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.

**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 microorganisms 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 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.

**METHODS**

**Bacterial strains and culture conditions.** *Escherichia coli* strain DH5α [supE44 lacU169 (Δ80 lacZM15) bsdR17 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). Nitritotriacetic acid (NTA) was added at a concentration of 4 mM or 1 mM. Kanamycin, streptomycin and spectinomycin were added at final concentrations of 200 µg 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 RNAeasy 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 µg) served as template for cDNA synthesis and random hexamers were used as primers. The resultant cDNA was amplified with primers: OxpG (5'-CTGC-AGGAAACCTCTACACC-3') and OxpGR (5'-GAACGTGACAGG-TCTGAGCGG-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 hybB gene, and 0.1 kb of downstream sequence. In creation of a *ompB* deletion mutant, primers Gsp1 (5'-GGCTCTGGATATAATTCCT-3') and Gsp2 (5'-GGGGTAAACCCCTTGCGGTAG-3') were used to amplify the sequence upstream of oxpG (576 bp; -572 to +38); primers Gsp3 (5'-GGGGCTGTAGGCTGCAGG-3') and Gsp4 (5'-GGTGTAATAGGGACCCCGTCG-3') were used to amplify the sequence downstream of oxpG (410 bp; +115 to 113 bp downstream from the stop site). A kanamycin resistance cassette was amplified from pBBR1MCS-2 (Kovach et al., 2005) with primers KanF (5'-GTAACCCGGAAGGTGTTAACCGACCGGTAGAATGTCAGCT-3') and KanR (5'-GCAAGGCCGACGCTCAGAGCCAAAAGCAGAATGTCAGCT-3'). Recombinant PCR was performed using primers Gsp1 and Gsp4. Electroporation, mutation 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 Simbacco et al. (1989). Probes were labelled with [α-²³P]-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'-GGTATTAGAATATCCTCTTG-3') and rgR2 (5'-GGATTCGCAAGGCTTCCCGC-3') were used to amplify a 0.5 kb fragment located upstream of *ompB*. Primers rgR3 (5'-CAGGGTTGATGAGCTTCCCG-3') and rgR4 (5'-GATGCTGATGAGCTTCCCG-3') were used to amplify a 0.5 kb fragment located downstream of *ompB*. The adaA spectinomycin resistance cassette was cloned from pS9895 (Sandler & Clark, 1994) using primers RgspRV1 (5'-CGATGATATTGCCGAGAGTAGAGCCCTAA-3') and RgspRV2 (5'-CGATGATATTGCCGAGAGTAGAGCCCTAA-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 Δgsp::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'-CCCCGGAAATTCCCGCAGAATGCGTGAACCC-3'; and reverse primer, 5'-CCCGGATCCGCGTGGTGAATGCGTGAACCC-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.
Identification and expression of oxpG

An ORF (GI-39996875) containing an N-terminal signal sequence, 81 % identical to the PilA gene of \textit{G. sulfurreducens}, was identified in the \textit{G. sulfurreducens} genome (Reguera \textit{et al.}, 2005). This gene was designated \underline{ox} \textit{d} \underline{re} \underline{du} \underline{cing} \underline{g} \underline{p} \underline{o} \underline{t} \underline{e} \underline{n} \underline{s} \underline{i} \underline{s} \underline{t} \underline{r} \underline{e} \underline{a} \underline{m} \underline{a} \underline{t} \underline{i} \underline{r} \underline{y} \underline{m} \underline{u} \underline{n} \underline{t} \underline{u} 

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<th>Non-haem proteins required for Fe(III) oxide reduction</th>
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<td>Identification of OmpB. The protein was excised from the SDS gel and digested with trypsin according Gharahdaghi \textit{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).</td>
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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 (KPDKTPKGPDTPDC) (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). |
Characterization and expression of OmpB

The deduced amino acid sequence of OmpB indicates that this protein is a putative multicopper oxidase having the 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,
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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{Pseudomonas putida} strain GB-1 (De Vrind et al., 2003) and the genomic region flanking the \textit{oxpG} gene cluster also has similarity to the \textit{xcmT3} gene cluster. Genetic studies have demonstrated that the XcmX secretion system in \textit{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 \textit{Shewanella putrefaciens} (DiChristina et al., 2002). When \textit{ferE}, the gspE homologue in the \textit{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 \textit{S. putrefaciens} and \textit{G. sulfurreducens}. For example, the secretion system required for Fe(III) reduction in \textit{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 \textit{S. putrefaciens} is identical to that of \textit{Vibrio cholerae} (Sandkvist, 2001b). In contrast, the arrangement of the genes surrounding \textit{oxpG} in \textit{G. sulfurreducens} is similar to that of the non-classical XcmX secretion system of \textit{P. putida}.

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<th>Abiotic control</th>
<th>Wild-type (ompB mutant)</th>
<th>Compl. (ompB in trans)</th>
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**Fig. 5.** Reduction of Mn(IV) by wild-type, \textit{ompB} mutant and \textit{ompB} mutant cells complemented with \textit{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 expG 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 expG deletion mutant, and the fact that the expG 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 expG-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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