A putative multicopper protein secreted by an atypical type II secretion system involved in the reduction of insoluble electron acceptors in *Geobacter sulfurreducens*

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Extracellular electron transfer onto Fe(III) oxides in *Geobacter sulfurreducens* is considered to require proteins that must be exported to the outer surface of the cell. In order to investigate this, the putative gene for OxpG, the pseudopilin involved in a type II general secretion pathway of Gram-negative bacteria, was deleted. The mutant was unable to grow with insoluble Fe(III) oxide as the electron acceptor. Growth on soluble Fe(III) was not affected. An analysis of proteins that accumulated in the periplasm of the *oxpG* mutant, but not in the wild-type, led to the identification of a secreted protein, OmpB. OmpB is predicted to be a multicopper protein, with highest homology to the manganese oxidase, MofA, from *Leptothrix discophora*. OmpB contains a potential Fe(III)-binding site and a fibronectin type III domain, suggesting a possible role for this protein in accessing Fe(III) oxides. OmpB was localized to the membrane fraction of *G. sulfurreducens* and in the supernatant of growing cultures, consistent with the type II secretion system exporting OmpB. A mutant in which *ompB* was deleted had the same phenotype as the *oxpG* mutant, suggesting that the failure to export OmpB was responsible for the inability of the *oxpG*-deficient mutant to reduce Fe(III) oxide. This is the first report that proposes a role for a multicopper oxidase-like protein in an anaerobic organism. These results further emphasize the importance of outer-membrane proteins in Fe(III) oxide reduction and suggest that outer-membrane proteins other than c-type cytochromes are required for Fe(III) oxide reduction in *Geobacter* species.

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INTRODUCTION

Microbial oxidation of organic matter coupled to the reduction of Fe(III) oxides is an important process in the carbon cycle of a variety of sedimentary environments (Lovley, 2000). Micro-organisms growing via Fe(III) reduction in subsurface environments can simultaneously reduce toxic metals such as U(VI), Tc(VII), Co (III) and V(V), which may be a useful strategy for the bioremediation of metal-contaminated subsurface environments (Anderson & Lovley, 2002; Anderson *et al.*, 2003; Caccavo *et al.*, 1994; Finneran *et al.*, 2002; Holmes *et al.*, 2002; Lloyd & Lovley, 2001; Ortiz-Bernad *et al.*, 2004). Furthermore, microbiological and geological evidence suggests that Fe(III) reduction

was an important form of respiration on early Earth (Lovley *et al.*, 2004; Tor *et al.*, 2004; Vargas *et al.*, 1998; Walker, 1987).

Molecular analysis has demonstrated that, with the exception of highly saline environments (Nevin *et al.*, 2003), *Geobacteraceae* are the predominant Fe(III)-reducing micro-organisms in a diversity of sedimentary environments in which Fe(III) reduction is an important process (Anderson *et al.*, 2003; Holmes *et al.*, 2002; North *et al.*, 2004; Röling *et al.*, 2001; Rooney-Varga *et al.*, 1999; Snoeyenbos-West *et al.*, 2000; Stein *et al.*, 2001). The primary form of Fe(III) in such environments is insoluble Fe(III) oxide (Lovley, 1991; Nevin & Lovley, 2002). The available evidence suggests that *Geobacter* species must directly contact Fe(III) oxides in order to reduce them (Childers *et al.*, 2002; Lovley *et al.*, 2004; Nevin & Lovley, 2000). Thus, electron transfer to Fe(III) oxides is expected to take place at or near the outer cell surface (Lovley *et al.*, 2004).

Most studies on electron transfer to Fe(III) in *Geobacter* and other dissimilatory Fe(III) reducers, such as *Shewanella*

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Abbreviations: MALDI-MS, matrix-assisted-laser-desorption/ionization time-of-flight mass spectrometry; NTA, nitrilotriacetic acid; PSD, post-source decay.

Four supplementary figures are available with the online version of this paper.

species, have focused on the role of *c*-type cytochromes (Lovley *et al.*, 2004; Richardson 2000). The genome of *G. sulfurreducens* reveals an exceptional number of putative *c*-type cytochromes (Methe *et al.*, 2003), many of which are predicted to be membrane-bound, and some of which are required for Fe(III) reduction (Leang *et al.*, 2003; Lloyd *et al.*, 2003; Reguera *et al.*, 2005; Mehta *et al.*, 2005).

However, proteins aside from cytochromes may also be important in Fe(III) oxide reduction. For example, pili are expressed during growth on Fe(III) oxide, but not soluble Fe(III) citrate, and a pilus-deficient mutant could not reduce Fe(III) oxides (Childers *et al.*, 2002; Reguera *et al.*, 2005). Studies on the pili of *G. sulfurreducens* revealed the presence of a homologue for *pilA*, the gene for the structural pilin protein. A phylogenetic analysis placed the protein among the bacterial pseudopilins of type II secretion systems (Reguera *et al.*, 2005).

In several Gram-negative bacteria, type II secretion systems are responsible for the translocation of proteins into or through the outer membrane (Arrieta *et al.*, 2004; Pugsley, 1993a; Sandkvist, 2001a). Classical type II general secretion pathways (GSPs), such as the pullulanase system (*pul*) of *Klebsiella oxytoca* (d'Enfert *et al.*, 1987), consist of 12–15 interacting proteins whose genes and genetic arrangement are well conserved among bacteria (Filloux, 1999; Pugsley, 1993b; Sandkvist, 2001b). Pseudopilins are proposed to form a pilus-like periplasmic structure that assists in the secretion of proteins either by providing a channel to the outside of the cell that proteins can pass through or by acting as a piston to push out proteins (Nunn, 1999; Vignon *et al.*, 2003).

Here we report that the pseudopilin gene of *G. sulfurreducens* is required for reduction of insoluble Fe(III) and Mn(IV) oxide, but not the reduction of soluble Fe(III) in *G. sulfurreducens*. One of the proteins secreted by this putative type II secretion system is OmpB, a putative multicopper, outer-membrane protein that is specifically required for the reduction of Fe(III) oxide, but not soluble Fe(III).

METHODS

Bacterial strains and culture conditions. *Escherichia coli* strain DH5 α [*supE44* Δ *lacU169* (ϕ 80 *lacZ* Δ M15) *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1*] was used for DNA manipulations (Hanahan, 1983; Woodcock *et al.*, 1989). Targeted gene disruption experiments were performed on *Geobacter sulfurreducens* strain DL1 to produce *oxpG::kan* and *ompB::spec* strains). *G. sulfurreducens* strain PCA (ATCC 51573) (Caccavo *et al.*, 1994) was obtained from our laboratory culture collection. Cells were cultured with acetate as the electron donor and either fumarate (40 mM), Fe(III) citrate (50 mM) or poorly crystalline Fe(III) oxide (100 mM) as the electron acceptor as previously described (Coppi *et al.*, 2001). Nitrotriacetic acid (NTA) was added at a concentration of 4 mM or 1 mM. Kanamycin, streptomycin and spectinomycin were added at final concentrations of 200 μ g ml⁻¹ when required.

DNA manipulations and RT-PCR conditions for RNA expression analysis. Genomic DNA was extracted with the Master Pure

Complete DNA and RNA purification kit (Epicenter Technologies). Plasmid purification was carried out with the Mini Plasmid purification kit (Qiagen). Total RNA was purified from mid-exponential-phase cultures with RNeasy Mini kits (Qiagen) followed by treatment with RNase-free DNase (Ambion). Reverse transcription was performed with the Omniscript RT kit (Qiagen) according to the manufacturer's instructions. Total RNA (1 μ g) served as template for cDNA synthesis and random hexamers were used as primers. The resultant cDNA was amplified with primers: *OxpGF* (5'-CTGC-AGGAAACCTCTACACC-3') and *OxpGR* (5'-GAACTGACAAGG-TCTGAGCCG-3') for the *oxpG* gene.

Construction of an *oxpG* deletion mutant. In order to create an *oxpG* mutant, recombinant PCR (Murphy *et al.*, 2000) was used to construct a linear DNA fragment containing the first 0.038 kb of the *oxpG* gene preceded by upstream sequence and followed by a kanamycin resistance cassette, the last 0.1 kb of the *hyaB* gene, and 0.1 kb of downstream sequence. In creation of a *oxpG* deletion mutant, primers Gsp1 (5'-GCCTCTGGATATAATTCCTTAC-3') and Gsp2 (5'-GGGTAAACCCCTTGCGGTTACG-3') were used to amplify the sequence upstream of *oxpG* (576 bp; -572 to +38); primers Gsp3 (5'-GGGCTCGTGAGGCTGTCTGCAGG-3') and Gsp4 (5'-CGTCGTTAAGGGCAGCCATGTGCGC-3') were used to amplify the sequence downstream of *oxpG* (410 bp; from +115 to 113 bp downstream from the stop site). A kanamycin resistance cassette was amplified from pBBR1MCS-2 (Kovach *et al.*, 1995) with primers KanF (5'-CGTAACCGCAAGGGGTTTACCCACCTGGGA-TGAATGTCAGCTA-3') and KanR (5'-GCAGGACAGCCTCACGA-GCCCAAGCCCAACCTTTCATAGA-3'). Recombinant PCR was performed using primers Gsp1 and Gsp4. Electroporation, mutant isolation and genotype confirmation using Southern hybridization were performed as described by Coppi *et al.* (2001). Southern hybridization with *G. sulfurreducens* genomic DNA was performed according to Sambrook *et al.* (1989). Probes were labelled with [α -³²P]dCTP (Perkin Elmer) with the Multiprime DNA labelling system (Amersham Pharmacia Biotech). One of the correct genotype mutants, designated DLTM2, was chosen as the representative strain.

Construction of an *ompB* deletion mutant. The *ompB*-deficient strain was created essentially the same way as for the *oxpG* mutant strain. Primers rgR1 (5'-GCTAGAATTCGGCGACGCGCTCCGAG-3') and rgR2 (5'-GCATGGATCCAGAACGGTTCCTGC-3') were used to amplify a 0.5 kb fragment located upstream of *ompB*. Primers rgR3 (5'-CAGGGTGTGCGACGAATTTCCGCAT-3') and rgR4 (5'-GCATAAGCTTGTAAAGCCGATTTGATG-3') were used to amplify a 0.5 kb fragment located downstream of *ompB*. The *aadA* spectinomycin resistance cassette was cloned from pJS985 (Sandler & Clark, 1994) using primers RGspcRV1 (5'-CGATGATATCGCACAGGAT-GACGCCTAAC-3') and RGspcRV2 (5'-GCGATGATATCGAAGC-GGCGTCGGCTTG-3'). The primers rgR1 and rgR4 were used to amplify the 1.9 kb fragment and used to perform single-step gene replacement according to the method of Leang *et al.* (2003). The mutation again was confirmed by Southern blot hybridization.

Expression of the *oxpG* and *ompB* genes in trans. The mutant Δ gsp::kan was complemented using the expression vector pCDS as described previously (Leang *et al.*, 2003). The primers used to amplify *oxpG* were: forward primer, 5'-CCCGGAATCCCCGAA-GTGGGTGAGCC-3'; and reverse primer, 5'-CCGGGATCCCGC-GCTGGTATGACATGG-3' (with *EcoRI* and *BamHI* restriction sites underlined). The *oxpG* coding sequence was digested with *EcoRI* and *BamHI* and inserted into the *EcoRI* and *BamHI* sites of the expression vector pCDS to generate pCDS-*oxpG*. The *oxpG* gene was sequenced to screen for PCR artefacts. The *oxpG* deletion mutant was electroporated with pCDS-*oxpG*. Streptomycin was used as the selection marker to screen for the insert. The strain was designated DLTM2C. Successful transformants were screened for the insert in the correct orientation.

When *ompB* was expressed *in trans*, an expression vector derived from pCM66 (Marx & Lidstrom, 2001) was constructed. Primers rgG1 (5'-CCGAGACATGTCAACAGCTGATTGCCCTTCAC-3') and rgG2 (5'-CCGAGGACGTCGCGAAAACCTCTCAAGGATCTTAC-3') were used to amplify a 1.8 kb fragment from pCD342 (Dehio *et al.*, 1998). The PCR product was digested with *Afl*III/*Aat*III and ligated to the 5.9 kb *Aat*III/*Afl*III fragment from pCM66 to create pRG2. Primers rgR5 (5'-GCGATGAATCTTACTGGATCGTCAGGTTGTTGAC-3') and rgR6 (5'-GCGATGAATCCACAAAGGAGACATTTGATC-3') were used to amplify the *ompB* gene and the native ribosome-binding site. The 4 kb PCR product was digested with *Eco*RI and *Hind*III and ligated with *Eco*RI/*Hind*III pRG2 to create pRG2ompB. Further procedures were identical to those of *oxpG* expression.

Analytical techniques. Cell suspension experiments were carried out as previously described (Leang *et al.*, 2003). Fe(III) reduction was monitored by measuring the accumulation of HCl-extractable Fe(II) with ferrozine as previously described (Lovley & Phillips, 1986). Growth on fumarate was monitored spectrophotometrically by measuring the turbidity at 600 nm. Cell densities of other cultures were determined by acridine orange staining and epifluorescence microscopy (Lovley & Phillips, 1988).

Isolation of periplasmic proteins. Cells grown with insoluble Fe(III) oxide as terminal electron acceptor supplemented with 4 mM NTA were grown to exponential phase. Cells were harvested after dissolving the insoluble Fe(III) oxide using oxalate solution (28 g ammonium oxalate l⁻¹ and 15 g oxalic acid l⁻¹). The cells were pelleted by centrifugation at 3000 g for 20 min, washed in PBS buffer and centrifugation was repeated. Cell pellets were resuspended in 25 mM HEPES (pH 7.5), 0.5 mM MgCl₂ and 20 % sucrose containing protease inhibitor. Cells were stirred for 20 min on ice. EDTA (pH 8.0) was added to a final concentration of 10 mM and the suspension was stirred on ice for 5–10 min. Lysozyme was added at a final concentration of 0.5 mg ml⁻¹ and stirring continued for another 30 min at room temperature to generate spheroplasts. The spheroplasts were pelleted by centrifugation at 5000 g at 4 °C for 15 min. The outer-membrane fraction was removed from this preparation by centrifugation at 50 000 g at 4 °C for 1 h. The supernatant was used as the periplasmic fraction.

Identification of OmpB. The protein was excised from the SDS gel and digested with trypsin according Gharahdaghi *et al.* (1999). Digests were concentrated using ZipTip C18 pipette tips (Millipore) according to the recommended protocol with the exception of 1 % formic acid instead of trifluoroacetic acid. MALDI-MS (matrix-assisted-laser-desorption/ionization time-of-flight) and PSD (post-source decay) data were obtained on a Kratos Axima CFR (Kratos Analytical).

Gel electrophoresis and Western blot analysis. Samples were electrophoresed on 10 % SDS polyacrylamide gels and visualized via Coomassie staining, with SeeBluePlus prestained proteins (Invitrogen) or subjected to Western analysis by transferring the proteins to Immuno blot PVDF membrane (Bio-Rad). The membrane was probed with a polyclonal antibody raised against a peptide of OmpB (KPDKTPIGPGDTPDC) (Sigma Genosys). Polyclonal alkaline phosphatase conjugated anti-rabbit antibody (Sigma) was used as secondary antibody, OmpB was visualized by SigmaFast 5-bromo-4-chloro-3-indolyl phosphatase/nitro blue tetrazolium tablets (Sigma).

RESULTS

Identification and expression of *oxpG*

An ORF (GI-39996875) containing an N-terminal signal sequence, 81 % identical to the PilA gene of *G. sulfurreducens*,

was identified in the *G. sulfurreducens* genome (Reguera *et al.*, 2005). This gene was designated oxide reducing pathway protein G (*oxpG*) because PSORT analysis (Nakai & Horton, 1999) and FingerPRINTScan (<http://www.bioinf.man.ac.uk/fingerPRINTScan/>) suggested that this ORF is similar to the genes for a general secretion pathway protein G related to type II general secretion pathways of Gram-negative bacteria. *oxpG* is not part of the classical type II secretion system, which has been described previously (Sandkvist, 2001b). The arrangement of the ORFs surrounding the putative *G. sulfurreducens oxpG* (see Supplementary Fig. S1, available with the online version of this paper) is like that around the gene *xcm*, part of a novel secretion system in *Pseudomonas putida* GB-1 that is required for secretion of a copper-containing manganese oxidase (De Vrind *et al.*, 2003). The gene arrangement surrounding the *G. sulfurreducens oxpG* is also found in *Geobacter metallireducens* (<http://www.jgi.doe.gov>).

RT-PCR analysis revealed that *oxpG* was expressed under all culture conditions studied, which included growth with Fe(III) citrate, fumarate, or insoluble Fe(III) or Mn(IV) oxides as the electron acceptor (see Supplementary Fig. S2).

Characterization of the *oxpG* deletion mutant

In order to identify the potential role of OxpG in Fe(III) respiration, an *oxpG* deletion mutant was constructed and was confirmed by Southern hybridization. Examination of the mutant by transmission electron microscopy demonstrated that it continued to produce pilin (data not shown).

The mutant reduced Fe(III) citrate (see Supplementary Fig. S3A) or fumarate (data not shown) as well as the wild-type. However, the *oxpG* deletion mutant was severely impaired in its ability to reduce insoluble Fe(III) (Fig. 1) and Mn(IV) oxides (data not shown). The mutant reduced Fe(III) in the Fe(III) oxide medium when the Fe(III) chelator NTA was added (Fig. 1). These results further suggested that the deletion of *oxpG* specifically affected the secretion of proteins required for reduction of insoluble electron acceptors. When *oxpG* was expressed in the deletion mutant *in trans*, the capacity for Fe(III) oxide reduction was restored (Fig. 1).

Identification of OmpB from the *oxpG* mutant

Mutations in genes involved in type II secretion in other organisms result in accumulation of exoproteins within the periplasm (Pugsley, 1993a). Therefore, in order to identify proteins secreted by the OXP-like system in *G. sulfurreducens*, the periplasmic protein fractions of mutant and wild-type cells were compared (Fig. 2). A protein with an approximate molecular mass of 140 kDa accumulated in the periplasm of the mutant cells (Fig. 2). The MALDI-MS analysis of this protein indicated that it was encoded by ORF GI-39996496 in the *G. sulfurreducens* genome (Methe *et al.*, 2003). The protein was named outer-membrane protein B (OmpB).

Fig. 1. Effect of *oxpG* deletion on the reduction of insoluble Fe(III) oxide: time-course of Fe(III) reduction by the wild-type strain (■), the *oxpG* mutant strain (●) and the *oxpG* mutant strain complemented with *oxpG* in trans (□) in the absence (a) and presence (b) of 4 mM NTA. The results are the means of triplicate incubations; error bars represent SD.

Characterization and expression of OmpB

The deduced amino acid sequence of OmpB indicates that this protein is a putative multicopper oxidase having the

Fig. 2. Comparison of protein patterns in the periplasmic fractions of wild-type and *oxpG* mutant cells. Proteins from the periplasmic fraction were separated on a 7.5% polyacrylamide-SDS gel. OmpB was excised and sequenced by MALDI-MS with PSD analysis. Lanes: M, molecular mass markers; 2, wild-type; 3, *oxpG* mutant.

highest similarity to a manganese oxidase (MofA) from *Leptothrix discophora* (see Supplementary Fig. S4). The sequence contains four deduced copper-binding sites, two near the amino terminus and two near the C-terminus. This arrangement is typical of copper oxidases (Brouwers *et al.*, 2000b). The protein consists of 1303 aa, with a calculated molecular mass of 139.6 kDa, and a pI of 4.47. A 26 aa signal peptide was predicted, indicating the importance of the secretion pathway in the transport of this protein. The protein is predicted to be extracellular (PSORTB at <http://www.expasy.org>). However, when the various cell fractions were run on a SDS-PAGE, OmpB was identified in both the membrane fraction and the extracellular fluid, but not in the soluble fraction of the cells (Fig. 3), suggesting that OmpB is cell-surface exposed. Western blot analysis also indicated that OmpB is expressed at the same levels during growth with soluble electron acceptors, or insoluble Fe(III) oxide or Mn(IV) oxide (Fig. 3).

Effect of *ompB* mutation on growth with different electron acceptors

A mutant in which *ompB* was deleted reduced Fe(III) citrate (see Supplementary Fig. S3B), and fumarate (data not shown) as well as the wild-type. The mutation did not affect the growth in either medium, as monitored by cell counting and optical density, respectively (data not shown). However,

Fig. 3. Expression of OmpB under various culture conditions and in subcellular fractions. Western blot analysis was carried out with cell lysate from Mn(IV) oxide- (lane 1), Fe(III) oxide- (lane 2), Fe(III) citrate- (lane 3) and fumarate-containing (lane 4) cultures. The presence of OmpB was tested in the soluble fraction of cell lysate (lane 5), membrane fraction (lane 6) and extracellular supernatant of fumarate cultures (10-fold concentrated) (lane 7). M, molecular mass markers. Each lane was loaded with 5 µg protein.

Fig. 4. Reduction of insoluble Fe(III) oxide (a) or Fe(III) oxide supplemented with 1 mM NTA (b) by cultures of wild-type strain (■), *ompB* deletion mutant strain (●), or *ompB* deletion mutant strain complemented with *ompB* in trans (□). The results are the means of triplicate incubations; error bars represent SD.

the mutant poorly reduced insoluble Fe(III) oxide (Fig. 4a) and Mn(IV) (Fig. 5). When the chelator NTA was added to the cultures containing Fe(III) oxide, there was no significant difference between the mutant and the wild-type in the reduction of Fe(III) (Fig. 4b). When the mutant was complemented with *ompB* in trans, the reduction of Fe(III) oxide was restored, but the rate was somewhat slower than that of the wild-type (Fig. 4a). The complementation of *ompB* in trans also restored the reduction of Mn(IV) (Fig. 5).

DISCUSSION

The results demonstrate that a type II secretion system is required in *G. sulfurreducens* for the reduction of insoluble Fe(III) and Mn(IV) oxides and that this is related, at least in part, to the need to export a putative multicopper-containing protein to the outer surface of the cell. As detailed below, these results further emphasize that reduction of insoluble Fe(III) oxides requires cell components not needed to reduce soluble, chelated Fe(III). This is an important distinction because most of the Fe(III) available for microbial reduction in soils and sediments is expected to be insoluble (Lovley, 1991; Nevin & Lovley, 2002). Furthermore, the results demonstrate that, in addition to the *c*-type cytochromes that are widely regarded to be important outer-membrane proteins for electron transport and/or Fe(III) reduction, other proteins that are not cytochromes are also necessary for this process.

Role of OxpG in Fe(III) oxide reduction

The results suggest that the OxpG is involved in exporting OmpB, and possibly other proteins, specifically required for the reduction of Fe(III) and Mn(IV) oxides. OxpG is most homologous to XcmT3, a type II pseudopilin from *Pseudomonas putida* strain GB-1 (De Vrind *et al.*, 2003) and the genomic region flanking the *oxpG* gene cluster also has similarity to the *xcmT3* gene cluster. Genetic studies have demonstrated that the XcmX secretion system in *P. putida*

strain GB-1 is involved in the secretion of a manganese-oxidizing enzyme (De Vrind *et al.*, 2003), which is also a multicopper oxidase (Brouwers *et al.*, 1999).

A type II secretion system is also required for Fe(III) reduction in the dissimilatory Fe(III) reducer *Shewanella putrefaciens* (DiChristina *et al.*, 2002). When *ferE*, the *gspE* homologue in the *S. putrefaciens* secretion system, was deleted, Fe(III) and Mn(IV) reduction were inhibited. However, there are some significant differences between the systems required for Fe(III) reduction in *S. putrefaciens* and *G. sulfurreducens*. For example, the secretion system required for Fe(III) reduction in *S. putrefaciens* is more closely related to the classical type II GSP systems of Gram-negative bacteria that are involved in translocating toxins and hydrolases (Sandkvist, 2001b). In fact, the gene arrangement in *S. putrefaciens* is identical to that of *Vibrio cholerae* (Sandkvist, 2001b). In contrast, the arrangement of the genes surrounding *oxpG* in *G. sulfurreducens* is similar to that of the non-classical XcmX secretion system of *P. putida*

Fig. 5. Reduction of Mn(IV) by wild-type, *ompB* mutant and *ompB* mutant cells complemented with *ompB* in trans. The reduction of Mn(IV) is indicated by reduced intensity of colour. The pictures were taken after 20 days growth.

(De Vrind *et al.*, 2003). The genome of *G. sulfurreducens* contains the classical type II secretion system, showing more similarity to those in *V. cholerae* and *S. putrefaciens* (Sandkvist, 2001b), but its role in Fe(III) reduction has not been evaluated.

Another difference between the *G. sulfurreducens* and *S. putrefaciens* secretion systems required for Fe(III) reduction is that deleting *ferE* from *S. putrefaciens* inhibited the reduction of both soluble and insoluble Fe(III) (DiChristina *et al.*, 2002), whereas deleting *oxpG* only inhibited Fe(III) oxide reduction in *G. sulfurreducens*. Preliminary evidence suggested that a haem-containing protein was not properly exported to the outer membrane in the secretion system mutant of *S. putrefaciens*, but the actual role of this cytochrome in Fe(III) reduction has yet to be determined (DiChristina *et al.*, 2002). An outer-membrane *c*-type cytochrome, OmcB, is also required for reduction of both soluble and insoluble Fe(III) in *G. sulfurreducens* (Leang *et al.*, 2003). OmcB was not observed to accumulate in the periplasm of the *oxpG* deletion mutant, and the fact that the *oxpG* deletion mutant could reduce soluble Fe(III) as well as the wild-type suggested that OmcB was properly positioned in the outer membrane. In contrast, the inhibition of Fe(III) and Mn(IV) oxide in the absence of a functioning secretion system in *G. sulfurreducens* could be attributed, at least in part, to the failure to export OmpB, which is not a *c*-type cytochrome.

Role of OmpB in Fe(III) and Mn(IV) oxide reduction

The results suggest that one reason why the *oxpG*-deficient mutant cannot reduce Fe(III) or Mn(IV) oxide is that OmpB is not exported across the outer membrane. Although previous studies on extracellular electron transport to Fe(III) and Mn(IV) oxides have primarily focused on the role of outer-membrane *c*-type cytochromes (Lovley *et al.*, 2004; Richardson, 2000), it is becoming increasingly apparent that other types of proteins may also be important. For example, MtrB, which is a non-haem-containing outer-membrane protein required for Fe(III) reduction in *S. putrefaciens* (Beliaev & Saffarini, 1998), has the likely role of properly positioning cytochromes in the outer membrane (Myers & Myers, 2002). Pilin is required for Fe(III) oxide reduction in *G. sulfurreducens*, with the probable role of facilitating the final step in electron transfer to Fe(III) oxides (Reguera *et al.*, 2005).

The protein most closely related to OmpB is MofA of the Mn(II) and Fe(II)-oxidizing micro-organism *Leptothrix discophora* (Brouwers *et al.*, 2000a; Corstjens *et al.*, 1997). Like MofA, OmpB contains four predicted copper-binding sites arranged in a manner commonly found in multicopper oxidases (Brouwers *et al.*, 2000b). MofA is considered to be responsible for the oxidation of Mn(II). The partially purified MofA catalyses the oxidation of Mn(II) with oxygen as the electron acceptor (Corstjens *et al.*, 1997). Mn(IV) is produced and accumulates in the sheath which surrounds

the cells, where the putative manganese-oxidizing system is also found (Brouwers *et al.*, 2000b). Mn(IV) oxide formation was also stimulated with the addition of copper to the medium, further suggesting the involvement of a copper protein.

Anaerobic functions for multicopper oxidases have not previously been proposed. It is not clear whether this protein is redox active, and what its substrates are. Further studies are needed to determine whether OmpB is directly involved in the reduction of Fe(III) oxide and Mn(IV), or whether it has a yet unknown function, which facilitates the reduction of these metals. The study of the amino acid sequence of OmpB reveals a unique feature of OmpB that is not found in other multicopper oxidases. It contains a fibronectin type III-like domain (see Supplementary Fig. S4), similar to those in bacterial hydrolases (Kataeva *et al.*, 2002; Lin & Xiong, 2004). The fibronectin domain occurs most frequently in cellulases and chitinases in bacteria (Lin & Xiong, 2004; Watanabe *et al.*, 1994). It is proposed that in those enzymes the fibronectin domain either facilitates the adhesion of the protein to polysaccharides or cell receptors, or it participates in the maintenance of the proper conformation at the binding and/or catalytic sites of the enzyme (Kataeva *et al.*, 2002; Watanabe *et al.*, 1994). OmpB also contains an EXXE motif, which may be a potential Fe-binding site. This motif was found to be responsible for Fe(III) binding in an Fe(III) transporter (FTR1) (see Supplementary Fig. S4) (Severance *et al.*, 2004). A plausible explanation is that both the potential Fe(III)-binding and fibronectin motifs of OmpB might be involved in association of the cell with Fe(III) oxide. Additional studies to further elucidate these properties and the possible redox activity of the copper are warranted.

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