

Evidence that OmcB and OmpB of *Geobacter sulfurreducens* are outer membrane surface proteins

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Introduction

It is important to understand the mechanisms of electron transfer to Fe(III) in *Geobacter* species because they are the most abundant Fe(III)-reducing microorganisms in a diversity of subsurface environments in which Fe(III) reduction is an important process (Rooney-Varga *et al.*, 1999; Snoeyenbos-West *et al.*, 2000; Röling *et al.*, 2001; Holmes *et al.*, 2002, 2005; Anderson *et al.*, 2003; North *et al.*, 2004; Ortiz-Bernad *et al.*, 2004; Vrionis *et al.*, 2005). The insoluble nature of Fe(III) oxides requires that *Geobacter* species transfer electrons outside the cell in order to reduce Fe(III) (Lovley *et al.*, 2004). In addition to Fe(III) oxides, *Geobacter* species are capable of reducing a variety of other extracellular electron acceptors, including Mn(IV) oxides (Lovley & Phillips, 1988), other metals (Lovley *et al.*, 1991, 1993; Caccavo *et al.*, 1994; Ortiz-Bernad *et al.*, 2004), humic substances (Lovley *et al.*, 1996), and electrodes (Bond *et al.*, 2002; Bond & Lovley, 2003; Lovley, 2006).

Most studies of extracellular electron transfer in *Geobacter* species have focused on *Geobacter sulfurreducens* because the complete genome sequence (Méthé *et al.*, 2003), a genetic

Abstract

The *c*-type cytochrome (OmcB) and the multicopper protein (OmpB) required for Fe(III) oxide reduction by *Geobacter sulfurreducens* were predicted previously to be outer membrane proteins, but it is not clear whether they are positioned in a manner that permits the interaction with Fe(III). Treatment of whole cells with proteinase K inhibited Fe(III) reduction, but had no impact on the inner membrane-associated fumarate reduction. OmcB was digested by protease, resulting in a smaller peptide. However, immunogold labeling coupled with transmission electron microscopy did not detect OmcB, suggesting that it is only partially exposed on the cell surface. In contrast, OmpB was completely digested with protease. OmpB was loosely associated with the cell surface as a substantial portion of it was recovered in the culture supernatant. Immunogold labeling demonstrated that OmpB associated with the cell was evenly distributed on the cell surface rather than localized to one side of the cell like the conductive pili. Although several proteins required for Fe(III) oxide reduction are shown to be exposed on the outer surface of *G. sulfurreducens*, the finding that OmcB is also surface exposed is the first report of a protein required for optimal Fe(III) citrate reduction at least partially accessible on the cell surface.

system (Coppi *et al.*, 2001), and an *in silico* genome-based metabolic model (Mahadevan *et al.*, 2006) are available. Furthermore, *G. sulfurreducens* can readily be cultured with soluble Fe(III) form, Fe(III) citrate, or fumarate as the electron acceptor.

Previous studies have identified various electron transfer components required for Fe(III) reduction by *G. sulfurreducens*. Several *c*-type cytochromes are required for optimal reduction of Fe(III) citrate as well as Fe(III) oxide. They localize in the inner membrane (Butler *et al.*, 2004) or periplasm (Lloyd *et al.*, 2003), as well as in the outer membrane including the *c*-type cytochrome, OmcB (Leang *et al.*, 2003; Leang & Lovley 2005; Kim *et al.*, 2006). However, other components are exclusively required for Fe(III) oxide reduction, but not the reduction of Fe(III) citrate. These include the *c*-type cytochromes OmcS and OmcE (Mehta *et al.*, 2005), the multicopper protein, OmpB (Mehta *et al.*, 2006), as well as the electrically conductive pili, known as 'microbial nanowires' (Reguera *et al.*, 2005). The pili are clearly displayed outside the cell (Reguera *et al.*, 2005) as are OmcS and OmcE (Mehta *et al.*, 2006). OmpB and OmcB are both considered to be located in the outer

membrane, but whether these proteins are exposed to the outer surface has not been determined previously.

The purpose of this study was to further localize OmpB and OmcB in order to better understand their role in Fe(III) reduction. The results suggest that whereas OmpB is highly exposed on the outer surface and is only loosely associated with the outer membrane, OmcB appears to be tightly associated with the outer membrane, with only a portion of the protein exposed to the extracellular environment.

Materials and methods

Bacterial strains and preparation of cell fractions

Wild type (DL1) as well as *omcB* (DL6, $\Delta omcB::cam$) (Leang *et al.*, 2003) and *ompB* ($\Delta ompB::spec$) (Mehta *et al.*, 2006) mutant strains of *G. sulfurreducens* are routinely maintained in the authors' laboratory. These pure cultures were grown under strict anaerobic conditions as described previously (Coppi *et al.*, 2001). Briefly, the growth medium consisted of a carbonate buffered minimal medium with 20 mM acetate as the electron donor and 40 mM fumarate as the electron acceptor. Cell growth was monitored by measuring the OD_{600 nm} (Genesys 2, Spectronic Instruments, Rochester, NY).

Wild type and mutant *G. sulfurreducens* cells in their late exponential growth phase were harvested by centrifugation (4000 g for 15 min at 4 °C). The supernatants were concentrated 10-fold with a centrifugal filtration system equipped with a 10 kDa-cutoff membrane. Cells from the cultures were disrupted by sonication (Sonic dismembrator F550; Fisher Scientific, PA), and the soluble and insoluble fractions were collected by centrifugation at 257 000 g for 60 min at 4 °C. The soluble fraction of disrupted cells contains cytosolic and periplasmic proteins whereas, the insoluble fraction contains membrane proteins. Membrane proteins were further fractionated into cytoplasmic membrane and outer membrane proteins by solubilizing the first in 1% (w/v) sodium laurylsarcosine, as described elsewhere (Nikaido, 1994; Kaufmann & Lovley, 2001).

Western blot analysis

Proteins were separated by electrophoresis in 10% sodium dodecylsulfate (SDS) polyacrylamide gels. Western blot analysis was performed by transferring the proteins to Immobilon blot polyvinylidene difluoride membranes (Bio Rad, CA). The membranes were probed with polyclonal antibodies raised against a peptide of OmcB or OmpB (Kim *et al.*, 2006; Mehta *et al.*, 2006). A polyclonal alkaline phosphatase-conjugated anti-rabbit antibody (Sigma, MO) was used as a secondary antibody. OmcB and OmpB were visualized by staining with a SigmaFast™ 5-bromo-4-chloro-3-indolyl phosphatase/nitroblue tetrazolium tablet (Sigma, MO).

Preparation of resting cell suspensions

The preparation of resting cell suspensions was carried out as previously described (Shelobolina *et al.*, 2007). Briefly, cells from late-exponential phase cultures were harvested by centrifugation as described above and washed twice in an osmotically balanced wash buffer (NaHCO₃, 2.5 g L⁻¹; NH₄Cl, 0.25 g L⁻¹; NaH₂PO₄·H₂O, 0.006 g L⁻¹; KCl, 0.1 g L⁻¹; NaCl, 1.75 g L⁻¹). Cells were resuspended in the wash buffer and a portion was subjected to proteinase K (Sigma, MO) treatment for 30 min, as described below. The other untreated portion served as a control. The proteinase K reaction was stopped by adding a protease inhibitor (Roche, NJ) to the sample. The cells were then washed twice and resuspended in wash buffer. Untreated and proteinase K-treated cell suspensions were incubated in a minimal buffer (NaHCO₃, 2.5 g L⁻¹, NH₄Cl, 0.25 g L⁻¹, and NaH₂PO₄·H₂O, 0.006 g L⁻¹) with 5 mM acetate as the electron donor and 20 mM Fe(III) citrate or 10 mM fumarate as the electron acceptors to assay Fe(III)- and fumarate-reducing activities, respectively. The rate of fumarate reduction by resting cell suspensions was monitored by measuring the concentration of fumarate over time by HPLC (Shimadzu LC-6A, Kyoto, Japan). Samples were eluted from an Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad Laboratories, CA) at a rate of 1 mL min⁻¹ for 20 min using 8 mM H₂SO₄ as an eluent. The fumarate concentration of the samples was monitored at a wavelength of 210 nm using matching retention times of fumarate standards of known concentration as controls. Fe(III) reduction was determined as the amount of HCl-extractable Fe(II) using a ferrozine assay, as previously described (Lovley & Phillips, 1988). Protein concentration was determined with the bicinchoninic acid method using bovine serum albumin as a standard (Smith *et al.*, 1985).

Protease susceptibility assay

Cells grown to the mid-exponential phase were harvested by centrifugation for 10 min at 6000 g, at 4 °C, washed twice, and resuspended in 10 mM HEPES (pH 7.5) containing 500 μM MgCl₂. The final cell density was 38.7 mg wet cells mL⁻¹. The cells were incubated with or without 1 U mL⁻¹ proteinase K at 37 °C, for different lengths of time (i.e. 10, 20, and 30 min). A protease inhibitor was then added to stop the proteolytic reaction. Cells were recovered by centrifugation at 6000 g for 1 min and washed twice in HEPES buffer containing the protease inhibitor. The washed cells were subjected to protein analyses by SDS-polyacrylamide gel electrophoresis (PAGE) followed by coomassie blue staining. The *c*-type cytochromes in the protein preparations were separated by Tricine-SDS PAGE, and visualized by heme-specific staining (Thomas *et al.*, 1976; Francis & Becker 1984).

Protein immunodetection by transmission electron microscopy (TEM)

For immunolocalization of the OmcB and OmpB proteins, mid-exponential phase cultures of the wild type and the *omcB* and *ompB* mutant strains were adsorbed onto carbon-coated copper grids and fixed with 1% glutaraldehyde. Immunolabeling was performed at room temperature using primary antibodies raised against the OmcB and OmpB proteins [1 : 100 in phosphate buffered saline–bovine serum albumin (PBS–BSA) buffer for 1 h] and NanoGold[®]-conjugated secondary antibodies (Nanoprobes; 1 : 50 in PBS–BSA buffer for 30 min) following the manufacturer's recommendations. After immunolabeling, cells were treated with the Goldenhance[™]-EM reagent (Nanoprobes, Yaphank, NY) for 5 min to increase the size of the gold particles and were negatively stained with the vanadium-based, Nanovan[™] stain (Nanoprobes, Yaphank, NY), also following the manufacturer's recommendations. Immunolabeling was also performed in cell suspensions following the manufacturer's recommendations. Briefly, cells were harvested by centrifugation (300 g for 5 min) and washed with PBS buffer containing 0.02 M glycine. Cells were incubated for 3 min with the primary antibody in a PBS–BSA buffer with gentle agitation and with the NanoGold[®]-conjugated secondary antibodies before treatment with the Goldenhance[™]-EM reagent (Nanoprobes, Yaphank, NY). The immunolabeled cells were washed twice in PBS–BSA and fixed with glutaraldehyde before being immobilized on the copper grid. Immunolabeled samples were examined with a JEOL 100S transmission electron microscope operated at 80 V. Samples containing strains in which the gene for OmcB (Leang *et al.*, 2003) or OmpB (Mehta *et al.*, 2006) was deleted were labeled, respectively, with the OmcB and OmpB anti-sera and used as controls for nonspecific binding of the primary antibody and background noise.

Results and discussion

Protease digestion of whole cells removes capacity for Fe(III) reduction

Information on the localization of proteins required for Fe(III) reduction in *Geobacter* species can aid in understanding the mechanisms of this process. One strategy for evaluating whether proteins are exposed on the outer surface of the cell is to determine whether these proteins are susceptible to protease digestion in whole-cell preparations.

Microscopic examination revealed that cells of *G. sulfurreducens* treated with proteinase K (1 U mL⁻¹) for up to 30 min remained intact (Fig. 1). The protease-treated cells reduced fumarate as well as controls, which were not treated with protease (Fig. 2a). Fumarate is reduced at the inner

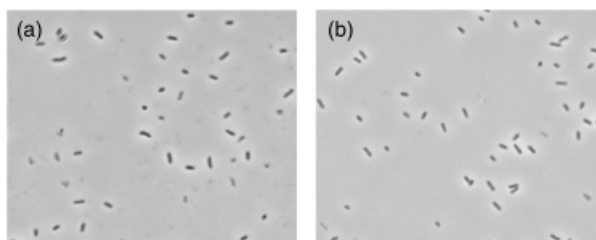


Fig. 1. Phase contrast microscopic images of untreated (a) and proteinase K-treated (b) cells of *Geobacter sulfurreducens* ($\times 100$ magnification).

membrane (Butler *et al.*, 2004). Thus, these results demonstrated that the protease treatment did not disrupt electron transfer processes within the cell. However, the rate of Fe(III) citrate reduction in protease-treated cells was only 21% of that in untreated controls (Fig. 2b). These results suggested that protease treatment removed outer surface proteins that were required for Fe(III) citrate reduction.

When the proteins in the proteinase K-treated cells and untreated cells were separated with SDS-PAGE, the total protein profile of the treated cell samples did not differ significantly (Fig. 3a), suggesting that proteinase K did not have access to the majority of the most abundant cell proteins. However, when the proteins were stained for heme there were notable differences in the composition and in the intensity of the bands (Fig. 3b). The intensity of the heme-containing proteins at high molecular mass range decreased with increasing time of proteinase K treatment, with a corresponding increase in the intensity of lower molecular weight bands. The 9.6 kDa periplasmic *c*-type cytochrome, PpcA (Lloyd *et al.*, 2003), remained intact throughout the proteinase K treatment (Fig. 3b), providing further evidence that the outer membrane was not disrupted during proteolysis.

Exposure of the *c*-type cytochrome OmcB on the outer cell surface

Although *G. sulfurreducens* is predicted to have a multitude of outer membrane *c*-type cytochromes, only one of these cytochromes that are predicted to be in the outer membrane, OmcB, has been definitely shown to be necessary for optimal reduction of soluble, chelated Fe(III) (Leang *et al.*, 2003; Kim *et al.*, 2006). As previously discussed (Leang *et al.*, 2003), topology prediction programs such as SIGNALP (<http://www.cbs.dtu.dk/services/SignalP/>), HMMTOP (<http://www.enzim.hu/hmmtop/>), and SOSUI (<http://www.proteome.bio.tuat.ac.jp/sosui/frame0.html>) indicate that OmcB is likely to be associated with the outer membrane of *G. sulfurreducens* because it contains a signal peptide homologous to those of lipoproteins, which is followed by a cysteine residue after the putative cleavage site that is thought to serve as the specific lipid attachment site of the protein to the membrane.

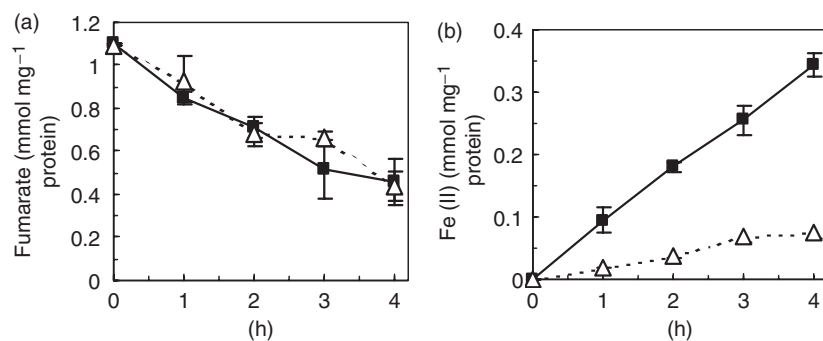


Fig. 2. Time-course of fumarate (a) and Fe(III) (b) reduction by proteinase K-treated (Δ) or untreated (■) cell suspensions of *Geobacter sulfurreducens*. Fumarate reduction was measured as a function of fumarate disappearance and Fe(III) reduction as a function of Fe(II) formation. Error bars are SDs from the mean of triplicate samples.

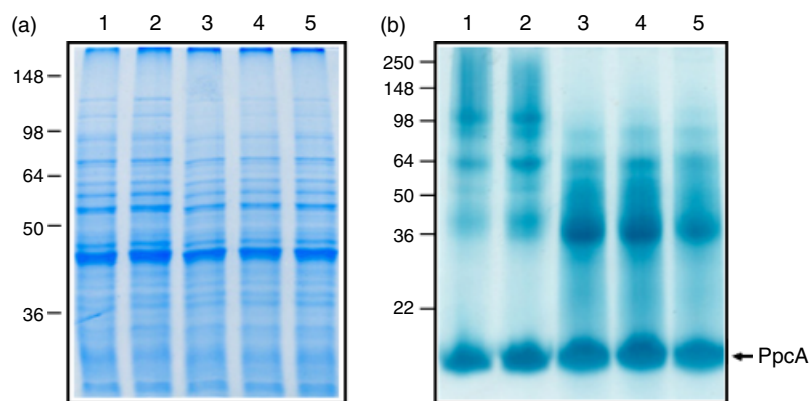


Fig. 3. Total protein (a) and heme-stained protein (b) profiles of whole cells treated with proteinase K. Lane 1, cells before protease treatment; lane 2, untreated control cells; lanes 3–5, whole cells treated with proteinase K (1 U mL⁻¹) for, respectively, 10, 20 and 30 min. Each lane contained 5 μg of protein.

Associated with the loss of the capacity for Fe(III) citrate reduction in the proteinase K-treated cells was an apparent protease-catalyzed digestion of OmcB (Fig. 4a). When the proteins of cells treated with proteinase K for different periods of time were separated on SDS-PAGE gels and treated with an antibody specific for OmcB, there was a progressive loss of the 85-kDa band associated with OmcB over time. There was no loss in untreated controls. The loss of OmcB from the protease-treated cells was accompanied by the appearance of an additional band with a lower molecular weight (*c.* 40 kDa) that reacted with the OmcB antibody (Fig. 4a). This suggested that only a portion of the OmcB in intact cells was accessible to proteinase K.

In order to further evaluate the localization of OmcB, whole cells were treated with OmcB antibodies and gold-conjugated secondary antibodies and examined with TEM (Fig. 5). OmcB was not detected even though other, surface-exposed proteins can be detected with this same technique. However, OmcB appeared to be primarily localized in the outer membrane because when the cell was fractionated the OmcB antibodies detected OmcB in the outer membrane fraction with only traces detected in the soluble cell fraction, and in culture supernatant fluids (Fig. 6a). OmcB was absent in the cytoplasmic membrane fraction. These results suggest that although OmcB is localized in the outer membrane, only a portion of OmcB is exposed outside the cell. The OmcB antibodies were developed with a peptide of 281

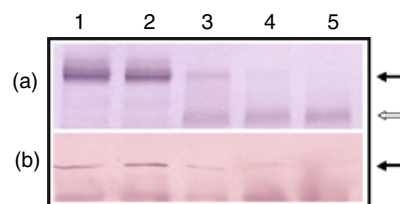


Fig. 4. Western blot analysis of OmcB (a) and OmpB (b) after the treatment of whole cells with proteinase K. Lane 1, cells before protease treatment; lane 2, control cells after 30 min treated in the same way as those of protease-treated cells but protease was excluded; lanes 3–5, Whole cells were treated with protease K (1 U mL⁻¹) for 10 min. (Lane 3), 20 min (lane 4), and 30 min (lane 5). In each lane, 5 μg of protein samples were loaded. OmcB and OmpB are indicated with solid arrows. The appearance of a new protein band on protease digestion of OmcB is indicated with a clear arrow in (a).

amino acids from the central section of the OmcB sequence (Kim *et al.*, 2006), which apparently is not accessible to the antibody when OmcB is localized in the intact outer membrane *in vivo*.

Localization of the putative multicopper protein, OmpB

In order to further evaluate the approaches used to localize OmcB, the putative multicopper protein, OmpB, was studied with similar techniques. A study in which the gene for

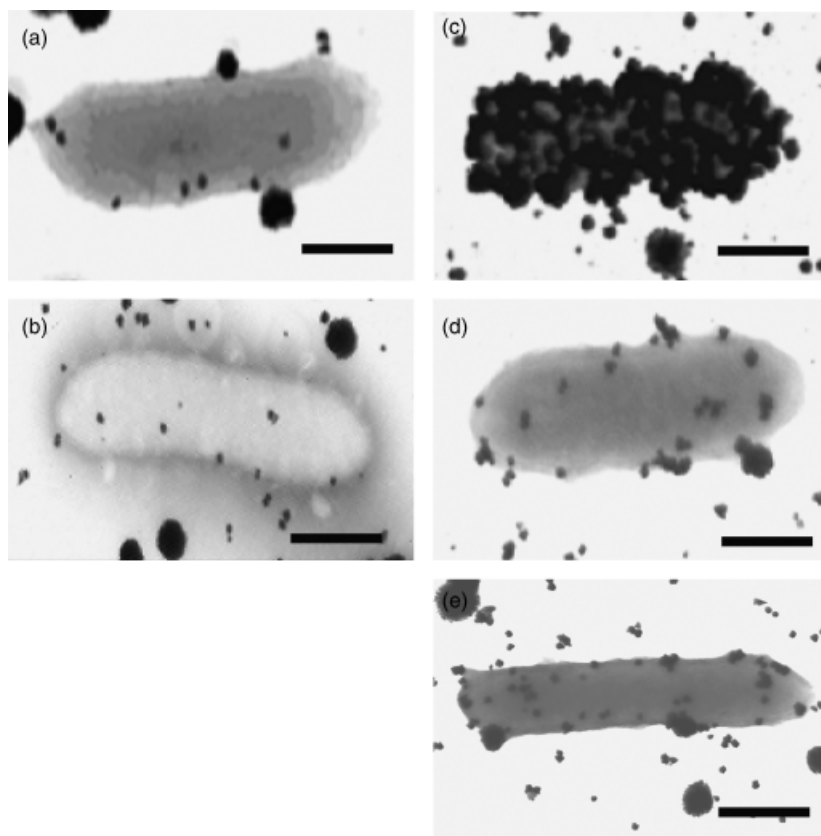


Fig. 5. TEM images of *Geobacter sulfurreducens* immunolabeled for OmcB (a, b) or OmpB (c–e). Cells of the wild type (a and c) were immobilized onto the copper grid support before immunolabeling. The *omcB* (b) and *ompB* (d) mutant strains served as controls for non-specific antibody binding. Wild-type cells in panel (e) were immunolabeled before immobilization on the copper grid. Scale bars represent 0.5 μm .

OmpB was deleted demonstrated that OmpB is required for the reduction of Fe(III) oxide, but not Fe(III) citrate (Mehta *et al.*, 2006). OmpB is predicted to be localized on the outer surface of *G. sulfurreducens* because when the gene necessary for the proper functioning of a type-II secretion system is deleted, OmpB accumulates in the periplasm (Mehta *et al.*, 2006).

Localization of OmpB with polyclonal antibodies raised against OmpB suggested that OmpB is loosely associated with the outer surface of *G. sulfurreducens*. OmpB was primarily detected in culture supernatant fluids (Fig. 6b). OmpB was also detected in the outer membrane but only in small amounts even after loading four times more protein than that used to detect OmcB in the outer membrane fractions (Fig. 6b). OmpB was only weakly present in the soluble cell fraction, and was absent in the cytoplasmic membrane fraction.

TEM-immunogold analysis revealed that the OmpB antibodies could access OmpB on the cell surface and that OmpB was uniformly distributed on the cell (Fig. 5c). In these studies, culture samples were directly applied and fixed to the TEM copper grids; thus, supernatant proteins were also present in the cell surroundings and OmpB was detected in the culture supernatant fluids of wild-type cells, in agreement with cell fractionation studies. When a strain in which the gene for OmpB had been deleted was treated

with the antibody, the signal was very weak, demonstrating low levels of antibody cross-reactivity (Fig. 5d). If the wild-type cells were washed before adsorption and fixation to the copper grids, the signal for OmpB was absent (Fig. 5e), demonstrating that OmpB is very loosely associated with the cell surface.

Proteinase K treatment of whole cells resulted in progressive loss of OmpB over time without the production of a secondary band (Fig. 4b). This is consistent with the results from the localization studies, which suggest that OmpB is primarily exposed outside the cell.

Topological and structural analyses with the SIGNALB, HMMTOP, and SOSUI programs predicted that OmpB is a soluble protein with a single transmembrane domain spanning amino acids 6 to 28. This transmembrane motif also includes the predicted cleavage site for OmpB's signal peptide (between the alanine and phenylalanine at positions 26 and 27, respectively), but, unlike OmcB, no cysteine residue that may function as a membrane anchor was found. This is consistent with the experimental data that OmpB is loosely bound to the outer membrane.

Implications

The results demonstrate for the first time that *G. sulfurreducens* requires protein(s) exposed on the outer surface of

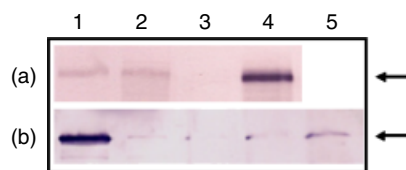


Fig. 6. Western blot analysis of OmcB (a) and OmpB (b) in the culture supernatant and different cell fractions. Lane 1, culture supernatant; lane 2, soluble fractions; lane 3, cytoplasmic membrane fraction; lane 4 and 5, outer membrane fraction. Each lane contains an amount of 2.5 μ g proteins in the case of OmcB (a). When OmpB (b) was tested, lanes 1–4 received 5 μ g of proteins and lane 5 received 10 μ g of proteins. OmcB and OmpB are indicated with arrows.

the cell in order to reduce soluble, Fe(III) citrate. Previous studies have demonstrated that several proteins especially required for the reduction of Fe(III) oxide are localized on the outer cell surface, but none of these have been required for the reduction of Fe(III) citrate. For example, pili are required for Fe(III) oxide reduction but not Fe(III) citrate reduction and, because of their electrical conductivity and specific binding of Fe(III) oxides, may be the final conduit for electron transfer from the cell to Fe(III) oxides (Reguera *et al.*, 2005). Deleting the genes for either of the outer membrane *c*-type cytochromes, OmcS or OmcE, inhibits Fe(III) oxide reduction, but has no impact on Fe(III) citrate reduction (Mehta *et al.*, 2005). Both of these cytochromes are readily sheared off the outer cell surface, suggesting that they are exposed on the outside of the cell (Mehta *et al.*, 2005). In a similar manner, deleting the gene for OmpB inhibits Fe(III) oxide reduction (Mehta *et al.*, 2006), but not the reduction of Fe(III) citrate, and the studies described here demonstrate that OmpB is on the outside of the cell and so loosely associated with the cell surface that it is often primarily recovered in the culture supernatant. The OmpB that is associated with the cell surface is evenly distributed, unlike the conductive pili that are also required for Fe(III) oxide reduction, but localized to one side of the cell (Reguera *et al.*, 2005).

Like OmcS, OmcE, and OmpB, OmcB can be considered to be an outer surface protein, but with some important differences. OmcB is required for optimal Fe(III) citrate reduction as well as Fe(III) oxide reduction (Leang *et al.*, 2003). Furthermore, OmcB appears to be much more tightly associated with the outer membrane than OmcS, OmcE, or OmpB. The results presented here demonstrate that although a portion of OmcB is exposed on the outer surface of the cell, a significant portion is likely to be embedded within the outer membrane.

These considerations are consistent with the different properties of insoluble Fe(III) oxides and soluble, chelated Fe(III). Access of redox proteins to Fe(III) oxides is expected to be much more sterically hindered than for soluble Fe(III). Therefore, in order for proteins to have the potential to transfer electrons to

Fe(III) oxide directly they must be significantly displayed outside the cell. In contrast, as long as a redox-active portion of a protein such as OmcB is exposed on the outer cell surface, it is likely to have the possibility of transferring electrons to soluble Fe(III). Further analysis of the localization of other proteins predicted to be in the outer membrane of *G. sulfurreducens* is warranted in order to better understand their potential role in extracellular electron transfer.

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