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Chlorobium ferrooxidans sp. nov., a phototrophic green sulfur bacterium that oxidizes ferrous iron in coculture with a *"Geospirillum"* sp. strain

Abstract A green phototrophic bacterium was enriched with ferrous iron as sole electron donor and was isolated in defined coculture with a spirilloid chemoheterotrophic bacterium. The coculture oxidized ferrous iron to ferric iron with stoichiometric formation of cell mass from carbon dioxide. Sulfide, thiosulfate, or elemental sulfur was not used as electron donor in the light. Hydrogen or acetate in the presence of ferrous iron increased the cell yield of the phototrophic partner, and hydrogen could also be used as sole electron source. Complexed ferric iron was slowly reduced to ferrous iron in the dark, with hydrogen as electron source. Similar to *Chlorobium limicola*, the phototrophic bacterium contained bacteriochlorophyll *c* and chlorobactene as photosynthetic pigments, and also resembled representatives of this species morphologically. On the basis of 16S rRNA sequence comparisons, this organism clusters with *Chlorobium*, *Prosthecochloris*, and *Pelodictyon* species within the green sulfur bacteria phylum. Since the phototrophic partner in the coculture KoFox is only moderately related to the other members of the cluster, it is proposed as a new species, *Chlorobium ferrooxidans*. The chemoheterotrophic partner bacterium, strain KoFum, was isolated in pure culture with fumarate as sole substrate. The strain was identified as a member of the ε-subclass of the Proteobacteria closely related to "*Geospirillum arsenophilum*" on the basis of physiological properties and 16S rRNA sequence comparison. The "*Geospirillum*" strain was present in the coculture only in low numbers. It fermented fumarate, aspartate, malate, or pyruvate to acetate, succinate, and carbon dioxide, and could reduce nitrate to dinitrogen gas. It was not involved in ferrous iron oxidation but possibly provided a thus far unidentified growth factor to the phototrophic partner.

Key words Iron metabolism · Green phototrophic bacteria · *Chlorobium ferrooxidans* · *Geospirillum* · Phototrophic iron oxidation · 16S rRNA sequence analysis

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Introduction

Iron is the fourth most important element in the Earth's crust, making up approximately 5% of the total crust mass (Ehrlich 1990). In biological systems, the redox change between the Fe(II) and the Fe(III) state is of utmost importance in redox reactions, especially in heme-containing proteins, iron-sulfur proteins, etc. (Neilands 1974). The redox change between Fe(II) and Fe(III) also plays an important role in redox processes in oxygen-limited environments such as sediments or water-logged soils. The redox potential of the Fe(II)/Fe(III) transition depends strongly on the prevailing pH: under strongly acidic conditions, the transition from Fe^{2+} to Fe^{3+} occurs at E_h = +0.77 V, whereas at pH 7.0 the transition occurs mainly between $FeCO_3$ and $Fe(OH)_3$, at E_h = +0.1 – +0.2 V (Stumm and Morgan 1981; Widdel et al. 1993). This comparably low redox potential caused us to check whether also anoxygenic phototrophic bacteria could oxidize ferrous iron to ferric iron compounds with concomitant reduction of CO_2 to cell material. Enrichment cultures have led to the isolation of several pure cultures of anoxygenic purple bacteria (Widdel et al. 1993), and some of these cultures have subsequently been described in more detail (Ehrenreich and Widdel 1994; Heising and Schink 1998). All isolated strains of ferrous iron oxidizers were Proteobacteria belonging either to the Chromatiaceae or to the non-sulfur purple bacteria. However, oxidation of ferrous iron to ferric iron should also be possible with the light reaction system of green phototrophs since the reaction centers of these bacteria accept electrons at a potential of +0.3 V (Gottschalk 1985).

In the present communication, we describe a novel green phototrophic bacterium related to *Chlorobium*, *Prosthecochloris*, and *Pelodictyon* species that oxidizes ferrous iron to ferric iron in coculture with a fermenting bacterium closely related to "*Geospirillum arsenophilum*".

Materials and methods

Sources of organisms

Enrichments for new cultures of anaerobic ferrous-iron-oxidizing bacteria were started with surface sediment samples from freshwater creeks and ditches close to Konstanz and Oldenburg (Germany). The binary mixed culture of strain KoFox and strain KoFum was obtained from a ditch close to the building of the Faculty of Biology, University of Konstanz (Germany). *Chlorobium limicola* f. *thiosulfatophilum* strain 6230 (DSM 249) and *Chlorobium vibrioforme* strain 6030 (DSM 260^T) were from our own culture collection (collection N. Pfennig, Konstanz, Germany).

Media and growth conditions

All strains were cultivated in a sulfide-free mineral medium designed for green sulfur bacteria (Trüper and Pfennig 1991) that was buffered with 30 mM bicarbonate and contained 1 mM sulfate as sulfur source. Vitamins were added from a stock solution (Pfennig 1978). Substrates were added from sterile, neutralized stock solutions. Ferrous iron was added to a final concentration of 8 mM from a 0.8 M $FeSO_4$ solution that was prepared and filter-sterilized under N_2 gas and kept under N_2 at 4°C. Reaction of ferrous iron with bicarbonate led to the formation of a white precipitate of siderite ($FeCO_3$). The pH was adjusted to 6.5 unless indicated otherwise.

Ferrous sulfide was prepared by precipitation of 0.2 M Na_2S solution in 10 mM NaOH with an equal volume of 0.2 M $FeSO_4$ in 10 mM HCl under N_2 atmosphere. The precipitate was washed twice with sulfide-free mineral medium (Ehrenreich and Widdel 1994). Amorphous ferric hydroxide $Fe(OH)_3$ was prepared by slow neutralization of 0.4 M $FeCl_3$ with 0.5 M NaOH. The precipitate was washed three times with distilled water to make a final suspension of approximately 0.4 M $Fe(OH)_3$ (Lovley and Phillips 1986). Fe(III)citrate was prepared as a 0.2 M stock solution from brown Fe(III)citrate monohydrate.

Bacteria were grown in 50-ml screw-capped bottles filled with liquid medium to the top. Utilization of H_2 was tested in rubber-sealed serum bottles with an H_2/CO_2 (80:20, v/v) headspace. Utilization of substrates was checked in 22-ml screw-capped tubes. Cultures were incubated at 18°C at a distance of approximately 40 cm from a 40-W bulb (equivalent to 14 $W \cdot m^{-2}$). For enrichment cultures, the conditions were varied with respect to pH (pH 6.5, 6.8, and 7.3), various trace element solutions, and light intensity (3–14 $W \cdot m^{-2}$). Enrichment cultures were incubated behind infrared filters that excluded light at wavelengths < 740 nm (Göttinger Farbfilter, Göttingen, Germany). Formation of orange-brown precipitates indicated ferrous iron oxidation. Bacteria were purified from enrichment cultures in deep-agar dilution series (Pfennig 1978). $FeSO_4$ was added as a sterile solution to the agar before the liquid medium was added in order to allow homogeneous distribution of $FeCO_3$ through the entire medium. Culture purity was checked at regular intervals by phase-contrast microscopy after growth in mineral medium or in complex media (AC medium, diluted 1:10; Difco, Detroit, Mich., USA).

In vivo absorption spectra were taken in cell suspensions mixed with 5 g sucrose per 3 ml culture fluid to avoid excessive light scattering (Pfennig and Trüper 1991). Spectra were taken with a Shimadzu UV-300 spectrophotometer (Shimadzu, Kyoto, Japan) in end-on position. Cells grown with ferrous iron were washed twice with 10 mM EDTA in 50 mM Tris-HCl (pH 7.0) before sucrose was added.

For extraction of pheophytenes formed from bacteriochlorophylls, culture volumes of 10 ml were centrifuged (20 min at 3,000 × *g*) and resuspended in 1 ml distilled water. After addition of 9 ml of 0.1 M citric acid in methanol, the mixture was shaken and incubated at 4°C for 30 min. After centrifugation (20 min at 3,000 × *g*), the supernatant contained pheophytenes that were analyzed by spectrophotometry (Kontron, Zurich, Switzerland). An absorption maximum at 667.5 nm was characteristic of bacteriochlorophyll *c*, a maximum at 657.5 nm of bacteriochlorophyll *d* (N. Pfennig, Konstanz, Germany, personal communication).

Carotenes were characterized by thin-layer chromatography and absorption spectra after extraction with a methanol/acetone (2:7, v/v) mixture (Schmidt 1971).

Analysis of 16S rRNA sequences

16S-rRNA-encoding DNA fragments were amplified and sequenced as described earlier (Springer et al. 1992). In the case of the phototrophic strain KoFox, which could only be grown in coculture with strain KoFum, the identity of the determined sequence was verified by in situ cell hybridization with fluorescent-specific probes (CY3- or fluorescein-labeled; MWG-BioTech, Ebersberg, Germany) that targeted terminal regions of the 16S rRNA. Standard procedures (Manz et al. 1992; Amann 1995) were applied for in situ hybridization and detection. The sequence data have been deposited at the EMBL database under accession nos. Y18254 for strain KoFum and Y18253 for the phototrophic strain of KoFox. The new 16S rRNA sequences were fitted into an alignment of approximately 16,000 homologous full or partial primary structures available in public databases (Ludwig 1995) using the respective automated tools of the ARB software package (Ludwig and Strunk 1997). Distance matrix, maximum parsimony, and maximum likelihood methods were applied for tree construction as implemented in the ARB software package. Different datasets varying with respect to the outgroup reference organisms (sequences) included and to the alignment positions were analyzed.

Assay of redox balances

Ten replicate 50-ml culture bottles with mineral medium containing 8 mM $FeSO_4$ as sole electron source were inoculated with 2.5 ml of an outgrown defined coculture. Two bottles were assayed at the beginning, two control bottles were incubated in the dark, and these and the other six bottles were assayed at the end of the oxidation process after 35 days. The content of each culture bottle was transferred under nitrogen gas into larger glass vials containing 5 ml 37% HCl and was incubated at 100°C for 10 min to dissolve all ferric hydroxides. After centrifugation (20 min at 3,000 × *g*), samples were taken for determination of ferrous iron, ferric iron, and protein content (Lowry et al. 1951). Bacterial biomass was calculated using the formula $<C_4H_7O_3>$ (Biebl and Pfennig 1978) and was assumed to be composed of 50% protein.

Growth curves were recorded in a similar manner with three replicate cultures in 250-ml bottles sealed with butyl rubber septa and containing 200 ml medium under an atmosphere of N_2/CO_2 (80:20, v/v). Samples for determination of ferrous iron, ferric iron, and protein content were taken with syringes at intervals of 4–5 days after intensive mixing of the bottle contents and were assayed as described above.

Analytical methods

For analysis of iron salts, 5 ml of precipitate-free culture fluid was removed from 50-ml culture bottles and was replaced with 5 ml 37% HCl to dissolve all iron precipitates. Ferrous iron was quantified by the ferrozine method (Stookey 1970); total iron was quantified by the same method after reduction with hydroxylamine. The

difference between the ferrous iron and total iron concentrations was taken as the ferric iron concentration (Stookey 1970). Sulfide was quantified colorimetrically according to Cline (1969). Acetate was assayed by gas chromatography (Platen and Schink 1987).

Chemicals

All chemicals were of analytical-grade purity and were acquired from Alfa Products (Karlsruhe), Boehringer (Mannheim), Fluka (Neu-Ulm), Merck (Darmstadt), Riedel de Haen (Seelze), and Serva (Heidelberg), all in Germany. Gases at purity standard 5.0 were supplied by Sauerstoffwerke (Friedrichshafen, Germany).

Results

Enrichment of iron-oxidizing bacteria

For enrichment of novel iron-oxidizing phototrophic bacteria, 50-ml screw-capped bottles with mineral medium containing 8 mM $FeSO_4$ as electron source (precipitated as $FeCO_3$) and inocula (approximately 2–3 ml each) from sediments of various ditches were incubated at 18°C in dim light behind filters that excluded light of <740 nm wavelength. After 4–6 weeks of incubation, the white siderite precipitates had turned brownish in 47 of more than 90 different enrichment cultures. Enrichments were most successful at pH 6.8 and low light intensity (3–6 W per m^2); enrichments at pH 6.5 or 7.3, or at higher light intensities (10 W per m^2) yielded active cultures in only 10–20% of all assays. The choice of the trace element solution [SL 10 or SL 12; see Pfennig and Trüper (1991)] had no influence on the outcome of the enrichments. Cultures were transferred five to ten times in the same medium at pH 6.8, with 8 mM $FeSO_4$ as sole electron source and trace element solution SL 10. Oxidation of ferrous iron was nearly complete in the subcultures after 2–3 weeks, and in most cultures a greenish color was observed in the culture liquid. In vivo absorption spectra exhibited a maximum at 756 nm, indicating that green phototrophic bacteria with bacteriochlorophyll *c* were present. Microscopy with UV light (390–420 nm excitation wavelength) gave no indication of red fluorescence as is typical of chlorophyll-*a*-containing cyanobacteria.

In deep-agar dilutions, the green phototrophic bacteria could not be purified with 8 mM $FeSO_4$ alone or in the additional presence of 1 mM acetate, 2 or 4 mM ascorbate, or 80% hydrogen in the head space. Dark green colonies grew only in the first dilution tube and always contained several morphological types of bacteria. In alternative dilution series with 4 mM sulfide as electron source, colonies were formed as far up as the third dilution tubes and were composed of green centers with white satellites, sometimes also with red components. Liquid cultures inoculated with cell material from these colonies oxidized ferrous iron within 3–4 weeks and contained at least three types of bacteria: short rods, motile vibrioid cells, and a few stalked cells reminiscent of *Rhodomicrobium vannielii* (Widdel et al. 1993). In subsequent dilution series with 2 mM sulfide and 0.5 mM $FeSO_4$, the stalked cells could be outdiluted. However, the other two cell types could not be separated in dilution experiments with either sulfide at various concentrations (0.5–2 mM) or hydrogen as additional electron source, and the same problem arose with five different enrichment cultures treated in the same manner. After growth in liquid medium with 8 mM $FeSO_4$, an enrichment culture from a ditch close to the biology building at the University of Konstanz showed a ratio of short rods to vibrios of approximately 1,000:1; this culture was used for further purification studies. Nonetheless, this culture also did not yield a pure culture of the phototrophic bacterium in dilutions in liquid or in agar medium. Therefore, we first purified the contaminating vibrioid bacterium.

Purification and properties of the vibrioid partner bacterium

Since the morphology of the vibrioid partner bacterium suggested a relationship to *Campylobacter* sp., we tried to purify this bacterium with fumarate as sole substrate. Transfers into liquid medium with 5 mM fumarate caused a dramatic increase of the vibrioid cells (over 90%) as compared to the short rods, and in two subsequent agar dilution series starting from the phototrophic enrichment culture, the vibrioid bacterium (strain KoFum) was isolated in pure culture with fumarate as substrate.

Cells of strain KoFum were highly motile vibriod rods, 0.5×2–4 μm in size, and often appeared in pairs or short chains (Fig. 1a). Cells were gram-negative upon staining (Bartholomew 1962) and KOH Gram typing (Gregersen 1978), carried one polar flagellum each, and were catalase-negative and oxidase-positive. The strain grew at 30°C with fumarate, with a doubling time of approximately 1 h, and fermented fumarate, malate, or aspartate completely to succinate, acetate, and butyrate roughly in a 7:1:0.3 ratio. Pyruvate was fermented to acetate, hydrogen, and carbon dioxide. Formate, acetate, lactate, ethanol, glucose, fructose, xylose, and arabinose were not oxidized or fermented; sulfate, thiosulfate, or elemental sulfur were not reduced. Nitrate was reduced to dinitrogen gas with hydrogen, fumarate, or ferrous iron as electron donor, and ferric iron was reduced only slowly with hydrogen as electron donor (approximately 7% reduced after 3 weeks of incubation).

Characterization of the phototrophic partner in the coculture

The phototrophic partner bacterium could be purified easily in deep-agar dilution series with 8 mM $FeCO_3$ in the presence of a background lawn of strain KoFum. In ferrous-iron-grown liquid cultures, strain KoFum amounted to approximately 0.1% of the total bacterial community (Fig. 1b). Cells of the phototrophic bacterium, strain KoFox, were 0.5×1 μm in size and often appeared in pairs (Fig. 1b). Cells were immotile and gram-negative according to staining and KOH Gram typing.

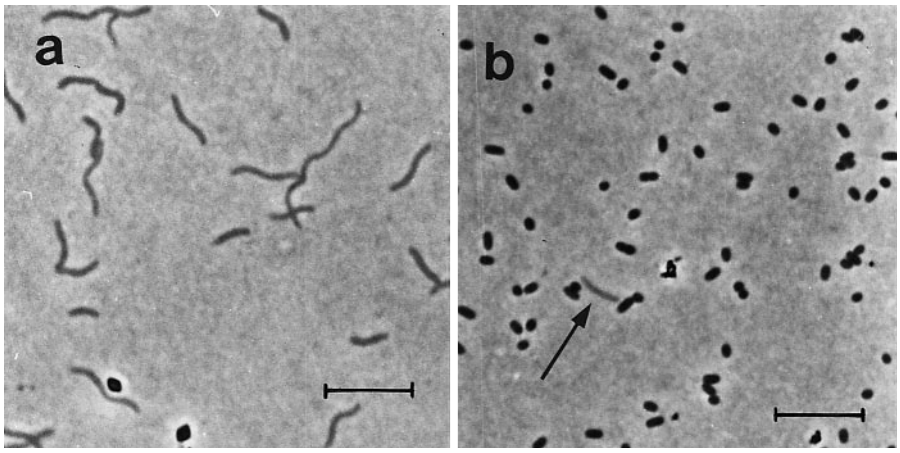

Fig. 1 Phase-contrast photomicrograph of strain KoFum and the coculture. **a** Strain KoFum grown with fumarate. **b** The binary mixed culture KoFox grown with ferrous carbonate. *Arrow* points at a cell of strain KoFum (*bar* 5 μm)

Growth with ferrous iron carbonate under optimal conditions was exponential, with a doubling time of 5.3 days (μ = 0.132 $days^{-1}$) – however, often with extended lag phases of 5–10 days. The optimum temperature for growth was 22–25°C; growth was possible at 18–30°C. The pH range was 6.3–7.1, with an optimum at pH 6.5–6.7.

The stoichiometry of ferrous iron oxidation and cell mass formation is documented in Table 1. Oxidations were always nearly 100%, with variations of 5–10%. The measured growth yield was 7.5 g cell mass per mol ferrous iron oxidized, as expected from calculations using the cell mass formula $<C_4H_7O_3>$ (Table 1). No ferrous iron oxidation was found in the dark or in noninoculated cultures.

Absorption spectra were taken with suspensions of living cells of the coculture and were compared to spectra obtained with *Chl. limicola* f. *thiosulfatophilum* grown with 2 mM sulfide. As shown in Fig. 2, the two absorption spectra were identical, with absorption maxima at wavelengths of 335, 457, and 756 nm. The maximum at 457 nm is due to the carotenoid chlorobactene, the maximum at 756 nm to bacteriochlorophyll *c* (Trüper and Pfennig 1991). The identity of bacteriochlorophyll *c* was confirmed after extraction with methanol/citric acid. The absorption spectrum exhibited a maximum at 668 nm, which is typical of bacteriopheophytene.

Although *Chl. limicola* f. *thiosulfatophilum* resembled the phototrophic component of the coculture, it did not oxidize ferrous iron in our hands; nor did it do so after addition of strain KoFum. Ferrous iron oxidation was also not observed with *Chl. vibrioforme*.

In order to optimize the growth conditions for the coculture, further possible substrates were provided either alone or together with ferrous sulfate. No growth was observed with sulfide (0.5–2 mM), thiosulfate (5 mM), or elemental sulfur alone or in various combinations with each other. Sulfide added at a concentration of 1 mM even inhibited growth with H_2. Ferrous sulfide (FeS) was oxidized very slowly in the course of incubation times of 2–3 months.

Oxidation of ferrous iron was accelerated by the presence of hydrogen, acetate, pyruvate, fumarate, cysteine, or thiosulfate (even though the latter was not used as sole electron donor); acetate and hydrogen specifically increased the number of the green bacteria in the coculture (Table 2). Nonetheless, we could not identify growth conditions that would allow growth and isolation of the phototrophic partner bacterium in pure culture; the following

Table 1 Growth and substrate oxidation balance of the binary mixed culture KoFox plus KoFum. $FeSO_4$ was added to all cultures to a final concentration of 8 mM

Growth conditions	Fe(II) oxidized (mM)	Cell dry mass measured ($mg \times l^{-1}$)	Cell dry mass expected ($mg \times l^{-1}$)	Growth yield found [mg cell dry mass × $(mmol\ FeSO_4)^{-1}$]	Electron balance (%)
Cultures incubated in the light[a]	6.31	47.9	47.3	7.6	101
	2.72	15.5	20.4	5.7	76
	2.43	20.0	18.2	8.2	110
	1.84	13.3	13.8	7.2	96
	6.99	54.7	52.4	7.8	104
	5.83	45.9	43.7	7.9	105
Sterile cultures incubated in the dark	0.1	2.8			
	0.08	–2.9			
Cultures incubated in the dark	0.00	0.2			
	0.16	–4.0			

[a] Results of six parallel experiments

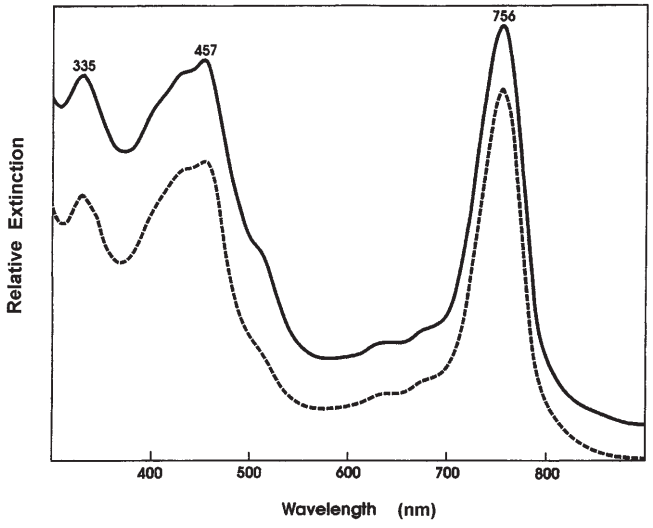

Fig. 2 Absorption spectra of living cells of the binary mixed culture (*solid line*) and *Chlorobium limicola f. thiosulfatophilum* (*broken line*). The mixed culture was grown with ferrous carbonate under a hydrogen/carbon dioxide atmosphere; *Chl. limicola* was grown with 2 mM sulfide

additions to the medium were tried without success: yeast extract, prefermented yeast extract, sediment extract, or filter-sterilized spent medium of the coculture or of strain KoFum, both maintained under strictly anoxic conditions before application.

Reduction of ferric iron in the dark

The coculture was grown phototrophically with $FeCO_3$. After incubation in the dark with hydrogen as electron donor, the formation of ferrous iron was examined, but no significant reduction of the previously produced ferric hydroxide was observed. However, from ferric nitrilotriacetate or ferric citrate, ferrous iron was produced at rates of up to 33 nmol Fe(II) × min × (mg protein)$^{-1}$, which is in the same range of electron transfer activity as observed during phototrophic growth with $FeCO_3$ [24 nmol Fe(II) × min × (mg protein)$^{-1}$, as calculated from the growth rate and the growth yield]. This activity may at least partly be due to strain KoFum in the coculture.

Taxonomic assignment of the isolates by 16S rRNA sequence analysis

Comparative 16S rRNA sequence analysis revealed a close relationship (99.4% overall 16S rRNA sequence similarity) of the vibroid strain KoFum and of "*Geospirillum arsenophilum*" (to date still not validly described) (Ellis et al. 1997. These two strains cluster with other "*Geospirillum*" (Lonergan et al. 1996), "*Dehalospirillum*" (Scholz-Muramatsu et al. 1995), and *Sulfurospirillum* (Finster et al. 1997) species (Fig. 3). The corresponding sequence similarity values were in the range of 91.9–99.4%, whereas similarities to the *Campylobacter* species of 86.3–88.6% indicate only a moderate relationship. Both phylogenetic groups are representatives of the ε-subclass of the Proteobacteria.

Modern standard techniques allow rRNA sequence determination for uncultured organisms from mixed cultures and complex environmental samples (Amann et al. 1995). However, specific probe design and in situ cell hybridization have to be performed in order to assign a sequence to a specific morphotype. Given that the phototrophic strain in KoFox can only be grown in coculture with strain KoFum, two specific oligonucleotide probes were designed that targeted regions close to the termini of the *Chlorobium*-type 16S rRNA sequence retrieved from the coculture (KoFox173, 5′-CCGCTGCATCATCTGGTA-3′, corresponding to *E. coli* 16S rRNA positions 173–190; KoFox1240, 5′-TGCCCTCTGTAGCTACCA-3′, corresponding to *E. coli* 16S rRNA positions 1240–1257). After in situ hybridization of the coculture, the fluorescent hybridization signals of both probes could clearly be assigned only to the *Chlorobium*-like cells. This result corroborates that the determined *Chlorobium*-type sequence originated from strain KoFox. Furthermore, given that the probes have distant target sites on the rRNA molecule, the results also document that no chimeric sequence was amplified from the original coculture.

The *Chlorobium*-like partner in the coculture KoFox was shown indeed to be a representative of the green sulfur bacteria phylum and is related to *Chlorobium* (Woese et al. 1990; Wahlund et al. 1991; Figueras et al. 1997; Overmann and Tuschak 1997), *Prosthecochloris* (Overmann and Tuschak 1997), and *Pelodictyon* (Overmann and Tuschak 1997) species (Fig. 4). However, no close relationship was found to any strain (including the type

Table 2 Influence of various substrates on iron oxidation and on the composition of the binary mixed culture during growth in the light with ferrous sulfate. An anoxic medium without an additional reducing agent was used under an N_2/CO_2 atmosphere

Cultures grown with 8 mM $FeSO_4$ plus:	Acceleration of iron oxidation	Cell numbers of green bacteria increased[a]	Cell numbers of strain KoFum increased
H_2/CO_2	+	+	–
Acetate (2 mM)	+	+	–
Pyruvate (2 mM)	+	+	+
Fumarate (5 mM)	+	+	+
Cysteine (2 mM)	+	–	–
$S_2O_3^{2-}$ (2 mM)	+	–	–

[a] As compared to cultures grown with 8 mM $FeSO_4$ as sole substrate

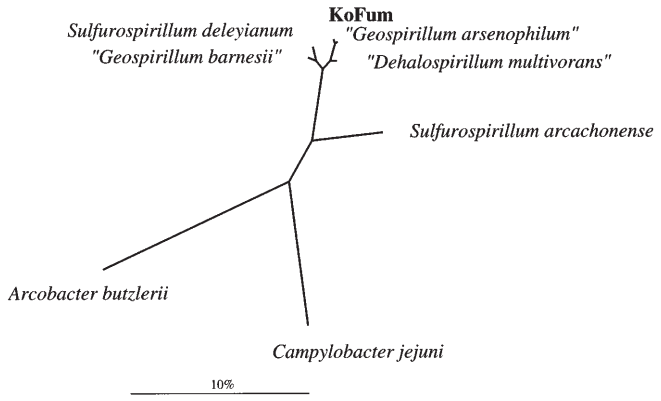

Fig. 3 16S-rRNA-based unrooted tree reflecting the phylogentic relationships of strain KoFum. The tree is based on the results of a maximum likelihood analysis of sequences from a selection of close and moderately related reference organisms of the ε-subclass of the Proteobacteria. The tree topology was evaluated by performing distance matrix and maximum parsimony analyses of all available small-subunit rRNA sequence data from representatives of the ε-subclass of the Proteobaceria and selected representatives of other major lines of descent or the full dataset. Almost complete sequences (comprising at least 90% of the gene in comparison with the corresponding *Escherichia coli* sequence) were used for the tree construction. Only sequence positions that share identical residues in at least 50% of all sequences from the *"Geospirillum"*, *"Dehalospirillum"*, and *Sulfurospirillum* cluster were included for the calculations (*bar* 10% estimated sequence divergence)

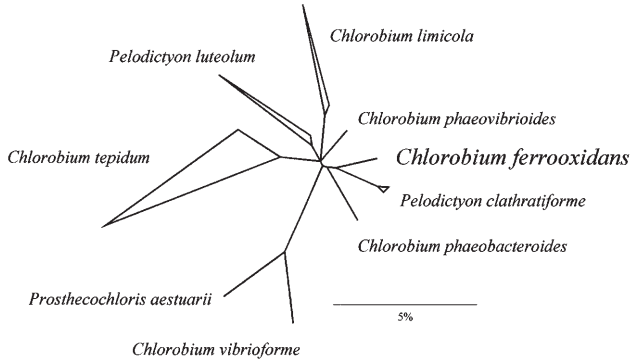

Fig. 4 16S-rRNA-based tree reflecting the phylogentic relationships of *Chlorobium ferrooxidans*, the phototrophic component of the coculture KoFox. The tree is based on the results of a maximum likelihood analysis of all available sequences from representatives of the green sulfur bacteria phylum and a selection of members of the *Cytophaga*/*Flavobacterium*/*Bacteroides* phylum. The tree topology was evaluated and corrected according to the results of distance matrix and maximum parsimony analyses of all available small-subunit rRNA sequence data from the green sulfur bacteria and *Cytophaga*/*Flavobacterium*/*Bacteroides* phyla and from selected representatives of other major lines of descent or the full dataset of the ARB sequence database (Ludwig and Strunk 1997). Multifurcations indicate that a common relative branching order was not significantly supported by application of the alternative treeing approaches. Almost complete sequences (comprising at least 90% of the gene in comparison with the homologous *Escherichia coli* sequence) were used for the tree construction. Only sequence positions that share identical residues in at least 50% of all sequences from the representatives of the green sulfur bacteria phylum were included for the calculations. The *triangles* indicate phylogenetic groups. The *Chlorobium tepidum* cluster comprises *Chl. tepidum* ATCC 49652^T, *Chl. limicola* strains DSM 246, UdG 6038, UdG 6040, UdG 6041, UdG 6042, UdG 6043, UdG 6044, and UdG 6045, and *Chlorobium vibrioforme* strains DSM 263, UdG 6026, and UdG 6043. The *Chlorobium limicola* cluster comprises *Chl. limicola* 6330 (DSM 245^T) and *Chlorobium phaeobacteroides* strains UdG 6046, UdG 6047, and UdG 6051. The *Chlorobium phaeovibrioides* branch comprises only *Chl. phaeovibrioides* strain 2631 (DSM 269^T). The *Pelodictyon clathratiforme* cluster comprises *Pld. clathratiforme* GP and Pelodictyon phaeoclathratiforme BU-1 (DSM 5477^T). The *Pelodictyon luteolum* cluster comprises *P. luteolum* 2530 (DSM273^T), *Chl. vibrioforme* 6132 (DSM 262), and Chl. phaeovibroides UdG 6035. The *Prosthecochloris aestuarii* cluster comprises *Chl. vibrioforme* DSM 260 and *P. aestuarii* SK 413 (DSM 271^T) (*bar* 5% estimated sequence divergence)

strains) for which rRNA sequence data currently are available. Overall 16S rRNA sequence similarity values of 91.4–96.7% indicate that our strain represents a separate line of descent within a *Chlorobium*/*Pelodictyon*/*Prosthecochloris* cluster.

Discussion

Ferrous iron oxidation by a green phototrophic bacterium

Oxidation of ferrous iron by phototrophic bacteria is still a novel phenomenon that to date has been observed only with phototrophic purple sulfur or non-sulfur bacteria (Widdel et al. 1993; Ehrenreich and Widdel 1994; Heising and Schink 1998). Phototrophic oxidation of ferrous iron has been discussed in a broader perspective mainly with reference to its possible implications for our understanding of the early evolution of life on Earth. The question arose whether the banded iron formations are really indicative of the first appearance of oxygen in the atmosphere (Walker et al. 1983; Beukes and Klein 1992) due to an oxygenic two-step photosynthesis by cyanobacteria, for example, or whether these oxidized layers could also have been produced by anoxygenic phototrophs using only one light reaction (Widdel et al. 1993; Ehrenreich and Widdel 1994). Our finding that ferrous iron can also be oxidized by green anoxygenic phototrophic bacteria may add to further speculations on the question of how banded iron formations originated; since the green phototrophs represent a separate group within the domain Bacteria, this may indicate that phototrophic ferrous iron oxidation was a widespread metabolic capacity in an early phase of evolution.

On the basis of comparisons of the redox potential of the couples $FeOOH/FeCO_3$ and the acceptor redox potential of green phototrophs (Widdel et al. 1993; Gottschalk 1985), ferrous iron oxidation by green anoxygenic phototrophic bacteria was expected to be possible. However, growth of these bacteria is slow, and green phototrophs can be enriched with ferrous iron only at a comparably low temperature (18°C). Incubation at higher temperatures (20–25°C) selects for growth of *Rhodomicrobium vannielii* (Widdel et al. 1993; Heising and Schink 1998). The defined coculture characterized in the present study was substantially slower (t_d = 5.3 days) than the pure cultures of purple bacteria described previously (doubling times of 2–4.5 days;

Ehrenreich and Widdel 1994). Thus, the green phototrophs would hardly have a chance of being enriched in conventional enrichment cultures with ferrous iron at higher temperatures, and this may explain why they have been overlooked in our earlier studies.

Oxidation of ferrous iron by the defined coculture was stoichiometrically coupled to biomass formation from CO_2 according to the equation:

$$17\ FeCO_3 + 29\ H_2O \rightarrow 17\ Fe(OH)_3 + <C_4H_7O_3> + 13\ CO_2.$$

Iron oxidation was nearly complete; a small amount of remnant ferrous iron (approximately 5% of the substrate provided) was probably not accessible to the bacteria, perhaps due to formation of mixed oxides, e.g., magnetite. Growth was exponential, although the free Fe^{2+} ion concentration in the medium in equilibrium with $FeCO_3$ is only approximately 3 μM (Ehrenreich and Widdel 1994). The green bacteria were obviously more efficient in the release of the insoluble ferric iron oxides than is *R. vannielii,* which encrusts itself in rusty layers that severely limit metabolic activity after growth with ferrous iron (Heising and Schink 1998). It has been postulated that the purple phototrophic strains L7 and SW2 transfer the electrons from ferrous iron by a carrier system across the periplasmic space to the reaction center in the cytoplasmic membrane (Ehrenreich and Widdel 1994), but this postulated electron carrier still awaits identification.

Compared to other green phototrophs, strain KoFox behaves atypically in not utilizing sulfide as sole electron source, but a thorough evaluation of the known strains of green phototrophs revealed that actually more such bacteria are sensitive to free sulfide (J. Overmann, Oldenburg, Germany, personal communication). Ferrous sulfide was utilized so slowly that this capacity can hardly be used as a reliable physiological criterion for taxonomic purposes.

The physiological importance of ferrous iron oxidation for our strain may be substantial since we could find only a few alternative electron sources that supported phototrophic growth of this strain. Whether the oxidized ferric iron hydroxides can act as electron acceptors for a respiratory metabolism in the dark still needs to be elucidated. We found ferric iron reduction only with chelated ion forms such as ferric citrate or ferric nitrilotriacetate. The ecological significance of these activities is questionable since chelated iron reacts unspecifically with many electron transfer systems. Due to this background, the reported iron reduction activities of *Rhodobacter capsulatus* (Dobbin et al. 1996) and of many heterotrophic iron reducers (Lovley 1993) also need to be critically revised because they have nearly exclusively been obtained with chelated ferric iron forms whose importance in natural environments is hard to assess.

Cooperation between the partners in the coculture

In spite of many efforts, we could not separate the green component of the coculture from its *"Geospirillum"*-like chemoheterotrophic partner organism. The number of the partner cells after growth with ferrous iron was so low that one cannot expect that these partners fulfilled any substantial function in energy generation comparable, for example, to that of the partners in syntrophic associations (Pfennig 1980; Schink 1997). Furthermore, the difference between the redox potentials of the $FeOOH/FeCO_3$ couple (+200 mV) and that of the reaction center of the green phototrophs (+300 mV) can hardly allow for a functional niche of a further bacterium in electron transfer between these two systems, and not even the supply of an iron-chelating agent could be expected from such a partner due to its low number. Rather, we have to assume that the chemoheterotrophic partner provides some trace nutrient(s) to the phototroph that we are thus far unable to provide with our medium. Such relationships have been reported earlier, e.g., for the extremely low-light-adapted culture *Chlorobium phaeobacteroides* from the redox interface of the Black Sea (Overmann et al. 1992), or for fermenting defined cocultures that degrade resorcinol (Tschech and Schink 1985) or dipicolinic acid (Seyfried and Schink 1990). In all these cases, vibrioid partner bacteria similar to strain KoFum have been present in low numbers and have been essential for growth although their function in the coculture has never been resolved.

Taxonomy of the two bacterial strains

On the basis of its morphology, pattern of substrate utilization and product formation, and lack of acetate oxidation capacity, the chemoheterotrophic bacterium strain KoFum was assumed to be related to the genus *Campylobacter*, which together with the genera *Arcobacter, Alvinella, "Dehalospirillum", "Geospirillum", Helicobacter, Wolinella,* and *Sulfurospirillum* represents the ε-subclass of the Proteobacteria. The comparative analysis of its 16S rRNA sequence data corroborated a moderate relationship to *Campylobacter* strains (Fig. 3); however, the closest relative is *"Geospirillum arsenophilum",* as indicated by 99.4% 16S rRNA sequence similarity. *"Dehalospirillum", "Geospirillum",* and *Sulfurospirillum* represent a phylogenetic sister group of *Campylobacter*.

The green component of the coculture was identified as a relative of a phylogenetic group comprising *Chlorobium*, *Pelodictyon,* and *Prosthecochloris* species. However, no close relative is known to date, and strain KoFox represents its own line of descent within this group. Based on comparison of 16S rRNA sequence similarity data, it has been shown previously that the various strains grouped in species of the genus *Chlorobium* appear to be a heterogeneous collection of different organisms (Figueras et al. 1997; Overmann and Tuschak 1997). Subgroups containing strains of different *Chlorobium* species have been defined by Figueras et al. (1997). The majority of these clusters is also supported by analysis of the enlarged dataset available today. However, there is no significant evidence for a separation of brown (*Chl. phaeobacteroides* and *Chl. phaeovibroides*) and green (*Chl. limicola, Chl. tepidum,* and *Chl. vibrioforme*) species as pos-

tulated by Figueras et al. (1997). These authors have found a clear correlation of 16S-rRNA-based phylogeny and the content of green and brown bacterial chlorophylls. According to their analyses, a clear separation of green- and brown-pigmented chlorobia is supported by the rRNA data. The comparative analysis of the now-enlarged dataset of 16S rRNA sequences does not support such a clear separation (Fig. 4); similar results have been obtained recently by Overmann and Tuschak (1997). Furthermore, not only strains of different *Chlorobium* species appear to be phylogenetically intermixed; a similar situation has also been found for *Prosthecochloris* and *Pelodictyon* strains (Overmann and Tuschak 1997).

Nonetheless, strain KoFox differs from all *Chlorobium* strains in its obvious lack of sulfide oxidation since species of the genus *Chlorobium* by definition oxidize sulfide, sulfur, and often thiosulfate. Utilization of acetate is typical of *Chlorobium* species (Pfennig and Trüper 1971). These differences are supported by (moderate) overall 16S rRNA sequence similarities of 91.4–96.3%.

Here, the new species *Chlorobium ferrooxidans* is proposed, with the phototrophic component of the culture KoFox as the type strain.

Formal description of *Chlorobium ferroxidans* sp. nov.

Chlorobium ferrooxidans (sp. nov.) fer.ro.ox'i.dans; *ferrum* L.n.n., iron; *oxidans* M.L.adj., oxidizing; *ferrooxidans*, iron-oxidizing.

Short, rod-shaped cells, 0.5x1.0–1.5 μm in size, with rounded ends, single or in pairs. Nonmotile, gram-negative, nonsporeforming.

Strictly anaerobic phototroph. Oxidizes ferrous iron provided in the medium as ferrous carbonate to ferric iron hydroxides. Hydrogen or acetate enhance ferrous-iron-dependent growth, and hydrogen is also used as sole electron source. Sulfide not oxidized. No assimilation of organic substrates other than acetate. Grows in freshwater medium with ferrous iron as sole electron source, in defined coculture with a "*Geospirillum*"-like bacterium, strain KoFum.

Selective enrichment from freshwater ditches and ponds at 18°C under dim light behind light filters that exclude light of 740 nm wavelength.

pH range, 6.3–7.1; pH optimum, 6.5–6.7. Temperature range, 18–30°C; temperature optimum, 22–25°C.

Habitat: anoxic sediments of shallow freshwater ditches.

Type strain: KoFox, deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

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