the Archean time in quantities sufficient for phototrophic energy metabolism. Under the conditions with approximate H_2 and CO_2

concentrations relevant to those proposed in ancient oceanic environments, we tested the

Fe(II) oxidation capability of TIE-1 and SW2 using pure cell suspensions in the lab. We

found that Fe(II) oxidation proceeds at a considerable rate under the maximum amount of

predicted H_2 concentrations, indicating that the phototrophic Fe(II) oxidation activity was

not likely to be inhibited by the presence of H_2 in the ancient atmosphere, consistent with

the hypothesis that phototrophic Fe(II)-oxidizing bacteria are involved in ancient BIF

deposition.

In order to study the molecular mechanism of phototrophic Fe(II) oxidation, we

need to have a model system to work with. However, prior to this research, none of the

previously isolated phototrophic Fe(II)-oxidizing bacteria were genetically tractable. To

address this problem, I first isolated a new phototrophic Fe(II) oxidizer that was able to

form colonies on agar plates. This isolate was identified as a strain of the purple non-

sulfur bacterium *Rhodopseudomonas palustris,* and was named strain TIE-1. The

isolation and characterization of TIE-1 are described in chapter 4, which was published as

a research article in *Applied and Environmental Microbiology*, August 2005, p. 4487--

4496, vol. 71, no. 8. Taking advantage of TIE-1's capability of forming colonies on agar

plates, I developed a genetic system in this organism, aiming to look for genes and gene

products essential for photoautotrophic growth on Fe(II). After random transposon

mutagenesis and screening of 12,000 mutants, six were found to be specifically impaired

in Fe(II) oxidation. Five of these mutants have independent disruptions in a gene

predicted to encode an integral membrane protein that appears to be part of an ATP-

dependent transporter; the sixth mutant has an insertion in a gene that is homologous to

cobS, encoding an enzyme involved in cobalamin (vitamin B$_{12}$) biosynthesis. The involvement of these proteins in Fe(II) oxidation remains unclear at this time.

Because we wanted to identify the genes that encode the catalysts for Fe(II) oxidation and none were readily apparent from our genetic screen, a biochemical approach was taken to compare protein expression profiles of TIE-1 grown on Fe(II) versus other substrates. In chapter 5, soon to be published as a research article in the *Journal of Bacteriology* (2007), I describe our discovery of two redox active proteins and a putative outer membrane protein that are the key players of Fe(II) oxidation in TIE-1. Using reverse genetics, a three-gene operon was identified and designated as the *pio* operon (for phototrophic iron oxidation). Expression of the first gene, named *pioA*, increased under Fe(II) growth conditions. PioA encodes a novel soluble decaheme *c*-type cytochrome. The second gene, *pioB,* encodes a putative outer membrane β-barrel protein, and the third gene, *pioC,* encodes a putative high-potential iron-sulfur protein (HiPIP). Mutational studies suggest these proteins interact and form a putative Fe(II) oxidoreductase complex, with PioA being the Fe(II) oxidoreductase.

Besides TIE-1, we have also examined the phototrophic Fe(II) oxidation system in another phototrophic Fe(II) oxidizer *Rhodobacter* sp. SW2. While this portion of the work was the primary work of Dr. Laura Coral, a former graduate student in the lab, I have made important contributions in showing FoxE is a *c*-type cytochrome, re-testing the Fe(II) oxidation activity of recombinant clones, and moving the cosmid library from *E. coli* into SB1003. This work was first described in Dr. Laura Croal's thesis but an updated version is presented in chapter 6 of this dissertation, and will also be published as

a research article in the *Journal of Bacteriology* (2007). SW2 is also a purple non-sulfur bacterium, but is not amenable to traditional genetic analysis. Thus, we took a heterologous expression approach by expressing a genomic cosmid library of SW2 in its close relative *Rhodobacter capsulatus* SB1003 and screened cell suspensions of each clone for enhanced light-dependent Fe(II) oxidation activity. We have identified a three-gene operon *foxEYZ*: *foxE* encodes a novel *c*-type cytochrome; *foxY* appears to encode a protein containing the redox cofactor pyrroloquinoline quinone; and *foxZ* appears to encode a protein with transport function. The results suggest that these Fox proteins play key roles in phototrophic Fe(II) oxidation in SW2.

In the final chapter of this thesis (chapter 7), I summarize the key findings of my research and provide some viewpoints and future directions pertaining to phototrophic Fe(II) oxidation in TIE-1.

As an extension of the genetic studies about *pio* and *fox* genes, I initiated biochemical characterizations of the proteins encoded by these genes. Preliminary results on the subcellular localization analysis of PioABC in TIE-1 and FoxE in SW2, are presented in Appendix A. The partial purification of PioC is presented in Appendix B.

Chapter 2

Motivation and Background

2.1   BIFS AND BIOMARKERS

A central goal of geobiology is to use the information preserved in the rock record to understand the interconnectivity between the evolution of Earth and the evolution of life (43, 46). Since microbial organisms, representing the earliest form of life, appeared on the planet, they have had an enormous impact on the geochemistry of Earth over geological time. Our goal is to examine the interplay between the metabolism of these microbes and the geochemical environment where they thrived by using modern organisms as model systems. The assumption is that the metabolism of modern organisms are good proxies of their ancestral forms.

One of the best examples lies in the examination of the physiology of phototrophic Fe(II)-oxidizing bacteria and their hypothesized role in the deposition of Banded Iron Formations (BIFs) (44, 49, 60). BIFs are a class of ancient sedimentary iron deposits formed during the Precambrian (29). They typically consist of alternating layers of chert and iron-bearing minerals such as magnetite (Fe_3O_4) and hematite (Fe_2O_3). BIFs are present on all the continents, representing one of the world's major sources of iron ore (58). The formation of the oxidized iron minerals in BIFs in an oxygen (O_2)-limited (or depleted) environment has been interpreted as the result of an anoxic process potentially catalyzed by phototrophic Fe(II)-oxidizing bacteria (61). In the absence of oxygen these

bacteria, belonging to a group of Purple and Green bacteria, use Fe(II) as an electron donor and couple Fe(II) oxidation with reductive CO_2 assimilation by using energy from light (14 and reference therein, 31, 66). Interpretation of phylogenetic analyses of 16S rDNA and bacterial chlorophyll biosynthesis genes combined with the prediction of abundant Fe(II) in ancient oceans have led to the suggestion that phototrophic Fe(II) oxidation is one of the most ancient forms of metabolism (45, 48, 61, 67, 68). Theoretical calculations based on the predicated depositional rate of iron oxides in the Hamersley basin of Australia and the measured rate of phototrophic Fe(II) oxidation by pure cultures suggest that these bacteria could have been capable of oxidizing and precipitating the large amounts of the Fe deposited in BIFs (37, 38). However, in order to test the hypothesis that ancient Fe(II)-oxidizing phototrophs are indeed responsible for early BIF formation, we need to look for biomarkers unique to this metabolism preserved in the rock record. Different approaches have been taken aimed at finding specific characteristics that could only be ascribed to this type of metabolism.

(1) The mineral products of phototrophic Fe(II) oxidation were examined for distinctive features, such as morphology and mineralogy, in hopes of distinguishing whether Fe(III) minerals were formed biologically or abiotically. The product of phototrophic Fe(II) oxidation is generally poorly crystalline ferrihydrite that forms outside the cell (57) that gradually transforms into more crystalline iron oxides, such as goethite and lepidocrocite (36). During diagenesis, goethite and lepidocrocite can be further transformed into more stable forms such as hematite, one of the major iron constituents in BIFs. Because physical and chemical properties of Fe(III) precipitates

formed by bacterial cultures are similar to their abiotic counterparts (11, 25, 36) and both reaction types are strongly dependent on the medium chemistry (34), it remains difficult to elucidate the exact role of microbes in BIF formation from this type of mineral analysis.

(2) Isotopic signatures have been examined for a few of the phototrophic Fe(II)-oxidizing bacteria with respect to their ability to fractionate Fe isotopes (15). Rate independent fractionation ($^{56}Fe/^{54}Fe$ ratio) of ~ +1.5‰ was observed in the oxidation product of poorly crystalline (hydro)ferroxide relative to aqueous Fe(II). This number is comparable to that observed for Fe isotope fractionation by abiotic Fe(II) oxidation by O_2 or dissimilatory Fe(III)-reducing bacteria, suggesting that use of Fe isotopes to identify phototrophic Fe(II) oxidation in the rock record is limited unless the precipitation of Fe(III) minerals can be decoupled with Fe(II) oxidation for Fe isotope measurements (4, 15).

(3) Given the results from studies of Fe(III) minerals and Fe isotope fractionation, it is evident that it is non-trivial to find a robust biosignature for phototrophic Fe(II) oxidation. The Fe(II) oxidation process can be divided into two steps: [1] biologically catalyzed Fe(II) oxidation and [2] passive nucleation and precipitation of (hydro)ferroxides. Previous to the work outlined in this dissertation, nothing was known about the molecular basis of phototrophic Fe(II) oxidation. However, the abiotic chemistry involved Fe(III) precipitation is well understood. We hypothesize that Fe(III), as a byproduct of Fe(II) phototrophy, is released from the cell in soluble, colloidal or ligand-bound forms. Fe(III) oxide nucleation takes place near the cell surface

driven by passive sorption and nucleation reactions (11, 63) through electrostatic attractions between the negatively charged cell wall and positively charged Fe(III) molecules (17, 24, 29).

Mineral encrustation of bacterial cell walls is not evident during Fe(II) oxidation for most of the phototrophic Fe(II) oxidizers including TIE-1 and SW2, which implies that biologically produced substances may be present to prevent cells from encrustation and cell death, since mineral precipitation on the surface is likely to act as a barrier for solute transport. Such biologically produced substances could be Fe(III) ligands (41, 62) or extracellular polymeric substances (EPS) (65 and reference therein). Ligands could keep positively charged Fe(III) from precipitating on the negatively charged cell wall. Similarly, EPS could provide templates for Fe(III) sorption and nucleation that are at a distance from the cell surface. It seem unlikely that large quantities of soluble strong Fe(III) ligands are present during phototrophic Fe(II) oxidation, based on examination of a few cultures of phototrophic Fe(II)-oxidizing bacteria (36). Thus, these Fe(III) ligands, if present, are probably either weak ligands or are not released into the bulk solution. Biologically produced organic rich extracellular polymers produced by neutrophilic Fe(II) oxidizing bacteria were shown to be able to promote Fe(III) mineral precipitation (10). However, to our knowledge, EPS production has not been linked to Fe(III) precipitation in Fe(II) phototrophy. Additionally, the protons generated during Fe(III) precipitation lower the pH of the local environment and may further reduce the chance of potential Fe(III) precipitation. Indeed, a decrease in pH from 6.50 to 6.25 was observed from the edge to the center of the colonies as a consequence of the metabolism of

phototrophic Fe(II) oxidation (36). Whether such a decrease in pH is sufficient to prevent Fe(III) precipitation remains unclear.

(4) In order to understand how the Fe(III) mineral nucleation and precipitation occurs and what Fe(III) ligands (organic or inorganic), if any, are involved in this process, we need to understand the molecular mechanisms of biologically catalyzed Fe(II) oxidation. This includes understanding where Fe(II) is oxidized, how Fe(II) and Fe(III) are trafficked intra- and extracellularly, the enzymes involved in Fe(II) oxidation, and how and where the last step of ferric (hydro)oxide precipitation occurs. In other words, elucidation of the molecular components of the phototrophic Fe(II) oxidation pathway is necessary both to constrain our interpretation of the Fe-isotopic fractionation produced by these bacteria and to identify biomarkers unique to this metabolism that might be preserved in the rock record. The results presented in this dissertation on the molecular basis of phototrophic Fe(II) oxidation provide important information for searching for imprints of this type of metabolism left behind from the ancient Earth.

2.2   PHOTOTROPHIC FE(II) OXIDATION

The first example of phototrophic Fe(II)-oxidizing bacteria was reported by Widdel et al. over a decade ago (66). In contrast to oxygenic photosynthesis (such as that carried out by plants) where water is split to produce O_2, these bacteria use Fe(II) as the electron donor, and no O_2 is produced in a process called anoxygenic photosynthesis. Besides Fe(II), many other reducing compounds can also support anoxygenic

photosynthesis, including H_2, reduced sulfur compounds, as well as various organic

compounds (7). During anoxygenic photosynthesis, these bacteria conduct a cyclical

light-driven electron transport chain through which a proton motive force is formed

across the cell membrane that is later used for ATP synthesis (7). Some electrons are

diverted out from the cyclic flow to NADH dehydrogenase, through which NAD(P)H is

generated for reductive CO_2 fixation. The electrons removed from the cyclic flow are

replaced by the oxidation of the external electron donor, such as H_2 or Fe(II).

Thermodynamic calculations of the redox potential of the iron couple [Fe(II)/Fe(III)] in a

bicarbonate system at neutral pH is in the range of -100 to $+200$ mV (34, 66), which is

low enough to donate electrons to the reaction center of purple (E_0', $+450$ mV) or green

bacteria (E_0', $+300$ mV) and therefore provide reducing power to sustain microbial

growth (7).

To date, the phototrophic Fe(II)-oxidizing bacteria that have been isolated are

phylogenetically diverse, including members of the purple sulfur (*Thiodictyon* sp. F4),

purple non-sulfur (*Rhodobacter* sp. SW2, *Rhodovulum iodosum* N1, *Rhodovulum*

robiginosum N2, *Rhodopseudomonas palustris* TIE-1) and green sulfur bacteria

(*Chlorobium ferrooxidans* KoFox) (20, 30, 34, 56, 57, 66). F4, a gas vesicle containing

strain, was isolated from the sediment surface of a freshwater pond in Woods Hole, MA

(20, 66). It grows on Fe(II) but not on sulfide, and cells are not physically associated

with the Fe(III) precipitates. SW2, isolated from a pond in Germany, can grow

phototrophically using Fe(II) or H_2 as electron donors, but not free sulfide (20, 66). One

mM of sulfide was used, but the inhibitory effect of sulfide at this concentration was not

tested. However, SW2 can grow on ferrous sulfide minerals (FeS, 5 mM) with the production of Fe(III) and sulfate (20), suggesting that growth inhibition from sulfide is concentration dependent. N1 and N2, isolated from the coastal sediments of the North Sea (Germany), are the only marine isolates found so far, and their cell growth is salt dependent (57). Although they are purple non-sulfur bacteria, they can oxidize various sulfur species including sulfide, elemental sulfur and thiosulfate. KoFox is, so far, the only isolate that belongs to the green sulfur group (20, 66) but does not exist in pure culture and is enriched with another chemoheterotrophic bacterium. Beside Fe(II), KoFox can also use H_2 for photosynthesis, but not reduced sulfur species (30). TIE-1 was isolated by our laboratory from an iron-rich puddle at Woods Hole, MA (see Chapter 3). In contrast to these isolates that can grow photoautotrophically on Fe(II), *Rhodomicrobium vannielii* BS-1 is a purple non-sulfur bacterium that is able to oxidize Fe(II) but not grow on Fe(II) alone, and cell growth is dependent on the presence of H_2 or organic compounds as co-substrates (31). BS-1 grows poorly on Fe(II) because the solid iron oxides accumulate on the cell surface and impede further Fe(II) oxidation after two or three generations of cell division.

Microbial Fe(II) oxidation is not restricted to phototrophs: acidophilic and neutrophilic Fe(II)-oxidizing bacteria can also gain energy from the oxidation of Fe(II), including bacteria that couple Fe(II) oxidation to the reduction of O_2 at either low (3 and references therein) or neutral pH (22, 54), and bacteria that couple Fe(II) oxidation to the reduction of nitrate at neutral pH (6, 56 and references therein). These microbial Fe(II) oxidizers

are widely distributed in the environment and play an important role in Fe cycling (19, 21, 23, 54, 56, 64).

In contrast to the comparatively limited reports on phototrophic Fe(II) oxidation, microaerobic Fe(II) oxidation at neutral pH, and nitrate-dependent Fe(II) oxidation, numerous studies have been reported concerning the molecular mechanisms of aerobic Fe(II) oxidation under acidic conditions due to their importance in the formation of acid mine drainage (AMD) (3, 35). AMD is a significant environmental problem worldwide, releasing high concentrations of acid and heavy metals, which result in devastating effects to fishing industries and drinking water. Acidophilic Fe(II)-oxidizing bacteria are the major driving force for AMD formation (3). These bacteria can grow chemolithotrophically using Fe(II) as the electron donor and O_2 as the terminal electron acceptor (33). Among all the acidophilic Fe(II)-oxidizers, *Acidithiobacillus ferrooxidans* (γ-Proteobacteria) is the only species whose Fe(II) oxidation system has been studied due to its ease of cultivation. However, its environmental relevance under AMD-generating conditions may have been overstated (3, 53). Our current knowledge about the Fe(II) oxidation system in *A. ferrooxidans* is briefly reviewed in the next section as well as in a recent review by Croal *et al.* (14).

2.3   FE(II) OXIDATION IN *A. FERROOXIDANS*

Acidithiobacillus ferrooxidans is an acidophilic chemolithotrophic bacterium, which can obtain energy through Fe(II) oxidation coupled to the reduction of O_2 at acidic

pH (33). Besides Fe(II), *A. ferrooxidans* can also use H_2, formate or various reduced

sulfur compounds under aerobic conditions (18, 40, 52, 55). Under anaerobic conditions,

it can also reduce Fe(III) with H_2 or sulfur as the electron donor (16, 47, 50, 51). Due to

the lack of genetic tools in manipulating acidophilic bacteria, most of our knowledge

about the Fe(II) oxidation system of *A. ferrooxidans* comes from biochemical studies.

Various redox active proteins that appear to be involved in Fe(II) oxidation have been

identified and characterized, but the electron transport pathway from Fe(II) to O_2 has not

been established and remains controversial.

The first protein recognized in the Fe(II) oxidation system of *A. ferrooxidans* is a

high potential iron sulfur protein (HiPIP) encoded by the monocistronically transcribed

gene, *iro* (39). Iro is believed to be involved in Fe(II) oxidation and has been proposed to

serve as the Fe(II) oxidoreductase due to the following reasons. Iro is stable at low pH,

shows *in vitro* Fe(II)-cytochrome *c*552 oxidoreductase activity (see below), and is a

feasible electron acceptor with a redox potential of +510 mV at neutral pH. This

potential is energetically favorable to facilitate electron transfer between Fe(II)/Fe(III)

(+650 mV when complexed with sulfate, an anionic species required for Fe(II) oxidation

by *A. ferrooxidans*) and H_2O/O_2 ($\Delta E_0' = 0.82$ V) (26, 69). Iro was characterized in both

strains of Fe-1 (26, 39) and BRGM (9). In addition, a HiPIP believed to be a homolog of

Iro from strain ATCC 33020 was overexpressed and purified from the periplasmic space

of a recombinant *E. coli* strain (8). The translation of this HiPIP to the periplasm of *E.*

coli is dependent on the TAT protein translocation pathway, and the purified HiPIP has

all the biochemical properties of the normal HiPIPs including a correctly inserted [4Fe-

4S] cluster (8).

Rusticyanin, a periplasmic blue copper protein, has also been extensively studied because of its high levels of expression under Fe(II) growth conditions. This high level of expression is correlated with a higher level of Fe(II) oxidation activity of whole cells grown under iron conditions (5, 12, 13, 32, 33, 73). Rusticyanin is generally believed to play an essential role in electron transport chain from Fe(II) to O_2 based on several observations. First, it gets reduced when intact cells are exposed to Fe(II). Also, the purified protein shows *in vitro* Fe(II) oxidation activity under acidic conditions. Rusticyanin has a high redox potential (+680 mV). Moreover, strong protein-protein interactions of rusticyanin were observed with another periplasmic protein cytochrome *c*4, showing a Fe(II)-cytochrome *c*4 oxidoreductase activity (27).

Using reverse genetics, the gene that encodes rusticyanin, *rus*, was found in an operon (called the *rus* operon) with several other genes that encode redox active proteins (2). As shown by reverse transcription and Northern hybridization analysis, expression of the *rus* operon is regulated by Fe(II) on the transcriptional level (70). The redox active proteins encoded by the *rus* operon include an outer membrane *c*-type cytochrome encoded by gene *cyc2*, a putative periplasmic *c*4-type cytochrome encoded by gene *cyc1*, four proteins encoded by *coxABCD* that are the subunits of an *aa*3-type cytochrome *c* oxidase, and a putative periplasmic protein of unknown function (1, 2, 72). Since Cyc2 is localized to the outer membrane and *A. ferrooxidans* is able to oxidize the insoluble mineral iron sulfide (pyrite, FeS_2), it has been proposed that Cyc2 is an Fe(II) oxidoreductase (72). Furthermore, an electron transport pathway from Fe(II) to O_2 has

been proposed based on the electron carrier proteins encoded by the *rus* operon alone. In this model, electrons are transferred in the order of Fe(II), Cyc2, rusticyanin, Cyc1, cytochrome oxidase and O_2 (2).

Various non-*rus*-operon-encoded *c*-type cytochromes have also been identified and proposed to be involved in Fe(II) oxidation in different strains of *A. ferrooxidans*. Not surprisingly, the overall cytochrome *c* content in *A. ferrooxidans* is high, representing ~10% of the total cellular protein (71). The midpoint redox potentials of several *c*-type cytochromes from *A. ferrooxidans* typically range between +330 and +360 mV at pH 7.0, and between +610 and +660 mV at pH 3.5 (42, 69, 71). A few examples of the *c*-type cytochromes identified are 1) a ~21 kDa membrane bound cytochrome *c*552 from strain ATCC 13661 (59), 2) a 14 kDa cytochrome *c*552 from strain BRGM and strain Fe-1, which is a soluble *c*-type cytochrome with similarity to the N-terminal region of cytochrome *c*2 (59, 69), 3) A ~68 kDa membrane bound cytochrome *c*550 from strain ATCC 13661 (59), and 4) a di-heme *c*4-type cytochrome from strain BRGM with redox potential between 400 and 500 mV at neutral pH (28).

2.4   SUMMARY

Microbial Fe(II) oxidation is ubiquitous and potentially plays an important role in Fe cycling in both modern and ancient environments. In contrast to acidophilic Fe(II) oxidizers, studies of phototrophic Fe(II) oxidation are limited. However, understanding this metabolism has significant interest well outside bacterial physiology because this

metabolic capability has been hypothesized to be involved in the deposition of Banded Iron Formations in the early history of the Earth. To test this hypothesis and to identify potential biomarkers unique to this metabolism, understanding the molecular mechanisms of phototrophic Fe(II) oxidation remains a prerequisite. In this dissertation, a study of the physiology and genetics of phototrophic Fe(II) oxidation in two purple non-sulfur bacteria are presented.

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Chapter 3

Phototrophic Fe(II) Oxidation in the Presence of H_2: Implication for Banded Iron Formation

3.1   ABSTRACT

Banded Iron Formations (BIFs) are an ancient class of iron ore deposits. Although their origins remain enigmatic, it has been proposed that phototrophic Fe(II)-oxidizing bacteria may have catalyzed the deposition of the most ancient BIFs. Both Fe(II) and H_2 can serve as electron donors for anoxygenic photosynthesis and these substrates are predicted to have been present in the atmosphere and ocean of the Archean in quantities sufficient for energy metabolism. If both Fe(II) and H_2 were present and H_2 were the preferred substrate, as might be predicted given its lower redox potential, the potential that these bacteria were involved in the BIF genesis may be lessened. Here we investigated the effects of H_2 on phototrophic Fe(II) oxidation by cell suspensions of two strains of Fe(II)-oxidizing purple non-sulfur bacteria, *Rhodopseudomonas palustris* TIE-1 and *Rhodobacter* sp. SW2. We found Fe(II) oxidation by these strains still proceeded at significant rates under an atmosphere containing approximately three times the maximum predicted concentration of H_2 in the Archean atmosphere when CO_2 was abundant. Thus, it seems unlikely that the presence of H_2 in an Archean atmosphere would have posed a barrier to phototrophic Fe(II) oxidation, consistent with the hypothesis that these bacteria may have catalyzed ancient BIF deposition.

3.2   INTRODUCTION

Recent debates in the literature have called into question the idea that evidence for the earliest life in the rock record can be inferred from morphology (Brasier et al., 2002; Schopf et al., 2002) or chemical composition alone (Fedo and Whitehouse, 2002; Lepland et al., 2005; Mojzsis et al., 1996). The value of a search strategy that considers not only morphology and chemical analyses (*e.g.,* isotopic compositions or rare earth element analyses), but also the ecophysiological context of the fossils in question is becoming increasingly appreciated (Tice and Lowe, 2004).

If our goal is to trace not just the presence of life, but more specifically the effects of a particular metabolism in the rock record, knowledge concerning how a metabolism of interest is regulated by environmental factors must be taken into account. It has been suggested that anoxygenic photoautotrophs able to use ferrous iron [Fe(II)] as an electron donor for photosynthesis were involved in the deposition of Banded Iron Formations (BIFs) that formed prior to the rise of O_2 (Hartman, 1984; Konhauser et al., 2002; Kopp et al., 2005; Widdel et al., 1993). This model assumes that these bacteria used Fe(II) as an electron donor for photosynthesis. However, many anoxygenic phototrophs, including those able to oxidize Fe(II), are capable of using a variety of electron donors for photosynthetic growth. One such donor that is broadly used by diverse phototrophic bacteria is hydrogen gas (H_2) (White, 2000).

The atmosphere of the early Earth is thought to have contained between 1,000 and 300,000 ppm of H_2 as a result of volcanic emissions and atmospheric photochemistry (Catling et al., 2001; Kasting, 1993; Tian et al., 2005). These quantities of H_2 are sufficient to support H_2-based photoautotrophy and it is possible that such quantities could have interfered with phototrophic Fe(II) oxidation leading to BIF formation, given that H_2 is a more thermodynamically favorable electron donor than Fe(II). Coupling an understanding of how phototrophic Fe(II) oxidation is regulated with biogeochemical/stratigraphic reconstructions of the ancient environment can help refine models that consider the role of these phototrophs in BIF deposition.

Fe(II) based phototrophy is thought to be an ancient metabolism (Croal et al., 2004) and we assume that extant organisms capable of this metabolism are representative of their ancient counterparts (Anbar and Knoll, 2002). Here, we investigate the effects of H_2 on the phototrophic Fe(II) oxidation activity of two strains of purple non-sulfur bacteria and show that Fe(II) oxidation is feasible at concentrations of H_2 and CO_2 similar to those that have been inferred for the Archean.

3.3   MATERIAL AND METHODS

Organisms and Cultivation *Rhodobacter* sp. strain SW2 was a gift from F. Widdel (Max Planck Institute for Marine Microbiology, Bremen, Germany) and *Rhodopseudomonas palustris strain* TIE-1 was isolated in our lab (Jiao et al., 2005). Methods for anaerobic medium preparation and the cultivation of SW2 and TIE-1 under

anoxic conditions followed those described by Widdel and Bak (Widdel and Bak, 1992).

Phototrophic cultures were maintained in a previously described anoxic minimal salts

medium for freshwater cultures that was adjusted to a pH of 6.8 (Ehrenreich and Widdel,

1994). Cultures of TIE-1 and SW2 were incubated at 30 °C and 16 °C, respectively, 20

to 30 cm from a 34 W tungsten, incandescent light source. Electron donors for

photosynthetic growth were added to the anaerobic, basal medium as follows: thiosulfate

was added from anoxic filter sterilized stocks to a final concentration of 10 mM and H_2

was provided as a headspace of H_2/CO_2 (80:20 [vol/vol]). For growth on Fe(II), 4 ml

anoxic filter sterilized Fe(II)$Cl_2\cdot H_2O$ stock solution (1 M) was added per liter of

anaerobic, basal medium. 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,

nitrilotriacetic acid disodium salt (NTA, Sigma), was supplied from an anoxic 1 M filter

sterilized stock solution. Various concentrations of NTA used in Figure 1 are listed in the

figure legend. For all other experiments, 5 and 10 mM of NTA were used for SW2 and

TIE-1, respectively.

Cell suspension assays All cell suspension assays were prepared under anoxic

conditions in an anaerobic chamber (Coy Laboratory Products, Grasslake, MI) to avoid

exposure to oxygen. Cells of SW2 or TIE-1 grown on H_2, thiosulfate, or Fe(II)-NTA

were harvested in early exponential phase (OD$_{600}$ ~0.15 to 0.18) by centrifugation

(10,000 rpm on a Beckman JLA 10.5 rotor for 20 min). Pellets were washed once with

an equal volume of 50 mM N-2-hydroxyethylpiperazine-N`-2-ethanesulfonic acid

(HEPES) buffer containing 20 mM NaCl at pH 7 (assay buffer) to remove residual

medium components and resuspended in assay buffer containing the appropriate amount

of $NaHCO_3$ and $Fe(II)Cl_2 \cdot H_2O$ or ferric (hydr)oxide to a final OD_{600} of 1.0.

Resuspending the cells to the same final OD_{600} ensured that the assays were normalized

to cell number, as verified by cell counts using a Petroff-Hauser counting chamber. For

Fe(II) oxidation assays, concentrations of $NaHCO_3$ and $Fe(II)Cl_2$ used were 1 or 20 mM

and 0.5, 1 or 2 mM, respectively. For Fe(III) reduction assays, ferric (hydr)oxide (final

concentration 0.5 mM) was substituted for $Fe(II)Cl_2$, and the $NaHCO_3$ concentration used

was 1 mM. Ferric (hydr)oxide was synthesized according to the method described by

Cornell and Schwertmann (Cornell and Schwertmann, 1979). Briefly, a 500 ml solution

of 40 g $Fe(NO_3)_3 \cdot 9H_2O$ was stirred vigorously, followed by the addition of 330 ml of 1 M

KOH solution to 7-8, which yielded a brown suspension. The last 20 ml of KOH was

added dropwise with constant checking of the pH. A washing procedure using

centrifugation to collect the particles and sonication to resuspend the particles in fresh

DDW was repeated 5 times. The pH of the washed suspension was approximately 7.0.

Unless otherwise stated, assay volumes were 3 ml and cell suspensions of TIE-1 and

SW2 were incubated at 30 °C and 16 °C, respectively, in 12 ml sealed serum bottles, 30

cm from a 34 W tungsten incandescent light bulb. The headspace of the assay bottles

contained either N_2/CO_2 (80:20 [vol/vol]) or H_2/CO_2 (80:20 [vol/vol]).

Analytical methods Fe(II) concentrations in the cell suspensions were measured in triplicate by the ferrozine assay (Stookey, 1970). To measure Fe(II), 10 μl cell suspension were added to 90 μl 1 N HCl, to which 100 μl ferrozine solution (0.1% (w/v) ferrozine in 50% (wt/v) ammonium acetate solution) were added. After 10 minutes, the absorbance at 570 nm was measured. To determine the concentration of Fe(III) in a sample, the total concentration of Fe in the sample was first determined by adding 10 μl of cell suspension to 90 μl of hydroxylamine hydrochloride (HA) solution (10% (w/v) HA in 1 M HCL). 100 μl ferrozine solution was then added and after 10 minutes, the absorbance at 570 nm was measured. The Fe(III) concentration in the sample was then calculated as the difference between the total and Fe(II) concentrations in the sample.

The protocol for measuring hydrogenase activity was adapted from Elsen et al. (Elsen et al., 2003). Benzyl viologen (Sigma) was added to the assay to a final concentration of 5 mM and the reduction of benzyl viologen was measured as a change in the absorbance at 570 nm.

H_2 was measured with a Hewlett Packard 5890 series II gas chromatograph equipped with a thermal conductivity detector and a 30-m bonded phase fused-silica DB 1 capillary column (inner diameter, 0.32 mm). The temperatures of the oven, injector, and detector were 80 °C, 80 °C, and 90 °C, respectively. Hamilton (Reno, NV) sample lock syringes were used to inject gas samples into the gas chromatograph.

The program MINEQL$^+$ (Environmental Research Software; http://www.mineql.com/homepage.html) was used to calculate the concentrations of the various Fe(II) and NTA species in the phototrophic minimal salts medium (pH 6.8) when

4 mM Fe(II)Cl$_2$·H$_2$O and 5 or 20 mM NTA were added. A closed system was assumed, the ionic strength of the solution was not considered, the temperature was set at 25 °C and component concentrations were: H$_2$O, 1×10^0; H$^+$, 1.58×10^{-7}; Ca^{2+}, 3.69×10^{-6}; Cl$^-$, 6.97×10^{-3}; CO$_3^{2-}$, 4.79×10^{-6}; Fe^{2+}, 5.73×10^{-8}; K$^+$, 3.55×10^{-3}; Mg^{2+}, 1.27×10^{-4}; NH$_4^+$, 5.47×10^{-3}; SO$_4^{2-}$, 1.81×10^{-3}; EDTA^{-4}, 1.36×10^{-14}; NTA^{-3}, 4.5×10^{-6}.

3.4   RESULTS AND DISCUSSION

3.4.1   Effects of NTA on Fe(II) oxidation in TIE-1 and SW2

The mineral products of Fe(II) oxidation by TIE-1 and SW2 are poorly crystalline ferric (hydr)oxide precipitates (Croal et al., 2004; Kappler and Newman, 2004). These precipitates greatly hinder our ability to harvest cells grown under these conditions. To prevent the precipitation of ferric phases in our cultures, we added varying concentrations of the Fe chelator NTA to our growth medium containing 4 mM Fe(II). A concentration of at least 5 mM NTA was necessary to keep Fe(III) in solution for both cultures. Similar to what has been observed previously for the Fe(II)-oxidizing phototrophic strain, *Rhodomicrobium vannielli* (Heising and Schink, 1998), the addition of NTA accelerated the rate of Fe(II) oxidation by both TIE-1 and SW2. For SW2, the rate of Fe(II) oxidation increased approximately 25% when 7.5 mM NTA was added, while the addition of 10 and 15 mM had adverse effects on the rate of Fe(II) oxidation (Figure 3.1A). For TIE-1, the addition of 5, 7.5, and 15 mM NTA increased the Fe(II) oxidation rate approximately 47, 48 and 44%, respectively (Figure 3.1B). No growth of TIE-1 or

SW2 was observed in control experiments where only NTA and no Fe(II) was added (data not shown).

When the Fe(II)-oxidizing phototroph, *R. vannielli*, grows on Fe(II) alone, the ferric precipitate products completely encrust the cells and impede further Fe(II) oxidation by this strain (Heising and Schink, 1998). Given that mineral encrustation is not evident with TIE-1 or SW2, the increased rate of Fe(II) oxidation upon addition of NTA may be due to a lowering of the redox potential of the Fe(II)/Fe(III) couple, or a change in the bioavailable Fe(II) species that effectively facilitates Fe(II) oxidation.

The concentration of NTA tolerated by the two strains differed. For SW2, concentrations of NTA higher than 7.5 mM were inhibitory whereas TIE-1 was not adversely affected by up to 15 mM NTA (Figure 3.1A and B). MINEQL$^+$ modeling of the chemical species in medium containing 4 mM Fe(II)Cl$_2 \cdot$H$_2$O and 5 or 20 mM NTA predicts that 99.8% of the total Fe(II) is present as the Fe[NTA] complex in both cases. Because the concentration of the Fe[NTA] complex does not change and is the dominant chemical species at all the NTA concentrations tested, the increased tolerance of NTA by TIE-1, relative to SW2, may result from differences in cell wall permeability or the efficiency/number of generalized solute efflux pumps. This would be consistent with the observation that TIE-1 is more resistant to a greater concentrations of the antibiotics kanamycin, gentamicin, tetracycline and chloramphenicol (the mechanisms of resistance of the latter two being via efflux pumps), than SW2 and most purple non-sulfur bacteria in general (Jiao et al., 2005).

Figure 3.1 Growth of SW2 and TIE-1 on 4 mM Fe(II)Cl$_2$·H$_2$O with varying concentrations of NTA. A. Data for SW2: ♦ - 0 mM NTA, ■ - 7.5 mM NTA, **+** - 10 mM NTA, ● - 15 mM NTA, ✳ - Abiotic + 15 mM NTA. B. Data for TIE-1: ♦ - 0 mM NTA, ▲ - 5 mM NTA, ■ - 7.5 mM NTA, ● - 15 mM NTA, ✳ - Abiotic + 15 mM NTA. Data for SW2 and TIE-1 are representative of triplicate and duplicate cultures, respectively.For SW2 data, the error bars represent the range of duplicate cultures; TIE-1 data is representative of duplicate cultures.

3.4.2   Phototrophic Fe(II) oxidation in the presence of a H$_2$ atmosphere

In a bicarbonate containing system of pH ~7 the relevant Fe couple, Fe(OH)$_3$ + HCO$_3^-$/FeCO$_3$, has a redox potential of +0.2 V (Ehrenreich and Widdel, 1994) while the relevant H$_2$ couple, 2H$^+$/H$_2$, has a redox potential of -0.41 V (Madigan et al., 2003), suggesting H$_2$ is a more thermodynamically favorable electron donor than Fe(II) under these conditions. This raises the question – in an environment where H$_2$ and Fe(II) coexist, would phototrophic Fe(II) oxidation still proceed? It is often assumed that bacteria preferentially use substrates according to the amount of free energy they can gain from them. To date, most studies that support this idea have focused on the hierarchical use of different electron acceptors in respiration (Ingledew and Poole, 1984). In

principle, the same logic also applies to electron donors. We note, however, that thermodyamics cannot provide the full story: the ability/efficiency of microbes to use substrates to obtain energy for growth also depends on kinetic issues such as substrate affinity, enzyme turnover rate, and substrate availability.

To investigate the effects of the presence of H_2 on phototrophic Fe(II) oxidation by TIE-1 and SW2, we measured the rates of Fe(II) oxidation in cell suspensions of these strains where the concentrations of Fe(II), $NaHCO_3$ and H_2 were comparable to those thought to be relevant for the Archean environment. Specifically, the initial Fe(II) concentration of ~0.4 to 0.45 mM is within the upper range of 0.054 to 0.54 mM predicted by Holland and Ewers (Ewers, 1983; Holland, 1973), the $NaHCO_3$ concentration of 20 mM is on the same order as the 70 mM predicted for an Archean ocean and an order of magnitude higher than the present day concentration of 2 mM (Grotzinger and Kasting, 1993), and the H_2 concentration of 800,000 ppm is also on the same order as the recently proposed concentration in the early atmosphere of 300,000 ppm (Tian et al., 2005).

In our experiments containing 1 mM $NaHCO_3$ in the absence of H_2, we observed initial rates of Fe(II) oxidation for TIE-1 and SW2 of ~0.07 mM/hr and ~0.15 mM/hr, respectively (Figure 3.2A and B). Under the same low $NaHCO_3$ conditions, in the presence of H_2, the rate of Fe(II) oxidation by TIE-1 decreased by ~43% compared to the absence of H_2 (Figure 3.2A, Table 3.1). SW2 showed an even more dramatic inhibition by H_2. During the first five hours of the assay, the rate of Fe(II) oxidation by SW2 in the

presence of H_2 decreased by ~80% as compared to the absence of H_2, and after 10 hours, only ~22% of the total Fe(II) added initially was oxidized (Figure 3.2B, Table 3.1).

In contrast, in the presence of 20 mM $NaHCO_3$, while the rates of Fe(II) oxidation decreased for both strains in the presence of H_2, the inhibition by H_2 was less severe as compared to the 1 mM $NaHCO_3$ conditions. For TIE-1, the initial rate of Fe(II) oxidation decreased ~31% as compared to that in the absence of H_2 (Figure 3.2A, Table 3.1). For SW2 the initial rate of Fe(II) oxidation decreased ~39% (Figure 3.2B, Table 3.1). When comparing the total Fe(II) oxidized under these conditions, for SW2 all of the Fe(II) was oxidized to completion within two hours and for TIE-1, after eight hours, the same amount of Fe(II) was oxidized as in the absence of H_2 (Figure 3.2A and B).

Figure 3.2 H_2 inhibits the Fe(II) oxidation activity of both SW2 (A) and TIE-1 (B) to varying degrees depending on the concentration of $NaHCO_3$. ▲ - H_2 + 1 mM $NaHCO_3$; ● - N_2 + 1 mM $NaHCO_3$; ♦ - H_2 + 20 mM $NaHCO_3$; ■ - N_2 + 20 mM $NaHCO_3$. Data are representative of two independent experiments. The volume of the assay was 1.5 ml, the headspace of the cell suspension was exchanged every 2 hours and the assay bottles were shaken vigorously at each time point to ensure maximal H_2 saturation of the cell suspension solution. Error bars represent the error on duplicate cell suspension assays for TIE-1 and triplicate assays for SW2.

Table 3.1 Decrease in rates of Fe(II) oxidation in the presence of H_2 relative to no H_2 conditions by cell suspensions of TIE-1 and SW2. 0.5 mM of Fe(II) was used for this assay. Under H_2 conditions the headspace contained H_2/CO_2 (80:20 [vol/vol] while under no H_2 conditions the headspace contained N_2/CO_2 (80:20 [vol/vol]).

Strain	$NaHCO_3$ [mM]	% decrease in rate relative to no H_2 condition
TIE-1	1	43
TIE-1	20	31
SW2	1	80
SW2	20	39

To verify that the apparent decrease in the rates of Fe(II) oxidation in the presence

of H_2 was not due to the ability of these organisms to couple H_2 oxidation to the

reduction of Fe(III) during the assay, we performed Fe(III) reduction activity assays with

TIE-1 and SW2. In H_2 pre-grown cell suspensions of TIE-1 and SW2 to which 1 mM

$NaHCO_3$ and 0.5 mM ferric (hydr)oxide were added, no Fe(II) was detected for either

TIE-1 and SW2 in the presence (Figure 3.3) or absence of H_2 (data not shown). This

suggests that neither SW2 nor TIE-1 have Fe(III) reduction activity under these

conditions, and thus the observed decrease in Fe(II) oxidation activity was not due to the

reduction of oxidized Fe(II).

Figure 3.3 No Fe(III) reduction activity was observed in cell suspension assays with H_2 grown cells of TIE-1 and SW2. 1 mM NaHCO3 and ~500 µM ferric (hydr)oxide were added at the beginning of the assay. △ - TIE-1, Fe(II); ▲ - TIE-1, Fe(III); □ - SW2, Fe(II); ■ - SW2, Fe(III). No Fe(II) increase was detected throughout the incubation period and Fe(III) concentration remained constant. Data shown is from samples incubated in the presence of H_2 under light. Similarly, controls that contained no H_2 or that were incubated in the dark did not exhibit any Fe(III) reduction (data not shown).

Together, these results indicate that phototrophic Fe(II) oxidation may be

inhibited by H_2 in modern environments where the concentration of bicarbonate is low

(~2 mM). However, if the concentration of bicarbonate is high (*i.e.*, >20 mM), as is predicted for the Archean ocean (Grotzinger and Kasting, 1993), even in an atmosphere containing 800,000 ppm H_2, Fe(II) oxidation by these phototrophs could still have proceeded at appreciable rates.

3.4.3   How does H_2 inhibit Fe(II) oxidation?

It is not clear how H_2 inhibits Fe(II) oxidation in our cell suspension assays. All the Fe(II) oxidation activity observed in H_2 pre-grown cells of SW2 and TIE-1 is light dependent (data not shown), suggesting that electron transfer from Fe(II) is linked to the photosynthetic electron transport system. To determine if the same held true for electrons derived from H_2, cell suspensions of H_2 grown TIE-1 and SW2 were incubated in the light and dark and H_2 consumption was followed over time. H_2 was only consumed by the cells exposed to light (Figure 3.4). Controls without light showed similar decreases in H_2 compared to no cell controls. We suspect the decrease of H_2 in the headspace of abiotic or dark controls is caused by diffusion into the buffer.

Figure 3.4 Relative H_2 consumption by cell suspensions of H_2-grown TIE-1 and SW2 show that this activity is light dependent. ♦ - SW2 + H_2 + 20 mM $NaHCO_3$ + light; ■ - SW2 + H_2 + 20 mM $NaHCO_3$ + dark; ▲ - TIE-1 + H_2 + 20 mM $NaHCO_3$ + light; ✶ - TIE-1 + H_2 + 20 mM $NaHCO_3$ + dark; ● - no cells + H_2 + 20 mM $NaHCO_3$ + light. All data were standardized to the rate of H_2 consumption by cells under light (defined as 100%).

Given that the utilization of Fe(II) and H_2 by these strains is light dependent, there are several possibilities by which H_2 might inhibit Fe(II) oxidation. 1) H_2 may inhibit the expression of the Fe(II) oxidoreductase, 2) H_2 may directly inhibit the Fe(II) oxidoreductase itself, or 3) hydrogenase is active in these cells and is effectively out-competing the Fe(II) oxidoreductase to donate electrons to the photosynthetic electron transport chain.

If H_2 were to inhibit expression of the Fe(II) oxidoreductase or the Fe(II) oxidoreductase enzyme itself, cells pre-grown on H_2 and transferred to assay conditions containing both Fe(II) and H_2 would be expected to have no Fe(II) oxidation activity (during H_2 pre-growth the Fe(II) oxidoreductase would be repressed and upon transfer to the assay containing H_2, the repression of the Fe(II) oxidoreductase would continue due

to the presence of H_2). On the contrary, in our experiments where H_2 was added to the

cell suspensions of H_2-grown cells to investigate its effects on Fe(II) oxidation, we see

that both TIE-1 and SW2 do have Fe(II) oxidation activity (albeit, less than the activity

observed for H_2 pre-grown cells transferred to an assay with only Fe(II) (Figure 3.2A and

B). This observation implies that the Fe(II) oxidoreductase is expressed and active in the

presence of H_2.

If both the hydrogenase and Fe(II) oxidoreductase enzymes are present and active

under our assay conditions, the observed inhibition of Fe(II) oxidation by H_2 may result

from competition between the two enzymes to donate electrons to the photosynthetic

electron transport chain and ultimately CO_2. Under conditions where the physiological

electron acceptor, CO_2, would be abundant (*i.e.*, 20 mM $NaHCO_3$), H_2 inhibition of Fe(II)

oxidation is observed as a slight decrease in the rate of Fe(II) oxidation for both TIE-1

and SW2 (Figure 3.2A and B, Table 3.1). If these enzymes compete to donate electrons

to the photosynthetic electron transport chain and ultimately CO_2, when the concentration

of the electron acceptor is low (*i.e.*, 1 mM $NaHCO_3$), we might expect the competition

between the two enzymes to become more intense and be manifested as a greater

inhibition of Fe(II) oxidation by H_2. In support of this hypothesis, we observe that the

rate of Fe(II) oxidation for both strains under low $NaHCO_3$ concentrations decreased

more in the presence of H_2 as compared to higher $NaHCO_3$ concentrations, particularly

for SW2 (Figure 3.2A and B, Table 3.1). In addition, assuming free iron is the species

used by SW2 and TIE-1 cells for Fe(II) oxidation (Croal et al., 2004; Kappler and

Newman, 2004), it is unlikely that the difference observed in the rate of Fe(II) oxidation

in the presence of different bicarbonate concentrations is due to a change in Fe(II)

speciation because MINEQL$^+$ modeling of the chemical species in the cell suspension

buffer containing 0.5 mM Fe(II)Cl$_2 \cdot$H$_2$O and 1 or 20 mM bicarbonate predicts that over

77% of the total Fe(II) is present as the free ion in both cases.

Figure 3.5 Hydrogenase and Fe(II) oxidation activity for SW2 and TIE-1 as measured by benzyl viologen (BV) reduction and the ferrozine assay, respectively. A & B. Hydrogenase activity for SW2 and TIE-1, respectively: ♦ - H$_2$ + 20 mM NaHCO$_3$ + 1 mM FeCl$_2 \cdot$H$_2$O + 5 mM BV; ● - H$_2$ + 20 mM NaHCO$_3$ + 5 mM BV. C & D. Fe(II) oxidation activity for SW2 and TIE-1, respectively: ▲ H$_2$ + 20 mM NaHCO$_3$ + 1 mM FeCl$_2 \cdot$H$_2$O; ● - N$_2$ + 20 mM NaHCO$_3$ + 1 mM FeCl$_2 \cdot$H$_2$O. The volume of the assay was 1 ml, and the assay bottles were shook vigorously at each time point to ensure maximal H$_2$ saturation of the cell suspension solution. Error bars represent the error on triplicate cell suspension assays.

To further test this competition hypothesis, we measured the hydrogenase activity of cell suspensions of TIE-1 and SW2 pre-grown on H_2 in the presence and absence of Fe(II) to determine if the hydrogenase enzyme is in fact present and active under the conditions of our assay. The H_2-dependent reduction of benzyl viologen observed indicates that the cells used for our assay do have an active hydrogenase, the activity of which does not seem to be greatly affected by the presence Fe(II) (Figure 3.5A and B). These findings are expected given that the cells are pre-grown on H_2, a condition where the hydrogenase is expected to be highly expressed (Vignais and Colbeau, 2004). Conversely, we found that H_2 did not significantly inhibit Fe(II) oxidation in cell suspensions of TIE-1 or SW2 pre-grown on Fe-NTA even at low bicarbonate concentrations, given that hydrogenase is not expected to be expressed under Fe(II) growth conditions (Figure 3.6).

The proposed flow of electrons from Fe(II) and H_2 to the photosynthetic electron transport chain and CO_2 is shown in Figure 3.7. The hydrogenase enzyme, presumably located in the cytoplasmic membrane (CM) of TIE-1 and SW2 by comparison to *Rhodobacter capsulatus* (Vignais and Colbeau, 2004), donates electrons from H_2 to the quinone pool. Enzymes involved in Fe(II) oxidation are not well studied. Recent findings from our lab suggest that redox active proteins located in the periplasm such as *c*-type cytochromes are involved in Fe(II) oxidation in SW2 and TIE-1 (Croal et al., 2007; Jiao and Newman, 2007). Electrons from H_2 or Fe(II) then flow through the photosynthetic electron transport chain in a cyclic fashion to produce ATP or feed into

NAD$^+$ reduction (catalyzed by the NADH dehydrogenase also located in the CM).

NADH can then be used to fix CO_2.

Figure 3.6 H_2 does not inhibit the Fe(II) oxidation activity of Fe(II)-NTA pre-grown TIE-1 or SW2 cell suspensions at low concentrations of $NaHCO_3$. Cells were pre-grown on Fe-NTA and washed three times in the assay buffer before resuspension in assay buffer containing 1 mM $NaHCO_3$ and ~0.5 mM $FeCl_2$, with an atmosphere of either N_2 or H_2. The assay volume used was 2 ml. ◆ - TIE-1 + N_2; ■ - TIE-1 + H_2; ▲ - SW2 + N_2; ● - SW2 + H_2. Error bars represent the error on triplicate cell suspension assays.

Figure 3.7 A cartoon representation of the flow of electrons from Fe(II) and H_2 to the photosynthetic electron transport chain and CO_2. The red lines, associated with k_1, represent the pathway and the overall rate of electron flow from Fe(II) to the photosynthetic electron transport chain. The blue lines, associated with k_2, represent the pathway and the overall rate of electron flow from H_2 to the photosynthetic electron transport chain. OM: outer membrane; PERI: periplasm; CM: cytoplasmic membrane; ICM: intracytoplasmic membrane; CYT: cytoplasm, RC: reaction center, UQ: ubiquinone, NADH DH: NADH dehydrogenase, H_2ase: hydrogenase, FOxRed: Fe(II) oxidoreductase.

The physiological basis for the difference in sensitivity to H_2 under low bicarbonate concentrations observed for the two strains remains to be determined. The decreased Fe(II) oxidation activity in the presence H_2 observed for SW2 (relative to TIE-1), may imply that the overall electron flow rate from Fe(II) to the photosynthetic

electron transport chain (represented by k_1) for SW2 is less efficient than that for TIE-1 (Figure 3.7). Such a scenario may result if the electron flow rate from Fe(II) (k_1) is less than the electron flow rate from H_2 (k_2) in SW2, and k_1 and k_2 are nearly equivalent in TIE-1, or if there is more hydrogenase relative to Fe(II) oxidoreductase in SW2 versus TIE-1. To further test the competition hypothesis, the dependence of the Fe(II) oxidation rate and/or the H_2 uptake rate on the bicarbonate concentration should be tested over multiple biocarbonate concentrations, both for the wild type and specific mutants that lack either the Fe(II) oxidase or the H_2 uptake hydrogenase. Details concerning the rates of individual reactions within a physiological pathway cannot be resolved with cell suspension experiments and require further investigations with purified enzymes. Thus, purification of the Fe(II) oxidoreductases is a priority for future work.

3.4.4   Implications for Banded Iron Formations

Inferences on mechanism aside, the implication of our results for BIF genesis are that when the physiological electron acceptor for photosynthesis (CO_2), is abundant (as is presumed to have been the case in an ancient Archaean ocean), Fe(II) oxidizing phototrophs have the capacity to oxidize Fe(II) even in the presence of the alternative electron donor, H_2. Thus, the presence of H_2 in an ancient atmosphere up to concentrations of even 800,000 ppm would not preclude Fe(II) oxidation by these bacteria. That our $NaHCO_3$ concentrations (20 mM) are lower than the predicted

concentrations in an Archean ocean by ~3.5 fold implies that the slight inhibitory effects of H_2 observed under our conditions might be even less at concentrations of 70 mM.

If we assume that photochemical reactions and volcanic emissions were the major source of H_2 and calculate the concentration of H_2 in a photic zone of 100 m over an area of 10^{11} m^2 (equivalent to the depositional basin of the Hamersley Group, which contains among the largest BIFs (Konhauser et al., 2002)), using a hydrogen mixing ratio of 30% (which is at the upper limit of what has been predicted (Tian et al., 2005)), and a Henry's constant for H_2 of $10^{-3.1}$ (Morel and Hering, 1993), we find the concentration of H_2 expected in this volume of ocean water to be 0.24 ppm (0.12 mM). Given that diffusion and H_2 consumption rates by other bacteria are not considered in this calculation, we expect the concentration of H_2 would be even less with depth. Additionally, the solubility of H_2 in water decreases with increasing temperature (Fernandez-Prini et al., 2003). If estimations of Archean ocean temperatures at $70\pm15°C$ are correct (Knauth and Lowe, 2003), our calculated value represents a maximum for the amount of H_2 dissolved in the photic zone of this basin and is several orders of magnitude less than those used for our experiments. Therefore, it is likely that at depths approaching 100 m in an ancient open ocean, H_2 would pose no barrier to Fe(II) oxidation by these anoxygenic phototrophs. Further, in sulfide depleted environments, which are thought to have prevailed in the ancient oceans prior to 1.8 Ga (Poulton et al., 2004), Fe(II) may have been the predominant inorganic electron donor for anoxygenic photosynthesis.

3.5   CONCLUSIONS

We find that even in the presence of 800,000 ppm H_2, Fe(II) oxidation still proceeds at appreciable rates by two species of Fe(II)-oxidizing purple non-sulfur phototrophs when the concentration of $NaHCO_3$ is 20 mM. Additionally, our calculations predict that the concentration of dissolved H_2 in the photic zone of an Archean ocean may have been less than 0.24 ppm. Based on our results, we predict this concentration would have had little effect on Fe(II) oxidation by anoxygenic phototrophs at depth within the photic zone based on our experiments. These results suggest that the presence of H_2 in an ancient atmosphere at the currently predicted values would not have precluded the involvement of these organisms in BIF deposition.

The molecular mechanism by which H_2 inhibits Fe(II) oxidation by these phototrophs when $NaHCO_3$ concentrations are low remains to be proven, but our favored explanation involves competition between the hydrogenase and the Fe(II) oxidoreductase in donating electrons to the photosynthetic electron transport chain and ultimately to CO_2. The physiological differences between TIE-1 and SW2 that result in differential sensitivity to H_2 at low $NaHCO_3$ concentrations are not known, but may result from differences in enzyme activities of the two pathways in both strains. Further studies that combine microbial physiology and geological approaches will enable biogeochemical reconstructions of ancient environments and help shed light on BIF deposition and the ecology of the Archean ocean.

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Chapter 4

Isolation and Characterization of a Genetically Tractable Photoautotrophic Fe(II)-Oxidizing Bacterium *Rhodopseudomonas palustris* strain TIE-1

4.1 ABSTRACT

We report the isolation and characterization of a phototrophic ferrous iron [Fe(II)]-oxidizing bacterium named TIE-1 that differs from other Fe(II)-oxidizing phototrophs in that it is genetically tractable. Under anaerobic conditions, TIE-1 grows photoautotrophically with Fe(II), H_2 or thiosulfate as the electron donor, and photoheterotrophically with a variety of organic carbon sources. TIE-1 also grows chemoheterotrophically in the dark. This isolate appears to be a new strain of the purple non-sulfur bacterial species *Rhodopseudomonas palustris* based on physiological and phylogenetic analysis. Fe(II) oxidation is optimal at pH 6.5 to 6.9. The mineral products of Fe(II) oxidation are pH dependent: below pH 7.0 goethite ($\alpha \cdot FeOOH$) forms, above pH 7.2 magnetite (Fe_3O_4) forms. TIE-1 forms colonies on agar plates and is sensitive to a variety of antibiotics. A hyperactive mariner transposon is capable of random insertion into the chromosome with a transposition frequency of ~10^{-5}. To identify components involved in phototrophic Fe(II) oxidation, mutants of TIE-1 were generated by transposon mutagenesis and screened for defects in Fe(II) oxidation using a cell suspension assay. Among approximately 12,000 mutants screened, six were identified

that are specifically impaired in Fe(II) oxidation. Five of these mutants have independent disruptions in a gene that is predicted to encode an integral membrane protein that appears to be part of an ABC transport system; the sixth mutant has an insertion in a gene is a homolog of CobS, an enzyme involved in cobalamin (vitamin B12) biosynthesis.

4.2   INTRODUCTION

Phototrophic Fe(II)-oxidizing bacteria were first reported over a decade ago (20, 57), but very little is known about how these bacteria oxidize Fe(II) at the molecular level. In part, this is due to the lack of a genetic system in any of these isolates. Accordingly, we set out to isolate a new Fe(II)-oxidizing species that would be amenable to genetic analysis. We were motivated to understand the process of phototrophic Fe(II) oxidation in detail because it likely represents one of the most ancient forms of photosynthesis and organisms with this metabolism may have catalyzed the deposition of Banded Iron Formations (BIFs), an ancient class of iron-containing sediments (20, 36, 57). To test the hypothesis that Fe(II)-oxidizing phototrophs played a role in BIF deposition at discrete intervals in earth history, we must be able to evaluate putative molecular biosignatures that are preserved in ancient rocks. These biosignatures generally fall into two classes: organic and inorganic. At present, there are no unique organic biomarkers associated with this physiology, nor are there clear inorganic biosignatures, although stable Fe-isotopes may hold promise in this regard (14). Given this, elucidation of the molecular components of the phototrophic Fe(II) oxidation

pathway is necessary both to constrain our interpretation of the Fe-isotopic fractionation produced by these bacteria and to identify biomolecules specific to Fe(II) oxidation that may be preserved over geologic time (14).

To date, the phototrophic Fe(II)-oxidizing bacteria that have been isolated are phylogenetically diverse and include members of the purple sulfur (*Thiodictyon sp.* strain F4), purple nonsulfur (*Rhodobacter ferrooxidans* strain SW2 and *Rhodovulum sp.* strains N1 and N2) and green sulfur bacteria (*Chlorobium ferroxidans* KoFox) (14, 20, 27, 53). In addition to the Fe(II)-oxidizing phototrophs, Fe(II) oxidation is also catalyzed by nitrate dependent Fe(II)-oxidizing bacteria (4, 37) and acidophilic or neutrophilic Fe(II)-oxidizing aerobic microorganisms (19, 22, 52, 55). Most of what is known about the molecular mechanisms of Fe(II) oxidation derives from biochemical studies of the acidophilic Fe(II)-oxidizing aerobe *Acidithiobacillus ferroxidans* (13). Proteins thought to be involved in the transfer of electrons from Fe(II) to O_2 include the blue copper protein rusticyanin (11, 12), a high redox potential Fe-S protein (23), an outer membrane porin (46), several types of cytochromes (1, 42, 56, 60), and one or more cytochrome oxidases (29). The exact role of each of these carriers in the electron transport pathway of Fe(II)-oxidation, however, is uncertain and controversial. In particular, there is debate on where Fe(II) oxidation takes place in the cell (2, 5, 58), although there is general agreement that the Fe(II) oxidase is located external to the cytoplasmic membrane.

Here, we describe the isolation and characterization of a genetically tractable Fe(II)-oxidizing phototroph that is closely related to the type strain of *Rhodopseudomonas palustris*. As a first step in the identification of the molecular

components of the phototrophic Fe(II) oxidation pathway, we performed a genetic screen

to identify genes involved in Fe(II) oxidation. The potential functions of these genes in

the process of phototrophic Fe(II) oxidation are discussed.

4.3   MATERIAL AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are

listed in Table 4.1.

Medium and culture conditions. Basal medium for phototrophic Fe(II)-oxidizing

bacteria was prepared as described by Ehrenreich and Widdel (20). Medium containing

dissolved Fe(II) but no Fe(II) precipitates (called filtered Fe(II) medium) was prepared by

adding $FeCl_2$ (to a final concentration of 10 mM) and subsequent filtration of the

precipitated Fe(II) minerals leaving ~4 mM Fe(II) in solution (for details see (13)). Fe(II)

containing medium refers to filtered Fe(II) medium unless specified otherwise. For

phototrophic growth with Fe(II) as the electron donor, cultures were incubated at 30 °C in

Fe(II)-containing medium with a N_2:CO_2 (80:20) headspace ~20 cm distance from a 40

W incandescent light bulb. For phototrophic growth with H_2 as the electron donor, H_2 is

supplemented in the headspace (H_2:CO_2 80:20). For aerobic growth, TIE-1 was grown in

YP medium that contains 0.3% yeast extract and 0.3% bactopeptone (Difco).

Escherichia coli strains were cultured in Luria-Bertani (LB) broth at 37 °C (44). *E. coli*

β2155 and WM3064 were supplemented with DAP (diaminopimelic acid, 300 μM final

concentration). Kanamycin and tetracycline were used at 200 and 50 μg/ml for TIE-1

and 50 and 15 μg/ml for *E. coli* respectively.

Table 4.1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or markers; characteristics and uses	Source or reference(s)
Bacterial strains		
E. coli β2155	Donor for bacterial conjugation; *ThrB1004 pro thi strA hsdS lacZΔM15* (F´ *lacZΔM15 lacI^q trajD36 proA^+ proB^+*) *ΔdapA::erm* (Erm^r) *pir*::RP4 [::*kan* (Km^r) from SM10]	(17)
WM3064	Donor strain for conjugation: *thrB1*004 *pro thi rpsL hsdS lacZ*ΔM15 RP4–1360 Δ(*araBAD)567 ΔdapA1341*::[*erm pir*(wt)]	W. Metcalf, Univ. of Illinois, Urbana
UQ950	*E. coli* DH5α λ (pir) host for cloning; FΔ*(argF-lac)169* Φ*80dlacZ58(*ΔM15*) glnV44*(AS) *rfbD1 gyrA96*(NalR) *recA1 endA1 spoT1 thi-1 hsdR17 deoR* λ*pir+*	D. Lies, Caltech
Rhodopseudomonas palustris CGA009	Wild type (ATCC BAA-98)	(34)
Plasmids		
pSC189	The transposon delivery plasmid. National Center for Biotechnology Information accession no. AY115560	(10)
pRK415	10.5 kb incP-1 (pK2) Tc^r lacZ	(32)
pT198	T198 PCR fragment, including the promoter region, cloned into the Xba I site of pRK415	This study
pT498	T498 PCR fragment, including the promoter region, cloned into the Xba I site of pRK415	This study

Isolation. Cultures of phototrophic Fe(II)-oxidizing bacteria were enriched by inoculating medium supplemented with 10 mM $FeCl_2$ (without filtration) with samples taken from an iron-rich mat from School Street Marsh in Woods Hole, MA. Enrichments were incubated at room temperature in the light. After a few days, rusty patches developed on the inner surface of the bottles. Cultures containing rusty patches were transferred successively to Fe(II) containing medium. After three transfers, the enrichments were subjected to an anaerobic agar dilution series where 6 ml of prewarmed medium supplemented with $FeCl_2$ and 1 ml of bacterial culture were mixed with 3 ml of 3% (w/v) molten agar in a test tube under a constant N_2 stream. Sequential dilutions were made by transferring 1 ml of bacteria/agar mix from one tube to the next until a series of 10 tubes were completed. The tubes were incubated in the light at room temperature (22°C). After 2 weeks, ovoid-shaped red colonies consisting of cells and rusty particles developed. Colonies were picked and subcultured in the filtered Fe(II)-containing medium. To select specifically for Fe(II)-oxidizing bacteria that could grow aerobically on agar plates, colonies formed in the agar dilution tubes were streaked to YP agar plates and incubated aerobically at 30 °C in the dark. Agar dilution series were repeated three times to obtain pure cultures; the purity was checked by phase contrast microscopy.

Analytical techniques. Fe(II) oxidation was monitored by measuring the consumption of Fe(II) over time. Fe(II) was quantified by the ferrozine assay (51). Cell mass was quantified by protein content determined by the method of Bradford (6), using reagents

obtained from Bio-Rad (Richmond, CA). The cell mass versus protein ratio was

determined with 200 ml of cell culture grown with 10 mM acetate phototrophically: cell

mass was dried completely at 70 °C. For protein measurement and microscopic cell

counts of Fe(II) grown cultures, the Fe(III) precipitates in 1 ml culture were dissolved by

addition of 800 μl of oxalate solution (28 g/L of ammonium oxalate and 15 g/L of oxalic

acid in 1 L ultra pure H_2O) plus 100 μl of ferrous iron ethylenediammonium sulfate (100

mM) as described by Suter et al. (54). Protein was precipitated by trichloroacetic acid

(0.5 M), collected by centrifugation and dissolved in NaOH (0.1 N) as described by

Ehrenreich and Widdel (20). Cells were counted by epifluorescence microscopy after

fixing with glutaraldehyde (2.5%) and staining with diamidino-2-phenylindole. The

mineral products of Fe(II) oxidation were identified by X-ray diffraction (XRD) analysis

as described by Kappler and Newman (31). Sample preparation and analysis by scanning

electron microscopy (SEM), energy dispersive spectroscopy (EDS) and transmission

electron microscopy (TEM) were done as described by Kappler and Newman (31).

MINEQL+ (Environmental Research Software) was used to calculate the Fe(II)

speciation of the medium at different pH values given the total soluble Fe(II)

concentration measured prior to inoculation. We assumed a closed system and the model

solution had basal component concentrations equal to the growth medium. We did not

account for the small decrease of phosphate concentration by the precipitation of

vivianite [$Fe_3(PO_4)_2 \cdot 8H_2O$] in our model, as the amount of phosphate removed from

solution was less than 4% (estimated by assuming that the decrease in soluble Fe(II)

measured at the highest pH tested, 7.5, was due to the precipitation of vivianite, and

calculating the corresponding amount of phosphate that would have been removed from solution). The total Fe(II) concentration was set to the experimentally measured total dissolved Fe(II) concentration at different pH values, and no solid was removed. The ionic strength of the solution was not considered and the temperature was set at 25 °C.

Determination of physiological and phototrophic characteristics. To test for growth with different carbon sources, sterile stock solutions of various carbon sources (acetate lactate, succinate, pyruvate, malate, fumarate, benzoate, formate and glucose) were added to the basal medium at a final concentration of 10 mM. Sulfide, sulfite or thiosulfate were tested as electron donor at 5 mM. Elemental sulfur (1 g/L) was added from an autoclaved suspension and growth was checked visually for pinkish turbidity. Growth of non-precipitate-containing cultures was monitored by increase of optical density at 600 nm. To determine the pH dependence of Fe(II) oxidation, the pH of the filtered Fe(II) containing medium (initial pH 6.8) was adjusted between 5.5 and 7.5 with 1 M HCl or 1 M Na_2CO_3. The headspace of the cultures for this experiment was initially flushed with H_2:CO_2 (80:20) to stimulate bacterial growth, and end point measurements of Fe(II) concentration and protein content were taken after 5 days when Fe(II) oxidation had not proceeded to completion. Whole cell absorption spectra were recorded in 40% (w/v) sucrose using a multidetection microtiterplate reader (Synergy HT, Bio-Tek, Winooski, VT). Carotenoids were extracted from phototrophically grown TIE-1 and *R. palustris* CGA009. Cells from 15 ml of cultures grown with acetate as the electron donor were harvested by centrifugation (10 min; 7,800 x *g*). For pigment extraction, 5 ml of a

mixture of acetone and ethanol (1:1) were added and all following procedures were done under extremely dim light to protect the pigments from photo transformations. The suspension was sonicated for 2 minutes and incubated in the dark at 30°C for 1 hour. The pigments were transferred to hexane by adding 3 ml of hexane and 0.5 ml of H_2O followed by vigorous mixing. The upper phase was collected and replaced several times until it stayed clear. The combined hexane fractions were concentrated ~10-fold under a stream of N_2 and stored at -20ºC before further analysis. The extracted pigments were separated using a normal-phase thin-layer chromatography (TLC) system with silica as adsorbent (Kieselgel 60, Merck, Darmstadt, Germany) and a mixture of petrolether and acetone (4:1) as the mobile phase. Absorbance spectra of the crude extracts were recorded in a quartz 96-well microtiterplate and identified by comparison to extracts from the closely related reference strain *R. palustris* CGA009 (7).

16S rDNA sequence determination. Cells grown in YP medium for 2 days were harvested by centrifugation. Genomic DNA was extracted using the DNeasy Kit (Qiagen). 16S rDNA was amplified using primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGATCC-3'). The PCR product was eluted in water after purification using a QIAquick PCR Purification Kit (Qiagen). DNA was sequenced by the DNA Sequencing Core Facility at the Beckman Institute at Caltech using primers 8F and 1492R, with 2X coverage. Sequence alignment was performed on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast/). Distance and

maximum likelihood phylogenetic trees were constructed using the ARB software

package (40).

Antibiotic sensitivity. Sensitivity of strain TIE-1 to antibiotics (chloramphenicol,

tetracycline, kanamycin, gentamycin and ampicillin) was determined by growth tests on

YP agar medium containing the antibiotics at various concentrations. 100 μl of a cell

suspension (~10^8 cells/ml) were spread on the agar plates with antibiotics and the number

of colonies formed was counted after aerobic incubation at 30°C in the dark for 5 days.

The minimal inhibitory concentration (MIC) was defined as the minimal antibiotic

concentration at which no colonies formed on the plate during the allotted incubation

time.

Genetic screen. To generate a library of transposon mutants to screen for Fe(II)-

oxidation defects, the plasmid pSC189, carrying the kanamycin resistant hyperactive

mariner transposon (10), was moved via conjugation from the donor strain *E.coli* β2155

to TIE-1. A deletion of the *dapA* gene of *E. coli* β2155 renders it unable to grow without

the exogenous addition of DAP to the growth medium (17). Thus, TIE-1 exconjugants

with transposon insertions can be selected on YP agar plates containing kanamycin (75

μg/ml) but no DAP. Transposon containing TIE-1 exconjugants were picked to 96-well

microtiter plates containing YP plus kanamycin and incubated aerobically at 30 °C

overnight with shaking. To test the transposon containing isolates for Fe(II) oxidation

activity by a cell suspension assay, 20 μl of these YP cultures was transferred to 96 well

microtiter plates containing 200 μl phototrophic basal medium without Fe(II). These plates were incubated anaerobically in the light under an atmosphere of $N_2:CO_2:H_2$ (80:15:5) in an anaerobic glove box. After 3 days of incubation, the plates were centrifuged and the supernatant was removed. Under anaerobic conditions, cell pellets were resupended in a buffer (50 mM Hepes, 20 mM NaCl, 20 mM $NaHCO_3$, pH 7) mixed with 200-300 μM of $FeCl_2$. After a 5-hour incubation in the light, 100 μl of ferrozine solution (1 g of ferrozine plus 500 g of ammonia acetate in 1 L of ddH_2O) was added into each well, and the OD_{570} was recorded to determine the concentration of the remaining Fe(II) (51). Putative mutants were identified in instances where the total Fe(II) removed from the system was less than ~50% relative to the wild type. At least three independent checks were performed for each mutant.

Southern blot. To verify that the mariner transposon inserted in a random fashion, we performed southern blot on 10 randomly selected mutants from different mating events. *Sma*I and *Sph*I digested genomic DNA from the mutants was separated on a 1% agarose gel and transferred to nylon membrane using a positive pressure blotting apparatus (Stratagene, CA) according to the manufacturer's instructions. Probe DNA was prepared from a gel-purified *Mlu*I restriction fragment of pSC189 that contained an internal part of the mariner transposon including the kanamycin resistance gene. Approximately 25 ng of probe DNA was labeled with 50 micro-Curries of alpha-P^{32}-dCTP using the Ready-To-Go labeling beads (Amersham Pharmacia Biotech). Prior to hybridization, unincorporated radioactive nucleotides were removed from the reaction by centrifugation

through sephadex columns (ProbeQuant G-50 Microspin columns, Amersham Pharmacia Biotech) according to the manufacturer's instructions. Nylon membranes were hybridized overnight at 65˚C. Hybridized membranes were washed 3 times for 5 minutes each in 2x SSC buffer (20 x SSC: 175.3 g/L NaCl plus 88.2 g/L of trisodium citrate) plus 0.1% SDS (sodium dodecyl sulfate) at room temperature, then twice of 15 minutes each with 0.1x SSC plus 0.1% SDS at 65˚C. The membrane was exposed to X-ray film at -80°C for 48 hr prior to development.

Cloning of mariner-containing fragments. To identify the DNA sequence flanking the transposon in the mutants, genomic DNA was digested with restriction enzyme *Sac*II followed by ligation at a DNA concentration (2-3 μg/ml) that favored intramolecular ligation (49). Ligated DNA was washed and concentrated using a DNA purification kit (Qiagen) and transformed into *E. coli* UQ950 cells. Plasmid DNA was extracted from overnight cultures of kanamycin resistant clones. The sites of transposon insertions of these mutants were determined by sequencing with primers Mar3 (5'-CTTCTTGACGAGTTCTTCTGAGC-3') and Mar4 (5'-TAGGGTTGAGTGTTGTTCCAGTT -3') that anneal near the ends of the mariner transposon in opposite directions. Sequence analysis and homology searches were performed by using the BLAST and the ORF Finder utilities that are available on the NCBI website (http://www.ncbi.nlm.nih.gov/blast/). Protein analysis including subcellular localization analysis and motif finding was done using TargetP and MotifScan on the ExPASy proteomics server (http://us.expasy.org/).

Complementation. Plasmid pT198 and pT498 were constructed to complement the genetic defect in mutants 76H3 and A2, respectively. Primers were designed based on the corresponding gene sequences in *R. palustris* CGA009 that were analogous to the disrupted genes in the mutants. For mutant 76H3, a 1.4 kb gene fragment was amplified through PCR from wild type TIE-1 with primers T198L (5'-GGCTCTAGATCAACCAGAAACCAGCTTCC-3') and T198R (5'-GGCTCTAGATGTGAGCCACTCTGTCATCC-3'). For mutant A2, a 1.3 kb gene fragment was generated with primers T498L (5'-GGCTCTAGACAATTGCGACAGCTTACGAC-3') and T498R (5'-GGCTCTAGA AGAACCGCCTTCTTGGTCT-3'). The purified PCR products were digested and ligated to the *Xba*I cloning site of the broad host plasmid pRK415 vector to generate the vectors pT198 and pT498 for complementation. pT198 and pT498 were introduced into *E. coli* UQ950 by transformation. Transformants with the inserts were isolated through a blue/white screen on LB plates with tetracycline (15 μg/ml). The plasmids pT198 and pT498 were purified from *E. coli* UQ950, transformed by heat shock into the *ΔdapA* donor strain *E. coli* WM3064 and moved via conjugation into the mutant TIE-1 strains. TIE-1 exconjugants containing vector pT198 and pT498 were selected on YP agar plates supplemented with 75 μg/ml of tetracycline. Colonies were picked and grown up in YP liquid medium with tetracycline (75 μg/ml). YP cultures were washed and subcultured in the basal medium plus tetracycline (75 μg/ml) with H_2 as the electron donor. Cells were

then collected by centrifugation and tested for complementation of Fe(II)-oxidation activity by the cell suspension assay as described.

4.4   RESULTS

4.4.1   Isolation

Phototrophic Fe(II)-oxidizing bacteria were enriched from samples taken from an iron-rich mat from School Street Marsh in Woods Hole, MA. The pH ranged from 6 to7 at this site. Rusty orange-brown crusts formed on the inner surface of the enrichment bottles incubated in the light but not in the dark, suggesting the presence of phototrophic Fe(II)-oxidizing bacteria. After several transfers in liquid medium and purification in agar dilution series, several isolates that looked microscopically identical were obtained. One isolate named TIE-1 was chosen for all further experiments. In the agar-shake tube, TIE-1 develops ovoid-shaped purple colonies containing orange-brown particles resembling iron rust. Phototrophically grown cells are motile and dumbbell shaped with a length of 1-4 μm (Figure 4.1A). The heterogenous cell morphology suggests that cells divide asymmetrically by budding, as is the case for closely related species (35). Electron microscopy reveals that phototrophically grown cells contain lamellar intracytoplasmic membranes (Figure 4.1B). TIE-1 forms colorless colonies with purple centers on YP agar plates aerobically in the dark (Figure 4.1C). TIE-1 is colorless in liquid medium under aerobic conditions, and purple when grown phototrophically.

4.4.2   Phylogeny

The 1,396 bp 16S rDNA sequence of TIE-1 was obtained and deposited in the Genbank database under the accession number AY751758. The 16S rDNA sequence was compared with the sequences from representative species of its close relatives, along with the phototrophic Fe(II)-oxidizing bacteria that have been isolated so far (Figure 4.1D). The 16S rDNA sequence of TIE-1 shares an identity of 98.9% and 99% to that of *Rhodopseudomonas palustris* ATCC17001[T] and strain CGA009, respectively, belonging to the alpha subdivision of the *Proteobacteria,* affiliated closely with the nitrogen fixing phototrophic rhizobia. TIE-1 clusters differently from the other known purple nonsulfur Fe(II) oxidizing phototrophs such as *Rhodobacter sp.* strain SW2 and *Rhodovulum sp.* strains N1 and N2.

Figure 4.1 A. Phase contrast micrographs showing the cell morphology of TIE-1 grown phototrophically with acetate as the electron donor. B. Lamellar intracytoplasmic membranes (arrow) of a cell from a one-week-old culture grown with Fe(II) as the electron donor. Fe(III) precipitates form outside the cell. C. Colony morphology on a YP agar plate incubated aerobically in the dark for 5 days. D. 16S rDNA based tree showing phylogenetic relationships between TIE-1 and related organisms. Bootstrap values are given at branch points. Anaerobic phototrophs able to oxidize Fe(II) are underlined. TIE-1 (boxed) is separated significantly from the other known purple nonsulfur Fe(II) oxidizing phototrophs such as *Rhodobacter sp.* strain SW2 and *Rhodovulum sp.* strains N1 and N2.

Figure 4.2 Phototrophic growth of strain TIE-1 with Fe(II) as the electron donor. Shown are Fe(II) concentration (◆), protein content (■), cell density (▲). Data are representative of three independent cultures. The increase in cell number is consistent with the increase of protein content and the progress of Fe(II) oxidation throughout the incubation. No Fe(II) oxidation occurs for the duration of the incubation in the abiotic control and bacterial growth and Fe(II) oxidation are light dependent (data not shown). Approximately 5.36 mg of biomass is produced per mmole of Fe(II) oxidized.

4.4.3 Phototrophic oxidation of Fe(II)

TIE-1 is able to grow photoautotrophically with Fe(II) as electron donor under

anaerobic conditions (Figure 4.2). The increase in cell number is concomitant with the

increase of protein content and the progress of Fe(II) oxidation throughout the incubation.

No Fe(II) oxidation occurs for the duration of the incubation in abiotic controls, and

bacterial growth and Fe(II) oxidation are light dependent (data not shown). Based on the

experimentally determined ratio of protein to cell mass of 47%, there is 2.52 mg of

protein, i.e., 5.36 mg of biomass produced per mmol of Fe(II) oxidized. This represents

~72% of the theoretical cell yield of 7.5 mg/mmol of Fe(II), based on the stoichiometry

of CO_2 reduction coupled to Fe(II) oxidation according to the equation: $4\ Fe^{2+} + HCO_3^- +$

$10\ H_2O = < CH_2O > + 7\ H^+ + 4\ Fe(OH)_3.$ This number is comparable to what has been

measured for a *Rhodomicrobium*-like isolate, which has 4.5 mg biomass produced per

mmole of Fe(II) oxidized (57).

The final product of Fe(II) oxidation accumulates exclusively outside the cell in

the form of Fe(III) precipitates. The spherical aggregates resembling poorly crystalline

Fe(III) (hydr)oxides appear the most in the youngest cultures (one week) and the more

crystalline needle-like structures resembling goethite appear only in the older cultures ($\geq$

3 weeks). Figure 4.3 shows that the Fe(III) precipitates are of two morphologies in a

three-week-old Fe(II) grown culture. One comprises small spherical aggregates of ~1 μm

in size (black arrow I) and the other has a needle-like shape (black arrow II). TEM and

XRD analysis suggest that the change of mineral morphology with time reflects mineral

transformation, consistent with previous findings for minerals in cultures of *Rhodobacter*

sp. strain SW2 (31). This type of mineral transformation is suggested to result from the

adsorption of Fe(II) onto ferric (hydr)oxide, promoting its transformation to the

thermodynamically more stable goethite (61). Elemental analysis of both types of

precipitates using EDS give signals only for iron and oxygen with the atomic ratio (O/Fe)

of 1.4 ± 0.1.

Figure 4.3 Scanning electron micrograph of ferric iron precipitates produced by TIE-1, showing the presence of two types of Fe(III) minerals including nano-spherical (black arrow I) and needle-like structures (black arrow II) in a two week old culture. The relative proportion of each morphological type varies with the age of the culture (see text). The white arrow indicates a TIE-1 cell.

4.4.4   Physiological and biochemical characterizations

Similar to other *R. palustris* strains, TIE-1 was able to grow aerobically in the dark with a doubling time of ~3 hours in YP medium (Figure 4.4A). TIE-1 could also grow photoautotrophically with H_2 as the electron donor (Figure 4.4B). Among sulfur compounds, TIE-1 uses thiosulfate, but not sulfide, elemental sulfur or sulfite as an electron donor to support photosynthetic growth. Phototrophic growth of TIE-1 can be supported by a number of organic substrates including acetate (Figure 4.4C), lactate, succinate, pyruvate, malate, fumarate and benzoate, but not formate or glucose (data not

shown). We also tested Fe(III) reduction by TIE-1 and found that TIE-1 was not able to reduce Fe(III) citrate with acetate as the electron donor in the dark (data not shown).

Fe(II) oxidation by TIE-1 is stimulated by the presence of H_2. In an atmosphere with 80% H_2, about 80% of the total Fe(II) in the system is oxidized within 5 days (data not shown), whereas the same amount of oxidation takes about 2 weeks when Fe(II) is as the sole electron donor (Figure 4.2). The cell density of a culture grown on H_2 (~10^9 cells/ml) is about 10 times as much as that when Fe(II) is the electron donor (Figure 4.2 and Figure 4.4B). Thermodynamically, this makes sense because the redox potential (ΔE_0') of the $2H^+/H_2$ redox couple (- 0.41 V) (41) is significantly lower than that of the $Fe(OH)_3/Fe^{2+}$ redox couple (- 0.11 V), calculated by setting $[Fe^{2+}] = 1$ mM and assuming equilibrium constants give by Morel and Hering (47); H_2, therefore, is expected to be the preferred electron donor, providing more free energy to support bacterial growth. Cells grown on H_2 alone can immediately oxidize Fe(II), i.e., H_2 does not reduce the rate of Fe(II) oxidation (data not shown). Together, these results suggest that the stimulation of Fe(II) oxidation in the presence of H_2 is due to the stimulation of bacterial growth by H_2.

Figure 4.4 Growth of TIE-1 chemoheterotrophically in YP medium aerobically in the dark (A), photoautotrophically with H2 as the electron donor (B), and photoheterotrophically with acetate as the electron donor (C). Doubling time (Td) is calculated for each growth condition from the slope of the curve over the exponential growth phase.

4.4.5 pH dependence of Fe(II) oxidation

To determine the optimal pH of Fe(II) oxidation by TIE-1, cells from Fe(II) grown

cultures were inoculated into Fe(II)-containing medium with H_2 in the headspace and the

pH of the medium was adjusted to values spanning 5.5 to 7.5. Bacterial growth and

dissolved Fe(II) were measured after a 5-day incubation. The pH was measured at the

end of the experiment, and in each case was within 0.2 units of the initial pH.

Appreciable Fe(II) oxidation occurred over the entire pH range tested, nevertheless,

under these conditions, the highest rate of Fe(II) oxidation occurred between pH 6.5 and

6.9 (Figure 4.5A), similar to the pH measured for other phototrophic Fe(II)-oxidizing

bacteria (20, 28). In contrast, the amount of bacterial growth (represented by the protein

content) was maximal and not appreciably different within the pH range 6.5 to 7.5. This

suggests that Fe(II) oxidation did not significantly contribute to cell growth in these

experiments (i.e., cells were growing on H_2). We also observed that the mineral product

of Fe(II) oxidation was pH dependent, with poorly crystalline ferric (hydr)oxide and goethite dominating at lower pH and magnetite at pH $> 7.2 \pm 0.2$ (Figure 4.3, Figure 4.5B). The same pH trend in iron mineralogy was observed for cultures grown on Fe(II) alone (data not shown).

Figure 4.5 A. pH dependence of phototrophic Fe(II) oxidation in the presence of H_2: dissolved Fe(II) concentration in the supernatant at the end of the experiment (◆), protein content after the incubation (☐). The optimal pH for Fe(II) oxidation occurs from 6.5 to 6.9. The total amount of Fe(II) oxidized decreases significantly at pH higher than 7.0, although the amount of bacterial growth represented by the protein content remains constant compared to that at pH 6.8. B. X-ray diffractograms of the product of Fe(II) oxidation by TIE-1 shows goethite and magnetite formation at medium pH of 6.8 and 7.5 respectively. For comparison, reference diffractograms of goethite and magnetite mineral standards are included.

Because the total amount of dissolved Fe(II) remained in excess of 1.5 mM at pH ≥ 6.9, it seems unlikely that the amount of Fe(II) oxidation measured over this time period was limited by soluble Fe(II). However, the possibility exists that changes in concentration of a minor species of Fe(II) might have controlled the rate of Fe(II)

oxidation. Considering this, we used MINEQL+ to calculate the equilibrium concentrations of soluble Fe(II) species over this pH range in the context of the composition of our medium. As pH increases from 6.5 to 7.5, MINEQL+ predicts that Fe^{2+} is the major Fe(II) species and only decreases by 0.4 mM over this pH range. Three minor Fe(II) species, including $Fe(OH)_3^-$, $Fe(OH)_{2(aq)}$ and $Fe(OH)^+$ increase in concentration 1000-, 80- and 10-fold, respectively. In the discussion, we consider how changes in these species' behavior with increasing pH might affect the overall Fe(II) oxidation rate.

4.4.6 Pigment characterization

The absorption spectrum of whole cells of TIE-1 grown with acetate as the electron donor shows three major peaks at 590, 805 and 871 nm similar to that of *R. palustris* CGA009, indicating the presence of bacteriochlorophyll a. The absorption spectrum obtained from TIE-1 grown with Fe(II) as the electron donor is similar and shows approximately the same peaks of absorbance as cells grown on acetate except that the peak at 871 nm is wider for the Fe(II) grow culture (Figure 4.6A). The absorption spectra of carotenoid extracts (Figure 4.6B) and thin-layer chromatography separations (data not shown) obtained from TIE-1 and CGA009 grown on acetate also look identical. The absorption spectra of carotenoid extracts show major peaks overlapping between 400 and 600 nm, suggesting the presence of carotenoids commonly present in *R. palustris* including spheroidene (450, 482, 514 nm), okenone (521 nm), lycopene and rhodopsin (463, 490, 524 nm) (43).

Figure 4.6 Pigment analysis: A. Absorption spectrum of cell suspension of strain TIE-1 grown with acetate (a) and Fe(II) (b) as the electron donor, and strain CGA009 (c) grown with acetate as electron donor under phototrophic conditions. All curves look similar and show major absorption peaks at 590, 805 and 871 nm, indicating the presence of bacteriochlorophyll a. B. Absorption spectra of carotenoid extraction from strain TIE-1 (a) and strain CGA009 (b) grown with acetate as the electron donor show major peaks overlapped intensively between 400 and 600 nm, indicating the presence of the normal spirilloxanthin series.

4.4.7   Characterization of antibiotic sensitivity

Growth of $\sim 10^7$ cells on YP solid or liquid medium is inhibited completely by

chloramphenicol (300 μg/ml), tetracycline (75 μg/ml), kanamycin (100 μg/ml),

gentamycin (300 μg/ml) and ampicillin (50 μg/ml). These results indicate that it should

be possible to select for the acquisition of these antibiotic resistant markers, which will facilitate genetic manipulation.

4.4.8   Transposon mutagenesis and mutant characterization

We used transposon mutagenesis to identify genes involved in phototrophic Fe(II) oxidation in strain TIE-1. The frequency of transposon insertion obtained for TIE-1 is ~10^{-5} with the mariner transposon. Southern blot analysis of 10 randomly selected isolates derived from independent transposition events indicated that the transposon integrates as a single event in random locations (data not shown).

We performed a limited screen of ~12,000 transposon insertion mutants for defects in phototrophic Fe(II) oxidation using a cell suspension assay. Based on the assumption that strain TIE-1 has the same number of genes as strain CGA009 and the transposition is purely random, this screen is ~88% saturated assuming a Poisson distribution (26). Fourteen mutants were identified as being defective in Fe(II) oxidation: eight mutants had general photosynthetic growth defects; the other six were specifically defective in Fe(II) oxidation. BLAST analysis performed on DNA sequences flanking the mariner insertions revealed that the sequence flanking the transposon has significant similarity to sequences from the genome of *R. palustris* strain CGA009 (38) in all cases.

The eight mutants exhibiting general growth defects grew at least 50% less on acetate or H_2 compared to the wild type (data not shown). Two of these mutants were disrupted in genes that are homologs of *bchZ* and *bchX,* known to encode proteins involved in bacteriochlorophyll synthesis (8). It is not surprising that our screen picked

up components of the general photosynthetic electron transport system given the large

variance in cell density in the step prior to the cell suspension assay. Two mutants,

however, were identified that are specifically defective in Fe(II) oxidation: 76H3 and A2.

76H3 is a representative of 5 mutants that have transposon insertions at different

locations in the same gene, whereas A2 was only isolated once. Both mutants exhibit

normal photosynthetic growth in minimal medium with H_2 as the electron donor, but their

ability to oxidize Fe(II) is less than 10% of the wild type (Figure 4.7 A and B).

Complementation of the disrupted genes indicates that their expression is necessary and

sufficient to restore nearly wild-type levels of activity, suggesting that Fe(II) oxidation

defects were not caused by the downstream genes (Figure 4.7 C and D).

Figure 4.7 Mutants 76H3 and A2 are specifically defective in Fe(II) oxidation. A. Normal growth of mutant 76H3 and A2 with H2 as the electron donor. Data are representative of two independent cultures. B. Defects in phototrophic Fe(II) oxidation for mutants 76H3 and A2 compared to wild type. Growth was stimulated with H2 present in the headspace initially. Data are representative of duplicate cultures. C. Mutant 76H3 and A2 carrying plasmids pT198 and pT498 respectively show 80% of Fe(II) oxidation compared to the wild type in the cell suspension assay. D. Organization of the genomic regions surrounding the mutated genes in mutants 76H3 and A2. The black arrows indicate the disrupted genes and the transposon insertion sites are marked by the open triangles. The numbers provided below the open reading frames (all arrows) are consistent with the numbers given for the identical regions from the CGA009 genome.

Because the sequence fragments from TIE-1 flanking the transposon insertions were highly similar to sequences from strain CGA009, we designed primers based on the CGA009 genome to sequence the regions surrounding the transposon insertions in 76H3 and A2 (Figure 4.7D). Both regions contained homologs of genes found in the same order in CGA009. Mutant 76H3 has a transposon insertion in a gene that shares 99% identity over the entire gene sequence (791 bp) to gene RPA0198 in *R. palustris* CGA009 that encodes a putative integral membrane protein. BLAST search predicts that the protein encoded by this gene shares 100% identity to a possible transport protein in *R. palustris* CGA009, 85% identity to a probable ABC transport permease in *Bradyrhizobium japonicum*, and 60% identity to a hypothetical transmembrane protein from *Magnetospirillum sp.* MS-1. It is predicted to encode a cytoplasmic-membrane protein with 6 internal helices based on sequence analysis with the Psort program (http://www.psort.org/). No known motifs could be identified in this protein by the Motifscan program (http://myhits.isb-sib.ch/cgi-bin/motif_scan). Based on the annotation of the CGA009 genome, the upstream genes encode a putative ABC transporter permease (RPA0197) and a putative ABC transporter ATP-binding protein (RPA0196). The downstream gene (RPA0199) encodes a putative phosphinothricin acetyltransferase.

Mutant A2 has a transposon insertion in a gene that shares 99% identity over the entire gene sequence (995 bp) to gene RPA0498 in *R. palustris* CGA009 that is annotated as a *cobS* gene. The translated protein sequence is 100% identical to a putative CobS in strain CGA009, 93% identical to a putative CobS from *Bradyrhizobium japonicum*, 80%

identical to a well-studied CobS from *Pseudomonas denitrificans* and 76% and 71%

identical to MoxR-like ATPases from *Rhodospirillum rubrum* and *Rhodobacter*

sphaeroides, respectively. Studies of CobS function in *P. dentitricians* have shown that

CobS is a cobaltochelatase-a cytoplasmic protein involved in cobalt insertion into

porphyrin rings (16). MoxR-like ATPases belong to a superfamily of proteins with

<u>a</u>ssociated <u>A</u>TPase <u>a</u>ctivity (AAA) (30). Not surprisingly, members of the MoxR family

function as chaperons/chelatases in the assembly of specific metal-containing enzymatic

complexes. Based on the annotation of the CGA009 genome, the genes downstream

appear to encode a N-acetylglutamate synthase and related acetyltransferases (RPA0497),

a CobT homolog (RPA0496), and a conserved hypothetical protein (RPA0495).

4.5   DISCUSSION

We have isolated and characterized a genetically tractable Fe(II)-oxidizing

bacterium, *Rhodopseudomonas palustris* strain TIE-1. Two Fe(II)-oxidizing strains of *R.*

palustris have been reported previously (20, 28, 57). Based strictly on morphological

characteristics, an Fe(II)-oxidizing *R. palustris*-like strain was first isolated from an iron-

rich ditch in Germany (20). This isolate did not oxidize iron completely and ceased to

grow at a grayish green intermediate oxidation state; it was not maintained in culture

collections (F. Widdel, personal communication). Although the type strain *R. palustris*

DSM123[T] was found to be incapable of Fe(II) oxidation by Ehrenreich and Widdel (20),

Heising and Schink claimed it was capable of Fe(II) oxidation (28). In this study, we

tested the Fe(II) oxidation capacity of strain *R. palustris* CGA009, whose genome has been sequenced. Strain CGA009 is not able to grow photoautotrophically on H_2 or Fe(II), however, photoheterotrophically-grown cells (using acetate as the electron donor) can slowly oxidize Fe(II) in the cell suspension assay at a similar rate to that achieved by acetate-grown cell suspensions of strain TIE-1 (unpublished data). In contrast, strain TIE-1 can grow photoautotrophically with H_2 and Fe(II), and H_2-grown cell suspensions readily oxidize Fe(II) at rates much higher than those achieved by acetate-grown cell suspensions.

Rates and products of phototrophic Fe(II) oxidation by TIE-1 are pH dependent. The amount of Fe(II) oxidation is significantly less at pH higher than 7.0, compared to that oxidized at optimal pH, but this is not due to a growth defect. Previous interpretations of a similar result by Heising and Schink (28) suggested that this might be due to the lower solubility of Fe(II) at higher pH (28). Although our measurements indicate that the total amount of dissolved Fe(II) in our system does not appreciably decrease as pH increases, more subtle species dynamics may control the bioavailability of Fe(II) under these conditions. The transformation from poorly crystalline ferric (hydr)oxides to more crystalline ferric (hydr)oxides is promoted by the adsorption of Fe(II) species onto the solid phase (61). Given that the point of zero charge (pzc) for ferric (hydr)oxide likely occurs at the upper end of our pH spectrum (3), we would expect cationic Fe(II) species to adsorb to the ferric (hydr)oxides as the pzc is reached and then exceeded with increasing pH. Interestingly, the abiotic oxidation rate of $Fe(OH)^+$ in both freshwater and seawater has been found to be 10^7 times greater than that of Fe^{2+} (45).

Moreover, $Fe(OH)_{2(aq)}$ is thought to be the most readily oxidized form of Fe(II) over a pH range from 6 to 8, and the rate-limiting step for the oxidation of Fe^{2+} under these conditions (45). Assuming $Fe(OH)_{2(aq)}$ or $Fe(OH)^+$ is the preferred species taken up or bound by TIE-1, the decrease in Fe(II) oxidation measured coincident with magnetite formation in our medium could be explained if sorption of these species by ferric (hydr)oxides out-competed their sorption/uptake by the cell. Alternatively, it is possible that the decrease in Fe(II) oxidation is due to the inactivation of a biomolecule involved in Fe(II) binding, uptake and/or oxidation at high pH.

Independent of whether magnetite formation affects the bioavailability of Fe(II), it is noteworthy that magnetite formation can be associated with this type of metabolism. Previous studies with other Fe(II)-oxidizing phototrophs only found various forms of ferric oxides (e.g., goethite and lepidocrocite) to accumulate in the culture medium over time; magnetite was never observed (14, 31, 52). In contrast, magnetite formation following an intermediate state of green rust was reported for the nitrate-dependent Fe(II)-oxidizing bacterium *Dechlorosoma suillum* strain PS (9). Magnetite formation has been reported for dissimilatory iron-reducing bacteria (DIRB) with magnetite formed through the reduction of ferric oxide (39). Magnetite formation by TIE-1 is unlikely to be formed through the re-reduction of Fe(III), however, based on the evidence that TIE-1 is unable to reduce Fe(III) citrate with acetate as the electron donor in the dark. Considering the differences in the chemistry of the medium used to grow these bacteria, the simplest way to account for magnetite formation in some, but not all of these cases, is that differences in medium chemistry controlled the amount and speed of Fe(II)

adsorption onto ferric (hydr)oxides (61). Because pH 7.5 is a reasonable pH value for ancient seawater (25), it is possible that the primary magnetite found in BIFs may record the activity of Fe(II)-oxidizing phototrophs. However, this interpretation does not exclude the possibility that magnetite in BIFs may also have been facilitated by DIRB or abiotic processes (48).

To begin to identify genes involved in phototrophic Fe(II) oxidation, we first needed to develop an efficient method for generating random chromosomal insertions in TIE-1. Transposon mutagenesis has been shown to work in the *Rhodospirillaceae* family, but with mixed success (18). For example, transposition by Tn5 derivatives were found to transpose in *R. capsulatus* and *R. rubrum* with frequencies of 10^{-4} to 10^{-5} (24, 33), however, for *R. palustris* strain CGA009 and strain EPT100*,* Tn5 derivatives were either not successful or very inefficient (21). The fact that the hyperactive mariner transposon used in this study transposes randomly and at high frequencies in TIE-1, suggests that this type of transposon may be an effective mutagenic tool for other *R. palustris* strains.

Out of a total of 12,000 mutants screened for their ability to oxidize Fe(II) in the cell suspension assay, only six were identified as being specifically defective in Fe(II) oxidation, and only two genes were implicated in this process. It is intriguing that both of these genes are also present in *R. palustris* strain CGA009, although this organism cannot grow on Fe(II). Given that photoheterotrophically-grown cells of CGA009 can oxidize Fe(II) in the cell suspension assay comparably to TIE-1 when grown under the same conditions, this indicates that Fe(II) oxidation can be decoupled from growth. However,

our cell suspension assay did not decouple Fe(II) oxidation from the photosynthetic

apparatus, as no Fe(II) oxidation occurred in the dark. It will be interesting to learn what

allows TIE-1 but not CGA009 to conserve energy from Fe(II) oxidation for growth. It is

possible that essential genes for this process are missing from CGA009, mutated, or not

expressed. To resolve this, a screen could be performed to identify TIE-1 mutants that

are incapable of phototrophic growth on Fe(II), or CGA009 could be complemented for

growth on Fe(II) through provision of genes from TIE-1 (13).

Although much remains to be learned about how TIE-1 oxidizes and grows on

Fe(II), the two mutants identified in this study provide important new information. Strain

A2 contains a disruption in a homologue of a cobalt chelatase (CobS). Because the

structures of cobaltochelatases and ferrochelatases (which insert Fe(II) into porphyrin

rings) are similar, it has been suggested that they have similar enzymatic activities (15,

50). While it is possible that the phenotype of A2 might be due to the disruption of an

enzyme that inserts Fe(II) into a protein or a cofactor that is involved in Fe(II)-oxidation,

this seems unlikely because cobatochelatases and ferrochelatases are typically different at

the amino-acid level (15). We hypothesize, instead, that a protein involved in Fe(II)

oxidation requires cobalamin as cofactor; if true, this would represent a novel use for

cobalamin (50). In contrast, strain 76H3 is disrupted in a gene that appears to encode a

component of an ABC transport system that is located in the cytoplasmic membrane.

While a variety of things could be transported by this system, whatever is being

transported (e.g., the Fe(II) oxidase or a protein required for its assembly) likely resides

at least momentarily in the periplasm. This raises the question of where Fe(II) is oxidized

in the cell? Because Fe(II) is known to enter the periplasmic space of gram negative

bacteria through porins in the outer membrane, it is conceivable that Fe(II) could be

oxidized in this compartment; alternatively, the Fe(II) oxidase could reside in the

outermembrane and face the external environment, as has been inferred for Fe(II)

oxidizing acidophilic bacteria (23, 59). Determining what catalyzes Fe(II) oxidation and

where it is localized are the most important next steps in our investigation of the

molecular basis of phototrophic Fe(II) oxidation. The isolation of the genetically

tractable strain TIE-1 will enable these studies.

4.6   ACKNOWLEDGEMENTS

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Chapter 5

The *pio* Operon Is Essential for Phototrophic Fe(II) Oxidation in *Rhodopseudomonas palustris* TIE-1

5.1   ABSTRACT

Phototrophic Fe(II) oxidizing bacteria couple the oxidation of ferrous iron [Fe(II)] to reductive CO_2 fixation using light energy, but until recently, little has been understood about the molecular basis for this process. Using *Rhodopseudomonas palustris* TIE-1 as a model organism, here we report the discovery of a 3-gene operon, designated as the *pio* operon (for phototrophic iron oxidation) that is necessary for phototrophic Fe(II) oxidation. The first gene in the operon, *pioA*, encodes a *c*-type cytochrome that is upregulated under Fe(II) growth conditions. PioA contains a signal sequence and shares homology with MtrA, a decaheme cytochrome c from *Shewanella oneidensis* MR-1. The second gene, *pioB,* encodes a putative outer membrane beta-barrel protein. PioB is a homologue of MtrB from *S. oneidensis* MR-1. The third gene, *pioC*, encodes a putative high potential iron sulfur protein (HiPIP) with a Tat signal sequence, and is similar to the putative Fe(II) oxidoreductase (Iro) from *Acidithiobacillus ferrooxidans*. Like PioA, PioB and PioC appear to be secreted proteins. Deletion of the *pio* operon results in loss of Fe(II) oxidation activity and growth on Fe(II). Complementation studies confirm that the phenotype of this mutant is due to loss of the *pio* genes. Deletion of *pioA* alone results in loss of almost all Fe(II) oxidation activity, however, deletion of either *pioB* or

pioC alone results in only a partial loss of Fe(II) oxidation activity. Together, these results suggest that proteins encoded by the *pio* operon are essential and specific for phototrophic Fe(II) oxidation in *R. palustris* TIE-1.

5.2   INTRODUCTION

One of the distinguishing features of microbial metabolism is its diversity: over billions of years of Earth history, microbes have evolved an impressive array of strategies to obtain energy for growth. The process of photosynthesis, for example, goes well beyond the ability to split water and produce oxygen. Different groups of microorganisms carry out "anoxygenic" photosynthesis, using substrates such as molecular hydrogen (H_2), various sulfur species, small organic molecules, or ferrous iron [Fe(II)] as an exogenous electron donor to drive reductive CO_2 fixation (6, 8, 12, 17). If we seek to understand the origins of the remarkable metabolic diversity that characterizes modern life on Earth, it is important to know how different types of metabolisms operate at the molecular level. This is necessary both to be able to compare the components of different metabolisms to each other, and to inform our search for biosignatures unique to these metabolisms in the rock record.

As a step towards this general goal, we have chosen to focus on the process of phototrophic Fe(II) oxidation, which can be described by the following equation:

$4Fe^{2+} + CO_2 + 11H_2O + h\nu = [CH_2O] + 4Fe(OH)_3 + 8H^+$.

This type of photosynthesis is interesting in the context of metabolic evolution for several

reasons. First, phototrophic Fe(II) oxidation is phylogenetically widespread, appearing in purple and green bacteria (10, 14, 21, 22, 65, 66); phylogenic comparisons of genes from different photosynthetic organisms suggest that anoxygenic photosynthesis is more ancient than oxygenic photosynthesis (57, 72). Second, iron has an intermediate redox potential ($\Delta E_0' = -0.11$ V) (28) compared to other substrates used as electron donors in photosynthesis (e.g. H_2 ($\Delta E_0' = -0.41$ V) or H_2O ($\Delta E_0' = 0.82$ V)) (28, 44). It has been suggested, therefore, that Fe(II)-based photosynthesis may represent a transition form of metabolism from anoxygenic to oxygenic photosynthesis (57). Third, Fe(II) is thought to have been the most widespread source of reducing power in the late Archean and early Proterozoic (3.8-1.6 billion years ago (Ga)) with an estimated concentration of about 0.1 to 1 mM in seawater (69); atmospheric oxygen seems to have appeared in significant amounts only after 2.4 Ga (15, 26, 30, 60).

Banded Iron Formations (BIFs), are an ancient class of iron ore deposits that may record the story of the evolution of photosynthesis. Because the use of Fe(II) results in the production of ferric iron [Fe(III)] minerals, it has been suggested that Fe(II)-based phototrophy might have been responsible for catalyzing BIF deposition early in Earth history (14, 34, 71). Later occurrences of BIFs (e.g., at 1.8 Ga), however, are believed to have resulted from Fe(II) oxidation catalyzed by molecular oxygen produced by cyanobacteria. Episodic deposition of BIFs throughout the Precambrian thus may reflect a transition from anoxygenic to oxygenic photosynthesis. How did ancient phototrophs evolve from using Fe(II) as an electron donor to using H_2O?

To address this question, we must understand the molecular machinery of

phototrophic Fe(II) oxidation. Discovered in the early 1990's by Widdel and co-workers (71), phototrophic Fe(II) oxidizing bacteria such as *Thiodictyon, Rhodobacter*, and *Chlorobium* species have been isolated from a wide variety of environments, including both freshwater and marine settings (14, 21, 29, 66, 71). However, very little is understood at the molecular level about the mechanism of Fe(II) oxidation in any of these organisms. In the companion paper to this article, we report the discovery of a *c*-type cytochrome and a putative pyrroloquinoline quinone containing enzyme from an Fe(II)-oxidizing strain-*Rhodobacter* SW2-that stimulates Fe(II) oxidation activity in its close relative, *Rhodobacter capsulatus* SB1003 (11). Because our ability to explore the mechanistic basis of Fe(II) oxidation in SW2 itself is limited due to the impracticality of direct mutational analysis (11), we established a genetic system in a different Fe(II)-oxidizing phototroph, *Rhodopseudomonas palustris* TIE-1 (28). In this report, we describe the identification of the *pio* operon, a 3-gene operon essential for phototrophic growth on Fe(II) by *R. palustris* TIE-1.

5.3   MATERIAL AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 5.1. *R. palustris* CGA010, derived from parent strain CGA009 after a frame shift in the *hupV* gene was repaired, was kindly provided by F. Rey and C. S. Harwood (University of Washington).

Media and culture conditions. For aerobic growth *R. palustris* strains were grown in

YP medium (0.3% yeast extract and 0.3% Bacto Peptone (Difco)) with shaking at 30˚C.

For anaerobic growth *R. palustris* strains were grown without shaking at 30˚C in FEM, a

defined basal medium for phototrophic Fe(II)-oxidizing bacteria (12). For

photoheterotrophic growth FEM was supplemented with 10 mM acetate. For

photoautotrophic growth electron donors were used such as thiosulfate (10 mM),

hydrogen (80% atmosphere), and soluble Fe(II). FEM Medium containing soluble Fe(II)

was prepared as previously described and the final Fe(II) concentration is in the range of

4 to 6 mM (28). Cultures were incubated 20 to 30 cm from a 34 W tungsten,

incandescent light source at 30°C. All phototrophic cultures, except those grown on

hydrogen, were grown in an atmosphere consisting of 80% N_2 and 20% CO_2.

Escherichia coli strains were cultured in lysogeny broth (LB) at 37°C. *E. coli* WM3064

was supplemented with 300 μM diaminopimelic acid (DAP). Kanamycin and

gentamicin were used at 100 and 200 μg/ml for *R. palustris* and 50 and 20 μg/ml for *E.*

coli, respectively.

Table 5.1 Bacterial strains and plasmids.

Strain or plasmid	Genotype or markers, characteristics [a]	Source or reference
E. coli stains		
WM3064	Donor strain for conjugation: *thrB1004 pro thi rpsL hsdS lacZ*ΔM15 RP4–1360 Δ(*araBAD*)567 Δ*dapA*1341::[*erm pir*(wt)]	W. Metcalf, Univ. of Illinois, Urbana
UQ950	*E. coli* DH5α (pir) host for cloning; F- Δ*(argF-lac)169* Φ*80dlacZ58(*ΔM15) *glnV44*(AS) *rfbD1 gyrA96*(NalR) *recA1 endA1 spoT1 thi-1 hsdR17 deoR λpir+*	D. Lies, Caltech
S17-1	*thi pro hdsR hdsM$^+$ recA*; chromosomal insertion of RP4-2 (Tc::Mu Km::Tn*7*)	(63)
R. palustris stains		
TIE-1	Isolated from Woods Hole, MA	(28)
CGA009	Wild type (ATCC BAA-98)	(32)
CGA010	*hupV$^+$* derivative of CGA009	F. Rey & C. S. Harwood, University of Washington
TIE-3	*R. palustris* str. TIE-1, Δ*pioABC*	This study
Δ*pioA*	*R. palustris* str. TIE-1, Δ*pioA*	This study
Δ*pioB*	*R. palustris* str. TIE-1, Δ*pioB*	This study
Δ*pioC*	*R. palustris* str. TIE-1, Δ*pioC*	This study
Plasmids		
pJQ200SK	Mobilizable suicide vector; *sacB* Gm^r	(59)
pYQABC	2-kb fusion PCR fragment containing ΔpioABC cloned into the *Spe* I site of pJQ200sk; used to make the TIE-3 *ΔpioABC* strain.	This study
pYQA	2-kb fusion PCR fragment containing ΔpioA cloned into the *Spe* I site of pJQ200sk; used to make the TIE-4 *ΔpioA* strain.	This study
pYQB	2-kb fusion PCR fragment containing ΔpioB cloned into the *Spe* I site of pJQ200sk; used to make the TIE-5 *ΔpioB* strain.	This study
pYQC	2-kb fusion PCR fragment containing ΔpioC cloned into the *Spe* I site of pJQ200sk; used to make the TIE-6 *ΔpioA* strain.	This study
pBBR1MCS-2	5.1-kb broad-host range plasmid: Km^r, *lacZ*	(31)
pYQ01	PCR fragment, including *pioABC*, generated using primers cyc-start and FeS-end cloned into the *Hind* III and *Spe* I sites of pBBR1MCS-2	This study
pYQ02	PCR fragment, including *pioA,* generated using primers cyc-start and cyc-end, cloned into the *Hind* III and *Spe* I sites of pBBR1MCS-2	This study
pYQ03	PCR fragment, including *pioB*, generated using primers MtrB-start and MtrB-end, cloned into the *Hind* III and *Spe* I sites of pBBR1MCS-2	This study
pYQ04	PCR fragment, including *pioC*, generated using primers FeS-start and FeS-end, cloned into the *Hind* III and *Spe* I sites of pBBR1MCS-2	This study

[a] Km, kanamycin; Gm, gentamicin.

Cell suspension assay. All cell suspension assays were conducted at room temperature in an anaerobic chamber containing an atmosphere of 80% N_2, 15% CO_2 and 5% H_2 (12, 28). Fe(II) cultures used for this assay contained 10 mM nitrilotriacetic acid (NTA) to prevent ferric iron precipitation. NTA alone does not support phototrophic growth of *R. palustris* (data not shown). Cells were pre-grown in the medium indicated until mid-exponential phase to an OD 660 nm of ~ 0.3 measured by 96-well plate reader (Synergy HT, Bio-Tek, Winooski, VT) with a volume of 200 μl. Cells were harvested by centrifugation (10,000 x g for 15 min) and washed in the same volume of HEPES buffer (50 mM N-2-hydroxyethylpiperazine-N`-2-ethanesulfonic acid with 20 mM NaCl, pH 7.0).   To start the assay, cells were resuspended in HEPES buffer containing 20 mM $NaHCO_3$ and either 400 μM or 1 mM (as indicated in Figure 5.1) of $FeCl_2$. Cells were concentrated approximately 3 times compared to the original growth culture and 100 μl of the cell suspension was aliquoted into a 96-well plate. The OD reading is about 0.7 measured by the 96-well plate reader. The plates were incubated at room temperature in the glove box under a 40 W tungsten light with light intensity of about 3000 lux. Over time 100 μl ferrozine solution (1 g of ferrozine plus 500 g of ammonia acetate in 1 L of ddH_2O) was added to the wells to monitor Fe(II) levels (64). The rate of Fe(II) oxidation was calculated based on the linear portion of the curves generated.

Extract preparation, SDS_PAGE analysis and heme staining. *R. palustris* TIE-1 was grown on either H_2, thiosulfate or Fe(II) plus NTA until mid-exponential phase and cells were harvested by centrifugation at 10, 000 *g* for 15 min. Cell pellets were resuspended

and washed 3 times in the same volume of HEPES buffer and resuspended in the same buffer containing Protease inhibitor cocktail (Roche) and 50 µM DNase (Roche) and incubated at 4 °C for 30 min. Cells were disrupted by French press (3 passes at 18,000 psi) and the cell lysate was clarified by centrifugation at 10,000 g for 15 min at 4 °C. The resulting supernatant was centrifuged at 200,000 g for 120 min at 4°C. The supernatant was defined as the soluble fraction and the pellet, which was resuspended in HEPES buffer, was defined as the membrane fraction. Protein concentrations were determined by the Bradford assay (7). SDS-PAGE was preformed by standard procedures according to Laemmli (38). Soluble and membrane fractions were incubated in loading buffer containing dithiothreitol at room temperature for 10 minutes without heating, and separated on a 12% Tris/HCl pre-cast gel (Bio-Rad). Coomassie staining was performed using BioRad standard staining protocol as described by the manufacturer. Gels stained for heme-containing proteins were performed according to the in-gel peroxidase activity assay as described (16).

Table 5.2 Sequence of the oligonucleotides

Oligonucleo tide	Length (bp)	Sequence (5'-3')
pioA1	28	GG<u>ACTAGT</u>CCGACATCGTACTCAACGAC
pioA1p	41	**TATTTAAATTTAGTGGATGGG**TACGAACAGCGACGAGATCC
pioA2	28	GG<u>ACTAGT</u>AGTATTGGCCGCTGAGTTTG
pioA2p	42	**CCCATCCACTAAATTTAAATA**TGCCAGAATTGTCACAACAAC
pioB1	28	GG<u>ACTAGT</u>GTTACTTCGTCGGCTCCAAG
pioB1p	41	**TATTTAAATTTAGTGGATGGG**TACGGTCACCACGGAGATTG
pioB2	28	GG<u>ACTAGT</u>TCGACGACGAAGGCTTCTAT
pioB2P	41	**CCCATCCACTAAATTTAAATA**GCGCAGTACTTCCAGGTCTC
pioC1	28	GG<u>ACTAGT</u>AACGCCGCTACGACAATTAC
pioC1p	40	**TATTTAAATTTAGTGGATGGG**GTCGTTGCGTTTGTCGTTC
pioC2	28	GG<u>ACTAGT</u>TCAGTTCATGTGCCAGCATC
pioC2p	39	**CCCATCCACTAAATTTAAATA**AGCCCGATCAGCGAGAAC
pioA-start	28	GG<u>AAGCTT</u>CCGACATCGTACTCAACGAC
pioA-end	28	GG<u>ACTAGT</u>GAGTCGTTCCATCACCCTTC
pioB-start	26	<u>AAGCTT</u>GAACGCTTGCCAGAATTGTC
pioB-end	26	<u>ACTAGT</u>GTCTGTCGTCCTCCCATTGT
pioC-start	28	GG<u>AAGCTT</u>CAGCCAACGTCAACAACAAT
pioC-end	28	GG<u>ACTAGT</u>GCAGATGACGTGATCAAAGC
RT-pioA-L1	20	TCAACGACACCTGCTACACC
RT-pioB-R1	20	TTACGGTCACCACGGAGATT
RT-pioB-L1	20	GCGCAGTACTTCCAGGTCTC
RT-pioC-R1	19	GTCGTTGCGTTTGTCGTTC
RT-pioAL	20	AGG TGA TGG ACA CCT GCT TC
RT-pioAR	20	ACG CAG GTG ATT TTC GTT TC
RT-pioBL	20	GCCTGAAGAAGAGCAACACC
RT-pioBR	20	GCATAGCCGAGCTTGAAATC
RT-pioCL	19	GAACGACAAACGCAACGAC
RT-pioCR	19	AGGCCTTCTTGGTGACCTG

The linker region in the primers for crossover PCR are in bold and the restriction sites are underlined.

RT-PCR. *R. palustris* TIE-1 was grown photoautotrophically on Fe(II) plus NTA until exponential phase. Total RNA was extracted as described previously (56). Briefly, cells were harvested and resuspended in 1 ml TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). Cells were disrupted using a Mini-BeadBeater-8 (BioSpec Products, Bartlesville) in 2 ml screw-capped tubes containing approximately 1 ml 0.1 mm zirconia/silica beads (BioSpec) for 1 min periods, with cooling on ice after each period with a total of 4 min. RNA extraction was then carried out using Qiagen RNA extraction kit. DNase digestion

was performed on the Mini-column with the Qiagen RNase-free DNase set. The RNA was eluted from the column, and a second DNase treatment was performed with Roche RNase-Free DNase. The RNA was finally resuspended in 40 µl nuclease-free water. cDNA was synthesized using BioRad iScript cDNA Synthesis Kit. Control PCR using RNA as template in the absence of reverse transcriptase confirmed that the isolated RNA was free of contaminating genomic DNA. Primers used for all RT-PCR reactions are listed in Table 5.2. To test if *pioABC* are cotranscribed, primers RT-pioA-L1 and RT-pioB-R1 were used to detect the presence of transcript *pioAB* and primers RT-pioB-L1 and RT-pioC-R1 were used to detect transcript *pioBC*. To test the transcription of *pio* genes in the mutant background *ΔpioA*, *ΔpioB* or *ΔpioC,* RT-pioAL and RT-pioAR was used to detect *pioA*, RT-pioBL and RT-pioBR for *pioB* and RT-pioCL and RT-pioCR for *pioC*.

Cloning, DNA manipulations, and mutant construction. Standard protocols were used for DNA cloning and transformation (28). Plasmids were purified on QIAprep spin columns (Qiagen, Chatsworth, CA). *R. palustris* TIE-1 chromosomal DNA was isolated using DNeasy kit (Qiagen). DNA was extracted from agarose gels using the Qiaquick gel extraction kit (Qiagen), and plasmid DNA was purified with the Qiaprep spin miniprep kit (Qiagen). DNA was sequenced at the Laragen DNA sequencing center (http://www.laragen.com/services.htm) by standard automated-sequencing technology.

Construction of deletion mutant. All primer sequences used in construction of the

mutants are listed in Table 5.2. For construction of *pio* operon deletion mutant TIE-3, a 1

kb DNA fragment upstream of *pioA* was produced by PCR with primers pioA1 and

pioA1p using TIE-1 genomic DNA as template. Similarly, a 1 kb PCR fragment

downstream of *pioC* was generated with primers pioC2 and pioC2p. The PCR products

were used as templates for another round of fusion PCR with primers pioA1 and pioC2.

The resulting 2 kb fusion PCR product was gel purified and restriction digested using

restriction enzyme Spe I, and cloned into the suicide vector pJQ200sk (59) to generate

pYQABC. pYQABC was mobilized into TIE-1 by conjugation from *E. coli* S17-1 (13).

Selection of single recombinants on PM plates containing 400 µg/ml of gentamicin

followed by selection of double recombinants on PM sucrose (10%) plates were

conducted as described (13). Individual gene deletion mutant *ΔpioA* and *ΔpioB* and

ΔpioC were made in a similar manner via suicide plasmids pYQA, pYQB and pYQC,

respectively. Primers used for generating pYQA are pioA1, pioA1p, pioA2 and pioA2p,

for pYQB are pioB1, pioB1p, pioB2 and pioB2p, for pYQC are pioC1, pioC1p, pioC2

and pioC2p. PCR was used to verify that the expected deletion had occurred.

Generation of complementing plasmids. The *pioABC* operon and the individual *pio*

genes were amplified from genomic DNA of TIE-1 using the FailSafe PCR kit

(Epicentre, WI). The PCR products were designed to have EcoR I and Hind III

restriction sites, and were ligated *in trans* into vector pBBRMCS-2 (35, 36) digested with

the same enzymes. The resulting plasmids were conjugated into *R. palustris* strains

indicated (28). The *pioABC* operon was amplified with primers pioA-start and pioC-end (pYQ01), *pioA* gene with primers pioA-start and pioA-end (pYQ02), *pioB* gene with primers pioB-start and pioB-end (pYQ03), pioC gene with primers pioC-start and pioC-end (pYQ04).

5.4   RESULTS

5.4.1   Identification of an Fe(II)-oxidation specific *c*-type cytochrome

With the goal of identifying proteins that are expressed when TIE-1 grows on Fe(II), we compared the Fe(II)-oxidation activity of cell suspensions that had been pre-grown photoautotrophically on different electron donors including H_2, thiosulfate and Fe(II). Cells were collected and resuspended in buffer containing Fe(II), and Fe(II) oxidation was followed by the ferrozine assay. When 1 mM initial Fe(II) was provided, approximately 0.8 mM of Fe(II) was oxidized within the first half hour with Fe(II)-grown cells, whereas only 0.2 mM of Fe(II) was oxidized with H_2- or thiosulfate-grown cells (Figure 5.1). Compared to the H_2- or thiosulfate-grown cells, Fe(II)-grown cells showed a 4-5 fold higher rate of Fe(II) oxidation activity, suggesting that specific proteins were induced under Fe(II)-grown conditions. Given these results, we assayed for differential protein expression with cells grown on Fe(II) compared to other electron donors. Crude cell extracts from cells grown on H_2, thiosulfate or Fe(II) were separated by SDS-PAGE. Although no significant differences were detected visually by coomassie staining (data not shown), a difference in expression of *c*-type cytochromes was observed by heme

staining. Accordingly, we characterized the expression profile of *c*-type cytochromes

from soluble and membrane fractions of cells grown on Fe(II), H$_2$ and thiosulfate (Figure

5.2). A unique *c*-type cytochrome (~40 kDa) appeared in significant quantity in the

soluble fraction only when cells were grown on Fe(II). Protein identification by mass

spectrometry indicated that peptide fragments of this protein match those of a putative

decaheme *c*-type cytochrome from *Rhodopseudomonas palustris* CGA009 (encoded by

gene RPA0746) (39).

Figure 5.1 Fe(II) oxidation activity of *R. palustris* TIE-1 tested by a cell suspension
assay with cells pre-grown phototrophically with Fe(II), H$_2$ or thiosulfate as the electron
donor. Approximately 5×10^9 cells/ml were used in the cell suspension assay. Compared
to the H$_2$- or thiosulfate-grown cells, Fe(II)-grown cells showed a 4 to 5 fold higher rate
of Fe(II) oxidation activity, suggesting that specific proteins were induced under Fe(II)-
grown conditions.

Figure 5.2 Heme staining of crude cell extract, soluble and membrane proteins of TIE-1 grown on Fe(II), H₂, and thiosulfate, separated by SDS-PAGE. A *c*-type cytochrome (~40 kDa) indicated by the black arrow is highly expressed in the soluble fraction of Fe(II)-grown cells. Approximately 100 mg of protein was loaded per lane. The dark diagonal line in the "soluble fraction thio" lane is a tear in the gel.

5.4.2 Identification and sequence analysis of *pio* genes

By designing primers based on the CGA009 genome, we were able to sequence a 5.7 kb region from TIE-1 that includes the decaheme *c*-type cytochrome open reading frame (ORF) as well as two downstream ORFs (Figure 5.3). We designate these genes *pioA, pioB* and *pioC*, where *pio* stands for p̲hototrophic i̲ron o̲xidation. The DNA sequence of *pioA*, *pioB* and *pioC* was deposited in the Genbank database under the accession numbers EF119739 , EF119740 and EF119741, respectively. The deduced protein sequences of *pioA*, *pioB* and *pioC* are about 98%, 97% and 100% identical to those of RPA0746, RPA0745 and RPA0744, respectively, indicating high sequence similarity between TIE-1 and CGA009 over this region, consistent with the highly

conserved sequences previously identified between these two strains (28). To test the hypothesis that genes *pioABC* form an operon, we carried out RT-PCR experiments using primers designed to amplify the intergenic regions. RT-PCR products were obtained for both intergenic regions in the cluster (Figure 5.3). No product was obtained in controls to which reverse transcriptase or template was omitted. These results show that *pioABC* are co-transcribed. An intergenic region of about 700 bp is present upstream of *pioA*, proceeded by an ORF encoding a protein homologous to a subunit of the putative sulfate ABC transporter CysA from *E. coli* K-12 (27). The ORF downstream of *pioC* transcribes in the opposite direction relative to the *pio* operon. Because of the presence of the large intergenic region upstream of the *pio* operon, as well as the opposite direction of transcription for the downstream ORF, it seems likely that the *pio* operon functions independently of the adjacent genes.

Figure 5.3 Organization of the *pio* genes on the *R. palustris* TIE-1 chromosome. Arrows indicate the direction of transcription. The gene numbers corresponding to these genes in *R. palustris* CGA009 are given. The small black arrows A, B, C and D indicate the locations of primers used for RT-PCR experiments. PCR products were obtained for both of the regions between the *pio* genes, indicating they constitute an operon: RT reactions (lane 1 and 5), control with no reverse transcriptase added to cDNA (lane 2 and 6), TIE-1 genomic DNA control (lane 3 and 7), and no template control (lane 4 and 8).

The deduced amino acid sequence of *pioA* consists of 540 amino acids with a putative signal sequence characteristic of secreted proteins through the Sec pathway; a cleavage site is predicted between residue 40 and 41, according to SignalP (http://www.cbs.dtu.dk/services/SignalP/). Lack of hydrophobic regions within PioA, with the exception of the signal sequence, as well as the observation that PioA is in the soluble fraction (Figure 5.2), suggest that PioA is likely to be a periplasmic protein. PioA contains 10 putative heme-binding sites (CXXCH) characteristic of *c*-type cytochromes. Comparison of PioA to sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/blast/) reveals that it is similar to several decaheme *c*-type cytochromes in *Shewanella, Vibrio* and *Geobacter* species (4, 40-43, 48, 51-54). In particular, it has 40% identity and 55% similarity over 285 amino acids to MtrA from *Shewanella oneidensis* MR-1, which is involved in metal (e.g., Fe(III) and Mn(IV)) reduction (5, 49, 50, 58), and this similarity is mostly due to the highly conserved nature of the heme-binding sites that are present close to the C-terminal end of PioA. However, approximately 270 amino acids close to the N-terminus of PioA have no homolog in the database. No significant similarity was found when comparing PioA to other proteins in the database.

The second ORF, *pioB*, is 99 nucleotides downstream of *pioA*. *pioB* encodes a protein of 810 amino acids and contains a putative signal peptide with a predicted cleavage site between residue 25 and 26 based on the SingalP program, suggesting it is also secreted through the Sec pathway. It has a putative porin motif close to the C-terminus according to InterProScan (http://www.ebi.ac.uk/InterProScan/) and is predicted

to be an outer membrane β-barrel protein according to Transmembrane Barrel Hunt (20) and PRED-TMBB programs (3). Comparison of PioB to sequences in the databases reveals similarities to several outer membrane proteins from *Shewanella* and *Geobacter* species. In particular, it has 21% identity and 38% similarity over 536 amino acids close to the c-terminus of an outer membrane protein MtrB from *Shewanella oneidensis* MR-1, which is involved in metal (e.g., Fe(III) and Mn(IV)) reduction (4, 51). However, approximately 120 amino acids at the N-terminus show no homology to anything in the database. According to the secondary structure predicted by PRED-TMBB (3), both PioB and MtrB are outer membrane porins with 28 transmembrane beta-strands, the largest number of beta-strands among all known outer membrane porins (9, 33, 62). Similar to other outer membrane porins, PioB and MtrB are predicted to have long loops protruding into the extracellular space and short turns on the periplasmic side, except that PioB has longer extracellular loops compared to MtrB, consistent with the sequence length difference between the two proteins. The conserved regions between PioB and MtrB mainly occur in the transmembrane regions, consistent with the idea that these regions are generally more conserved than the loop regions among outer membrane porins (62).

The third ORF, *pioC*, is 140 nucleotides downstream of *pioB*. *pioC* encodes a putative <u>hi</u>gh <u>p</u>otential <u>i</u>ron-sulfur <u>p</u>rotein (HiPIP) that contains an iron-sulfur binding site. The deduced amino acid sequence of *pioC* consists of 94 amino acids with a predicted twin-arginine translocation (Tat) signal sequence at the N-terminus, suggesting export into the periplasm through the Tat protein translocation pathway. A signal

sequence cleavage site was predicted between residues 37 and 38 based on the SignalP

program. Because there is no transmembrane region other than the signal peptide

predicted by HMMTOP (http://www.enzim.hu/hmmtop/html/submit.html), we predict

PioC resides in the periplasm. Comparison of PioC to sequences in the database reveals

similarities to HiPIPs from several bacteria, with most of the similarity occurring over

approximately 50 amino acid residues close to the C-terminus spanning the iron-sulfur

cluster binding site. PioC is 47% identical and 52% similar over 48 amino acids to a

HiPIP from *Rhodopila globiformis* (1), is 32% identical to a hypothetical protein encoded

by gene RPA3566 from *R. palustris* CGA009, and is 44% identical and 53% similar over

51 amino acids to a HiPIP from *Acidithiobacillus ferrooxidans*, a putative iron

oxidoreductase known as the "Iro" protein (19, 37).

5.4.3   *pioABC* are specifically required for phototrophic Fe(II) oxidation

To determine whether the *pio* operon is necessary for growth on Fe(II), we

constructed a mutant (TIE-3) in which all 3 genes in the *pio* operon were deleted from the

chromosome by homologous recombination. We tested the ability of mutant TIE-3 to

grow on different substrates. When Fe(II) was provided as the electron donor for

photoautotrophic growth, very little Fe(II) was oxidized by strain TIE-3 in a period of 2

weeks (Figure 5.4A). In contrast, wild type strain TIE-1 oxidized Fe(II) to completion

within this time period. End point measurements of total protein content in the cultures

indicated that TIE-3 did not grow over the course of incubation in contrast to TIE-1

(Figure 5.4A). To determine if TIE-3 was specifically defective for growth on Fe(II), we

tested growth on substrates other than Fe(II). Photoautotrophic growth of TIE-3 on H_2 or

thiosulfate and photoheterotrophic growth on acetate were tested by measuring cell OD.

TIE-3 grew on these substrates as well as TIE-1 (Table 5.3). These results indicate that

the *pioABC* operon is essential and specific for growth on Fe(II).

Figure 5.4 A. Defect in growth and phototrophic Fe(II) oxidation in the *pio* operon
deletion mutant TIE-3. Data are representative of triplicate cultures. Whereas TIE-1
oxidized Fe(II) to completion in a period of 2 weeks, very little Fe(II) was oxidized by
TIE-3. End point measurements of total protein content in these cultures revealed that
TIE-3 did not grow during the course of incubation, in contrast to TIE-1. B. TIE-3 is
defective in Fe(II) oxidation activity measured by the cell suspension assay compared to
TIE-1. Complementation with the *pio* operon on a plasmid (pYQ01) restored TIE-3's
Fe(II) oxidation activity to about 50% of that of TIE-1, whereas a vector control
(pBBRMCS-2) had no effect (data not shown).

Table 5.3 Comparison of doubling time of *R. palustris* TIE-1 and TIE-3 grown on different substrates.

Substrates	TIE-1 (hours)	TIE-3 (hours)
Fe(II)	80±10	-
H_2	40±5	36±7
Thiosulfate	55±7	57±6
Acetate	8±2	8±3

"-" means no growth observed.

To further characterize the *pioABC* operon deletion mutant, with respect to its Fe(II) oxidation phenotype, we performed a cell suspension assay. In this assay, H_2 grown cells of wide type and mutant TIE-3 were washed then incubated with Fe(II) under light in the anaerobic chamber. Fe(II) oxidation activity was followed using ferrozine assay. The Fe(II) oxidation activity we observed was light dependent (Figure 5.4B). Over a period of several hours for the equivalent density of H_2 grown cells, 400 μM of Fe(II) was oxidized to completion by TIE-1, but very little Fe(II) was oxidized by TIE-3. This indicates that the *pio* operon is responsible for almost all the Fe(II) oxidation activity in H_2-grown TIE-1. Considering the initial rate of Fe(II) oxidation, the activity of TIE-3 could be restored to about 50% of the wide type level by complementation with the entire *pio* operon (Figure 5.4B); the total amount of Fe(II) that was oxidized over a period of 9 hours was the same between wide type TIE-1 and the complemented strain. The vector alone did not affect Fe(II) oxidation by TIE-3 or TIE-1 (data not shown). However, complementation with each individual gene (*pioA, pioB,* or *pioC*), did not restore any Fe(II) oxidation activity (data not shown). This suggests that more than one gene in the *pio* operon is necessary for this activity.

Because the *pio* operon is so highly conserved between strain CGA009 and TIE-

1, we checked whether the *pio* operon also confers Fe(II) oxidation to CGA009. Deletion

of the genes corresponding to *pioABC* (i.e., RPA0746, RPA0745 and RPA0744) in

CGA009 resulted in a large defect in Fe(II) oxidation activity (data not shown), similar to

that observed in TIE-1. Strain CGA009 shows a similar amount of Fe(II) oxidation

activity in the cell suspension assay as H_2-grown TIE-1. However, it does not show

measurable growth over the same time period as TIE-1, therefore, we chose to work with

strain TIE-1 for further analysis

Figure 5.5 Growth on Fe(II) by individual *pio* deletion mutants (ΔpioA, ΔpioB, and ΔpioC) when Fe(II) is provided as the sole electron donor. Data are representative of triplicate cultures. Whereas the wild type (TIE-1) oxidized Fe(II) to completion in a period of 3 weeks, very little Fe(II) was oxidized by each mutant. No growth occurred for any of these mutants based on measurement of protein content (data not shown).

To access the relative importance of the individual *pio* genes for Fe(II) oxidation,

we constructed three individual deletion mutants, Δ*pioA*, Δ*pioB,* and Δ*pioC*. We

confirmed that the mutations were nonpolar by RT-PCR (data not shown) using primers

listed in Table 5.2. Neither growth nor Fe(II) oxidation occurred for any of these mutants during growth assay on Fe(II) (Figure 5.5); growth of these mutants on other substrates such as H_2, thiosulfate or acetate was unaffected (data not shown). In contrast, $\Delta pioA$ lost almost all Fe(II) oxidation activity in the cell suspension assay with H_2-grown cells, similar to TIE-3, whereas $\Delta pioB$ and $\Delta pioC$ only partially lost Fe(II) oxidation activity, exhibiting approximately 10% and 40% of the initial rate of Fe(II) oxidation of wild type level (Figure 5.6A). The partial defect in Fe(II) oxidation by $\Delta pioC$ may be explained by functional substitution of other small soluble electron carriers in the cell (e.g., the other HiPIP encoded by the homolog of RPA3566). Complementation by the respective wild-type copies of the genes restored Fe(II) oxidation activity to different extents in the mutants. In comparing the total amount of Fe(II) oxidized after 12 hours, complementation of $\Delta pioA$, $\Delta pioB$ and $\Delta pioC$ resulted in 85%, 60% and 99% of that achieved by TIE-1 in the same amount of time (Figure 5.6B). The reason for the relatively low extent of complementation for $\Delta pioB$ compared to TIE-1 is not clear. Perhaps it is caused by different levels of expression of *pioB* when expressed on a vector driven by a non-native promoter versus when expressed from the endogenous promoter. Together, these results indicate that all 3 Pio proteins are required for full Fe(II) oxidation activity in *R. palustris* TIE-1.

Figure 5.6 A. Fe(II) oxidation activity by individual *pio* deletion mutants (Δ*pioA*, Δ*pioB,* and Δ*pioC*) in the cell suspension assay. Δ*pioA* lost nearly all Fe(II) oxidation activity, similar to the *pio* operon deletion mutant TIE-3. Δ*pioB* and Δ*pioC* mutant showed approximately 10% and 40% of the activity compared to TIE-1 (as measured by calculating the rate of Fe(II) oxidation for the linear portion of the curve). Data represent the mean ± standard deviations of 3 independent cultures. B. Complementation by the respective wild-type copies of the *pio* genes restored Fe(II) oxidation activity to different extents in the mutants. In comparing the total amount of Fe(II) oxidized after 12 hours, complementation of Δ*pioA,* Δ*pioB* and Δ*pioC* resulted in 85%, 60% and 99% of that achieved by TIE-1 in the same amount of time.

5.5   DISCUSSION

Iron is thought to have been an important substrate for microbial metabolism on the early Earth, including ancient types of photosynthesis. Although the molecular basis of Fe(II) oxidation by acidophilic bacteria has been studied for decades (67, 68, 74, 75, 77), it is only very recently that Fe(II) oxidation has been examined in anoxygenic phototrophs (11, 28). Because photoautotrophic Fe(II) oxidation is likely to have been one of the most ancient forms of microbial Fe(II) oxidation (12), understanding the molecular basis of this metabolism is not only relevant for understanding the evolution of

photosynthesis, but for understanding the evolution of other Fe(II) oxidizing systems.

C-type cytochromes with a wide range of redox potentials are involved in Fe(II) oxidation by *A. ferrooxidans* (2, 68, 76, 77) and *Rhodobacter sp.* SW2 (11) as well as dissimilatory Fe(III) reduction by *Shewanella* and *Geobacter* species (4, 40, 42, 43, 73). Consistent with this, we found a *c*-type cytochrome to be upregulated when *R. palustris* TIE-1 was grown photoautotrophically on Fe(II). By reverse genetic analysis, we identified a 3-gene operon (the *pio* operon) that seems likely to encode the phototrophic Fe(II) oxidoreductase complex. Detailed biochemical studies are needed to confirm this and understand the mechanism of electron transfer from Fe(II), however, based on the results of this study, we can suggest potential functions for the Pio proteins.

The first gene in the *pio* operon encodes PioA, a putative decaheme *c*-type cytochrome. Because the ΔpioA mutant lost almost all its Fe(II) oxidation activity, similar to the *pio* operon deletion mutant TIE-3, this suggests that PioA plays an essential role during Fe(II) oxidation. We postulate that it receives electrons directly from Fe(II), serving as the Fe(II) oxidoreductase. This function would be analogous to that of *c*-type cytochromes in *S. oneidensis* and in *A. ferrooxidans* (2, 54) that serve as direct electron donors to Fe(III) and direct electron acceptors from Fe(II), respectively. Although confirmation of protein localization is necessary, sequence analyses suggest that PioA is a soluble protein that resides in the periplasm.

The second gene in the operon encodes PioB, a putative outer membrane beta barrel protein with no obvious redox active prosthetic groups. While not as severe as the ΔpioA phenotype, deletion of *pioB* caused a large defect in Fe(II) oxidation, suggesting that

PioB also plays an important role in this process. We suggest that it functions as an iron transporter, given its similarity to other known outer membrane porins (55, 62) and its lack of redox-cofactor binding motifs. However, at this stage, neither the transport direction nor the substrate (e.g., an Fe(II) or Fe(III) complex) of PioB is known. The closest relative of PioB is MtrB from *S. oneidensis* MR-1, which is involved in dissimilatory Fe(III) reduction (4, 48, 51, 52, 54). It has been suggested that MtrB helps localize the Fe(III) reductase complex in *S. oneidensis* MR-1 to the outside of the cell (51). By analogy, it is also possible that PioB may assist in the localization of other proteins involved in Fe(II) oxidation that remain to be identified.

The third gene in the operon encodes PioC, a putative HiPIP. Given that PioC is required for growth on Fe(II), we suggest that it functions as an electron carrier from PioA to the photosynthetic reaction center. Based on the redox potential of a HiPIP (0.345 V) measured from *Rhodopseudomonas marina* (23, 47), the calculated iron couple $Fe(OH)_3/Fe^{2+}$ (-1.1V) (28) and the measured reaction center (0.4 to 0.5 V) in purple bacteria (57), a HiPIP is a reasonable candidate for this function because its redox potential falls between that of the iron couple and the reaction center (RC). Spectroscopic and kinetic experiments have shown that HiPIPs can mediate electron transfer to the RC directly or via a RC-bound cytochrome in various purple bacteria (24, 25, 45, 46, 61). In this way, HiPIPs can functionally substitute for cytochrome $c2$, a common electron carrier in the periplasm of purple bacteria that shuttles electrons between the cytochrome *bc1* complex and the RC during cyclic electron flow (47). In the case of *R. palustris* CGA009, genome annotation predicts the presence of cytochrome c2 (encoded by gene

RPA1535) , along with another HiPIP (encoded by gene RPA3566). The fact that $\Delta pioC$ does not have a phototrophic growth defect on H_2 suggests that PioC has a function specific for Fe(II) phototrophy. Interestingly, a HiPIP has been demonstrated to serve as the electron acceptor for a thiosulfate:tetrathionate oxidoreductase during phototrophic growth of *Chromatium vinosum* on thiosulfate (18). PioC is also homologous to a HiPIP (Iro) found in *A. ferrooxidans*, an acidophilic Fe(II) oxidizing bacterium that couples Fe(II) oxidation to the reduction of oxygen at low pH. Because of its high redox potential, *in vitro* ability to oxidize Fe(II) and donate electrons to cytochrome c-552, as well as its stability under acidic conditions, Iro was proposed to catalyze Fe(II) oxidation in *A. ferrooxidans* (19, 37); whether this applies *in vivo* has been disputed, however (76, 77). Nevertheless, the finding that a HiPIP is involved in Fe(II) oxidation in both *R. palustris* and *A. ferroxidans* suggests some evolutionary relationship between the two Fe(II) oxidation systems.

In summary, the *pio* operon appears to encode proteins that are responsible for Fe(II) oxidation in *R. palustris* TIE-1. Determining their localization will be important for gaining insight into how this organism traffics in iron. Although much is understood about Fe(III) acquisition for assimilatory purposes when Fe(II) is limiting (70), *R. palustris* presents an opportunity to understand the opposite problem: how does a cell dispose of Fe(III) when it is growing on Fe(II)? Interestingly, in phototrophic Fe(II) oxidizing bacteria, the Fe(III) mineral product appears to be deposited exclusively outside the cell (28, 29); this make sense because precipitation of ferric minerals inside the cell could be fatal given the highly insoluble nature of Fe(III) at neutral pH. If our

predictions are correct, and the Fe(II) oxidoreductase complex resides in the periplasm, how then does the cell avoid this problem? Are there specific ligands that keep Fe(III) soluble? Or are there protein complexes that bind and transport Fe(III) out of the cell so efficiently that internal ferric mineral precipitation is precluded? We hope that future biochemical studies of the Pio proteins and their associated partners will address these questions.

5.6   ACKNOWLEDGEMENTS

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Chapter 6

The Fox Operon from *Rhodobacter* sp. SW2 Promotes Phototrophic Fe(II) Oxidation in *Rhodobacter capsulatus* SB1003

6.1 ABSTRACT

Anoxygenic photosynthesis based on Fe(II) is thought to be one of the most ancient forms of metabolism and is hypothesized to represent a transition step in the evolution of oxygenic photosynthesis. However, little is known about the molecular basis of this process because, until recently (27), most phototrophic Fe(II)-oxidizing bacteria have been genetically intractable. In this study, we circumvent this problem by taking a heterologous complementation approach to identify a three-gene operon (the *foxEYZ* operon) from *Rhodobacter* sp. strain SW2 that confers enhanced light-dependent Fe(II) oxidation activity when expressed in its genetically tractable relative, *Rhodobacter capsulatus* SB1003. The first gene in this operon, *foxE*, encodes a *c*-type cytochrome with no significant similarity to other known proteins. Expression of *foxE* alone confers significant light-dependent Fe(II) oxidation activity to SB1003, but maximal activity is achieved when *foxE* is expressed with the two downstream genes, *foxY* and *foxZ*. In SW2, the *foxE* and *foxY* genes are co-transcribed in the presence of Fe(II) and/or hydrogen, with *foxZ* being transcribed only in the presence of Fe(II). Sequence analysis predicts that *foxY* encodes a protein containing the redox cofactor pyrroloquinoline quinone and

that *foxZ* encodes a protein with transport function. Future biochemical studies will permit the localization and function of the Fox proteins in SW2 to be determined.

6.2 INTRODUCTION

Oxygenic photosynthesis, the biological process by which water is oxidized to molecular oxygen (O_2) using solar energy with the concomitant fixation of inorganic carbon, has had a profound impact on the biology and chemistry of the Earth, however, its origin remains enigmatic (7, 21, 31). Lipid biomarker and stratigraphic geochemical analyses of stromatolite assemblages may date the existence of the first cyanobacteria to ~2.7 Ga (9, 11); however, whether this is indeed the case is debatable (12, 18). Recent phylogenetic studies using several photosynthesis-related genes suggest that the anoxygenic form of photosynthesis evolved before the oxygenic form, with the purple photosynthetic group representing the most ancient taxon (53). In addition, the structural and biophysical similarities between the reaction centers of purple phototrophs and the oxygen evolving reaction center (PSII) of cyanobacteria are hypothesized to reflect a common ancestry (41, 43). Thus, it has been proposed that PSII evolved from the reaction center of purple bacteria via a series of transitional stages involving reaction centers able to accept electrons from compounds such as Fe(II), Mn-bicarbonate clusters or H_2O_2 (5, 8, 19, 40). Fe(II) is thought to have been abundant in the ancient ocean making it likely that Fe(II)-oxidizing organisms evolved early on (15, 50). Further, today Fe(II) oxidation is performed by phylogenetically diverse organisms including purple and

green phototrophic bacteria—a phylogenetic distribution that may reflect the antiquity of this form of photosynthesis (14, 15). In this context, understanding the molecular mechanisms by which anoxygenic bacteria oxidize Fe(II) phototrophically is of evolutionary interest.

Although first discovered over a decade ago, the majority of Fe(II)-oxidizing phototrophs are not currently amenable to genetic analyses and little is known about how they oxidize Fe(II) (14, 52). Recent studies of the newly isolated *Rhodopseudomonas palustris* TIE-1, where the ability to generate mutants has been developed, revealed that a homolog of a cobalt chelatase and an integral membrane protein that is likely a component of an ABC transport system are required for photoautotrophic Fe(II) oxidation (26). The roles of these proteins in Fe(II) oxidation by *R. palustris* TIE-1 remain unclear, but given their lack of redox cofactor binding motifs, it seems unlikely that they are involved directly in the transfer of electrons from Fe(II). In chapter 5, I report the discovery of a three gene operon from TIE-1, designated the *pio* operon; deletion of this operon leads to a specific growth defect on Fe(II). One of the genes in this operon, *pioA*, encodes a *c*-type cytochrome that is predicted to function in the periplasm of this strain as the Fe(II) oxidoreductase (Jiao et al, companion paper ref. (27)). Several years ago, at least two *c*-type cytochromes were found have increased expression in *Rhodomicrobium vannielii* strain BS-1 during phototrophic growth on Fe(II), but whether these cytochromes were specific to or required for Fe(II) oxidation was not determined (25).

Beyond serving as an electron donor for photosynthesis, Fe(II) can support the growth of both aerobic and anaerobic chemolithoautotrophs (14 and references therein). Yet, very little is understood about how Fe(II) oxidation works at the molecular level for these organisms either. To date, our deepest understanding of microbial Fe(II) oxidation comes from genetic and biochemical studies of *Acidithiobacillus ferrooxidans*, a bacterium that couples Fe(II) oxidation to the reduction of O_2 at low pH. Several proteins, including the blue copper protein, rusticyanin (13), a high-redox potential Fe-S protein (34), an outer membrane porin (37), and several types of cytochromes (4, 36, 48, 54, 55) have been implicated in the enzymatic oxidation of Fe(II) by *A. ferrooxidans*. How these proteins work together, however, is uncertain and may differ strain-by-strain (4, 34, 55). Nevertheless, in *A. ferrooxidans* strain ATCC33020, the protein proposed to be the primary acceptor for electrons from Fe(II), Cyc2, is a *c*-type cytochrome. In *A. ferrooxidans* strain ATCC33020 this cytochrome is known to be localized to the outer membrane (4, 55), which stands in contrast to the predicted periplasmic localization of PioA in TIE-1 (27). Thus, while knowledge of the mechanism of Fe(II) oxidation by aerobic acidophiles can inform our studies of anoxygenic phototrophs, the mechanism of Fe(II) oxidation between these organisms is likely to be different given that anoxygenic phototrophs oxidize Fe(II) under anaerobic conditions at neutral pH where the ferric iron product of this metabolism is a mineral, whereas *Acidithiobacillus* grows in aerobic acidic environments where the ferric iron product is soluble.

Here, we use a heterologous complementation approach to identify genes involved in phototrophic Fe(II) oxidation in the genetically intractable Fe(II)-oxidizing

photosynthetic bacterium, *Rhodobacter* sp. strain SW2. We identify the *foxEYZ* operon - a three gene operon from SW2 that confers enhanced light-dependent Fe(II) oxidation activity to *Rhodobacter capsulatus* SB1003.

6.3 MATERIAL AND METHODS

Strains, vectors, and growth conditions. The bacterial strains, plasmids, and cosmids used or constructed in this study are described in Table 6.1. For aerobic growth of *Rhodobacter capsulatus* SB1003 (SB1003), YP medium was used (51). For phototrophic growth of SB1003 and *Rhodobacter* sp. strain SW2 (SW2), a previously described anoxic minimal salts medium for freshwater cultures (pH 6.8) was used (20). As substrates for phototrophic growth, H_2 was provided as a headspace of 80% H_2:20% CO_2 and acetate was added to a final concentration of 10 mM.

To test SB1003 for its ability to grow phototrophically on insoluble Fe(II) alone or in the presence of added co-substrates, this strain was first grown phototrophically on H_2 and then transferred to fresh phototrophic medium containing the following substrates for growth: [1] 9 mM Fe(II)Cl$_2$·H$_2$O; [2] 9 mM Fe(II)Cl$_2$·H$_2$O + 0.1% Yeast Extract; [3] 9 mM Fe(II)Cl$_2$·H$_2$O + H_2. To test SB1003 for its ability to grow phototrophically on Fe(II) under a condition where both the Fe(II) substrate and the Fe(III) product of this metabolism remained dissolved, SB1003 was grown phototrophically on H_2 and transferred to fresh phototrophic medium containing 3-4 mM Fe(II)Cl$_2$·H$_2$O + 5 mM NTA. These concentrations of Fe(II) and NTA were used because higher concentrations

of NTA were toxic to SB1003 and, at this at this concentration of NTA, higher concentrations of Fe(II) resulted in Fe(III) mineral precipitation as bacteria Fe(II) oxidation progressed. To test the SB1003 strains carrying the Fe(II) oxidation activity conferring cosmids for their ability to grow phototrophically on Fe(II), these strains were first grown phototrophically on H_2 and then transferred to fresh phototrophic medium containing $Fe(II)Cl_2·H_2O$ as the sole electron donor. Concentrations of both 4 and 9 mM $Fe(II)Cl_2·H_2O$ were tested for these cosmid carrying stains. In all of these cultures, Fe(II) oxidation was used as a proxy for growth and was monitored via the ferrozine assay (46).

Table 6.1 Strains, cosmids, and plasmids used in this study.

Strain or plasmid	Genotype, markers, characteristics and uses	Source and/or reference(s)
Bacterial Strains		
E. coli WM3064	Donor strain for conjugation; *thrB1004 pro thi rpsL hsdS lacZΔM15 RP4–1360 Δ(araBAD)567 ΔdapA1341::[erm pir(wt)]*	W. Metcalf (UI-Urbana-Champaign)
E. coli DH10β	Host for *E. coli* cloning; F- *mcrA Δ(mrr-hsdRMS-mcrBC) Δ80dlacZΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ(ara leu)7697 galU galK rpsL nupG* (Str^R)	Invitrogen (Carlsbad, CA)
Rhodobacter capsulatus SB1003	*rif-10*	R. Haselkorn (U. Chicago), (56)
Rhodobacter sp. strain SW2	Wild type	F. Widdel (MPI, Bremen, Germany), (20)
Cosmids and Plasmids		
pLAFR5	21.5 kb broad-host-range cosmid cloning vector derivative of pLAFR3, *ori* RK2 (Tc^R, *lacZα*)	(30)
9E12	Contains SW2 genomic DNA cloned into the *Bam*HI site of pLAFR5 that confers Fe(II) oxidation activity to SB1003	This work
pBBR1MCS5	Gm^R derivative of pBBR1	(33)
pP3-gm1	Contains a ~9.4 kb *Pst*I fragment from 9E12 in pBBR1MCS5 (Gm^R)	This work
pP3-gm2	Contains a ~9.4 kb *Pst*I fragment from 9E12 in pBBR1MCS5 (Gm^R). Insert is cloned in the opposite orientation to that of pP3-gm1	This work
pBBR1MCS2	Kn^R derivative of pBBR1	(33)
pAK20	Derivative of pBBR1MCS2 (Kn^R). Inserts can be expressed from a *tac* promoter fused to GFP	Komeili, A. (UC-Berkeley), (32)
p*foxEYZ*	Contains a 3235 bp PCR product derived from 9E12 containing the *foxE*, *foxY* and *foxZ* genes cloned into the *Eco*RI/*Spe*I sites of pAK20	This work
p*foxE*	Contains a 1273 bp PCR product derived from 9E12 containing the *foxE* gene cloned into the *Eco*RI/*Spe*I sites of pAK20	This work
p*foxY*	Contains a 1351 bp PCR product derived from 9E12 containing the *foxY* gene cloned into the *Eco*RI/*Spe*I sites of pAK20	This work

| p*fox*Z | Contains a 1290 bp PCR product derived from 9E12 containing the *fox*Z gene cloned into the *Eco*RI/*Spe*I sites of pAK20 | This work |

Phototrophically grown cultures of SB1003 and SW2 were incubated under continuous illumination ~30 cm from a 34 W incandescent light at 30˚C and 16˚C, respectively. Luria-Bertani (LB) medium was used for routine culturing of *E. coli* strains DH10β and WM3064 at 37˚C. 0.2 mM diaminopimelic acid (DAP) was added to permit growth of WM3064 cultures. Antibiotic concentrations were as follows: for SB1003, 1 μg/ml tetracycline (Tc), 5 μg/ml kanamycin (Kn), and 3 μg/ml gentamicin (Gm) were used; for *E. coli* strains, 15 μg/ml Tc, 50 μg/ml Kn, and 20 μg/ml Gm were used. Phototrophically grown cultures supplemented with Tc were incubated behind UV light filters to minimize light mediated degradation of this drug (17).

***Rhodobacter* sp. strain SW2 genomic cosmid library construction.** Genomic DNA was isolated from SW2 according to standard protocols (16). Plasmid and cosmid DNA were purified using Qiagen Mini or Maxi Kits, respectively. After purification, the cosmid vector pLAFR5 (30) was digested sequentially with *Sca*I and *Bam*HI. SW2 genomic DNA was partially digested with *Sau*3AI, dephosphorylated and ligated with digested pLAFR5 at a 9:1 molar ratio of insert to vector in the presence of 5 mM ATP. The ligation was packaged into recombinant λ phage using the Stratagene Gigapack III XL packaging extract and *E. coli* WM3064 was infected with the resultant phage lysate.

Cosmids from WM3064 were transferred to SB1003 via conjugation. Selection against the donor strain was achieved by omitting DAP from the medium. The resulting library contained 1536 clones with an average insert size of 23.5 kb. Based on comparison to the genome sizes of *R. sphaeroides* and *R. capsulatus*, which are ~4.5 and 3.6 Mb, respectively, we estimate that our library represents 5-6 times coverage of the SW2 genome (24, 35).

Cell suspension assay for Fe(II) oxidation activity.   All cell suspension assays were prepared and conducted at room temperature in an anaerobic chamber containing an atmosphere of 5% H_2:80% N_2:15% CO_2. A light intensity of ~ 500 lux from an incandescent bulb was used for light-incubated assays. Our initial screen to identify cosmids able to enhance Fe(II) oxidation was an end-point assay. Here, SB1003 transconjugants were grown photoautotrophically on H_2 in 96 well plates, washed once with an anoxic buffer containing 50 mM HEPES (N-2-hydroxyethylpiperazine-N`-2-ethanesulfonic acid) and 20 mM NaCl at pH 7 (assay buffer) and resuspended in 100 μl of assay buffer containing ~0.2 mM of Fe(II)Cl_2·H_2O and 20 mM $NaHCO_3$. After a ~20 hour incubation in the light, the concentration of Fe(II) remaining in each clone-containing well was measured by adding 100 μl of ferrozine solution (0.1% (w/v) ferrozine in 50% (wt/v) ammonium acetate solution) (46). Cosmids from clones that showed less purple color than the negative control, SB1003+pLAFR5, were purified, moved into a clean SB1003 genetic background, and the resultant strains were re-tested

quantitatively for their Fe(II) oxidation activity through a time-course cell suspension assay.

All time-course cell suspension assays contained the same number of cells. To prepare these assays, ~50 ml of an early-exponential phase culture was harvested by centrifugation, washed once with an equal volume of assay buffer, resuspended to a final OD_{570} of ~0.3 in assay buffer containing 0.5 mM $Fe(II)Cl_2 \cdot H_2O$ and 20 mM $NaHCO_3$ and dispensed in 300 μl aliquots to a 96 well plate. For each Fe(II) measurement, 10 μl of cell suspension was transferred to 90 μl of 1M HCl. 100 μl of ferrozine solution was added and the OD_{570} was read after 10 min. Samples for total Fe measurements were diluted 1/10 with a solution of hydroxylamine hydrochloride (10% (w/v) in 1 M HCL) and incubated at 65 °C overnight to facilitate the reductive dissolution of Fe(III) precipitates. 100 μl of these samples were combined with 100 μl of ferrozine solution and the OD_{570} was read after 10 min. Fe concentration measurements were corrected for cell interference and rates of Fe(II) oxidation were calculated through the initial linear portion of the Fe(II) oxidation curves.

Cloning, sequencing, annotation and RT-PCR. Cosmid 9E12 was digested with *Pst*I and the subsequent restriction fragments were gel purified, ligated with *Pst*I digested pBBR1MCS5 (33) and transformed into *E. coli* DH10β. Strains of SB1003 with representative plasmids containing the correct size insert in both transcriptional orientations, were constructed and tested for light-dependent, Fe(II)-oxidation activity.

Shotgun cloning and sequencing of P3 were performed by Laragen (Los Angeles, CA). To annotate the sequence, an initial set of putative ORFs was identified using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Initial functional assignments and homology identifications were made by comparison of the translated ORFs to proteins in the BLAST database using BlastP (http://www.ncbi.nlm.nih.gov/blast/). Additional protein analyses (e.g., membrane-spanning domains, subcellular localization and motif identification) were performed using the tools on the ExPASy proteomics server (http://us.expasy.org/). Predicted operons, promoters and terminators were identified using the tools at Softberry (http://www.softberry.com/berry.phtml). The *R. capsulatus* genome was accessed at the Integrated Genomics website (http://www.integratedgenomics.com/). The sequence of the *fox* operon has been deposited in GenBank (accession number DQ381537).

Table 6.2 Oligonucleotides used in this study. Nucleotides in lowercase denote engineered restriction site sequences. Oligonucleotides 9, 13, 17, and 21 have a MfeI and a PstI restriction site at the 5' end. Oligonucleotides 10, 14, and 18 have a SpeI restriction site at the 5' end.

Oligonucleotide Name	Description	Sequence (5'-3')
9	Forward primer used to amplify p*foxE* insert	cgcccaattgctgcagTTATTGCGCCCTTTTTCATC
10	Reverse primer used to amplify p*foxE* insert	ggcgactagtAGGCGATCCTCACCATAGG
13	Forward primer used to amplify p*foxY* insert	cgcccaattgctgcagTCCACGAGCTGAACCTGAC
14	Reverse primer used to amplify p*foxY* insert	ggcgactagtGTTATAGGTGGCGGTTGCTG
17	Forward primer used to amplify p*foxZ* insert	cgcccaattgctgcagTCAGACCACCGATTACGACA
18	Reverse primer used to amplify p*foxZ* insert	ggcgactagtGGTTTGAGTTTGAGGCAGGA
21	Forward primer used to amplify p*foxEYZ* insert	cgcccaattgctgcagAGGAAGTGCTGACCGACATC
18	Reverse primer used to amplify p*foxEYZ* insert	ggcgactagtGGTTTGAGTTTGAGGCAGGA
1	Used for RT-PCR	AAGGTGTTCCAGCACCTGAC
2	Used for RT-PCR	GGCATAGGCGATGATGGTAT
3	Used for RT-PCR	CCTTTGACGGCAAGCTTTAT
4	Used for RT-PCR	GTGATCACCTTGACCAGCAG
5	Used for RT-PCR	CGATCAAGGAATGGATCCTG
6	Used for RT-PCR	GGCACACCGATCTGAATCTT
7	Used for RT-PCR	CGAGTTGTGGAGCTTTTACG
8	Used for RT-PCR	CAGGGCGTTGGAGAAGAAC
54	Forward primer used to verify transcription of *foxZ* from p*foxZ* in SB1003	TTTCATCAACTCGCAACTGG
55	Reverse primer used to verify transcription of *foxZ* from p*foxZ* in SB1003	ATAAAGCTTGCCGTCAAAGG
29	Forward primer used to verify transcription of *foxY* from p*foxY* in SB1003	GACCCTGTGCTATGCCATC
51	Reverse primer used to verify transcription of *foxY* from p*foxY* in SB1003	GGCACCTGGTAGTTCGACAG

The insert fragments of p*foxE*, p*foxY*, p*foxZ*, p*foxEY* and p*foxEYZ* were generated by PCR amplification from 9E12 using Mix D from the FailSafe PCR System by Epicentre and the primers listed in Table 6.2. Primers were designed with *Spe*I or *Mfe*I and *Pst*I restriction sites to facilitate cloning. PCR products were digested with *Spe*I and *Mfe*I and ligated into *EcoRI* and *Spe*I digested pAK20 (32) to create p*foxE*, p*foxY*, p*foxZ*, p*foxEY*, p*foxEYZ*. After insert sequence verification, strains of SB1003 carrying these plasmids were constructed and tested for Fe(II) oxidation activity.

For RT-PCR experiments, total RNA was extracted from H_2-grown SW2 cells and H_2-grown SW2 cells incubated in the light in assay buffer containing 0.5 mM Fe(II)Cl$_2$·H_2O and 20 mM NaHCO$_3$ for ~30 min (Fe-induced cells) using the RNeasy Protect Bacteria Mini Kit (Qiagen). cDNA was synthesized using the BioRad iScript cDNA Synthesis Kit. Control reactions contained no reverse transcriptase. These cDNA, as well as genomic DNA from SW2, served as templates for PCR amplification using the primers described in Table 6.2. The same cDNA template was used for the data presented in Figure 6.5B and C and the same cDNA template was used for the data presented in Figure 6.5D and E.

To confirm expression of the p*foxY* and p*foxZ* inserts, RT-PCR was performed on total RNA extracted from YP grown cultures of SB1003 carrying these plasmids using primers integral to these two genes. A product was obtained in both cases (data not shown).

Biochemical methods. For total protein extraction, cells from 1 liter (L) cultures of acetate and H_2-grown SW2 and H_2-grown SB1003+p*foxE* and SB1003+pBBRMCS2 were harvested in exponential phase by centrifugation and washed once with assay buffer. Because the ferric precipitates that form during phototrophic growth of SW2 on Fe(II) alone preclude the harvesting of cells for protein extraction, to approximate Fe(II)-growth conditions and obtain sufficient cells for total protein extraction, cells from a 1 L culture of H_2-grown SW2 were harvested, washed with assay buffer and resuspended in 20 ml of assay buffer containing 2 mM Fe(II)Cl$_2 \cdot$H$_2$O and 20 mM NaHCO$_3$. This suspension was incubated in the light until all Fe(II) was oxidized and cells were then harvested by centrifugation. Cell pellets from all conditions were resuspended in 3 ml of assay buffer to which a protease inhibitor cocktail (Roche, Complete EDTA-free) was added. Cells were lysed via passage three times though a French pressure cell at 16,000 psi. DNase (Sigma) was added to the extracts followed by a 30 minute (min) incubation on ice. Cell debris and unbroken cells were removed by low speed centrifugation (10,000x*g* for 20 min). Crude membrane and soluble fractions were separated by ultracentrifugation (200,000x*g* for 90 min) and the membrane pellet was resuspended in assay buffer. Protein concentration was measured using the Bio-Rad protein assay. SDS-PAGE was performed by standard procedures according to the Laemmli method (1). Cytochrome *c* bands were detected according to the in-gel peroxidase activity assay of Francis and Becker (22). Bands of interest were cut from the heme stained gels and submitted to the Protein/Peptide MicroAnalytical Laboratory/Facility at Caltech for LC/MS/MS analysis.

6.4   RESULTS

6.4.1   Identification of four cosmids that enhance Fe(II) oxidation in SB1003

The Fe(II)-oxidizing phototroph, *Rhodobacter* sp. strain SW2, is not amenable to traditional genetic analysis (14) but is closely related to *Rhodobacter capsulatus* SB1003, which is genetically tractable (14). SB1003 is not able to grow photoautotrophically on Fe(II) alone (Figure 6.1A) or in the presence of additional substrates such as 0.1% yeast extract (Figure 6.1B) or H_2 (Figure 6.1C). However, in the presence of Fe(II)-nitrotriacetic acid (NTA), both Fe(II) oxidation (Figure 6.1D) and growth occur (data not shown); NTA does not support phototrophic growth in the absence of Fe(II) (data not shown). Therefore, to identify genes from SW2 that conferred Fe(II) oxidation activity, we expressed a genomic cosmid library of SW2 in SB1003 under conditions where Fe(II) oxidation by SB1003 was kinetically slow. Under the conditions used, SW2 oxidized, on average, 6-fold more Fe(II) than SB1003. This difference in activity could be clearly visualized via our colormetric, end-point ferrozine assay allowing for the identification of putative SB1003 clones with enhanced Fe(II) oxidation activity (Figure 6.2).

Figure 6.**1** Fe(II) oxidation activity by Rhodobacter capsulatus SB1003 under different conditions. Cultures of SB1003 containing Fe(II)Cl2 as the sole electron donor show no Fe(II) oxidation over a 27-day period (A). Addition of 0.1% Yeast Extract (YE) or H_2 as co-substrates does not stimulate Fe(II) oxidation by SB1003 (B and C, respectively) as has been found for other Rhodobacter capsulatus strains (20, 25). When SB1003 is inoculated into medium containing Fe(II)Cl2+NTA, however, rapid and complete oxidation of Fe(II) is observed (D). The data in A, B, and C are representative of multiple independent cultures and the error bars are the standard deviation for triplicate ferrozine assays. Data shown in D are the average of three independent SB1003 cultures and error bars represent the standard deviation of this average. The legend in A applies to all graphs.

Figure 6.2 Screen for enhanced Fe(II) oxidation activity in SB1003. After a ~20-hour incubation in assay buffer containing 0.2 mM Fe(II), SW2 oxidizes an average of ~6-fold more Fe(II) than SB1003. This difference in Fe(II) oxidation is clearly visualized upon the addition of the colormetric reagent ferrozine, where wells containing Fe(II) turn purple and wells with no Fe(II) remain clear. Likewise, during the screen, Fe(II)-oxidizing clones like SB1003+9E12 are identified as clear wells upon addition of ferrozine.

In our screen of 1536 cosmid-carrying SB1003 clones, four clones with enhanced

Fe(II) oxidation activity were identified. To further characterize this activity, Fe(II) and

total Fe concentrations were followed for 3 hours in cell suspensions of these strains

incubated in the light and dark. This shortened incubation time, as compared to the screen

(~20 hours), magnified the differences in Fe(II) oxidation activity between the putative

clones and the control strains. As shown by the representative clone, SB1003+9E12,

there is an ~1000-fold increase in the amount of Fe(II) oxidized after three hours in light-

incubated SB1003+9E12 cell suspensions compared to the control strain

SB1003+pLAFR5 (Figure 6.3A). In terms of rate, in the light-incubated cell suspensions

of SB1003+9E12, the amount of Fe(II) decreased at a rate ~45 times faster than

SB1003+pLAFR5 (Figure 6.3A) while the total amount of Fe stayed constant (data not shown). In comparison, Fe(II) and the total amount of Fe remained constant when cell suspensions of SB1003+9E12 were incubated in the dark (Figure 6.3B and total Fe data not shown), demonstrating the light dependence of this reaction. The light dependence of this Fe(II) oxidation activity may be due to the involvement of the photosynthetic reaction center complex in generating a proton gradient across the cytoplasmic membrane, which in turn drives electron flow from Fe(II) to NAD(P)+; it is not due to a need for light in accepting electrons from Fe(II) per se.

Although the 9E12 cosmid significantly enhanced light-dependent, Fe(II) oxidation activity in SB1003 in the concentrated cell suspension assay, this cosmid did not enable this strain to grow photoautotrophically on Fe(II) alone over a period of 40 days, nor did the other cosmids identified (data not shown).

Figure 6.3 Cosmid 9E12 confers enhanced light-dependent Fe(II)-oxidizing activity to SB1003. When incubated in the light, the concentration of Fe(II) in cell suspensions of SB1003+9E12 decreases at a rate ~45 times faster than that of SB1003 carrying the control cosmid, pLAFR5 (A). In contrast, no decrease in Fe(II) is observed when SB1003+9E12 is incubated in the dark (B). This demonstrates that cosmid 9E12 confers enhanced light-dependent Fe(II) oxidation in SB1003. Legend in B also applies to A. Data shown are the averages of biological triplicates for each strain, and error bars represent the standard deviation.

6.4.2 Identification and characterization of the *fox* operon

Restriction site mapping of the four cosmids revealed a ~9 kb *Pst*I restriction fragment (designated P3) common to three of the cosmids, a portion of which was found in the fourth cosmid (data not shown). P3 was cloned from 9E12 into the broad-host range vector, pBBR1MCS5, in two transcriptional orientations to create pP3-gm1 and pP3-gm2. Both of these constructs conferred equivalent enhanced light-dependent, Fe(II) oxidation activity to SB1003 (data shown for P3-gm2 in Figure 6.4A and B), suggesting that the gene(s) responsible for the observed activity was expressed from its endogenous promoter. The rates of Fe(II) oxidation by these clones were equivalent to that of SB1003+9E12, indicating that P3 contained all the genes necessary to confer maximal Fe(II) oxidation activity (Figure 6.3A and 6.4A).

Figure 6.4 Stimulation of light-dependent Fe(II) oxidation activity in SB1003 by the fox genes. When incubated in the light, cell suspensions of SB1003 strains carrying the 9E12 subclones, pP3-gm2, pfoxEYZ, and pfoxE show a significant increase in light-dependent Fe(II) oxidation activity, whereas SB1003 strains carrying pfoxY, pfoxZ, and the vector controls, pBBR1MCS2 and pBBR1MCS5, do not (A). When incubated in the dark, the concentration of Fe(II) (B) remains constant in cell suspensions of all the 9E12 subclone-carrying strains of SB1003, showing the light dependence of this Fe(II) oxidation. Legend in B also applies to A. Data shown are the averages of biological triplicates for each strain, and error bars represent the standard deviation.

In purple non-sulfur bacteria, of which *Rhodobacter* is one type, the capacity to use inorganic electron donors such as sulfide or H_2 as reductants for NAD(P)+ is enabled by redox active enzymes that are able to accept electrons from these substrates and subsequently donate them to the cyclic electron transport chain (23, 49). Thus, our primary criterion to identify open reading frames (ORFs) in the P3 sequence encoding putative Fe(II)-oxidizing proteins was the predicted presence of redox cofactor binding sites. Among the 10 ORFs identified in P3, ORFs 2 and 3 were the most promising candidates (Figure 6.5A). ORF2 is 876 base pairs (bp) and is predicted to encode a soluble, 291 amino acid (aa) protein with two C-X-X-C-H peptide motifs - the classic sequence suggestive of covalent heme attachment (47). A signal sequence characteristic of secreted proteins is found at the N-terminus of this protein, predicting transport across the cytoplasmic membrane. The predicted product of ORF2 shows no significant similarity to other proteins in the BLAST database or the SB1003 genome. ORF3 is 1089 bp and appears to encode a soluble 362 aa protein with conserved repetitive domains similar to those found in eukaryotic and bacterial WD-repeat regulatory proteins as well as bacterial dehydrogenases and serine/threonine kinases containing the redox cofactor pyrroloquinoline quinone (PQQ) (3, 42, 44). A putative signal sequence is also found in this protein.

Figure 6.5 In SW2, foxE, foxY and foxZ are co-transcribed in the presence of Fe(II). A Cloning strategy to identify foxE, foxY and foxZ, genes from SW2 that enhance light dependent, Fe(II) oxidation activity in SB1003. Numbered arrows identify the positions of the oligonucleotides used for RT-PCR experiments (described in Table 6.2). The 1 kb scale bar applies to P3 segment only. Using total RNA extracted from H2-grown, Fe(II)-induced SW2 as a template for cDNA synthesis and oligonucleotides 5 and 6 (B) and 7 and 8 (C), PCR products were obtained for both of the regions between the fox genes (+RT lanes). Using total RNA extracted from H2-grown SW2 as a template for cDNA synthesis and oligonucleotides 5 and 6 (D) and 7 and 8 (E), a PCR product was obtained for the region between foxE and foxY, but not between foxY and foxZ. Additional experiments with oligonucleotides 1, 2, 3, and 4 confirmed the results in B, C, D and E (data not shown). –RT lanes: controls with no reverse transcriptase added to the cDNA reactions, + lanes: controls with SW2 genomic DNA; – lane: no template controls.

ORFs 2 and 3 are predicted to be part of the same transcriptional unit along with the downstream ORF4. ORF4 is 906 bp and is predicted to encode a 301 aa cytoplasmic membrane protein with 10 transmembrane domains, a signal peptide and two domains of unknown function conserved among known drug/metabolite transporters like PecM from *Erwinia chrysanthemi* (DUF6, Pfam accession number PF00892). A putative σ^{70} promoter consensus sequence resides 113 bp upstream of ORF2 (TTACCG(12 bp)CGGTATATT) and a predicted Rho-independent bacterial terminator is found upstream of this promoter (Figure 6.5A). ORFs 2, 3, and 4 could be PCR amplified from

all four of the identified cosmids (data not shown). With RT-PCR using RNA from Fe(II) induced, H_2-grown cells of SW2, we verified that these three genes are co-transcribed and thus form an operon (Figure 6.5B, C). We designate ORFs 2, 3 and 4 as *foxE*, *foxY* and *foxZ* (Fe(II) oxidation), respectively. ORF5 and 6 are predicted to be transcribed in the opposite direction relative to ORF4 and are thus segregated from the *fox* operon. The vectors p*foxEYZ*, p*foxE*, p*foxY*, and p*foxZ* were constructed (Figure 6.5A) to test the gene products for their effects on light-dependent Fe(II) oxidation activity in SB1003. SB1003+pfox*EYZ* showed light-dependent Fe(II) oxidation activity at a rate equivalent to SB1003+pP3-gm2, suggesting that *foxEYZ* are sufficient to confer this activity (Figure 6.4A and B). Of the individually cloned genes, only SB1003+p*foxE* showed significant light-dependent Fe(II) oxidation activity, at a rate ~20% that of SB1003+p*foxEYZ*, while SB1003+p*foxY* and SB1003+p*foxZ* behaved similarly to the control, SB1003+pBBR1MCS2, showing little Fe(II) oxidation activity (Figure 6.4A and B). These experiments show that *foxE* is sufficient to promote Fe(II) oxidation activity in SB1003, but they do not demonstrate that the *fox* genes are required for phototrophic growth of SW2 on Fe(II). Because we cannot delete genes in SW2, the closest we can come to addressing whether the *fox* genes are involved in phototrophic Fe(II) oxidation in SW2 is to determine whether they are differentially expressed under different growth conditions. RT-PCR revealed that the *fox* genes are co-transcribed in SW2 in the presence of Fe(II) (Figure 6.5B and C). In contrast, under H_2-grown conditions, *foxZ* is not transcribed, whereas *foxE* and *foxY* are (Figure 6.5D, E). Although sequences with weak similarity to promoters are predicted 305 and 805 bp upstream of *foxZ*, no

corresponding Rho-independent termination sequences are identified, making it unlikely

that this difference in *foxZ* transcription results from its independent transcription in the

presence of Fe(II). The cause of this layered transcriptional regulation is not yet clear.

6.4.3 FoxE is a *c*-type cytochrome

Cytochromes of the *c*-type are characterized by the covalent attachment of

heme(s). In these proteins, heme is bound via thioether bonds to the two cysteines of a

conserved sequence motif. Although less common sequence motifs have been identified,

the classic sequence motif for heme-binding sites in a *c*-type cytochrome is C-X-X-C-H

(6, 47). The predicted protein sequence of *foxE* has two of these classic heme-binding

motifs (Figure 6.6A). To test if *foxE* encodes a *c*-type cytochrome, we performed in-gel

heme-peroxidase activity stains of crude protein extracts from H_2-grown SB1003+p*foxE*.

These stains revealed the presence of a ~25 kDa *c*-type cytochrome that was not present

in extracts of the control strain, SB1003+pBBR1MCS2, when an equivalent amount of

total protein was loaded (Figure 6.6B). A *c*-type cytochrome of the same approximate

size was identified in crude protein extracts of SW2 grown photoautotrophically on H_2

with an Fe(II) induction (Figure 6.6B). Mass spectrometry analysis confirmed that these

bands from SW2 and SB1003+*pfoxE* contained peptide fragments that matched

fragments predicted from the FoxE sequence and these fragments showed no similarity to

any protein in the BLAST database or SB1003 genome. Heme stain analyses of the

soluble and membrane fractions of these crude extracts showed FoxE to be present in the

soluble fraction (data not shown). FoxE was also present in crude cell extracts of H_2-

grown SW2 cells without an Fe(II) induction, as identified by heme stains and confirmed

by mass spectrometry, but was not detected in extracts of cells grown

photoheterotrophically on acetate (Figure 6.6B). Detection of FoxE in H_2-grown SW2

cells with and without an Fe(II) induction is consistent with our RT-PCR data showing

that *foxE* is expressed under both conditions.

A. predicted signal sequence

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MTLWEDEMLDRMKGGFAATALLCLGLANPLAADTRTLSQQYLDDVRSGAI  50
VIEGDSAAVSELILKRDIPIPYSYIAQLFATPNAFGSGPACIICHGSNNP  100
THAYRGLNLSTCDGLRNGSTEQPARAIFTPGEDPKNAIIGRRLRANRMPL  150
GIAFNNPTDSAPILAIKEWILAGAPNDEHFTKEILPLFATDNTFGPDTPH  200
CTTCHFSNQEPPSFHELNLTTYEGIMLGADSVAKGVDNATKVIIPGDPEA  250
SKVFQHLTEDRMPPGIDPSEDRDHPNTQILFAWIKQGAKCE           291
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Figure 6.6 FoxE is a c-type cytochrome. (A) Amino acid sequence of FoxE. In bold are two consensus motifs suggestive of covalent heme binding sites and the predicted signal sequence of this protein is 32 amino acids long. (B) Heme-peroxidase stains of crude cell extracts separated by SDS-PAGE. SB1003 lanes: pfoxE and C – SB1003+pfoxE and SB1003+pBBR1MCS2, respectively, grown phototrophically on H_2 and induced with Fe(II). SW2 lanes: H_2/Fe – SW2 grown phototrophically on H_2 with an Fe(II) induction; H_2 – SW2 grown phototrophically on H_2; Ac – SW2 grown phototrophically on acetate. The c-type cytochrome, FoxE, is indicated by the black arrow. Approximately 120 µg of total protein was loaded per lane and the molecular weight marker (lane M) is the broad range standard from Bio-Rad.

6.5   DISCUSSION

Using a heterologous expression system, we identified an operon from the genetically intractable *Rhodobacter* sp. strain SW2 that confers a significant increase in light-dependent Fe(II) oxidation activity when heterologously expressed in *R. capsulatus* SB1003. This operon, which we designate the *fox* operon, contains three genes: *foxE*, which encodes a novel *c*-type cytochrome; *foxY*, predicted to encode a protein with repetitive domains similar to WD-repeat family proteins and proteins that bind the redox cofactor PQQ; and *foxZ*, predicted to encode a protein with transport function.

Given the precedent for a *c*-type cytochrome Fe(II) oxidoreductase in *A. ferrooxidans* and our observation that the *foxE* gene from SW2 encodes a novel *c*-type cytochrome that significantly enhances light-dependent, Fe(II) oxidation activity in SB1003 when expressed alone, it is possible that FoxE is the native Fe(II) oxidoreductase in SW2. Although we are unable to prove this via deletion of the *foxE* gene in SW2, it is perhaps not surprising that mutational analysis of *R. palustris* strain TIE-1 demonstrates that a soluble *c*-type cytochrome (PioA) is essential for phototrophic growth on Fe(II) in that organism (Jiao et al., companion paper ref. (27)). Given that ours is a heterologous expression system, it is possible that FoxE stimulates Fe(II) oxidation indirectly in SB1003. By drawing analogy to PioA, however, the simplest interpretation is to predict that FoxE stimulates Fe(II) oxidation directly by serving as the Fe(II) oxidoreductase. In both strains, *c*-type cytochromes are found in an operon with another putative electron carrier (FoxY in SW2, PioC in TIE-1). Although potentially similar in function, the *fox*

and *pio* gene products are not homologs. Interestingly, *foxZ* is dispensable with respect to stimulating Fe(II) oxidation in SB1003, yet its expression in SW2 correlates specifically with the presence of Fe(II). More work needs to be done to understand the regulation of the *fox* genes, and biochemical experiments are required to elucidate their functions.

Electrons derived from Fe(II) must ultimately make their way to NAD(P)+ via electron carriers in the intracytoplasmic membrane of Fe(II) phototrophs. Because these bacteria oxidize Fe(II) at neutral pH, where the mineral product of this metabolism is insoluble, the question of where the Fe(II) oxidoreductase is localized is important to consider because intracellular precipitation of the Fe(III) mineral product of this metabolism presents a hazard to these organisms. We have previously reported that Fe(III) does not precipitate inside SW2 during growth on Fe(II) (29), and in line with this, others have proposed that Fe(II) oxidation might occur on the cell surface (20). This prediction is consistent with the model for Fe(II) oxidation by the acidophilic Fe(II) oxidizing bacterium *A. ferrooxidans* ATCC33020, where the purported Fe(II)-oxidizing enzyme, Cyc2, is localized to the outer membrane (55). FoxE, however, appears to lack β-sheets or lipoprotein attachments characteristic of known integral or associated outer membrane *c*-type cytochromes like Cyc2 or OmcA and OmcB from *Shewanella oneidensis* MR-1 (39, 55). In addition, our detection of FoxE only in the soluble fraction of cell extracts suggest that it resides either in the cytosol or periplasmic space. Although the localization of FoxE remains to be experimentally determined, the fact that *c*-type cytochrome maturation occurs in the periplasm in gram-negative bacteria, and we know

of no examples of bacterial *c*-type cytochromes that function in the cytosol, we

hypothesize that FoxE functions in the periplasm of SW2 (2).

If FoxE functions as the Fe(II) oxidoreductase in the periplasm of SW2, this

implies that SW2 has a mechanism for preventing the precipitation of Fe(III) in the

periplasmic space. While Fe(III) may be released as a soluble inorganic species (45) and

quickly transported out of the cell, it is also possible that the cell produces organic

ligands that aid in this process. This hypothesis has been previously put forth to explain,

in part, the Fe isotope fractionations produced by Fe(II)-oxidizing phototrophs (15, 29). If

such cell-associated ligands exist and are stable over geological time scales, they may

provide a means to identify traces of this metabolism in the rock record, enabling studies

directed at understanding the environmental impact of these organisms over time (15,

28). For this to be possible, these ligands would need to be extractable from organic

remains in ancient rocks, and their structures would need to be recognizable. While of

course this cannot be guaranteed, the fact remains that organic geochemists have

unearthed a plethora of "orphan" biomarkers whose functions are unknown (10). As the

structures of molecules required for Fe(II) oxidation in contemporary organisms are

determined, with luck, perhaps a match will be found. Additionally, if SW2 has a

mechanism to avert Fe(III) precipitation in the periplasm that SB1003 does not, this may

help explain why SB1003 can only use Fe(II) as an electron donor for growth when an Fe

chelator, such as NTA, is added to the medium (Figure 6.1A, B, C and D). This is not the

only scenario, however. Addition of NTA to the medium will decrease the redox

potential of iron (38), which could facilitate Fe(II) oxidation by SB1003 by an Fe(II)

oxidoreductase optimized for substrates with low redox potentials. Elucidation of the native Fe(II) oxidoreductase from SB1003 should be possible with traditional genetic analysis and is one of our future goals.

The roles of *foxY* and *foxZ* in Fe(II) oxidation by SW2 are currently unclear. Sequence analyses predict that FoxY contains motifs suggestive of PQQ binding. If FoxY does bind PQQ, its role in phototrophic Fe(II) oxidation by SW2 may be to assist FoxE in electron transfer to a component of the cyclic electron transfer chain. Recent findings support a role for a quinoprotein in Mn(II)-oxidation by *Erythrobacter* sp. strain SD21, providing precedent for the involvement of PQQ-containing enzymes in metabolisms involving metal oxidation (H. Johnson and B. Tebo, personal communication). Sequence analysis of FoxZ predicts that it is a cytoplasmic membrane protein with transport function. The involvement of a putative cytoplasmic membrane transporter is consistent with the finding that a homolog of the permease subunit of an ABC-type transporter is necessary for full Fe(II) oxidation activity by *R. palustris* TIE-1 (26).

The discovery of a putative Fe(II) oxidoreductase from the neutrophilic, anoxygenic phototroph, SW2, that is different in its predicted cellular localization from that involved in Fe(II) oxidation by the acidophilic aerobe *A. ferrooxidans* is curious, as one might have expected the opposite based on the respective growth environment of these organisms (e.g., Fe(III) is more soluble at acidic pH compared to neutral pH). Moreover, evidence from studies with *R. palustris* strain TIE-1 suggests that the putative Fe(II) oxidoreductase from this strain also functions in the periplasm (Jiao et al, companion paper ref. (27)). While an emerging theme among the Fe(II) oxidoreductases

from both phototrophs and acidophilic bacteria is that they involve *c*-type cytochromes, it seems that the topology of the components involved is quite different. Whether these differences are reflective of differing evolutionary origins for biologically-catalyzed Fe(II) oxidation remains to be explored. For such studies, more knowledge of the mechanisms of Fe(II) oxidation by diverse organisms is needed so that comparative studies can be conducted. Our work demonstrates that a heterologous complementation approach can be used to identify genes involved in Fe phototrophy even from genetically "intractable" species.

6.6   ACKNOWLEDGEMENTS

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Chapter 7

Conclusions and Outlook

7.1   CONCLUSIONS

During my doctoral studies I have examined the mechanism and geological relevance of phototrophic Fe(II) oxidation by anoxygenic phototrophic bacteria. These bacteria grow photoautotrophically with Fe(II) as the electron source, coupling Fe(II) oxidation to reductive CO_2 fixation while harvesting light energy. Important advances were made in understanding which genes are involved in Fe(II) oxidation in two of these bacteria, *Rhodopseudomonas palustris* TIE-1 and *Rhodobacter* sp. SW2. In addition, by mimicking the environmental parameters comparable to conditions thought to be relevant for the ancient Earth when these bacteria might have thrived, we were able to constrain the geological role of these phototrophic Fe(II) oxidizers. Major findings of my thesis are briefly summarized below:

1) Both Fe(II) and H2 can serve as the electron donor for anoxgyenic photosynthesis and these substrates are predicted to be present in significant amount in the early Earth history. Since H_2 has a more negative redox potential than Fe(II) under physiological conditions, one would expect that H_2 is a better choice of electron donor for phototrophic Fe(II) oxidizers when both are present. Under the constraint of H_2 and CO_2 concentrations comparable to values predicted under

the ancient oceanic conditions, pure cell suspensions of TIE-1 and SW2 showed significant amounts of phototrophic Fe(II) activity without inhibition by the presence of H_2. This suggests that phototrophic Fe(II) oxidizers may have thrived, consistent with the hypothesis that these bacteria may have played a role in the deposition of Banded Iron Formations (BIFs) in the early Earth history.

2) The Fe(II) oxidation system was investigated in the purple non-sulfur bacterium *Rhodobacter* sp. SW2, which is not amenable to traditional genetic analysis. Through heterologous expression of a genomic library of SW2 in its close relative *Rhodobacter capsulatus* SB1003 (which has minimal amount of Fe(II) oxidation activity under the conditions tested), the *foxEYZ* operon of genes was identified to be critical for the phototrophic Fe(II) oxidation activity in SW2. FoxE was identified as a novel *c*-type cytochrome localized to the periplasm, FoxY a protein containing a putative redox cofactor pyrroloquinoline quinone, and FoxZ a putative transporter of unknown function. While *foxE* alone confers light-dependent Fe(II) oxidation activity to SB1003, maximum activity is achieved when *foxE* is expressed with *foxY* and *foxZ*.

3) The first genetically tractable phototrophic Fe(II) oxidizing bacterium *R. palustris* TIE-1 was isolated and characterized. A genetic system was developed in the organism, aiming to identify genes and gene products essential for photoautotrophic growth on Fe(II).

4) Random transposon mutagenesis and screening of 12,000 mutants was carried out in TIE-1 to identify mutants specifically defective in Fe(II) oxidation. Of the six mutants obtained, five of have independent disruptions in a gene that is predicted to encode an integral membrane protein with similarity to an ATP-dependent transporter. The sixth mutant has an insertion in a gene that is homologous to *cobS*, encoding an enzyme involved in cobalamin (vitamin B$_{12}$) biosynthesis. The involvement of these proteins in Fe(II) oxidation remains unclear.

5) A three-gene operon designated the *pio* operon was identified in TIE-1. The first gene, named *pioA*, encodes a *c*-type cytochrome that was upregulated under Fe(II) growth conditions. The second gene, *pioB,* encodes a putative outer membrane beta-barrel protein, and the third gene, *pioC,* encodes a putative high-potential iron-sulfur protein (HiPIP). Mutational analysis showed that the *pio* operon is essential and specific to phototrophic Fe(II) oxidation in TIE-1. Both PioA and PioC are soluble proteins, and based on the presence of signal sequences, both PioA and PioC are likely to reside in the periplasm.

6) PioC was overexpressed and partially purified from *E. coli.*

7.2   OUTLOOK

Perhaps the most exciting aspect of my research is that it provides an opportunity for a detailed understanding of the molecular mechanisms of phototrophic Fe(II) oxidation. Using two purple non-sulfur bacteria *Rhodopseudomonas palustris* TIE-1 and *Rhodobacter* sp. SW2 as model systems, novel genes and proteins involved in phototrophic Fe(II) oxidation were identified. Because of the limitations of genetic analysis in SW2, future studies will mainly involve TIE-1. With the identification of the Pio proteins, continued research will enable us to reveal the remarkable nature of this metabolism with respect to the mechanisms of energy conservation. Some efforts have already been made regarding the subcellular localization and purification of the Pio proteins (see appendixes A and B). Future work concerning the biochemical characterization of the purified Pio proteins includes measuring their redox potentials, investigating the molecular interactions between the Pio proteins, and electron transfer between the Pio proteins, Fe(II) and other electron carriers that are also potentially involved.

Lessons should be learned from the studies on the electron transport pathway of Fe(II) oxidation in *Acidithiobacillus ferrooxidans*. Although many efforts were made in addressing this question, the electron carriers involved are still debatable. These studies were carried out mainly by testing *in vitro* assays for Fe(II) oxidation activity with purified proteins. Does it mean that the complexity and redundancy of the Fe(II) oxidation system in *A. ferrooxidans* preclude the pure biochemical methods without the

aid of the genetic tools? One aspect is reflected by the unspecific nature of the redox

reactions between Fe and *c*-type cytochromes. Therefore, in TIE-1, both biochemistry

and genetics should be applied to more efficiently address the electron transport process

in phototrophic Fe(II) oxidation; *in vitro* biochemical studies with purified proteins,

membrane vesicles, and whole cells, should be combined with *in vivo* mutational

analysis.

It is interesting to note the similarities between the Pio proteins involved in

phototrophic Fe(II) oxidation in TIE-1 and the Mtr proteins involved in the dissimilitary

Fe(III) reduction in *Shewanella oneidensis* (see Chapter 5). In both systems, a decaheme

c-type cytochrome (PioA and MtrA) and an outer membrane beta-barrel protein (PioB

and MtrB) are involved. Does this suggest an evolutionary connection between the

enzymes for Fe(II) oxidation and Fe(III) reduction? In order to address this question, a

sufficient number of Fe(II) oxidizing and Fe(III) reduction enzymes from physiologically

diverse bacteria must first be obtained. However, our current knowledge about the

enzymes involved in phototrophic Fe(II) oxidation precludes any significant phylogenetic

or functional predictions be made from such a limited sample set.

The functions of PioA and PioB are rather intriguing. The predicted function of

PioA as an Fe(II) oxidoreductase located in the periplasm of TIE-1 suggests that Fe(III)

would form in this cell compartment. Then, how does TIE-1 prevent potential Fe(III)

precipitates from forming? If Fe(III) ligands are involved in Fe(III) transport, how does

Fe(III)-ligand association and dissociation happen before and after crossing the outer

membrane? Such knowledge is of great importance in understanding the unique features

of extracellular biomineralization catalyzed by phototrophic Fe(II)-oxidizing bacteria. Due to the structural prediction of PioB as a beta-barrel outer membrane protein similar to general porins and genetic localization to *pioA* and *pioC*, we predict that PioB may function as an Fe(III) transporter. In contrast to all known Fe transport systems involved in Fe acquisition that are repressed in the presence of Fe, PioB is induced when Fe is abundant. Understanding the function and regulation of PioB will broaden our understanding of transporters in general, including specific insights into the key elements that are responsible for the specificity of these porins to different metals.

The discovery of Pio and Fox proteins involved in phototrophic Fe(II) oxidation in two purple non-sulfur bacteria TIE-1 and SW2, respectively, suggests that the phototrophic Fe(II) oxidation system are diverse, even within this one group of microorganisms. Ultimately, elucidation of the mechanisms of phototrophic Fe(II) oxidation of bacterial isolates of different phylogenetic groups and environments will increase our knowledge about the diversity of phototrophic Fe(II) oxidation systems. Experiments that seek to address the contribution phototrophic Fe(II)-oxidizers in the global Fe cycle include an examination of the environmental distribution of TIE-1, SW2, and their respective iron oxidation systems couple with detailed kinetic measurements of the expression and activity of key indicator genes and enzymes.

The work presented in this thesis will lead to detailed understanding of the mechanisms of phototrophic Fe(II) oxidation, which is a necessary to test the involvement of these phototrophs in the deposition of BIFs in the early Earth history. The putative Fe(II) oxidoreductase, Pio and Fox proteins, found in TIE-1 and SW2,

respectively, permit a more fundamental understanding of the biomineralization process catalyzed by phototrophic Fe(II)-oxidizing bacteria. In addition, the discovery of the Pio proteins may lead to findings of novel Fe transporters within biological systems.

Appendix A

Subcellular Localization of PioABC in *Rhodopseudomonas palustris* TIE-1 and FoxE in *Rhodobacter* sp. SW2

A.1 INTRODUCTION

In establishing protein function, it is important to determine the subcellular environment in which the protein resides. Subcellular localization influences protein function in several aspects by determining the local chemical environment such as pH and redox potential, availability of interaction partners and substrates, and where reaction products will form, which may serve as the substrates for subsequence reactions. Although many efforts have been made predicting the subcellular localization of hypothetical proteins through bioinformatic approaches, definitive subcellular localizations for most proteins should be tested experimentally, especially for those that have no significant homologs in the database.

Recently our lab has made some progress in identifying molecular components important for phototrophic Fe(II) oxidation in two model organisms, *Rhodopseudomonas palustris* TIE-1 and *Rhodobacter* sp. SW2. In TIE-1, we discovered the three-gene *pio* operon to be essential for phototrophic Fe(II) oxidation (see Chapter 5). Expression of the first gene in the operon, *pioA*, is increased when TIE-1 is grown under conditions of Fe(II) phototrophy. PioA is a putative decaheme *c*-type cytochrome present in the cytoplasm or periplasm. The second gene, *pioB*, encodes a putative outer membrane β-

barrel protein. Both *pioA* and *pioB* contain a signal sequence characteristic of proteins

secreted across the cell membrane through the Sec protein secretion pathway (37). The

third gene, *pioC*, encodes a putative high redox potential iron sulfur protein (HiPIP) with

a twin-arginine translocation (TAT) signal sequence, suggesting PioC is secreted across

the cell membrane through the TAT protein secretion pathway (27). Our hypothetical

model for phototrophic Fe(II) oxidation in TIE-1 based on the Pio proteins is as follows:

PioA is a periplasmic protein that binds and oxidizes Fe(II). PioB interacts with PioA

and/or serves as an Fe(III) transporter to remove the Fe(II) oxidation product [Fe(III)]

across the outer membrane. PioC is in the periplasm and shuttles electrons from PioA to

the photosynthetic reaction center (RC), either directly or indirectly via cytochrome $c2$ as

the electron mediator (for details about functional role of PioC, see Appendix B).

The three-gene *fox* operon from SW2 was found to confer enhanced light-

dependent Fe(II) oxidation activity when heterologously expressed in *Rhodobacter*

capsulatus SB1003 (see Chapter 6). The first gene in this operon, *foxE*, encodes a

soluble novel *c*-type cytochrome. The second gene, *foxY*, appears to encode a putative

soluble periplasmic protein with a redox active cofactor. The third gene, *foxZ*, appears to

encode a cytoplasmic membrane protein with 10 transmembrane domains. A signal

sequence characteristic of secreted proteins is found at the N-terminus of all three Fox

proteins. Our model for phototrophic Fe(II) oxidation in SW2 based on the Fox proteins

is as follows: FoxE is a soluble periplasmic protein that serves as the Fe(II)

oxidoreductase. FoxY is also a soluble periplasmic protein containing a pyrroloquinoline

quinone redox active cofactor and assists FoxE in electron transfer to a component of the cyclic electron transfer chain.

Subcellular localization analysis of proteins is a key step towards elucidating their functional roles. Although genetic analysis showed that Pio and Fox proteins play an important role in phototrophic Fe(II) oxidation in TIE-1 and SW2, respectively (see Chapter 5 and 6), the individual functions of these proteins remain unclear. The subcellular location of these proteins, especially of the putative Fe(II) oxidoreductases PioA and FoxE, has substantial influence on our model of phototrophic Fe(II) oxidation. Since PioA and FoxE are both c-type cytochromes (which mature exclusively in the periplasm in Gram-negative bacteria (32, 37)), we hypothesize that PioA and FoxE function in the periplasm of TIE-1 and SW2, respectively. The location of PioA and FoxE will determine where their interactive partners are localized and how electrons enter the cyclic electron flow. In TIE-1, PioC is thought to be the electron acceptor for PioA, and in SW2 this electron carrier is predicted to be FoxY. The Fe(II) oxidoreductase is likely located where Fe(II) is readily available. Free ions of Fe(II) present in the growth medium (see Chapter 4) can readily diffuse into the periplasmic space through general porins on the outer membrane (33, 34) into the periplasm where Fe(II) oxidation by PioA and FoxE would occur.

Based on these premises, the Fe(II) oxidation product, Fe(III), will likely form in the periplasmic space. Due to the low solubility of Fe(III) at neutral pH, precipitation of Fe(III) in the periplasm would be expected to occur. The absence of Fe(III) precipitation in the periplasm of any phototrophic Fe(II) oxidizing bacterium including TIE-1 and

SW2 (21, 22) suggests mechanisms to overcome Fe(III) mineral precipitation, possibly with the involvement of specialized Fe(III) chelators and/or transporters. If such Fe(III) ligands exist and are stable over geological time scales they may provide a means to identify traces of this metabolism in the rock record and thereby enable studies directed to understanding the environmental impact of these organisms over geological time.

In this chapter of my thesis, I describe the results of several biochemical experiments performed to localize PioABC and FoxE proteins in their native organisms. Continued studies into this area will be required to fully elucidate the localization and function of these proteins in phototrophic Fe(II) oxidation.

A.2 MATERIAL AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table A.1. *R. palustris* CGA010 (CGA010), derived from parent strain CGA009 where a frameshift in the *hupV* gene was repaired, was kindly provided by F. Rey and C. S. Harwood (University of Washington).

Table A.1 Bacterial strains and plasmids (^{a}Gm, gentamicin).

Strain or plasmid	Genotype or markers, characteristics [a]	Source or reference
E. coli stains		
WM3064	Donor strain for conjugation: *thrB1*004 *pro thi rpsL hsdS lacZ*ΔM15 RP4–1360 Δ*(araBAD)567* Δ*dapA*1341::[*erm pir*(wt)]	W. Metcalf, Univ. of Illinois, Urbana
UQ950	*E. coli* DH5α (pir) host for cloning; F- Δ*(argF-lac)169* Φ*80dlacZ58(*ΔM15*) glnV44*(AS) *rfbD1 gyrA96*(NalR) *recA1 endA1 spoT1 thi-1 hsdR17 deoR λpir+*	D. Lies, Caltech
R. palustris stains		
TIE-1	Isolated from Woods Hole, MA	(21)
CGA009	Wild type (ATCC BAA-98)	(24)
CGA010	*hupV*$^+$ derivative of CGA009	F. Rey & C. S. Harwood, U. of Washington
PioA-HA	*R. palustris* str. TIE-1, HA tagged to the C-terminus of *pioA*	This study
PioB-HA	*R. palustris* str. TIE-1, HA tagged to the C-terminus of *pioB*	This study
PioC-HA	*R. palustris* str. TIE-1, HA tagged to the C-terminus of *pioC*	This study
PioA-3HA	*R. palustris* str. TIE-1, 3-HA tagged to the C-terminus of *pioA*	This study
Plasmids		
pJQ200SK	Mobilizable suicide vector; *sacB* Gm^r	(23)
pPioA-HA	2-kb fusion PCR fragment containing PioA-HA cloned into the *Spe*I site of pJQ200SK; used to make the PioA-HA strain.	This study
pPioB-HA	2-kb fusion PCR fragment containing PioB-HA cloned into the *Spe*I site of pJQ200SK; used to make the PioB-HA strain.	This study
pPioC-HA	2-kb fusion PCR fragment containing PioC-HA cloned into the *Spe*I site of pJQ200SK; used to make the PioC-HA strain.	This study
pLARS	A derivative of pBBR1MCS2 (25, 26); insertion *mamA* gene is under the control of a *tac* promoter and fused to a 3xHA tag at its 3' end.	L. Dietrich, Caltech
pPioA-3HA	2.3-kb fusion PCR fragment containing PioA-3HA cloned into the *Xba*I site of pJQ200sk; used to make the PioA-3HA strain.	This study

Media and culture conditions. For aerobic growth *R. palustris* strains were grown in YP medium (0.3% yeast extract and 0.3% Bacto Peptone) at 30 ˚C with shaking. For anaerobic growth *R. palustris* strains were grown without shaking at 30 ˚C in FEM, a defined basal medium for phototrophic Fe(II)-oxidizing bacteria (14). Hydrogen (80% H_2: 20% CO_2) was used for photoautotrophic growth of TIE-1 and SW2. Cultures were incubated at a distance of ~15 cm from a 34 watt tungsten incandescent light source. *Escherichia coli* strains were cultured in LB at 37 °C with shaking. *E. coli* WM3064 was supplemented with 300 μM diaminopimelic acid (DAP). Kanamycin and gentamicin were used at 100 and 200 μg/ml (or 400 μg/ml as indicated) for TIE-1 and 50 and 20 μg/ml for *E. coli*, respectively.

Table A.2 Sequence of the oligonucleotides.

Oligonucleotide	Length (bp)	Sequence (5'-3')
HA-PioA1	28	GG**ACTAGT**ATGGTTCGACCGAGAACTTG
HA-PioA1p	57	<u>AGCGTAGTCTGGAACGTCGTATGGGTA</u>CGATCCTCTTCGGTGCCAGCGCGATCCGGA
HA-PioA2	57	AGAGGATCG<u>TACCCATACGACGTTCCAGACTACGCT</u>TAGCTCGGAGCCGGGGACAGC
HA-PioA2p	28	GG**ACTAGT**CATCGTGGTGTACTGGAACG
HA-PioB1	28	GG**ACTAGT**TCGCTGTCGATGTCCTACAC
HA-PioB1p	57	<u>AGCGTAGTCTGGAACGTCGTATGGGTA</u>CGATCCTCTCCATTTCGCGTTGAGCGAGAC
HA-PioB2	28	GG**ACTAGT**TCGACGACGAAGGCTTCTAT
HA-PioB2p	57	AGAGGATCG<u>TACCCATACGACGTTCCAGACTACGCT</u>TGAGCGGCGGCGGCGGCGCCA
HA-PioC1	28	GG**ACTAGT**GCCTGAAGAAGAGCAACACC
HA-PioC1p	57	<u>AGCGTAGTCTGGAACGTCGTATGGGTA</u>CGATCCTCTTGCCTTGCCGGCGTAGAGGCG
HA-PioC2	28	GG**ACTAGT**TCAGTTCATGTGCCAGCATC
HA-PioC2p	57	AGAGGATCG<u>TACCCATACGACGTTCCAGACTACGCT</u>TAACGCGGCTTTGATCACGTC
3HA-PioA1-Hind	28	GG**AAGCTT**ATGGTTCGACCGAGAACTTG
3HA-PioA1p-BamH	29	GG**GGATCC**TCGGTGCCAGCGCGATCCGGA
3HA2-PioA2-Spe	29	GG**ACTAGT**TAGCTCGGAGCCGGGGACAGC
3HA-PioA2p-Xba	28	GG**TCTAGA**CATCGTGGTGTACTGGAACG
3HA-PioA1-Xba	28	GG**TCTAGA**ATGGTTCGACCGAGAACTTG

* HA linker regions in the primers for crossover PCR are underlined and restriction sites are shown in bold.

Construction of HA-tag on C-terminus of *pioA*, *pioB* and *pioC*. All primer sequences used in making the constructs are listed in Table A.2. In-frame hemagglutinin (HA) tags on the C-terminal end (right before the stop codon) of *pioA*, *pioB* and *pioC* were engineered to the chromosome of the native strain of TIE-1 by double recombination events using suicide vectors followed by *sacB* selection. The HA-tagged constructs were made by overlap extension PCR. Briefly, ~1 kb DNA was amplified at the 5'- direction

near of the C-terminus of *pioA* just prior to the stop codon, using primers HA-PioA1 and

HA-PioA1p. The HA tag was engineered on the reverse primer, HA-PioA1p. Similarly,

a ~1 kb region downstream of *pioA* starting from the stop codon was amplified using

primers HA-PioA2 and HA-PioA2p. The two resulting PCR products were then used as

templates for another round of PCR with primers HA-PioA1 and HA-PioA2p. The final

PCR product was cloned into the *Spe*I sites of pJQ200sk (39), a suicide vector encoding

gentamicin resistance and sucrose sensitivity, to generate pPioA-HA. pPioA-HA was

transformed into *E. coli* WM3064 by heat shock and introduced into TIE-1 by

conjugative matings (13). Single recombination events were selected on PM agar (20)

containing 10 mM succinate and 400 μg/ml of gentamicin. Colonies that had undergone

a second recombination event were selected for growth on PM agar containing 10 mM

succinate and 10% (w/v) sucrose. The resulting HA-tagged clone, PioA-HA, was

checked by sequencing. Clones PioB-HA and PioC-HA were made in a similar manner

with primers HA-PioB1, HA-PioB1p, HA-PioB2 and HA-PioB2p for clone PioB-HA,

primers PioC1, HA-PioC1p, HA-PioC2 and HA-PioC2p for clone PioC-HA,

respectively.

Construction of 3xHA-tag on the C-terminus of *pioA*. The length of a primer

containing 3xHA (three epitopes of HA peptide) exceeds what is feasible by direct PCR

amplification. For making this construct, the PCR product amplified from TIE-1

genomic DNA using primers 3HA-PioA1-Hind and 3HA-PioA1p-BamH (Table A.2) and

cloning vector pLARS were digested by *Hind*III and *BamH*I restriction enzymes and then

ligated with T4 DNA ligase. A second PCR product was obtained with primers 3HA2-

PioA2-Spe and 3HA-PioA2p-Xba using TIE-1 genomic DNA as template and

subsequently inserted into the above construct via the corresponding restriction sites *Spe*I

and *Xba*I. With the resulting plasmid as a PCR template, a second round of PCR was

done with primers 3HA-PioA1-Xba and 3HA-PioA2p-Xba and the resulting PCR product

was cloned into *Xba*I sites of vector pJQ200sk (39) to generate plasmid pPioA-3HA.

Plasmid pPioA-3HA was introduced into TIE-1 to generate strain PioA-3HA following

the same procedure as described for making clone PioA-HA.

Homogenate fractions. 50 ml of TIE-1 cells grown photoautotrophically on H_2 were

harvested at mid-exponential phase (OD660, ~0.4) by centrifugation at 10,000 *g* for 15

minutes. Cells were washed and resuspended in 3-ml HS Buffer (50 mM HEPES, 20

mM NaCl, pH 7.0), lysed by four passages through a French Press at 18,000 psi, and

lysate clarified by centrifugation at 10,000 *g* for 15 minutes at 4 °C. Soluble and

membrane fractions from this clarified cell lysate were obtained by ultracentrifugation at

200,000 *g* for 1.5 hours.

Periplasmic fractionation of *Rhodobacter* sp. SW2. Periplasmic proteins from SW2

were obtained by generating spheroplasts, using a lysozyme-EDTA treatment adapted

from Vasquez-Laslop et al. (44). Lysozyme hydrolyzes repetitive N-acetylglucosamine-

B-1 and 4-N-acetylmuramic acid bonds present in the bacterial cell wall, and

ethylenediamine tetracetic acid (EDTA) weakens the cell by binding divalent cations, such

as Mg^{2+} and Ca^{2+}, from the cell envelope and to cause the release of phospholipids and protein-lipopolysaccharide complexes needed for membrane stability. Briefly, 50 ml of SW2 cells grown photoautotrophically on H_2 to a mid-exponential phase were harvested by centrifugation and washed once in TRIS buffer (50 mM Tris-HCl, pH 8.0). The cell pellet was then resuspended in 2 ml of the TRIS buffer containing 0.5 M sucrose. 5 μl of 50 mM EDTA stock solution and 5 μl of 60 mg/ml of freshly made lysozyme stock solution were slowly added into the cell suspension. The components were mixed by gentle pipetting and inversions followed with incubation on ice for 30 minutes. Spheroplast formation was periodically checked by microscopy over time. After 30 minutes, the suspension was centrifuged at 6,000 *g* for 15 minutes at 4 °C. Periplasmic proteins collected in the supernatant were concentrated (about five fold v/v) using a Microcon centrifugal membrane filter with a 10 kDa cut off (Millipore) before electrophoresis. This concentrated solution was analyzed for alkaline phosphatase activity and heme-containing proteins (see below).

Periplasmic fractionation of TIE-1. General protocols attempted for obtaining the periplasmic fraction of TIE-1 have not been successful. Subcellular fractionation methods for *R. palustris* strain No. 7 was previously published in one study (38) which modified protocols from previous studies on *Rhodobacter spheroides* and *E. coli* (42, 46), but could not be reproduced with *R. palustris* TIE-1 or *R. palustris* CGA010 in our hands. In the literature, two methods described for obtaining periplasmic proteins through

spheroplast formation for Gram-negative cells include a treatment with lysozyme-EDTA

and a treatment with osmotic shock (11, 44). The lysozyme-EDTA treatment protocol is

similar to what is described above for periplasmic fraction of SW2, with variations of

lysozyme and EDTA concentrations and incubation temperature and time (17, 19, 40, 41,

44, 46). Following the basic principle of the protocol, the harshest condition tested for

TIE-1 used lysozyme at 1 mg/ml and EDTA at 10 mM, incubated overnight at 37 °C.

The majority of the cells still remained intact when checked by microscopy and the

protein concentration of the supernatant after centrifugation at 6,000 g for 15 minutes was

below the detection limit using the Bradford assay.

In an attempt to lyse cells by osmotic shock treatment, several published protocols

were tested with variations in EDTA concentration, incubation temperature, and

incubation time (6, 28, 35, 42). Briefly, cells washed in 30 mM Tris-HCl, pH 7.5 were

resuspended in the osmotic buffer (30 mM Tris-HCl, pH 7.5, 20% sucrose, 10 mM

EDTA). After 10 or 30 minute incubations on ice or at room temperature, cells were

pelleted by centrifugation for 20 minutes at 6,000 g at 4 °C and gently resuspended in 1

ml of ice-cold water or 1 ml of ice-cold 5 mM $MgSO_4$. These cells were then incubated

on ice for 10 minutes or more. The cell suspension was monitored by microscopy over

time but no spheroplast formation was observed after one hour of incubation.

Biochemical analysis of proteins. 24 μl of the SW2 periplasm protein solution was

supplemented with 6 μl of 5x sample buffer (containing DTT) and analyzed by standard

Laemmli sodium dodecyl sulfate (SDS) electrophoresis using 12% Pre-cast

ployacrylamide gels (BioRad) in a BioRad Mini-Protein apparatus. BioRad Precision

plus protein standards (dual color) were used as molecular weight markers. *C*-type

cytochrome bands were detected according to the in-gel peroxidase activity assay of

Francis and Becker (15). Bands of interest were cut from the heme-stained gels and

submitted to the Protein/Peptide MicroAnalytical Laboratory/Facility at Caltech for

LC/MS/MS analysis.

Alkaline phosphatase activity of the periplasmic fractions from SW2 was tested as

described with *E. coli* as a positive control (5, 8). 90 μl of the periplasmic fraction from

SW2 or *E. coli* was mixed with 10 μl of freshly prepared *p*-nitrophenyl phosphate

(PNPP) solution (0.5 M Tris-HCl, 4% PNPP, 0.5 mM $MgCl_2$, pH 9.0) in a 96 well plate,

and incubated at room temperature. The absorbance at 405 nm was followed over time

with a Synergy HT, Bio-Tek, plate reader (Winooski, VT).

Western blot analysis. SDS-PAGE gels were transferred to a Immobilon p^{SQ}

transmembrane (Millipore) by electrophoresis overnight at 22 volts at 4° C. The

membrane was blocked in the TBST solution (20 mM Tris-HCl, pH 8.0 ,125 mM NaCl ,

0.1% Tween 20) containing 5% non-fat dry milk, and washed three times in TBST

solution for five minutes. 100 μl of primary antibody (rabbit anti-HA antibody, Sigma)

stock solution was mixed into 50 ml of TBST solution containing 5% non-fat dry milk.

The primary antibody was bound at room temperature for two hours after which the

membrane was washed three times in TBST solution for five minutes before binding to

the secondary antibody (goat anti-rabbit HPR conjugate, BioRad). The secondary

antibody solution was prepared by mixing 25 μl of antibody stock solution into 50 ml of

TBST solution containing 5% non-fat dry milk. The secondary antibody was bound for

one hour at room temperature with gentle shaking and washed three times in TBST

solution for five minutes for one hour at room temperature on a rocker. Immobilon

western chemiluminescent horseradish peroxidase substrate (Millipore) and a BioRad

imager were used for detection following the manufacture's instructions.

A.3   RESULTS AND DISCUSSION

A.3.1   Western blot for detecting HA-tagged Pio proteins

Among all the TIE-1 clones generated only clone PioC-HA could be detected by

Western analysis with a HA antibody (Figure A.1). Separation of the soluble and

membrane fractions by ultracentrifugation of the clarified cell extract showed that PioC is

localized in the soluble fraction in H_2 grown TIE-1 (Figure A.1). In future studies, the

resuspended membrane faction after ultracentrifugation should also be included as a

negative control in Western analysis. TIE-1 derivatives containing HA-versions of the

Pio proteins showed wild-type levels of Fe(II) oxidation activity in the cell suspension

assay (Figure A.2). This suggests that the HA-tag did not significantly affect the

functions of the Pio proteins nor their subcellular locations.

Proper in-frame HA tag insertions were confirmed in all clones by DNA

sequencing. While it is unclear why PioA-HA and PioB-HA clones could not be detected

by Western blot analysis, the following explanations are possible:

1) Low expression of PioA and PioB in TIE-1 under H_2 phototrophy. As shown in Chapter 5, expression of PioA increases during Fe(II) phototrophy as detected through heme-staining. However, it is unclear at this stage whether regulation is at the level of transcription, translation or post-translation. Since the expression of Pio proteins may be dependent on Fe(II), it is worthwhile repeating the Western blot with Fe(II) grown or H_2 grown and Fe(II) induced cells of these HA-tagged constructs.

Figure A.1 Western blot of HA-tagged Pio proteins and subcellular localization of PioC. All proteins were extracted from cells grown phototrophically on H_2. Lane 1: soluble fraction of PioC-HA (i.e., supernatant fraction after ultracentrifugation); Lane 2: clarified cell extract from PioA-HA; Lane 3: clarified cell extract from PioB-HA; Lane 4: clarified cell extract from PioA-3HA; Lane 5: clarified cell extract from PioC-HA. Bands of ~10 kDa detected in lane 1 & 5 are probably PioC, but mass spectrometry analysis should be done for confirmation. These results demonstrate that PioC is expressed in the soluble fraction of H_2 grown TIE-1.

2) The C-terminus of PioA and PioB are not surface exposed in the protein (or protein complex) structure formed during cooling and refolding, and therefore, the HA tags at these positions are not likely to be detected by the antibody. Although protein samples were boiled at 95 °C for 5 to 10 minutes for complete denaturation before loading on the gel, protein refolding might have occurred during the cooling process. If this is the case, other denaturing conditions could be tested, e.g., using urea instead of heating for protein denaturation (4).

Figure A.**2** Endpoint measurements for testing the Fe(II) oxidation activity of the HA tagged clones made in the study through cell suspension assay using wild type TIE-1 and *pio* operon deletion mutant TIE-3 as positive and negative controls, respectively. About 4X concentrated H_2 grown cultures (OD660 nm ~0.3) were used for this assay and cells were normalized by OD. Measurements shown were made after incubation for two hours under light.

3) Other technical reasons that could have accounted for the failure to detect PioA-HA and PioB-HA on the Western include low detection sensitivity of the Western blot, which could be improved by obtaining more concentrated protein samples, prolonging antibody incubation time, and modifying the stringency of the washing steps,

such as omitting Tween 20 from the TBST buffer.

Because PioA-HA and PioB-HA constructs could not be detected by Western analysis, a variant of PioA was constructed with three epitopes of the HA tag fused to the C-terminal end of the protein. This PioA-3HA construct was also not detected by Western analysis (Figure A.1). An N-terminal fusion to PioA and B could be tested in the future. However, such a construct could interfere with the transport of these proteins due to the presence of the putative signal sequences on the N-termini. For PioA, two putative signal sequence cleavage sites were predicted: one between residues 32 and 33 and the other between residues 40 and 41. Construction of a 3xHA tagged PioA in both locations is underway.

To overcome potential issue of low expression, future studies could employ immunogold labeling of the HA-tagged clones. In this method, the secondary antibody is conjugated with 10 nm gold particles, and whole cells or cry-ultrathin sections of cells are visualized using transmission electron microscopy (TEM) (18). Alternatively, after we have purified PioA and PioB, we could raise antibodies directly against these proteins or their peptide fragments.

A.3.2 Heme staining of periplasmic fraction of SW2

Because SW2 is not amenable for genetic analysis, we did not construct fusion proteins for Western blot analysis. However, the predicted Fe(II) oxidoreductase in SW2, FoxE, is a *c*-type cytochrome (see Appendix A) which can be detected by heme-staining (15). To determine the cellular localization of FoxE, the periplasmic fraction

from H_2 grown SW2 cells was isolated by spheroplast formation. Alkaline phosphatase activity was assayed as a periplasmic marker protein, with the periplasmic fraction from *E. coli* serving as a positive control (Figure A.3). In future studies a cytoplasmic marker protein such as malate dehydrogenase, should also be included in future studies (1, 11). Periplasmic proteins from SW2 were separated by SDS-PAGE followed with an in-gel heme-peroxidase activity stain, which revealed the presence of a ~25 kDa heme-containing protein (Figure A.4), similar to the reported size for FoxE (see Chapter 6). This band was excised and analyzed by tandem mass spectrometry analysis. One peptide (sequence: VIIPGDPEASK) identified from this band corresponded to FoxE, but ideally, two or three peptides are needed for definitive identification. In summary, detection of FoxE in the periplasmic fraction of H_2-grown SW2 cells is consistent with our prediction. Future work should include more rigorous controls for subcellular fractionation and also optimize the fractionation protocol to obtain pure periplasmic proteins at high concentrations for mass spectrometry analysis.

Figure A.3 Alkaline phosphatase activity (measured by the increase in absorbance at 405 nm) was tested on the periplasmic fraction (~400 µg/ml) of H_2 grown SW2, using the periplasmic fraction from *E. coli* as a positive control.

Figure A.4 Detection of FoxE in the periplasmic fraction of H2 grown SW2 by heme staining of SDS-PAGE. The ~25 kDa weak band is similar to the reported size for FoxE, and is partially confirmed by mass spectrometry analysis (see text).

A.3.3 Alternative methods for spheroplast formation in *R. palustris*

It is not clear what is responsible for the inert nature of the *R. palustris* cell wall that makes it resistant to cell lysis and fractionation. Similar levels of resistance to standard lysis and fractionation treatments was observed in both TIE-1 and CGA010, suggesting a general phenomenon for *R. palustris*. However, the difficulty encountered here with *R. palustris* is not unique. Resistance to lysozyme-EDTA treatment was also observed with the fermenting Gram-negative bacterium *Brevibacterium ketosoreductum* (45), suggesting that the resistance mechanism of *R. palustris* to lysozyme is unlikely to be due to the presence of laminar intracytoplasmic membranes of phototrophic grown cells.

In depth understanding of the *R. palustris* cell wall structure will be necessary to develop protocols for efficient spheroplast formation. Many unknown factors could be responsible for the recalcitrant nature of *R. palustris* cell walls. An example of the complex nature of cell wall structure is illustrated by a study examining the basis of chlorhexidine resistance in *Pseudomonas*. The nitrogen and phosphorus content of the cell walls was compared to elucidate the nature of chlorhexidine resistance (12). The chlorhexidine resistant strain of *Pseudomonas* contained 8-fold less lipopolysaccharide phosphorus than a chlorhexidine sensitive strain of *P. aeruginosa*. Since the antimicrobial effects of chlorhexidine are associated with the attraction between the positively charged antibiotic with the negatively charged bacterial cells, the increase in chlorhexidine resistance is likely due to a decrease in surface charge from fewer phosphorus groups (12). Until information on the cell wall composition is available for

R. palustris, it is still prudent to assay various methods already developed for making spheroplasts in other organisms. Below is a list of conditions one may consider.

1) Although the addition of sucrose in the lysozyme-EDTA treatment was reported to accelerate and stabilize spheroplast formation in *E. coli* and other bacteria (29), this does not always hold true in other systems. For example, in *Acetobacter melanogenus* and *Gluconobacter oxydans*, the presence of sucrose in lysozyme-EDTA solution inhibited spheroplast formation (45). Further, a heat shock treatment following the lysozyme-EDTA treatment was reported to achieve more efficient spheroplast formation in *E. coli* (30). In addition, varying salt concentrations in the lysis solution or pre-treatment of cells in salt solution may also facilitate spheroplast formation (36).

2) Various lysis reagents that "soften" the cell wall are reported to be useful in spheroplast formation. One example is polymyxin B, a peptide antibiotic particularly active against gram-negative bacteria, which damages the outer membrane and allows lysozyme to reach and digest the cell wall (16). Short exposure to polymixn B followed by lysozyme incubation produced *E. coli* spheroplasts efficiently (10). Chloroform has also been used to release periplasmic proteins in *E. coli* (2), and because of the relative ease of this procedure, it should be explored in *R. palustris*. In case chloroform alone is not effective, low concentrations of SDS may also be added. In a trial run with TIE-1, one drop of 0.1% SDS and one drop of chloroform were added into 1 ml of ~5X concentrated H_2 grown TIE-1 cells, and gently vortexed for 5 seconds. This procedure did not yield spheroplasts. However, optimization of this protocol by varying SDS, chloroform, or cell concentration might still be useful. Following the same logic, 1X

BugBuster reagent with recombinant lysozyme (EMD Biosciences, Madison, WI) was tested and yielded promising preliminary results. 50 ml of H_2 grown TIE-1 cells were harvested and washed in 50 mM Tris-HCl, pH 8.0, before the cell pellet was resuspended in the 1X BugBuster solution and incubated at room temperature. A fraction (10%-20%) of cells transformed into spherical shapes after incubation for 30 minutes.

   3) Other reagents have been supplied in the growth medium or fractionation solution in order to facilitate spheroplast formation of bacteria with stronger/thicker cell walls. For example, the addition of L-glycine to the growth medium of *Mycobacteium smegmatis* was required for spheroplasts formation (43). Many efforts have been made in the cell wall disruption of gram-positive bacteria due to their importance in the food industry and clinical applications (31). Supplementing 10 mM L-threonine (weakens cell wall cross-links) or 10 mM L-lysine (mechanism of action currently unknown) in the growth medium increases sensitivity to lysozyme treatment in *Streptocooci mutans* (9). Moreover, mutanolysin is generally used to liberate reducing sugars and free amino acids from the peptidoglycan layers of the cell wall. Pre-digestion with mutanolysin (120 U/ml) for one hour at 37 °C followed by a half hour treatment with lysozyme led to spheroplast formation in *Rhodococcus* species (3).

   4) It is generally considered that stationary-phase cells are more resistant to lysozyme than those collected from actively growing cultures, due to increases in cell wall O-acetyl groups and decreases in N-acetyl groups as cells age (7). However, the presence of polyethylene glycol appears to improve breakage even for stationary-phase cells of *Lactobacillus* and *Streptococcus* (9).

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Appendix B

Overexpression and Purification of PioC in *E. coli*

B.1   INTRODUCTION

Rhodopseudomonas palustris TIE-1 uses Fe(II) as an electron donor for

photoautotrophic growth. Genetic studies show that a putative high redox potential iron-

sulfur protein (HiPIP), encoded by gene *pioC*, last gene in the *pio* operon, is important in

phototrophic Fe(II) oxidation (18). The first gene in this operon, *pioA,* encodes a putative

decaheme *c*-type cytochrome that is up-regulated during Fe(II) phototrophy. An in-frame

deletion of *pioC* resulted in a decrease in phototrophic Fe(II) oxidation activity in the cell

suspension assay and the inability to grow on Fe(II), while growth on other substrates

was not affected. Protein sequence analysis predicts that *pioC* encodes a putative HiPIP

94 amino acids in length, containing an iron-sulfur binding site, a twin-arginine

translocation (TAT) signal sequence, and a cleavage site between residues 37 and 38.

Based on observations that [1] no hydrophobic regions are predicted other than the signal

peptide, [2] the presence of the TAT signal sequence at the N-terminus, and [3] detection

of PioC in the soluble fraction of crude cell extracts of TIE-1 grown photoautotrophically

on H_2, we predict that PioC resides in the periplasmic space (see appendix A for more

detail). Comparison of PioC to sequences in the NCBI sequence database revealed

similarity to bacterial iron-sulfur proteins, with greatest homology occurring near the

iron-sulfur cluster binding site. PioC is 47% identical and 52% similar over 48 amino

acids to the HiPIP from *Rhodophiala globiformis* (2), and is 44% identical and 53%

similar over 51 amino acids to a putative HiPIP iron oxidoreductase, encoded by gene

iro, involved in aerobic Fe(II) oxidation from *Acidithiobacillus ferrooxidans* (9, 19).

HiPIPs are soluble electron carrier proteins containing a [4Fe-4S] cluster with

redox potentials mostly ranging from 260-450 mV, albeit sometimes a low as 50-100

mV. They are present in great abundance in the periplasmic space in both photosynthetic

and non-photosynthetic Proteobacteria (5, 6) including a variety of purple sulfur and

purple non-sulfur bacteria (32, 36). With divergent primary sequence, the greatest

homology of HiPIP proteins has the greatest homology near iron-sulfur cluster binding

site (36).

Based on relatively high redox potentials, being small soluble proteins localized

to the periplasmic space, HiPIPs are thought to serve the same functional role as

cytochrome $c2$ (redox potentials ranging between 260 and 330 mV) in purple bacteria,

transferring electrons from the cytochrome *bc1* complex to the photosynthetic reaction

center (RC, with redox potentials of 450-500 mV) to complete the cyclic electron flow (5,

36) during phototrophic growth. This prediction is consistent with spectroscopic and

kinetic evidence showing that HiPIPs can be photooxidized by bacteriochlorophyll in

whole cells and membrane vesicles (21-24, 33).   Although electron transfer distance and

free energy may be sufficient to allow electron transfer *in vitro*, they do not necessarily

address *in vivo* cellular chemistry. For example, the RCs from both *Rubrivivax*

gelatinosus and *Rhodocyclus tenuis* interact with and accept electrons from cytochrome

$c2$, HiPIP and horse heart cytochrome *c in vitro* (29, 30). However, under physiological

conditions *R. gelatinosus* only utilizes HiPIP (33) and *R. tenuis* utilizes HiPIP or

cytochrome $c8$ as electron donors, depending on ambient redox potentials present in the

photoinduced cells (24).

The functional role of HiPIP in photosynthetic electron transfer in purple bacteria

is further complicated by the structural difference of the RC within the purple bacteria

(26). Based on the subunit composition of the RC complexes, RCs are divided into two

groups including Groups I and II (5). Both Groups I and II RCs contain three core

subunits, L, H and M subunits, but Group II RCs possess an additional *c*-type

cytochrome subunit (usually a tetraheme *c*-type cytochrome) peripherally bound to the

periplasmic face of the complex. A combination of several mutagenesis, kinetic, and

crystal structure studies in *Blastochloris viridis* and *Thermochromatium tepidum* have

demonstrated that under physiological conditions the cytochrome subunit in the Group II

RC serves as the electron mediator from the soluble electron carriers (such as cytochrome

c2 and HiPIP) to the RC (10-14, 27). Biophysical analysis of the Group I RC from

Rhodobacter sphaeroides co-crystallized with cytochrome $c2$ (1, 3) have sown that the

physiological electron donor docked within the proximity of the special pair of the

chlorophyll molecules of the RC for direct electron transfer (4, 34). Although HiPIP has

been shown to function as a mediator between cytochrome *bc1* complex and the

tetraheme *c*-type cytochrome subunit in Group II RCs (16, 23, 24, 29, 30, 33), to our

knowledge, the role of HiPIP in group I RC system has not been addressed. It may

simply be due to the fact that the commonly studied purple bacteria with Group I RCs,

such as *Rhodobacter sphaeroides, Rhodobacter capsulatus* or *Rhodospirillum rubrum,* do

not contain HiPIPs.

$R.$ *palustris*, however, contains a Group I RC in which no *c*-type cytochrome subunit is attached to the RC complex (5) and genome analysis predicts the presence of both cytochrome *c*2 and HiPIPs. This bacterium, to our knowledge, is the only photosynthetic bacterium containing a Group I reaction center and HiPIP electron carriers identified to date (23, 25, 32, 36). Besides the cytochrome *c*2 encoded by gene RPA1535 on the *R. palustris* CGA009 genome, another homolog of cytochrome *c*2 (encoded by gene RPA3693) is also present. Furthermore, besides the *pioC* present in the *pio* operon, the *R. palustris* CGA009 genome predicts the presence of another gene (RPA3566) that also encodes for a putative HiPIP, which, based on genomic organization, seems unrelated to the *pio* operon. A previous study also revealed the presence of two HiPIPs in *Rhodospirillum salinarum*, however, but no functional analysis has been reported (2).

In addition to a direct role of HiPIP in cyclic electron transfer, it was found that HiPIP could serve as an electron acceptor for a thiosulfate-tetrathionate oxidoreductase in *Chromatium vinosum* (15). This suggests, in at least some cases, HiPIP can provide electrons to the photosystem from an external electron donor. Following this logic, we hypothesize that PioC carries electrons from the Fe(II) oxidoreductase, PioA, to the cyclic electron transport chain via RC or cytochrome *c*2, based on the following reasons: 1) Cytochrome *c*2 is predicted to be present in the *R. palustris* genome, which can serve as the electron carrier from the cytochrome *bc*1 complex to the reaction center in most purple bacteria; 2) PioC is only required for phototrophic growth on Fe(II), but not other substrates such as H_2 or thiosulfate; 3) The partial defect of Fe(II) oxidation activity

observed in the Fe(II) oxidation activity with whole cells can be explained by the presence of another putative HiPIP from the *R. palustris* genome (see Chapter 3). However, this model does not rule out the possibility that PioC may have a dual function in the cyclic electron flow mediating electron transfer from the cytochrome *bc1* complex to the RC, interchangeable with cytochrome *c*2. Furthermore, the substitution of cytochrome c2 by PioC may depend on redox potentials of the external electron donors supplied in the growth medium, similar to the system in *R. tenuis* (24).

To test our hypothesis that PioC is the electron shuttle between PioA and the RC during Fe(II) dependent phototrophic growth of TIE-1 we hope to construct an *in vitro* electron transfer pathway for phototrophic Fe(II) oxidation. In this chapter of my thesis, I describe my preliminary work towards purification of PioC.

B.2   MATERIAL AND METHODS

Bacterial stains, plasmids and growth conditions. Wild-type *Rhodopseudomonas palustris* TIE-1 was used (17). *E. coli* strain BL21 was obtained from Stratagene, TX. Over-expression vector pET32h (35) was kindly provided by Dr. Christian Ungermann (Osnabrueck, Germany). TIE-1 was grown at 30 °C under aerobic condition in YP medium (0.3% peptone plus 0.3% yeast extract). *E. coli* was grown at 37 °C in LB under aerobic conditions, and in LB plus 20 mM lactate under anaerobic conditions. Ampicilin (Ap) was used at 100 µg/ml for *E. coli*.

Cloning of *pioC* Amplification of *pioC* was achieved by PCR with forward primer (#159) PioC-N-NcoCap (5'-CATGCCATGGGTATGAACGACAAACGCAAC-3') and reverse primer (#160) PioC-C-XhoCap (5'-CCGCTCGAGTTATGCCTTGCCGG CGTAGA-3'), using genomic DNA extracted from aerobically grown TIE-1 as template. The PCR product was digested with restriction enzymes *Nco*I and *Xho*I and ligated into pET32h vector (35) to generate plasmid pET32hPioC. The ligation product was transformed into *E. coli* strain UQ950 (31) and colonies were selected on LB-Ap agar plates. DNA sequencing of the pET32hPioC insert verified that *pioC* was successfully cloned. For recombinant expression of PioC, pET32hPioC was then introduced into *E. coli* BL21, which carries the T7 RNA polymerase structural gene under the control of the IPTG-inducible *lac* promoter.

Purification of the recombinant PioC. One colony of *E. coli* BL21 carrying the pET32hPioC plasmid was picked and grown in 10 ml LB-Ap overnight at 37 °C. This 10 ml overnight culture was inoculated into 1 L of LB-Ap. After incubating under the same condition for about 4 hours until the OD (absorbance at 600 nm) reached 0.5-0.6, 1 ml of 1 M IPTG stock was added into the 1 L culture (final IPTG concentration was 1 mM and incubated for an additional 2-hour induction. Cells were then collected by centrifugation at 8,000 g for 20 min at 4 °C, and resuspended in 4 ml of TS buffer (50 mM Tris-HCl, 0.3 M NaCl, pH 7.0). 60 µl of 5 mg/ml DNase stock was added into the cell suspension and incubated for 30 minutes on ice before cells were passed 3 times through a French pressure cell at 20,000 psi. An additional 8 ml of TS buffer was added into the cell lysate

to a total volume of 12 ml before the lysate was centrifuged at 10,000 g for 30 minutes to remove unbroken cells and cell debris. The resultant supernatant is called clarified cell lysate.

TALON Superflow metal affinity resin (cobalt based, Clontech) was gently stirred before 1 ml slurry solution was taken out, washed three times with filtered TS buffer by gentle resuspension and centrifugation at 800 g for three minutes. For protein binding, 1 ml of washed cobalt resin was added into the clarified cell extract in a 15 ml conical tube, and incubated at 4 °C for 2 hours with gentle swirling. The resin was then removed by centrifugation at 800 g for 5 minutes, and the supernatant is called "binding waste solution". The pelleted resin was washed three times with 15 ml of TS buffer containing 20 mM imidazole by resuspension, incubated for 10 minutes at 4 °C, and centrifuged at 800 g for 5 minutes at 4 °C. The protein was eluted from the resin with 3 ml of filtered TS buffer containing 200 mM imidazole followed by incubation at 4 °C for 10 minutes.

Because of the presence of the engineered thrombin protease cleavage site in the construct, thrombin protease was used to remove the thioredoxin and 6-His tags. Different conditions for thrombin protease cleavage were tested by varying incubation temperature (room temperature or at 4 °C) and protease concentration (1 to 10 unit per 100 μg of protein).

B.3   RESULTS AND DISCUSSION

The entire *pioC* gene was cloned into the *Noc*I and *Xho*I sites of pET32h,

resulting in a thioredoxin and His tag on the N-terminal end of PioC with a thrombin

protease cleavage site between the His tag and PioC (Figure B.1). Because of the

presence of His tag on the N-terminal of PioC, we expect that the over-expressed PioC in

E. coli resides in the cytosol, and the putative TAT signal sequence of PioC is not cleaved

in the purified protein. In other words, the purified PioC using this construct is probably

different from the native PioC in *R. palustris* TIE-1 where the TAT signal sequence is

cleaved during secretion into the periplasmic space. A solution for obtaining native PioC

is to clone the gene from TIE-1 minus the predicted signal sequence. The signal

sequence cleavage sit for PioC is predicted to be between amino acid 37 and 38.

Figure B.1 Illustration of the overexpression construct pET32hPioC (not to scale). The
entire *pioC* gene was cloned into the *Noc* I and *Xho* I restriction sites of pET32h,
resulting in thioredoxin and 6-His tag to the N-terminal end of PioC, with a thrombin
protease cleavage site in between.

Conditions for the over-expression of PioC in recombinant *E. coli* cells were

tested by varying oxygen availability, temperature, and IPTG concentration (Figure B.2).

Cells grown under different conditions were incubated to an OD of ~ 0.5 before IPTG at

indicated concentrations was added. Induction was allowed to proceed for two hours

before an equal volume of cell cultures (16 μl) were withdrawn and analyzed by SDS-

PAGE (Figure B.2). Since recombinant *E. coli* exhibited different growth rates under the

conditions tested, we did not necessarily load the same amount of protein in each lane. In IPTG-induced BL21/pET32hPioC, a band of ~25 kDa was detected on SDS-PAGE, in contrast to the non-IPTG-induced controls (Figure B.2). Based on the protein sequence, the predicted mass of PioC is ~ 9.9 kDa before cleavage (precursor form) and ~6.2 kDa after signal sequence is cleaved (mature form). The thioredoxin is ~12 kDa, and therefore we expect a ~22 kDa over-expression product with our construct. The ~25 kDa product shown in Figure B.2 was confirmed to be PioC by mass spectrometry analysis with ~60% coverage of the PioC protein. These results showed that under all the conditions tested, the expression of PioC in the recombinant *E. coli* is inducible and under tight regulatory control. Qualitatively, the expression of PioC was improved under aerobic conditions than anaerobic conditions. Under aerobic conditions, greater expression was observed at 37 °C than at lower temperatures (Figure B.2). Therefore, we chose the following growth and induction conditions for all further experiments: aerobic growth at 37 °C and induction with 1 mM IPTG (varying IPTG concentration was not tested in this study). Under these conditions, the protein concentration of the final PioC eluate was estimated to be 300 μg/ml in a total volume of 3 ml.

Figure B.2 Test of induction in the over-expression of PioC (the protein bands of ~25 kDa) in the recombinant *E. coli* BL21 under varying growth and induction conditions. Upper gel: aerobic conditions in LB-Ap medium. "--": no IPTG; "+": 1 mM IPTG. Lane 1: dual color Precision plus protein standards; Lane 2 & 3: 16 °C; lane 4 & 5: 25 °C; lane 6 & 7: 30 °C; lane 8 & 9: 37 °C. Lower gel: anaerobic conditions in LB-Ap medium supplemented with 10 mM lactate. Two temperatures were tested, 16 °C and 25 °C, respectively, with IPTG concentration at 0, 25, 100 and 1000 µM, respectively. In IPTG-induced BL21/pET32hPioC, PioC (proteins bands of ~25 kDa) was detected, in contrast to the non-IPTG-induced controls. The best over-expression was obtained under aerobic conditions at 37 °C.

Figure B.3 Test of thrombin protease cleavage in the purification of PioC. Left gel: lane clear: clarified cell extract; bind: binding waste solution (see text); w1, w2 and w3: three washes with 20 mM of imidazole; ellu: elute with 200 mM of imidazole; 2 hrs, 4 hrs and 8 hrs: After thrombin protease proceeded for 2, 4 and 8 hours in the cold room at enzyme concentration of 1 unit per 100 μg of protein; bf and af: the mixture was loaded on the gel before and after passing through the GST column, respectively. These results show that the thrombin cleavage works poorly under these conditions, because the protein bands at ~25 kDa (indicated by black arrows) are expected to be cut into 2 smaller bands with size of 10 and 12 kDa, respectively.

Because of the presence of the engineered thrombin protease cleavage site in our construct, we were able to use thrombin protease to cleave off the thioredoxin and 6-His tag from PioC. Different conditions for thrombin protease cleavage were tested by varying cleavage temperature and enzyme concentration (Figure B.3 and B.4). Since the thrombin protease cleavage site is between PioC and thioredoxin, we expect a ~ 12 kDa (thioredoxin) and a ~ 10 kDa (PioC) product after thrombin protease cleavage. The protein mixture eluted from the cobalt column was mixed with of 1 unit of thrombin protease per 100 μg of PioC protein at 4° C (Figure B.3). Three milliliter volumes was extracted from the mixture after two, four, and eight hours, and individually applied to a glutathione *S*-transferase (GST) chromatography column to separate the cleaved

thioredoxin from PioC.

Figure B.4 Optimization of thrombin protease cleavage conditions for purification of PioC: incubated overnight at room temperature or at 4 °C with varying thrombin protease concentration at 0, 1, 4 and 10 units per 100 µg of protein. The disappearance of ~25 kDa bands representing PioC plus the thioredoxin tag (indicated by the upper arrow) and the appearance of ~10 kDa bands representing PioC by itself (indicated by the lower arrow) suggest that the best cleavage can be obtained by at 4°C with thrombin protease at 10 units per 100 µg of protein.

Protein mixtures before and after GST-mediated separation were examined by SDS-PAGE to evaluate the efficiency of protease cleavage (Figure B.3). Thrombin cleavage was relatively poor under the conditions tested, and therefore the GST column binding affinity was not clear. To determine optimal conditions for cleavage a series of reactions were performed varying concentrations of protease added from 0 to 10 units per 100 µg of protein in incubating at room temperature or 4 °C overnight (Figure B.4). With prolonged incubation time and increased protease concentration the efficiency of

cleavage was greatly improved. The best cleavage was observed after incubating the protein mixture with 10 units of thrombin protease enzyme per 100 μg of protein overnight at 4 °C.

Based on these preliminary results, it seems that over-expression and purification of PioC in *E. coli* is relatively straightforward. However, additional attention is needed in the following areas:

1) As described in the results section, the purified PioC with this construct is likely to yield a PioC precursor with the putative TAT signal sequence attached. The signal sequence can be checked by mass spectrometry analysis of the purified protein. Alternatively, expression of PioC in the periplasmic space of *E. coli* can also be examined. The periplasmic fraction of *E. coli* can be obtained through spheroplast formation by a method of lysozyme-EDTA treatment (7).

2) Tests need to be done to see if the over-expressed PioC contains the [4Fe-4S] cluster. Because proteins secreted through the TAT pathway do have the cofactors assembled and attached to the apoprotein in the cytosol before secretion (20), it is likely that the overexpressed PioC will have the Fe-S cluster attached, as observed with purification of Iro from *A. ferrooxidans* (8). Electron paramagnetic resonance (EPR) analysis can be used to confirm the iron sulfur (Fe-S) cofactor in the purified protein (8). If we find the purified PioC lacking the Fe-S center, *in vitro* reconstitution of the Fe-S can be achieved (28).

3) Once the purified protein with the Fe-S cluter is obtained, redox potentials of PioC can be measured by cyclic voltammetry, UV/Vis titration, and EPR analysis (21).

4) With the purified protein Fe(II) oxidation activity can be measured through direct and indirect Fe(II) oxidation activity assays. Direct oxidation activity assay can be done by mixing Fe(II) and oxidized PioC in an anaerobic cuvette and following the reduction of PioC spectrophotometrically. Additionally, we can also test if Fe(II) can be oxidized by PioA with PioC as the electron acceptor. This experiment can be performed with purified PioA, or by comparing the rate of Fe(II) oxidation or PioC reduction with total cell extract from TIE-1 and the *pioA* deletion mutant. A double mutant of *pioA* and *pioC* should be used to replace the *pioA* deletion mutant for this experiment if PioC itself shows high Fe(II) oxidation activity.

5) In addition to testing the Fe(II) oxidation activity of PioC, as described in the Introduction, the ability to oxidizing cytochrome $c2$ and/or reducing the RC should be examined. Mixtures of purified cytochrome $c2$, PioC, and whole cells or membrane vesicles containing the RC prepared from TIE-1 can be used for these experiments: PioC is first reduced and combined with oxidized cytochrome $c2$, and then both the oxidation of PioC and reduction of cytochrome $c2$ can be followed spectrophotometrically. Alternatively, reduced PioC can be mixed with whole cells or membrane vesicles containing the RC, and then flash-induced oxidation of PioC or reduction of the RC can be followed spectrophotometrically (24).

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