Anaerobic Oxidation of Ferrous Iron by Purple Bacteria, a New Type of Phototrophic Metabolism

ARMIN EHRENREICH AND FRIEDRICH WIDDEL
Max-Planck-Institut für Marine Mikrobiologie, D-28359 Bremen, Germany

Received 22 July 1994/Acepted 29 September 1994

Anoxic iron-rich sediment samples that had been stored in the light showed development of brown, rusty patches. Subcultures in defined mineral media with ferrous iron (10 mmol/liter, mostly precipitated as FeCO₃) yielded enrichments of anoxygenic phototrophic bacteria which used ferrous iron as the sole electron donor for photosynthesis. Two different types of purple bacteria, represented by strains L7 and SW2, were isolated which oxidized colorless ferrous iron under anoxic conditions in the light to brown ferric iron. Strain L7 had rod-shaped, nonmotile cells (1.3 by 2 to 3 μm) which frequently formed gas vesicles. In addition to ferrous iron, strain L7 used H₂ + CO₂, acetate, pyruvate, and glucose as substrates for phototrophic growth. Strain SW2 had small rod-shaped, nonmotile cells (0.5 by 1 to 1.5 μm). Besides ferrous iron, strain SW2 utilized H₂ + CO₂, monocarboxylic acids, glucose, and fructose. Neither strain utilized free sulfide; however, both strains grew on black ferrous sulfide (FeS) which was converted to ferric iron and sulfate. Strains L7 and SW2 grown phototrotopically without ferrous iron were purple to brownish red and yellowish brown, respectively; absorption spectra revealed peaks characteristic of bacteriochlorophyll a. The closest phototrophic relatives of strains L7 and SW2 so far examined with a photoautotrophic utilization of ferrous iron as electron donor for CO₂ fixation. Dependence of ferrous iron oxidation on light and CO₂ was also demonstrated in dense cell suspensions. In media containing both ferrous iron and an organic substrate (e.g., acetate, glucose), strain L7 utilized ferrous iron and the organic compound simultaneously; in contrast, strain SW2 started to oxidize ferrous iron only after consumption of the organic electron donor. Ferrous iron oxidation by anoxygenic phototrophs is understandable in terms of energetics. In contrast to the Fe⁺⁺/Fe⁺⁺⁺ pair (Eₒ = +0.77 V) existing in acidic solutions, the relevant redox pair at pH 7 in bicarbonate-containing environments, Fe(OH)₂⁺HCO₃⁻/FeCO₃⁻, has an Eₒ' of +0.2 V. Ferrous iron at pH 7 can therefore donate electrons to the photosystem of anoxygenic phototrophs, which in purple bacteria has a midpoint potential around +0.45 V. The existence of ferrous iron-oxidizing anoxygenic phototrophs may offer an explanation for the deposition of early banded-iron formations in an assumed anoxic biosphere in Archean times.

Iron is the fourth most abundant element in the Earth’s crust (21) and the most abundant of the metals which naturally undergo redox changes. In oxic environments, only the ferric state is energetically stable; common forms are, e.g., the poorly crystalline ferrhydrite [usually written as Fe(OH)₃] and the crystalline goethite (α-FeOOH) and hematite (α-Fe₂O₃) (21, 40). In anoxic habitats, reduction to the ferrous state occurs either chemically, e.g., by sulfide or certain organic compounds (20, 34), or biologically in direct contact with dissimilatory iron-reducing bacteria (20, 31, 32). Specialized lithoautotrophic bacteria such as acidophilic Thiobacillus ferrooxidans (5, 15) or neutrophilic Gallionella ferruginea (22, 23) gain energy for growth from the oxidation of ferrous iron with O₂ as terminal electron acceptor. G. ferruginea has to compete with the autooxidation of ferrous iron, which is rapid under neutral conditions.

The reducing power of ferrous iron is strongly influenced by the OH⁻ ion activity and thus by the pH. The standard redox potential of the soluble Fe⁺⁺/Fe⁺⁺⁺ pair (Eₒ = +0.77 V) is relevant only below pH 2 to 3. At higher pH values, ferrous ions form compounds with OH⁻ ions. The stable forms around pH 7 are practically insoluble, neutral hydroxy and oxy compounds with a thermodynamic equilibrium concentration of Fe⁺⁺⁺ of ≤10⁻¹⁷ M (19, 41). In comparison, ferrous minerals, apart from ferrous sulfide (FeS) and pyrite (FeS₂), are relatively soluble; e.g., FeCO₃ (siderite) at pH 7 and a bicarbonate concentration of 0.02 M has an equilibrium concentration of Fe⁺⁺⁺ in the micromolar range. As a result, the ferric-ferrous pair shows a marked shift in the redox potential towards the negative range above pH 2 to 3. For instance, the Eₒ' of the pair Fe(OH)₂⁺HCO₃⁻/FeCO₃⁻ is +0.2 V. (For details concerning solubilities and E-pH diagrams see, e.g., references 3, 19, 41, 44, and 46.)

Several authors have discussed the possibility of a naturally occurring oxidation of ferrous iron in the absence of free oxygen. The knowledge of such a process would have implications that bear on our understanding of the origin of banded-iron formations (BIFs). BIFs were deposited during the Archean and Proterozoic ages over thousands of square kilometers (4, 10, 25, 26, 44). Most models for BIF deposition assume a chemical oxidation of dissolved ferrous iron in ocean water with free oxygen from early oxygenic photosynthesists such as cyanobacteria (4). Alternatively, however, an experimentally proven UV light-driven reaction of ferrous iron with water yielding ferric iron and H₂ has been proposed to explain BIF deposition (7, 9, 18, 35). Furthermore, there have been speculations about a direct biological oxidation of ferrous iron by

* Corresponding author. Mailing address: Max-Planck-Institut für Marine Mikrobiologie, Fahrenheistr. 1, D-28359 Bremen, Germany. Phone: 49 421-2208-122. Fax: 49 421-2208-130.
photosynthetic organisms without an intermediate appearance of free oxygen (11, 24, 38, 43). Cohen (11) could measure a ferrous iron-dependent photoassimilation of CO₂ by cyanobacteria from mats while inhibiting the oxygenic photosystem. Recently, isolation of purple bacteria which grew autotrophically by using ferrous iron as sole electron donor for anoxygenic photosynthesis was reported (14, 46); because such phototrophs possess only one photosystem they may have been involved in BIF deposition before the onset of oxygenic photosynthesis via the highly evolved two-step photosynthesis.

In the present paper we describe the growth physiology of two different isolates of ferrous iron-oxidizing phototrophs and their relationships to known purple bacteria.

**MATERIALS AND METHODS**

**Sources of organisms.** Various freshwater mud samples for enrichments were taken from brown, iron-rich ditches near Lübeck (source of strain L7) and from ponds in Schaumberg Wald, Hanover region (source of strain SW2); Schöngesinger Forst near Munich (source of strain SF4); and the town area of Munich, Germany. Marine samples were from the mud flat of Jadebusen, North Sea. Only the upper few centimeters of the sediments were collected.

Thiodictyon elegans DSM 232, Thiodictyon bacillorum DSM 234, Rhodobacter capsulatus DSM 1710, and Rhodopseudomonas palustris DSM 123 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

**Medium and cultivation methods.** Techniques of medium preparation and cultivation of phototrophic bacteria under anoxic conditions were as described elsewhere (45), however, the medium composition was modified. For freshwater cultures, the following salts were dissolved in 1 liter of distilled water: 0.3 g of NH₄Cl, 0.5 g of KH₂PO₄, 0.5 g of MgSO₄·7H₂O (in sulfate-free medium replaced by 0.4 g of MgCl₂·6H₂O), and 0.1 g of CaCl₂·2H₂O. Medium for marine enrichments contained in addition (per liter) 22 g of NaCl, 3.5 g of MgCl₂·6H₂O, 0.5 g of KCl, and CaCl₂·2H₂O increased to 0.15 g. After autoclaving and cooling under N₂-CO₂ (90:10 [vol/vol]) the following components were added (per liter) from sterile stock solutions described in detail (45): 1 ml of trace element mixture containing sodium EDTA, 22 ml of 1 M NaHCO₃ solution (autoclaved under CO₂), 1 ml of vitamin mixture (p-aminobenzoate, biotin, nicotinate, pantothenate, and pyridoxin), 1 ml of thiamine solution, and 1 ml of vitamin B₁₂ solution (all vitamins were filter sterilized). The pH was adjusted to 7.0. The batch was then aseptically dispensed so as to fill 4/5 of the volume of tubes (20 ml) or flat bottles (100, 250, and 500 ml); the headspace was gassed with N₂-CO₂ (90:10 [vol/vol]) and sealed with black rubber stoppers. FeSO₄₃H₂O was added to each tube or bottle from a 1.0 M solution (autoclaved and maintained under N₂) to a final concentration of 10 mM; if required, the resulting drop in pH was compensated for by the addition of 8 mM Na₂CO₃ (final concentration) from a 1 M stock solution. The Fe⁺⁺ ions reacted, probably with carbonate and phosphate in the medium, to form a fluffy, colorless precipitate which gradually became crystalline. For growth with hydrogen, a bigger headspace (1/2 of total volume), which was gassed with H₂-CO₂ (80:20 [vol/vol]), was used. For cultivation of strain L7 in the absence of ferrous iron, the medium was reduced with 4 mM ascorbate added from a 1 M solution (ascorbic acid neutralized with NaOH, filter sterilized, and maintained under N₂). Organic substrates were added from sterile 1 M stock solutions to the final concentrations indicated. For growth with FeS a concentrated suspension was added to a final concentration of 5 mM/liter. The suspension of FeS was prepared by aseptic mixing of equal volumes of 0.2 M FeSO₄ and 0.2 M Na₂S solutions under an atmosphere of N₂; the black precipitate was washed aseptically by repeated sedimentation, removal of the supernatant, and addition of sterile medium; finally, the suspension was adjusted to 0.2 mol of FeS per liter. Aliquots were taken with sterile pipettes while the suspension was magnetically stirred under N₂.

Culture tubes and bottles were incubated horizontally on a Plexiglas plate and illuminated with two tungsten lamps (25 W) installed above and below the cultures at a distance of 30 cm; agar dilutions for isolation of gas vesicle-containing phototrophs were incubated in dim light obtained from one tungsten lamp (25 W) at a distance of 50 to 60 cm. Unless otherwise indicated, the incubation temperature was 18°C.

Growth of oxygenic phototrophs in enrichment cultures was prevented by using light filters (RG 780; Helioplan, Gräfelfing, Germany) excluding wavelengths shorter than approximately 780 nm.

To check utilization of oxygen as terminal electron acceptor for chemotrophic growth in the dark, 0.1 ml of a grown culture was mixed at 41°C with 9 ml of medium containing 0.8% (wt/vol) molten agar and an organic substrate. The solidified medium was incubated in a jar containing air with 10% CO₂.

**Isolation.** Pure cultures were isolated by the agar dilution method (45) which was slightly modified. To 3 ml of 3% (wt/vol) agar molten in a test tube (160 by 16 mm), 0.11 ml of a 1 M FeSO₄ solution was aseptically added, and the agar was gently mixed. Then, 8 ml of prewarmed (41°C) medium containing 2 mM acetate and 4 mM ascorbate was added. Ferrous minerals precipitating during this procedure remained homogeneously distributed because of the viscosity of the agar. After stepwise dilution of the inoculum at 41°C, the agar tubes were immediately cooled in a water bath, sealed anoxically, and incubated in the light. Colonies were again subject to agar dilutions and finally transferred to liquid medium.

Purity was verified microscopically after growth of isolated strains in medium with yeast extract (0.5 g/liter) and pyruvate (3 mM), glucose (2 mM), or acetate (4 mM). Also, homogeneity of colonies in agar dilutions was checked.

**Suspension experiments.** Cells of strain L7 for experiments in dense suspensions were grown on ferrous iron (10 mmol/liter) under an atmosphere of H₂-CO₂ (80:20 [vol/vol]). Cells were separated from insoluble iron minerals in a separatory funnel; red aggregates of the phototrophic bacteria settled prior to the inorganic precipitate. Cells were then resuspended in 50 ml of 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, pH 7.0, with 20 mM NaCl and 1 mM NaHCO₃, so as to achieve the 10-fold cell density of the original culture.

**Analytical procedures.** Ferrous iron was quantified photometrically at 510 nm after chelation with 2 mM o-phenanthroline in 0.7 M sodium acetate buffer, pH 5, in a test volume of 1 ml (modified from reference 16). Samples were withdrawn from agitated rubber-sealed culture bottles by means of anoxic syringes, acidified with concentrated HCl (final concentration, 1 M) and heated to 100°C for 10 min; the concentration of ferrous iron in the assay was adjusted between 10 and 60 μM. Ferric iron was determined by the same method after reduction with 0.28 M hydroxyammonium chloride in the acidified sample; the ferrous iron concentration determined before reduction was subtracted.

Sulfate was determined on a Sykam (Gilching, Germany) ion chromatograph equipped with an LCA 09 (Sykam) anion-exchange column, an acidic suppressor column, and a conduc-
tivity detector as described previously (1); the eluent contained 10 mM K_2CO_3 and 10% (vol/vol) ethanol in water.

Prior to acetate and glucose determination, samples with a volume of 0.2 ml were mixed with 0.8 ml of sodium phosphate buffer, pH 7.0, and centrifuged to remove iron minerals. Acetate was determined enzymatically employing acetyl coenzyme A synthetase, adenylate kinase, pyruvate kinase, and lactate dehydrogenase (12). Glucose was measured enzymatically with glucose oxidase, peroxidase, and diammonium 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonate) (28).

Cell mass was determined by protein. Growth cultures were acidified with concentrated HCl to a final concentration of 2 M. After iron minerals had dissolved at 40°C within 5 min, trichloroacetic acid was added to a final concentration of 0.5 M. Precipitated protein was centrifuged for 10 min at 30,000 × g, dissolved in 0.1 M NaOH at 60°C during 6 min, and determined with the biuret reagent (33). The cell mass/protein ratio was measured in a separate experiment with cells grown on acetate and then dried to constant weight at 70°C.

Absorption spectra were recorded with a Beckman (Palo Alto, Calif.) DU 640 spectrophotometer. An acetate-grown culture of strain SW2 was concentrated 10-fold by centrifugation; 1 ml of suspension was added to a solution of 5 g of saccharose in 3 ml H_2O to minimize defraction. Since strain L7 formed aggregates, cells from a glucose-grown culture were disrupted by sonication in potassium phosphate buffer (50 mM, pH 7.0) and centrifuged for 10 min at 10,000 × g to remove cell debris. The pigmented supernatant was centrifuged for 1 h at 150,000 × g; the membrane pellet was resuspended and homogenized in the indicated buffer.

**Analysis of rRNA.** Preparation of genomic DNA, in vitro amplification of 16S rRNA genes, and direct sequencing of amplified DNA were performed as described elsewhere (6). The sequenced rRNA gene fragments are homologous to positions 62 to 1508 (strain L7) or 54 to 1512 (strain SW2) of the *Escherichia coli* 16S rRNA gene (8). Phylogenetic relationships were calculated with W. Ludwig, Munich, Germany. Obtained sequences were inserted into an alignment with those from other bacteria, including phototrophs. Phylogenetic trees were reconstructed by applying the maximum-likelihood method using the program fastDNAml (29); trees were scrutinized by maximum parsimony and distance matrix analyses using the corresponding tools of the PHYLIP program package (17).

**Nucleotide sequence accession numbers.** Data are deposited under EMBL accession numbers X78718 (strain L7) and X78717 (strain SW2).

**RESULTS**

**Enrichment and isolation.** Samples of freshwater and marine sediment for crude enrichment of phototrophic bacteria were incubated in anoxic jars next to a tungsten lamp. In several samples the expected phototrophic sulfur bacteria developed as characteristic purple and green, often refractile patches. In some of the samples, however, rusty deposits resembling oxidized iron developed on the illuminated side of the glass wall within 4 weeks. If samples from naturally iron-rich ditches with ochre-brown water were incubated in the light, rusty patches appeared in the dark sediment even within 1 to 2 weeks. Development of chlorophyll a-containing, oxygenic phototrophs such as cyanobacteria or algae that could account for iron oxidation by oxygen production was not detected by epifluorescence microscopy (excitation wavelength, 430 nm).

To study the obviously light-dependent formation of ferric iron under more defined conditions, subsamples with particles from rust layers were transferred to mineral media with added FeSO_4 (10 mmol/liter) or FeS (synthetic, 5 mmol/liter). Freshwater and marine subcultures (inoculum, 5% [vol/vol]) converted the colorless precipitate formed from FeSO_4 within 5 to 10 days completely to ferric iron; the reaction was strictly light dependent. Iron oxidation also occurred under a long-wavelength (>780-nm) filter which excludes oxygenic phototrophs. Enrichments of freshwater origin exhibited good growth with iron only if incubated below 20°C. A marine enrichment grew also well at 28°C. The precipitate in the enrichments first turned greyish green before it assumed its final brown, rusty color. Bacterial cells were observed between or attached to fluffy and compact precipitates. All well-growing freshwater enrichments contained rod-shaped to oval, mostly aggregated cells with gas vesicles as the most striking morphological type. Upon addition of H_2CO_3 (80/20 [vol/vol]) part of the aggregates became buoyant and accumulated as tiny purple flocs near the medium surface. In addition, smaller nonmotile and motile rod-shaped cell types, partly with an asymmetric mode of division, were detected. The marine enrichment contained various small oval and rod-shaped cells. The intensely black FeS added to parallel enrichments disappeared completely in the light within 4 weeks and yielded a beige sediment.

For isolation of the cell type with gas vesicles, the buoyant purple aggregates formed upon addition of H_2 to iron cultures were washed by two consecutive passages through sterile medium, broken in a small-pastele tissue grinder, and used as the starting inoculum for agar dilutions. The efficiency of colony development of the gas vesicle-containing type in agar was poor. In three parallel dilution series, only four separate colonies consisting of gas vesicle-containing cells were detected after 4 weeks among colonies of other phototrophs. A pure culture designated strain L7 was used for further studies.

When inocula for agar dilutions were directly taken from liquid and precipitate of freshwater enrichments, a luxuriant development of yellowish brown and red colonies occurred within 2 weeks. When incubated further in the light, many colonies turned opaque and greyish brown because of deposition of coarse crusts which probably consisted of ferric minerals. Ten isolates were purified from the yellowish brown colonies. Even though originating from three different locations these isolates resembled each other by their morphology, color, and nutritional properties (see following section). All isolates consisted of tiny, sometimes elongated rods. Two representatives, strains SW2 and SF4, were chosen for more detailed experiments. A further type of phototrophic bacterium was isolated from the red colonies. By their color, size, and asymmetric mode of cell division, these isolates resembled *R. palustris* (37). Isolates of this type, in contrast to the gas-vaculated and the tiny rod-shaped strains, never oxidized ferrous iron completely but ceased to grow at a greyish green intermediate oxidation state.

Even though the marine ferrous iron-oxidizing enrichment grew as fast as the freshwater enrichments, marine isolates obtained thus far developed only poorly in iron medium.

**Morphology and color of pure cultures.** Cells of strain L7 (Fig. 1A) were 1.3 μm in diameter and 2 to 3 μm in length. Motility was never observed. In freshly inoculated cultures, cells were usually rod shaped and had small or no gas vesicles. Towards the end of growth all cells formed large gas vesicles (Fig. 1A, lower section) with all cells formed large gas vesicles (Fig. 1A, upper section) in the stationary phase. Cells occurred mostly in short chains or aggregates. Strain SW2 (Fig. 1B), SF4, and the other isolates from yellowish brown colonies had...
FIG. 1. Phase-contrast photomicrographs of the newly isolated ferrous iron-oxidizing phototrophic bacteria. Precipitates are visible as granular or amorphous precipitates. (A) Strain L7 from a growing culture (lower panel) and stationary culture (upper panel). (B) Strain SW2. To increase the cell density for the photomicrograph, cultures of strain L7 and SW2 on ferrous iron were supplemented with H₂ and acetate (1 mM), respectively, as additional substrates. Bar, 10 μm (applicable to all photomicrographs).

FIG. 2. Progressive oxidation by strain L7 of ferrous iron (10 mmol/liter) in anoxic tubes. (A) Sterile iron medium; (B) tube after inoculation; (C and D) successive intermediate oxidation states; (E) fully oxidized iron. Cultures were shaken immediately before the photograph was taken.

greyish green intermediate state (Fig. 2), as already observed in the enrichments. There were slight differences in the appearance of the ferric precipitates formed by the two types of iron-oxidizing phototrophs at the end of growth. The ochre to light brown precipitate formed by strain L7 was partly finely grained and more compact than the precipitate formed by strains SW2, SF4, and the similar isolates. The latter strains formed an orange-brown precipitate with a more fluffy appearance. Ferromagnetic precipitates were never observed.

The color of phototrophic bacteria grown with ferrous iron as the only electron donor was completely masked by their oxidation product. On organic substrates without iron the isolates exhibited the same colors as observed in young colonies, namely purple to brownish red for strain L7 and yellowish brown for strains SW2, SF4, and the other isolates of this type. Absorption spectra of strains L7 and SW2 (Fig. 3) exhibited peaks in the long wavelength range characteristic of bacteriochlorophyll a (37).

Optimal growth conditions and nutritional characteristics. All iron-oxidizing freshwater isolates had a relatively low temperature range for growth with an optimum at 18 to 20°C. No growth occurred above 24°C. The pH range for growth of strain L7 was 6.0 to 7.5, with an optimum at 7.0; the pH range for strain SW2 was 5.5 to 7.0, with an optimum at 6.5.

In addition to ferrous iron, H₂ served as electron donor for autotrophic growth of all isolates.

All iron-oxidizing isolates also grew on FeS and formed ferric iron and sulfide (see section on quantitative growth studies with FeS). During growth, cells of strain L7 formed refractile inclusions resembling sulfur droplets. No growth occurred on free sulfide; the added sulfide concentration (1 mM) did not inhibit growth of strain L7 on acetate. Growth on thiosulfate (4 mM) was never observed.

relatively small, rod-shaped, nonmotile cells with a length of 1 to 1.5 μm and a diameter of 0.5 μm.

When ferrous iron was the only electron donor, isolates exhibited different tendencies to attach to the formed ferric mineral. The large cells of strain L7 were not or only loosely associated with insoluble precipitates and easily detected between particles. In contrast, strain SW2 and the other yellowish brown isolates associated tightly with precipitated flocs of ferric iron and were rarely seen as free cells. Only in the case of strain SF4 was a significant portion of free cells observed in the medium. Upon addition of hydrogen or organic growth substrates, a high proportion of free cells developed in all cultures (Fig. 1). In cultures of strain L7 with an additional electron donor, such as H₂, purple to brownish red aggregates became visible to the naked eye; in some cultures part of these aggregates became buoyant.

Iron oxidation in the pure cultures occurred through a
Conversion of colorless manganese(II) (added as MnSO₄, 10 mmol/liter) to higher, dark oxidation states was never observed in any pure culture.

Photoheterotrophic growth was tested with a variety of low-molecular-weight compounds (millimolar concentrations are given in parentheses). Strain L7 utilized acetate (4), pyruvate (3), and glucose (2) as organic substrates but did not grow on formate (5), propionate (2), butyrate (2), ethanol (2), lactate (4), succinate (4), fumarate (4), malate (4), or fructose (2). Strain SW2 was more versatile; it grew on formate (5), acetate (4), propionate (2), butyrate (2), lactate (4), glucose (2), fructose (2), alanine (4), aspartate (4), and glutamate (4); no growth occurred on methanol (2), ethanol (2), succinate (4), fumarate (4), malate (4), or benzoate (2). Substrate tests with strain SF4 and another yellowish brown isolate yielded exactly the same results.

Strain SW2, in contrast to strain L7, grew in the dark on acetate or glucose with oxygen as electron acceptor. Neither strain utilized glucose anaerobically in the dark by either fermentation or nitrate reduction.

**Genealogic relationships.** Relationships derived from 16S rRNA sequences of strains L7 and SW2 are depicted in Fig. 4. Strain L7 grouped within the gamma subclass of the proteobacteria; among the phototrophs with known 16S rRNA sequences *Chromatium* species were the closest relatives. The closest phototrophic relatives of strain SW2 were *Rhodobacter* species of the alpha subclass of the proteobacteria.

Quantitative growth studies with FeCO₃. Production of ferric iron by strains L7 and SW2 under autotrophic conditions was monitored during incubation in 500-ml cultures. The inoculum was 25 ml.

Fastest growth under autotrophic conditions was observed with strain L7 (Fig. 5A). After a lag phase of 4 to 5 days, iron oxidation occurred within 10 days. In smaller tubes (20 ml), iron was usually oxidized after 8 days including the lag period. Strain SW2 exhibited a considerable lag phase under autotrophic conditions (Fig. 5B).

No iron oxidation occurred in inoculated media in the dark or in sterile controls in the light during an incubation time of 3 months. Growth was never observed in media without addition of ferrous iron, H₂, or organic substrates. The dependence of ferric iron oxidation on light was also shown in dense cell suspensions; in addition, the dependence of iron oxidation on the presence of CO₂ could be demonstrated in such suspensions (Fig. 6). The amount of cell dry mass (approximate formula, CH₂O₅) formed per mol of ferrous iron oxidized (Table 1) is close to the theoretical value (7.5 g/mol) for an autotrophic metabolism according to the following equation:

\[
4 \text{Fe}^{2+} + \text{CO}_2 + 11 \text{H}_2\text{O} \rightarrow 4 \text{Fe(OH)}_3 + (\text{CH}_2\text{O}) + 8 \text{H}^+ \tag{1}
\]

With consideration of the dominating minerals and ions in bicarbonate-containing medium near neutrality, the actual net reaction is better approximated by

\[
4 \text{FeCO}_3 + 10 \text{H}_2\text{O} \rightarrow 4 \text{Fe(OH)}_3 + (\text{CH}_2\text{O}) + 3 \text{HCO}_3^- + 3 \text{H}^+ \tag{2}
\]

Phosphate scavenging from the medium by iron minerals is not
considered here. Since the exact nature of the formed ferric iron is unknown, the product is simply expressed as ferric hydroxide.

Autotrophic growth and iron oxidation was stimulated by H₂ as additional electron donor. The time required for complete oxidation of iron was almost half as long as with iron alone. The increase in cell yield due to H₂ was demonstrated with strain L7. After iron oxidation was complete, the formed cell dry mass was almost 7 times higher than in cultures with iron alone (Table 1).

Iron oxidation was also stimulated by organic compounds as additional substrates, as demonstrated with acetate and glucose in cultures of strains L7 and SW2. Strain L7 utilized ferrous iron simultaneously with acetate (data not shown) or glucose, even at a glucose concentration as high as 10 mM (Fig. 7A). Strain SW2 started to oxidize ferrous iron only after consumption of acetate (Fig. 7B) or glucose (data not shown). With a low acetate concentration (1 mM), oxidation of ferrous iron started immediately after acetate consumption and was complete within 5 days. The apparent acceleration of iron oxidation by organic substrates may be mainly due to the increased density of cells available for iron oxidation.

Quantitative growth studies with FeS. Oxidation of ferrous sulfide was quantified under autotrophic conditions. Tubes containing sulfate-free medium with 5 mmol of FeS per liter were inoculated with strains L7 (inoculum, 20% [vol/vol]) and SW2 and SF4 (each inoculum, 10% [vol/vol]); precultures had also been grown on FeS. The intensely black FeS in the horizontally incubated tubes disappeared completely in the light within 3 weeks and yielded a beige to yellowish sediment. A net sulfate production of 4.5 to 4.8 mmol/liter was measured in illuminated cultures. All acid-soluble iron after growth was in the ferric state. In dark controls, the black FeS and the sulfate concentration added with the inoculum (≤ 1 mmol/liter) remained unchanged. Also in sterile controls incubated in the light, FeS did not disappear. Results indicate a phototrophic oxidation of both components of FeS, according to

\[
4 \text{FeS} + 9 \text{HCO}_3^- + 10 \text{H}_2\text{O} + \text{H}^+ \rightarrow 4 \text{Fe(OH)}_3 + 4 \text{SO}_4^{2-} + 9 (\text{CH}_2 \text{O})
\]

Growth tests with known species of phototrophic bacteria. *T. elegans, T. bacillosum, or R. palustris* did not oxidize ferrous

### TABLE 1. Anaerobic oxidation of ferrous iron and synthesis of cell mass by anoxicogenic phototrophic bacteria under autotrophic conditions

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Fe(II) oxidized (mmol/liter)</th>
<th>Cell mass produced (mg of dry mass/liter)</th>
<th>Growth yield (g of dry mass/mol of Fe)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L7</td>
<td>9.5</td>
<td>72</td>
<td>7.6</td>
</tr>
<tr>
<td>L7 with H₂*</td>
<td>9.6</td>
<td>500</td>
<td>ND</td>
</tr>
<tr>
<td>SW2</td>
<td>9.5</td>
<td>72</td>
<td>7.6</td>
</tr>
<tr>
<td>SF4d</td>
<td>9.3</td>
<td>82</td>
<td>8.8</td>
</tr>
</tbody>
</table>

* 10.0 mmol of Fe(II) per liter was added to all strains as FeSO₄ reacts with carbonate and phosphate in the medium to yield a colorless precipitate.
* Added as H₂-CO₂ mixture (80:20 [vol/vol]).
* ND, not determined because H₂ consumption was not quantified.
* Isolate similar to strain SW2.
ANAEROBIC OXIDATION OF FERROUS IRON BY PURPLE BACTERIA

Vol. 60, 1994

FIG. 7. Growth of phototrophic bacteria on a mixture of ferrous iron and an organic electron donor. (A) Strain L7 growing on ferrous iron and glucose; (B) strain SW2 growing ferrous iron and acetate. Symbols: ■, ferric iron; ○, glucose or acetate.

Iron. *R. capsulatus* oxidized most (>80%) of the ferrous iron within 4 weeks when hydrogen or acetate was present as an additional electron donor; the process did not continue in subcultures with ferrous iron alone.

**DISCUSSION**

**Physiological and bioenergetic aspects.** Utilization of ferrous iron as electron donor for light-driven assimilation of CO$_2$ presents a new mode of photoautotrophic growth. The considerable amount of cell dry mass (>70 mg/liter) (Table 1) formed with ferrous iron in defined mineral medium and the CO$_2$ dependence of ferrous iron oxidation (Fig. 6) can be explained only by utilization of CO$_2$ as carbon source and acceptor of reducing equivalents for cell synthesis. The only organic compounds added were vitamins with a total concentration of as low as 0.04 mg/liter. Also the high cell yield (500 mg/liter) (Table 1) in defined medium with H$_2$ as additional electron donor is a proof of the capacity for autotrophic growth. The occurrence of bacteriochlorophyll $a$ (Fig. 3) and the genealogical relationships as revealed by 16S rRNA sequencing show that the new isolates indeed belong to the anoxygenic purple bacteria which possess only one photosystem.

In such organisms, ferrous iron can only be oxidized directly without involvement of free oxygen as an intermediate.

Utilization of ferrous iron by organisms with one photosystem is understandable from a bioenergetic point of view. For free Fe$^{2+}$ ions at a concentration of 10 mM being oxidized at pH 7 to Fe(OH)$_3$, viz., for the redox pair Fe(OH)$_3$/Fe$^{2+}$, a redox potential as low as E' = −0.1 V is calculated (all values derived from data given in reference 41). At pH 7.0 and the bicarbonate concentration applied in the medium (20 mM) the added ferrous ion (10 mM) is essentially present as FeCO$_3$ rather than as Fe$^{2+}$, according to the solubility product, and to the dissociation constants of aqueous CO$_2$ and HCO$_3^-$ (41); the equilibrium concentration of Fe$^{2+}$ under these conditions is around 3 μM. Formation of ferrous phosphates is not considered here because of the relatively low phosphate concentration (3.7 mM). The bioenergetically relevant redox pair should therefore be Fe(OH)$_3$ + HCO$_3^-$ /FeCO$_3$ which at pH 7.0 and a concentration of inorganic carbon (CO$_2$ + HCO$_3^-$ + CO$_3^{2-}$) of 20 mM has an E' of approximately +0.1 V (for standard activities, E$'_a$ = +0.2 V). Even though ferrous iron under these conditions is a weaker reductant than many other electron donors used by phototrophs (e.g., S$_2$/H$_2$S, E' = −0.18 V with 1 mM sulfide), an electron transfer to the photosystem of anoxygenic phototrophs is energetically feasible; the reaction center of purple bacteria in its nonexcited state has a midpoint potential around +0.45 V (13). A possible electron flow scheme is proposed in Fig. 8. An electron donor which is energetically comparable to ferrous iron is dimethylsulfide (dimethylsulfoxide/dimethylsulfide, E$'_{o}$ = +0.16 V [47]), which can also be utilized by certain purple bacteria (48).

The topography of the ferrous iron-oxidizing system is of particular interest. Oxidation of ferrous iron in the periplasm or the cytoplasm would cause an accumulation of immense quantities of insoluble ferric iron (around 14 g of ferric hydroxide per g of cell dry mass) which would distort the cell and probably impede regular cell function. An export of formed ferric iron by means of chelators (30) would be metabolically expensive, apart from the problem that ferric iron would finally have to be released from the chelator. One has therefore to postulate that ferrous iron is oxidized on the cell surface in direct contact with the outer membrane; in this way, the insoluble product remains outside the cell. Indeed, cells of the new isolates never showed any sign of intracellular accumulations when grown in iron media. In the proposed model, the outer membrane would have to contain a redox-active component extracting electrons from Fe$^{2+}$ ions. Electrons may then be carried via a periplasmic transport system to the reaction center. It appears possible that cytochrome c$_2$ accepts electrons from the redox component in the outer membrane. An analogous periplasmic electron flow from a redox protein in the outer membrane to the cytoplasmic membrane with involvement of rusticyanin as an electron shuttle is thought to occur in the acidophilic iron oxidizer *T. ferrooxidans* (5, 15). Iron-reducing bacteria thriving under neutral conditions (31, 32) are also confronted with the insolubility of ferric iron which in this case is the electron acceptor. In iron-reducing *Shewanella putrefaciens* a cytochrome localized in the outer membrane is assumed to reduce extracellular ferric iron with electrons transported through the periplasm (36).

**Paleoecological implications.** The finding of a photosynthesis based on ferrous iron as the electron donor has implications for our understanding of the origin of BIFs. BIFs present the world’s greatest iron deposits and economically major source of iron ore (26). They are known from every continent and may expand over hundreds of kilometers with a thickness of more
than hundred meters (10, 26, 44). Being sedimentary in origin, BIFs usually show a laminated structure formed by alternating iron-rich and iron-poor, mainly siliceous layers. Iron is present in both the ferric and the ferrous state. Deposition of BIFs occurred during the Archean and Proterozoic ages, until approximately \(1.8 \times 10^9\) years ago; earliest BIFs have been dated as from \(3.8 \times 10^9\) years ago and belong to the oldest known sedimentary deposits (44).

There is general agreement that BIFs were precipitated from ocean waters by oxidation of dissolved ferrous iron derived from deep oceanic hydrothermal sources (4, 25, 44). However, an adequate mechanism of iron oxidation which can account for the immense depositions is still a subject of discussion.

According to a recently explained, comprehensive view, ferrous iron oxidation took place in deep marine environments at the chemocline of a density-stratified ocean, with anoxic, reducing deep waters and overlying oxic waters (4). Ferric iron precipitation in such a system would have been by mere chemical reaction with oxygen. Such a model not only implies transfer of oxygen from sites of its production to sites of iron precipitation; one would also have to assume the existence of free oxygen in the atmosphere and surface waters already since early Archean times. The oxygen is thought to have arisen from photosynthesis in early cyanobacteria, because the rates of oxygen production by abiotic photolysis of water was too low to account for BIF deposition (44).

Other models are based on the assumption that the biosphere was anoxic and reducing until \(2.5 \times 10^9\) years before present, the time which marks the transition from the Archean to the Proterozoic age. This conclusion was drawn from the observation that red beds of terrestrial origin, which would indicate atmospheric oxygen, are rare or unknown from the Archean record (44). Furthermore, the existence of Archean conglomerates containing the insoluble, oxygen-sensitive tetravalent uranium is regarded as an indicator of an anoxic biosphere at that time (44). Ferric iron in the early anoxic biosphere could therefore precipitate only in the immediate vicinity of oxygenic phototrophic populations. Oxygen reacted with the surrounding ferrous iron and did not accumulate or at most remained restricted to localized “oxygen oases” (27). In such models, ferric iron would have been precipitated only in surface waters receiving enough light; appropriate sites would have been shallow basins which were open to the ocean.

Also the view of an iron deposition due to an abiotic, UV light-driven reaction of ferrous iron and \(H_2O\) to ferric iron and \(H_2\) (7, 9, 18, 35) assumes iron precipitation in the upper waters supplied with light. Former claims that during such photo-chemical reactions \(CO_2\) is reduced to formaldehyde were withdrawn (35). Ferrous iron oxidation without an involvement of \(O_2\) would mean that the appearance of ferric iron in the geological record cannot be regarded as a reliable indicator of the onset of oxygen production by phototrophs.

Another possibility to produce ferric iron without free oxygen, namely by direct utilization of ferrous iron as electron donor for photosynthesis, has been considered by a number of
authors (11, 24, 38, 43). Since ferrous iron has been sometimes regarded as a high potential electron donor at any pH, early phototrophs with two photosystems have been suggested as candidates for direct iron oxidation (38). However, taking the insoluble hydroxy and oxy compounds as the energetically stable forms of ferric iron under neutral conditions into account, the actual redox potential of the ferric-ferrous pair is around +0.1 V (see preceding section); this value does not appear particularly high if compared to that for O\textsubscript{2}/H\textsubscript{2}O (E\textsubscript{0} = +0.82 V). Any anoxygenic phototroph with only one photosystem should in principle be able to derive electrons for photosynthesis from ferrous iron. The present investigation substantiates such a process with two types of purple bacteria. Phototrophs with only one photosystem may be regarded as metabolically simpler and evolutionary older than phototrophs with two photosystems (38). It therefore appears possible that anoxygenic phototrophic bacteria such as isolated in the present study or metabolically similar types with one photosystem were causatives of early BIF deposition in the Archean era before free oxygen became significant as an oxidant. Sites for such an anoxygenic oxidation of ferrous iron might also have been upper waters with supply of light, preferentially shallow basins open to the ocean. Filamentous microfossils from Archean times (39) must not necessarily present cyanobacteria, viz., oxygenic phototrophs; also, certain anoxygenic phototrophs are filamentous, e.g. the genus Chloroflexus (38). If and to what extent anoxygenic phototrophs may have contributed to BIF deposition when oxygen began to accumulate remains a subject of speculation.

An observation that has to be explained in view of a biologic origin of BIFs is their low content in preserved organic carbon and their enrichment in \textsuperscript{12}C-carbonate versus the average of global carbonate (40). On the one hand, the low organic carbon values have provided an argument in support of an abiotic mode of ferric iron precipitation. In this case the \textsuperscript{12}C-rich carbonate in BIFs is supposed to originate directly from dissolved inorganic carbon with a similar isotopic composition in the Archean deep-sea water (4). On the other hand, it has been proposed that cell material formed by photosynthesis and buried with ferric iron was gradually degraded and oxidized in the dark during diagenesis by anaerobic chemooorganotrophs reducing ferric to ferrous iron (2, 42, 43). In a closed system, the assimilation reactions (equations 1 and 2) could theoretically be inverted. However, partial hydrodynamic separation of the heavier ferric mineral from the lighter cells (43) or a loss of cell carbon from precipitates due to lytic and fermentative processes was likely to occur; therefore, only part of the ferric iron could be reduced again after sedimentation, resulting in the observed preservation of both ferrous and ferric iron. Since cell carbon assimilation from CO\textsubscript{2} always enriches the lighter isotope, CO\textsubscript{2} and carbonate formed by reoxidation would also be rich in \textsuperscript{13}C.

Apart from having been an abundant redox system during Precambrian times, ferrous and ferric iron in aquatic habitats could in addition have provided a protection against UV radiation which must have been intense under an atmosphere with no or little oxygen (38). Not only dissolved iron but also insoluble ferric iron as it occurs under neutral conditions has been shown to effectively absorb UV light (38).

ACKNOWLEDGMENTS

We thank Ralph S. Wolfe, Urbana, Ill., for encouraging discussions about bioenergetics of iron. We are indebted to A. Teske for his advice for the sequencing work and to W. Ludwig, Munich, Germany, for calculation of phylogenetic trees. We also thank Silke Heising and Bernhard Schink, Konstanz, Germany, for stimulating discussions.

This work was supported by the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, and the Max-Planck-Gesellschaft.

REFERENCES


Downloaded from http://aem.asm.org/ on November 15, 2016 by guest


34. Luther, G. W., J. E. Kostka, T. M. Church, B. Sulzberger, and W. Stumm. 1992. Seasonal iron cycling in the salt-marsh sedimentary environment: the importance of ligand complexes with Fe(II) and Fe(III) in the dissolution of Fe(III) minerals and pyrite, respectively. Mar. Chem. 40:81–103.


