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NOTES

Direct Involvement of Type II Secretion System in Extracellular Translocation of *Shewanella oneidensis* Outer Membrane Cytochromes MtrC and OmcA▽

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MtrC and OmcA are cell surface-exposed lipoproteins important for reducing solid metal oxides. Deletions of type II secretion system (T2SS) genes reduced their extracellular release and their accessibility to the proteinase K treatment, demonstrating the direct involvement of T2SS in translocation of MtrC and OmcA to the bacterial cell surface.

Decaheme *c*-type cytochromes (*c*-Cyts) MtrC (also known as OmcB) and OmcA of the gram-negative bacterium *Shewanella oneidensis* are lipoproteins anchored at extracellular side of the outer membrane (OM) (20, 21), where they are hypothesized as the terminal reductases of the insoluble form of metals, such as iron [Fe(III)] and manganese [Mn(IV)] (hydr)oxide (for reviews, see references 10, 13, 23, and 26). Consistent with this hypothesis, MtrC and OmcA are predicted to possess a putative binding site for solid metal oxide (17) and purified MtrC and OmcA bind and directly transfer electrons to the oxide (10). The MtrC and OmcA also are found in the extracellular polymeric substance produced by the bacterial cells under anaerobic, uranyl-reducing conditions, where they are spatially colocated with nano-domain uraninite (UO_2) (18). However, it is still unclear how MtrC and OmcA are translocated across the OM to the extracellular environment.

Type II secretion system (T2SS) is involved in bacterial reduction of solid Fe(III)/Mn(IV) oxides. The *Shewanella* mutants without key components of T2SS, such as GspD, GspE, or GspG, had impaired ability to reduce Fe(III) or Mn(IV) oxide and resulted in absence of a heme-containing protein with apparent molecular mass of 91 kDa in the KCl cell extracts or absence of *c*-Cyts in the protruding structures or domains formed on the bacterial surface when the bacteria were cultured under the electron acceptor-limited conditions (1, 2, 6, 12). Deletion of the *oxpG* gene (a homolog of *xcmT*/*xcpT*/*gspG* family) from one of the T2SSs of *Geobacter sulfurreducens* also impaired the bacterial ability to reduce Fe(III) oxide. The T2SS of *Geobacter* involved in reducing metals differed from that of *S. oneidensis*, whose system contains all the core components (i.e., GspCDEFGHIJKLMO) (4), since (i) it was a Xcm-like T2SS and (ii) the exoproteins translocated by this type T2SS, such as OmpB of *G. sulfurreducens* and CumA of *Pseudomonas putida* GB-1, were multicopper proteins in which CumA was an extracellular Mn(II) oxidase (3, 5, 19). OmpB is required for reduction of insoluble metal oxides, but whether it is a functional metal reductase has yet to be determined (19). Despite its involvements in reducing metals, the role of T2SS in extracellular translocation of MtrC and OmcA has never been experimentally investigated.

Translocation of exoproteins by T2SS is a two-step process. The exoproteins are first translocated from the cytoplasm across the inner membrane (IM) to the periplasm via either the Sec or Tat secretion pathway and then across the OM via T2SS (for reviews, see references 4, 8, 15, 22, and 24). Both MtrC and OmcA possess the signal peptides that target them to the periplasm through the Sec pathway. Inside the periplasm, MtrC and OmcA undergo an extensive maturation process that includes (i) acylation at the cysteine residue of their N termini and (ii) covalent insertion of 10 heme groups into each of their polypeptides (14, 16, 21, 25, 27). After maturation, MtrC and OmcA that lack the +2 Asp sorting signal for their IM retention are subsequently repositioned to the extracellular side of OM via a previously uncharacterized mechanism (18, 20). The focus of this research, thus, was to investigate the role of T2SS in the extracellular translocation of MtrC and OmcA.

GspD and GspG affect extracellular release of native MtrC and OmcA. We first measured the release of MtrC and OmcA into the growth medium by the mutants with an in-frame deletion of *gspD* (Δ*gspD*) or *gspG* (Δ*gspG*) (Fig. 1A) in comparison to the wild type (wt) of *S. oneidensis*. Cultures were grown in 200-ml sealed serum bottles with air in the headspace and 69 ml of chemically defined medium in which lactate (20 mM) was

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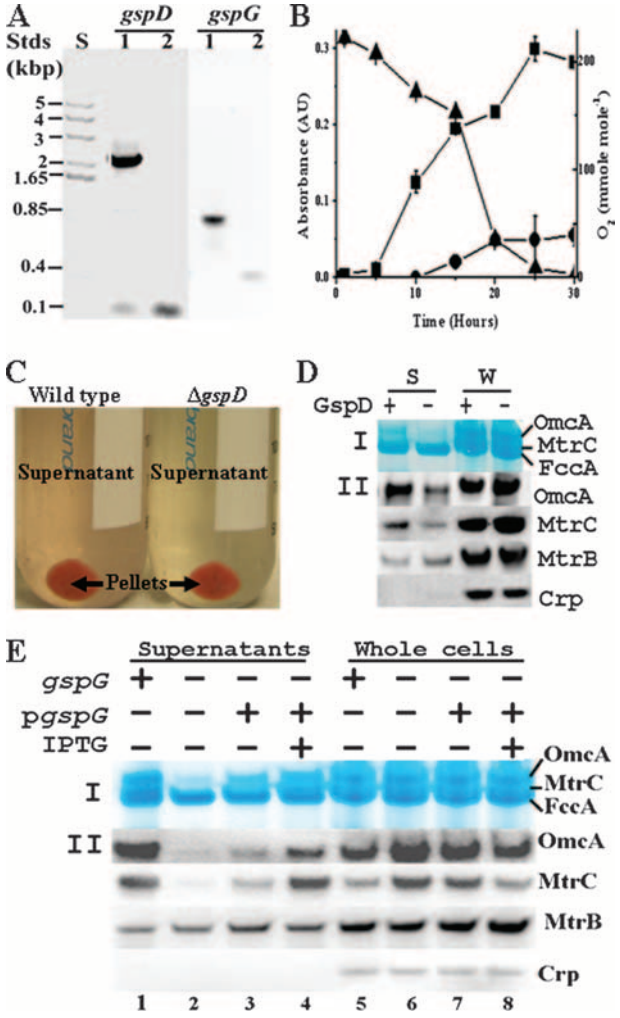

FIG. 1. Influence of deletion of *gspD* or *gspG* on extracellular release of native MtrC and OmcA. (A) Agarose gel showing size of DNA standards (Stds) in kilobase pairs (lane S) and PCR products of the *gspD* or *gspG* amplified from the wt (lanes 1) and the respective mutants (lanes 2). (B) Bacterial culture of wt. The optical density at 600 nm of cell culture (■), the optical density at 525 nm of the filtrate of cell culture (●), and the O_2 concentration (▲) in the headspace of serum bottle during the time course of the study were measured. For points without an error bar, the error was smaller than the symbol ($n = 3$). (C) Colors of the supernatants of wt and Δ*gspD* cultures under O_2-limited growth condition. (D and E) Heme staining and Western blot analyses of the effects of deleting *gspD* (D) or *gspG* (E). After 2 μg of concentrated supernatant proteins (lanes S [D] and lanes 1 to 4 [E]) or 10 μg (D) or 5 μg (E) of whole-cell lysate proteins (lanes W [D] and lanes 5 to 8 [E]) from wt (lanes +) and respective mutant (lanes –) were separated by SDS-PAGE, they were either visualized by heme staining (I panel) or probed with the respective antibodies (II panels).

used as the sole carbon and energy source (12). As the cultures grew, the O_2 in the headspace of the bottles decreased from 225 to 2.7 mmol of O_2 per mol of air (mmol mol^{-1}) during the 30-h incubation period (11). When O_2 in the headspace decreased to 153 mmol mol^{-1} (usually after 15 h of culture), the wt culture became pink and exhibited significant absorbance at 525 nm (Fig. 1B). After the cultures from four bottles were pooled and centrifuged (6,000 × *g*, 15 min, 4°C), the supernatant of the wt remained pink (Fig. 1C). In contrast, the supernatant of the Δ*gspD* or Δ*gspG* culture was pale yellow (only that of Δ*gspD* is shown in Fig. 1C). The color of the Δ*gspG* supernatant, which was complemented with the *gspG* cloned in a plasmid where the expression of *gspG* was controlled by an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter, was also pale yellow in the absence of IPTG but was pink when IPTG was added to the medium at a final concentration of 1 mM (data not shown). All of these results suggested that deletion of *gspD* or *gspG* was preventing the release of pigmented proteins to the medium. No major difference was observed between the wt and the mutants at the end of 16 h of culture in terms of (i) their cell densities, since the CFU/ml values were $2.3 \pm 0.4 \times 10^8$ for the wt, $2.1 \pm 0.5 \times 10^8$ for the Δ*gspD* mutant, and $2.0 \pm 0.4 \times 10^8$ for the Δ*gspG* mutant ($n = 6$), and (ii) their cell colors (only those of the wt and the Δ*gspD* mutant are shown in Fig. 1C). These results indicate that deletion of *gspD* or *gspG* has little or no effect on cell growth and pigmentation under the conditions used in this study. Filtration of the supernatant through 0.2-μm-pore-size filters to remove any remaining cells did not change the supernatant color.

To determine whether MtrC and OmcA were the pigmented proteins whose secretion was affected by deleting *gspD* or *gspG* gene, we concentrated the filtered supernatants from the wt, Δ*gspD* mutant, Δ*gspG* mutant, Δ*gspG* mutant plus complement, and Δ*gspG* mutant plus complement plus IPTG with Millipore concentrators with a molecular mass cutoff of 7 kDa and analyzed the proteins in the supernatants and in the whole-cell lysates by heme staining and Western blotting after they were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The intensity of OmcA bands of heme staining from the supernatants of both mutants was much weaker compared to the wt. Uninduced, complemented Δ*gspG* mutant only showed a slight increase in the intensity of OmcA heme-staining band on an SDS-PAGE gel, while the addition of IPTG significantly increased its intensity. The amount of MtrC in the supernatants, however, was too low to be visualized by heme staining (Fig. 1D, panel I, lanes S; Fig. 1E, lanes 1 to 4). Western blot analysis showed not only that OmcA and MtrC in the supernatants were less abundant in the mutants than in the wt (Fig. 1D, panels II, lanes S; Fig. 1E, lanes 1 to 2) but also that they were more abundant in the whole cells of the mutants than in those of wt (Fig. 1D, panels II, lanes W; Fig. 1E, lanes 5 to 6). Complementation of Δ*gspG* mutant with functional GspG reversed the effects of deleting *gspG* on the relative abundances of OmcA and MtrC in both supernatants and whole cells, demonstrating that the effect of *gspG* gene deletion on MtrC and OmcA is nonpolar (Fig. 1E). The reason for the observed difference between the *gspD* and *gspG* deletions on MtrC and OmcA (Fig. 1D and E) is unclear, but this result indicates that the elimination of GspD or GspG might have different impacts on localization of these *c*-Cyts. The abundances of an integral OM protein MtrB and a cytoplasmic protein Crp, meanwhile, were present in approximately equal abundance in the supernatants and/or whole cells of both the wt and the mutants, suggesting that the deletion of *gspD* or *gspG* had little effect on the abundances of these proteins (Fig. 1D and E). Likewise, no apparent difference was observed between the mutants and wt on the intensities of the periplasmic fumarate reductase FccA bands stained for hemes from the supernatants and the whole cells, demonstrating in-

TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant genotype or sequence (5′–3′)	Purpose	Source or reference
Strains			
MR-1	Wild type		ATCC
ΔSO0166	Δ*gspD*		D. Saffarini
ΔSO0169	Δ*gspG*		D. Saffarini
ΔSO0169 complement	Δ*gspG* + p*gspG*		D. Saffarini
MR-1 + pLS132	Wild type + p*omcA*		This study
MR-1 + pLS138	Wild type + p*mtrC*		This study
MR-1 + pLS146	Wild type + p*mtrC*		This study
MR-1 + pLS147	Wild type + p*omcA*		This study
ΔSO0166 + pLS132	Δ*gspD* + p*omcA*		This study
ΔSO0166 + pLS138	Δ*gspD* + p*mtrC*		This study
ΔSO0166 + pLS146	Δ*gspD* + p*mtrC*		This study
ΔSO0166 + pLS147	Δ*gspD* + p*omcA*		This study
ΔSO0169 + pLS132	Δ*gspG* + p*omcA*		This study
ΔSO0169 + pLS138	Δ*gspG* + p*mtrC*		This study
ΔSO0169 + pLS146	Δ*gspG* + p*mtrC*		This study
ΔSO0169 + pLS147	Δ*gspG* + p*omcA*		This study
Plasmids			
pLS132	Coding sequence for *omcA*		25
pLS138	Coding sequence for *mtrC*		28
pLS146	Coding sequence for the signal peptide of *mtrB* and the mature *mtrC* that lacks a lipid anchor		This study
pLS147	Coding sequence for the signal peptide of *mtrB* and the mature *omcA* that lacks a lipid anchor		7
Primers			
SO0166F	CAATAACACGCGTTAGGGG	Validation of Δ*gspD*	
SO0166R	CCTCGACTTGGGATACTTG	Validation of Δ*gspD*	
SO0169F	GTTACGCTCGCCCTCGG	Validation of Δ*gspG*	
SO0169R	TACGGTTTCATCGAGCAC	Validation of Δ*gspG*	
SO1776EF1	CACCTAAGAAGGAGATATACATCCCATGAAATTTAAACTC AATTTGATCAC	Construction of pLS146	
SO1776SR	ATTACCATCGCTTCCACCATCAGCAGCGACGGCCAAG	Construction of pLS146	
SO1778EF3	GGTGGAAGCGATGGTAATAAC	Construction of pLS146	
SO1778ER3	CTTGCAACAACCAGGGCAACATGCAGCAAGCTTGTCATCG TCATCCATTTTCACTTTAGTGTGATCTGC	Construction of pLS146	

tegrity of the OM in the T2SS mutants with regard to their permeability to the periplasmic proteins (Fig. 1D and E). Thus, the absence of either GspD or GspG, two key components of the T2SS, significantly decreases the extracellular release of native MtrC and OmcA. The release of MtrC and OmcA serves as a convenient assay system to test the role of T2SS in extracellular translocation of MtrC and OmcA, even though the nature and mechanisms of their release remain obscure.

GspD and GspG affect the extracellular release of recombinant MtrC and OmcA. Among the T2SS exoproteins that have been investigated to date, pullulanase (PulA) of *Klebsiella oxytoca* is the only lipoprotein whose secretion by T2SS has been well characterized. Recombinant PulA with addition of six histidine residues (His_6 tag) in its C terminus and lacking a lipid-anchor (i.e., nonacylated) in its N terminus was still efficiently translocated to the extracellular environment by the T2SS of *K. oxytoca* (9). To investigate whether recombinant MtrC and OmcA behaved similarly to recombinant PulA with respect to their extracellular secretion, we tested the effects of deletions of *gspD* or *gspG* gene on the secretion of the recombinant MtrC and OmcA that contained a V5/His_6 tag in their C termini and were either acylated or not in their N termini. The procedures for making these constructs were described previously (7, 25) and the strains, plasmids, and oligonucleotide primers used in this study are listed in Table 1.

Similar to the results with native MtrC and OmcA, the supernatants of wt (with respect to the T2SS) containing recombinant MtrC or OmcA were pink, in sharp contrast to the pale yellow supernatants from the Δ*gspD* or Δ*gspG* mutants. The cultures containing whole cells, meanwhile, were red (only those of recombinant/acylated are shown in panels S and W of Fig. 2A). Because they were overexpressed by addition of L-arabinose at a final concentration of 1 mM, the recombinant MtrC and OmcA were the predominant heme proteins in the supernatants (Fig. 2B and C, I panels). The FccA band was very faint or undetectable in the samples containing recombinant MtrC or OmcA (Fig. 2B and C, panels I, lanes 1 and 4), in contrast to earlier observations with native MtrC and OmcA (Fig. 1D and E). Compared to that in wt, little or no recombinant MtrC or OmcA was detected in the supernatants of Δ*gspD* or Δ*gspG* by heme staining (Fig. 2B and C, I panels, lanes 2, 3, 5, and 6) or Western blot (Fig. 2B and C, II panels, lanes 2, 3, 5, and 6) under the conditions tested. There was significant accumulation of recombinant/nonacylated MtrC and OmcA in the cells of the T2SS mutants relative to the wt (Fig. 2C, lanes 7 to 12). In contrast to these results, as well as

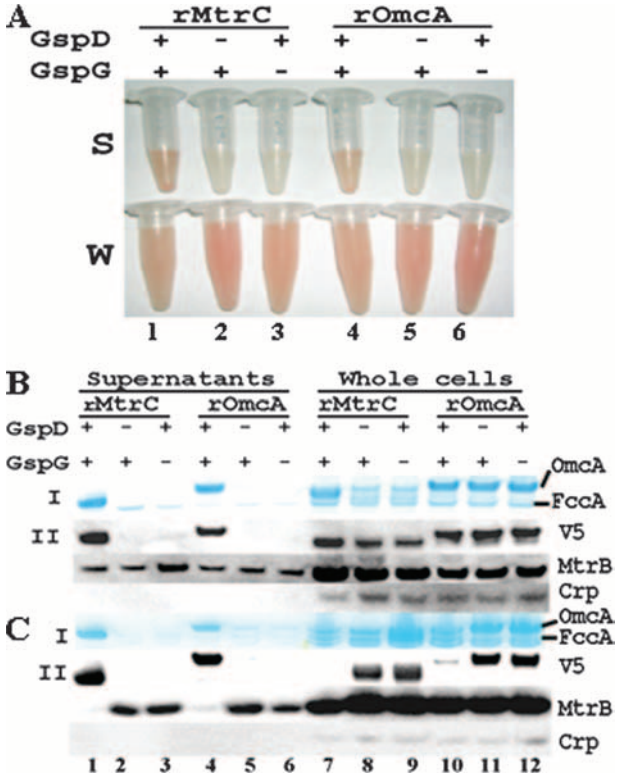

FIG. 2. Influence of deletion of *gspD* or *gspG* on extracellular release of recombinant MtrC and OmcA. (A) The concentrated supernatants (panel S) and whole cells (panel W) of wt (lanes 1 and 4), Δ*gspD* mutant (lanes 2 and 5), and Δ*gspG* mutant (lanes 3 and 6) in which recombinant (r)/acylated MtrC and OmcA were overexpressed. (B and C) Heme staining and Western blot analyses of the gene deletion effects on recombinant/acylated MtrC and OmcA (B) and recombinant/nonacylated MtrC and OmcA (C). A total of 1 μg (B) or 0.5 μg (C) of concentrated supernatant proteins (lanes 1 to 6) or 5 μg of whole-cell lysate proteins (lanes 7 to 12) from wt (lanes 1, 4, 7, and 10), Δ*gspD* mutant (lanes 2, 5, 8, and 11), and Δ*gspG* mutant (lanes 3, 6, 9, and 12) were separated by SDS-PAGE and were then either visualized by heme staining (I panels) or probed with the respective antibodies (II panels).

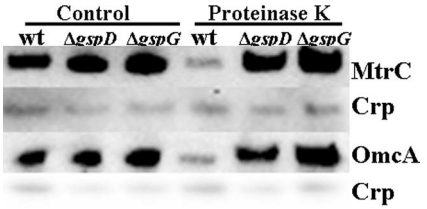

FIG. 3. Proteinase K accessibility of MtrC and OmcA in wt, Δ*gspD* mutant, and Δ*gspG* mutant cells. The preparations of cells were as described in the text. The whole-cell lysates (~10^6 cells) were separated by SDS-PAGE and then probed with the respective antibodies.

those with native MtrC and OmcA, there was no apparent excess accumulation of recombinant/acylated MtrC or OmcA in the cells of Δ*gspD* or Δ*gspG* relative to the wt. Heme staining showed overexpression of the MtrC in the cells with a functional T2SS but little in either Δ*gspD* or Δ*gspG* cells. It also showed that the abundance of OmcA was nearly identical among wt, Δ*gspD*, and Δ*gspG* cells. While the results of Western blot analysis of the recombinant/acylated OmcA were consistent with that of heme staining, they also showed the presence of recombinant/acylated MtrC in all cell types. After normalizing against MtrB, the difference in MtrC abundance between wt and Δ*gspD* or Δ*gspG* mutant was much less apparent than that from heme staining (Fig. 2B, lanes 7 to 12). These results demonstrated that recombinant/acylated MtrC was expressed in Δ*gspD* and Δ*gspG* mutants. Given the profound differences in the abundance of the recombinant/acylated MtrC and OmcA in the supernatants of the wt and either Δ*gspD* or Δ*gspG* mutant in concert with similar abundances in the cells, we conclude that T2SS is involved in the extracellular release of the recombinant/acylated forms of MtrC and OmcA. The reason for no apparent accumulation of recombinant/acylated MtrC or OmcA in the cells of T2SS mutants is currently unknown.

Because they are not membrane associated (7; L. Shi, unpublished data), the extracellular release of recombinant/nonacylated MtrC and OmcA into the growth medium should be a single-step process: their translocation across the OM, similar to that of recombinant/nonacylated PulA from *K. oxytoca* (9). The effects of GspD and GspG on the recombinant/nonacylated *c*-Cyts therefore suggest that T2SS plays a direct role in extracellular secretion of MtrC and OmcA by translocating them across the OM.

GspD and GspG affect accessibility of MtrC and OmcA to proteinase K. To confirm the findings that T2SS directly affects the translocation of MtrC and OmcA across the OM, we treated the cells of wt, Δ*gspD*, and Δ*gspG*, individually, with proteinase K (New England Biolabs, Ipswich, MA) in the buffer (100 mM Tris [pH 7.4], 150 mM NaCl, 1 mM $CaCl_2$, 50 μM $MgCl_2$) that contained 0.5 mg of the protease/ml and 10^9 cells/ml at room temperature for 60 min. Compared to those in controls without the treatment, the abundances of MtrC and OmcA in wt cells were much lower after treatment with proteinase K under the conditions tested. In contrast to wt, the treatment of Δ*gspD* and Δ*gspG* mutants with proteinase K had little effect on the abundance of MtrC and OmcA (Fig. 3). These results are not only consistent with the previous findings that MtrC and OmcA are cell surface exposed (20) but also confirm that T2SS indeed regulates directly the translocation of MtrC and OmcA across the OM.

Conclusions. Although the nature or the mechanisms of extracellular release of acylated MtrC and OmcA into the growth medium under the condition tested in this study are currently unknown, the results of this research demonstrate for the first time the direct role of the T2SS in translocating MtrC and OmcA across the OM, since deletion of T2SS genes (i) impairs the ability of *S. oneidensis* to release of MtrC and OmcA to the growth medium and (ii) makes the MtrC and OmcA resistant to the proteinase K treatment. Furthermore, the effects of T2SS mutations on MtrC and OmcA are very similar to those on PulA of *K. oxytoca*. Based on these and previous findings, the sequential steps of extracellular translocation of MtrC and OmcA in *S. oneidensis* should include their (i) biosyntheses in the cytoplasm, (ii) translocation across the IM via the Sec pathway, (iii) maturation in the periplasm, and (iv) translocation across the OM via the T2SS.

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