Phototrophic oxidation of ferrous iron by a Rhodomicrobium vannielii strain

Silke Heising and Bernhard Schink

Author for correspondence: Bernhard Schink. Tel: +49 7531 882140. Fax: +49 7531 882966. e-mail: Bernhard.Schink@uni-konstanz.de

Oxidation of ferrous iron was studied with the anaerobic phototrophic bacterial strain BS-1. Based on morphology, substrate utilization patterns, arrangement of intracytoplasmic membranes and the in vivo absorption spectrum, this strain was assigned to the known species Rhodomicrobium vannielii. Also, the type strain of this species oxidized ferrous iron in the light. Phototrophic growth of strain BS-1 with ferrous iron as electron donor was stimulated by the presence of acetate or succinate as cosubstrates. The ferric iron hydroxides produced precipitated on the cell surfaces as solid crusts which impeded further iron oxidation after two to three generations. The complexing agent nitrilotriacetate stimulated iron oxidation but the yield of cell mass did not increase stoichiometrically under these conditions. Other complexing agents inhibited cell growth. Ferric iron was not reduced in the dark, and manganese salts were neither oxidized nor reduced. It is concluded that ferrous iron oxidation by strain BS-1 is only a side activity of this bacterium that cannot support growth exclusively with this electron source over prolonged periods of time.

Keywords: iron metabolism, phototrophic bacteria, Rhodomicrobium vannielii, iron complexation, nitrilotriacetate (NTA)

INTRODUCTION

Iron is the fourth most important element in the Earth’s crust, making up about 5% of the total crust mass (Ehrlich, 1990). In biological systems, the redox change between the Fe(II) and Fe(III) state is of utmost importance in redox reactions, especially in haem-containing proteins, iron—sulfur proteins, etc. (Neiilands, 1974). The redox change between Fe(II) and Fe(III) also plays an important role in the mineralization of biomass in oxygen-limited environments such as sediments, water-logged soils or contaminated aquifers (Lovley, 1993). The redox potential of the Fe(II)/Fe(III) transition depends strongly on the prevailing pH: at strongly acidic conditions, the transition occurs at the standard redox potential of +0.77 V, whereas at pH 7.0, the transition redox potential is between +0.1 and +0.2 V (Stumm & Morgan, 1981; Widdel et al., 1993). This comparatively low redox potential caused us to check whether anoxicogenic phototropic bacteria could oxidize ferrous to ferric iron compounds with concomitant reduction of CO₂ to cell material. Enrichment cultures led to the isolation of several pure cultures of anoxygenic purple bacteria, including a Rhodomicrobium vannielii-like isolate (Widdel et al., 1993). Other strains of purple phototrophs able to oxidize ferrous iron are strain L7, a non-motile rod with gas vacuoles which is related to the genus Chromatium, and strain SW2, a non-motile rod related to the genus Rhodobacter. Both strains can use not only FeCO₃ but also FeS as electron source, and can oxidize it completely to Fe(III) and sulfate (Ehrenreich & Widdel, 1994).

In the present communication, a purple non-sulfur bacterium is described in detail which was isolated with ferrous iron as sole energy source in the light (Widdel et al., 1993). The strain was characterized as belonging to the species Rhodomicrobium vannielii, and the type strain of this species was also found to be able to oxidize ferrous iron.

METHODS

Sources of organisms. Strain BS-1 was enriched from the sediment of a ditch close to Tübingen-Bebenhausen, Germany. The following strains were obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany: Rhodobacter capsulatus DSM 155, Rhodobacter sphaeroides DSM 158T, Rhodobacter sulfidophilus (Rhodosulcum sulfidophilum) DSM 1374T, Rhodomicrobium vannielii DSM 162T, Rhodopseudomonas acidophila DSM.
Enrichment of iron-oxidizing bacteria

Sediment samples from a ditch close to Tübingen-Bebenhausen were used as inocula in mineral medium with 2 mM FeSO$_4$ as electron source at 25 °C in dim light (14 W m$^{-2}$). After 1–2 weeks incubation, brownish precipitates formed on the bottle surfaces, and precipitated material collected at the bottom. Transfers were made into subcultures with some of the surface-attached material and also with free culture liquid. In subcultures, mainly stalked bacteria developed which were associated with the bottle surfaces and with ferric iron precipitates. After three to four transfers, the cell material was diluted in deep-agar series for purification. Colonies developing in these cultures were deep brown and either round with entire margins or fluffy. Under microscopic examination, both types of colonies were dominated by stalked bacteria. Since isolation and resuspension of ferric-hydroxide-containing colonies proved to be difficult, strains were isolated in deep-agar dilution series with 4 mM succinate as substrate. The developing colonies were deep red and contained the same type of stalked bacteria as observed in the enrichment cultures. Strain BS-1 was characterized in detail.
During exponential growth, cells of strain BS-1 were either motile rods or stalked cells, 0.7 μm wide and 2 μm long. After growth with succinate, cells formed thin stalks which aggregated in nets, reminiscent of the morphology of *Rhodoferax vannielli* (Fig. 1a). In the stationary phase, polyhedral exospores developed. Cultures of strain BS-1 with 8 mM FeSO₄ plus 1 mM acetate or butyrate as substrate required 6–8 weeks for iron oxidation to cease. Cells formed brownish layers on the inner glass surfaces of the culture flasks. Phase-contrast micrographs showed highly refractive cells that were obviously surrounded by iron oxides, including the stalks (Fig. 1b).

Also, the type strain of *Rhodoferax vannielli* oxidized ferrous iron in the light. A detailed comparison of strain BS-1 with the *Rhodoferax vannielli* type strain revealed that strain BS-1 behaves similarly to *Rhodoferax vannielli* in utilization of substrates. Differences were found only in the utilization of benzoate, tartrate, aspartate and glutamate, which were utilized exclusively by strain BS-1, and sulfide, methanol and ethylene glycol, which were used only by *Rhodoferax vannielli* (Table 1). The pigmentation of both strains was examined by recording in vivo absorption spectra after photoheterotrophic growth with succinate. Absorption spectra of these strains (Fig. 2) were identical, with maxima at 375, 592, 803 and 868 nm, typical of bacteriochlorophyll a (Pfennig & Trüper, 1991), and carotenoid absorption maxima at 460, 487 and 523 nm, indicating the presence of lycopene and rhodopene (Pfennig & Trüper, 1991). Both cultures appeared orange–brown after growth with succinate. Strain BS-1 was also able to grow aerobically in the dark with succinate as carbon and energy source.

**Growth of strain BS-1 with ferrous iron**

Addition of 8 mM FeSO₄ to the bicarbonate-buffered medium caused the formation of a white precipitate of FeCO₃. Growth in mineral medium with ferrous iron as

---

**Table 1. Utilization of substrates by strain BS-1 and *Rhodoferax vannielli* type strain**

<table>
<thead>
<tr>
<th>Substrates tested for support of phototrophic growth</th>
<th>Strain BS-1</th>
<th><em>Rhodoferax vannielli</em> type strain*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂/CO₂, acetate, propionate, butyrate, valerate, caproate, caprylate, lactate, pyruvate, ethanol, malate, malonate, fumarate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose, fructose, citrate, glycolate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Benzoate, tartrate, aspartate, glutamate</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>HS⁻, methanol, ethylene glycol</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Glyoxylate, mercaptoethanol, cyclohexane carboxylate, cysteine</td>
<td>-</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Data from Whittenbury & Dow (1977). ND, Not determined.
sole electron source was slow; after 8 weeks, approximately 50% of the added ferrous ions was oxidized. Addition of an organic cosubstrate enhanced growth substantially; these cosubstrates were provided at concentrations releasing equivalent amounts of electrons for photosynthetic electron transport (1 mM acetate, 9.6 mM succinate, 0.7 mM malate, 0.8 mM pyruvate or 0.4 mM butyrate). No difference in iron oxidation activity was found if the HCl-based solution SL10 or the EDTA-based solutions SL9 and SL12 were used as trace element solutions.

As the solubility of iron salts depends strictly on the prevailing pH, the pH-dependence of growth and iron oxidation was studied. Whereas the growth rate with acetate as substrate increased continuously with increasing pH, iron oxidation activity exhibited an optimum at approximately pH 6.4–6.7, and no iron oxidation was observed at pH >7.1 (Fig. 3). The in vivo absorption spectra of intact cells grown with either acetate alone or 1 mM acetate plus 8 mM FeSO₄ were identical (results not shown).

Strain BS-1 did not oxidize Mn(II) salts provided as 4 mM MnSO₄, which precipitated in the medium as reddish-white MnCO₃. Formation of dark-stained oxidation products was not observed, neither in the presence nor in the absence of acetate as organic cosubstrate.

Strain BS-1 did not reduce Fe(III) salts in the dark with acetate or succinate as electron donor. Neither exogenously provided amorphous FeOOH nor Fe(III) oxo-hydroxides produced by the cells themselves were reduced under such conditions. The same was true for the Rm. vannielli type strain. In both strains, MnO₂ was not reduced in the dark with acetate as electron donor.

**Effect of complexing agents on iron oxidation activity**

Several complexing agents were checked for possible support of iron oxidation activity by strain BS-1. EDTA (0.2 mM, 0.5 mM) and NTA (1 mM) stimulated iron oxidation rates by >50% as compared to a chelator-free control. NTA or EDTA alone did not serve as electron source. Citrate had no stimulating effect at 1–10 mM concentration. In the presence of 15 mM NTA, the FeSO₄-containing medium stayed entirely clear and optical analysis of time courses of growth and iron oxidation became possible. However, NTA at 15 mM concentration decreased the acetate- or iron-dependent growth rates by approximately 30%. Moreover, iron oxidation in these cultures was not associated with stoichiometric cell mass increase: after acetate consumption, the cell mass did not increase any further, but iron was oxidized slowly, without concomitant cell mass formation (Fig. 4).

Ultrathin sections of cells grown with ferrous iron plus acetate in the presence or absence of NTA revealed clear...
differences in cell ultrastructure (Fig. 5a, b). Whereas cells grown with FeCO₃ were covered with numerous crystalline, electron-dense precipitates inside the cells and on the cell surface, cells grown in the presence of NTA did not show such precipitates but exhibited the lamellar intracytoplasmic membrane structures typical of the genus *Rhodobacterium*.

Cell-free extracts of cells grown with ferrous iron plus NTA, H₂ plus NTA, or with H₂ plus ferrous iron plus NTA were subjected to SDS-PAGE. The band pattern of all three extracts did not differ qualitatively, but at least two bands in the molecular mass range 12–14 kDa were significantly thicker in extracts of iron-grown cells than in extracts of cells grown in the absence of ferrous iron (Fig. 6a). These could be stained by haem staining, indicating that they represent cytochromes (Fig. 6b).

### Iron oxidation by other strains of purple non-sulfur bacteria

Several pure cultures of purple non-sulfur bacteria were checked for their ability to oxidize Fe(II) salts provided either as FeCO₃ or FeS. As well as strain BS-1 and *Rm. vannieli*, Fe(II) was also oxidized by *Rhodobacter capsulatus*, *Rhodopseudomonas palustris* and *Rhodospirillum rubrum*, either alone or in the presence of H₂ or yeast extract as cosubstrates. With *Rhodobacter*...
sphaeroides and Rhodopseudomonas acidophila, no iron oxidation activity was observed.

DISCUSSION

Unlike most bacteria, the newly isolated strain BS-1 could easily be affiliated with an existing species, mainly on morphological grounds. The formation of motile swimmer cells, stalked cells attached to surfaces, as well as polyhedral exospores indicated that this strain had to be affiliated with the existing genus and species Rm. vanniellii. This assumption was further supported by the lamellar arrangement of intracytoplasmic membranes typical of this species (Imhoff & Trüper, 1991), and by the absorption spectrum, which was identical to that of the type strain. Also, the substrate utilization spectra of the type strain of Rm. vanniellii and of strain BS-2 were sufficiently similar to allow affiliation of strain BS-1 with the species Rm. vanniellii. Finally, the type strain of Rm. vanniellii was also found to be able to grow with ferrous iron as electron donor.

Utilization of ferrous iron as electron donor is limited by the low solubility of Fe²⁺ in the growth medium. At pH >7.2, the concentration of free Fe²⁺ in the presence of excess bicarbonate is in the range of a few micromolar (Ehrenreich & Widdel, 1994). At higher pH, the solubility decreases drastically. This relationship may be the reason for the observed pH dependence of ferrous iron oxidation by strain BS-1 (Fig. 3): the pH optimum of ferrous-iron-dependent photosynthesis was substantially lower than that of succinate-dependent photosynthesis, and there was hardly any ferrous iron oxidation at pH >7.0. Organic cosubstrates (acetate, butyrate, malate, succinate, pyruvate) increased the rate of iron oxidation considerably, perhaps by complexation of Fe³⁺, but probably more by enhanced growth with additional electron sources and increased cell surface areas, which increased in turn the contact area with the ferrous substrate.

The products formed by iron oxidation [FeOOH and Fe(OH)₃] have even lower solubility products \( K_{sp} = 6 \times 10^{-36} \) for Fe(OH)₃ (Mortimer, 1987) than the substrate FeCO₃ \( K_{sp} = 2.1 \times 10^{-11} \). The ferric oxohydroxides precipitated on the cell surfaces, forming crystals of various sizes. It is evident that ferrous iron is oxidized at the cell surface, and the insoluble oxohydroxides precipitated exactly there, forming thick, refractile crusts which impede further oxidation after prolonged cultivation.

Other ferrous-iron-oxidizing phototrophs (strains SW2 and L7; Ehrenreich & Widdel, 1994) form ferric iron oxohydroxides separate from the cells which prevent enclosure of the cells with insoluble rust covers. It is evident that there are individual differences between the various strains of ferrous-iron-oxidizing phototrophs with respect to the site of ferric oxide deposition, e.g. due to excretion of short-chain carboxylic acids which may act as chelating agents for Fe³⁺. The solubility of ferrous iron ions in culture supernatants from different strains of ferrous-iron-oxidizing phototrophs differed by about two orders of magnitude, indicating that complexing agents were excreted (R. Warthmann & B. Schink, unpublished results). Culture supernatants of strain BS-1 contained very little ferrous iron, which may be the reason for the observed accrustation of ferric iron oxohydroxides on the cell surfaces, and which may also explain the comparatively slow growth of this strain with ferrous iron.

Aerobic mixotrophic iron-oxidizing bacteria such as Sphaerotilus natans and Leptothrix discophora precipitate ferric oxohydroxides in sheets of acidic exopolymers on the cell surface (van Veen et al., 1978; Ghiorse, 1984). Obviously, these bacteria also oxidize ferrous iron at the cell surface but the thick sheets provide sufficient protection to the cells to allow further metabolic activity.

We tried to avoid the ferric hydroxide precipitations by addition of complexing agents, especially NTA, to the media. Although ferrous and ferric iron ions could be maintained in solution with 15 mM NTA, and the cells still exhibited active growth with acetate as electron source, they were obviously damaged by this complexing agent, perhaps by removal of divalent cations from the outer membrane. Thus ferrous iron oxidation in the presence of 15 mM NTA was not coupled to stoichiometric cell matter formation but may have led to synthesis of organic acids that were subsequently excreted into the surrounding medium through leaky membranes.

The acidophilic aerobic ferrous-iron-oxidizing bacteria Thiobacillus ferroxidans and Leptospirillum ferrooxidans produce specific proteins for oxidation of ferrous iron which are located in the periplasm (Blake et al., 1993). By analogy to these aerobic ferrous iron oxidizers, we assume that iron oxidation in the phototrophic bacteria is localized on the outside of the cytoplasmic membrane as well. Periplasmic electron carriers similar to the rusticyanins of the aerobic acidophilic iron oxidizers may transfer these electrons to cytochrome c₉ and with this to the photosystems (Ehrenreich & Widdel, 1994). If such carriers were present only in insufficient amounts, part of the iron oxidation process would also occur in the periplasm, with the observed detrimental effect for the cells.

The protein band pattern of cell-free extracts of cells grown with ferrous iron differed only quantitatively from those grown with hydrogen in the absence of ferrous iron. Obviously, there was no difference in the photosynthetic apparatus used in the presence or absence of ferrous iron, and there was no indication of a specific cytochrome that was specifically involved in iron oxidation. However, at least two protein bands that probably corresponded to cytochromes were substantially increased in iron-grown cells, indicating that such a carrier is induced to higher activity in ferrous iron oxidation. The fact that only few phototrophic bacteria among those tested turned out to be able to use ferrous iron as electron donor indicates that this metabolic
capacity depends on a specific arrangement of carrier systems that is not present in every phototrophic bacterium. The difference may be due to a specific arrangement of carrier systems, perhaps to a specific exposure of such carriers to the periplasmic space which allows access of ferrous iron ions only with certain strains, or to an enhanced synthesis of a periplasmic (?) cytochrome that could mediate electron flow from outside across the periplasmic space.

The physiological advantage of ferrous iron oxidation by strain BS-1 is still enigmatic. Since ferrous iron crusts deposited on the cell surface were not reduced or redissolved upon exposure to organic substrates in the dark, this process appears to be an irreversible reaction that impairs the cell physiology on a long-term basis. Also, the phototrophic isolates L7 and SW2 did not reduce ferric iron precipitates in the dark (Ehrenreich & Widdel, 1994). On the other hand, iron oxidation must provide some advantage to strain BS-1 because it could be enriched with iron as sole electron donor from sediments in the presence of trace amounts of organic acids in the inoculum material during the first enrichment steps. We assume that in nature, strain BS-1 finds ways to redissolve thin crusts of ferric iron hydroxides after oxidative precipitation, but we were unable to mimic such culture conditions in the laboratory.

We conclude that ferrous iron oxidation by strain BS-1 may be only a side activity of minor physiological importance. Ferrous iron oxidation may be of advantage to the cells in nature as long as sufficient concomitant cell surface increase is secured by organic cosubstrates to avoid complete coverage of the cell surface by rusty layers.

ACKNOWLEDGEMENTS

The authors are indebted to Professor Dr Frank Mayer, Göttingen, for taking the electron micrographs. Advice by Professor Dr Norbert Pfennig, Konstanz, on aspects of the physiology and cultivation of phototrophic bacteria is greatly appreciated. This study was supported by the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, in its special investigation of the hydrogen-oxidizing acetate-forming anaerobic bacterium Acetobacterium woodii. Arch Microbiol 115, 207–213.

REFERENCES


Received 27 February 1998; revised 20 April 1998; accepted 22 April 1998.