# Evaluation of electron-shuttling compounds in microbial ferric iron reduction

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**Abstract**

Iron-reducing bacteria can transfer electrons to ferric iron oxides which are barely soluble at neutral pH, and electron-shuttling compounds or chelators are discussed to be involved in this process. Experiments using semipermeable membranes for separation of ferric iron-reducing bacteria from ferric iron oxides do not provide conclusive results in this respect. Here, we used ferrihydrite embedded in 1% agar to check for electron-shuttling compounds in pure and in enrichment cultures. *Geobacter sulfurreducens* reduced spatially distant ferrihydrite only in the presence of anthraquinone-2,6-disulfonate, a small molecule known to shuttle electrons between the bacterial cell and ferrihydrite. However, indications for the production and excretion of electron-shuttling compounds or chelators were found in ferrihydrite-containing agar dilution cultures that were inoculated with ferric iron-reducing enrichment cultures.
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*Keywords:* Ferric iron-reducing bacteria; *Geobacteraceae*; Electron-shuttling compounds; Ferrihydrite in agar

## 1. Introduction

Dissimilatory ferric iron-reducing bacteria and (some) fermenting bacteria can transfer electrons to ferric iron, an electron acceptor which is barely soluble at neutral pH. Although the importance of bacterial ferric iron reduction has been recognized, the details of electron transfer to ferric iron oxides are still unknown. It is not clear whether an immediate physical contact between the bacterial cells and the ferric iron oxides is necessary and/or sufficient to allow electron transfer [1–3]. Electron-shuttling compounds or iron chelators are thought to be involved in electron transfer with some species of ferric iron-reducing bacteria [4,5]. A few years ago, it was speculated primarily based on in vitro assays that a small *c*-type cytochrome could act as electron shuttle with *Geobacter sulfurreducens* [6]. This hypothesis has been questioned repeatedly [7,8] and this example emphasizes the need for methods to test for electron-shuttling compounds or iron chelators in vivo.

In several studies, semipermeable membranes were used which physically separated ferric iron from ferric iron-reducing bacteria to determine whether bacteria released electron-shuttling compounds or iron chelators (e.g. [9–11]). In all these studies, there was no indication of ferric iron reduction if the ferric iron oxide was separated from the cells. These results suggested that ferric iron-reducing bacteria do not produce or release electron-shuttling compounds or iron chelators. Recently, it was demonstrated with *G. metallireducens* and anthraquinone-2,6-disulfonate (AQDS) as electron carrier that experiments with semipermeable membranes might be misleading: In experiments with semipermeable membranes which separated the cells from ferrihydrite, ferrihydrite was not reduced, not even if AQDS (previously reported to stimulate ferric iron reduction) was added [8]. In the same study, Nevin and Lovley introduced ferrihydrite-containing microporous alginate beads as an alternative test system. However, these microporous beads have a molecular-mass cutoff of 12 kDa, which allows testing only for low-molecular-mass electron-shuttling compounds or iron chelators.

Serial dilutions in anoxic agar medium are widely used for isolation of anaerobic bacteria [12]. In 1% agar, the pore size in the solidified agar medium is too small for most bacteria to penetrate, but big enough to allow diffu-

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sion even of large molecules. We used ferrihydrite embedded in agar to test for the presence of electron-shuttling or iron-chelating compounds in vivo with pure and with enrichment cultures.

## 2. Materials and methods

### 2.1. Microorganisms and medium composition

*Geobacter sulfurreducens* (DSM 12127) was obtained from D.R. Lovley; *Geobacter bremensis* (DSM 12179) and *Geobacter pelophilus* (DSM 12255) were from subcultures that had been kept since the isolation of the organisms [13,14]. A ferrous iron-oxidizing, nitrate-reducing enrichment culture [15] had been kept in our laboratory since it was established.

Inocula for enrichment cultures were collected from freshwater ditches in Konstanz and from soil in Hilden (Germany).

Techniques for preparation of media and cultivation of bacteria under anoxic conditions have been described elsewhere [12]. In the present study, a defined, bicarbonate-buffered freshwater medium was used containing (per liter of distilled water): 0.3 g of $NH_4Cl$, 0.025 g of $MgSO_4 \times 7H_2O$, 0.4 g of $MgCl_2 \times 6H_2O$, 0.6 g of $KH_2PO_4$, and 0.1 g of $CaCl_2 \times 2H_2O$. After autoclaving and cooling under an atmosphere of $N_2/CO_2$ (80/20, vol/vol), 30 ml $NaHCO_3$ solution (1 M, autoclaved under $CO_2$), vitamins, a non-chelated mixture of trace elements, and a selenite and tungstate solution [12] were added. The pH was adjusted to 7.0. Cultures were incubated at 28°C in the dark.

### 2.2. Preparation of cell suspensions

For cell suspension experiments, bacteria were grown with 10 mM acetate, 40 mM fumarate, and 2 mM cysteine as reducing agent. Cultures (500 ml) were harvested in the late exponential growth phase by centrifugation at $6000 \times g$ and 4°C for 15 min (*G. sulfurreducens*) or 20 min (*G. bremensis* and *G. pelophilus*) under anoxic conditions, and washed once with 50 mM *N*-(2-hydroxyethyl)-piperazine-*N*'-(2-ethane) sulfonic acid (HEPES), pH 7.0. Cells were resuspended in bicarbonate-buffered medium to a final concentration of approximately 2.5 mg dry matter per ml.

### 2.3. Microbial production of ferrihydrite

A lithotrophic, nitrate-reducing enrichment culture oxidized ferrous iron to 2-line ferrihydrite [13,15]. The produced ferrihydrite was washed five times with the 10-fold volume of distilled water to remove medium components. The resulting ferrihydrite suspension was deoxygenated by stirring under $N_2$ and repeated exchange of the atmosphere in a tightly sealed flask. The suspension was autoclaved and stored in the same tightly sealed flask under $N_2$.

### 2.4. Ferrihydrite embedded in agar

Agar powder was washed, melted, dispensed as 3% (wt/vol) solution in aliquots of 3 ml to test tubes sealed with aluminum caps, autoclaved, and stored at 4°C [12]. For preparation of ferrihydrite containing agar plugs, agar in tubes was melted again and kept liquid in a water bath (60°C). Aluminum caps were replaced by butyl rubber stoppers. Medium (6–8 ml) and biologically produced ferrihydrite (10 mM) were added and gently mixed with the agar. After mixing, 5 ml portions were transferred into screwcap tubes that were closed with butyl rubber stoppers and placed in an ice bath, either almost horizontally or vertically (Fig. 1). Immediately after solidification of the agar, the atmosphere was exchanged with $N_2/CO_2$ (80/20, vol/vol).

Agar dilution series with ferrihydrite (10 mM) as electron acceptor, acetate as electron donor (5 mM), and no reducing agent were prepared according to Widdel and Bak [12]. Alternative dilution series contained in addition 100 µM AQDS as electron shuttle.

### 2.5. Analytical methods

Concentrations of *c*-type cytochrome(s) were estimated by spectral analysis with a Uvikon 930 spectrophotometer (Kontron): air-oxidized samples showed spectra with absorption maxima at 408 to 410 nm. For calculations, the specific absorption coefficient ($\varepsilon = 137$ $mM^{-1}$ $cm^{-1}$) of the γ band of a small *c*-type cytochrome of *G. sulfurreducens* was used [16].

Ferrous iron was quantified photometrically at 562 nm after reaction with ferrozine [17]. Immediately before with-

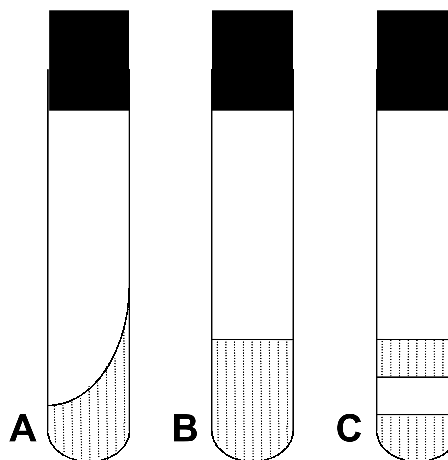


Fig. 1. A,B: Scheme of different set-ups with ferrihydrite embedded in agar. Depending on the orientation of the tube during solidification of the agar, agar plugs of different shapes were obtained. C: Alternatively, two layers of ferrihydrite-containing agar were separated by a layer of ferrihydrite-free agar.

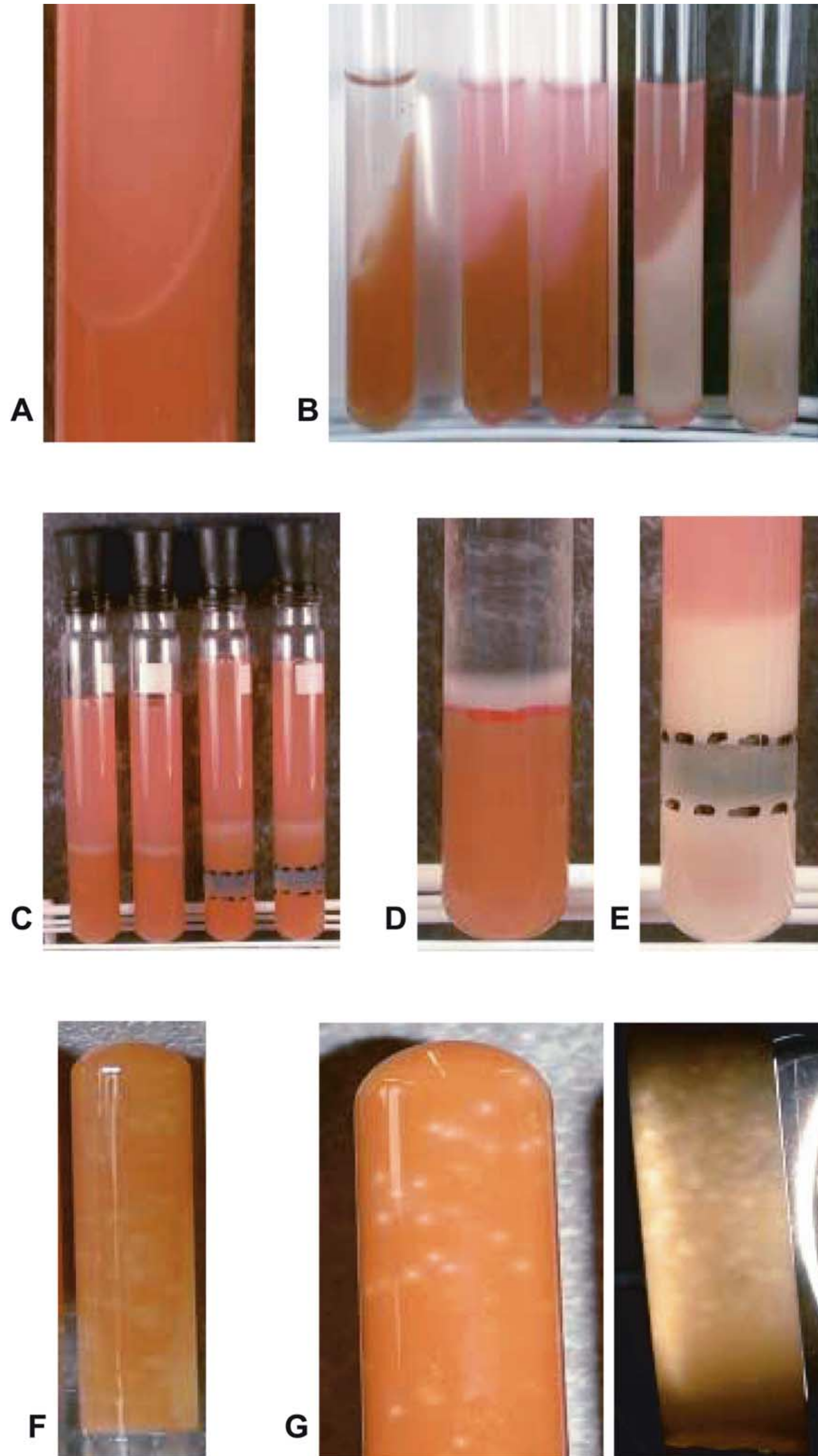


Fig. 2. A–E: Cell suspension experiments with *G. sulfurreducens* and ferrihydrite embedded in agar. A: After 3 h of incubation in the presence of 200 μM AQDS. B: After 4 days of incubation with AQDS (both tubes on the right) or without AQDS (second and third tube from the left); control with medium plus AQDS only (tube on the left). C: After 5 h of incubation in the presence of 200 μM AQDS. D: After 5 days of incubation; cells were already removed after 5 h of incubation. E: After 5 days of incubation in the presence of AQDS. F: Tube from an agar dilution series with *G. sulfurreducens*, ferrihydrite embedded in agar, acetate and 100 μM AQDS. G: Tubes of agar dilution series inoculated with two different ferric iron-reducing enrichment cultures. Note: the ferrihydrite-free agar is translucent and the background shines through.

drawing samples, cultures were agitated to disperse iron precipitates homogeneously. Samples were taken with anoxic syringes and were immediately acidified by 10-fold dilution in 1 M HCl.

## 3. Results and discussion

### 3.1. c-*Type cytochromes in* Geobacter *species*

A few years ago it was proposed that a small *c*-type cytochrome of *G. sulfurreducens* could act as an electron shuttle between the bacterial cell and ferric iron oxides [6]. This hypothesis was based mainly on the observations that cytochromes were detected in the supernatant of growing cultures at considerable concentrations, and that purified cytochrome reduced ferrihydrite (among other electron acceptors) at high rates. Similar *c*-type cytochromes were detectable in supernatants of growing cultures of *G. bremensis* and *G. pelophilus* at concentrations ranging from 120 to 150 nM. Nevertheless, further experiments with cell suspensions shed doubt on the proposed shuttle function: Purified cytochrome added to washed suspensions of *G. sulfurreducens* cells did not stimulate ferrihydrite reduction ([7], Jan Kaden, Konstanz, unpublished results). Furthermore, release of cytochromes into the medium could be stimulated in cell suspension experiments of *G. sulfurreducens*, *G. bremensis*, and *G. pelophilus* by addition of 25 mM NaCl, 25 mM $Na_2SO_4$, or 25 mM $NaNO_3$; none of these strains can use sulfate or nitrate as electron acceptor, but all three compounds increase the ionic strength of the medium. In all three *Geobacter* species, we found in addition a relationship between the cytochrome concentrations in the supernatants and the way how supernatants were obtained: five times higher cytochrome concentrations were detected in filtrates – after separation of cells by filtration through a 0.2-μm filter – than after centrifugation ($20\,800 \times g$, 15 min). Finally, after ultracentrifugation ($100\,000 \times g$, 60 min) of supernatants which were obtained from growth experiments with all three *Geobacter* species, traces of extracellular cytochromes (approximately 20–30 nM) were found only in the stationary growth phase. These results indicate that the cytochromes found in supernatants were artificially released from the cells during preparation of the supernatants, rather than intentionally released by the bacteria as electron-shuttling molecules. This observation exemplifies that putative shuttling compounds can be released artificially by inappropriate preparation procedures.

### 3.2. *Ferrihydrite embedded in agar*

Anaerobic bacteria are often isolated by dilution in growth media containing 1% agar. At this agar concentration, the pores of the solidified agar are approximately 150 nm in diameter, too small for most bacteria to penetrate but big enough to allow diffusion even of large molecules [18]. For comparison, cytochrome *c* isolated from horse heart with 12 kDa has a diameter of 1.5 nm, and β-galactosidase of *Escherichia coli* with 464 kDa has a diameter of 5.1 nm [18]. We adapted the technique of agar dilution series to prepare tubes with agar plugs that contain ferrihydrite (Fig. 1). We used ferrihydrite produced by ferrous iron-oxidizing, nitrate-reducing bacteria because it mixed with agar more homogenously than chemically precipitated ferrihydrite. Furthermore, it was shown to be a suitable electron acceptor for ferric iron-reducing bacteria, which reduced it completely to the ferrous state [13]. Concomitant with this complete reduction, a striking color change was observed: The biologically produced ferrihydrite was orange-brown in color, and during reduction white ferrous iron phosphates and carbonates were formed. In our set-up with agar-embedded ferrihydrite, orange-brown agar still contained a large percentage of ferrihydrite, whereas white agar contained mainly ferrous iron precipitates. Monitoring of the iron concentrations in the liquid medium showed that ferrihydrite was not lost from the agar; however, it cannot be ruled out that a small amount of ferrous iron diffused out of the agar (Figs. 2B,D and 3).

In order to examine whether the *c*-type cytochrome of *G. sulfurreducens* can shuttle electrons in vivo to spatially distant agar-embedded ferrihydrite, cell suspension experiments were performed in tubes with ferrihydrite-containing agar plugs at the bottom (Fig. 2A–E). Due to a high content of *c*-type cytochromes, cell pellets and cell suspensions of *G. sulfurreducens* were reddish-pink in color (Fig. 2A–C,E). To control the viability of the cells and to stimulate the metabolism, a small amount of ferrihydrite was included in the liquid medium; with acetate as electron donor, ferrihydrite was reduced in the presence or absence of AQDS (200 μM), and AQDS stimulated ferrihydrite reduction (Fig. 3). However, ferrihydrite embedded in the agar was reduced only in the presence of AQDS

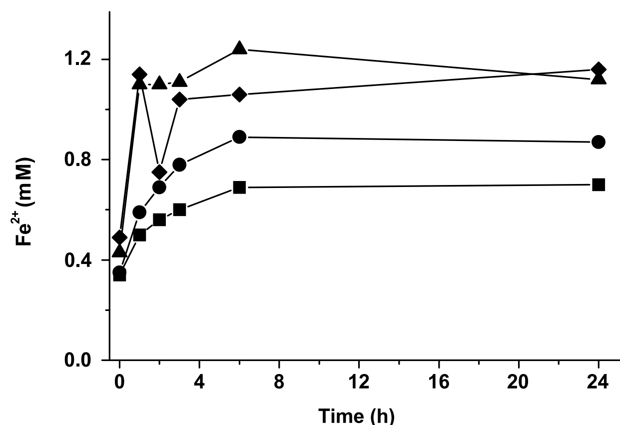


Fig. 3. Ferrous iron production in the liquid medium of a cell suspension experiment with *G. sulfurreducens* and ferrihydrite embedded in agar (same experiment as shown in Fig. 2B) in the presence (▲, ◆) or absence (●, ■) of 200 μM AQDS.

(Fig. 2A–E). This reduction of ferrihydrite embedded in agar was visible already after 2–3 h as a thin white band formed at the top of the agar plug (Fig. 2A). Complete reduction of the embedded ferrihydrite was accomplished in 4–5 days (Fig. 2B,E). AQDS alone in the absence of cells did not reduce ferrihydrite, and when cells were removed during the experiment, reduction of ferrihydrite in the agar stopped (Fig. 2B,D). In another control experiment, two layers of ferrihydrite-containing agar were separated by a layer of ferrihydrite-free agar (Figs. 1C and 2E); ferrihydrite in both agar layers was reduced. This finding indicates that actually AQDS diffused through the agar and was recycled approximately 25 times. Furthermore, in agar dilution series with *G. sulfurreducens*, *G. bremensis*, or *G. pelophilus* supplied with ferrihydrite plus acetate, cleared zones developed only in the presence of AQDS due to its shuttling capacity (Fig. 2F). In conclusion, these results demonstrate that electron-shuttling compounds can be studied in vivo with ferrihydrite embedded in agar, and that *G. sulfurreducens*, *G. bremensis*, and *G. pelophilus* did not produce or excrete any electron-shuttling molecules under these conditions.

For enrichment cultures, medium supplied with 10 mM microbially produced ferrihydrite as electron acceptor and acetate as electron donor and carbon source was inoculated with samples from two different habitats: a freshwater ditch and a water-saturated soil. In all enrichment cultures ferrihydrite reduction was observed. As it is known that humic substances can act as electron shuttle between ferrihydrite and bacteria [19,20], cultures were repeatedly transferred to outdilute possible electron-shuttling compounds from the sample material. Agar dilution series with ferrihydrite plus acetate were inoculated from the fifth transfers. In nine out of twelve agar dilution series we observed cleared zones in the agar (Fig. 2G), suggesting that these iron-reducing bacteria excreted electron-shuttling compounds or chelators. We are presently trying to isolate such organisms.

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