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Ferrihydrite reduction by *Geobacter* species is stimulated by secondary bacteria

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Abstract *Geobacter* species such as *G. bremensis*, *G. pelophilus*, and *G. sulfurreducens* are obligately anaerobic and grow in anoxic, non-reduced medium by fast reduction of soluble ferric citrate. In contrast, insoluble ferrihydrite was either only slowly or not reduced when supplied as electron acceptor in similar growth experiments. Ferrihydrite reduction was stimulated by addition of a reducing agent or by concomitant growth of secondary bacteria that were physiologically and phylogenetically as diverse as *Escherichia coli*, *Lactococcus lactis*, or *Pseudomonas stutzeri*. In control experiments with heat-inactivated *Geobacter* cells and viable secondary bacteria, no (*E. coli*, *P. stutzeri*) or only little (*L. lactis*) ferrihydrite was reduced. Redox indicator dyes showed that growing *E. coli*, *P. stutzeri*, or *L. lactis* cells lowered the redox potential of the medium in a similar way as a reducing agent did. The lowered redox potential was presumably the key factor that stimulated ferrihydrite reduction by all three *Geobacter* species. The observed differences in anoxic non-reduced medium with ferric citrate versus ferrihydrite as electron acceptor indicated that reduction of these electron acceptors involved different cellular components or different biochemical strategies. Furthermore, it appears that redox-sensitive components are involved, and/or that gene expression of components needed for ferrihydrite reduction is controlled by the redox state.

Keywords Ferrihydrite reduction · *Geobacter bremensis* · *Geobacter pelophilus* · *Geobacter sulfurreducens* · Secondary bacteria · *Escherichia coli* · *Lactococcus lactis* · *Pseudomonas stutzeri* · Redox potential · Redox indicator dyes

Dedicated to Prof. Dr. Dr. h.c. mult. Hans Günter Schlegel on the occasion of his 80th birthday.

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Introduction

Dissimilatory ferric-iron-reducing bacteria catalyze the reduction of ferric iron oxides to ferrous iron in anoxic habitats and are thought to play an important role in the geochemistry of iron (Thamdrup 2000). The biochemical details of electron transfer to ferric iron oxides have not yet been elucidated. In particular, it is unclear whether electron transfer requires direct physical contact between the bacterial cell and ferric iron oxide (Lovley 2000; Straub et al. 2001). Nonetheless, ferric iron reduction is stimulated by electron-shuttling molecules such as humic substances or anthraquinone-2,6-disulfonate (AQDS), a humic acid analogue (Lovley 2000). In the last decade, many species of ferric-iron-reducing bacteria have been isolated, several of them belonging to the genus *Geobacter* within the δ-subgroup of Proteobacteria (e.g. Lovley 1997; Snoeyenbos-West et al. 2000). *Geobacter sulfurreducens*, *Geobacter bremensis*, and *Geobacter pelophilus* were isolated from freshwater sediments and are able to grow by reduction of ferrihydrite, an important ferric iron oxide in nature (Caccavo et al. 1994; Straub et al. 1998; Straub and Buchholz-Cleven 2001). All three species are obligate anaerobes. Routinely, they are cultivated in the presence of a reducing agent such as cysteine. Since it is currently discussed whether cysteine can serve as an electron shuttle in the reduction of ferrihydrite (Nevin and Lovley 2000; Doong and Schink 2002), we sought for a possibility to replace cysteine in the cultivation of ferric-iron-reducing bacteria. However, most other chemical reducing agents (e.g., sulfide, dithionite) reduce ferrihydrite directly.

Removal of oxygen may not be sufficient to allow growth of obligate anaerobic bacteria since most of them require, in addition, a low redox potential. Many strictly anaerobic bacteria are inhibited at E_h values higher than –100 mV and very sensitive strains even require E_h values below –330 mV for growth. Hence, the redox potential is important among the physicochemical factors that affect growth of anaerobic bacteria (Breznak and Costilow 1994). For facultative anaerobes, the redox potential is

also important since it controls, via redox-sensing molecules and regulators, the expression of genes that are involved e.g., in nitrogen and/or carbon fixation, or the utilization of hydrogen (reviewed by Bauer et al. 1999). A well-studied example is the regulator FNR in *Escherichia coli* which is responsible for controlling aerobic–anaerobic regulation of over 120 target genes (Sawers et al. 1988; Guest et al. 1996; Unden et al. 2002).

Many bacteria can lower the redox potential of a medium prior to or during growth (Jacob 1970). In particular, facultatively anaerobic bacteria can therefore substitute for reducing agents as they scavenge residual oxygen and lower the redox potential of the medium. Smith and Hungate (1958) isolated methanogenic bacteria using *E. coli* as a secondary bacterium. It should be noted that an oxygen-free, non-reduced growth medium is not "buffered" with respect to its redox properties, and that release or surface exposure, even of very few redox-active molecules, may dramatically change the measurable redox potential.

In the present study, we show that *Escherichia coli*, *Pseudomonas stutzeri*, or *Lactococcus lactis* as secondary bacteria stimulate ferrihydrite reduction by different *Geobacter* species. *E. coli* belongs to the γ-subdivision of Proteobacteria and grows either by respiration or by fermentation. *P. stutzeri* also belongs to the γ-subdivision of Proteobacteria, and its metabolism is restricted to aerobic or anaerobic respiration (nitrate reduction). In contrast, *L. lactis* is a gram-positive bacterium belonging to the *Bacillus/Clostridium* group that is able to grow only by fermentation, lacks cytochromes, and does not require iron for growth. These three physiologically and phylogenetically different bacteria were chosen as secondary bacteria in order to ensure that they did not interfere specifically with iron reduction catalyzed by the *Geobacter* species studied.

Materials and methods

Sources of bacteria

G. bremensis (DSM 12179) and *G. pelophilus* (DSM 12255) were obtained from subcultures that had been kept since the isolation of these organisms (Straub et al. 1998; Straub and Buchholz-Cleven 2001). *G. sulfurreducens* (DSM 12127) was obtained from D.R. Lovley (Amherst, USA), *E. coli* strain K12 MC 4100 (DSM 6574) from R. Dippel (Konstanz), *L. lactis* subsp. *lactis* (DSM 20481) from D. Schmitt-Wagner (Konstanz), and *P. stutzeri* (DSM 5190) from S. Gerhardt (Konstanz).

Medium composition and growth conditions

Techniques for preparation of media and cultivation of bacteria under anoxic conditions have been described elsewhere (Widdel and Bak 1992). In the present study, a defined, bicarbonate-buffered freshwater medium was used; it contained per liter of distilled water: 0.3 g NH_4Cl, 0.025 g $MgSO_4 \cdot 7H_2O$, 0.4 g $MgCl_2 \cdot 6H_2O$, 0.6 g KH_2PO_4, and 0.1 g $CaCl_2 \cdot 2H_2O$. After autoclaving and cooling under an atmosphere of N_2/CO_2 (80/20 v/v), 30 ml of 1 M $NaHCO_3$ (autoclaved under CO_2), vitamins, a non-chelated mixture of trace elements, and a selenite plus tungstate solution were added (Widdel and Bak 1992). The pH was adjusted to 7.0.

Growth experiments were usually inoculated with 1% (v/v) of a fully grown pre-culture. Pre-cultures of *Geobacter* species were grown with fumarate (20–40 mM) as electron acceptor, acetate (5–10 mM) as electron donor and carbon source, and cysteine (2 mM) as reducing agent. Cultures were incubated at 28°C in the dark. For cultures growing with insoluble ferrihydrite, tubes were incubated horizontally and shaken every other day to ensure homogeneous distribution of bacteria and iron minerals.

E. coli was grown with 5 mM glucose, *L. lactis* with 5 mM glucose plus 0.1% yeast extract, and *P. stutzeri* with 5 mM acetate plus 5 mM nitrate. For heat inactivation, samples were incubated for 10 min in a water bath at 80°C; heat-inactivation was checked by growth tests. Filtrates were obtained by filtration of grown cultures through 0.2-μm filters under anoxic conditions in an anaerobic chamber; in addition, filtrates were heat-inactivated and checked for viable cells by growth tests.

Redox indicator dyes

The following redox indicator dyes were used to estimate redox potentials in growth media: methylene blue, resorufin, nile blue, phenosafranine, neutral red, or benzyl viologen (reviewed by Jacob 1970). Stock solutions (2.5 mM) were prepared in 20 mM potassium phosphate buffer (pH 7.0), filter sterilized, and stored in the dark at 4°C; the final concentration of the dyes was 25 μM each. The different dyes were tested separately.

Synthesis of ferrihydrite

Synthetic ferrihydrite was produced as described by Lovley and Phillips (1986). The product was washed five times with a tenfold volume of distilled water. The resulting ferrihydrite suspension was deoxygenated by stirring under N_2 and repeated flushing of the headspace in a tightly sealed flask. The suspension was autoclaved and stored in the same tightly sealed flask under N_2. According to electron diffraction analysis, the pattern of the ferrihydrite was identical before and after autoclaving. Furthermore, all three *Geobacter* species reduced autoclaved ferrihydrite at the same rate as non-autoclaved ferrihydrite (K.L. Straub, unpublished data).

Analytical methods

Ferrous iron was quantified photometrically at a wavelength of 562 nm after reaction with ferrozine (Stookey

1970). Immediately before sampling, cultures were agitated to disperse iron precipitates homogeneously. Samples were taken with anoxic syringes and were immediately acidified by tenfold dilution in 1 M HCl. Ferric iron was determined similarly after reduction with 0.28 M hydroxyl ammonium chloride (final concentration in the sample); the ferrous iron concentration determined before reduction was subtracted.

Results

Oxygen-sensitivity of the three *Geobacter* species

The three *Geobacter* species differed in their sensitivity towards oxygen. *G. pelophilus* was the most and *G. sulfurreducens* the least oxygen-sensitive species. With 20 mM fumarate as electron acceptor and 5 mM acetate as electron donor, *G. pelophilus* never grew and *G. bremensis* grew sometimes in anoxic non-reduced medium, even when the medium had been freshly prepared, i.e., was less than 24 h old. In contrast, *G. sulfurreducens* also grew in non-reduced medium. However, growth was best in freshly prepared medium, and *G. sulfurreducens* lowered the redox potential in the medium during growth to approximately –110 mV as determined with redox indicator dyes. In aged medium, growth of *G. sulfurreducens* was poor, and biofilms were formed on the glass walls of the test tubes. When a reducing agent such as cysteine (2 mM) or ascorbate (2 mM) was added to the medium, all three *Geobacter* species grew well with fumarate as electron acceptor; maximal optical densities were reached after approximately 2 days of incubation.

Reduction of ferric citrate or ferrihydrite in anoxic non-reduced medium

All three *Geobacter* species reduced soluble ferric citrate in freshly prepared anoxic non-reduced medium: approximately 3.5 mM ferric citrate was reduced in growth experiments within 2–3 days. Ferric citrate was not reduced in heat-inactivated control assays (Fig. 1). When ferrihydrite was used as electron acceptor, little or no direct ferrihydrite reduction was observed in anoxic non-reduced medium (Fig. 2a,b). In some long-term growth experiments, only *G. sulfurreducens* eventually reduced the provided ferrihydrite (6 mM) completely after 8–10 weeks of incubation. Addition of 0.1 mM AQDS to similar growth experiments stimulated ferrihydrite reduction by all three *Geobacter* species; with AQDS, approximately 6 mM ferrihydrite was completely reduced within 3–4 days.

Stimulation of ferrihydrite reduction by cysteine or by secondary bacteria

Addition of 2 mM cysteine to growth media stimulated ferrihydrite reduction by all three *Geobacter* species: approximately 5 mM ferrihydrite was completely reduced within 2 weeks. Alternatively, secondary bacteria were tested for a possible growth-stimulating effect. In experiments with anoxic non-reduced medium, *Geobacter* species as primary ferric-iron-reducing bacteria and *E. coli* or *L. lactis* as secondary bacteria, ferrihydrite reduction was completed after 1–2 weeks of incubation. Growing cells of *P. stutzeri* also stimulated reduction of ferrihydrite by *Geobacter* species in anoxic non-reduced medium although not as strongly as growing cells of *E. coli* or *L. lactis*. Irrespective of the type of secondary bacterium, the pH values dropped in these growth experiments, and pH values between 6.6 and 6.8 were determined at the end of the respective experiments. Ferrihydrite reduction by *G. pelophilus* was most reliably stimulated by growth of secondary bacteria; representative growth experiments are shown in Fig. 2a. Stimulation of ferrihydrite reduction was also repeatedly observed with *G. sulfurreducens* (Fig. 2b). Only *G. bremensis* did not always respond positively upon the addition of secondary bacteria.

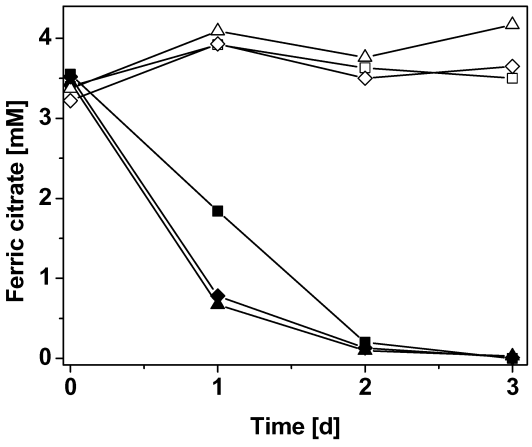

Fig. 1 Reduction of ferric citrate in growth experiments with *Geobacter bremensis* (*filled triangles*), *G. pelophilus* (*filled diamonds*), and *G. sulfurreducens* (*filled squares*) in anoxic non-reduced medium. *Open symbols* show respective experiments with heat-inactivated inoculum. Means of duplicate determinations are shown for representative cultures

Reduction of ferrihydrite by *E. coli*, *L. lactis*, or *P. stutzeri*

Neither *E. coli* nor *P. stutzeri* was able to use ferrihydrite as electron acceptor in the presence of 5 mM acetate as electron donor and carbon source; no reduction of ferrihydrite was noted after 6 weeks of incubation. In addition, *E. coli* did not reduce ferrihydrite during fermentative growth on 5 mM glucose (Fig. 3). In contrast, *L. lactis* reduced approximately 1 mM ferrihydrite during fermentative growth for 1 week with 5 mM glucose (Fig. 3). Addition of a mixture of heat-inactivated cells of the three *Geobacter* species to growth assays with *E. coli*, *L. lactis*, or *P. stutzeri* did not stimulate ferrihydrite reduction (Fig. 3).

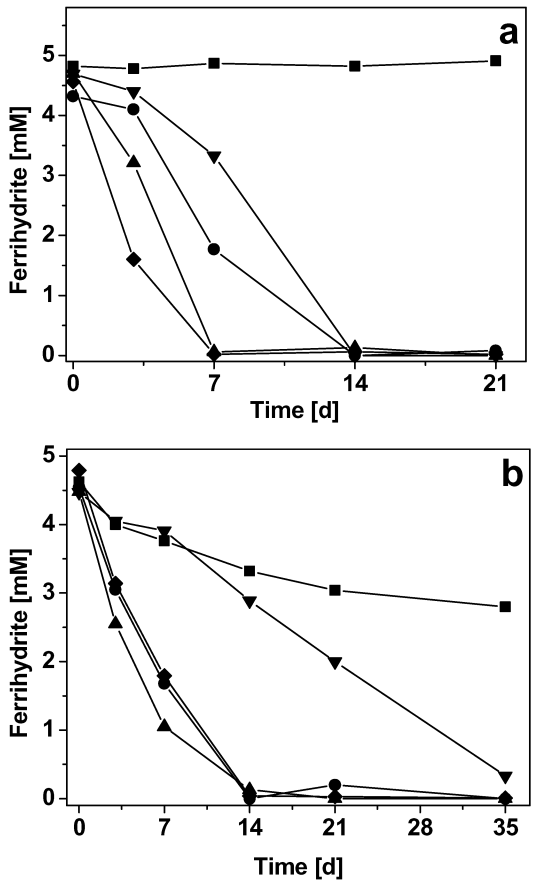

Fig. 2 Reduction of ferrihydrite in growth experiments with **a** *G. pelophilus* and **b** *G. sulfurreducens* in anoxic non-reduced medium. Experiments without further additions (*filled squares*), addition of 2 mM cysteine (*filled circles*), or concomitant growth of *Escherichia coli* (*filled triangles*), *Lactococcus lactis* (*filled diamonds*), or *Pseudomonas stutzeri* (*filled inverted triangles*) were run in parallel. Means of duplicate determinations are shown for representative cultures

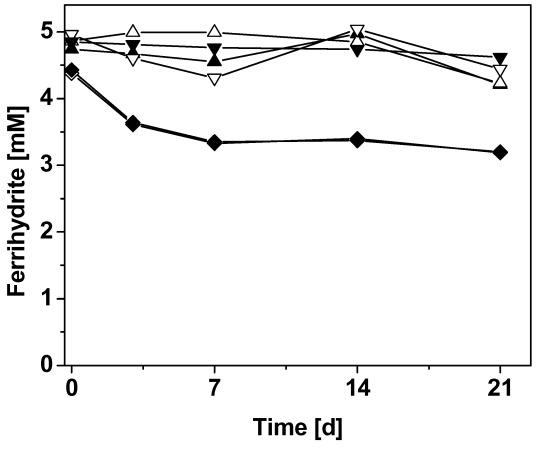

Fig. 3 Reduction of ferrihydrite in growth experiment with *E. coli* (*filled triangles*), *L. lactis* (*filled diamonds*), or *P. stutzeri* (*filled inverted triangles*). *Open symbols* show respective experiments with the addition of a mixture of heat-inactivated cells of the three *Geobacter* species. Means of duplicate determinations are shown for representative cultures

Table 1 Stimulation of ferrihydrite reduction in growth experiments with *G. pelophilus*. All growth experiments were supplied with 5–6 mM ferrihydrite as electron acceptor and 5 mM acetate as electron donor. In co-culture experiments with secondary bacteria additional substrates were supplied: 5 mM glucose for *E. coli*, 5 mM glucose plus 0.1% yeast extract for *L. lactis*, or 5 mM acetate plus 5 mM nitrate for *P. stutzeri*. Viable or heat-inactivated cells, or culture filtrate were added at a ratio of 10% (v/v). Symbols: (++) complete reduction of ferrihydrite within 7 days, (+) complete reduction of ferrihydrite within 14 days, (–) no stimulation of ferrihydrite reduction

Additions to growth experiments	***E. coli***	***L. lactis***	***P. stutzeri***
Inoculum plus additional substrates	++	++	+
Viable cells	+	–	+
Heat-inactivated cells	+	–	–
Anoxic heat-inactivated culture filtrate	–	+	–

Growth experiments with *G. pelophilus* and various preparations of secondary bacteria

To further investigate the stimulation of ferrihydrite reduction by *G. pelophilus* with secondary bacteria, experiments were set up with different preparations of secondary bacteria. At a ratio of 10% (v/v) each, either viable cells, heat-inactivated cells, or heat-inactivated anoxic filtrate of grown cultures of either *E. coli*, *L. lactis*, or *P. stutzeri* was added to anoxic non-reduced medium inoculated with *G. pelophilus*. The only substrates provided in these experiments were 6 mM ferrihydrite plus 5 mM acetate. In control experiments in the presence of substrates for the secondary bacteria plus the respective inoculum, ferrihydrite reduction by *G. pelophilus* was stimulated as described before (Fig. 2a; Table 1). Furthermore, ferrihydrite reduction was stimulated by addition of viable or heat-inactivated cells of *E. coli* or by viable cells of *P. stutzeri*. However, ferrihydrite reduction under these conditions was significantly slower than in the control experiments with growing secondary bacteria. Surprisingly, addition of heat-inactivated anoxic filtrate of *L. lactis* cultures stimulated ferrihydrite reduction by *G. pelophilus* as well. No stimulation of ferrihydrite reduction was observed when the pH was adjusted to 6.7 or upon the (sole) addition of 5 mM glucose, 0.1% yeast extract, 5 mM nitrate, or 5 mM lactate to anoxic non-reduced medium. Results for *G. pelophilus* are summarized in Table 1. Similar results were obtained in the same type of experiments with *G. sulfurreducens*. In contrast, *G. bremensis* responded inconsistently in such experiments.

Assessment of redox potentials with redox indicator dyes

In order to estimate the redox potentials in various cultures, the redox dyes methylene blue (E_0'=+11 mV), resorufin (E_0'=–51 mV), nile blue (E_0'=–142 mV), phenosafranine (E_0'=–252 mV), neutral red (E_0'=–325 mV), or benzyl viologen (E_0'=–359 mV) were used. Controls showed that these dyes were neither toxic at the applied concen-

Table 2 Approximate values of redox potentials determined with a combination of different redox indicator dyes. As substrates were supplied: 5 mM glucose for *Escherichia coli*, 5 mM glucose plus 0.1% yeast extract for *Lactococcus lactis*, or 5 mM acetate plus 5 mM nitrate for *Pseudomonas stutzeri*. Viable or heat-inactivated cells, or culture filtrate were added at a ratio of 10% (v/v)

Additions to anoxic non-reduced medium	***E. coli*** (mV)	***L. lactis*** (mV)	***P. stutzeri*** (mV)
Inoculum and substrates	<–390	~–390	~–200
Viable cells	<–390	~–50	~–50
Heat-inactivated cells	~–200	>+70	>+70
Heat-inactivated anoxic culture filtrate	>+70	>+70	>+70

trations nor were they used as substrate by any of the strains studied. In sterile anoxic non-reduced medium, the redox potential was above +70 mV and was not altered by addition of ferrihydrite. when 2 mM cysteine was added, the redox potential in the medium dropped to approximately –110 mV within few hours. Redox potentials were determined in accordance with the growth experiments with different *Geobacter* species (Tables 1, 2). Growth of *E. coli*, *L. lactis*, or *P. stutzeri* lowered the redox potential in the medium to below –390 mV, around –390 mV, and around –200 mV, respectively. Even when only viable cells of *E. coli* were added to the medium without glucose, a decrease in the redox potential to below –390 mV was noted. Addition of viable *L. lactis* or *P. stutzeri* cells to sterile medium decreased the redox potential only to around –50 mV. However, the redox indicator dyes responded much faster in actively growing cultures. The addition of heat-inactivated cells of *E. coli* still decreased the redox potential of medium slowly to around –200 mV. Upon addition of heat-inactivated anoxic filtrates obtained from grown cultures of *E. coli*, *L. lactis*, or *P. stutzeri*, no change in the redox potential was observed. These results are summarized in Table 2.

Discussion

The three dissimilatory ferric-iron-reducing species *G. bremensis*, *G. pelophilus*, and *G. sulfurreducens* are strict anaerobes and are unable to grow with oxygen as electron acceptor (Caccavo et al. 1994; Straub et al. 1998). With all three species, only slow or no direct reduction of insoluble ferrihydrite was observed in anoxic non-reduced medium (Fig. 2). However, when ferric citrate was used as electron acceptor instead of ferrihydrite, all three *Geobacter* species grew well in freshly prepared anoxic non-reduced medium (Fig. 1). Ferric citrate differs from ferrihydrite in two important aspects: it is soluble at pH 7 and its reduction to the ferrous form occurs at a much higher redox potential (+372 mV) than ferrihydrite (–100 mV to +100 mV; Brookins 1988; Widdel et al. 1993; Thamdrup 2000). *Geobacter* species were also able to grow in freshly prepared anoxic non-reduced medium when soluble AQDS mediated the reduction of ferrihydrite. These differences between the reduction of soluble ferric citrate and AQDS, on one hand, and the reduction of insoluble ferrihydrite, on the other, indicate that different cellular components are involved in these processes.

All three *Geobacter* species showed good growth in the presence of a reducing agent such as cysteine, irrespective of whether ferrihydrite or fumarate was supplied as the electron acceptor. Stimulation of ferrihydrite reduction by *G. sulfurreducens* upon addition of cysteine was previously observed and it was speculated that the redox couple cystine/cysteine could act as an electron shuttle between the cells and ferrihydrite (Doong and Schink 2002). In that study, the function of cysteine as a reducing agent, i.e., the scavenging of oxygen and lowering of the redox potential, was not taken into consideration. Although fumarate is a soluble electron acceptor at pH 7 and the enzymatic reduction probably proceeds directly, reduction of fumarate also was stimulated by the addition of cysteine in all three *Geobacter* species. This observation indicated that cysteine might be more important as a reducing agent than as an electron shuttle when *Geobacter* species are grown with ferrihydrite as electron acceptor. This kind of stimulation of cellular activities by reducing agents was reported earlier for anaerobic bacteria and/or their enzymes that are active in the extracellular degradation of insoluble macromolecules such as cellulose, chitin, or keratin (e.g., Forsberg and Groleau 1982; Johnson et al. 1982; Giuliano and Khan 1984; Pel and Gottschal 1987; Riffel et al. 2003). These stimulations by reducing agents were often attributed to protection of enzymes from oxidation or to regulatory effects. However, reducing agents can chemically alter substrates and facilitate their utilization this way; in the case of keratin degradation by *Chryseobacterium* sp. strain kr6, the reducing agent probably reductively destabilized the substrate polymer (Riffel et al. 2003).

Some facultatively anaerobic bacteria scavenge oxygen and lower the redox potential in the medium and thus can act as reductants similar to chemical reducing agents (Smith and Hungate 1958; Breznak and Costilow 1994). The possible importance of oxygen-free conditions and of a low redox potential for efficient reduction of ferrihydrite in the three *Geobacter* species was further investigated with secondary bacteria as substitutes for reducing agents, most of which (e.g., sulfide, dithionite, ascorbate, cysteine) reduce ferrihydrite themselves. In order to avoid that a secondary bacterium specifically interfered with the reduction of ferrihydrite, three physiologically and phylogenetically diverse bacteria were chosen for co-cultivation experiments: *E. coli*, *L. lactis*, and *P. stutzeri*. Ferrihydrite reduction by *Geobacter* species in anoxic non-reduced medium was stimulated by concomitant growth of all three secondary bacteria (Fig. 2). This implies that secondary bacteria actually can replace reducing agents and that cysteine is more important as a reducing agent than as an electron shuttle in ferrihydrite reduction by *Geobacter* species. Furthermore, it is unlikely that the stimulation of ferrihydrite reduction in the presence of cysteine was due to a chemical modification of ferrihydrite caused by cysteine, e.g., changes in the surface structure.

Co-cultivation experiments with *E. coli* provided the most extensive results. *E. coli* can consume oxygen as electron acceptor, and during fermentative growth on 5 mM glucose the redox potential in the medium dropped below –390 mV (Table 2). Furthermore, the *E. coli* strain used in this study was unable to reduce ferrihydrite (Fig. 3). Stimulation of ferrihydrite reduction by *Geobacter* species was very strong in co-cultures with *E. coli* (Fig. 2; Table 1). When *E. coli* cells were alive but without substrate or were heat inactivated, ferrihydrite reduction by *Geobacter* species was still stimulated and the redox potential in the medium was lowered by addition of either preparation (Tables 1, 2). No stimulation of ferrihydrite reduction was observed upon addition of an anoxic filtrate obtained from a grown *E. coli* culture; when added to plain medium, this filtrate had no effect on the redox potential either (Tables 1, 2). Neither the sole addition of glucose or lactate nor adjusting the pH to 6.7 in anoxic non-reduced medium stimulated ferrihydrite reduction by *Geobacter* species. Taking all these observations into account, it is unlikely that the strong stimulation of ferrihydrite reduction in a co-culture with *Geobacter* species was caused by the *E. coli* substrate glucose, the major fermentation product lactate, or lowering of the pH, which accompanied the growth of *E. coli* on glucose. Furthermore, it is unlikely that *E. coli* interacted actively with ferrihydrite by any kind of association since heat-inactivated *E. coli* cells still stimulated ferrihydrite reduction by *Geobacter* species. It is also unlikely that *E. coli* cells produced and excreted a stimulating factor such as siderophores, chelators, or shuttling molecules since addition of anoxic culture filtrates had no positive effect on the rates of ferrihydrite reduction. The only positive correlation throughout all the experiments was the effect that different *E. coli* preparations had on the redox potential of the medium and the stimulation of ferrihydrite reduction by *Geobacter* species (Tables 1, 2). In active co-cultures, *E. coli* presumably effectively scavenged oxygen and lowered the redox potential in the medium sufficiently to allow efficient reduction of ferrihydrite by *Geobacter* species. We have no explanation for the inconsistent response of *G. bremensis*, which contrasted with the reproducible positive response of *G. pelophilus* and *G. sulfurreducens* to growth of *E. coli*.

Experiments with *Geobacter* species and *L. lactis* or *P. stutzeri* as secondary bacteria showed a similar correlation between redox potentials and stimulation of ferrihydrite reduction as outlined in detail for experiments with *E. coli*, with one remarkable difference: anoxic culture filtrate of *L. lactis* did not lower the redox potential in medium but stimulated ferrihydrite reduction (Tables 1, 2). We only can speculate that by filtering a grown culture of *L. lactis* through 0.2-µm filters, some cellular components were artificially released, and stimulated ferrihydrite reduction by *Geobacter* species. After all, *L. lactis* was able to transfer some electrons to ferrihydrite by a so far unknown mechanism (Fig. 3). For *Geobacter* species it was shown before that filtration can indeed lead to an artificial release of cytochromes from cells (Straub and Schink 2003).

In natural habitats characterized by complex bacterial communities, bacterial ferric iron reduction typically proceeds under oxygen-free reduced conditions (Thamdrup 2000). In that respect, the observed necessity for oxygen-free conditions and a low redox potential in the medium for effective ferrihydrite reduction by *Geobacter* species is not surprising. This observation can be explained by two possibilities, which even may be complementary: either oxygen-sensitive and/or redox-sensitive cellular compounds are involved in ferrihydrite reduction, or gene expression of cellular components involved in ferrihydrite reduction is controlled by the redox state. These hypotheses remain to be examined in future studies.

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