Characterization of a Neutrophilic, Chemolithoautotrophic Fe(II)-Oxidizing \(\beta\)-Proteobacterium from Freshwater Wetland Sediments

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A neutrophilic Fe(II)-oxidizing bacterium (FeOB) isolated from Fe-rich freshwater wetland sediments has been phylogenetically and physiologically characterized. The 16S rRNA gene sequence of this organism (designated strain TW2) places it among the Rhodocyclus group within the \(\beta\)-proteobacteria. The closest known relative to strain TW2 is the heterotrophic perchlorate reducer Dechlorosoma suillum, with 94\% 16S rRNA gene identity. TW2 grows chemolithoautotrophically with Fe(II) as an electron donor and \(O_2\) as an electron acceptor. Inorganic carbon fixation during growth on Fe(II) was demonstrated via \(H^14CO_3^-\) fixation experiments. The organism can also grow organotrophically with acetate as the sole carbon and energy source, and can utilize acetate as an auxiliary source of fixed carbon which enhances cell yield (2–3-fold) during lithotrophic growth on Fe(II). No other electron donors and no electron acceptors other than \(O_2\) were utilized. The organism’s ability to grow with Fe(II) and acetate, along with its limitations with respect to electron acceptor utilization, suggests a specific adaptation to microaerobic niches in redox interfacial environments. The unique metabolism of strain TW2, together with the 16S rRNA sequence data, suggests that this organism represents a novel taxonomic group at the genus level.

Keywords Fe(II) oxidation, freshwater sediments, chemolithotrophy, \(\beta\)-proteobacteria, 16S rRNA gene phylogeny

INTRODUCTION

Although prokaryotic microorganisms have been associated with the precipitation of Fe(III) oxyhydroxides in natural environments for over 100 years (Winogradski 1888), their physiology and systematics has remained enigmatic (Ghirose 1984). Early studies tended to group all bacteria associated with the Fe(III) oxide precipitation within a single taxonomic unit, further subdivided on the basis of ecology (e.g., acidophilic vs. neutrophilic) and morphology of the organisms (Gorlenko et al. 1977). Studies of 16S rRNA gene sequences indicate that the aerobic (lithotrophic or otherwise) Fe(II)-oxidizing bacteria (FeOB) are found among virtually every major group of prokaryotes, including \(\alpha\)-, \(\beta\)- and \(\gamma\)-proteobacteria (Hallbeck and Pedersen 1991; Emerson and Moyer 1997; Kelly and Wood 2000; Sobolev and Roden 2001), low-GC gram positive bacteria (Johnson et al. 2001), Actinobacteria (Johnson and Roberto 1997) and Nitrospira group (Lane et al. 1992). However, until recently, the cultured neutrophilic FeOB were limited to the lithotrophic bacterium Gallionella ferruginea and the heterotrophic sheathed bacteria of the Leptothrix-Sphaerotilus group within the \(\beta\)-proteobacteria (Emerson 2000). Recent discoveries of chemolithoautotrophic FeOB within the \(\gamma\)-proteobacteria (Emerson and Moyer 1997) suggest that diversity within this physiological group is greater than was previously recognized.

The ability of bacteria to derive energy for chemolithotrophic growth from Fe(II) oxidation has been a point of controversy, in terms of both kinetic and free energy considerations. At circumneutral pH, Fe(II) undergoes rapid chemical oxidation by \(O_2\) (Stumm and Morgan 1996), so that neutrophilic FeOB face strong competition with abiotic oxidation processes. In addition, although the oxidation of Fe(II) by \(O_2\) is slow at the low pH, the free energy yield of the Fe(II) oxidation at the acidic pH is barely sufficient to synthesize ATP (Ehrlich 1990). Thus, it appears that the biological Fe(II) oxidation is limited by either kinetic or free energy constraints, depending on the pH of the medium. Theoretical calculations show, however, that while rates of circumneutral abiotic Fe(II) oxidation decrease dramatically with decreasing partial pressure of oxygen, the free energy of the reaction remains essentially unchanged at ca. \(-90\ \text{kJ mol Fe(II)}^{-1}\) (Roden et al. 2002). Conclusive evidence for chemolithotrophy in microorganisms associated with the Fe(II) oxidation at circumneutral pH has been obtained, both for pure cultures (Hallbeck and Pedersen 1991; Emerson and Moyer 1997), as well as mixed natural communities (Emerson and Revsbech 1994).
Recent studies indicate that the lithotrophic FeOB, when grown in opposing gradients of Fe(II) and O₂, can successfully compete with the abiotic oxidation of Fe(II), and thereby catalyze over 90% of total Fe(II) oxidation in a model gradient system (Sobolev and Roden 2001). Furthermore, these FeOB have been shown to promote bacterial Fe cycling in an artificial system designed to model conditions in Fe-rich aquatic sediments (Sobolev and Roden 2004). Together these findings suggest that, in contrast to conventional wisdom (Davison and Seed 1983), bacteria might play a significant role in Fe(II) oxidation in circumneutral environments.

The emerging recognition of the phylogenetic diversity of neutrophilic FeOB, coupled with their potentially major role in environmental Fe cycling, highlights the need for physiological studies of such organisms, particularly with respect to environmental regulation of their role in redox transformation of Fe-bearing minerals. The nature of this regulation is important, as at least some of the FeOB are capable of heterotrophic (Sobolev and Roden 2001) and mixotrophic (Hallbeck and Pedersen 1991) growth, and thus may or may not function as Fe(II) oxidizers in a particular environment.

In this paper we present a physiological and phylogenetic characterization of a neutrophilic FeOB isolated from Fe-rich freshwater wetland sediments in north central Alabama, USA. The organism is capable of lithoautotrophic growth with Fe(II) as an electron donor, as well as heterotrophic growth with acetate, using oxygen as electron acceptor. The physiological properties of the isolate suggest a specific adaptation to life at oxic-anoxic interfaces that are ubiquitous in hydromorphic soils and aquatic sediments.

**MATERIALS AND METHODS**

**Enrichment and Isolation**

A neutrophilic Fe(II)-oxidizing bacterium (designated strain TW2) was isolated by repeated dilution to extinction in semi-solid, agar-stabilized Fe(II)-O₂ opposing gradient medium (see Figure 1A) as previously described (Sobolev and Roden 2001).

**Morphological Studies**

Overall cell morphology was assessed by differential interference contrast light (Nomarski) microscopy. Flagellar arrangement was determined by transmission electron microscopy of the acetate-O₂ grown cells stained with 2% w/v uranyl acetate (Beveridge et al. 1994).

**DNA Amplification and Sequencing**

Nucleic acids were extracted from the Fe(III) oxide-rich growth band of an opposing-gradient culture according to a protocol developed for soil samples (Zhou et al. 1996). A fragment of the 16S rRNA gene ca. 1,500 base pairs in length was amplified with universal eubacterial primers fD1 and rD1 (Weisburg et al. 1991). The PCR cycler was set to 35 cycles, each with a denaturation temperature of 94°C for 15 s, initial annealing temperature of 55°C for 15 s, decreasing to 45°C over 20 cycles (1°C decrease every other cycle), and an elongation temperature of 72°C for 45 s. Amplified 16S rDNA was precipitated with isopropanol (0.6 volume) and resuspended in the 1 × Tris-EDTA (TE) buffer at 10 ng/µL. Ligation of the gene fragment into the pCR 2.1 plasmid and chemical transformation of competent Escherichia coli INVαF™ cells was conducted according to the TOPO-TA cloning kit (Invitrogen) instructions. Transformants were selected on LB agar with 50 µg mL⁻¹ of ampicillin and 40 µg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Sambrook et al. 1989) and subcultured in Luria broth with ampicillin (25 µg mL⁻¹). Plasmid extracts were obtained with Stratagene Clear Cut kit, according to the manufacturer’s instructions. The extracts were used as templates for further PCR amplification with primers specific to the vector immediately outside the insertion site (M13F, M13R, Operon Technologies stock primers, see Table 1). Selection of primers specific to the plasmid sequence outside the insertion region prevented amplification of the vector.

**Table 1**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Tm, °C</th>
<th>Sequence reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>fD1</td>
<td>AGAGTTTGATCCTGGCTTCAG</td>
<td>59.9</td>
<td>Weisburg et al. 1991</td>
</tr>
<tr>
<td>rD1</td>
<td>AAGGAGGTGATCCAGCC</td>
<td>59.9</td>
<td>Weisburg et al. 1991</td>
</tr>
<tr>
<td>M13 (−20) Reverse</td>
<td>GTAATAACGACGCAGCATG</td>
<td>56.9</td>
<td>Operon™</td>
</tr>
<tr>
<td>M13 (−24) Forward</td>
<td>AACAGCTATAGCCATG</td>
<td>50.9</td>
<td>Operon™</td>
</tr>
<tr>
<td>P2</td>
<td>ATTACGCGGCCTGCTCG</td>
<td>61.9</td>
<td>Muyzer et al. 1993</td>
</tr>
<tr>
<td>P3</td>
<td>CGCCGCCTCGCCCGCGCCGGGCGGGGCGGGGGCGGGCAGG</td>
<td>61.9</td>
<td>Muyzer et al. 1993</td>
</tr>
<tr>
<td>TW377F</td>
<td>TCTGAGAGGATGATCTG</td>
<td>54.9</td>
<td>This study</td>
</tr>
<tr>
<td>TW856F</td>
<td>GCACGAAACGCTGGGAG</td>
<td>64.9</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Original designation, where applicable.

5’ to 3’.

Operon Technologies Tm calculator at [http://www.operon.com/oligos/toolkit.php].

Operon Technologies stock primers.

GC-clamp not included in Tm calculation.
any *E. coli* DNA that could have contaminated the plasmid. The amplified insert was concentrated to 20 ng/µL and submitted for sequencing to the University of Georgia, Athens. Since the insert length was greater than the sequence length normally obtained in a single sequencing run, primer walk was justified. After each sequencing run, resulting in ca. 600 unambiguously resolved nucleotide bases, unique primers (designated as TW341F, TW856F, see Table 1) were designed to amplify DNA beginning at approximately 100 bases inwards from the 3' end of unambiguously resolved sequence stretch. Sequence fragments were aligned by use of Pairwise BLAST search (Altschul et al. 1997) and then combined by using the XeSee sequence editor. The completed sequence was compared to the database sequences using Advanced BLAST search (Altschul et al. 1997).

**Culture Purity Verification via DGGE**

Total DNA extracted from the cultures was amplified as described above, except that a set of primers specific to ca. 200-bp fragment of the 16S rRNA with a GC-clamp attached (P2, P3 from Muyzer, Waal, and Uitterlinden (1993), see Table 1) was used. The fragment length consistency and PCR yield were checked by agarose gel electrophoresis (as above), PCR product was concentrated to ca. 25 ng/µL and resuspended in 1 × Tris-acetate-EDTA (TAE) buffer. Ten µL of the resuspended PCR product was loaded per lane of DGGE gel (6% acrylamide/bis-acrylamide, denaturant gradient concentration 20%–60% (Jackson et al. 1998) and subjected to electrophoresis for 5 hr at constant voltage and temperature (7.5 V/cm, 65°C) in 1 × TAE buffer (Bio-Rad DCode unit). Upon completion of electrophoresis, the gel was retrieved, stained with Ethidium Bromide (as above), visualized under the UV transilluminator and photographed with a digital camera.

**Phylogenetic Analysis**

The most closely related sequences (90% or greater sequence identity) retrieved by the BLAST were uploaded into the
Electron Donor and Acceptor Utilization

After 12 d of growth of TW2 in an opposing gradient culture, the oxide layer containing bacteria (ca. 1 mL) was collected with a pipette and resuspended in 9 mL of 30 mM bicarbonate buffer (pH 7). This suspension was used to inoculate (1% vol:vol) all experimental systems.

Electron donor/acceptor utilization experiments (see Table 2) were conducted in 25 mL pressure tubes with 10 mL of medium containing NaHCO$_3$ (2.5 g/L), K$_2$HPO$_4$ (0.06 g/L), NH$_4$Cl (0.01 g/L), minerals and vitamins (Lovley and Phillips 1986). The medium was sparged with O$_2$-free N$_2$:CO$_2$ (80:20 v/v) for 5 minutes and headspace flushed with the same gas mix for one minute. Whenever experimental design called for the presence of oxygen, 7 mL of headspace gas was withdrawn and replaced with sterile air. Electron donors, other than Fe(II), and HS$^-$ (see later) were added at 10 mM, except benzoate (1 mM) and hydrogen (50% headspace volume at atmospheric pressure). Electron acceptors were present at 10 mM, except oxygen (equilibrium concentration ca. 140 µM) and solid-phase amorphous Fe(III) oxide (10 mmol L$^{-1}$). Oxygen was used as the electron acceptor for all electron donors tested in this study, and acetate was the electron donor for all tested electron acceptors.

Mn(II) utilization was tested in 200-mL bottles, which included 50 mL of the PIPES-buffered medium with 10 mM MnSO$_4$. Controls were set up in identical fashion, except for the addition of 1 mM Na-azide. Growth was assessed visually in comparison to uninoculated or electron donor-free controls, with final scoring at 4 weeks.

Sulfide utilization was assessed in a gradient system similar to that of Nelson et al. (Nelson et al. 1986), except that the Na$_2$S concentration was increased to 10 mM. Growth was assessed by measuring O$_2$ profiles with a microelectrode in live vs. azide-killed cultures with a microelectrode (Revsbech 1989).

Growth experiments with acetate/O$_2$ were conducted in 50 mL of medium with 110 mL of headspace, which was flushed daily with sterile air. Growth was determined by measuring optical density at 600 nm wavelength. In one experiment, the influence of O$_2$ concentration on growth with acetate/O$_2$ was examined in cultures with a headspace of 100% air and with 10% air/90% N$_2$.

Growth experiments with Fe(II) as an electron donor were conducted in opposing gradient cultures set up in rubber stoppered culture tubes (Sobolev and Roden 2001) with an air headspace. The cultures were inoculated with 0.1 mL of the cell suspension (above), which amounted to 1.5 × 10$^6$ to 2.5 × 10$^6$ cells/tube or 2.5 nmol biomass C (assuming 20 fg C per cell, see later). Triplicate tubes were sacrificed at each sampling point. The headspace was flushed with N$_2$ for 10 min and the tubes restopped and homogenized by shaking for 20 min. This procedure resulted in formation of no additional Fe(III), as indicated by time-zero Fe measurements (see Figure 7). Samples were then collected for Fe analysis via 0.5 M HCl extraction and Ferrozine determination of Fe(II) and total Fe and direct bacterial counts with acridine orange (Emerson and Moyer 1997). Bacterial cell counts were converted into biomass carbon assuming 20 fg C per cell (Hoff 1993).

The opposing gradient cultures were also used to assess the influence of acetate on lithotrophic growth with Fe(II). The cultures were set up and inoculated as described above, except that the source plug (the bottom solid layer with 1.5% agar) contained either 10 mM Na acetate, 50 mM Fe(II) (as FeCl$_2$), or both acetate and Fe(II). Controls, identical to the treatments in all respects except for the lack of Fe(II) or acetate in the source plug, were used to account for any bacterial growth that might have occurred on agar impurities. Bacterial counts and Fe measurements (where applicable) were conducted as described previously. All statistical analyses were performed using functions in Microsoft Excel.

**Table 2**

<table>
<thead>
<tr>
<th>Electron donor$^a$</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>+</td>
</tr>
<tr>
<td>Formate</td>
<td>−</td>
</tr>
<tr>
<td>Lactate</td>
<td>−</td>
</tr>
<tr>
<td>Methanol</td>
<td>−</td>
</tr>
<tr>
<td>Glucose</td>
<td>−</td>
</tr>
<tr>
<td>Benzoate</td>
<td>−</td>
</tr>
<tr>
<td>H$_2$</td>
<td>−</td>
</tr>
<tr>
<td>Fe(II)</td>
<td>+</td>
</tr>
<tr>
<td>Mn(II)</td>
<td>−</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Electron acceptor$^b$</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrous Feric Oxide</td>
<td>−</td>
</tr>
<tr>
<td>Fe(III)-NTA</td>
<td>−</td>
</tr>
<tr>
<td>Nitrate</td>
<td>−</td>
</tr>
<tr>
<td>Perchlorate</td>
<td>−</td>
</tr>
<tr>
<td>Sulfate</td>
<td>−</td>
</tr>
<tr>
<td>AQDS$^c$</td>
<td>−</td>
</tr>
<tr>
<td>Oxygen</td>
<td>+</td>
</tr>
</tbody>
</table>

$^a$All tests conducted with oxygen as an electron acceptor.

$^b$All tests conducted with acetate as an electron donor.

$^c$Anthraquinone-2,6-disulfonate.
**Lithoautotrophic Carbon Fixation**

H$^{14}$CO$_3^-$ fixation was tested in Fe(II)-O$_2$ opposing gradient cultures (as above) set up in scintillation vials with 4 mL of bottom source layer and 10 mL of top mineral medium layer with 30 mM bicarbonate buffer. Controls consisted of systems (i) lacking Fe(II), (ii) lacking Fe(II) but containing 10 mM acetate, (iii) containing both Fe(II) and acetate, and (iv) containing Fe(II) but lacking bacterial inoculum. NaH$^{14}$CO$_3$ with a specific activity of 56 mCi mmol$^{-1}$ (0.5 µCi or 8.9 × 10$^{-3}$ µmol $^{14}$C per sample) was added immediately before pouring the top layer. Due to the high specific activity of the radioactive bicarbonate, its influence on the total pool of the inorganic carbon was ignored in calculations of HCO$_3^-$ fixation rates. The top layer was allowed to cool and then inoculated as described above for the Fe(II)-O$_2$ growth experiments. At the conclusion of the experiment, the cultures were cored with a detipped 5 mL syringe. The cored material was incubated in 2 mL of 0.5 M HCl overnight to drive off the unassimilated H$^{14}$CO$_3$ mixed with 5 mL of liquid scintillation cocktail (Fisher), and radioassayed with Beckman scintillation counter, using external $^{14}$C standards.

**RESULTS**

**Morphology**

Strain TW2 is a long, slightly curved rod, ca. 1 µm in diameter and 4–6 µm in length with a single polar flagellum (Figure 1B,C).

**Culture Purity**

DGGE analysis of a 200 bp of the 16S rRNA gene fragment amplified from a Fe(II)-O$_2$ opposing gradient TW2 culture showed only a single band (Figure 1D), which verified the purity of the isolate.

**DNA Amplification and Sequencing**

A ca. 1500 bp fragment of the 16S rRNA gene was successfully amplified with the universal primers, resulting in a single band of DNA on an agarose gel. Sequencing of the fragment cloned into a plasmid and reamplified with the primers matching the target sequences just outside the insertion site yielded over 500 unambiguously resolved base pairs per run, and the whole gene sequence was completed in three runs, with two unique primers designed and synthesized during the course of sequencing (see Table 1). The resulting fragments comprised total sequence length of 1,485 bp.

**Phylogenetic Position**

Based on its 16S rRNA gene sequence, TW2 belongs to the *Rhodocyclus* group within the β-proteobacteria. According to the phylogenetic analysis, members of the *Rhodocyclus* group form a monophyletic sister taxon with the *Galionella* group of which *G. ferruginea* is the only representative (Figure 2). The closest relative to TW2 (ca. 94 16S rRNA gene sequence similarity) is the heterotrophic perchlorate-reducing bacterium *Dechlorosoma suillum* (Coates et al. 1999). Also closely related to TW2 are the phototroph *Rhodocyclus tenius* (Hiraishi 1994), and *Ferribacterium limneticum*, a dissimilatory iron-reducing bacterium (Cummings et al. 1999). The 16S rRNA gene sequence has been assigned accession number AF503539 in GenBank.

**Electron Donor and Acceptor Utilization**

Among the electron donors tested, only acetate and Fe(II) (in opposing gradient cultures) were utilized to support cell growth. Acetate was therefore used as an electron donor in electron acceptor tests. Under the conditions tested, only oxygen was utilized as an electron acceptor by TW2 (Table 2).

TW2 exhibited a ca. 24-h lag phase when grown in Fe(II)-O$_2$ opposing gradient cultures (Figure 3), during which time Fe(III) accumulation was apparently associated with abiotic Fe(II) oxidation. Between 48 and 144 h, cell growth was directly correlated with Fe(III) production (Figure 4), with a doubling time, calculated as described by Turley (1993), of ca. 24.6 h for the faster of two experiments. Growth leveled off by 168 h at ca. 2 × 10$^8$ cells per tube. The presence of 10 mM acetate in Fe(II)-O$_2$ opposing gradient cultures increased the growth rate (compared to a parallel experiment) and the final biomass 2–3-fold (Figure 3). No cell growth occurred in control systems lacking acetate and Fe(II), which ruled out the possibility of heterotrophic growth of TW2 on agar impurities. A small amount of cell growth occurred in gradient cultures containing acetate alone, with a doubling time of ca. 60 h.

Growth of TW2 with acetate as an energy source was slow compared to growth on Fe(II) in opposing gradient cultures (Figure 5), with a doubling time on the order of 71 h. No difference in growth rate was observed in cultures with 10% vs. 100% air saturation (data not shown), which suggests that TW2 does not require microaerophilic conditions for efficient organotrophic growth with acetate.

**Lithoautotrophic Carbon Fixation**

A significant increase (2–5-fold) in apparent H$^{14}$CO$_3^-$ fixation was detected in Fe(II)-O$_2$ opposing gradient cultures compared to abiotic, electron donor-free, and acetate-only controls (Figure 6). The presence of acetate in Fe(II)-O$_2$ opposing gradient cultures decreased H$^{14}$CO$_3^-$ fixation to a level equal to that in acetate-only controls.

**Biotic vs. Abiotic Fe(II) Oxidation**

Comparable rates and quantities of Fe(III) deposition occurred in control cultures lacking FeOB and cultures inoculated with TW2 (Figure 7). These results suggest that the Fe(II) oxidation in the diffusion-mediated opposing gradient cultures was limited by Fe(II) transport, as opposed to the reaction rate
between Fe(II) and O$_2$. This result agrees well with that of Emerson and Moyer (1997), who found no difference between the rate of Fe(II) deposition in gradient cultures in which FeOB (strains ES1 and ES2) were present versus abiotic controls. However, both our work (Sobolev and Roden 2001) and that of Emerson and Moyer (1997) have shown that the zone of the Fe(II) oxidation and Fe(III) deposition is more compact and localized in the presence of microbial catalysis compared to abiotic systems (see Figure 1A).

**DISCUSSION**

**General Physiology of TW2**

The capacity of TW2 to grow with acetate as a carbon and energy source clearly distinguishes it from the obligately lithotrophic FeOB strains ES1 and ES2 isolated by Emerson and Moyer (1997). Although we found no evidence that microaerophilic conditions enhanced organotrophic growth with acetate (not shown), a significant enhancement of growth (both rate and the final biomass) was observed when acetate was present in Fe(II)-O$_2$ opposing gradient cultures, as opposed to Fe(II)-only or acetate-only treatments (Figures 3 and 4). The poor growth of TW2 in acetate-only gradient cultures suggests that Fe(II) oxidation was the dominant mode of energy generation to support growth in the Fe(II)-plus-acetate opposing gradient systems. It is likely that the enhanced growth observed in the presence of acetate can be attributed to utilization of acetate as an energy source.
Figure 4. Relationships between Fe(III) accumulation (Fe(II) oxidation) and biomass production in Fe(II)-O₂ gradient cultures. See text for description of the conversion from cell numbers (Figure 3) to biomass C. Data points represent means of triplicate cultures. Experiments 1 and 2 for the Fe(II) only systems refer to separate growth experiments.

auxiliary source of fixed carbon, which reduced the amount of energy required for CO₂ fixation and thereby increased overall cell yield. It makes sense that TW2 can utilize acetate in this capacity, since acetate is produced in anaerobic sediments and is thus likely to be available (e.g., as a result of upward diffusion) at least in small quantities at the aerobic-anaerobic interface. In general, TW2’s physiology suggests a specific adaptation to the ecological niche of microaerobic sedimentary redox interfacial environments in which FeOB are known to proliferate (Emerson 2000) and from which the organism was isolated.

Strain TW2’s ability to grow mixotrophically with Fe(II) and acetate is analogous to the capacity of G. ferruginea to utilize sugars such as glucose and thereby enhance cell yield during lithotrophic growth on Fe(II) (Hallbeck and Pedersen 1991). Although we did not test for the ability of TW2 to utilize sugars during growth on Fe(II), its inability to utilize glucose as a sole carbon and energy source for organotrophic growth (Table 2) suggests that it is unlikely to be able to do so. To our knowledge, the potential for G. ferruginea to grow under strictly
organotrophic conditions (i.e., in the absence of Fe(II)) has not been systematically evaluated, nor has its ability to utilize smaller organic substrates such as acetate for mixotrophic growth on Fe(II). More detailed studies of the comparative physiology of these two related organisms is warranted.

**Growth Yield on Fe(II) and O$_2$**

A linear relationship between Fe(III) accumulation and TW2 biomass carbon (C) accretion suggested that cell growth was directly coupled to Fe(II) oxidation and associated Fe(III) oxide deposition (Figure 4). Biomass yield during the growth period between 48 and 144 h indicated that oxidation of 1 µmol of Fe(II) resulted in accumulation of 0.014–0.024 µmol of biomass C (results from two separate experiments, see Figure 4). These growth yields are comparable, on a per-electron-transferred basis, to yields obtained during chemolithoautotrophic growth of *Beggiaota* in HS$^-$-O$_2$ opposing gradient cultures (Nelson et al. 1986).

Formation of 1 mol of biomass C required oxidation of ca. 42 to 73 mol of Fe(II), which is about half as much as that estimated by Ehrlich (1990) and Beck (1960) for the acidophilic Fe(II) oxidizer *Thiobacillus ferrooxidans*. Assuming the following overall reaction stoichiometry

$$\text{Fe}^{2+} + 0.25\text{O}_2 + 2.5\text{H}_2\text{O} \rightarrow \text{Fe(OH)}_3 + 2\text{H}^+$$

and assuming that 57.5 mol Fe(II) was oxidized per one mol of biomass formed (average of our two experiments), ca. 5060 kJ would be liberated during Fe(II) oxidation per mol of biomass C formed. Since it takes ca. 502 kJ to fix 1 mol of CO$_2$ (Silverman and Lundgren 1959), chemolithoautotrophic carbon fixation is thermodynamically feasible for these organisms. In fact, only 10% of the energy liberated during the Fe(II) oxidation would account for all the cellular carbon observed in the cultures. Our estimate of the inorganic carbon fixation efficiency fits well with the published range of free energy efficiencies for acidophilic FeOB, which has been reported to be as low as 3.2% (Temple and Colmer 1951) and as high as 30% (Lyalikova 1958), with intermediate values of 4.8% to 10.6% (Beck and Eldsen 1958) and around 20% (Silverman and Lundgren 1959). It is important to note, however, that the biomass C as estimated by the cell count does not necessarily account for all the organic C formed by the FeOB in our system. TW2 has been hypothesized to excrete a chelating agent (Sobolev and Roden 2001), possibly in order to retard or delay cell encrustation with solid-phase Fe(III) oxides. Results suggested that this chelator was capable of complexing mM levels of Fe(III) (Sobolev and Roden 2001). Obviously, such an agent could not be accounted for by microscopic methods. Although the percentage of the total C fixed by the TW2 that may be allocated to synthesis of this chelator is unknown, it is reasonable to expect that the actual C fixation yield is higher than the value calculated on the basis of the cell counts.

Experiments with H$_4$CO$_3^-$ demonstrated the capacity of TW2 to fix inorganic carbon while growing in Fe(II)-O$_2$ opposing gradient cultures (Figure 6). Given the specific activity of the H$_4$CO$_3^-$ in the culture, we estimate (after correcting for the background amount of H$_4$CO$_3^-$ fixation detected in the no electron donor and abiotic controls) that ca. 0.513 µmol C was assimilated over the 120 h of the experiment. Conversion of this amount of biomass C into cell numbers assuming 20 fg C per cell yields a value of 3.1 × 10$^8$ cells, a number ca. 50% greater than the numbers obtained in independent Fe(II)-O$_2$ growth experiments (Figure 3). Thus, autotrophic CO$_2$ fixation can account for all of the biomass C formed in the cultures. It is worthwhile to note that H$_4$CO$_3^-$ fixation nearly ceased when acetate was added to opposing gradient cultures (Figure 6), which supports the argument that TW2 uses acetate mainly as an auxiliary source of fixed carbon which enhances cell yield (2–3-fold; see Figures 3 and 4) during lithotrophic growth on Fe(II). This situation is analogous to the near complete inhibition of inorganic C fixation by *G. ferruginea* in the presence of glucose at concentrations of 10 µM or higher (Hallbeck and Pedersen 1991).

**Phylogeny of TW2**

Unlike the unicellular chemolithotrophic FeOB isolated by Emerson and Moyer (Emerson and Moyer 1997), which belong to the $\gamma$-proteobacteria, phylogenetic analysis of the TW2 culture indicates that this organism belongs to the *Rhodocyclus* group within the $\beta$-proteobacteria lineage. There are no known neutrophilic aerobic Fe(II) oxidizers immediately related to strain TW2. The taxonomic group most closely related to TW2 includes all known strains of the perchlorate-reducing *Dechlorosoma* genus (Achenbach et al. 2001), with *Dechlorosoma saillum* having ca. 94% 16S rRNA gene similarity to TW2. This group also includes an uncultured microorganism associated with trichlorobenzene transformation (Wintzingerode et al. 1999). It is important to note that although there was a significant support for grouping TW2 with known strains of *Dechlorosoma* (jackknife value 97), the resolution of our analysis was insufficient to separate the relationships among the *Dechlorosoma*-like organisms. This observation suggests that TW2 and *Dechlorosoma* represent distinct evolutionary branches. The fact that strain TW2 has no known relatives within its own branch (in contrast to the *Dechlorosoma* genus), suggests that the organism represents a a novel taxonomic group at the genus level.

Members of the *Rhodocyclus* group possess a surprisingly wide range of metabolic capabilities in view of their close phylogenetic relatedness (Achenbach and Coates 2000). The affiliation of TW2 with this group suggests that the organism belongs to an evolutionary branch which is either inherently physiologically plastic and/or particularly prone to lateral gene transfer. Lateral gene transfer, a process ubiquitous among the bacteria (Ragan 2001), provides a possible explanation for the capacity of TW2 to grow lithoautotrophically via Fe(II) oxidation, despite its close phylogenetic linkage to a group not known to
be capable of lithoautotrophic metabolism. Thus, lateral gene transfer from, e.g., a Gallionella-like organism to a Dechlorosoma ancestor could account for presence of lithotrophic Fe(II) oxidation capability within the Rhodocyclus group. Support for this idea comes from the fact that organisms from the genus Dechlorosoma have been shown to oxidize Fe(II) with nitrate as an electron acceptor (Chaudhuri et al. 2001), although neither autotrophy nor Fe(II) oxidation with O₂ as the electron acceptor has been demonstrated for this genus.

Independent evolution of different lineages provides an alternative explanation for the presence of the Fe(II)-dependent lithoautotrophy in evolutionarily distinct groups. The fact that the ability to oxidize Fe(II) is present in organisms as diverse as heterotrophic bacteria of the Sphaerotilus-Leptothrix group (Ghiorei 1984) and acidophiles such Thiobacillus (Kelly and Wood 2000) suggests the possibility that as many as three independently evolved systems exist for Fe(II) oxidation: (1) chemolithotrophic neutrophilic oxidation, as expressed in TW2 (Sobolev and Roden 2001) and Gallionella (Hallbeck and Pedersen, 1991) in the β-proteobacteria or strain ES1 (Emerson and Moyer 1997) and its relatives in the γ-proteobacteria; (2) heterotrophic neutrophilic oxidation as expressed in Sphaerotilus-Leptothrix, perhaps as a way to detoxify metabolic byproducts (Dubinina 1978), oxygen (Ghiorei 1984) or Fe(II) (Hallbeck and Pedersen 1991, 1995); and (3) chemolithothrophic acidiphilic oxidation, as expressed in the genus Thiobacillus and physiologically similar organisms (Johnson 1998; Rawlings 2001). However, Emerson and Moyer (1997) show Thiobacillus caldus (later reclassified as Acidithiobacillus caldus, see Kelly and Wood 2000), a γ-proteobacterium, as a group ancestral to both γ-proteobacteria (such as Beggiatoa or strain ES-1) and β-proteobacteria, such as Gallionella. Combining this fact with information presented in Rawlings (2001), which indicates a relatively recent separation between the β- and γ-proteobacteria on an evolutionary timescale, suggests a common evolutionary history for the Fe(II) oxidation in acidophilic and neutrophilic FeOB within β- and γ-subdivisions of the proteobacteria. Final judgment on this issue will require identification and sequence analysis of the genes involved in Fe(II) oxidation in these organisms.

REFERENCES


