Characterization of the highly conserved VFMGD motif in a bacterial polyisoprenyl-phosphate *N*-acetylaminosugar-1-phosphate transferase

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Abstract: Polyisoprenyl-phosphate *N*-acetylaminosugar-1-phosphate transferases (PNPTs) constitute a family of eukaryotic and prokaryotic membrane proteins that catalyze the transfer of a sugar-1-phosphate to a phosphoisoprenyl lipid carrier. All PNPT members share a highly conserved 213-Valine-Phenylalanine-Methionine-Glycine-Aspartic acid-217 (VFMGD) motif. Previous studies using the *MraY* protein suggested that the aspartic acid residue in this motif, D267, is a nucleophile for a proposed double-displacement mechanism involving the cleavage of the phosphoanhydride bond of the nucleoside. Here, we demonstrate that the corresponding residue in the *E. coli* *WecA*, D217, is not directly involved in catalysis, as its replacement by asparagine results in a more active enzyme. Kinetic data indicate that the D217N replacement leads to more than twofold increase in $V_{\max}$ without significant change in the K_m for the nucleoside sugar substrate. Furthermore, no differences in the binding of the reaction intermediate analog tunicamycin were found in D217N as well as in other replacement mutants at the same position. We also found that alanine substitutions in various residues of the VFMGD motif affect to various degrees the enzymatic activity of *WecA* *in vivo* and *in vitro*. Together, our data suggest that the highly conserved VFMGD motif defines a common region in PNPT proteins that contributes to the active site and is likely involved in the release of the reaction product.

Keywords: GlcNAc phosphate transferase; lipopolysaccharide; membrane protein; O-antigen

Introduction

The synthesis of lipid-linked glycan precursors is essential for the production of glycoconjugates in prokaryotes and eukaryotes, such as polysaccharides, glycopeptides, and a wide range of glycosylated proteins.^{1,2} The enzymes that catalyze the initiation of the synthesis of these lipid-linked glycans fall into two broad families of membrane proteins: (i) the polyisoprenyl-phosphate *N*-acetylaminosugar-1-phosphate transferases (PNPTs) and (ii) the polyisoprenyl-phos-

phate hexose-1-phosphate transferases (PHPTs). These enzymes catalyze the transfer of a sugar-1-phosphate from a nucleoside sugar diphosphate to a phosphoisoprenyl lipid carrier resulting in the formation of a phosphoanhydride bond.^{1,2} Enzymes of the PHPT family occur only in bacteria, and they catalyze the synthesis of O-antigen,^{3–8} various exopolysaccharides,^{8,9} and glycans precursors for protein glycosylation.^{10–13}

PNPT proteins occur in prokaryotes and eukaryotes. The eukaryotic members are UDP-*N*-acetylglucosamine (UDP-GlcNAc):dolichyl-phosphate (Dol-P) GlcNAc-1-P transferases and reside in the rough endoplasmic reticulum membrane.¹⁴ In contrast to the eukaryotic members, bacterial PNPTs use several different diphospho *N*-acetylaminosugar

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Figure 1. Topological model of the *E. coli* WecA. **A:** Refined topological model of the *E. coli* WecA. This model was previously established by a combination of bioinformatics, substituted cysteine accessibility experiments, and reporter gene fusions.²⁰ A dotted ellipse indicates the highly conserved V₂₁₃FMGD₂₁₇ motif, and its topology has been determined in this work (see Results section). The highly conserved residues that are important for function based on previous research^{20,23,24} are circled. Shading indicates D217. The dotted circle indicates the residue S220. Squares show the control residues for cysteine scanning accessibility experiments used in this work, G181 and S362. **B:** ClustalW alignment of PNPT members shows the highly conserved VFMGD motif. Alignment shows protein sequences from *E. coli* MraY, *B. subtilis* MraY, *Homo sapiens* GPT, Fission Yeast GPT, and *E. coli* WecA. The completely conserved amino acids are marked with an asterisk (*), and partially conserved amino acids are marked with a colon (:).

nucleoside substrates.¹⁵ The eukaryotic PNPTs are specific for Dol-P,¹⁶ whereas the bacterial enzymes function only with undecaprenyl-phosphate (Und-P).¹⁷ WecA and MraY are prototypic bacterial PNPTs; the former is an UDP-GlcNAc:Und-P GlcNAc-1-P transferase¹⁸ that initiates the synthesis of O-antigen and enterobacterial common antigen, and the latter is a UDP-*N*-acetylmuramyl (MurNAc)-pentapeptide:Und-P MurNAc-pentapeptide-1-P transferase that initiates the synthesis of cell wall peptidoglycan.¹⁹

These enzymes have multiple transmembrane (TM) helices, and several studies suggest that all the cytosolic loops contribute residues to form a putative catalytic site.^{19,20} Enzyme kinetics studies on MraY have suggested that the reaction involves the formation of an enzyme-phospho-MurNAc-pentapeptide intermediate prior to its transfer to Und-P.²¹ However, recent experiments using purified MraY

proteins suggests a one-step catalytic mechanism in which a base contributed by the side chain of a conserved aspartic acid residue would permit the deprotonation of a hydroxyl of the Und-P terminal phosphate. This deprotonated hydroxyl group would be involved in the nucleophilic attack on the β -phosphate of the UDP-MurNAc-pentapeptide.¹⁹

Despite the differences in the PNPT family regarding *N*-acetylaminosugar and lipid specificities, these proteins share highly conserved amino acid motifs, suggesting a common enzymatic mechanism.²² To better understand the catalytic mechanism of PNPT enzymes, several of these conserved regions have been characterized in MraY and WecA. For example, the DDxxD motif in the cytoplasmic loop II of WecA has been proposed to bind the cofactor Mg²⁺ [Fig. 1(A)].²⁰ The highly conserved D156 residue has been postulated to play a critical role in the catalytic mechanism as mutations to this residue

causes the protein to lose all activity *in vivo* and *in vitro*.^{20,23} A conserved HHH motif present in loop V is predicted to be part of a carbohydrate recognition domain.^{15,24} Another highly conserved region shared among eukaryotic and prokaryotic PNPT family members is the 213-Valine-Phenylalanine-Methionine-Glycine-Aspartic acid-217 (VFMGD) motif [Fig. 1(B)].²² Lloyd *et al.*²⁵ proposed that the highly conserved aspartic acid residue 267 (D267) in the VFMGD motif of MraY contains a catalytic nucleophile, according to a proposed double-displacement catalytic mechanism that involves the cleavage of the pyrophosphate bond of the nucleoside.²¹ The comparable aspartic acid residue in the VFMGD motif of WecA is D217, which was predicted to be located in a TM region.²⁰ In this study, we investigated the topology and function of the WecA V₂₁₃FMGD₂₁₇ motif. Our results demonstrate that D217 and the rest of the residues in the motif are located facing the cytosol, but D217 is not a catalytic nucleophile. However, the polarity and size of amino acid side chains at the D217 position are important for enzymatic activity, as were the replacements of the V213, F214, G216, and D217 residues with alanine. We propose that the highly conserved VFMGD motif defines a region in PNPT proteins that contributes to the active site, likely involved in the binding and/or recognition of the nucleotide moiety of the nucleoside phosphate precursor.

Results

The substituted cysteine accessibility method reveals that aspartic acid residue D217 is exposed to the cytosol

In silico analyses using topology prediction programs for membrane proteins placed the aspartic acid residue D217 in the eighth predicted TM (TM VIII) segment of WecA.²⁰ However, the predicted location of this residue did not agree with its proposed function as a nucleophile.^{22,25} D217C and S220C derivatives of WecA were constructed in a cysteine-less WecA-FLAG-Hisx7 protein.²⁰ Bacterial cells expressing the WecA proteins with the Cys replacements were preincubated with 2-(trimethylammonium)ethyl methanethiosulfonate bromide (MTSET), a membrane-impermeable thiol-blocking reagent, and subsequently treated with the membrane-permeable biotin maleimide. Biotinylated proteins were visualized as described in the "Materials and Methods" section. As controls, aliquots of the same cells were treated with biotin maleimide only. We also used G181C (periplasmic) and S362C (cytoplasmic) replacement mutants as topology controls.²⁰ The results demonstrated that the Cys residue at position D217, similar to the residue at position S362 (cytoplasmic control), was labeled with biotin maleimide irrespective of pretreatment with MTSET. In

Figure 2. Sulfhydryl labeling accessibility of the cysteine replacement in WecA_{D217C}. The top panel shows the results obtained with biotin maleimide labeling. The second and fourth panels show Western blots with anti-FLAG antibodies. The third panel shows the results of biotin maleimide labeling with MTSET-pretreated samples. WecA was purified by Co²⁺ affinity chromatography and separated by 14% SDS-PAGE. After transfer to a nitrocellulose membrane, biotin maleimide labeling was detected by a streptavidin-conjugated fluorophore (green signal), and WecA-FLAG proteins were detected using anti-FLAG antibodies (red signal). Biotinylated WecA-FLAG proteins are observed by the merged images, which appear yellow using Odyssey LI-COR Scanner. The cysteine replacement of D217 in the cysteine-less WecA-FLAG protein is labeled with biotin maleimide irrespective of MTSET pretreatment, suggesting that this residue faces the cytosol.

contrast, the periplasmic control Cys residue at position G181 was labeled with biotin maleimide; however, as expected due to its periplasmic location, labeling was blocked by pretreatment with the membrane-impermeable agent MTSET (Fig. 2).²⁰ Therefore, we conclude that D217 is exposed to the cytoplasmic space. Interestingly, the replacement of S220 with Cys could not be labeled with biotin maleimide, suggesting that this residue may reside at the membrane boundary or within TM VIII [Fig. 1(A)].

Substitution of aspartic acid residue D217 by nonacidic but polar residues results in a more active enzyme

To investigate the role of D217, we constructed WecA_{D217N} and assessed the function of this protein *in vivo* by determining its ability to restore O7 Ag synthesis in the *E. coli* *wecA*-defective strain MV501.¹⁸ The Asn replacement did not abolish the synthesis of polymeric O-antigen [Fig. 3(A)], suggesting that this aspartic acid residue is not involved in catalysis. We also tested the enzymatic activity of membrane extracts containing WecA_{D217N} by measuring the incorporation of ¹⁴C-GlcNAc into a lipid fraction [Fig. 3(B)]. Surprisingly, WecA_{D217N} had increased *in vitro* transferase activity relative to the parental enzyme. This result was confirmed by densitometry quantitation of the reaction product after separating the lipid fractions by thin layer chromatography (TLC) [Fig. 4(A)]. The results were normalized by the amount of WecA protein present in the

Figure 3. *In vivo* and *in vitro* complementation of the D217 replacement mutants. **A:** Complementation of O7 LPS expression in *E. coli* MV501 by plasmids encoding parental WecA (WT) and various D217 replacement mutants, as determined with silver-stained polyacrylamide gels. The pBAD24 cloning vector was used as a negative control. **B:** GlcNAc-1-P transferase activity assays performed with total membrane extracts prepared from *E. coli* MV501 cells carrying pBAD24 or plasmids encoding parental WecA (WT) and D217 replacement mutants. Bars represent means and standard errors of triplicate experiments. **C:** Expression of the WecA proteins in the membrane extracts used for enzymatic assays, as detected by Western blotting using anti-FLAG antibodies.

membrane extract. The densitometric ratios (pixel density of Und-P-P-GlcNAc product formed per amount of WecA protein) were 1.75 for the parental WecA and 3.38 for the D217N mutant enzyme [Fig. 4(B,C)]. Together, these experiments demonstrate that D217 does not function as a catalytic nucleophile.

To further explore the role of D217, we replaced this residue with amino acids Glu, Ala, Lys, and Ser. Mutant proteins were tested for expression in total membrane fractions [Fig. 3(C)] and for functional ac-

tivity *in vivo*. WecA_{D217E} and WecA_{D217S} supported polymeric O-antigen synthesis, although the amount of O-antigen produced was reduced relative to that obtained with parental WecA [Fig. 3(A)]. MV501-expressing WecA_{D217A} could only produce a very faint band corresponding to one O-antigen unit, whereas WecA_{D217K} was not functional [Fig. 3(A)]. The absence of polymeric O-antigen with WecA_{D217A} suggests that this mutant enzyme is severely impaired in its enzymatic activity (see below), resulting in undetectable polymeric O-antigen. The *in vitro* transferase activity in membrane fractions showed that the replacement of D217 by glutamic acid (WecA_{D217E}) did not affect the *in vitro* enzyme activity. As for D217N, the D217S replacement resulted in a WecA protein with higher transferase activity than the parental enzyme, whereas replacements with Ala and Lys resulted in decreased activity and loss of activity, respectively [Fig. 3(B)], in agreement with the *in vivo* functional data. None of

Figure 4. The WecA_{D217N} protein mediates increased Und-P-P-GlcNAc production when compared with parental WecA. **A:** TLC of lipid extractions from MV501 total membranes containing parental WecA (WT) and the D217N mutant WecA total proteins that were incubated with ¹⁴C-UDP-GlcNAc. **B:** Western blot of parental WecA (WT) and D217N mutant proteins using anti-FLAG antibodies. **C:** Graph depicting the ratio of Und-P-P-GlcNAc product formed per WecA-FLAG protein.

Figure 5. GlnAc-1-P transferase activity of the parental WecA (A) and the D217N mutant (B). About 40 μg of total membranes were incubated with increasing amounts of ^{14}C -UDP-GlcNAc for 30 min at 37°C. The lipid fraction was extracted, and then the counts were quantified in a scintillation counter. The enzyme units are expressed as picomoles of UDP-GlcNAc incorporated per milligram of protein. The experiment was performed in triplicate.

these differences could be attributed to differential protein expression, as the parental and mutated forms of WecA were expressed at similar levels in the membrane protein extracts used for the enzymatic analysis [Fig. 3(C)].

The D217N WecA mutant has a higher V_{max} but no significant change in K_m

The differences observed above prompted us to investigate whether WecA_{D217N} produces more Und-P-P-GlcNAc than the parental WecA due to increased binding affinity for UDP-GlcNAc. Enzyme kinetics was determined using increasing amounts of ^{14}C -labeled UDP-GlcNAc (Fig. 5), and data were analyzed by nonlinear regression of the Michaelis-Menten equation to calculate V_{max} and K_m values (Table I). The parental WecA and WecA_{D217N} have V_{max} values of 2.6 ± 0.3 pmol/(min mg) and 6.7 ± 0.7 pmol/(min mg), respectively, whereas the K_m val-

Table I. Kinetic Parameters of UDP-GlcNAc for Parental WecA and D217N Mutant Enzymes

Protein	K_m (μM)	V_{max} [pmol/(min mg)]	V_{max}/K_m
Parental WecA	0.08 ± 0.04	2.6 ± 0.3	32.5
D217N	0.07 ± 0.03	6.7 ± 0.7	95.7

Transfer assays were performed with total membrane extracts prepared from MV501 cells carrying the plasmid expressing parental or mutant WecA as described in the "Materials and Methods" section.

ues of the two proteins did not change significantly (Table I). These results demonstrate that the Asp to Asn replacement results in approximately threefold increased catalytic efficiency when compared with the parental WecA, in agreement with the observed increased product conversion [Fig. 4(A)]. In general, tunicamycin is an inhibitor of the transfer reaction that acts as a putative transition-state analog inhibitor of WecA and PNPT enzymes.²⁶ Therefore, to gain more information on the function of the residue at position 217, we assessed the ability of WecA mutant proteins D217N, D217E, D217A, and D217K to bind tunicamycin at subinhibitory concentrations, an assay previously used to estimate UDP-GlcNAc binding in WecA mutant proteins.²⁴ In this assay, the level of transferase activity of the parental WecA is a function of the residual tunicamycin concentration in the supernatant after exposure to membranes containing the appropriate WecA mutant protein. The results show no differences in the binding capacity of the mutants relative to the parental enzyme (Fig. 6), suggesting that the change in enzymatic activity is likely not attributed to a change in

Figure 6. Tunicamycin binding assay using parental WecA and D217 replacement mutants. About 40 μg of total membranes containing the various D217 mutant proteins were incubated with a concentration of tunicamycin that inhibits 100% activity of 20 μg of total membranes containing wild-type WecA. The residual tunicamycin, unbound by the mutant proteins, was determined by adding the supernatants to 20 μg of total membranes containing wild-type WecA. The data were represented as a percentage of WecA-FLAG-binding tunicamycin activity. Data represent duplicates from two independent experiments.

Figure 7. *In vivo* and *in vitro* complementation of alanine replacement mutants in the VFMD motif. **A:** Complementation of O7 LPS in *E. coli* MV501. **B:** GlcNAc-1-P transferase activity of parental WecA and alanine replacement mutant proteins. Enzyme units are expressed as picomoles of UDP-GlcNAc incorporated per milligram of protein. Experiments were performed in triplicate. **C:** Expression of the mutant proteins in total membranes as detected by Western blotting using anti-FLAG antibodies.

the ability of the enzyme to bind a putative transition-state intermediate.

Alanine replacements of other residues of the VFMD motif result in diminished enzymatic activity

To assess the *in vivo* activity of alanine replacements in other VFMD residues, mutant proteins were expressed in MV501 and tested for membrane expression and their ability to restore O7 Ag synthesis. All mutant proteins were detected in the total membrane fraction by anti-FLAG Western blotting, indicating that they are correctly expressed at similar levels and exported to the bacterial membrane [Figs. 3(C) and 7(C)]. The lipopolysaccharide (LPS) was extracted from cells expressing these mutant enzymes, separated by SDS-PAGE and stained with silver nitrate [Fig. 7(A)]. The WecA_{V213A} and WecA_{M215A} complemented similar to the parental

enzyme; however, the WecA_{D217A} could only produce one O-antigen unit and WecA_{G216A} produced a reduced amount of polymeric O-antigen. Although F214A mutant enzyme could only produce one O-antigen unit, the F214Y can complement O-antigen synthesis similar to the wild-type WecA (data not shown), suggesting that the hydrophobicity of this residue is important for function. The alanine replacement of the completely conserved residues, V213, F214, G216, and D217, also had dramatic effects on the *in vitro* transferase activity [Fig. 7(B)], whereas the replacement of the M215, the less conserved residue of the motif, did not have any effect on *in vitro* transferase activity. The G216A mutation also revealed a severe effect on activity, suggesting that this residue plays a structural role. Together, these data indicate that the highly conserved residues of the VFMD motif may delineate a structural scaffold of the WecA's substrate active site rather than being directly involved in the catalytic process.

Discussion

The VFMD motif is a highly conserved region among eukaryotic and prokaryotic PNPT family members [Fig. 1(B)]. Our initial hypothesis was that this region and in particular the conserved acidic residue D217 of the *E. coli* WecA are critical to the enzyme's catalytic mechanism, as it was suggested for the analogous residue D267 in the *E. coli* MraY.²⁵ However, the WecA VFMD motif was predicted to be part of TM VIII, which is inconsistent with a putative catalytic role of D217. In this work, we provide experimental evidence based on substituted cysteine mutagenesis that D217 resides in the cytoplasmic space. Confirming this conclusion, the S220C replacement could not be labeled with biotin maleimide (Fig. 2), indicating that S220 resides within TM VIII, likely at the boundary between membrane and cytosolic space. Therefore, we have updated the topology model for WecA²⁰ by shifting D217 and the rest of the VFMD motif to the cytoplasmic space in loop IV and placing S220 at the border of TM VIII [Fig. 1(A)]. This topology refinement is consistent with the cytoplasmic location of the VFMD motif in MraY.²⁷

Further experiments to assess the functional role of D217 demonstrated that D217N and D217A replacements did not abolish the *in vitro* enzymatic activity of the mutant WecA proteins [Fig. 3(B)] or their ability to support O-antigen synthesis *in vivo* [Fig. 3(A)], as it would be expected for a site involved in catalysis. In contrary, Asn and Ser replacements at the 217 position resulted in enhanced enzymatic activity, as determined by approximately twofold increase in the reaction product [Fig. 4(C)]. These differences could not be attributed to changes in the ability of the mutant proteins

Table II. Characteristics of the Bacterial Strains and Plasmids Used in This Study

Strain or plasmid	Relevant properties	Reference
Strains		
DH5 α	<i>E. coli</i> K-12 F ⁻ Φ 80 <i>lacZ</i> AM15 <i>endA recA</i> <i>hsdR</i> (r _K ⁻ m _K ⁻) <i>supE thi gyrA relA</i>	Laboratory stock
MV501	<i>E. coli</i> VW187; <i>wecA</i> ::Tn10 Tet ^R	18
VW187	O7:K1; clinical isolate	32
Plasmids		
pBAD24	Cloning vector, inducible by arabinose; Amp ^R	33
pBAD-His	His ₆ inserted into pBAD24; Amp ^R	20
pKV1	Expresses WecA-FLAG-5xHis from <i>wecA</i> -FLAG cloned into pBAD-His	20
pJL7	pKV1 expressing cysteine-less WecA-FLAG-7xHis	20
pJL7-G181C	pJL7 expressing cysteine-less WecA-G181C-FLAG-7xHis	20
pJL7-S362C	pJL7 expressing cysteine-less WecA-S362C-FLAG-7xHis	20
pSEF8	pKV1 expressing WecA-D217N-FLAG-5xHis	This study
pSEF9	pKV1 expressing WecA-D217E-FLAG-5xHis	This study
pSEF17	pKV1 expressing WecA-D217A-FLAG-5xHis	This study
pSEF18	pKV1 expressing WecA-D217K-FLAG-5xHis	This study
pSEF36	pJL7 expressing cysteine-less WecA-D217C-FLAG-7xHis	This study
pSEF37	pJL7 expressing cysteine-less WecA-S220C-FLAG-7xHis	This study
pSEF43	pKV1 expressing WecA-V213A-FLAG-5xHis	This study
pSEF44	pKV1 expressing WecA-F214A-FLAG-5xHis	This study
pSEF45	pKV1 expressing WecA-M215A-FLAG-5xHis	This study
pSEF46	pKV1 expressing WecA-G216A-FLAG-5xHis	This study
pSEF48	pKV1 expressing WecA-D217S-FLAG-5xHis	This study

to bind the UDP-GlcNAc substrate when compared with the parental WecA (Fig. 5). Indeed, kinetic experiments revealed that parental and D217N WecA proteins have no significant difference in their K_m values, which is also consistent with the results of tunicamycin binding experiment (Fig. 6).

Despite no differences in K_m values, kinetic data showed that V_{max} of WecA_{D217N} was 2.5-fold greater than that of the parental WecA (Table I). Changes to the velocity of the enzyme without alteration in the affinity for the substrate may suggest a faster release of the reaction product.^{28–31} However, we have no direct proof that this is the mechanism behind the differences between parental WecA and the D217N mutant. Additional mutagenesis of the other residues in the VFMGD motif supports a structural role for this motif instead of a direct role in enzyme catalysis. This stems from the following observations: (i) the replacements showing a severe defect in the ability of WecA to produce O-antigen and to perform catalysis *in vitro* did not affect the localization of the mutated protein to the membrane; (ii) the lack of function of the F214A replacement was rescued by a replacement with tyrosine, suggesting that a bulky hydrophobic residue is required at this position; and (iii) the inability of G216A WecA mutant to remain functional, despite a relatively conservative substitution from Gly to Ala. Furthermore, our results do not support the notion that the VFMGD motif contains a catalytic nucleophile, as reported earlier for *MraY*.²⁵ These authors have reported a 10% residual transferase activity in the *MraY*_{D267N} mutant, which is inconsistent with the conclusion of residue D267 being a catalytic

nucleophile, as its mutation should result in complete loss of enzyme activity. In contrast, the decreased *in vitro* enzymatic activity of purified *MraY*_{D267N} may reflect an altered active site conformation, in agreement with our data indicating that this region in WecA may be highly structured and contribute to the active site. *MraY* D267 may also play a role in product release, which could not be elucidated from the experiments conducted by Lloyd *et al.*²⁵ Previous work in our laboratory demonstrated that the WecA residue D156, located in cytosolic loop 3, is likely a catalytic nucleophile, as it is absolutely required for WecA activity *in vivo* and *in vitro* when replaced with Asn.²⁰ A comparable residue in *MraY* has not been identified.

In conclusion, we propose that the conserved VFMGD motif provides a structural scaffold to a putative catalytic site. This model is consistent with our results showing that subtle side-chain changes at position D217 and neighboring residues have drastic effects on enzyme activity without changing the binding affinity for UDP-GlcNAc, which suggests that the VFMGD motif may contribute to the structure of the enzyme's active site.

Materials and Methods

Bacterial strains, plasmids, media, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table II. Bacteria were grown aerobically at 37°C in Luria-Bertani (LB) medium (10 mg/mL of tryptone, 5 mg/mL of yeast extract, and 5 mg/mL of NaCl; Difco Laboratories, Sparks, MD).

Growth medium was supplemented with 100 µg/mL of ampicillin and/or 20 µg/mL of tetracycline. DH5α cells were transformed with plasmids by calcium chloride method,³⁴ and MV501 cells were transformed with plasmids by electroporation.³⁵

Site-directed mutagenesis

Site-directed mutations were made in pKV1, an arabinose-inducible pBAD vector expressing the WecA-FLAG-Hisx5 protein. For sulfhydryl labeling experiments, novel cysteines were introduced in pJL7, similar to pKV1 but expressing the cysteine-less WecA-FLAG-Hisx7 protein. Replacements were introduced by the polymerase chain reaction (PCR) using primers containing the desired mutations and *Pfu* AD polymerase (Stratagene, Santa Clara, CA). *Dpn*I was added to PCR reactions for overnight digestion of parental plasmid DNA at 37°C. The resulting DNA was introduced into *E. coli* DH5α by transformation, and transformants were selected on LB-agar containing 100 µg/mL of ampicillin.

Growth conditions of cells for protein preparation

Briefly, MV501 cells containing the appropriate arabinose-inducible plasmids were grown overnight in LB containing 100 µg/mL of ampicillin and 20 µg/mL of tetracycline. From overnight cultures, cells were diluted to an optical density measured at 600 nm (OD₆₀₀) of 0.1 in LB broth containing the respective antibiotics. Cells were grown at 37°C until an OD₆₀₀ of 0.5, and cells were induced with 0.2% arabinose for 3 h at 37°C. Cells were harvested by centrifugation at 8000g, and cell pellets were frozen at -20°C until needed.

Total membrane preparation and immunoblotting

Cells were resuspended in 50 mM of TAE (pH 8.5) with protease inhibitors and lysed by French press (Thermo Scientific, Rockville, MD). Cell debris and unlysed cells were pelleted at 27,216g. Total membranes were isolated by centrifugation in microfuge tubes at 39,191g and resuspended in 1× TAE, unless otherwise stated. Protein concentrations were determined by Bradford protein assay (Bio-Rad, Hercules, CA). Total membrane fractions were used for *in vitro* transferase assays and immunoblotting. Immunoblotting was performed by separating total membrane fractions by 14% SDS-PAGE and transferring to a nitrocellulose membrane. Membranes were blocked overnight in 5% Western blocking reagent (Roche Diagnostics Canada, Laval, QC, Canada) and TBS. The primary antibody, 4.6 mg/mL anti-FLAG M2 monoclonal antibody (Sigma, Saint Louis, MO), was diluted to 1:10,000 and applied for 1.5 h, and the secondary antibody, 2 mg/mL goat anti-mouse Alexa fluor 680 IgG antibodies (Invitrogen Molecular

Probes, Eugene, OR), was diluted to 1:20,000 and applied for 20 min. Western blots were developed using LI-COR Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). Bio-Rad Precision Plus Protein Standards were used for all Western blots.

Sulfhydryl labeling of cysteine residues using biotin maleimide and protein purification

MV501 cells expressing cysteine-less WecA mutant proteins were grown as described in the "Growth conditions of cells for protein preparation" section. Cells were centrifuged at 8000g, washed twice with 0.1M sodium phosphate buffer (pH 7.2) and resuspended in this buffer. Whole cells were either pretreated with 0.1M MTSET (Toronto Research Chemicals, Toronto, ON, Canada), a membrane-impermeable blocking reagent or directly labeled with the membrane-permeable 0.5 mM *N*^ε-(3-maleimidylpropionyl)biocytin (biotin maleimide; Invitrogen Molecular Probes). The reaction was terminated by adding 2% 2-mercaptoethanol. Cells were washed three times with 0.1M sodium phosphate buffer (pH 7.2) and resuspended in sodium phosphate buffer prior to lysing by French press.

Total membranes were prepared and quantified as described above and solubilized for 16 h in 350 µL of 0.1M sodium phosphate buffer (pH 7.8), 8M urea, and 0.5% Triton X-100 at 4°C. Solubilized samples were centrifuged at 39,000g for 35 min. Supernatants, containing soluble WecA-FLAG-Hisx7 protein, were applied to Co²⁺ charged sepharose beads for 2 h at 4°C. Beads were washed twice with wash buffer [0.1M sodium phosphate buffer (pH 7.8), 8M urea, 0.5% Triton X-100, 300 mM NaCl, and 50 mM imidazole (pH 8)]. Samples were eluted with elution buffer [0.1M sodium phosphate buffer (pH 7.8), 8M urea, 0.5% Triton X-100, 300 mM NaCl, and 400 mM imidazole (pH 8)]. Purified samples were separated by 14% SDS-PAGE and transferred to nitrocellulose membrane. Western blotting using anti-FLAG antibodies was performed as described above, and a 1:25,000 dilution of streptavidin-conjugated infrared 800 dye (Rockland Immunochemicals, Gilbertsville, PA) was used to detect biotinylated proteins.

LPS analysis

In vivo transferase activity was assessed by observing the ability of mutant WecA proteins to complement O7 Ag synthesis in the *wecA*-defective strain MV501 (*wecA*::Tn10). MV501 cells harboring the respective plasmids were grown overnight in 5 mL of LB broth and induced with 0.002% arabinose. The OD₆₀₀ of overnight cultures were adjusted to a turbidity of 2, and cells were lysed by boiling and treated with proteinase-K. LPS samples were extracted using a hot phenol method as described by Marolda *et al.*³⁶ LPS samples were separated by

14% acrylamide gels using a Tricine/SDS buffer system and stained with silver nitrate.³⁶

In vitro transferase assay

Total membranes were prepared from MV501 cells containing plasmids expressing wild-type and mutant WecA proteins. About 40 or 60 µg of membranes, containing endogenous Und-P, were incubated for 30 min at 37°C with 78.8 pmol of ¹⁴C-labeled UDP-GlcNAc in a 250-µL reaction buffer containing 50 mM of Tris-HCl (pH 8), 40 mM of MgCl₂, 0.5 mM of EDTA, 0.5% CHAPS, 5 mM of 2-mercaptoethanol, and 50 mM of sucrose. For kinetic curves, 7.88–709.2 pmol of radiolabeled sugar was used. The lipid fraction was extracted twice with butanol and washed with water, and then the butanol phase (containing the lipid fraction) was added to scintillation cocktail (Ecolume, MP Biomedical, Solon, OH) and measured by scintillation counter (Beckman Coulter Canada, Mississauga, ON, Canada) to determine the radioactive counts per minute.

Thin layer chromatography

To determine the radioactive lipid products formed in the *in vitro* transferase assay, lipid extractions from these assays (described above) were dried overnight and resuspended in 20 µL of 2:1 chloroform-methanol solution. The extractions were spotted on Whatman TLC plates (silica gel, type PE SIL G) and placed in a saturated TLC tank with diisobutylketone, acetic acid, and water (80:50:10) solvent. The TLC plate was removed, dried, and exposed to a PhosphorImager storage screen overnight. The screen was imaged using PhosphorImager (Storm 840; Amersham Biosciences), and pixel densities were measured by ImageJ computer software.

Tunicamycin binding activity of WecA

The *in vitro* binding activity of proteins was assessed using a tunicamycin competition assay.²⁴ This assay is based on the ability of the WecA proteins to bind and be irreversibly inhibited by tunicamycin, a substrate-product transition-state analog.²⁶ About 40 µg of total membranes containing the mutant proteins were incubated for 10 min with a concentration of tunicamycin that inhibits 100% activity of 20 µg of total membranes containing wild-type WecA-FLAG enzyme. After which the membranes were centrifuged at 39,191g and the supernatants were added to 20 µg of total membranes containing wild-type WecA. The *in vitro* transferase assay was performed (as described above). Any residual tunicamycin that was not bound by the mutant WecA protein will inhibit the wild-type enzyme activity and can be described as a percentage of tunicamycin bound by wild-type WecA.

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