[4] Enrichment and Isolation of Ferric-Iron- and Humic-Acid-Reducing Bacteria

By KRISTINA L. STRAUB, ANDREAS KAPPLER, and BERNHARD SCHINK

Abstract

In anoxic habitats, ferric iron oxides and humic acids are widespread, and ferric-iron- and humic-acid-reducing microorganisms presumably play an important role in the oxidation of organic matter. Representative strains of ferric-iron- or humic-acid-reducing bacteria were isolated from a wide range of freshwater or marine environments. Most of them are strict anaerobes, and facultatively anaerobic microorganisms reduce ferric iron oxides or humic acids only after oxygen has been consumed. Hence, anaerobic techniques have to be used for the preparation of media as well as for the cultivation of microorganisms. Furthermore, special caution is needed in the preparation of ferric iron oxides and humic acids.

Introduction

Iron is the most prevalent element on Earth and the fourth most abundant one in the Earth's crust. In soils and sediments, it can make up a few percent per weight of the total dry mass. Different ferric iron oxides provide the ochre to the brown color of soils in temperate zones or the red color of tropical soils (Cornell and Schwertmann, 1996). Because iron is present in soils and sediments in the range of several 10 m*M*, ferric iron Fe(III) is the most important electron acceptor for microorganisms under anoxic conditions, especially in water-logged soils, natural wetlands, rice paddies, and freshwater lake sediments. However, ferric iron oxides are only poorly soluble at neutral pH with concentrations of soluble Fe^{3+} $\leq 10^{-9}$ *M* (Kraemer, 2004). Hence, microbial reduction of ferric iron oxides under these conditions has to cope with a practically insoluble electron acceptor. With increasing acidity, the solubility of ferric iron oxides increases, and Fe^{3+} is well soluble at pH < 2.5. Coincidentally with this solubility change, the actual redox potential of the transition between Fe(II) and Fe(III) changes dramatically, from about 100 mV at pH 7 to 770 mV below pH 2.5 (Fig. 1). Therefore, microorganisms involved in the reduction of ferric iron at neutral or at acidic pH are essentially dealing with entirely different substrates (Straub *et al.*, 2001).

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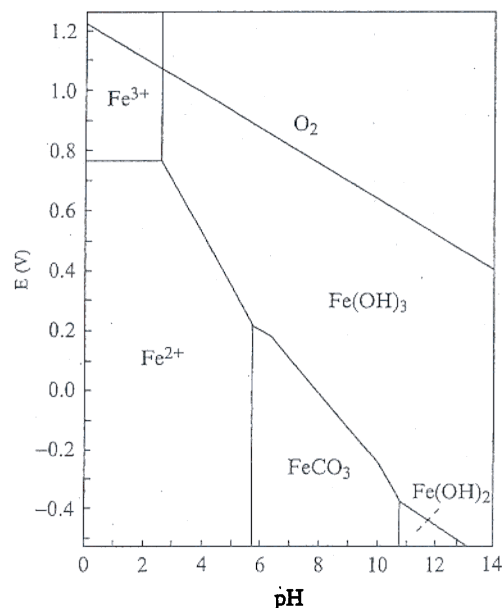

FIG. 1. Eh–pH diagram considering iron and carbonate at concentrations of 10 m*M* each, and oxygen at atmospheric pressure (0.21 atm). The thin line in the upper right refers to reduction of oxygen to water.

To overcome experimental problems of low ferric iron solubility at neutral pH, many laboratories use complexed iron species, i.e., Fe(III)-citrate, Fe(III)-NTA, or Fe(III)-EDTA as substitutes of ferric iron minerals. This may be justified for certain purposes, e.g., to grow major amounts of cell mass. However, one should be aware of pitfalls associated with the fact that a dissolved electron carrier might enter the periplasm and perhaps also the cytoplasm and may therefore be reduced in a different way than insoluble ferric iron oxides are (Straub and Schink, 2004a).

Humic compounds represent the majority of all organic matter, not only in soils but also in aquatic environments; e.g., more than 95% of all dissolved organic carbon in lake water is present as a yellow to brownish polymeric material, which is formed by the polymerization of aromatic and aliphatic precursors and is slowly decomposed by microbial activity or by photochemical reactions (Wetzel, 2001). Humic substances can be reduced by many microorganisms, such as fermenting, ferric-iron- and sulfate-reducing bacteria, and methanogenic, or hyperthermophilic archaea (Benz *et al.*, 1998; Cervantes *et al.*, 2002; Coates *et al.*, 1998; Kappler *et al.*, 2004; Lovley *et al.*, 1996, 1998, 2000). They contain quinoid structures, which

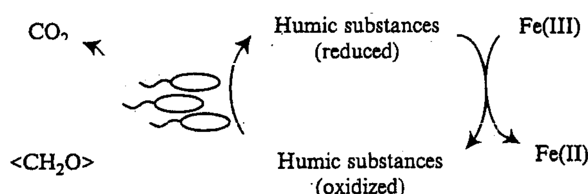

FIG. 2. Electron shuttling by humic substances: microbial oxidation of organic matter (CH_2O) with humic substances as electron acceptor and subsequent chemical reduction of Fe(III) by the reduced humic substances.

were suggested to represent their main electron-accepting moieties (Nurmi and Tratnyek, 2002; Scott *et al.*, 1998). However, complexed metal ions may also be involved in accepting electrons (Benz *et al.*, 1998; Lovley and Blunt-Harris, 1999; Struyk and Sposito, 2001), as well as conjugated aromatic constituents, which could take up electrons in delocalized π electron systems (Chen *et al.*, 2003).

Reduced humic compounds can transfer electrons directly to dissolved Fe^{3+} and various ferric iron minerals such as ferrihydrite, hematite, goethite, and ferruginous smectite (Chen *et al.*, 2003; Lovley *et al.*, 1998; Royer *et al.*, 2002; Szilágyi, 1971) (Fig. 2). Because humic substances are reoxidized during this chemical reaction, humic substances can serve again as electron acceptors for microorganisms, thus acting as electron shuttles. This allows an indirect reduction of solid-phase ferric iron without direct contact to the bacterial cells in the presence of low concentrations of humic substances, thus enhancing the rates of microbial metal reduction (Kappler *et al.*, 2004; Lovley *et al.*, 1996, 1998).

This chapter describes methods for the enrichment and cultivation of bacteria involved in the reduction of ferric iron oxides, either directly or indirectly via humic compounds. We focus primarily on the reduction of iron oxide minerals; growth media applying complexed iron, e.g., Fe(III)-citrate, have been described elsewhere (e.g., Lovley, 2000).

Preparation and Application of Ferric Iron Oxides

General Aspects

Sixteen different ferric iron oxides, hydroxides, or oxide hydroxides are known today, and they are often collectively referred to as iron oxides (Cornell and Schwertmann, 1996). Goethite, lepidocrocite, hematite, and ferrihydrite represent major iron oxides that are widespread in soils and sediments. Accordingly, these iron oxides are used frequently in laboratory

studies. In particular, ferrihydrite, the least crystallized ferric iron oxide, is used in most studies on microbial ferric iron reduction. The term ferrihydrite embraces a variety of structurally ill-defined, poorly crystallized ferric iron species, which are often also termed amorphous iron oxide, hydrous ferric oxide, or ferric (hydr)oxide. Goethite, hematite, and lepidocrocite can be either purchased or prepared in the laboratory (Schwertmann and Cornell, 1991). In contrast, ferrihydrite is not available commercially and always needs to be synthesized in the laboratory. We recommend verifying the identity of the prepared ferric iron oxides (e.g., by X-ray or electron diffraction analysis), particularly if one is not yet experienced in synthesizing ferric iron oxides.

Ferrihydrite is frequently also a by-product in the synthesis of goethite, hematite, or lepidocrocite. Ferrihydrite impurities can therefore hamper studies with the better crystallized ferric iron oxides. Hence, it is recommended to check for ferrihydrite impurities by selective dissolution (e.g., with hydroxylamine hydrochloride) and extraction from better crystallized ferric iron oxides. Extraction methods using oxalate or hydroxylamine hydrochloride as extracting agents have been described by various authors (e.g., Chao and Zhou, 1983; McKeague and Day, 1966; Schwertmann *et al.*, 1982). In control experiments with artificial mixtures of ferrihydrite and better crystallized ferric iron oxides (e.g., goethite), we found that both extracting agents leached most but not all ferrihydrite. It is therefore important to check the extraction efficiency and to consider quantitative aspects of experiments, i.e., to verify that ferrous iron was produced from the bulk ferric iron oxide and not only from small ferrihydrite impurities.

For storage and usage, synthesized ferric iron oxides can either be (freeze-) dried and added dry to experiments or be kept in aqueous suspension. We prefer to keep ferric iron oxides in suspension because suspensions can be deoxygenated thoroughly by stirring under vacuum and repeated flushing of the headspace with N_2. Ferric iron oxides are added from stock suspensions to cultures by means of syringes.

Except for short-term experiments (e.g., cell suspension experiments), it is usually necessary to sterilize ferric iron oxides. Unfortunately, no sterilization method is ideally suited because heat, pressure, or radiation might induce changes of the ferric iron crystal structure, particle size, and surface area. To limit possible changes, we sterilize ferric iron oxide suspensions only after thorough deoxygenation. Ferrihydrite after autoclaving once for 15 min was reduced by *Geobacter* species (*G. bremensis* and *G. pelophilus*) at the same rate as ferrihydrite that was not autoclaved. However, when we autoclaved ferrihydrite a second or third time the reduction rates declined notably. Alternatively, we boil ferric iron oxide suspensions for 30 min, incubate the suspensions for 2 days at 28° (to allow

spores to germinate), boil the suspension again for 30 min, and repeat the incubation and boiling procedure once more.

Preparation of Ferrihydrite

As ferrihydrite transforms with time to the better crystallized iron oxides goethite and/or hematite, we prefer to synthesize small amounts of ferrihydrite frequently and not to store them too long (≤ 6 months). For synthesis of ferrihydrite, we modified protocols of Ryden *et al.* (1977) and Lovley and Phillips (1986).

Prepare a 0.4 *M* solution of $FeCl_3$ in distilled water. This solution will be acidic, orange in color, and transparent. Stir the solution vigorously and adjust the pH slowly to 7.0 by adding 1 *M* NaOH drop by drop. We prefer to add NaOH at this low concentration and low rate because pH values above 7 (even only locally or momentarily) will induce the formation of other ferric iron oxides. Upon addition of NaOH, precipitates form, which dissolve again at the beginning of the neutralization process. Later the precipitates will persist and the color of the suspension changes to dark brown. When the pH has stabilized at pH 7.0, the synthesis of ferrihydrite is completed. After washing the produced ferrihydrite five times with a 10-fold volume of distilled water to remove sodium and chloride, the precipitate is spun down by centrifugation (5000*g*, 10 min). After the last centrifugation step, the ferrihydrite is resuspended in an adequate volume of distilled water to obtain a suspension of appropriate concentration, e.g., 0.5 *M*. The resulting suspension is transferred into glass test tubes together with a stirring bar. Each tube is sealed with a thick butyl rubber septum, and the ferrihydrite suspension is deoxygenated carefully by stirring under vacuum and repeated flushing of the headspace with N_2. Finally, the suspension is sterilized by autoclaving at 121° for 15 min.

Considerations on Ferrihydrite Concentrations

In batch culture experiments with iron-reducing bacteria, ferrihydrite is often used at concentrations in the range of 50 to 100 m*M*. At these concentrations, ferrihydrite gives a dark brown color to the medium, and the typical end product of ferrihydrite reduction under these conditions is magnetite, a black mixed-valence iron oxide (Fe_3O_4). Accordingly, the color of the medium changes from dark brown to black, and therefore other iron mineral phases that form transiently or as end product(s) may be masked and overlooked. The reason why magnetite forms under these conditions is still being discussed (Bell *et al.*, 1987; Brookins, 1988; Glasauer *et al.*, 2003; Jeon *et al.*, 2003). However, magnetite is not

necessarily the only end product of microbial ferrihydrite reduction: In growth experiments supplied with ferrihydrite at lower concentrations (5 to 10 m*M*), ferrihydrite was reduced completely to the ferrous state by various *Geobacter* species (*G. bremensis*, *G. metallireducens*, *G. pelophilus*, and *G. sulfurreducens*; Straub *et al.* 1998; Straub and Schink, 2004a). Medium containing only 5 to 10 m*M* ferrihydrite is orange-brown in color. Concomitant with complete reduction of ferrihydrite, the color of the iron precipitates changes from orange-brown to white because of the formation of ferrous iron precipitates with carbonate (siderite, $FeCO_3$) and phosphate [vivianite, $Fe_3(PO_4)_2$].

Enrichment of Ferric-Iron-Reducing Bacteria

Ferric iron oxides are reduced predominantly in anoxic habitats, and most ferric-iron-reducing bacteria are strict anaerobes. Facultatively anaerobic bacteria use ferric iron as an electron acceptor only after oxygen has been consumed. Hence, techniques for the preparation of media and cultivation of bacteria under strictly anoxic conditions should be used in studies on microbial ferric iron reduction; these techniques have been described in detail elsewhere (e.g., Widdel and Bak, 1992). In anoxic habitats, ferric-iron-reducing bacteria coexist with anaerobic bacteria that grow by fermentation or by anaerobic respiration, e.g., by reduction of nitrate or sulfate. To limit growth of fermentative bacteria in enrichment cultures, we use defined mineral media with electron donors/carbon sources that cannot be fermented easily, e.g., alcohols or fatty acids, and avoid complex nutrients such as yeast extract or peptone.

Medium Composition and Growth Conditions

Most media (freshwater, brackish, or seawater) contain substantial amounts of sulfate according to the composition of natural waters and/or sulfur source. In order to limit the growth of sulfate-reducing bacteria in enrichments for ferric-iron-reducing bacteria, the sulfate concentration is lowered to 0.1 m*M*, which is a sufficient sulfur supply for cell mass formation. Replacement of sulfate by organic sulfur sources (e.g., cysteine) is problematic in enrichment cultures, as they may be used as an additional source of organic substrate. Even if the sulfate concentration of the medium is as low as 0.1 m*M*, an indirect reduction of ferric iron oxides via sulfur cycling cannot be ruled out; it is therefore necessary to check isolated strains for direct or indirect ferric iron reduction (Straub and Schink, 2004b). Table I describes the mineral composition of freshwater and artificial seawater medium used in the enrichment and isolation of

TABLE I

COMPOSITIONS OF MINERAL MEDIA USED FOR ENRICHMENT AND ISOLATION OF FERRIC-IRON-REDUCING BACTERIA

Compound	Freshwater medium (g per liter)	Artificial seawater medium (g per liter)
KH_2PO_4	0.6	0.4[a]
NH_4Cl	0.3	0.25[a]
$MgSO_4 \times 7H_2O$	0.025	0.025
$MgCl_2 \times 6H_2O$	0.4	11
$CaCl_2 \times 2H_2O$	0.1	1.5
KBr		0.09
KCl		0.7
NaCl		26.4

[a] Autoclaved separately in 500-fold concentrated stock solutions and added to the medium after cooling.

ferric-iron-reducing bacteria. These media resemble other media that have been used successfully by other laboratories. Routinely, our media are buffered with 30 m*M* bicarbonate/CO_2 and are supplemented further with a mixture of seven vitamins, selenite, tungstate, and a solution of eight trace elements (Widdel and Bak, 1992). We prefer to use trace element solutions without chelators such as ethylene diamine tetraacetate (EDTA) or nitrilotriacetate (NTA), as they might unintentionally facilitate microbial ferric iron reduction via the formation of iron complexes.

Addition of a reducing agent will allow growth of oxygen-sensitive ferric-iron-reducing bacteria. Usage of an organic reducing agent such as cysteine or ascorbate is not recommended in enrichment cultures as it would represent an additional source of organic substrate. As an alternative, ferrous iron (2 m*M*) can be used as a mild reducing agent. To avoid an increase in sulfate concentration, we use $FeCl_2$ instead of $FeSO_4$, although the $FeCl_2$ salt is less stable. $FeCl_2$ is highly hygroscopic, and moist $FeCl_2$ oxidizes rapidly. Oxidation of ferrous iron salts is accompanied by a color change from green to orange or brown. To avoid oxidation, we store $FeCl_2$ salts or solutions under anoxic conditions (e.g., in an anoxic glove box).

Ferric-iron-reducing cultures are preferentially incubated in flat bottles or tubes, which are placed horizontally to provide a large surface area of the ferric iron oxides. Because it is still unclear how ferric-iron-reducing bacteria deliver electrons to ferric iron oxides, we avoid exposing the cells to excessive shearing on shakers, but rather shake them gently by hand every other day to allow an even distribution of iron minerals and bacteria and to avoid diffusion limitation.

Considerations on Redox Potentials

Ferric iron oxides differ in their midpoint potentials of reduction (e.g., Thamdrup 2000). In general, chelated ferric iron forms, e.g., Fe(III)-citrate or Fe(III)-NTA, have considerably higher redox potentials than ferric iron oxide minerals (Table II). With ferric iron minerals, the situation is even more complex because their particle size also influences their actual redox potential; e.g., the redox potentials for the reduction of goethite particles of 10 or 100 nm are −200 or −270 mV, respectively (Thamdrup, 2000). The redox potentials of ferric iron oxides are influenced further by the pH of the medium as they increase by 59 mV per unit decrease in pH (Thamdrup, 2000). Figure 1 gives an overview of stability fields and changes in redox potential in dependence of the prevailing pH for a system that includes iron (with ferrihydrite as the most generally used iron oxide), carbonate, and oxygen. More Eh-pH diagrams that involve further ferric iron species (e.g., magnetite, hematite) and/or additional elements (e.g., silicon, sulfur) are available in the literature (e.g., Bell *et al.*, 1987; Brookins, 1988). The redox potentials of the ferric iron oxides have to be considered for the choice of an appropriate electron donor or vice versa; e.g., at pH 7.0, molecular

TABLE II
REDOX POTENTIALS FOR REDUCTION OF VARIOUS IRON OXIDES AT pH 7.0 AND 25°[a]

System	E_0' (mV)	Reference
Fe(III)-NTA/Fe(II) NTA[b]	385	Thamdrup (2000)
Fe(III)-citrate/Fe(II) citrate	372	Thamdrup (2000)
Fe(III)-EDTA/Fe(II) EDTA	96	Wilson (1978)
Ferrihydrite/Fe^{2+}	−100 to 100	Brookins (1988); Thamdrup (2000); Widdel *et al.* (1993)
γ-FeOOH (lepidocrocite)/Fe^{2+}	−88	Thamdrup (2000)
α-FeOOH (goethite)/Fe^{2+}	−274	Thamdrup (2000)
α-Fe_2O_3 (hematite)/Fe^{2+}	−287	Thamdrup (2000)
Fe_3O_4 (magnetite)/Fe^{2+}	−314	Thamdrup (2000)

[a] Redox potentials depend strongly on pH value, temperature, concentration of reactants, and thermodynamic data chosen for calculations. For details, see related references.
[b] Nitrilotriacetate.

hydrogen ($2H^+/H_2$, $E_0' = -414$ mV) or formate (CO_2/formate, $E_0' = -432$ mV) can serve as an electron donor even for the reduction of better crystallized ferric iron oxides such as goethite, hematite, or magnetite, whereas the oxidation of acetate ($2CO_2$/acetate, $E_0' = -290$ mV) is energetically not favorable with these ferric iron oxides.

Monitoring Microbial Ferric Iron Reduction

Enrichment of ferric-iron-reducing bacteria can be monitored by measuring the production of ferrous iron. Before samples are taken with syringes under oxygen exclusion, cultures should be shaken vigourously to ensure a homogeneous distribution of ferrous and ferric iron species. Samples are acidified immediately with HCl (final concentration 1 *M*) to stabilize ferrous iron and to detach adsorbed ferrous iron from mineral phases. Ferrous iron can be quantified by a colorimetric assay with ferrozine (Stookey, 1970) or *o*-phenanthroline (Fachgruppe Wasserchemie, 1991; Straub *et al.*, 1999). Both assays allow quantification of ferrous iron in the micromolar range. *o*-Phenanthroline is classified as cancerogenic and hence is not so popular in ferrous iron determination as is ferrozine. Ferrozine stock solutions are prepared in distilled water with ammonium acetate (50%, w/v) containing 0.1% (w/v) ferrozine. As the ferrozine stock solution is light sensitive, it should be stored at 4° in the dark and can then be kept for approximately 2 weeks. As the sensitivity of the photometric assay with ferrozine is high, it is often necessary to dilute the samples with 1 *M* HCl before measurement. A sophisticated method to quantify ferrous and ferric iron by ion chromatography was developed by Schnell *et al.* (1998).

Isolation of Ferric-Iron-Reducing Bacteria

In general, methods that separate single cells on or inside solidified media are superior for the isolation of pure cultures over a simple dilution to extinction in liquid medium. Ferric-iron-reducing bacteria may be purified by streaking on ferric iron-containing agar plates that are incubated in anoxic jars. All preparations should be carried out inside an anoxic chamber, as highly oxygen-sensitive strains may die upon exposure to air. However, not all bacteria grow on surfaces, and a dilution inside semisolid agar might be necessary for the separation of single cells. The technique of anoxic agar dilutions has been described in detail by Widdel and Bak (1992). Unfortunately, mineral ferric iron oxides are opaque and therefore cannot be used in agar dilution series. Although one may see cleared zones in the ferric iron-containing agar (indicating ferric-iron-reducing bacteria),

one will not recognize faint neighboring colonies formed by noniron-reducing contaminants. It is also difficult to recognize such faint colonies in agar dilutions prepared with soluble ferric iron [e.g., Fe(III) citrate], as such medium is transparent but colored. To circumvent this problem, alternative electron acceptors that are soluble and colorless (e.g., fumarate, nitrate, dimethyl sulfoxide) are used for purification in agar dilution series. For example, *G. bremensis* and *G. pelophilus* were isolated with fumarate as an alternative electron acceptor (Straub *et al.*, 1998). After isolation of ferric-iron-reducing bacteria with an alternative electron acceptor, cultures are transferred back to the ferric iron oxide that was chosen initially for enrichment. Stock cultures should always be grown with ferric iron minerals as electron acceptor to maintain a selective pressure to use these insoluble electron acceptors.

Properties of Humic Substances

Humic substances, colloquially also called humus or humics, are redox-active polymeric organic compounds formed during the degradation of biopolymers such as lignin, proteins, and carbohydrates and are present in virtually all aquatic and terrestrial environments (Aiken *et al.*, 1985; Frimmel and Christman, 1988). A representative humic substance molecule is shown in Fig. 3 illustrating the heterogeneous structure of these compounds. Based on their solubility, humic substances are grouped into different fractions: humins (insoluble at alkaline pH), humic acids (soluble at alkaline and insoluble at acidic pH), and fulvic acids (soluble at alkaline and acidic pH) (Stevenson, 1994). The borderlines between these operationally defined fractions are not sharp and depend strongly on the exact pH and on the concentration and ionic strength of the solution/suspension. Chemical analyses showed that humins are more hydrophobic, have less polar functional groups, and a higher molecular weight than humic acids, which in turn are less soluble, higher in molecular weight, and less polar than fulvic acids (Stevenson, 1994).

Because of their heterogeneous structure and composition, which both depend on the chemical characteristics of the precursor compounds (Hayes *et al.*, 1989; Stevenson, 1994), humic substances contain various redox-active functional groups of different redox potentials. Thus, no defined redox potential can be assigned to humic substances but rather a range of redox potentials over which humic substances can accept or release electrons. Using different methods and different humic substances, ranges of redox potentials at pH 7 from −200 to 300 mV (Straub *et al.*, 2001) but also exact values of 320 to 380 mV (Visser, 1964), 700 mV (Matthiessen, 1995; Szilagyi, 1971), and 778 mV (at pH 5; Struyk and Sposito, 2001) have been

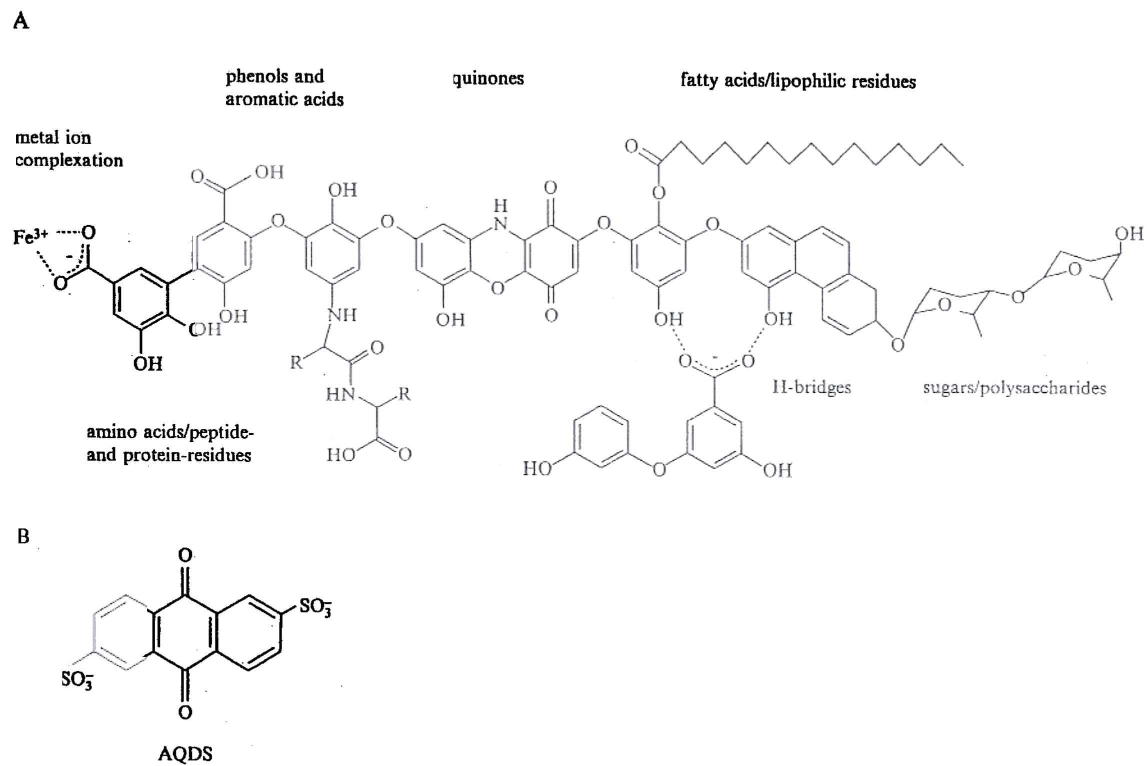

FIG. 3. Molecular structures of a representative humic substance molecule (A) and anthraquinone-2,6-disulfonate (AQDS), a model compound for quinone moieties in humic substances (B).

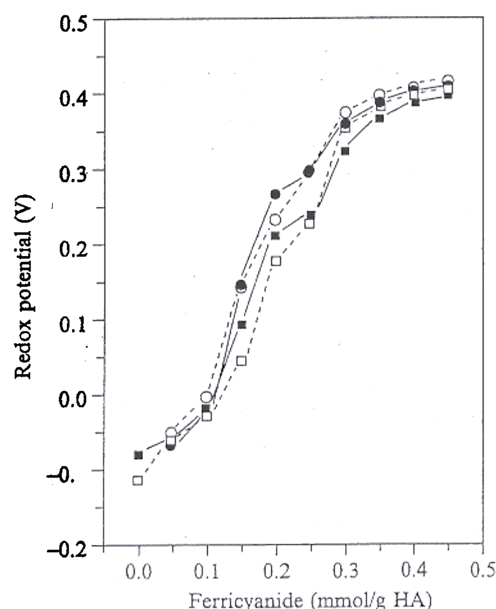

FIG. 4. Redox potentials recorded with a Pt redox electrode during titration of Pd/H_2-reduced humic acid preparations (Aldrich, 1 g/liter, phosphate buffer, pH 7) with potassium ferricyanide $\{K_3[Fe(CN)_6]\}$. Four replicates are shown.

reported. This wide range of values illustrates the difficulties in assigning accurate redox potentials to an insufficiently defined class of organic compounds. Because of their structural and compositional heterogeneity, a range of redox potentials between −200 and 300 mV is most likely, also with respect to a possible involvement in electron transfer to ferric iron oxides. Titration of reduced humic acid preparations with $K_3[Fe(CN)_6]$ and concomitant recording of the apparent redox potential showed that indeed most of the reducing equivalents present in reduced humic acids are transferred to Fe(III) at redox potentials between −100 and 400 mV (Fig. 4).

Isolation, Purification, and Application of Humic Substances

Isolation of Humic Substances

Humic substances are isolated from soils and sediments after the removal of small rocks and roots by alkaline extraction with 0.1 *M* NaOH at a solvent-to-soil ratio of 4:1 to 10:1 (v/w) for 24 h at 30° under shaking, if

possible, under anoxic conditions to avoid oxidative polymerization and degradation reactions. Inorganic constituents and the insoluble humin fraction are removed by centrifugation (30 min at 12,000*g*), and the supernatant is acidified with 1 *M* HCl to pH ~1 and kept at 4° for 24 h to precipitate humic acids. The fulvic acids remain in solution. The precipitated humic acids are separated by centrifugation and freeze-dried to be stored as a dry powder. To isolate the fulvic acids, the supernatant is passed through a column filled with XAD-8. This nonionic, macroporous methyl methacrylate ester resin should be washed with 1 *M* NaOH and rinsed with distilled water several times before use. The effluent is discarded, and the adsorbed fulvic acids are washed with 0.5 column volumes of distilled water. The fulvic acids are removed from the column by elution with 0.1 *M* NaOH. The eluate is freeze-dried either directly to obtain the Na-salt or after a passage through a cation-exchange resin where the Na^+ is replaced by H^+.

To isolate fulvic and humic acids from natural waters, the water is filtered (0.45 μm membrane filter) and the pH is adjusted to ~2 with HCl. The sample is passed over an XAD-8 column, and the sorbed fulvic and humic acids are eluted by 0.1 *M* NaOH. After acidification of the eluate to pH 1 with 1 *M* HCl, the precipitated humic acids can be separated by centrifugation. The fulvic acid fraction is passed through an XAD-8 column, washed with one column volume of distilled water to remove the salt, and eluted with 0.1 *M* NaOH. After passing the eluate through a cation-exchange resin column, the proton-saturated fulvic acids are obtained as powder by freeze drying.

In addition to separation of humic substances according to their solubility, humic substances can also be separated into polyphenolic and carbohydrate-rich fractions (Chen *et al.*, 2003; Gu and Chen, 2003).

Purification of Humic Substances

Because humic compounds after this isolation still contain significant amounts of inorganic constituents (quantifiable by the ash content), further purification may be necessary. The International Humic Substance Society (IHSS) provides a protocol that includes the removal of inorganic constituents by hydrofluoric acid treatment and dialysis to obtain highly pure humic substances (Swift, 1996). However, because these inorganic constituents may be important for the activities of humic substances in the environment, extensive purification is usually not required for experiments studying the effect of humic compounds on microbial redox processes. Furthermore, the IHSS offers standard humic compounds purified after the IHSS protocol (http://www.ihss.gatech.edu/) that have been well characterized and were already used in many studies.

Inexpensive humic compounds are also available commercially in large quantities (Aldrich). However, because this material is prepared from lignite, it represents highly humified and altered organic material and contains high amounts of inorganic compounds; thus, it is hardly representative of humic compounds from recent soils and waters (Malcolm and MacCarthy, 1986).

Determination of the Redox State of Humic Substances

Depending on how many redox-active groups are reduced/oxidized, humic substances possess a certain capacity to take up electrons (by the oxidized functional groups) or to release electrons from reduced functional groups to an electron acceptor of a more positive redox potential. The reducing and electron-accepting capacity of a certain humic substance sample can be determined by quantifying the reducing capacity (with ferric iron as an electron acceptor) of a native (untreated) vs a chemically reduced humic substance sample. For that, humic substances are dissolved (1 mg/ml) in 50 m*M* anoxic phosphate buffer (pH 7.0). Two separate 6-ml aliquots are incubated under H_2 and N_2 atmospheres, both in air-tight vessels in the presence of a Pd catalyst (5% Pd on activated charcoal; 1 mg/ml) on a rotary shaker at 30° for 24 h. After reduction, the H_2 headspace is replaced by N_2 by several cycles of vacuum degasing and N_2 flushing. Of both aliquots, 1 ml is mixed with 0.5 ml of a 3 m*M* potassium hexacyanoferrate ($K_3[Fe(CN)_6]$) solution (three replicates) and incubated on a shaker in the dark at 30°. After 24 h, 1 ml is taken per sample, mixed with 1 ml of HCl (1 *M*), and incubated for 30 min to precipitate the humic acids. After centrifugation (15,000*g*, 5 min), the absorbance at a wavelength of 416 nm is measured. Two 1-ml samples of humic acids to which water is added instead of hexacyanoferrate (followed also by the HCl precipitation step) give the background absorption of the humic acids that stay in solution after precipitation. A sample set with phosphate buffer and 0–3 m*M* hexacyanoferrate serves as calibration standard. The electron-accepting capacity of the humic acids can be calculated as the difference between the amounts of electrons transferred to hexacyanoferrate by the reduced and the nonreduced humic acid preparation.

A wide range of electron-accepting capacities of 0.1–0.9 mequiv electrons per gram sedimentary or soil humic acid were described (e.g., Chen *et al.*, 2003; Kappler and Haderlein, 2003; Royer *et al.*, 2002; Scott *et al.*, 1998; Struyk and Sposito, 2001). By electrochemical reduction of humic compounds and subsequent transfer of electrons to ferric iron or by chemical reduction and subsequent titration with I_2 as oxidant, electron-accepting capacities of 5.6 (Kappler and Haderlein, 2003) or even 11.5 mequiv/g

humic acids (Struyk and Spositio, 2001) were determined for natural humic acids, indicating that the obtained values depend on the reduction method (Pd/H_2 vs electrochemical) and the oxidant [Fe(III) vs I_2] used.

The redox state of microbially reduced dissolved humic compounds can be quantified after filtration (to remove the cells) by incubation of the filtrate with Fe(III)-citrate for 15 min and quantification of ferrous iron formed by the ferrozine method (see earlier discussion).

Application of Humic Substances

For microbiological experiments with humic substances, mostly the humic acid fraction has been used because humic acids can be obtained easily at large amounts and are well soluble at neutral pH at concentrations of up to ~1 mg/ml (remember that they are isolated by alkaline extraction and precipitation at acidic pH). In contrast, fulvic acid concentrations in soils/sediments are usually much lower, and the humin fraction is not soluble and difficult to handle.

For microbiological experiments with defined cultures of bacteria or under nongrowth conditions (e.g., cell suspension experiments), humic compounds are usually used without sterilization. Nonetheless, for enrichments or isolation, humic substances can be sterilized either by autoclaving or by filtration (membrane filter, 0.2 μm). Because of the limited solubility of humic acids at neutral pH, they can form precipitates and/or colloids at high concentrations and thus cause material loss during filtration. For autoclaving, humic acids are suspended in water or in a buffer solution similar to the buffer system used in the culture medium (e.g., 30 m*M* anoxic bicarbonate or phosphate buffer, pH 7.0) to 50 mg/ml under a N_2 headspace, and autoclaved at 121° for 25 min (Kappler *et al.*, 2004).

Enrichment, Isolation, and Quantification of Humic-Acid-Reducing Bacteria

In principle, media and cultivation techniques described earlier for ferric-iron-reducing bacteria can also be applied for the enrichment and cultivation of humic acid-reducing bacteria. However, some specific aspects of humic substances and humic acids need consideration. After purification of humic substances, all low molecular weight compounds have been removed, and the remaining polymeric humic compounds are not degradable to any significant extent by microorganisms (as shown, e.g., for *Shewanella alga* in the presence/absence of lactate; Lovley *et al.*, 1996). It is not advisable to use humic substances directly as sole electron acceptors for enrichment or isolation because of (i) the relative low solubility of

humic substances at neutral pH, leading to a relative low electron accepting capacity by the humic substances; (ii) the problems of measuring protein content or counting cells in the presence of significant concentrations of humic substances; and (iii) the high cost of large amounts of highly purified humic substances. Therefore, anthraquinone-2,6-disulfonic acid (AQDS, 5 m*M*) in combination with 2 m*M* acetate was used to obtain humic acid-reducing bacteria from diverse sediments in enrichment cultures (Coates *et al.*, 1998). AQDS (Fig. 3) is a model compound for quinone moieties in humic substances (it is a functional analogue, not a structural analogue or a model humic acid as claimed frequently in the literature). AQDS reduction can be followed easily by the development of the intense orange color of AHQDS (anthrahydroquinone-2,6-disulfonic acid), the reduced form of AQDS, which can be quantified photometrically at a 450-nm wavelength. After several transfers in liquid medium, dilution series in semisolid agar medium containing AQDS as the electron acceptor give colonies that can be isolated and transferred to liquid medium. Unfortunately, humic acid-reducing bacteria such as fermenting bacteria or *Shewanella* sp. that depend on more complex substrates as an electron donor (Benz *et al.*, 1998; Lovley *et al.*, 1998) cannot be grown selectively by this approach. They could be included by using their respective substrates, e.g., lactate, but care has to be taken to ensure that the observed growth is not caused by (AQDS-independent) fermentative growth.

Although AQDS has been used in many studies, results have to be interpreted with caution. The redox potential of AQDS ($E_0' = -184$ mV) is at the lower end of the redox potentials determined for humic substances, and bacteria that are able to transfer electrons to humic substances with redox potentials substantially higher than −184 mV can be overlooked when using AQDS. Furthermore, AQDS was shown to be taken up into the cell by a so far unknown mechanism (Shyu *et al.*, 2002). This is hardly possible with humic substances because of their greater size. Consequently, cells able to reduce AQDS do not necessarily reduce humic substances. Furthermore, a potential toxicity of AQDS was reported for pure cultures and indigenous microbial communities (Nevin and Lovley, 2000; Shyu *et al.*, 2002).

To count humic acid-reducing bacteria in sediment or soil, the most-probable number technique (dilution series) can be used. Humic acid concentrations of 0.5 g/liter are used (Kappler *et al.*, 2004). The simultaneous presence of 40 m*M* ferrihydrite allows reoxidation of the humic acids by electron transfer to ferric iron and thus a much higher turnover of organic substrates, e.g., acetate or lactate. However, this strategy to count humic acid-reducing bacteria in dilution series works only if the number of bacteria reducing ferric iron directly is substantially smaller than the

number of humic acid-reducing bacteria; therefore, the number of ferric-iron-reducing bacteria has to be determined in parallel in the absence of humic compounds.

Growth of humic substance-reducing bacteria has to be determined as an increase in biomass/cell numbers. To achieve a measurable amount of substrate oxidation and biomass formation, high concentrations of humic substances have to be used. Unfortunately, because of formation of cell-polymer aggregates accompanied by precipitation processes, direct microscopic cell counts are not possible. However, cell counts after the reduction of humic substances have been done if the bacteria can also grow aerobically on plates. After the reduction of humic substances, the cell number in the cultures was determined by plate counts with aerobic heterotrophic medium (e.g., *Shewanella* sp.; Lovley *et al.*, 1996), although the authors did not mention if the cells aggregated with humic substances and how they solved this problem. If the bacteria do not grow aerobically on plates, the growth of humic substance-reducing bacteria can be quantified using 5 m*M* AQDS as the electron acceptor instead of humic substances allowing microscopic cell counting (Lovley *et al.*, 1998). Attempts to measure protein content in humic acid-reducing cultures were not successful because of extensive interference of the protein assay with humic acids (Lovely *et al.*, 1996).

Reduction of humic substances by pure cultures can be shown either by quantifying the redox state of humic substances or by quantifying the oxidation of radiolabeled substrates (e.g., [^{14}C]acetate). When incubating cell suspensions (~1 mg protein/ml) with [^{14}C]acetate (0.2 m*M*) and humic acids (2 g/liter), the formation of $^{14}CO_2$ can be monitored over time (Coates *et al.*, 1998; Lovley *et al.*, 1996, 1998). With an electron-accepting capacity of >0.1 mequiv electrons per gram sedimentary or soil humic acid (see earlier discussion), this corresponds to >0.2 mequiv electrons that can be accepted by humic acids per liter of medium. This means that with 2 g/liter humic acids, at least 0.025 m*M* acetate can be oxidized to CO_2. This method will be useful only in exceptional cases.

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[5] Enrichment, Cultivation, and Detection of Reductively Dechlorinating Bacteria

By Frank E. Löffler, Robert A. Sanford, and Kirsti M. Ritalahti

Abstract

Strategies and procedures for enriching, isolating, and cultivating reductively dechlorinating bacteria that use chloroorganic compounds as metabolic electron acceptors from environmental samples are described. Further, nucleic acid-based approaches used to detect and quantify dechlorinator (i.e., *Dehalococcoides*)-specific genes are presented.

Introduction

A diverse group of bacteria use (poly)chlorinated aromatic and aliphatic compounds as terminal electron acceptors in their energy metabolism (Löffler *et al.*, 2003). This process, termed metabolic reductive dechlorination, involves energy capture from reductive dechlorination reactions. Other terms, including chlororespiration, dehalorespiration, and chloridogenesis, have also been applied to describe growth-coupled reductive dechlorination. Chlororespiration (Löffler *et al.*, 1996) and dehalorespiration

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