Proteome Response of a Metabolically Flexible Anoxygenic Phototroph to Fe(II) Oxidation

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ABSTRACT The oxidation of Fe(II) by anoxygenic photosynthetic bacteria was likely a key contributor to Earth’s biosphere prior to the evolution of oxygenic photosynthesis and is still found in a diverse range of modern environments. All known phototrophic Fe(II) oxidizers can utilize a wide range of substrates, thus making them very metabolically flexible. However, the underlying adaptations required to oxidize Fe(II), a potential stressor, are not completely understood. We used a combination of quantitative proteomics and cryogenic transmission electron microscopy (cryo-TEM) to compare cells of Rhodopseudomonas palustris TIE-1 grown photoautotrophically with Fe(II) or H₂ and photoheterotrophically with acetate. We observed unique proteome profiles for each condition, with differences primarily driven by carbon source. However, these differences were not related to carbon fixation but to growth and light harvesting processes, such as pigment synthesis. Cryo-TEM showed stunted development of photosynthetic membranes in photoautotrophic cultures. Growth on Fe(II) was characterized by a response typical of iron homeostasis, which included an increased abundance of proteins required for metal efflux (particularly copper) and decreased abundance of iron import proteins, including siderophore receptors, with no evidence of further stressors, such as oxidative damage. This study suggests that the main challenge facing anoxygenic phototrophic Fe(II) oxidizers comes from growth limitations imposed by autotrophy, and, once this challenge is overcome, iron stress can be mitigated using iron management mechanisms common to diverse bacteria (e.g., by control of iron influx and efflux).

IMPORTANCE The cycling of iron between redox states leads to the precipitation and dissolution of minerals, which can in turn impact other major biogeochemical cycles, such as those of carbon, nitrogen, phosphorus and sulfur. Anoxygenic phototrophs are one of the few drivers of Fe(II) oxidation in anoxic environments and are thought to contribute significantly to iron cycling in both modern and ancient environments. These organisms thrive at high Fe(II) concentrations, yet the adaptations required to tolerate the stresses associated with this are unclear. Despite the general consensus that high Fe(II) concentrations pose numerous stresses on these organisms, our study of the large-scale proteome response of a model anoxygenic phototroph to Fe(II) oxidation demonstrates that common iron homeostasis strategies are adequate to manage this. The bulk of the proteome response is not driven by adaptations to Fe(II) stress but to adaptations required to utilize an inorganic carbon source. Such a global overview of the adaptation of these organisms to Fe(II) oxidation provides valuable insights into the physiology of these biogeochemically important organisms and suggests that Fe(II) oxidation may not pose as many challenges to anoxygenic phototrophs as previously thought.
Anoxygenic phototrophic Fe(II) oxidizers fix CO₂ by oxidation of Fe(II) (equation 1 [+hv indicates that the reaction is light dependent]).

\[
\text{HCO}_3^- + 4\text{Fe}^{2+} + 10\text{H}_2\text{O} + \text{hv} \rightarrow (\text{CH}_2\text{O}) + 4\text{Fe(OH)}_3 + 7\text{H}^+
\]

This process is thought to have fueled the Earth’s biosphere prior to the evolution of oxygenic photosynthesis and is likely to have been a key contributor in the deposition of vast formations of layered Fe(II)-Fe(III) and Si-rich rock during Earth’s early history (1–3). Organisms capable of this metabolism are still found in many modern environments, including Fe(II)-rich (4–7) and Fe(II)-poor (8) stratified lakes, as well as both freshwater (9, 10) and marine sediments (11–14). These microorganisms belong to the purple sulfur bacteria (Gammaproteobacteria), purple nonsulfur bacteria (Alphaproteobacteria), and green sulfur bacteria (Chlorobi).

Anoxygenic phototrophs show remarkable versatility with regard to their metabolism. They can fix CO₂ using Fe(II), H₂, or H₂S as an electron donor (photoautotrophy), or they can use organic compounds as a carbon source (photoheterotrophy) (9, 10, 12, 14, 15). Some isolates can also grow chemoheterotrophically with dioxygen without any light at all, e.g., *Rhodopseudomonas palustris* TIE-1 (Jiao et al. [9]). Not only can these organisms switch metabolisms, but they can also use multiple metabolisms simultaneously. Previous studies with purple nonsulfur bacteria have shown that Fe(II) and H₂ can be utilized simultaneously, although this led to a lower rate of Fe(II) oxidation than that with Fe(II) alone (16). The simultaneous use of organic carbon and CO₂ fixation with Fe(II) as an electron donor has also been observed and depends on the identity of the carbon source. For example, *Rhodopseudomonas palustris* strain TIE-1 utilizes Fe(II) and lactate simultaneously but preferentially utilizes acetate before commencing Fe(II) oxidation (17). The cellular processes which facilitate this metabolic flexibility are not well constrained. For example, the proteins responsible for Fe(II) oxidation itself have only been determined in two anoxygenic phototrophic Fe(II) oxidizers (*Rhodopseudomonas palustris* TIE-1 and *Rhodobacter ferrooxidans* SW2) (18–21). However, the proteins identified are different even between these two purple nonsulfur bacteria and cannot be used as general markers for active Fe(II) oxidation.

Metabolic flexibility is clearly beneficial in soils, sediments, and aquatic habitats where anoxygenic phototrophs are found, as redox conditions and substrate availability can continuously fluctuate due to changes in light conditions (thus oxygen production), precipitation, physical disturbance, or competition with other organisms or abiotic reactions. Physical mixing of sediments, e.g., by wave action or bioturbation, can also act to redistribute organisms within the sediment column such that they are outside their favored redox zone. This has been shown to occur with Fe(II)-oxidizing bacteria in marine sediments where the bacteria are found to be equally abundant at all sediment depths regardless of redox or substrate conditions (11). Metabolic flexibility is a powerful strategy for coping with such varied environmental conditions, as these organisms increase their chances of being able to proliferate in any environment to which they are exposed.

Of all of the potential metabolisms these bacteria could use, Fe(II) oxidation would appear to pose the most challenges. First, they must be able to avoid encrustation of their outer membranes by Fe(III) minerals which form during Fe(II) oxidation (22, 23). Furthermore, they may also have to compete with Fe(III) minerals for trace nutrients which readily sorb to mineral surfaces (24). High iron concentrations may also induce stress on the cells. A growing body of literature suggests that iron can be harmful even under an anoxic atmosphere (25–28), despite the lack of oxygen radicals produced via the Fenton reaction which drives iron toxicity in oxic environments (29). So far, stress resulting from high Fe(II) concentrations under anoxic conditions has been suggested to be related to the formation of organic radicals, such as glutathione radicals,
which subsequently transfer electrons to cellular biopolymers (28) and to oxygen-independent photochemical reactions with Fe minerals, which can abiotically produce reactive oxygen species (30, 31). The process of Fe(II) oxidation itself has even been proposed as a defense mechanism against the stressful effects of high concentrations of Fe(II) (25). For example, the highest Fe(II) oxidation rates are observed when *Rhodopseudomonas palustris* TIE-1 cells are precultured on Fe(II) (18), suggesting that, whatever the cause, an adaptive response to Fe(II) stress is required.

If we are to better understand how these important organisms adapt to all of the challenges associated with metabolic flexibility and high Fe(II) concentrations in particular, it is essential to take a holistic view of Fe(II) oxidation. In order to construct an overall impression of the adaptations anoxygenic phototrophs require to conduct Fe(II) oxidation, we have adopted a shotgun quantitative proteomics approach with the goal to analyze, on a large scale, the protein complement of a model anoxygenic phototroph, *Rhodopseudomonas palustris* TIE-1, grown with either Fe(II) (10 mM), H$_2$ (80:20 H$_2$/CO$_2$), or acetate (10 mM). Large-scale analysis of the proteins comprising these cells provides a snapshot of the processes which are active under that condition, as opposed to transcriptome analysis, which does not account for any posttranscriptional regulation. By combining this technique with visual comparisons of the cells using cryogenic transmission electron microscopy (cryo-TEM), we can gain a detailed overview of how the cells adapt to different substrate conditions. *Rhodopseudomonas palustris* TIE-1, a purple nonsulfur bacterium, is arguably the best-studied Fe(II)-oxidizing isolate available (9, 18, 26, 32, 33). The entire genome has been sequenced and is essential for extracting meaningful functional insights from proteomics data, and the mechanism for iron oxidation is well constrained, making TIE-1 an excellent model for our study.

**RESULTS**

Global overview of proteomes in *Rhodopseudomonas palustris* TIE-1 cultures grown with different substrates. In order to gain a holistic overview of the cellular processes which facilitate Fe(II) oxidation in *Rhodopseudomonas palustris* TIE-1, we compared the proteomes of cells from parallel cultures grown with Fe(II), H$_2$, and acetate. The time taken before the cultures reached the mid-exponential phase, and thus the point of cell harvest, was substrate dependent. Photoheterotrophic cultures (grown on acetate) grew quickly and reached the mid-exponential phase after 18 h. Photoautotrophic cultures [grown on Fe(II) or H$_2$] grew more slowly, with the iron-grown cultures reaching the mid-exponential phase after 140 h and with H$_2$-grown cultures after 257 h.

Following protein extraction, mass spectrometry analysis, and subsequent downstream processing, 2,049 protein groups were identified across all substrates, with a false discovery rate of 0.01. Overall, there was very good reproducibility between triplicates (as seen in Fig. 1A, with average correlation $>$95%). High similarity was observed between both photoautotrophic cultures, i.e., those grown with Fe(II) or H$_2$ (average correlation, 83%; clustering shown in Fig. 1B). Cells grown with Fe(II) had an average correlation of 63% compared to cells grown with acetate, and those with H$_2$ had an average correlation of 73%. In a comparison of proteins which show significantly different expression when grown on Fe(II) (significance criteria outlined in Materials and Methods), we identified 445 proteins which showed higher expression than in acetate-grown cells and 189 proteins which showed higher expression than in hydrogen-grown cells. Fe(II)-grown cells had 241 proteins which were significantly lower in abundance than in acetate-grown cells and 164 proteins lower in abundance than in hydrogen-grown cells. Hydrogen-grown cells had 361 proteins with higher expression and 212 proteins with lower expression than in those grown with acetate. Many proteins were significantly more abundant in both Fe(II)- and H$_2$-grown cells than in those grown with acetate; similarly, many proteins were also lower in abundance in both Fe(II)- and H$_2$-grown cells than in those grown with acetate (Fig. 2). The similarities between both autotrophic cultures is reflected in the large number of proteins which were only
quantified under H$_2$- and Fe(II)-grown conditions (Fig. 2) and not under the acetate-grown conditions (Fig. 2).

**Commonalities and differences between autotrophic and heterotrophic metabolisms.** The main factor driving the differences between conditions was the carbon source, i.e., organic carbon as acetate and inorganic carbon as CO$_2$ (Fig. 2). Therefore, if we want to better understand the adaptations required for Fe(II) oxidation, we need to first understand the adaptation to an organic or inorganic carbon source. To determine which proteins are important specifically for autotrophic metabolism, we identified proteins which were significantly higher in abundance under both the Fe(II)

![Image of PCA and Venn diagrams](image-url)
and H₂ conditions than under the acetate conditions (258 proteins; see Data Set S1 in the supplemental material). These include proteins for a diverse range of functions, including energy and electron transport processes, amino acid synthesis, and motility. PioA, thought to be the iron oxidase in *Rhodopseudomonas palustris* TIE-1 (18), was significantly higher in both Fe(II)-grown and hydrogen-grown than in acetate-grown cells (adjusted *P* = 2.1 × 10⁻⁶ and 2.7 × 10⁻⁶, respectively), as was PioB, a membrane porin thought to anchor PioA in the outer membrane [adjusted *P* = 0.003 in Fe(II) versus acetate and 0.005 in H₂ versus acetate]. PioC, a high-potential iron-sulfur protein associated with the oxidation of Fe(II) (33), was not significantly different in abundance between any condition [i.e., Fe(II) versus acetate, Fe(II) versus H₂, or H₂ versus acetate].

There were 98 proteins significantly lower in abundance under both autotrophic conditions [i.e., lower in both Fe(II) and H₂ than in acetate; Data Set S2]. These were primarily general housekeeping proteins, including those for protein synthesis. Gene set enrichment analysis showed significant enrichment of ribosomal proteins in acetate-grown cells relative to those grown with H₂ or Fe(II) (data not shown). We also observed a significantly lower abundance of the following 4 proteins related to pigment synthesis: BchB and BchN for light-independent chlorophyll synthesis, 1 protein related to bacteriochlorophyll synthesis (Rpal_1711), and a geranylgeranyl reductase involved in chlorophyll biosynthesis (Rpal_1721). A putative photosynthetic complex assembly protein (Rpal_1737) was also lower in abundance under both the Fe(II) and hydrogen conditions than under acetate conditions.

In *Rhodopseudomonas palustris* TIE-1, much of the light-harvesting machinery is located on long folded intracytoplasmic membranes. Therefore, we used cryo-TEM to assess if the differences in the expression of proteins for pigments in the photoautotrophic compared to the phototrophic conditions was manifested in differences in these membranes. Cells grown with acetate have long thick intracellular membranes which occupy, on average, 113 μm² (standard deviation [SD], 38 μm², *n* = 16; including the intramembrane space), which are observed on both sides of the cell in 50% of the acetate-grown cells imaged (Fig. 3). In contrast, cells grown with H₂ or Fe(II) show that the intracytoplasmic membranes are limited to smaller regions within the cell [for H₂, 22 μm²; SD, 18 μm², *n* = 33; for Fe(II), 19 μm²; SD, 16 μm², *n* = 19]. Only 11% of the Fe(II)-grown cells and 9% of the H₂-grown cells imaged had membranes on both sides. No acetate cell was imaged without visible membranes, whereas 26% of the Fe(II) cells imaged had no visible membranes, as did 21% of hydrogen-grown cells (Fig. 3). No differences in the lengths of the cells were observed under any condition, and there is no clear difference observable by cryo-TEM between the H₂ and Fe(II) growth conditions. Intracellular round electron-dense polyphosphates were observed under all growth conditions but not necessarily in all cells observed (most clearly seen in Fig. 3A).
TABLE 1 Selection of proteins of interest with significantly higher abundance in Fe(II) than in both H₂ and acetate, indicating that they are important for adaptation to Fe(II) oxidation

<table>
<thead>
<tr>
<th>Protein by functional group</th>
<th>Description</th>
<th>Gene</th>
<th>Locus tag</th>
<th>log FC</th>
<th>adj. P value</th>
<th>Fe(II) vs acetate</th>
<th>Fe(II) vs H₂</th>
<th>adj. P value</th>
<th>adj. P value</th>
<th>Unique Fe(II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Efflux</td>
<td>Efflux pump membrane transporter</td>
<td>Rpal_0100</td>
<td>B3QG33</td>
<td>1.61</td>
<td>1.1E–02</td>
<td>1.53</td>
<td>1.3E–02</td>
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<td></td>
<td>Porin</td>
<td>Rpal_0539</td>
<td>B3QBE5</td>
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<td>1.7E–03</td>
<td>0.39</td>
<td>1.3E–03</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Efflux transporter, RND family, MFP subunit</td>
<td>Rpal_1604</td>
<td>B3QG56</td>
<td>1.23</td>
<td>5.8E–03</td>
<td>1.25</td>
<td>5.4E–03</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Efflux transporter, RND family, MFP subunit</td>
<td>Rpal_4297</td>
<td>B3QH9F</td>
<td>0.50</td>
<td>2.3E–03</td>
<td>0.32</td>
<td>2.0E–02</td>
<td>0.04</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Heavy-metal efflux pump</td>
<td>czcA</td>
<td>B3QG90</td>
<td>2.17</td>
<td>3.6E–05</td>
<td>1.71</td>
<td>1.4E–04</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Membrane processes

| B3QG5 | 3-hydroxyacyl-CoA dehydrogenase NAD binding domain protein | Rpal_0887 | B3QG5 | 1.81 | 1.5E–03 | 1.06 | 2.1E–02 | + |
| B3QCT3 | Phosphatidylethanolamine N-methyltransferase | Rpal_2217 | B3QCT3 | 1.30 | 5.3E–03 | 1.37 | 4.0E–03 | + |
| B3QL0 | Lipoprotein-releasing system ATP-binding protein | Rpal_3274 | B3QL0 | 0.12 | 1.4E–03 | 0.13 | 9.1E–04 | + |
| B3QD34 | Polysaccharide export protein | Rpal_3782 | B3QD34 | 0.94 | 1.7E–04 | 0.68 | 9.6E–04 | + |
| B3QF30 | UDP-N-acetylmuramyl-L-alanine ligase | Rpal_4047 | B3QF30 | 0.93 | 8.7E–03 | 1.32 | 1.5E–03 | + |
| B3QJ20 | Uncharacterized protein | Rpal_4480 | B3QJ20 | 1.17 | 7.9E–05 | 1.24 | 5.6E–05 | + |
| B3QH66 | Polysaccharide export protein | Rpal_5320 | B3QH66 | 0.33 | 5.1E–03 | 0.26 | 1.5E–02 | + |

Motility

| B3QJ3 | Putative methyl-accepting chemotaxis sensory transducer | Rpal_0260 | B3QJ3 | 0.24 | 9.0E–04 | 0.27 | 4.8E–04 | + |
| B3Q47 | Methyl-accepting chemotaxis sensory transducer | Rpal_0924 | B3Q47 | 1.56 | 1.0E–02 | 1.81 | 5.2E–03 | + |
| B3Q73 | Uncharacterized protein | Rpal_4453 | B3Q73 | 1.07 | 2.6E–02 | 1.00 | 3.4E–02 | + |
| B3Q57 | Flagellar basal body protein | Rpal_4455 | B3Q57 | 0.37 | 2.2E–03 | 0.25 | 1.6E–02 | + |
| B3QK6 | Methyl-accepting chemotaxis sensory transducer | Rpal_4682 | B3QK6 | 1.08 | 2.6E–02 | 1.25 | 1.4E–02 | + |

Energy metabolism

| B3Q17 | Cytochrome c oxidase, cbb₃ type, subunit I | Rpal_0019 | B3Q17 | 0.26 | 3.2E–02 | 0.30 | 1.8E–02 | + |
| B3Q48 | ATP synthase subunit delta | Rpal_0174 | B3Q48 | 0.35 | 3.4E–05 | 0.34 | 3.6E–05 | + |
| B3Q34 | ATP synthase subunit-b1 | Rpal_0911 | B3Q34 | 0.30 | 2.4E–05 | 0.30 | 2.2E–05 | + |
| B3Q1 | Cytochrome b | Rpal_1385 | B3Q1 | 0.27 | 2.2E–02 | 0.42 | 2.6E–03 | + |
| B3Q71 | Photosynthetic reaction center L subunit | Rpal_1716 | B3Q71 | 0.89 | 2.0E–03 | 0.82 | 3.1E–03 | + |
| B3Q871 | Reaction center protein M chain | Rpal_1724 | B3Q871 | 0.18 | 3.4E–02 | 0.19 | 2.7E–02 | + |
| B3Q68 | Cytochrome c class I (cytochrome c₅₅₃) | Rpal_1724 | B3Q68 | 0.18 | 3.4E–02 | 0.19 | 2.7E–02 | + |
| B3Q67 | Cytochrome B₅₆₁ | Rpal_2736 | B3Q67 | 0.31 | 1.0E–02 | 0.44 | 1.6E–03 | + |
| B3Q12 | K⁺-insensitive pyrophosphate-energized proton pump | Rpal_3012 | B3Q12 | 0.93 | 2.4E–02 | 1.04 | 1.5E–02 | + |

*For each comparison, log FC is the log 10-fold change between Fe(II) and the condition of interest; adj. P value is the adjusted P value for the Tukey test. Column “Unique Fe(II)” indicates which proteins, in addition to being significantly higher under the Fe(II) condition relative to both H₂ and acetate conditions, were detected only under the conditions with Fe(II). Proteins are grouped broadly into functional groups.

Given that one of the primary differences between the acetate and H₂/Fe(II) conditions (i.e., photoheterotrophy versus photoautotrophy) was the carbon source, we mapped protein expression onto the KEGG pathway for “carbon fixation in photosynthetic organisms” (Fig. S1). This showed no clear increase in abundance of proteins of this pathway despite the fact that many were detected, even under heterotrophic conditions. Similarly, overrepresentation analysis for KEGG orthology (KO) did not show any significant overrepresentation of carbon fixation in photoautotrophic cultures, i.e., when Fe(II) or H₂ was compared to acetate.

Proteins differing in abundance with Fe(II). We confidently identified 92 proteins which were significantly higher in abundance under Fe(II) conditions than under both H₂ and acetate-grown conditions, and 52 proteins which were significantly lower. Lists of selected proteins of interest are found in Tables 1 and 2, and a complete list can be found in Data Sets S3 and S4. Among the proteins with higher abundance are those related to motility, including those for pilus and flagellar formation and for chemotaxis. Metal efflux proteins were also highly abundant under this growth condition with Fe(II)
as an electron donor relative to both other conditions, including two resistance-nodulation-division (RND) family efflux transporter subunits (Rpal_1604 and Rpal_4297), a CzcA family heavy-metal efflux pump (Rpal_4972), and an efflux pump membrane transporter (Rpal_0100). These are accompanied by proteins associated with processes in the outer membrane (Table 1), such as membrane biogenesis (Rpal_4047) and export of polysaccharides (Rpal_3782 and Rpal_5320). Also increased in abundance are proteins involved in the respiratory electron transport chain, including a number of cytochromes, ATP synthases, and reaction center proteins (Table 1).

Of the 52 proteins which are significantly lower in Fe(II) than in both hydrogen and acetate, many are related to iron uptake. Decreased abundance of Fur, the global negative regulator of iron uptake, is observed in the Fe(II)-grown cells, as well as decreased abundance of a TonB-dependent siderophore receptor and TonB system transport protein ExbD. FeoA, an iron uptake protein, and hemin import ATP-binding protein HmuV, as well as 10 proteins annotated as ribosome constituents, are also lower with Fe(II) than under both other conditions. Some key proteins showing lower abundance in Fe(II)-grown cells than under both other conditions are displayed in Table 2.

**DISCUSSION**

**Physiology of TIE-1 under photoautotrophic and photoheterotrophic conditions.** Our results show that distinctive proteome profiles are obtained under different substrate conditions and that the main factor driving the difference between the conditions is the carbon source. However, we see few significant differences in the abundance of carbon fixation proteins between heterotrophically and autotrophically grown cells. During photoheterotrophic growth on acetate, which is slightly less reduced than average biomass molecules, the partial oxidation of some acetate to generate electrons for biosynthesis produces more reduced redox cofactors than can be used for these biosynthetic processes, which leads to a redox imbalance inside the cell. It is known that in *Rhodopseudomonas palustris*, CO₂ fixation pathways are used to

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**TABLE 2 Selection of proteins of interest with significantly lower abundance in Fe(II) than in both H₂ and acetate, indicating that they are important for adaptation to Fe(II) oxidation**

<table>
<thead>
<tr>
<th>Protein by functional group</th>
<th>Descriptionᵃ</th>
<th>Gene</th>
<th>Locus tag</th>
<th>log FC</th>
<th>adj. P value</th>
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<td>Iron</td>
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<tr>
<td>B3QAC8</td>
<td>Ferric uptake regulation protein</td>
<td>fur</td>
<td>Rpal_0454</td>
<td>-0.37</td>
<td>5.7E-03</td>
<td>-0.48</td>
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<td>B3QB1</td>
<td>Hemerythrin HHE cation binding domain protein</td>
<td>Rpal_1855</td>
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<td>1.3E-03</td>
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<td>B3QEK7</td>
<td>Hemin import ATP-binding protein HmuV</td>
<td>hmuV</td>
<td>Rpal_2409</td>
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<td>B3QKE1</td>
<td>TonB-dependent siderophore receptor</td>
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<td>B3QEV8</td>
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<td>Rpal_3966</td>
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<td>-1.00</td>
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<tr>
<td>B3QB63</td>
<td>FeoA family protein</td>
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<td>Photosynthesis</td>
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<td>B3Q7A8</td>
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<td>Rpal_1714</td>
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<td>B3Q7D2</td>
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<td>Ribosome</td>
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<td>B3Q725</td>
<td>50S ribosomal protein L21</td>
<td>rplU</td>
<td>Rpal_0151</td>
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ᵃFor each comparison, log FC is the log 10-fold change between Fe(II) and the condition of interest; adj. P value is the adjusted P value for the Tukey test. Proteins are grouped broadly into functional groups.
reoxidize these cofactors and play an essential role in recycling excess electrons (34, 35).

Our observation that carbon fixation pathways are expressed when acetate is provided as a carbon source are consistent with the use of carbon fixation to balance intracellular redox conditions during phototrophic growth.

Differences in proteome profiles appear instead to be more generally related to the effect of organic carbon substrates on growth. Microbial growth under phototrophic conditions is much slower than that with acetate [with H₂-grown cultures reaching mid-exponential phase after 10 days and Fe(II)-grown cultures after 6 days, compared to less than 1 day with acetate]. Acetate-grown cells also appear to be able to divert more resources toward light harvesting. This is evidenced by the extensive intracytoplasmic photosynthetic membranes observed in acetate-grown cells using cryo-TEM and in the higher abundance of proteins for pigment synthesis. Phototrophic cells, however, show short spatially limited intracytoplasmic membranes (Fig. 3). It is known that anoxygenic phototrophs expand the area dedicated to intracellular cytoplasmic membranes in response to low light levels and oxygen tension (36, 37). Our results suggest that carbon source also has an important role to play in the formation of these light-harvesting membranes.

**Specific adaptations of *Rhodopseudomonas palustris* TIE-1 to growth with Fe²⁺.**

Expression profiles of the Fe(II)-grown Fe(II)-oxidizing cultures are characterized by control over the influx and efflux of metal ions into the cell. Numerous metal efflux proteins are highly abundant during growth on Fe(II). One of these is a copper efflux protein, an effect also observed by Bird et al. (26) when documenting the transcriptional response of acetate-grown *Rhodopseudomonas palustris* TIE-1 cells to sudden Fe(II) shock. In that study, Bird et al. demonstrated that Fe(II) acts synergistically with copper (commonly found as a contaminant in Fe salts) to arrest growth in cells which were growing phototrophically on acetate. Our results further show that such efflux proteins are not only required for a short-term response but continue to be highly expressed even after several transfers on Fe(II) and when the cells are using Fe(II) for growth. Our results also show that high Fe(II) concentrations cause the cells to reduce the number of transporters for siderophores or Fe(II). Together, the increase in efflux proteins alongside the decrease in proteins for iron influx clearly show the two aspects involved in managing intracellular metal concentrations, where cells must select a suitable and tolerable balance of influx and efflux. This is consistent with the observed decrease in abundance of the ferric uptake regulator (Fur), a master regulator of the iron response in a huge diversity of bacteria.

On first glance, this appears to be exactly what would be expected from the action of well-characterized iron response regulators. However, this response becomes more intriguing when we appreciate that almost everything known about iron homeostasis in bacteria comes from cells grown under oxic conditions, where iron toxicity is observed at much lower concentrations (38). Only a few studies have investigated iron stress under anoxic conditions, where bacteria can tolerate much higher iron concentrations in the millimolar range (25). It has been proposed that under such conditions, stress results from the formation of glutathione radicals (28) and reactive oxygen species by oxygen-independent photochemical reactions with Fe minerals (30, 31). However, we see no evidence of an oxidative stress response in Fe(II)-grown cells that might be consistent with this. Furthermore, minerals formed during Fe(II) oxidation are often observed to precipitate in the periplasm or on the cell surface in other metabolic types of Fe(II) oxidizers, but this is not observed in phototrophic Fe(II)-oxidizing bacteria. The reasons for the lack of encrustation are not fully resolved, but we may reasonably expect that the potential for encrustation would lead to an adaptive response involving changes in cell membrane structure; however, there is no strong evidence for this in our proteome data. Additionally, the formation of iron minerals which readily sorb nutrients, such as phosphorus, can remove essential nutrients from the growth media; however, we see no evidence for phosphorus limitation in the proteome of Fe(II)-grown cells. Indeed, despite the fact that Fe(II) oxidation is regarded as a novel metabolism which will induce numerous stresses, Fe(II)-grown cells do not
differ drastically from cells grown autotrophically with hydrogen. We would suggest that the only adaptations required by anoxicogenic phototrophic Fe(II) oxidizers to thrive at these very high Fe(II) concentrations is a mechanism of Fe(II) oxidation and a typical suite of proteins for regulation of intracellular metal concentrations. The main challenge associated with utilizing Fe(II) as an electron donor, in our opinion, comes from the need to obtain all the carbon needed to build biomass solely from CO₂.

In addition to invoking a proteome response geared toward iron homeostasis, Fe(II)-grown cells show increased abundance of a number of key proteins in the photosynthetic electron transport chain. This is somewhat contradictory to our observation of short photosynthetic membranes and low abundance of pigment synthesis proteins under photoautotrophic conditions. One such protein is cytochrome C₉, which is a soluble high-potential iron-sulfur protein essential for photosynthesis. Its main function is in cyclic electron transfer for photosynthetic energy generation by shuttling electrons from the cytochrome bc₁ complex to the reaction center (33). Three proteins involved in proton transport across the membrane are also increased, with two subunits of the ATP synthase, and HppA, a pyrophosphate-energized proton pump. In addition to these are additional cytochromes (a cbb₃-type cytochrome c oxidase, cytochrome b, and cytochrome B561) and two photosynthetic reaction center subunits. Proteins involved in electron transport typically contain iron in their redox centers and are subsequently often shown to be downregulated as part of an iron-sparing response during iron limitation (39). A higher abundance of such proteins may suggest that the opposite strategy is employed under iron excess, such that iron is built into biomass as an attempt to reduce free iron within the cell.

Proton pumps, such as the F-type ATPases, and inorganic H⁺-pyrophosphatases, such as HppA, which we observe to be upregulated, are also known to have a role in pH homeostasis by maintaining the optimum pH gradient between the intracellular and extracellular environments. Changes in pH occur during Fe(II) oxidation due to the production of protons (equation 1). This can lead to a low-pH microenvironment around anoxicogenic phototrophic cells during Fe(II) oxidation and may be involved in the avoidance of Fe(III) mineral encrustation (22). While it is still to be determined whether such a pH management is passive or active, these provide promising targets for the investigation of pH homeostasis during Fe(II) oxidation.

Implications for detection of anoxicogenic phototrophic Fe(II) oxidation in complex communities. One challenge which currently hinders the study of Fe(II) oxidation by anoxicogenic phototrophs in the environment is the lack of general markers for this process. Some of the proteins thought to be associated with Fe(II) oxidation in this organism are detected under both photoautotrophic conditions. Jiao and Newman (18) established that proteins encoded by the pio operon are essential for this process. These consist of PioA (a periplasmic decaheme cytochrome), PioB (an outer membrane porin), and PioC (a periplasmic high-potential iron-sulfur cluster protein). PioAB is higher in abundance during growth on both Fe(II) and H₂ than on acetate in our study, whereas PioC shows no significant differential abundance between conditions. This is consistent with the results of Bose and Newman (32), who observed that pioABC is expressed under all photoautotrophic conditions they tested, yet its deletion only appears to be problematic in the case of growth on Fe(II). This result highlights the difficulties surrounding the detection of Fe(II) oxidation in the environment. The expression of the pio operon may give us a clue that photoautotrophy is the dominant metabolism in an environmental sample including *Rhodopseudomonas palustris* TIE-1, but it is not an indicator of Fe(II) oxidation. An alternative method to recognize photoautotrophy would, however, be useful, as our results show that the expression of carbon fixation pathways is not an indicator of photoautotrophy, as this is not over-represented under any condition. The use of proteomics to create metabolic profiles for each substrate may provide an alternative route for determining the dominant metabolism in a complex environment. Based on this study, we could suggest that an Fe(II) oxidation-dominated system would be characterized by high pio gene expression with small intracellular membranes, coupled with high expression of efflux proteins and low
expression of siderophores. However, it must still be determined how these profiles are influenced by substrate concentrations, or by the presence of multiple substrates simultaneously, before proteomics could be confidently used to detect Fe(II) oxidation in the environment.

**Conclusions.** Our results show that each substrate utilized by the metabolically versatile *Rhodopseudomonas palustris* TIE-1 [Fe(II), H₂, or acetate] gives rise to a unique set of proteins. The main difference between conditions is driven by differences in carbon source, not because of altered abundance of proteins related to carbon metabolism, but because of lower growth rates with an inorganic carbon source. This may be related to difficulties in producing photosynthetic machinery from inorganic carbon sources. The response of the cells grown with very high concentrations of Fe(II) is consistent with well-characterized mechanisms for the management of iron homeostasis and shows no evidence for associated oxidative stress responses or unusual mechanisms of defense. This leads us to conclude that Fe(II) oxidation need not be considered a very specialized metabolism, so long as a mechanism of Fe(II) oxidation and some ability to control intracellular iron levels are present.

Many open questions remain to be answered in order to fully understand the adaptations anoxicogenic phototrophic Fe(II) oxidizers implement to utilize these different substrates and to predict and detect which metabolism will be dominant in a given environment. Detailed understanding of the adaptation of anoxicogenic phototrophic Fe(II) oxidizers to Fe(II) oxidation will require elucidation of the mechanisms by which iron is sensed and regulated in these cells. We must also know whether the proteome profile which arises during adaptation to Fe(II) is similar among all phototrophs or whether each species has a unique adaptation method. Second, to determine the dominant metabolism at play in a given environment, we must gain a better understanding of substrate preference and proteome adaptation when multiple substrates are available simultaneously, particularly at low concentrations more relevant to environmental systems. In tackling these problems, we may eventually be able to reach the ultimate goal of directly determining metabolic type and thus its role in biogeochemical cycling in complex natural environments.

**MATERIALS AND METHODS**

**Bacterial growth conditions.** Basal medium was prepared according to Jiao et al. (9), which included the addition of 1 ml per liter trace element solution, 1 ml per liter vitamin B₁₂ solution, and 10 ml/liter of an altered vitamin solution (10), which contained 50 mg per liter riboflavin (17).

All experiments were conducted in 50-ml serum bottles with 25 ml medium and a headspace of either 90:10 N₂-CO₂ [for growth on acetate or Fe(II) experiments] or 80:20 H₂-CO₂ [for growth on H₂]. For iron experiments, 10 mM FeCl₂ was added, and for acetate experiments, 10 mM sodium acetate (CH₃COONa) was added. In H₂ experiments, the headspace was exchanged every 2 to 3 days. Experiments were inoculated with 10% of an early stationary-phase preculture and placed approximately 30 cm from a 40 W halogen light bulb at 21°C. *Rhodopseudomonas palustris* TIE-1 cells are routinely maintained in our culture collection on Fe(II). Prior to the experiment, this stock culture was transferred on the chosen substrate a minimum of 6 times before the proteomics experiment. For the proteomics analysis, triplicate experiments for each substrate were conducted. Optical density, as a proxy for cell density, in H₂ and acetate-grown cultures was monitored by the transfer of 100 μl of culture to 96-well plates prior to the measurement of absorbance at 660 nm (Speklo 1300; Analytik Jena). Fe(II) oxidation was monitored using the ferrozine assay following fixation in 1 M HCl (1:10 sample/HCl). Cells were harvested in the mid-exponential phase when H₂ cultures reached an optical density at 660 nm (OD₆₆₀) of 0.38 to 0.43. acetate cultures reached an OD₆₆₀ of 0.25 to 0.3, and Fe(II) cultures had a remaining concentration of 2.9 to 4.6 mM Fe(II) (where 10 mM was originally added). At the time of harvest, 20 ml of culture from each serum bottle was centrifuged at 7,000 rpm (5,752 × g) and 4°C for 30 min. Cell pellets were immediately frozen at −20°C until protein extraction.

**Protein extraction and quantification.** Frozen cell pellets were dissolved in an SDS protein extraction buffer containing 4% (wt/vol) SDS, 10 mM Tris-HCl (pH 8.0), 5 mM glycerol-2-phosphate, 5 mM sodium fluoride, 5 mM sodium orthovanadate, and 10 mM EDTA (pH 8.0). This solution was incubated at 95°C for 10 min and then placed in a sonicating bath, on a moderate setting, for 2 min. For the reduction of cysteine disulfide bonds, dithiothreitol was added to a final concentration of 10 mM. Alkylation of the reduced cysteine bonds was achieved by the addition of 5.5 mM iodoacetamide and shaking (650 rpm) for 45 min at room temperature in the dark. Nonsoluble cell debris was pelleted by centrifugation at 7,000 rpm (5,752 × g) and 4°C for 10 min.

Proteins were precipitated by mixing the supernatant with eight sample volumes of ice-cold acetone and one sample volume of ice-cold methanol. The mixture was vortexed and incubated at −20°C until precipitation.
The precipitated proteins were centrifuged at 1,000 rpm (117 × g) for 5 min, and the pellet washed 3 times with ice-cold acetone. After the final wash step, the pellet was air-dried at room temperature.

**Protein quantification and data analysis.** The protein pellet was rehydrated in urea buffer containing 6 M urea and 2 M thiourea in 100 mM Tris-HCl (pH 7.5). Proteins (10 μg per condition) were purified on an SDS-PAGE gel, and Coomassie-stained gel pieces were digested in gel with trypsin, as described previously (40). After desalting using C18 Stage Tips (41), extracted peptides were separated on an EasyLC nano-high-performance liquid chromatograph (HPLC; Thermo Scientific) coupled to an LTQ Orbitrap XL (Thermo Scientific), as described elsewhere (42), with slight modifications: the peptide mixtures were injected onto the column in HPLC solvent A (0.1% formic acid) at a flow rate of 500 nl/min and subsequently eluted with a 227-min segmented gradient of 5:33:50:90% of HPLC solvent B (80% acetonitrile in 0.1% formic acid) at a flow rate of 200 nl/min. Precursor ions were acquired in the mass range from m/z 300 to 2,000 in the Orbitrap mass analyzer at a resolution of 60,000. An accumulation target value of 10^6 charges was set, and the lock mass option was used for internal calibration (43). The 10 most intense ions were sequentially isolated and fragmented in the linear ion trap using collision-induced dissociation (CID) at the ion accumulation target value of 5,000 and default CID settings. Sequenced precursor masses were excluded from further selection for 90 s.

The acquired MS spectra were processed with the MaxQuant software package version 1.5.2.8 (44) with the integrated Andromeda search engine (45). The database search was performed against a target-decoy *Rhodopseudomonas palustris* TIE-1 database obtained from UniProt, containing 5,247 protein entries, and 285 commonly observed contaminants. Endoprotease trypsin was defined as protease with a maximum of two missed cleavages. Oxidation of methionine and N-terminal acetylation were specified as variable modifications, whereas carbamidomethylation on cysteine was set as a fixed modification. Initial maximum allowed mass tolerance was set to 4.5 ppm (for the survey scan) and 0.5 Da for CID fragment ions. Peptide, protein, and modification site identifications were reported at a correlation that assumes that the statistical distribution of each sample is the same) using the MSnbase package (49). Similarity between replicates and between different conditions was calculated by taking the average of the Spearman correlation calculated for each possible comparison (between triplicates, n = 3; between conditions, n = 9). In order to statistically compare protein abundances across conditions (even when the protein was not detected under one of the conditions), imputation was used on the data using a mixed model of nearest-neighbor averaging and left-censored missing data from a truncated distribution (49). Comparisons in which both conditions contained imputed values were removed. Proteins which were significantly different between conditions were identified using analysis of variance (ANOVA), followed by the Tukey post hoc test. Significance was set at an adjusted *P* value of 0.05 following Benjamini-Hochberg multiple-correction testing. Functional information, namely, gene ontology (GO) and KEGG orthology (KO), for *Rhodopseudomonas palustris* TIE-1 proteins was retrieved using the UniProt.ws package (50). Overrepresentation testing for GO and KO was done for each comparison via the clusterProfiler package based on hypergeometric distribution (adjusted *P* = 0.05) (51).

**Cryogenic transmission electron microscopy.** In order to observe visual differences in cell structure and morphology in cells grown on each of the three substrates, we used cryogenic transmission electron microscopy (cryo-TEM). TEM allows for investigation of the internal structure of cells and, when coupled to cryogenic sample preparation, allows observation of the cells in their hydrated state. For cells grown on cryo-TEM analysis, 4 μl of sample was added onto a Cu-holey carbon (300 mesh) grid (Agar Scientific Ltd., Essex, England). Blotting paper was lightly touched onto the front side of the sample to remove excess water immediately prior to vitrification by flash-freezing in liquid ethane (around −183°C). This plunge-freezing protocol results in a very thin layer of ice, where the vast majority of cells lie flat against the grid. Samples were transferred into a cryo-holder (Gatan, ETVy, France) and kept at liquid nitrogen temperature (around −196°C). Samples were inserted into a JEOL 2100 transmission electron microscope (TEM) equipped with an LaB6 cathode working at an accelerating voltage of 200 kV. Images were captured with a Gatan Ordress system.

To quantify the extent of iron oxidation which had occurred in these cultures, 100 μl of sample was removed in an anoxic glove box and centrifuged to pellet the cells before resuspending the cell pellet in anoxic MQ H2O. To remove any salts which would interfere with the analysis. Some samples, especially from the early phase cultures where low cell numbers were present, were concentrated by centrifugation at this step. For cells grown with Fe(II), 1 ml of sample was removed in an anoxic glove box and centrifuged to pellet the cells before resuspending the cell pellet in anoxic MQ H2O. To quantify the extent of iron oxidation which had occurred in these cultures, 100 μl of sample was extracted in 900 μl of 1 M HCl, and the Fe(II) concentration was measured using the ferrozine assay (53). Samples were processed for subsequent analysis as quickly as possible after removal from the glove box.
were recorded under low-dose conditions (10 electrons per Å²) at a nominal magnification ranging from 5,000 to 40,000 (spatial resolution ranging from 485 to 3,883 pixels · μm⁻¹) using a 2 K-by-2 K charge-coupled device (CCD) camera (JUS 1000; Gatan). For acetate-grown cells, 69 images of cells were collected over 4 growth stages. For iron-grown cells, 71 images were collected across 5 growth stages. For hydrogen-grown cells, 102 images were collected over 4 growth stages. The contrast and brightness of the images presented in Fig. 3 were adjusted slightly in ImageJ. To compare the sizes of the intracellular membranes, images were selected in which the entire cell was contained within the image and in which the intracellular membranes (or lack thereof) were easily distinguishable. As no difference between different growth phases was observed, samples from all stages were counted together in this analysis. The area covered by the intracellular membrane was measured manually using ImageJ. For cells in which membranes appeared on both sides of the cell, the measurement for each side was combined to provide the total area of the cell covered by intracellular membrane. The length of the cells was also measured manually in ImageJ, with no significant differences observed.

Data availability. The mass spectrometry proteomics data have been deposited with the ProteomeXchange Consortium via the PRIDE (52) partner repository under the dataset identifier PXD009629 (http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD009629).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01166-18.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.2 MB.

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We declare no conflicts of interest.

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