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The influence of chelating agents upon the dissimilatory reduction of Fe(III) by *Shewanella putrefaciens*

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The ability of *S. putrefaciens* to reduce Fe(III) complexed by a variety of ligands has been investigated. All of the ligands tested caused the cation to be more susceptible to reduction by harvested whole cells than when uncomplexed, although some complexes were more readily reduced than others. Monitoring rates of reduction by a ferrozine assay for Fe(II) formation proved inadequate using Fe(III) ligands giving Fe(II) complexes of low kinetic lability (e.g. EDTA). A more suitable assay for Fe(III) reduction in the presence of such ligands proved to be the observation of associated cytochrome oxidation and re-reduction. Where possible, an assay for Fe(III) reduction based upon the disappearance of Fe(III) complex was also employed. Reduction of all Fe(III) complexes tested was totally inhibited by the presence of O_2, partially inhibited by HQNO and slower in the absence of a physiological electron donor. Upon cell fractionation, Fe(III) reductase activity was detected exclusively in the membranes. Using different physiological electron donors in assays on membranes, relative reduction rates of Fe(III) complexes complemented the data from whole cells. The differences in susceptibility to reduction of the various complexes are discussed, as is evidence for the respiratory nature of the reduction.

Keywords: cytochrome oxidation, dissimilatory Fe(III) reduction, Fe(III) chelators, membrane-bound Fe(III) reductase, *Shewanella putrefaciens*

Introduction

It is now firmly established that dissimilatory reduction of Fe(III) to Fe(II) by microorganisms is of major importance in biogeochemical cycling (Lovley 1991, 1993). The facultative anaerobe *Shewanella putrefaciens* (formerly *Alteromonas putrefaciens*) has the ability to grow with Fe(III) as the sole terminal electron acceptor present, either in the form of insoluble amorphic Fe(III) oxide or soluble Fe(III) citrate (Lovley *et al.* 1989). Anaerobic growth of *S. putrefaciens* using Fe(III) or other electron acceptors (Myers & Nealson 1988, 1990, Morris *et al.* 1990) is not essential for expression of Fe(III) reductase activity. Cells grown under highly aerobic conditions are capable of Fe(III) reduction, but activity increases when growth is at low oxygen tension (Arnold *et al.* 1986a,b, 1988, 1990, DiChristina 1992). Recent work indicates that the ability of *S. putrefaciens* to grow on Fe(III) is governed by the gene *etrA*, the amino acid sequence of which has a high level of identity to the Fnr of *Escherichia coli* (Saffarini & Nealson 1993). However, *etrA* mutants retain the ability to reduce Fe(III) at rates comparable to the wild-type, reinforcing the hypothesis that more than one Fe(III) reductase may exist (Arnold *et al.* 1986a, 1990). *S. putrefaciens* mutants deficient in menaquinone and methylmenaquinone have been demonstrated to completely lose the ability to reduce Fe(III) (Myers & Myers 1993b). The enzyme or enzymes responsible for Fe(III) reduction in *S. putrefaciens* are yet to be characterized, but activity has been located in the cell membranes (Myers & Myers 1993a).

Literature concerning the importance of Fe(III) speciation in aqueous solution with respect to the reduction of the cation by *S. putrefaciens* is limited. In assays of Fe(III) reductase activity, the presence of the tetradentate aminocarboxylate ligand nitrilotriacetic acid (NTA) has been shown to accelerate the formation of Fe(II) ions using either Fe(III) chloride (Arnold *et al.* 1986b) or Fe(III) oxides (Arnold *et al.* 1988), whilst the reduction of Fe(III) citrate has been demonstrated to be faster than that of Fe(III) chloride (DiChristina 1992). These results indicate that soluble complexed forms of Fe(III) are more prone to

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reduction by the microorganism than solid or uncomplexed aqueous forms. However, based on a mathematical treatment of Fe(III):NTA speciation, it has been suggested that complexed forms of Fe(III) differ in their susceptibility to reduction by *S. putrefaciens* (Arnold *et al.* 1986b). In this paper we report the influence of a range of ligands upon the reduction of Fe(III) by *S. putrefaciens*. The ligands vary in both the number and nature of donor atoms (Figure 1), as well as affinities for Fe(III) (Table 1). Rates of Fe(III) reduction were followed by three distinct assays on harvested whole cells and isolated cell membranes.

Materials and methods

Bacterial growth and cell fractionation

The type strain of *S. putrefaciens* (NCIMB10471; ATCC8071) was purchased from The National Collections of Industrial and Marine Bacteria (Aberdeen, UK). Aerobic growth was achieved at 28–30 °C using minimal media previously described (Myers & Nealson 1990) with 50 mM DL-lactate as the carbon and energy source. Cultures (100 ml) in 250 ml foam-plugged conical flasks were shaken at 160 r.p.m. until the optical density at 650 nm was at least 1.0. Cells were harvested by centrifugation at 4 °C and washed twice with 100 mM NaHEPES, pH 7.0, the resulting pellet being homogenized with 5 ml of the same buffer prior to assays for Fe(III) reduction.

For cell fractionation, 400 ml cultures were grown aerobically in an identical manner to 100 ml cultures. After harvesting and washing with 100 mM Tris–HCl, pH 7.6, cells were suspended in 40 ml 0.5 M sucrose/5 mM EDTA/100 mM Tris–HCl, pH 7.6 containing 40 mg lysozyme and incubated at 30 °C for 1 h. Following centrifugation at 20000 r.p.m. for 1 h the periplasmic fraction was decanted and the spheroplast thoroughly homogenized in 10 ml 100 mM NaHEPES, pH 7.0, containing 10 mg deoxyribonuclease. The resulting suspension was added to 50 ml of water and stirred at 4 °C for 1 h prior to centrifugation at 20000 r.p.m. for 1 h. After decanting the cytoplasmic fraction, the cell membranes were homogenized in 15 ml 100 mM NaHEPES, pH 7.0, prior to assays for Fe(III) reduction.

Table 1. Thermodynamic stability constants of ligands used in this study for Fe(III) and Fe(II)

Ligand	$\log K_1$ Fe(III)	$\log \beta_3$ Fe(III)[a]	$\log K_1$ Fe(II)
NTA	15.9	–	8.8
Citrate	11.6	–	4.4
EDTA	25.1	–	14.3
DTPA	27.5	–	16.0
TTHA	26.8	–	NA
EGTA	20.5	–	11.8
DFO	30.6	–	[b]
Acetohydroxamic acid	11.4	28.3	4.8
Benzohydroxamic acid	11.1	27.8	NA
Maltol	11.1	28.5	NA
DMHP	14.9	35.1	NA
Kojic acid	9.2	24.4	NA
Salicylic acid	15.8	35.3	6.6
Catechol	20.0	43.8	8.0

Data from Martell & Smith (1974, 1977), Perrin (1979), Hider & Hall (1991). NA, data unavailable.
[a] $\log \beta_3 = \log K_1 + \log K_2 + \log K_3$.
[b] $\log K(Fe^{2+} + HDFO \rightleftharpoons FeHDFO^{+}) = 7.2$.

Preparation of Fe(III) complexes

All Fe(III) ligands used in this study are commercially available with the exception of 1,2-dimethyl-3-hydroxy-4-pyridinone (DMHP), *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)-1,2-diaminoethane (TPEN) and *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)-1,4-diaminobutane (TPBN), which were synthesized by literature procedures (Toftlund & Yde-Andersen 1981, Kontoghiorghes & Sheppard 1987). Stock solutions of complexes were prepared using a 3:1 molar ratio of bidentate ligand to Fe(III) or a 1:1 molar ratio of tri-, tetra- or hexadentate ligand to Fe(III).

Respiratory inhibitors and protonophores

Stock solutions of the respiratory inhibitors 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) (3.85 mM) and myxothiazol (410 μM), and the protonophores carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (both 100 μM), were prepared in methanol. Each was added 5 min before Fe(III) complex to an assay mixture and the cuvette thoroughly shaken.

Assay for Fe(II) formation using ferrozine

Ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine) is a bidentate ligand commonly used as a spectrophotometric reagent for Fe(II) (Stookey 1970). The high stability (thermodynamic and kinetic) and extinction coefficient (λ_{max} 562 nm; resulting from low-energy metal-to-ligand charge transfer) of the 3:1 ferrozine:Fe(II) complex combine to give extreme sensitivity. As with all chelators based on the ferroin moiety, the affinity of ferrozine for Fe(III) is negligible. Dailey & Lascelles (1977) first used ferrozine in an *in situ* assay for bacterial Fe(III) reduction and the following is a modification of their method. A 300 μl aliquot of whole cell or membrane suspension was initially added to 2.535 ml of N_2 purged 45 mM NaHEPES, pH 7.0, and 105 μl 10 mM ferrozine in a 3 ml glass cuvette containing a magnetic stirrer bar. The cuvette was subsequently sealed with a rubber septum and purged with N_2 for 5 min. An Aminco DW2000 spectrophotometer set in split beam mode at a constant wavelength of 562 nm was used to monitor the formation of 3:1 ferrozine:Fe(II) complex with respect to time after anaerobic addition of 30 μl 50 mM electron donor (DL-lactate for whole cells, formate for membranes) followed by 30 μl 10 mM Fe(III) complex. The reference

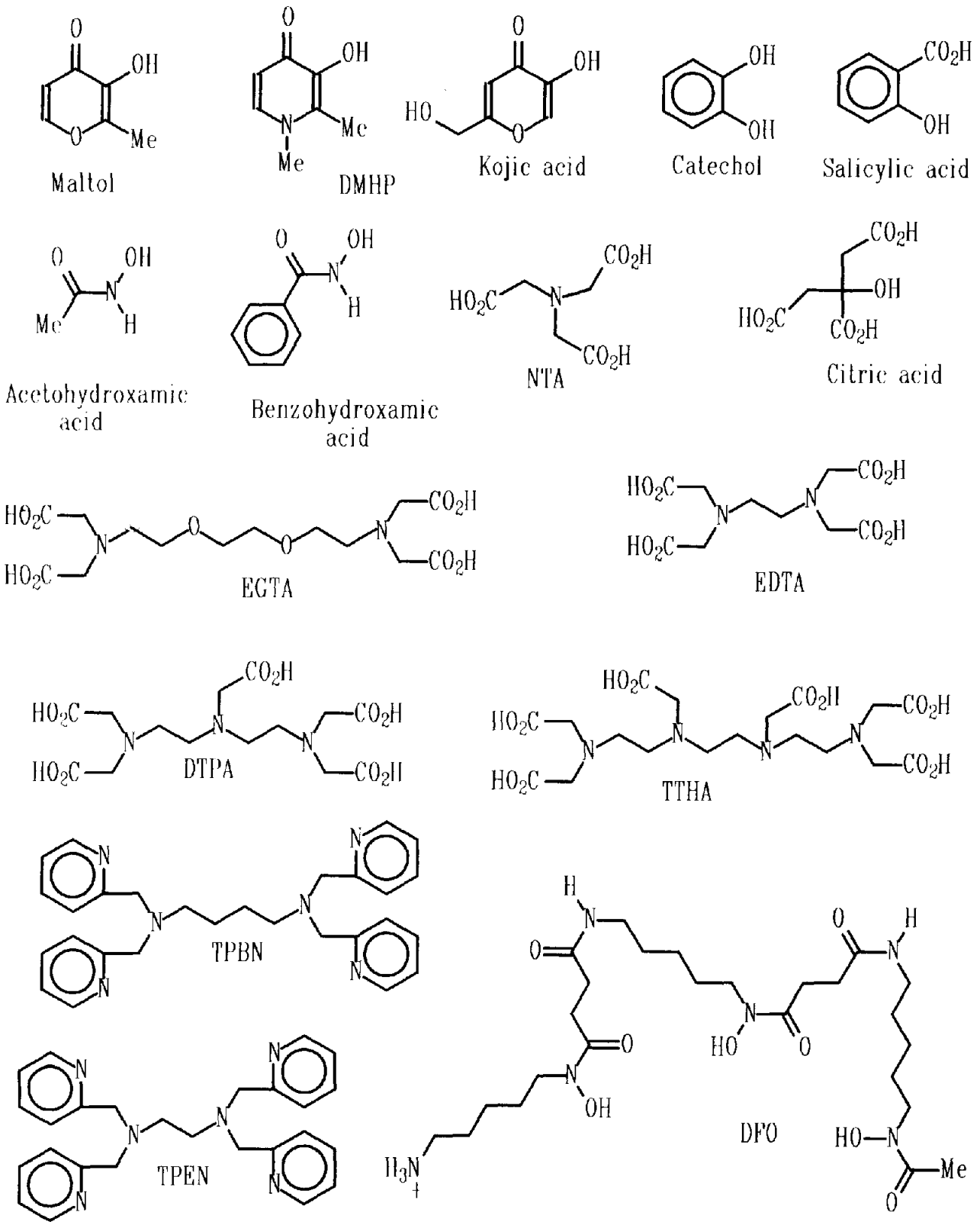

Figure 1. Structures of Fe(III) ligands used in this study.

cuvette contained a 300 μl aliquot of cell suspension and 2.7 ml of 45 mM NaHEPES, pH 7.0. Control assays in the absence of cells showed no formation of Fe(II)(ferrozine)$_3$ over a period of 30 min with any of the Fe(III) chelates, indicating that ferrozine does not act as a reductant of Fe(III) under the conditions imposed.

Assays for disappearance of Fe(III) complexes

Complexes of oxidizing cations with reducing ligands often possess ligand-to-metal charge transfer bands low enough in energy to be apparent in the visible region, and by virtue of this Fe(III) species with phenolic-like ligands such as maltol and acetohydroxamic acid are highly colored. Since the corresponding Fe(II) chelates are essentially colorless and moreover of low stability, the rate of disappearance of such Fe(III) complexes in the presence of *S. putrefaciens* should correlate strongly with the rate of Fe(III) reduction provided that facile oxidation of the Fe(II) formed does not occur. In assays for Fe(III) complex disappearance the same general protocol was followed as for assays of Fe(II) formation with the ferrozine being omitted. To initiate the reaction, which was monitored at or near the λ_{max} value of the complex, 150 μl of 10 mM Fe(III) complex was added.

Assays for cytochrome oxidation

Figure 2(A) shows a spectrum of *S. putrefaciens* whole cells (1 ml suspension in a 1 ml cuvette) under anaerobic conditions acquired on an Aminco DW2000 spectrophotometer set in split-beam mode with 1 ml 100 mM NaHEPES, pH 7.0, in the reference cuvette. The peaks at 552 and 523 nm are indicative of reduced *c*-type cytochromes whilst the broad nature of the spectrum suggests that *b*-type cytochromes are also present (Obuekwe & Westlake 1982). Addition of 50 μl 50 mM Fe(III) ligated by EDTA to the cells resulted in a bleaching of the spectral bands that was consistent with the oxidation of cytochromes by the Fe(III) (Figure 2B). Since these cytochrome oxidations were found to be reversible and moreover of a duration proportional to the amount of

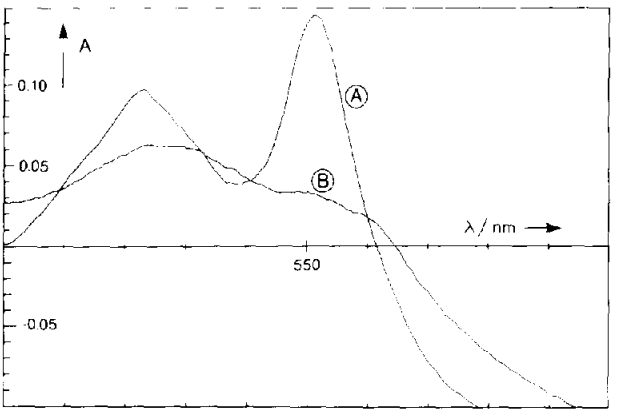

Figure 2. Whole cell cytochrome spectra of *S. putrefaciens* (**A**) reduced by anaerobicity and (**B**) oxidized by Fe(III) complexed with EDTA.

Fe(III) added, it was decided to use the transition time as a measure of Fe(III) reduction rates in the various chelates. Hence for whole cells a 1 ml aliquot of suspension was placed in a 1 ml glass cuvette, which was subsequently sealed with a rubber septum and purged with N_2 for 5 min. An Aminco DW2000 spectrophotometer set in split-beam mode at a constant wavelength of 552 nm was used to monitor the oxidation and re-reduction of *c*-type cytochromes with respect to time after anaerobic addition of 50 μl 10–100 mM Fe(III) complex. The reference cuvette contained 1 ml 100 mM NaHEPES, pH 7.0. Similar methodology was employed to monitor cytochrome oxidations in the membrane fraction (cytochromes initially reduced by anaerobic addition of 50 μl 50 mM formate to 1 ml suspension) and periplasmic fraction (reduced with excess dithionite then passed down a Pharmacia PD10 column).

Results

Fe(III) reduction by whole cells

Results pertaining to the effects of various Fe(III) ligands upon Fe(II)(ferrozine)$_3$ formation in the whole cell ferrozine assay are presented in Table 2, together with data regarding the influence of lactate and HQNO. Plots obtained using NTA (± lactate) are shown in Figure 3. In addition, Fe(II)(ferrozine)$_3$ formation was negligible over a period of 30 min using Fe(III) complexed with EDTA, DTPA, TTHA and TPEN. Data was found to be consistent for different cell batches, e.g. for uncomplexed Fe(III) and for Fe(III) ligated by maltol steady-state rates were 0.50 ± 0.09 ($n = 7$) and 55 ± 10 ($n = 7$) nmol Fe(II)(ferrozine)$_3$ formed (min mg dry weight cells)$^{-1}$ respectively. Using maltol (a model ligand for accelerated Fe(III) reduction), the presence of 500 μM formate or pyruvate increased the rate of Fe(II) complex formation by 190 and 120% respectively relative to the absence of an electron donor, whilst acetate caused no increment. Also using maltol, the rate of Fe(II)(ferrozine)$_3$ formation was slowed by the presence of 1.7 μM FCCP or CCCP to 18 and 45% respectively of that observed in the absence of lactate. Myxothiazol (1 or 10 μM) had insignificant effect upon rates of Fe(II) complex formation from either uncomplexed Fe(III) or Fe(III) complexed with maltol. Performing the assay in the absence of lactate without sparging the buffer or cuvette with N_2 (i.e. in the presence of dissolved O_2) resulted in a variable time lag before Fe(II)(ferrozine)$_3$ formation, which then proceeded at a comparable rate to that observed in an equivalent anaerobic assay. The presence of nitrate, nitrite or fumarate (all 100 μM) had no effect on rates of Fe(II)(ferrozine)$_3$ formation from either uncomplexed Fe(III) or Fe(III) complexed with maltol.

Some of the differences in reduction rates of the various Fe(III) complexes indicated by the ferrozine assay were not apparent in the cytochrome assay. For example, typical traces obtained using 10 mM stock solution Fe(III) complexed by DTPA and NTA are shown in Figure 4(A

Table 2. The effects of various Fe(III) ligands on the rate of Fe(II)(ferrozine)$_3$ formation in the whole cell ferrozine assay

Ligand	Steady-state rate of Fe(II)(ferrozine)$_3$ formation		
	Lactate present [nmol (min. mg dry weight cells)$^{-1}$][a]	Lactate absent (% of when lactate present)[b]	Lactate absent/40 μM HQNO present (% of when lactate absent)[b]
None	0.42	64	22
NTA	92	21	NP
Citrate	18	40	NP
EGTA	66	49	NP
TPBN	2.1	29	NP
DFO	0.79	58	NP
Acetohydroxamic acid	56	25	NP
Benzohydroxamic acid	80	20	4.1
Maltol	61	19	13
DMHP	6.1	42	6.6
Kojic acid	76	16	NP
Salicylic acid	2.7	47	NP

[a]Data from a single cell batch.
[b]Data from different cell batches.
NP, experiment not performed.

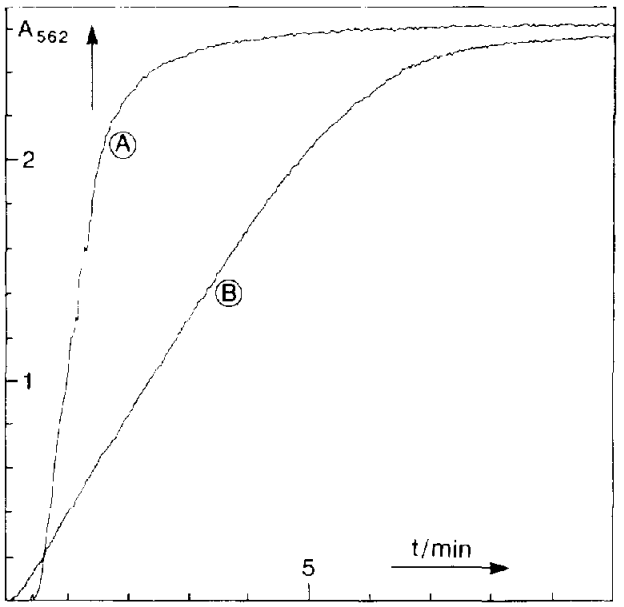

Figure 3. Whole cell ferrozine assay using NTA with (**A**) lactate present and (**B**) lactate absent. A_{562} 100 μM Fe(II)(ferrozine)$_3$ = 2.70.

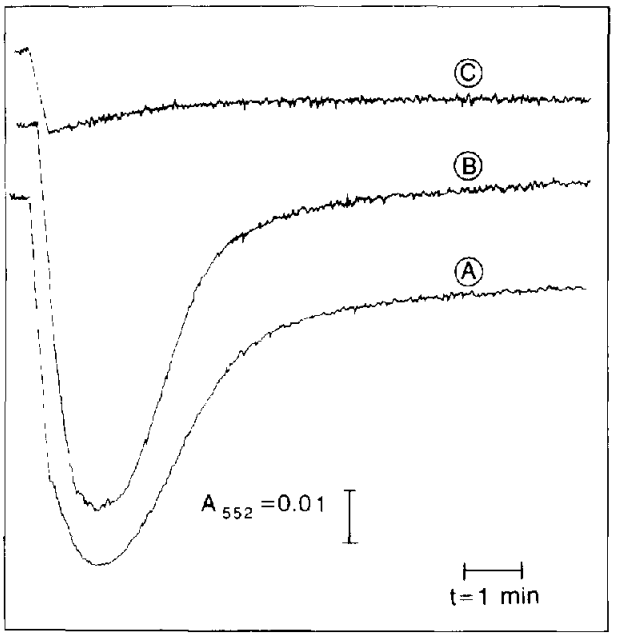

Figure 4. Whole cell cytochrome assay using 10 mM stock Fe(III) (**A**) complexed with DTPA, (**B**) complexed with NTA and (**C**) uncomplexed. The dotted line corresponds to injection of the Fe(III) complex.

and B respectively). Magnitudes of ΔA_{552} and transition times were not significantly different to the above for addition of 10 mM stock Fe(III) complexed by citrate, EDTA, TTHA, EGTA and acetohydroxamic acid. Smaller ΔA_{552} values (by $> 50\%$) and longer, less well defined transition times were, however, observed using equivalent concentrations of Fe(III) complexed by salicylic acid, TPEN and TPBN, whilst for Fe(III) in the absence of ligand no defined transition time was evident (Figure 4C).

The absorbances at 552 nm possessed by Fe(III) complexes with benzohydroxamic acid, maltol, DMHP, kojic acid and DFO at the concentrations employed in this assay precluded the collection of adequate data. Using 10 mM stock Fe(III) ligated by EDTA, the effect of the presence of 40 μM HQNO is shown in Figure 5. Similar though less marked transition time lengthening was observed in the presence of 1.7 μM CCCP or FCCP, whilst 1 or 10 μM myxothiazol had an insignificant effect. Addition of

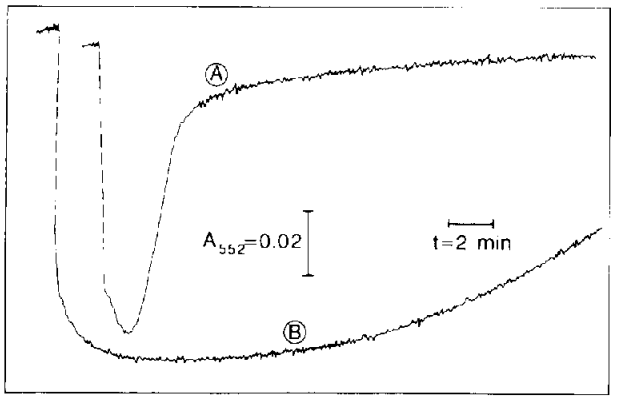

Figure 5. Whole cell cytochrome assay using 10 mM stock Fe(III) complexed by EDTA with (**A**) no inhibitor present and (**B**) 40 μM HQNO present. The dotted line corresponds to injection of the Fe(III) complex.

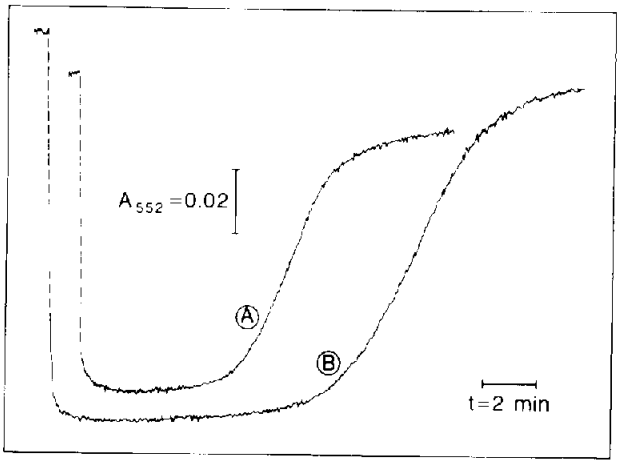

Figure 6. Whole cell cytochrome assay using (**A**) 10 mM stock fumarate and (**B**) 5 mM stock nitrite instead of Fe(III). The dotted line corresponds to injection of the terminal electron acceptor.

lactate, pyruvate or formate (final concentrations 2.5 mM) during assays using 50 mM stock Fe(III) complexed with EDTA resulted in an increase in ΔA_{552} followed by transition time shortening, whilst similar addition of acetate exerted a negligible influence. The effects of using fumarate and nitrite instead of Fe(III) in the assay are illustrated in Figure 6. Transition times for these electron acceptors were also lengthened by the presence of HQNO, FCCP and CCCP but unaffected by myxothiazol. Addition of 5 mM stock nitrate solution resulted in a transition time in excess of 30 min. When nitrite or fumarate (final concentrations 500 μM) were added during an assay using 50 mM stock EDTA complexed with Fe(III) the ΔA_{552} value was unaltered but the transition time increased.

Results obtained in the whole cell assay for the disappearance of Fe(III) complexes are presented in Table 3, together with data on the influence of lactate and HQNO. Only the ligands listed formed Fe(III) complexes possessing sufficiently high extinction coefficients for the assay to be confidently performed.

Fe(III) reduction by cell membranes

In the cytochrome assay using isolated cell membranes, relative magnitudes of ΔA_{552} and transition times using Fe(III) complexed with different ligands complemented data from the whole cell assay. Transition times were rendered longer by the presence of 40 μM HQNO but unaffected by 1 or 10 μM myxothiazol. Fumarate and nitrite reductase activities were also located in the cell membranes. As well as formate, lactate and NADH served to reduce the cytochromes present whilst acetate and pyruvate did not. Slow donation of electrons from NADPH was noted, the cytochromes taking some 20 min to reach the state of reduction attained in less than 1 min using NADH. Dithionite reduced periplasmic cytochromes were not oxidized by uncomplexed Fe(III) or Fe(III) complexed with EDTA or NTA.

Results pertaining to the effects of various Fe(III) ligands upon Fe(II)(ferrozine)$_3$ formation in the cell membrane ferrozine assay are presented in Table 4.

Table 3. Data from the whole cell assay for disappearance of Fe(III) complexes

Ligand	Wavelength at which complex with Fe(III) monitored (nm) [absorbance of 500 μM complex at that wavelength]	Steady-state rate of Fe(III) complex disappearance[a]		
		Lactate present [nmol(min.mg dry weight cells)$^{-1}$]	Lactate absent (% of when lactate present)	Lactate absent/40 μM HQNO present (% of when lactate absent)
Acetohydroxamic acid	400 [0.98]	60	11	13
Benzohydroxamic acid	437 [1.77]	75	14	14
Maltol	450 [1.82]	93	18	NP
DMHP	450 [2.36]	4.3	NP	NP
Kojic acid	402 [1.65]	130	25	NP
DFO	430 [1.39]	1.0	NP	NP

[a]Two cell batches were used in experiments, one for the first two and the other for the last four ligands.
NP, experiment not performed.

Table 4. The effects of various Fe(III) ligands on the rate of $Fe(II)(ferrozine)_3$ formation in the membrane ferrozine assay

Ligand	Steady-state rate of $Fe(II)(ferrozine)_3$ formation [nmol(min.mg dry weight cells)$^{-1}$]
NTA	15
Citrate	3.5
Acetohydroxamic acid	5.0
Benzohydroxamic acid	8.7
Maltol	9.0

Negligible differences in the rates of Fe(II) complex formation from Fe(III) complexed by NTA were noted using formate, lactate or NADH as an electron donor. In the presence of 40 μM HQNO, a lag of 10 min preceded the formation of $Fe(II)(ferrozine)_3$ from Fe(III) ligated by maltol, which then proceeded at a rate 4% of that recorded in the absence of the inhibitor.

In assays with the same batch of cell membranes used for the Fe(II) formation experiments reported in Table 4, Fe(III) complexed with maltol was found to disappear at a steady-state rate of 11 nmol (min mg dry weight cells) ^{1} with formate, lactate or NADH as the electron donor.

Discussion

Under the aerobic conditions imposed, cultures of *S. putrefaciens* developed an orange color during the logarithmic phase of growth. Since it has previously been noted that this pigmentation is related to cytochrome content and Fe(III) reductase activity (Obuekwe & Westlake 1982, DiChristina *et al.* 1988), cells were only harvested in the late logarithmic or stationary phase. Clearly the cultures become microaerobic as their density increases, causing not only induction of Fe(III) reductase activity but also nitrate, nitrite and fumarate reductase activities. Our attempts to grow *S. putrefaciens* anaerobically with lactate as the electron donor and Fe(III) citrate as the electron acceptor (Lovley *et al.* 1989) resulted in insufficient biomass for subsequent experiments.

The use of Fe(III) chloride as a standard for comparing rates of reduction with different chelators is questionable if it is considered that during the course of the ferrozine assay the speciation of the Fe(III) present will be constantly changing. In aqueous solution, the mononuclear hexa-aquo Fe(III) complex only exists under highly acidic conditions; as pH increases, protons are lost from the ligating water molecules, leading to the formation of poorly soluble oxo- and hydroxo-bridged polymeric Fe(III) structures (Powell 1993, Powell & Heath 1994). Fe(III) speciation is also variable if tri- and tetradentate ligands such as citrate and NTA are complexed with the cation. Apart from the existence of several monomeric species (Hider & Hall 1991), polymeric oxo- and hydroxo-bridged structures will also form (Heath *et al.* 1992, Powell & Heath 1994). With bidentate ligands the situation is simplified in that polynuclear Fe(III) species are much less likely to form (a possible exception being if the ligand is bulky; Hoveyda *et al.* 1993), although 3:1, 2:1 and 1:1 ligand:Fe(III) mononuclear species may exist. However, at neutral pH and with a 3-molar ratio of ligand to Fe(III) present, the 3:1 species has been found to be vastly predominant for all of the bidentates featured in this study at the concentrations employed, with the exception of salicylic acid and catechol (Hider & Hall 1991). Hexadentate ligands are in general also unlikely to form polymeric oxo- and hydroxo-bridged Fe(III) structures unless the pH is alkaline or an excess of the cation is present (Powell & Heath 1994). Furthermore, equimolar ratios of hexadentate aminocarboxylates have been demonstrated to be fully ligated at pH 7 to Fe(III) at the concentrations used in this study (Hider & Hall 1991). An exceptional case is EDTA, a ligand which by virtue of its size is incapable of forming an Fe(III) chelate of octahedral stereochemistry. The complex Fe(III)(EDTA) hence possesses a water molecule ligated at a seventh coordination site, resulting in the tendency for an oxo-bridged dimer to form. The significant contribution of this binuclear species to neutral solutions containing equimolar quantities of EDTA and Fe(III) is indicated by its precipitation when elevated concentrations of the components are present (Henderson 1993).

Results obtained from the ferrozine assay for reduction of Fe(III) complexed with NTA and citrate by whole cells of *S. putrefaciens* are in agreement with previous findings that these ligands cause an increase in Fe(II) formation compared with Fe(III) chloride (Arnold *et al.* 1986b, DiChristina 1992), although the enhancements are some order of magnitude greater in this study. However, making a direct comparison between the effects of these ligands on Fe(III) reduction rates based solely on the data from the ferrozine assay is complicated by the fact that they both interact quite strongly with Fe(II) (Table 1). If formed in a complex with NTA or citrate, Fe(II) has to be removed from that complex by ferrozine and thus the kinetic lability of the complex will influence the rate of $Fe(II)(ferrozine)_3$ formation. Although NTA has a higher thermodynamic stability constant for Fe(II) than citrate, the Fe(II) species formed with citrate after Fe(III) reduction may be less kinetically labile than those with NTA due to steric effects. Indeed, the ability of competing ligands to remove Fe(III) from complexes with NTA faster than from complexes with citrate has been noted (Hider 1984). The similar traces obtained for oxidation and re-reduction of *c*-type cytochromes in whole cell *S. putrefaciens* on addition of 10 mM stock Fe(III) complexed with NTA and citrate suggest that the true rates of Fe(II) formation may be closer than those given by the ferrozine assay.

More pronounced evidence for the effects of kinetic lability is apparent in data obtained using hexadentate aminocarboxylate ligands, with only Fe(III)(EGTA) appearing prone to reduction by *S. putrefaciens* in the ferrozine assay. In contrast the cytochrome assays using 10 mM stock Fe(III) complexed to EDTA, DTPA, TTHA and EGTA give similar results, and this suggests that the

former three ligands do not readily become unbound from Fe(II) in the presence of ferrozine. The far greater lability of Fe(II)(EGTA) is probably due to the extended distance between nitrogen atoms, allowing space for the competing ligand to approach the cation. Cytochrome assays for TPEN and TPBN also indicate a similar rate of reduction by *S. putrefaciens* of Fe(III) complexed with either of these hexadentate aminopyridine ligands. Although no information is available in the literature concerning the stability, spin-state or speciation of Fe(III) complexes likely to be formed, the ferrozine assays using TPEN and TPBN imply low kinetic lability of the resultant Fe(II) species with TPEN, suggesting that all donor atoms are ligated.

In the case of the hexadentate tris-hydroxamate ligand DFO (desferrioxamine-B), data obtained from the ferrozine and Fe(III) complex disappearance assays correlate strongly, suggesting that reduction of Fe(III)(DFO) is slow in comparison with Fe(III) complexed with NTA. The low thermodynamic stability of Fe(II)(DFO) (Table 1) means that the cation is readily lost to competing ferrozine or indeed water ligands. DFO is a naturally occurring bacterial siderophore, i.e. a member of a class of compounds synthesized by microorganisms to scavenge iron needed for growth from the environment (Hider 1984, Raymond 1990). Once inside the cell, iron has to be released from the siderophore complex to serve as a nutrient and the major pathway for this process is currently believed to be assimilatory Fe(III) reduction mediated by ferrisiderophore reductases (Fontecave *et al.* 1994). It is thus logical that Fe(II)(DFO) should be a very labile species, so that Fe(II) may be easily acquired for biosynthetic purposes. The slow dissimilatory reduction of Fe(III)(DFO) by *S. putrefaciens* with respect to Fe(III) complexed with NTA and citrate and the 1:1 Fe(III):hexadentate aminocarboxylate species indicates the former chelate to be a less suitable substrate for the enzyme or enzymes responsible, and this may simply be due to a lower reduction potential for Fe(III) when chelated by DFO. However, it must be considered that the siderophores produced by *S. putrefaciens* might be hexadentate hydroxamates similar to DFO and that Fe(III) needed for assimilatory reduction should perhaps not be more susceptible to dissimilatory reduction. A parallel could indeed be drawn with plant membrane Fe(III) reductases which reduce the cation in complexes with synthetic ligands but not in complexes with siderophores, leading eventually to iron accumulation in the plant root (Raymond 1990).

As with Fe(II)(DFO), the low thermodynamic stability of Fe(II)(bidentate hydroxamate)$_3$ complexes (Table 1) results in strong agreement between data obtained from the ferrozine and Fe(III) complex disappearance assays. Faster reduction of Fe(III)(benzohydroxamate)$_3$ compared with Fe(III)(acetohydroxamate)$_3$ might be due to a difference in reduction potentials, but the relative hydrophobicities of the complexes could also be of importance. Both species will be neutral, but the benzohydroxamate complex will be more lipophilic and this may be a significant factor for access to the membrane-bound Fe(III) reductase.

By evidence from the ferrozine and disappearance assays, Fe(III)(maltol)$_3$ is reduced considerably faster by *S. putrefaciens* than Fe(III)(DMHP)$_3$. Since these closely related species are both uncharged and of comparable sizes, the difference noted almost certainly results from Fe(III)(DMHP)$_3$ having a lower reduction potential than the corresponding complex with maltol. This supports clinical data which suggest that the high susceptibility of Fe(III)(maltol)$_3$ to reduction is of importance in its use as a dietary iron supplement (Barrand *et al.* 1990), whilst the lower reduction potential of Fe(III)(DMHP)$_3$ could be a factor in the value of this class of ligand for protecting against cellular damage induced by redox cycling chemicals (van der Waal *et al.* 1992).

The primary factor causing the reduction potential of Fe(III)(DMHP)$_3$ to be lower than Fe(III)(maltol)$_3$ might simply be the higher thermodynamic stability of the complex (Table 1). Maltol has a similar affinity for Fe(III) to the bidentate hydroxamic acids and the complexes are indeed reduced at comparable rates by *S. putrefaciens*. Moreover, the less thermodynamically stable 3:1 complex of the more hydrophilic 3-hydroxy-4-pyrone ligand kojic acid with Fe(III) is reduced faster than Fe(III)(maltol)$_3$. Salicylic acid has an affinity for Fe(III) similar to DMHP, but although reduction of the cation in the presence of both ligands is slow, a direct comparison cannot confidently be made if it is considered that (i) Fe(III)(salicylate)$_3$ bears a net charge of -3 and (ii) considerable quantities of 1:1 and 2:1 salicylate:Fe(III) species will be present in the pH 7 assay mixtures (a consequence of the high affinity of the ligand for protons). Equating the reduction rates for Fe(III)(DFO) (net charge +1) and the various Fe(III)(hexadentate aminocarboxylate) complexes (net charges negative) is also questionable. Nevertheless, the thermodynamic stability of an Fe(III) complex appears to be a major influence upon its susceptibility to reduction by *S. putrefaciens*.

The bidentate ligand having the highest affinity for Fe(III) is catechol (Table 1). However, this moiety is readily oxidized to semiquinone and quinone (which both chelate Fe(II) and Fe(III)), and below pH 9 mixtures of various Fe(II) and Fe(III) complexes exist in aqueous solution (Hider 1986). By virtue of this, the use of catechol in the assays described for monitoring Fe(III) reduction by *S. putrefaciens* is inappropriate: in the ferrozine assay all Fe(III) was reduced to Fe(II) within seconds in the absence of cells, the disappearance assay cannot be based on a single species and the cytochrome oxidation/re-reduction traces only account for Fe(III) not reduced by the ligand.

Evidence for the reduction of the various Fe(III) species used in this study being part of the respiratory process for *S. putrefaciens* was provided by the use of different terminal electron acceptors, the presence or absence of physiological electron donors and the use of inhibitors of respiratory chains. The time lag caused by the presence of O_2 in the ferrozine assay mixture before formation of

Fe(II) is registered is consistent with the microorganism respiring O_2 in preference to Fe(III). Furthermore, the fact that the presence of the alternative electron acceptors nitrate, nitrite and fumarate causes no such lag nor slowing of the rate of Fe(II) formation implies that Fe(III) is being respired in preference to these anions. The inhibition of Fe(III) reduction by nitrate and nitrite noted by previous workers (Obuekwe *et al.* 1981, DiChristina 1992) was not apparent in our results.

The similar magnitudes of ΔA_{552} obtained in the cytochrome assays for Fe(III) complexed by NTA, citrate, acetohydroxamic acid and the hexadentate aminocarboxylates and nitrate, nitrite and fumarate suggest comparable electron flow through the cytochromes to all these species via the respective terminal reductases. That the transition time is shorter for the Fe(III) complexes is a reasonable finding if it is considered that the reductions of fumarate to succinate and nitrate to nitrite are two electron processes, whilst nitrite is reduced to nitric oxide which might be further reduced to nitrogen gas via nitrous oxide by *S. putrefaciens*. Taking into account the non-inhibition of Fe(III) reduction by nitrate, nitrite and fumarate in the ferrozine assay, the fact that the ΔA_{552} values are not increased on addition of these alternative electron acceptors whilst the transition times are lengthened implies simultaneous electron flow to more than one terminal reductase is unlikely to occur. The small value of ΔA_{552} and long transition time observed using uncomplexed Fe(III) in the cytochrome assay suggests slow flow of electrons to the poorly soluble cation and supports data from the ferrozine assay.

The finding that the absence of lactate decreases the rate of reduction of uncomplexed Fe(III) by whole cells of *S. putrefaciens* could be due to reduced flow of electrons through the respiratory chain, but it is also possible that the solubility of the Fe(III) present is being increased by the lactate acting as a ligand for the cation (the $\log K_1$ value of lactate for Fe(III) is 2.9; Hider & Hall 1991). However, using Fe(III)(maltol)$_3$, lactate will not compete effectively with maltol for the cation and hence the decreased rate of Fe(III) reduction in the absence of the electron donor must be due to diminished electron supply. By this method formate and pyruvate were also demonstrated to act as electron sources for the respiratory chain, whilst acetate proved ineffective. These data support anaerobic growth studies of *S. putrefaciens* on Fe(III) oxide and Fe(III) citrate using different electron donors (Lovley *et al.* 1989, 1992). The increases in ΔA_{552} and transition time shortening caused by the addition of lactate, formate and pyruvate in the whole cell cytochrome assay with Fe(III)(EDTA) are also indicative of increased electron flow through the respiratory chain.

Inhibitory effects of HQNO upon reduction of uncomplexed Fe(III) by whole cell *S. putrefaciens* and upon reduction of Fe(III) complexed with citrate by isolated membranes have previously been noted (Obuekwe *et al.* 1981, Myers & Myers 1993a). Data from this study suggests that the reduction of all Fe(III) complexes tested by whole cells is partially inhibited by HQNO, with apparent reduction rates from the ferrozine and Fe(III) complex disappearance assays being slower and cytochrome transition times being longer in the presence of the compound. Transfer of electrons from the quinone pool to cytochromes thus appears to be of importance for the reduction of Fe(III) complexes by the microorganism, as well as for the reduction of nitrite and fumarate. That myxothiazol has no significant effect upon reduction rates of Fe(III) complexes, nitrite and fumarate suggests that should *S. putrefaciens* possess a cytochrome bc_1 complex, it is not involved in electron transport to any of these terminal electron acceptors. However, 16S rRNA sequencing has demonstrated *S. putrefaciens* to share a close phylogenetic relationship with *Escherichia coli* (Kita-Tsukamoto *et al.* 1993), a bacterium known to lack a cytochrome bc_1 complex.

The location of formate-dependent HQNO-sensitive Fe(III) reductase activity to the membranes of *S. putrefaciens* is in agreement with the work of Myers & Myers (1993a). These authors also found NADH to be a suitable electron donor for the enzyme in purified outer membranes, but noted lactate and NADPH to be ineffective. That the relative reduction rates of the various Fe(III) complexes using whole cells are consistent with those using cell membranes by evidence from ferrozine, disappearance and cytochrome assays suggests that chemical properties associated with the whole cell exert no influence.

If reduction of an Fe(III) complex by *S. putrefaciens* is truly respiratory then consequent extrusion of protons across the cytoplasmic membrane will be achieved, serving to create a pH gradient and associated electronic potential. Such proton translocation by *S. putrefaciens* has been noted to occur in response to the addition of Fe(III) oxide or Fe(III) citrate by a pH electrode technique (Myers & Nealson 1990). Collapsing the membrane potential with a protonophore such as FCCP or CCCP should in theory cause an Fe(III) complex to be reduced at a faster rate by *S. putrefaciens*. However, data from the ferrozine and cytochrome assays demonstrate that these compounds act as inhibitors of Fe(III) reduction as well as nitrite and fumarate reduction, probably by interaction with a component of the respiratory chain. Inhibition of denitrification reactions mediated by *Pseudomonas denitrificans* and *Pseudomonas aeruginosa* with FCCP and CCCP has previously been noted (Walter *et al.* 1978).

The ability of *S. putrefaciens* to reduce Fe(III) complexed by a wide variety of ligands implies that the enzyme or enzymes responsible lack substrate specificity. This is a property shared with ferrisiderophore reductases (Fontecave *et al.* 1994), and suggests that similar enzymes may be involved in dissimilatory and assimilatory Fe(III) reduction, although ferrisiderophore reductases tend to be soluble proteins. The fact that the outer membrane of *S. putrefaciens* possesses Fe(III) reductase activity (Myers & Myers 1993a) together with a large complement of cytochromes (Myers & Myers 1992) is clearly of importance for the reduction of insoluble forms of Fe(III) (reductive dissolution). The faster reduction of soluble Fe(III) complexes could in part result from the ability of

these species to access a dissimilatory Fe(III) reductase associated with the cytoplasmic membrane. Membrane-bound Fe(III) reductase activity has also been found in *Geobacter metallireducens* (formerly GS-15) (Gorby & Lovley 1991), *Spirillum intesonii* (Dailey & Lascelles 1977) and *Staphylococcus aureus* (Lascelles & Burke 1978). Since *G. metallireducens* can grow anaerobically with Fe(III) as the sole terminal electron acceptor present (Lovley & Phillips 1988) and *S. itersonii* translocates protons in response to Fe(III) (Short & Blakemore 1986), then these Fe(III) reductase activities are most likely to be dissimilatory. Monitoring reduction rates of Fe(III) complexed with various ligands will aid elucidation as to whether the Fe(III) reductases present in these micro-organisms are similar to that in *S. putrefaciens*.

The use of bi- and hexadentate ligands as opposed to tri- or tetradentate ligands or no ligand is clearly preferable in assays of Fe(III) reduction by *S. putrefaciens*, and hence the former are being employed in more detailed kinetic studies. An investigation of the susceptibility to reduction of small polynuclear Fe(III) clusters which model the oxo- and hydroxo-bridged forms of the cation present in the environment (Powell & Heath 1994) is also being performed.

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