Ferric reductase A is essential for effective iron acquisition in *Paracoccus denitrificans*

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Based on N-terminal sequences obtained from the purified cytoplasmic ferric reductases FerA and FerB, their corresponding genes were identified in the published genome sequence of *Paracoccus denitrificans* Pd1222. The *ferA* and *ferB* genes were cloned and individually inactivated by insertion of a kanamycin resistance marker, and then returned to *P. denitrificans* for exchange with their wild-type copies. The resulting *ferA* and *ferB* mutant strains showed normal growth in brain heart infusion broth. Unlike the *ferB* mutant, the strain lacking FerA did not grow on succinate minimal medium with ferric 2,3-dihydroxybenzoate as the iron source, and grew only poorly in the presence of ferric sulfate, chloride, citrate, NTA, EDTA and EGTA. Moreover, the *ferA* mutant strain was unable to produce catechols, which are normally detectable in supernatants from iron-limited wild-type cultures. Complementation of the *ferA* mutation using a derivative of the conjugative broad-host-range plasmid pEG400 that contained the whole *ferA* gene and its putative promoter region largely restored the wild-type phenotype. Partial, though significant, restoration could also be achieved with 1 mM chorismate added to the growth medium. The purified FerA protein acted as an NADH:FMN oxidoreductase and catalysed the FMN-mediated reductive release of iron from the ferric complex of parabactin, the major catecholate siderophore of *P. denitrificans*. The deduced amino acid sequence of the FerA protein has closest similarity to flavin reductases that form part of the flavin-dependent two-component monooxygenases. Taken together, our results demonstrate an essential role of reduced flavins in the utilization of exogenous ferric iron. These flavins not only provide the electrons for Fe(III) reduction but most probably also affect the rate of siderophore production.

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INTRODUCTION

Enzyme-catalysed reduction of Fe(III) to Fe(II) is generally accepted as a prerequisite for iron incorporation into cellular components (Schroder *et al.*, 2003). However, due to a relatively high chemical reactivity of Fe(III) complexes, it is often difficult to establish a unique inter-relationship between iron assimilation on the one hand and the Fe(III)-reducing activity of a particular enzyme on the other. The situation seems relatively clear-cut for systems where the reductive step precedes the import of iron into cells. *Saccharomyces cerevisiae*, for example, uses two externally directed, plasma-membrane ferric reductases, FRE1 and FRE2, to generate ferrous iron that can be accessed by the uptake proteins (Dancis *et al.*, 1990; Georgatsou & Alexandraki, 1994). Deletion of the *FRE1* and *FRE2* genes from the yeast genome not only completely abolishes

membrane-associated ferric reductase activity, but also renders the cell incapable of growing for an extended period of time in iron-deficient media. Regarding cytoplasmic Fe(III) reductases, only one analogous observation has been reported to date. FhuF, a cytoplasmic 2Fe–2S protein of *Escherichia coli*, has been shown to be indispensable for growth on ferrioxamine B as the sole iron source. FhuF in its [Fe²⁺–Fe³⁺] state can directly reduce ferrioxamine B-bound Fe(III); this suggests its function as a specific reductase for this type of siderophore (Matzanke *et al.*, 2004; Muller *et al.*, 1998).

Paracoccus denitrificans is a Gram-negative, strictly respiring, facultative anaerobic bacterium that is found in soil, sewage and sludge (for a review on its metabolism and genetics, see Baker *et al.*, 1998). When the cells experience iron deficiency, they produce a mixture of catechol-containing compounds (Tait, 1975). One of them, now called parabactin, was identified as *N*-[3-(2,3-dihydroxybenzamido)propyl]-*N*-[4-(2,3-dihydroxybenzamido)butyl]-2-(2-hydroxyphenyl)-*trans*-5-methyloxazoline-4-carboxamide (Person & Neilands,

A sequence comparison of FerA with some homologous proteins identified by BLAST searching is available with the online version of this paper.

1979). Fe(III) from the environment binds to parabactin via the four oxygens of the two catechol groups and two additional ligands (N and O) associated with the 2-(2-hydroxy)oxazoline group. The ferric parabactin–chelate complex formed then targets to a highly specific receptor in the outer membrane (Bergeron *et al.*, 1988). After internalization, further intracellular processing may involve hydrolysis of the parabactin oxazoline ring to give an open structure with a lower affinity for Fe(III) and/or enzymic reduction of Fe(III) to Fe(II) (Robinson & McArdle, 1981; Tait, 1975). It should be noted that *P. denitrificans* also acquires iron independently of siderophore production via a poorly defined low-affinity uptake pathway (Wee *et al.*, 1988).

P. denitrificans possesses two soluble enzymes, FerA and FerB, capable of reducing a number of Fe(III) complexes at the expense of NADH (Mazoch *et al.*, 2004). Both of them interact with flavins, but in a different manner. FerA can be classified as an NADH:flavin oxidoreductase with a sequential reaction mechanism. It requires the addition of FMN or riboflavin for activity on Fe(III) substrates. In these reactions, the apparent substrate specificity of FerA seems to originate exclusively from different chemical reactivities of Fe(III) compounds with the free reduced flavin produced by the enzyme. In contrast to FerA, FerB contains a non-covalently bound redox-active FAD coenzyme, uses NADPH as well as NADH, does not reduce free FMN, and displays a ping-pong type kinetic pattern with NADH and Fe(III)-nitrilotriacetate as substrates.

In the present study we have cloned and characterized the genes encoding FerA and FerB and have undertaken phenotypic analyses of strains mutated in these genes in

order to get a fundamental understanding of their physiological role in bacterial iron metabolism.

METHODS

Bacterial strains, plasmids and growth media. The strains and plasmids employed in this study are listed in Table 1. *P. denitrificans* strains were grown in Brain Heart Infusion (BHI) broth (Oxoid) or in minimal medium (Tait, 1975) containing 50 mM succinate. When appropriate, the media were rendered iron-deficient by the addition of 2,2'-bipyridyl at a minimum concentration that still fully prevented bacterial growth without an externally added iron source. For cultivation of *Escherichia coli* strains, Yeast-tryptone (YT) broth (Oxoid) was used. Selection for resistance to antibiotics was performed with rifampicin (20 or 80 µg ml⁻¹), kanamycin (25 µg ml⁻¹), streptomycin (25 µg ml⁻¹) and tetracycline (12.5 µg ml⁻¹) (all Sigma).

Purification of ferric reductases. FerA and FerB were purified from the cytoplasmic fraction of succinate-grown *P. denitrificans* by preparative HPLC chromatography as described previously (Mazoch *et al.*, 2004).

Preparation of ferric parabactin. Parabactin was isolated from cell-free supernatants of *P. denitrificans* cultures by extraction with ethyl acetate (Neilands, 1983). The 1:1 Fe(III) complex was formed by mixing a 40 mM solution of parabactin in 1 M NaOH with an equal volume of 40 mM FeCl₃ in 1 M HCl.

PCR and DNA sequencing. Oligonucleotide primers (Table 2) were designed using Oligo 4.0 (MBI) and synthesized commercially by Invitrogen (via KRD, Prague, Czech Republic). Primers were based on sequence data from the *P. denitrificans* Pd1222 sequencing project (<http://genome.ornl.gov/microbial/pden/>). Standard PCR amplifications utilized *Taq* DNA polymerase (TopBio). Successful PCR

Table 1. Strains and plasmids

Strain/plasmid	Relevant characteristic	Source/reference
<i>E. coli</i>		
TG1	<i>supE hsdD5 thi Δ(lac-proAB) F'(traD36 proAB lac^f lacZΔM15)</i>	Sambrook <i>et al.</i> (1989)
HB101/RH2020	<i>supE44 hsdS20(r_B⁻ m_B⁻) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 leuB6 thi-1; Tet^r</i>	Boyer & Roulland-Dussoix (1969)
<i>P. denitrificans</i>		
Pd1222	DSM 413; Rif ^r Spc ^r ; enhanced conjugation frequency	de Vries <i>et al.</i> (1989)
Pd12122	Pd1222 derivative with pEG4122	This study
Pd20121	Pd1222 derivative; <i>ferA</i> truncated; Kan ^r	This study
Pd20021	Pd1222 derivative; <i>ferB</i> truncated; Kan ^r	This study
Pd20122	Pd20121 derivative with pEG4122	This study
Pd30121	Pd1222 derivative; pPr121 integrated	This study
Pd30021	Pd1222 derivative; pPr021 integrated	This study
Plasmids		
pBK11	Str ^r ; <i>lacZ</i> promoter-probe vector	Kessler <i>et al.</i> (1992)
pPr121	pBK11 derivative; <i>PferA-lacZ</i>	This study
pPr021	pBK11 derivative; <i>PferB-lacZ</i>	This study
pEG400	<i>incP</i> broad-host-range vector; Str ^r Spc ^r ; pUC12mcs	Gerhus <i>et al.</i> (1990)
pEG4122	pEG400 derivative with <i>ferA</i> locus	This study
pRVS3	<i>oriT</i> (RK2); Kan ^r (Tn903); pBSII (KS) derivative	van Spanning <i>et al.</i> (1995)
pRVS3121	pRVS3 derivative, <i>ferA::Kan^r</i>	This study
pRVS3021	pRVS3 derivative, <i>ferB::Kan^r</i>	This study

Table 2. Oligonucleotide primers used in this study

Primer	Sequence (5'–3')*	Target	Location†
1	AAAGGATCCGCCGTCTTCCCCCGCGACCC	<i>ferA</i>	+ 5 to + 25
2	AAAGAGCTCGGGATCACCGGCACGCCCTCG	<i>ferA</i>	+ 380 to + 360
3	AAAGAGCTCAGACCGTCGCCGTCATGGTGGG	<i>ferB</i>	+ 8 to + 29
4	AAAGGATCCAGCGCCGC-ACCGATCACGCCCG	<i>ferB</i>	+ 362 to + 341
5	ATGCGCTTAAGCTTGGCCCCGGCCGCT	Upstream of <i>ferA</i>	–191 to –176
6	ATGATCTGGGATCCAGGCCTAGCCGGCAT	Downstream of <i>ferA</i>	+ 536 to + 561
7	GCAGACGACGACAGGGCGCGAACATG	<i>ferA</i>	–23 to + 3
8	AAACCGCAAGAGGAGATCAGGCATGG	<i>ferB</i>	–23 to + 4
9	AACGCCAGGGTTTCCCAGTCACGAC	pRVS3	
10	AATTTACACAGGAAACAGCTATGAC	pRVS3	
11	AAAGAAATTCGCGCCGCCGCTCAAGCGCGGCTT	Upstream of <i>ferA</i>	–377 to –356
12	AAAGAAATTCGCCGGCTCACGGCCAGAAGCTG	Upstream of <i>ferB</i>	–378 to –356
13	AAAGGATCCTCGCGCAGCAGCCGTGCCTCGGC	<i>ferA</i>	+ 67 to + 91
14	AAAGGATCCCAGCCGCCCTCGGCCAGTTTC	<i>ferB</i>	+ 78 to + 99
15	AAACGACGGCCAGTGAATCCGTA	<i>lacZ</i> (PBK11)	

*Sequences in italics denote restriction sites for *Bam*HI (1, 4, 6, 13, 14), *Sac*I (2, 3), *Hind*III (5) and *Eco*RI (11, 12).

†Position +1 corresponds to the first base of the relevant ORF.

amplification and digestion by restrictases were confirmed by agarose gel electrophoresis. DNA sequencing was carried out commercially by Genomac.

DNA manipulations and transformations. Recombinant DNA procedures were performed according to standard procedures (Sambrook *et al.*, 1989). Restriction endonucleases and T4 ligase were from New England BioLabs. Purification of DNA following PCR or restriction was achieved using the QIAEX II Gel Extraction kit (Qiagen). Plasmid DNA was purified from *E. coli* using the QIAprep Spin Miniprep kit (Qiagen). Chemically competent *E. coli* TG1 cells were transformed by heat-shock in accordance with the manufacturer's instructions.

Construction of *ferA* and *ferB* mutant strains. Internal 350 bp fragments of the *ferA* and *ferB* genes were PCR-amplified using *Taq* DNA polymerase with the primer pairs 1/3 and 2/4 (Table 2). The resulting fragments were ligated into pGEM-T Easy and these constructs were then transferred to *E. coli*. Once the identity of the insert had been confirmed by sequencing across the junction sites, the *Bam*HI–*Sac*I fragment was excised and cloned into the *Bam*HI/*Sac*I sites of suicide vector pRVS3 (van Spanning *et al.*, 1995). The resulting plasmids pRVS3121 (for inactivation of *ferA*) or pRVS3021 (for inactivation of *ferB*) were mobilized from *E. coli* TG1 to Pd1222 via triparental mating using *E. coli* HB101(pRK2020) as the helper strain (de Gier *et al.*, 1994). Clones of *P. denitrificans* that inserted the suicide vector successfully into their chromosome by homologous recombination were selected on medium with rifampicin and kanamycin. The mutant strains obtained in this way were checked for correct integration of the respective vectors by PCR using primer pairs 1/9 and 7/9 for *ferA* inactivation, and 3/10 and 8/10 for *ferB* inactivation.

Construction of a complemented strain of the *ferA* mutant. The *ferA* gene was amplified with primers 5 and 6 (Table 2). The 800 bp product of this amplification was digested with *Hind*III and *Bam*HI, and cloned into broad-host-range vector pEG400 (Gerhus *et al.*, 1990), yielding pEG4122. Plasmid pEG4122 was transferred from *E. coli* TG1 to the *ferA* mutant strain Pd20121 via triparental mating. The resulting strain was designated Pd20122. As a control, the same

plasmid was also transferred to the wild-type strain Pd1222, resulting in strain Pd12122.

Construction of promoter-*lacZ* fusions. DNA regions upstream of the coding sequences of the *ferA* and *ferB* genes were PCR-amplified using *Taq* DNA polymerase with the primer pairs 11/13 and 12/14, respectively (Table 2). The fragments obtained were ligated into the pGEM-T Easy vector (Promega) and transferred to *E. coli* TG1. The *Eco*RI–*Bam*HI fragments of the pGEM derivatives were then excised and inserted into the *Eco*RI/*Bam*HI-restricted vector pBK11 (Kessler *et al.*, 1992). The resulting promoter-*lacZ* reporter vectors, designated pPr121 and pPr021, were individually transferred into *P. denitrificans* Pd1222 via triparental mating. Clones of *P. denitrificans* that inserted the vector successfully into their chromosome were selected on plates with rifampicin and streptomycin. Colonies were checked for proper integration of the promoter-*lacZ* fusion by site-specific PCR using the forward primers 11 or 12 in combination with the reverse primer 15, specific for the *lacZ* gene. The strains kept for further studies were designated Pd30121 and Pd30021.

Growth kinetics. Growth parameters of *P. denitrificans* strains were quantified using a Bioscreen Microbiological Growth Analyser (Bioscreen C; Labsystems). Overnight cultures in BHI were inoculated 1:300 in triplicate into microtitre plates containing 0.3 ml of appropriate medium. The plates were then sealed and incubated for 5 days at 30 °C with continual shaking. The optical density at 600 nm was measured every 20 min. The lag phase duration and specific growth rates were calculated from plots of log(OD₆₀₀) versus time, using a method similar to that described by Warringer & Blomberg (2003).

Assays. Ferric reductase activity was measured spectrophotometrically in 25 mM Tris/HCl, pH 7.4, at 30 °C, with either 0.15 mM NADH or NADPH as electron donor, 0.2 mM ferric nitrilotriacetate as electron acceptor, and 0.8 mM ferrozine as a chromogenic Fe(II)-chelating agent (Mazoch *et al.*, 2004). Additionally, where indicated, 0.05 mM FMN was also included. β -Galactosidase activity was assayed by the method described by Miller (1972). The concentration of catechol-derived compounds was determined by Arnow's assay (Arnow, 1937) using 2,3-dihydroxybenzoic acid as a standard. Total protein was determined by the Lowry method.

RESULTS

Identification of the genes encoding FerA and FerB

The N-terminal amino acid sequences of the FerA and FerB proteins of *P. denitrificans* strain CCM 982 determined previously (SRLPPATRDKLADITFFPA and MVKTVAV-MVGLSRKDSLAKMLMKVLQK, respectively; see Mazoch *et al.*, 2004) were used as probes to search the *P. denitrificans* strain Pd1222 genome database (<http://genome.ornl.gov/microbial/pden/>). Significant matches (90 and 96 %) were found to the putative coding regions 2689 and 4071, respectively. At the predicted N terminus of the product from the 546 bp gene fragment, the FerA sequence is preceded by a methionine, which is probably cleaved off after translation. The mature protein is expected to be 180 aa long and have a relative molecular mass (M_r) of 18 898 and a pI of 6.2. This corresponds well with our earlier experimental estimates of M_r (18 814 and 18 917) and pI (6.9). The FerB protein encoded by the 549 bp gene fragment starts with a sequence identical to that of the published FerB, and appears to consist of 182 aa residues, has an M_r of 20 222 and a pI of 6.5. Again, these values are in agreement with those found previously for the purified FerB protein (20 196 and 5.5, respectively). Minor differences may be due to the employment of a different strain of *P. denitrificans* compared to the original source for ferric reductase purification.

The genetic organization of the regions surrounding the *ferA* and *ferB* genes is shown in Fig. 1. *ferA* is located downstream of three ORFs, two of which (ORF2 and ORF3) are predicted to encode inositol monophosphatase and a modulator of DNA gyrase, i.e. proteins with no obvious relationship to iron metabolism. Inspection of the 5' region of *ferA* revealed the sequence 5'-TTGATG-N₁₇-TAGAAA-3' that conforms closely (8 of 12 bases) to the canonical *E. coli* σ^{70} motif 5'-TTGACA-N₍₁₅₋₁₉₎-TATAAT-3' (Hawley & McClure, 1983). This finding may indicate that *ferA* has its own promoter. All ORFs located in the vicinity of *ferB* have the opposite transcriptional polarity, arguing against *ferB* being a part of an operon. Upstream of the *ferB* gene, only a Shine-Dalgarno sequence (5'-AGGAGA-3') was apparent from -7 to the translation start; no consensus sequences typical for *E. coli* promoters were identified at the appropriate positions.

Activity of the promoter regions of ferric reductase genes

In an effort to establish the functionality of the putative promoters of the *ferA* and *ferB* genes, we fused these regions to the *lacZ* gene of *E. coli* (see Methods) and followed the production of β -galactosidase in cells growing in either iron-rich or iron-limiting conditions (succinate minimal medium with either 100 or 10 μ M ferric sulfate). We consistently observed relatively high basal levels of *lacZ* expression that could not be further enhanced by iron

Fig. 1. Genetic organization of regions surrounding the *ferA* and *ferB* genes. Arrowed boxes indicate the size and direction of transcription of the ORFs. The position of the first bases of the ORFs are given with respect to the first translated base of the *ferA* gene. ORF numbering corresponds to that of the *P. denitrificans* genome database at <http://genome.ornl.gov/microbial/pden/>. ORF2 and ORF3 encode a putative inositol monophosphatase and a modulator of DNA gyrase, respectively; no sequences homologous to ORF1 were found by BLAST searching. The putative σ^{70} motif upstream of *ferA* and the Shine-Dalgarno sequence upstream of *ferB* are also shown.

limitation. At the two concentrations of iron used, the respective β -galactosidase activities were estimated to be 710 ± 50 and 690 ± 60 Miller units for strain Pd30121, and 1300 ± 200 and 1200 ± 200 Miller units for strain Pd30021 (means $\pm$ SD, three replicates taken from exponential-phase cultures at an OD₆₀₀ of 0.6). These results suggest that both *ferA* and *ferB* are probably expressed constitutively.

Disruption of the ferric reductase genes

The available nucleotide sequences allowed us to design primers specific for inner parts of the ferric reductase genes (primer pairs 1/2 and 3/4; see Table 2). These were then used in PCRs with genomic DNA of *P. denitrificans* Pd1222 as template to generate internal *ferA* and *ferB* fragments for the directed plasmid integration procedure outlined in Methods. The cell extracts of *ferA* (Pd20121) and *ferB* (Pd20021) mutant strains were tested for ferric reductase activity with Fe(III) nitrilotriacetate as electron acceptor, and NADH or NADPH as electron donors (Table 3). In the extract of the *ferA* mutant strain, both NADH and NADPH supported the reaction, but FMN had no effect, clearly demonstrating the absence of FerA. In the extract of the *ferB* mutant strain, the reaction rates with NADH or NADPH were about half compared to the control, and there was an approximately threefold further stimulation of NADH-dependent activity upon addition of 50 μ M FMN. To confirm that the activity in the absence of FMN was not due to residual FerB, the extracts from the wild-type cells

Table 3. Ferric reductase activity in cytosol from the parental (Pd1222), *ferB* mutant (Pd20021), *ferA* mutant (Pd20121) and complemented *ferA* mutant (Pd20122) strains

Activities are given in nmol Fe(II) formed s⁻¹ (mg protein)⁻¹ (means ± SD of triplicates).

Strain	-FMN		+ 50 µM FMN	
	NADH	NADPH	NADH	NADPH
Pd1222	17 ± 3	17 ± 5	40 ± 2	20 ± 2
Pd20021	11 ± 2	10 ± 2	45 ± 6	15 ± 2
Pd20121	56 ± 2	61 ± 2	66 ± 3	50 ± 5
Pd20122	39 ± 5	21 ± 4	57 ± 5	22 ± 1

and from the mutant were chromatographed on a HP10/10 Mono Q column (Pharmacia) with a salt gradient from 0 to 1 M NaCl. While the elution profile for Fe(III) reductase activity of the wild-type cell extract consisted of a peak corresponding to FerA (elution at 0.05–0.15 mM NaCl) and a peak corresponding to FerB (elution at 0.25–0.35 mM NaCl), the latter peak was missing from the extract of the *ferB* mutant. In summary, these data confirm that both insertional inactivations had resulted in the exclusive inability to express FerA (in the *ferA* mutant strain) or FerB (in the *ferB* mutant strain).

Growth responses of wild-type and mutant strains, and production of catechols

To find out whether FerA or FerB might be involved in iron assimilation, cells of the wild-type and both mutant strains were examined for growth and catechol production in succinate medium (Fig. 2). A full growth response of the *ferA* mutant strain was observed at a concentration of ferric sulfate that was almost 10-fold higher as compared to the wild-type and *ferB* mutant strains. At subsaturating initial concentrations of Fe, the growing wild-type and *ferB* mutant cells excreted catechol-containing compounds in amounts up to about 30 µM, whereas only traces of catechols were detectable in culture medium of the FerA-deficient cells. Differences in utilization of various iron sources are shown in Table 4. The *ferA* mutant strain failed to grow in the presence of 50 µM ferric 2,3-dihydroxybenzoate, and grew only poorly on other ferric complexes of the same concentrations. Contrary to what was seen in the synthetic medium (Fig. 2, Table 4), the growth characteristics of both mutant strains growing in brain heart infusion (BHI) broth were indistinguishable from those of the wild-type strain (results not shown).

Genetic complementation tests for *ferA*

Next, experiments were performed to confirm that the phenotype of the *ferA* mutant is due to the specific loss of functional FerA, but not due to the occurrence of a mutation elsewhere in the genome. To this end, the whole

Fig. 2. (a) Specific growth rate during the exponential phase, (b) maximum optical density, and (c) concentration of catechols accumulated as functions of the initial concentration of Fe(III). Bacteria were inoculated into 100 ml shaking flasks containing 15 ml succinate medium supplemented with the indicated concentration of ferric iron (added as sulfate), and growth kinetics were monitored. After reaching stationary phase, cultures were analysed for the concentration of catechols. □, Parental strain (Pd1222); ◇, *ferA* mutant (Pd20121); △, complemented *ferA* mutant (Pd20122); ○, *ferB* mutant (Pd20021).

Table 4. Comparison of the specific growth rates (μ) and lag times (t_{lag}) for the parental (Pd1222), *ferA* mutant (Pd20121) and complemented *ferA* mutant (Pd20122) strains

The values are means $\pm$ SD from three independent cultivations. NG, No growth.

Growth parameter	μ (h^{-1})			t_{lag} (h)		
	Pd1222	Pd20121	Pd20122	Pd1222	Pd20121	Pd20122
Ferric substrate (50 μM)						
$\text{Fe}_2(\text{SO}_4)_3$	0.21 ± 0.03	0.14 ± 0.01	0.17 ± 0.03	<0.3	30 ± 10	8 ± 4
FeCl_3	0.18 ± 0.02	0.13 ± 0.01	0.16 ± 0.03	<0.3	22 ± 10	7 ± 2
FeNTA	0.27 ± 0.04	0.13 ± 0.03	0.17 ± 0.03	<0.3	22 ± 11	8 ± 1
Fe citrate	0.28 ± 0.02	0.11 ± 0.02	0.20 ± 0.02	<0.3	20 ± 9	9 ± 2
FeEDTA	0.20 ± 0.02	0.12 ± 0.01	0.15 ± 0.02	<0.3	41 ± 17	19 ± 7
FeEGTA	0.19 ± 0.02	0.13 ± 0.02	0.16 ± 0.03	<0.3	31 ± 14	17 ± 2
$\text{Fe}(2,3\text{-DHB})_3$	0.22 ± 0.04	NG	0.15 ± 0.02	<0.3	NG	17 ± 8
$\text{Fe}(3,4\text{-DHB})_3$	0.19 ± 0.05	NG	0.14 ± 0.03	<0.3	NG	18 ± 5

ferA gene and a region encompassing 250 bp upstream of the *ferA* translational start codon were ligated into broad-host-range plasmid pEG400, and the resulting vector was transferred into *P. denitrificans* Pd20121. The presence of this plasmid largely reversed the effect of the *ferA* mutation on growth in minimal succinate media and on production of siderophores (Fig. 2, Table 4). In confirmation of the expected expression of active FerA, the total NADH-linked ferric reductase activity in the cell-free extract from the complemented *ferA* mutant cells became more responsive to further stimulation by FMN (Table 3).

Complementation of *ferA::Kan^r* with the *ferA* gene expressed *in trans* had to be performed in the presence of streptomycin to maintain the pEG4122 plasmid. Since the antibiotic itself may adversely affect cellular function even in resistant strains, it is not very meaningful to directly compare physiological responses of the complemented mutant strain with those observed for the wild-type strain when streptomycin was absent (Table 4). To make this comparison as valid as possible, we introduced pEG4122 also into the original, unmutagenized strain Pd1222. The resulting strain, Pd12122, grew on medium containing streptomycin with kinetics virtually identical to that of the complemented *ferA* mutant strain (data not shown). Taken together, these complementation data clearly demonstrated that the defective phenotype of *ferA::Kan^r* was solely caused by the absence of FerA.

Search for revertants

Growth recovery of the *ferA* cells observed after a prolonged period of incubation with no selective antibiotic pressure (kanamycin absent) could in principle result from a spontaneous reversion to the wild-type. To check this possibility, Pd20121 was grown in mineral medium without antibiotics and reinoculated three times from stationary-phase cultures. The growth pattern with a prolonged lag phase continued to occur during these growth cycles. The resulting culture was spread and grown

on BHI agar plates to give single colonies; ten of them were randomly picked for PCR analysis. In all cases, amplification of a PCR product of 446 bp was obtained with the primer pair 7/9, revealing the presence of the suicide plasmid in the bacterial genome at the proper insertion site. These data showed that the observed behaviour of the *ferA* mutant cultures was due to the mutant itself and not to the appearance of a true or pseudo-wild-type revertant.

Effect of added chorismate

Because the synthesis of catecholate siderophores starts with the formation of isochorismic acid from chorismic acid (Knaggs, 1999), we wondered whether the severely impaired production of parabactin in the *ferA* mutant might be attributable to a reduced availability of chorismate. We therefore repeated the growth experiments on the *ferA* cells under the same conditions as before (Table 4), but in the presence of 1 mM chorismate. There was, indeed, a partial reversal of the mutant phenotype as manifested by a decreased lag phase (10 ± 5 h), elevated growth rate ($0.17 \pm 0.03 \text{ h}^{-1}$) and production of a significant level of catechols ($9 \pm 1 \mu\text{M}$) (mean $\pm$ SD, data from three parallel cultivations). In contrast, neither cell growth nor siderophore accumulation was affected by the addition of chorismate to the wild-type cell cultures. The above hypothesis of a limited supply of chorismate in *ferA* cells thus appears to be substantiated.

Parabactin reduction catalysed by FerA

Having recognized the potential importance of FerA for iron assimilation, we decided to examine the activity of the purified enzyme towards the Fe(III) complex of parabactin. The assay mixture contained ferrozine to trap the released Fe(II) in the form of a coloured complex. When the initial concentration of Fe(III) parabactin was varied at 150 μM NADH, 50 μM FMN, 800 μM ferrozine, 3.9 μg FerA, a saturation curve was obtained from which non-linear regression gave an apparent half-saturation constant of $6 \pm 1 \mu\text{M}$ and a limiting rate of

$2.1 \pm 0.1 \text{ nmol s}^{-1} (\text{mg protein})^{-1}$. The reaction did not proceed when the enzyme or any of the reagents was omitted. These observations clearly demonstrated the capability of FerA to deal with a reductive release of iron from ferriparabactin under the mediation of a free flavin.

Sequence homology of FerA

The amino acid sequence of FerA was subjected to a BLASTP search against the non-redundant protein database (Swiss-Prot/TREMBL). This resulted in the identification of 10 sequences with 40–45 % identity and 50–60 % similarity to FerA. Most of them are oxidoreductases with known participation of flavins. A multiple sequence alignment of the most homologous regions of four representatives of these proteins is shown in Supplementary Fig. S1, available with the online version of this paper.

DISCUSSION

Our previous studies on purified FerA and FerB have established that only FerA reduces the ferric 2,3-dihydroxybenzoate complex at an appreciable rate (Mazoch *et al.*, 2004). Since *P. denitrificans* exclusively synthesizes siderophores of the catechol type (Tait, 1975), we considered FerA as a better candidate than FerB to be a member of the iron assimilatory pathway. This hypothesis is now verified by the results of our disruption-complementation experiments, which demonstrate a marked growth deficit of the *ferA* mutant strain in minimal medium containing ferric salts. The observation that the *ferA* mutant grows normally in BHI medium probably means that *P. denitrificans* possesses more than one pathway for iron uptake, one of which is operative under nutrient-rich growth conditions and does not rely on FerA.

It seems of interest to note that in *E. coli* there is an NAD(P)H:flavin oxidoreductase (Fre) with catalytic properties similar to those of *P. denitrificans* FerA (Coves & Fontecave, 1993). An *E. coli* mutant lacking the active *fre* gene was found to grow at the same rate as wild-type cells, both in rich or minimal media. Consequently, Fre was apparently dispensable under the experimental conditions used (Coves *et al.*, 1993). This behaviour differs from that of *P. denitrificans* and may be related to a relatively high level of an additional flavin/Fe(III) reductase activity that apparently resides in the α -subunit of the *E. coli* sulfite reductase. In the case of *P. denitrificans*, chromatographic analyses of their cytoplasmic fraction have given no clear hint of the presence of a third Fe(III) reductase besides FerA and FerB.

Based on the sequence analysis (Fig. S1), FerA is most similar to component B of the enzyme complex that oxidatively splits nitrilotriacetate (NTA) into iminodiacetate and glyoxylate. This complex, first purified from the bacterium *Aminobacter aminovorans* (formerly *Chelatobacter heintzii*) (Uetz *et al.*, 1992), has a heterodimeric AB structure. Component A is an NTA monooxygenase that uses

FMNH₂ and O₂ to oxidize NTA, and component B is an NADH:FMN oxidoreductase that provides FMNH₂ for NTA oxidation. The two components are only loosely linked to one another, and B can easily be replaced by flavin reductases from other sources (Xu *et al.*, 1997). It remains uncertain whether these features relate in any way to FerA, since *P. denitrificans* does not seem to be able to utilize NTA as sole nitrogen source (V. Sedláček, unpublished observations), and we currently have no evidence for a physical coupling between FerA and any other enzyme.

We postulate that FerA provides reducing equivalents for Fe(III) reduction *in vivo*. We base this postulation on the fact that we have demonstrated that FerA effectively catalyses an FMN-mediated reductive release of iron from the ferric chelate of the authentic *P. denitrificans* siderophore parabactin, even when the latter complex is present at only micromolar concentrations. FerA thus can be added to the list of flavin reductases known to use bacterial siderophores as substrates (Schroder *et al.*, 2003). From cyclic voltametric data, the reduction potential of Fe(III)-parabactin was calculated to be -0.673 V , versus a standard hydrogen electrode, at pH 7.0 (Robinson & McArdle, 1981), i.e. well below the range of physiological reductants. Nevertheless, as discussed by Boukhalfa & Crumbliss (2002), reduction of even very stable ferric complexes can still proceed provided that the ferrous ion formed subsequently binds to an appropriate ligand. Most of the *in vitro* ferric reductase assays use ferrozine as a specific Fe(II) chelator to pull the redox reaction forward. The nature of the ligand exchange reactions within the bacterial cell remains to be established, although there is some evidence that Fe(II) in *E. coli* can transiently interact with a phosphorylated sugar derivative (Bohnke & Matzanke, 1995).

We also postulate that the absence of FerA negatively affects the production of siderophores. In many micro-organisms, the expression of genes involved in siderophore biosynthesis is regulated via the ferric uptake regulator (Fur) protein, which acts as an iron-responsive, DNA-binding repressor protein. Binding of Fur-Fe(II) to operator sequences results in repression of these genes, while under low-iron conditions, Fur is released and transcription takes place (Lee & Helmann, 2007). If the same holds true for *P. denitrificans*, then it might be predicted that deletion of FerA would promote derepression of siderophore formation as a consequence of decreased intracellular concentration of FMNH₂ and hence also of Fe(II). Our failure to observe this derepression may indicate that reduced flavins take part directly in the reaction(s) of the aromatic biosynthesis pathway. Especially noteworthy in this respect is the conversion of 5-enolpyruvylshikimate-3-phosphate to chorismate, because virtually all chorismate synthases studied to date require FMNH₂ for their activity (Macheroux *et al.*, 1999). It is therefore conceivable that in the *ferA* mutant strain, chorismate is still synthesized, but at a rate insufficient to sustain a massive build-up of siderophores. In line with the foregoing interpretation is our finding that

the provision of extra chorismate has the effect of increasing catecholate production.

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