Dissimilatory iron(III) reduction by *Rhodobacter capsulatus*

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The photosynthetic proteobacterium *Rhodobacter capsulatus* was shown to be capable of dissimilatory Fe(III) reduction. Activity was expressed during anaerobic phototrophic and microaerobic growth with malate as the carbon source, but not during equivalent aerobic growth. A variety of Fe(III) complexes were demonstrated to act as substrates for intact cells and membrane fractions of strain N22DNAR⁺ using a ferrozine assay for Fe(II) formation. Rates of reduction appeared to be influenced by the reduction potentials of the Fe(III) complexes. However, Fe(III) complexed by citrate, which is readily reduced by *Shewanella putrefaciens*, was a poor substrate for dissimilation by *R. capsulatus*. The Fe(III)-reducing activity of *R. capsulatus* was located solely in the membrane fraction. The reduction of Fe(III) complexes by intact cells was inhibited by 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), suggesting the involvement of ubiquinol:cytochrome *c* oxidoreductases in the electron transport chain. Lack of sensitivity to myxothiazol plus data from mutant strains implies that the cytochrome *bc*₁ complex and cytochrome *c*₂ are not obligatory for dissimilation of Fe(III)(maltol)₃. Alternative pathways of electron transfer to Fe(III) must hence operate in *R. capsulatus*. Using strain N22DNAR⁺, the reduction rate of Fe(III) complexed by nitrilotriacetic acid (NTA) was elevated compared to that of Fe(III)(maltol)₃, and moreover was sensitive to myxothiazol. However, these differences were not observed in the absence of the electron donor malate. The governing factor for the reduction rate of Fe(III)(maltol)₃ thus appears to be the limited Fe(III)-reducing activity, whilst the reduction rate of Fe(III) complexed by NTA is controlled by the flux of electrons through the respiratory chain. The use of mutant strains confirmed that the role of the cytochrome *bc*₁ complex in Fe(III) reduction becomes apparent only with the superior substrate. The energy-conserving nature of Fe(III) reduction by *R. capsulatus* was demonstrated by electrochromic measurements, with the endogenous carotenoid pigments being employed as indicators of membrane potential generation in intact cells. Using Fe(III)EDTA as electron acceptor, periods of membrane potential generation were directly proportional to the quantity of complex added, and were extended in the presence of HQNO. Fe(III)-dependent carotenoid bandshifts were abolished by addition of the protonophoric uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Keywords: dissimilatory Fe(III) reduction, *Rhodobacter capsulatus*, membrane-bound Fe(III) reductase activity, iron chelators, carotenoid bandshift

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Abbreviations: DFO, desferrioxamine-B; DMHP, 1,2-dimethyl-3-hydroxy-4-pyridinone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; ferrozine, 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; maltol, 2-methyl-3-hydroxy-4-pyrone; NTA, nitrilotriacetic acid.

INTRODUCTION

The bacterial dissimilation of Fe(III) in anaerobic and microaerobic environments can have a significant influence on a number of biogeochemical element cycles (Lovley, 1991, 1993, 1995; Nealson & Saffarini, 1994). Despite the wealth of ecological data, there is still relatively little information available concerning Fe(III) respiration in pure cultures. The most widely studied species of Fe(III)-dissimilating bacteria are *Geobacter metallireducans* and *Shewanella putrefaciens*, which can both grow anaerobically with Fe(III) as the sole terminal electron acceptor (Lovley & Phillips, 1988; Lovley *et al.*, 1989). Fe(III) reductase activity for both these organisms has been located in cell membranes (Gorby & Lovley, 1991; Myers & Myers, 1993a). Other bacteria found to possess a membrane-bound Fe(III) reductase include *Spirillum itersonii* (Dailey & Lascelles, 1977) and *Staphylococcus aureus* (Lascelles & Burke, 1978), the former organism having been demonstrated to translocate protons in response to Fe(III) (Short & Blakemore, 1986). Work concerning the pathways of electron transport to Fe(III) during dissimilation of the cation has thus far been focused on *S. putrefaciens*, with membrane-bound cytochromes, menaquinone and methylmenaquinone, and a [3Fe-4S] centre all shown to be involved in the reductive process (Myers & Myers, 1992, 1993b; Tsapin *et al.*, 1994).

The photosynthetic proteobacterium *Rhodobacter capsulatus* is an attractive candidate in which to study dissimilatory Fe(III) reduction for a variety of reasons. Although it has a particularly complicated electron-transport system that includes components of cyclic photosynthetic electron transport and aerobic respiration, *R. capsulatus* has been demonstrated to utilize numerous terminal electron acceptors under anaerobic conditions (Ferguson *et al.*, 1987; McEwan, 1994). This is a property shared with the highly versatile facultative anaerobe *S. putrefaciens* (Nealson & Saffarini, 1994). Furthermore, a number of respiratory mutant strains of *R. capsulatus* are available (Bell *et al.*, 1992), allowing evidence to be gleaned as to components of the electron transport chain to any Fe(III) reductase. Finally, conservation of energy during anaerobic respiration can be easily investigated in *R. capsulatus*, with the electrochromic response of the endogenous carotenoid pigments providing a convenient non-invasive technique for monitoring membrane potential *in vivo* (McEwan *et al.*, 1982, 1983, 1985; Bell *et al.*, 1992; Richardson *et al.*, 1994).

When elucidating rates of Fe(III) reduction by bacteria, the question of Fe(III) speciation must be addressed. In aqueous solution, the mononuclear hexa-aquo Fe(III) complex exists only 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) species (Powell & Heath, 1994). During recent work on *S. putrefaciens*, it was noted that the presence of certain Fe(III) chelators can increase the rate of reduction of the cation by two orders of magnitude compared to uncomplexed Fe(III) in

Fig. 1. Structures of Fe(III) ligands used in this study. When present in an equimolar ratio, the hexadentates EDTA and DFO give predominantly a 1:1 complex with Fe(III) in pH 7.0 aqueous solution. The bidentates maltol, acetohydroxamic acid and DMHP give predominantly a 3:1 complex with Fe(III) if the ligand is present in a threefold molar excess at pH 7.0, but the high affinity of salicylate for protons results in considerable quantities of 2:1 and 1:1 complexes forming in similar aqueous solutions. Citric acid and NTA do not compete effectively enough with hydroxide for Fe(III) at pH 7.0 to permit the formation of a 2:1 ligand:cation complex unless a massive excess of ligand is present. In solutions of equimolar citric acid or NTA and Fe(III), speciation will be very complicated due to formation of oxo- and hydroxo-bridges between cations, which results in polymeric structures. Data on the affinity of the ligands for Fe(III) plus a more detailed discussion of complex speciation in aqueous solution are given by Hider & Hall (1991), whilst some oxo- and hydroxo-bridged polynuclear Fe(III) clusters formed with tri- and tetradentate ligands are reviewed by Powell & Heath (1994).

pH 7 buffered assays on whole cells (Dobbin *et al.*, 1995). In the present study a number of these Fe(III) chelators (Fig. 1) have been used to demonstrate that *R. capsulatus* is capable of dissimilatory Fe(III) reduction.

METHODS

Bacterial strains. *R. capsulatus* strains MT1131, MTG4S4, MTCBC1 and MTGS18 were a generous gift from Dr F. Daldal, University of Pennsylvania, USA. The mutants MTG4S4, MTCBC1 and MTGS18 lack cytochrome c_2 , the cytochrome bc_1 complex, and cytochrome c_2 /cytochrome bc_1 , respectively, whilst MT1131 is the parent strain (Daldal *et al.*, 1986, 1987). Strains H123 and 37b4 were obtained from Dr H. Hudig, University of Freiburg, Germany, and are a cytochrome c' deficient mutant and the wild-type, respectively (Hudig *et al.*, 1986). N22DNAR⁺ is a nitrate-respiring isolate (McEwan *et al.*,

1982). The anaerobic respiratory properties of all these strains are fully described by Bell *et al.* (1992).

Growth conditions. Cultures of strains MT1131, MTG4S4, N22DNAR⁺, 37b4 and H123 were grown to late exponential phase under anaerobic phototrophic conditions at 30 °C in RCV medium (Weaver *et al.*, 1975), with malate as carbon source and ammonium as nitrogen source (Richardson *et al.*, 1988). For strains MTCBC1 and MTGS18, which are incapable of phototrophic growth, 100 ml cultures were grown microaerobically at 30 °C in 250 ml foam-plugged conical flasks by shaking at 160 r.p.m. until late exponential phase was reached. Microaerobic growth of the photosynthetic strains was performed in total darkness. A 20 ml culture of N22DNAR⁺ was grown aerobically, at 30 °C and in darkness, in a 2 l foam-bunged conical flask by shaking at 250 r.p.m. until late exponential phase was attained. Antibiotics were added to the medium where appropriate (Bell *et al.*, 1992).

Cell fractionation. For the preparation of a periplasmic fraction, a 100 ml culture of *R. capsulatus* strain N22DNAR⁺ was harvested and washed with 100 mM Tris/HCl, pH 7.6, prior to suspension in 10 ml 0.5 M sucrose, 5 mM EDTA, 100 mM Tris/HCl, pH 7.6, containing 40 mg lysozyme. Following incubation at 30 °C for 30 min, debris was removed by ultracentrifugation at 45000 r.p.m. for 1 h in a Beckman type 70Ti rotor. For membrane preparation, a 100 ml culture of strain N22DNAR⁺ was harvested and washed with 10 mM Tris/HCl, pH 8.1, prior to suspension in 30 ml of the same buffer. Following two passes through a chilled French pressure cell operated at 1000 p.s.i. (6.9 MPa), lysozyme and MgCl₂ were added to final concentrations of 0.5 mg ml⁻¹ and 1 mM respectively and the mixture stirred at 20 °C for 10 min. A few crystals of DNase I and RNase A were then added and stirring was continued for a further 10 min. The membranes were pelleted by ultracentrifugation at 45000 r.p.m. for 1 h in a Beckman type 70Ti rotor.

Ferrozine assay for Fe(III) reduction. This assay was described and validated fully by Dobbin *et al.* (1995); it is based upon monitoring the formation of the highly stable 3:1 ferrozine:Fe(II) complex at its $\lambda_{\max}$ value of 562 nm. An Aminco DW2000 spectrophotometer set in split-beam mode was employed. Rates of formation of Fe(II)(ferrozine)₃ correlate strongly with rates of dissimilatory Fe(III) reduction if the Fe(III) complex under investigation is reduced to an Fe(II) complex of low kinetic and/or thermodynamic stability. EDTA is unsuitable for use in the assay since ferrozine does not compete effectively for Fe(II) if the cation is complexed by this hexadentate aminocarboxylate.

Electrochromic measurements. The underlying theory of and basic methodology for the measurement of carotenoid pigment absorption changes in intact cells of *R. capsulatus* as an indicator of membrane potential generation in response to light or the introduction of external electron acceptors are detailed by Clark & Jackson (1981) and McEwan *et al.* (1982). In this study a Shimadzu UV-3000 dual-wavelength/double-beam spectrophotometer was used with wavelength pairs of 503–487 nm for strain N22DNAR⁺ and 528–511 nm for strain 37b4. Fe(III)(maltol)₃ is unsuitable for use in electrochromic measurements owing to the absorbance spectrum of the complex interfering with carotenoid signals.

Chemicals. All Fe(III) ligands used in this study are commercially available with the exception of DMHP, which was synthesized according to 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). Stock solutions of the

respiratory inhibitors HQNO (3.85 mM) and myxothiazol (410 μ M), and the protonophore FCCP (100 μ M), were prepared in methanol.

RESULTS

Reduction of Fe(III) monitored by the ferrozine assay using intact cells of strain N22DNAR⁺

The dissimilatory reduction of Fe(III) could be readily detected in harvested intact cells of *R. capsulatus* strain N22DNAR⁺ grown under anaerobic phototrophic conditions using the ferrozine assay to monitor Fe(II) formation. For example, a time-course observed using Fe(III) complexed by NTA, in the presence and absence of malate, is shown in Fig. 2. Steady-state (linear) rates of Fe(II)(ferrozine)₃ formation were calculated with data from the first 100 s of these kinetic traces. Rates obtained in assays using a variety of Fe(III) complexes are presented in Table 1, together with data regarding the influence of malate, HQNO and myxothiazol. Malate was found not to catalyse Fe(III) reduction with no cells in the assay mixture. In the absence of electron donor or inhibitor, Fe(III) complexed by NTA was reduced slightly faster by intact cells of strain N22DNAR⁺ than Fe(III)(maltol)₃. However, significantly greater enhancement of reduction rate was achieved upon adding malate to the assay mixture using Fe(III) complexed by NTA compared to Fe(III)(maltol)₃. Addition of HQNO caused similar inhibition of Fe(II) formation from Fe(III)(maltol)₃ and Fe(III) complexed by NTA. In comparison to Fe(III)(maltol)₃, Fe(III) complexed by citrate was reduced much more slowly by intact cells of strain N22DNAR⁺, with the rate of Fe(II) formation being only 2.4 % of that obtained with the former Fe(III) species. Furthermore, the reduction rate of Fe(III) complexed by citrate was not influenced by malate or HQNO. Whilst

Fig. 2. Ferrozine assay on whole cells of *R. capsulatus* strain N22DNAR⁺ using Fe(III) complexed by NTA with (A) no electron donor and (B) 500 μ M malate present. Fe(II)(ferrozine)₃ formation can be seen to commence upon injection of substrate. Concentrations of NaHEPES, Fe(III), maltol and ferrozine were 50 mM, 100 μ M, 300 μ M and 350 μ M, respectively [A_{562} of 100 μ M Fe(II)(ferrozine)₃ = 2.70]. Harvested and washed cells were resuspended in 1 ml 100 mM NaHEPES, pH 7.0, per 20 ml culture, with 300 μ l aliquots subsequently being used in constantly stirred 3 ml assays. Full details are given by Dobbin *et al.* (1995).

Table 1. Effects of various Fe(III) ligands on the rate of Fe(II)(ferrozine)₃ formation in the whole-cell ferrozine assay using strain N22DNAR⁺

These representative data correspond to the same cell batch. Experiments were repeated on a further two batches, and all formation rates were within 12 % of those presented. NP, Experiment not performed.

Ligand	Steady-state rate of Fe(II)(ferrozine) ₃ formation [nmol min ⁻¹ (mg dry weight cells) ⁻¹]			
	No addition	500 µM malate present	40 µM HQNO present	500 µM malate and 5 µM myxothiazol present
Maltol	4.5	5.4	3.1	5.4
NTA	5.1	8.6	3.2	6.0
Citric acid	0.11	0.11	0.10	NP
Acetohydroxamic acid	4.9	NP	NP	NP
Salicylic acid	0.42	NP	NP	NP
DMHP	0.47	NP	NP	NP

Table 2. Effects of various Fe(III) ligands on the rate of Fe(II)(ferrozine)₃ formation in the ferrozine assay using membranes of strain N22DNAR⁺, with 500 µM NADH present

These representative data correspond to the same membrane batch. Experiments were repeated on a further two batches, and all formation rates were within 7 % of those presented. NP, Experiment not performed.

Ligand	Steady-state rate of Fe(II)(ferrozine) ₃ formation [nmol min ⁻¹ (mg dry weight cells) ⁻¹]	
	No addition	40 µM HQNO present
Maltol	0.19	0.17
NTA	0.17	0.15
Citrate	0.017	NP
Acetohydroxamic acid	0.14	NP
DFO	0.011	NP

Fe(III)(acetohydroxamate)₃ was reduced at a rate comparable to Fe(III)(maltol)₃, rates of Fe(II) formation from Fe(III) complexed by salicylate and Fe(III)(DMHP)₃ were an order of magnitude lower.

Additional ferrozine assays indicated the presence of myxothiazol (1, 5 or 10 µM) to have insignificant effect upon rates of Fe(II) formation from either Fe(III)(maltol)₃ or Fe(III) complexed by NTA in the absence of malate (data not tabulated). However, with 500 µM malate in the assay mixture, the presence of 5 µM myxothiazol inhibited Fe(II)(ferrozine)₃ formation from Fe(III) complexed by NTA, but had no effect upon the malate-stimulated reduction of Fe(III)(maltol)₃ (Table 1). The presence of FCCP (1.7 µM) slowed the reduction rate of Fe(III)(maltol)₃ to 64 % of that obtained in the absence of the protonophore (data not tabulated). When HQNO was used at lower concentrations (10 or 20 µM), Fe(III) reduction rates did not vary significantly from the values quoted in Table 1 using 40 µM of this inhibitor.

The rates of Fe(II) formation from Fe(III)(maltol)₃ and Fe(III) complexed by NTA using strain N22DNAR⁺ grown microaerobically were 90 % and 87 % respectively of those obtained using phototrophic anaerobic cells.

No formation of Fe(II)(ferrozine)₃ from Fe(III) complexed by NTA or Fe(III)(maltol)₃ was noted in assays with aerobically grown cells of strain N22DNAR⁺.

Fe(III) reduction by membranes of strain N22DNAR⁺

Upon fractionation of strain N22DNAR⁺, all Fe(III) reductase activity was found in the membranes. No formation of Fe(II) from either Fe(III)(maltol)₃ or Fe(III) complexed by NTA was observed using a periplasmic fraction pre-reduced with dithionite. Comparative rates of Fe(II)(ferrozine)₃ formation in the ferrozine assay using N22DNAR⁺ membranes with NADH as electron donor and various Fe(III) chelates (Table 2) showed slight variation from intact cell data. Whilst Fe(III)(maltol)₃ was

Table 3. Rates of Fe(II)(ferrozine)₃ formation in the ferrozine assay using whole cells with Fe(III) complexed by NTA or Fe(III)(maltol)₃, and 500 µM malate present

The representative data in each row correspond to the same cell batch. Experiments were repeated on a further two batches for each strain, and all formation rates were within 12% of those presented.

Strain	Mutant phenotype	Steady-state rate of Fe(II)(ferrozine) ₃ formation [nmol min ⁻¹ (mg dry weight cells) ⁻¹]	
		From Fe(III) complexed by NTA	From Fe(III)(maltol) ₃
MT1131	Parent strain	15	7.2
MTG4S4	cyt <i>c</i> ₂ ⁻	13	5.4
MTCBC1	cyt <i>bc</i> ₁ ⁻	8.4	8.0
MTGS18	cyt <i>c</i> ₂ ⁻ /cyt <i>bc</i> ₁ ⁻	3.1	3.0
37b4	Parent strain	9.1	6.2
H123	cyt <i>c</i> ' ⁻	12	8.0

Fig. 3. Electrochromic bandshifts of the carotenoid pigments of whole cells of strain N22DNAR⁺: (a) in response to illumination from a 100 W tungsten bulb, followed by additions of 25 µl 100 mM NaHEPES, pH 7.0, and Fe(III)EDTA to concentrations of 40 µM and 80 µM; (b) in response to 40 µM Fe(III)EDTA subsequent to additions of 10 µM HQNO and 1 µM FCCP. The shift upon illumination was some five times the magnitude of that upon injection of Fe(III)EDTA. The shift upon injection of buffer is probably due to a small amount of dissolved oxygen being present. Harvested and washed whole cells were suspended in 1 ml 100 mM NaHEPES, pH 7.0, per 60 ml culture, a 100 µl aliquot being used in constantly stirred 2.5 ml assays with the same buffer. Further details are given by Clark & Jackson (1981).

reduced more slowly than Fe(III) complexed with NTA and Fe(III)(acetoxyhydroxamate)₃ in whole cells, the converse was found using membranes. Furthermore, HQNO caused insignificant inhibition of Fe(II) formation from Fe(III)(maltol)₃ and Fe(III) complexed by NTA using membranes. However, similarly to intact cells, reduction of Fe(III) complexed with citrate by membranes was slow in comparison to Fe(III)(maltol)₃ (i.e. 7.4%). The rate of reduction of Fe(III)DFO by membranes was also some order of magnitude lower than that of Fe(III)(maltol)₃. Succinate (500 µM) served as an electron donor comparable in efficiency to NADH for the reduction of Fe(III)(maltol)₃ and the cation complexed by NTA (data

not presented). Neither NADH nor succinate catalysed Fe(III) reduction in the absence of membranes. Performing ferrozine assays on membranes with the detergent Triton X-100 (0.01–0.25%) present gave no variation in Fe(II) formation rates from either Fe(III)(maltol)₃ or Fe(III) complexed by NTA (data not presented).

Fe(III) reduction by mutant strains

Using Fe(III) complexed by NTA and Fe(III)(maltol)₃, and in the presence of malate, rates of Fe(II)(ferrozine)₃ formation for *R. capsulatus* strains MT1131, MTG4S4,

MTCBC1, MTGS18, 37b4 and H123 are presented in Table 3. Dealing first with MT1131 and mutants derived from this strain, variations in Fe(III) reduction rates were apparent in both sets of assays. For Fe(III) complexed by NTA, Fe(II) was formed fastest from the parent strain and slowest from the cytochrome *bc*₁ and cytochrome *c*₂ double mutant MTGS18. Furthermore, the absence of the cytochrome *bc*₁ complex caused a significant drop in reduction rate, whilst the absence of cytochrome *c*₂ exerted no such effect. The Fe(II) formation rate from Fe(III)(maltol)₃ was also considerably slower for the double mutant MTGS18 than MT1131. However, the absence of cytochrome *c*₂ or the cytochrome *bc*₁ complex alone caused insignificant variations in Fe(III)(maltol)₃ reduction rate relative to the parent strain. In comparison to the phototrophic anaerobic cultures, cells of strains MT1131 and MTG4S4 grown under microaerobic conditions formed Fe(II) from Fe(III) complexed by NTA at rates of 89% and 80%, respectively, and from Fe(III)(maltol)₃ at rates of 105% and 92%, respectively (data not tabulated). For the cytochrome *c*' deficient mutant H123, reduction rates of Fe(III) complexed by NTA and Fe(III)(maltol)₃ were comparable to those obtained with the parent strain 37b4 (Table 3).

Electrochromic carotenoid bandshifts induced during Fe(III) reduction

The generation of Fe(III)-dependent carotenoid bandshifts, indicative of a cytoplasmic membrane potential being created, was observed for *R. capsulatus* strain N22DNAR⁺ using EDTA to chelate the cation. Fig. 3(a) presents bandshifts obtained following illumination, addition of NaHEPES or addition of Fe(III)EDTA. The bandshift observed with buffer was insignificant compared to those with Fe(III)EDTA. Doubling the concentration of Fe(III) added caused an increase in bandshift duration. However, the long periods of decay for the Fe(III)-induced bandshifts disallow any confident calculation of reduction rates. Fig. 3(b) shows bandshifts induced with 40 µM Fe(III)EDTA being extended by HQNO and abolished by the uncoupling agent FCCP. Lowering of the baseline was observed on addition of both HQNO and FCCP. Upon addition of EDTA not complexed with Fe(III), a bandshift similar to that upon addition of NaHEPES was observed (data not presented). Fe(III)-dependent electrochromic carotenoid bandshifts were also obtained using *R. capsulatus* strain 37b4 (data not presented).

DISCUSSION

The results presented in this work clearly show that *R. capsulatus* can reduce Fe(III) chelates in reactions which are coupled to the generation of a membrane potential. Fe(III) can thus be added to the extensive list of anaerobic electron acceptors for the bacterium, which includes *S*-oxides, *N*-oxides and *N*-oxyanions (Ferguson *et al.*, 1987; McEwan, 1994).

Influence of chelating agents on reduction of Fe(III) by intact cells

With the exception of Fe(III) complexed by citrate, comparative rates of Fe(II)(ferrozine)₃ formation from the various Fe(III) complexes using whole cells of *R. capsulatus* strain N22DNAR⁺ were in broad agreement with data obtained from *S. putrefaciens* (Dobbin *et al.*, 1995), although specific activities were an order of magnitude lower in this study. These data are consistent with the view that the kinetics of Fe(III) reduction are controlled to some extent by the reduction potential of the Fe(III) chelate. Thus the rate of reduction of high-potential Fe(III)(maltol)₃ was an order of magnitude faster than that of low-potential Fe(III)(DMHP)₃, in spite of the fact that these closely related Fe(III) species are both uncharged and of similar size. Using *S. putrefaciens*, whole-cell ferrozine assays indicated that Fe(II)(ferrozine)₃ formed from Fe(III) complexed by citrate at a rate approximately one-third of that from Fe(III)(maltol)₃ (Dobbin *et al.*, 1995), and this was also found to be the case when bacterial cells were replaced with the chemical reducing agent ascorbic acid (P. S. Dobbin & D. J. Richardson, unpublished results). The comparable figure from the present study was 2.4% for strain N22DNAR⁺, which demonstrates that Fe(III) complexed by citrate is a poor substrate for dissimilation by *R. capsulatus*. Slower flux of electrons to Fe(III) complexed by citrate is also illustrated by the lack of influence exerted by the presence of malate or HQNO upon reduction rates compared to Fe(III)(maltol)₃ and the cation complexed by NTA. That Fe(III) complexed by citrate is a poor electron acceptor in *R. capsulatus* but not *S. putrefaciens* may indicate differences to exist between any specific Fe(III)-dissimilating enzymes in the two bacteria. A biological (as opposed to physicochemical) reason for the slow reduction of Fe(III) complexed by citrate could be the role of the ligand as a siderophore in *R. capsulatus*. Indeed, citrate has been implicated in iron assimilation for the close phylogenetic relation *Rhodobacter sphaeroides* (Moody & Dailey, 1984, 1985). By analogy with *Escherichia coli* (Wagge & Braun, 1981), an outer-membrane receptor which transports Fe(III) complexed by citrate into the periplasmic compartment may well exist for *R. capsulatus*. In view of the high initial concentration of Fe(III) in the ferrozine assays, it seems unlikely that this receptor will utilize all the citrate-chelated cation at the expense of dissimilatory reduction.

Influence of chelating agents on reduction of Fe(III) by membranes

Again with the exception of citrate, relative Fe(II) formation rates from different Fe(III) chelates in assays using membranes of *R. capsulatus* strain N22DNAR⁺ generally concurred with the *S. putrefaciens* membrane data (Dobbin *et al.*, 1995). Moody & Dailey (1984, 1985) have found DFO not to act as a siderophore for *R. sphaeroides*. If this is also the case for *R. capsulatus*, then the extremely low rate of formation of Fe(II)(ferrozine)₃ observed from Fe(III) chelated by DFO with N22DNAR⁺

membranes is probably a consequence of low reduction potential. The ability to utilize NADH and succinate as electron donors indicates localization of Fe(III) reduction at the cytoplasmic membrane, whilst the range of Fe(III) chelates reduced, which vary in overall size, charge and hydrophobicity, suggests that any specific Fe(III) reductase might possess a periplasmic active site. For *S. putrefaciens*, the reduction rates of Fe(III)(maltol)₃ and the cation complexed with NTA by membranes compared to intact cells were 14 % and 16 %, respectively (Dobbin *et al.*, 1995). However, from the present study on strain N22DNAR⁺, the corresponding figures were 3.5 % and 2.0 % (using the intact-cell rates obtained with malate present). The most probable explanation for this finding is that more of the membranes will form as inverted vesicles from cells of *R. capsulatus*, and hence less of the active site is available for reaction with substrate in fractions prepared from this bacterium. Such a phenomenon is related to the specialized structure of the intracytoplasmic membrane of *R. capsulatus*, which is formed by a process of invagination to incorporate photosynthetic pigments and pigment-binding proteins (McEwan, 1994). A possible reason for the faster reduction of Fe(III)(maltol)₃ compared to Fe(III)(acetohydroxamate)₃ and the cation complexed by NTA is that the former species is both uncharged and reasonably lipophilic, and thus might penetrate the membrane vesicle to access any active site inside. However, attempts to validate this hypothesis using Triton X-100 to puncture the membrane vesicles were unsuccessful.

Evidence for a specific Fe(III) reductase

The carotenoid bandshift studies undertaken here demonstrate that reduction of Fe(III)EDTA by *R. capsulatus* strains N22DNAR⁺ or 37b4 is coupled to the generation of a transmembrane proton electrochemical gradient. Increasing quantities of Fe(III)EDTA extended duration of carotenoid bandshifts, and this was correlated with a longer period of Fe(III) reduction. The presence of HQNO slowed electron flow to Fe(III), resulting in protons being extruded over a longer period, whilst FCCP uncoupled Fe(III) reduction from energy conservation, leading to bandshifts being abolished. The lowering of the baseline observed upon addition of HQNO and FCCP is indicative of uncoupling, although in the presence of HQNO the proton electrochemical gradient clearly persisted with reduction of Fe(III)EDTA. The inhibition of Fe(II) formation by FCCP observed in the ferrozine assay is not consistent with a role of dissipating the protonmotive force, as a thermodynamic backpressure should be removed in uncoupled cells leading to a stimulatory effect upon Fe(III) reduction. However, inhibition of Fe(III) reduction by FCCP using *S. putrefaciens* has been noted (Dobbin *et al.*, 1995), as well as inhibition of the denitrification reactions of other bacteria by a range of protonophores (Walter *et al.*, 1978).

The carotenoid bandshifts obtained in this present work with Fe(III)EDTA cannot be quantified easily and H⁺/e⁻ ratios were not calculated. We are thus not in a position to

say whether any specific Fe(III) reductase of *R. capsulatus* functions as a proton pump. Indeed, we have not obtained any irrefutable evidence for the presence of a physiological Fe(III) reductase in the bacterium. However, some of our data do suggest that a specific enzyme donates electrons to Fe(III) as opposed to non-specific reduction of the cation by one or more electron-transport components. Primarily, the fact that activity was not present in aerobically grown cells but was inducible under phototrophic anaerobic and microaerobic conditions strongly suggests that a specific Fe(III) reductase is expressed under oxygen-limited conditions.

Amongst the electron-transport components of *R. capsulatus* which may non-specifically reduce Fe(III) are cytochromes. Evidence for this is provided by the finding that horse-heart cytochrome *c* reduces certain Fe(III) species at appreciable rates, with the cation complexed by NTA being reduced faster than Fe(III)(maltol)₃ (P. S. Dobbin & D. J. Richardson, unpublished results). However, data obtained here using a periplasmic fraction of strain N22DNAR⁺ suggest that the non-specific oxidation of water-soluble cytochromes by Fe(III) is of no importance for *R. capsulatus*, and moreover lends support to the view that a specific Fe(III) reductase is operating. The periplasmic cytochrome *c*₃ of *Desulfovibrio vulgaris* has recently been implicated in both Fe(III) reduction and U(VI) reduction by this bacterium (Lovley, 1993; Lovley *et al.*, 1993), although whether or not the protein is a physiological Fe(III) reductase is yet to be established (Lovley, 1995). It seems unlikely that such a system could be coupled to vectorial proton translocation since the primary electron donor is thought to be periplasmic hydrogenase.

A final piece of evidence which supports the hypothesis that *R. capsulatus* expresses a specific Fe(III) reductase is the slow rate of reduction for Fe(III) complexed by citrate. Reduction rates of Fe(III) by a non-specific reducing agent (such as ascorbic acid) would probably be governed by the reduction potentials of an Fe(III) complex. This is not true for the reduction of Fe(III) complexed by citrate in *R. capsulatus*, and thus dissimilation of Fe(III) by the bacterium may be suggested to function via a specific enzyme complex.

Components of the electron transport chain to Fe(III)

The inhibition of electron transport to Fe(III) in *R. capsulatus* strain N22DNAR⁺ by HQNO suggests the involvement of a cytochrome *b* dependent quinol-oxidizing complex. However, the insensitivity to myxothiazol of Fe(II) formation from Fe(III)(maltol)₃ appears to indicate that the cytochrome *bc*₁ complex is not involved in electron transport to this substrate, whilst the comparable reduction rates of Fe(III)(maltol)₃ obtained using MT1131 and its cytochrome *bc*₁ deficient mutant MTCBC1 support such a theory. The operation of an alternative ubiquinol:cytochrome *c* oxidoreductase activity in *R. capsulatus* for the reduction of this substrate is moreover suggested. However, it has been shown with strains of *R. capsulatus* possessing a poor capacity for

nitrous oxide reduction that the flux of electrons from an alternative ubiquinol:cytochrome c oxidoreductase is sufficient to render respiration of this substrate near insensitive to myxothiazol (Richardson *et al.*, 1989). The dependence of the reaction on the cytochrome bc_1 complex was only revealed when the terminal nitrous oxide reductase activity was high, and parallel pathways of ubiquinol oxidation were subsequently proposed to exist for electron transport to the substrate. Therefore, if Fe(III)-reducing activity is sufficiently low to govern the rate of reaction, then the flow of electrons to Fe(III)(maltol)₃ in *R. capsulatus* may not be independent of the cytochrome bc_1 complex.

In contrast to results with Fe(III)(maltol)₃, reduction rates of Fe(III) complexed by NTA were approximately halved in strain MTCBC1 compared to the parent MT1131. Moreover, whilst Fe(III) complexed by NTA was reduced at around twice the rate of Fe(III)(maltol)₃ in MT1131, reduction rates for the two species are similar in MTCBC1. These data, from internally controlled experiments, strongly suggest that the cytochrome bc_1 complex is involved in electron transport to Fe(III) complexed by NTA, and are corroborated by the finding with strain N22DNAR⁺ that myxothiazol significantly inhibited reduction of the species in the presence of malate. Fe(III) complexed by NTA thus appears to be a superior substrate for reduction by *R. capsulatus*, with Fe(III) reducing activity being sufficiently high for the inhibition of the cytochrome bc_1 complex to depress reaction rate. That the reduction rate of Fe(III) complexed with NTA by strain N22DNAR⁺ in the absence of malate was unaffected by myxothiazol demonstrates availability of electrons from the ubiquinol pool to also be a controlling factor. It is conceivable that some component of the cytochrome bc_1 complex is responsible for reduction of Fe(III) complexed by NTA, and that similar non-specific reduction of Fe(III)(maltol)₃ does not occur owing to differences in reduction potentials between the two Fe(III) species.

For the cytochrome c_2 deficient mutant MTG4S4, only slight lowering of Fe(II) formation rates compared to MT1131 were noted from both Fe(III)(maltol)₃ and the cation complexed by NTA. This suggests that the electron flow to Fe(III) in *R. capsulatus* can operate independently of cytochrome c_2 . Previous work has shown cytochrome c_2 to be obligatory for electron transport to the nitrous oxide reductase of the bacterium (Richardson *et al.*, 1991), but reduction of nitric oxide by MTG4S4 has been found to occur at a rate comparable with that using MT1131 (Bell *et al.*, 1992). Hence another mediator of electron transport from the cytochrome bc_1 complex to the nitric oxide reductase was proposed by Bell *et al.* (1992). McEwan (1994) has recently suggested that the redox protein in question could be cytochrome c_x , which is thought to operate in parallel with cytochrome c_2 during cyclic electron transport (Jones *et al.*, 1990).

The formation of Fe(II) from Fe(III)(maltol)₃ using the doubly deficient mutant MTGS18 was at a rate less than half that using the parent MT1131, whilst a similar drop in reduction rate of Fe(III) complexed by NTA using MTGS18 as opposed to cytochrome bc_1 deficient

MTCBC1 was noted. These data show that cytochrome c_2 is not obligatory for electron transport from the alternative ubiquinol:cytochrome c oxidoreductase to Fe(III). However, the differences in reduction rates between strains are not sufficient for firm conclusions to be drawn regarding the relative abilities of cytochrome c_2 and the alternative redox protein to mediate electron transport in the absence of the cytochrome bc_1 complex. The similar rates of Fe(II) formation from Fe(III) complexed by NTA and Fe(III)(maltol)₃ in MTGS18 reinforce the hypothesis that the cytochrome bc_1 complex contributes to reduction of the former species in wild-type cells.

Data collected with the parent strain 37b4 and its mutant H123 suggest that the pentacoordinate cytochrome c' plays no role in Fe(III) respiration. Similar experiments have previously shown this protein not to be involved in the reduction of nitric oxide by *R. capsulatus* (Bell *et al.*, 1992).

Physiological role of Fe(III) reduction by *R. capsulatus*

It is now widely regarded that the physiological importance of redox poisoning by auxiliary oxidants is a primary reason for the expression of a variety of terminal dissimilatory reductases operative under anaerobic conditions in *R. capsulatus* (McEwan, 1994). During anaerobic phototrophic growth on the highly reduced carbon substrates butyrate and propionate terminal electron acceptors act as electron sinks, whilst growth on the more oxidized substrates succinate and malate is enhanced at low intensities of light by auxiliary oxidants (Richardson *et al.*, 1988; Jones *et al.*, 1990). Unfortunately, we have thus far been unable to reproducibly demonstrate an increase in biomass when growing batch cultures of *R. capsulatus* on propionate or butyrate in the presence of Fe(III). We believe this may be a consequence of the toxicity of the ligands at the concentrations required (60 mM maltol, 20 mM NTA or EDTA). Indeed, we have failed to grow *S. putrefaciens* on any Fe(III) species apart from the cation complexed by citrate. Detailed studies in continuous cultures of *R. capsulatus*, with the concentration of Fe(III) ligands being maintained at sub-toxic levels, are thus required to firmly establish whether the cation can support phototrophic growth on highly reduced carbon substrates.

R. capsulatus cells obtained from both phototrophic anaerobic and microaerobic cultures were found to be capable of dissimilatory Fe(III) reduction, suggesting that respiration of the cation might occur under oxygen-limited conditions. Hence Fe(III) dissimilation could be of major importance for the survival of the bacterium in natural light-limited and/or micro-oxic environments.

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