Functional Characterization and Membrane Topology of *Escherichia coli* WecA, a Sugar-Phosphate Transferase Initiating the Biosynthesis of Enterobacterial Common Antigen and O-Antigen Lipopolysaccharide

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Received 18 December 2006/Accepted 15 January 2007

WecA is an integral membrane protein that initiates the biosynthesis of enterobacterial common antigen and O-antigen lipopolysaccharide (LPS) by catalyzing the transfer of N-acetylgalactosamine (GlcNAc)-1-phosphate onto undecaprenyl phosphate (Und-P) to form Und-P-P-GlcNAc. WecA belongs to a large family of eukaryotic and prokaryotic prenyl sugar transferases. Conserved aspartic acids in putative cytoplasmic loops 2 (Asp90 and Asp91) and 3 (Asp156 and Asp159) were targeted for replacement mutagenesis with either glutamic acid or asparagine. 

The initiation reaction is catalyzed by two different classes of integral membrane proteins (52). One class, the polysoprenyl-phosphate N-acetylhexosamine-1-phosphate transferases (PNPTs), comprises proteins that are found in both prokaryotes and eukaryotes (14, 24, 25, 52). The prototype bacterial PNPT is WecA, a tunicamycin-sensitive UDP-GlcNAc:Und-P GlcNAc-1-phosphate transferase (37). WecA initiates O-unit synthesis in many *Escherichia coli* O types (1, 45, 58), *Klebsiella pneumoniae* O1 (11), *Shigella dysenteriae* (22), *Shigella flexneri* (58), and *Salmonella enterica* serovar Borreze (21), and it is also required for synthesis of the enterobacterial common antigen (38). Prokaryotic and eukaryotic PNPTs exhibit significant amino acid sequence similarity in certain regions of the protein (3, 4), underscoring the functional conservation of this class and presumably a common enzymatic mechanism (14, 28, 42, 52). Eukaryotic PNPTs are in the endoplasmic reticulum membrane, where they catalyze the transfer of GlcNAc-1-phosphate to dolichol phosphate, the first step in N-linked glycoprotein biosynthesis (25). Bacterial members of this class, such as WecA, MraY, WbpL, and WbcO, can utilize different N-acetylhexosamine substrates, and they also differ in their susceptibilities to selective inhibitors (6, 42). The other class of prenyl sugar transferases includes the polysoprenyl-phosphate hexose-1-phosphate transferases, and the prototype is WbaP (formerly RfbP) of *S. enterica*, in which the initiating sugar is galactose (54). The polysoprenyl-phosphate hexose-1-phosphate transferases are unique to prokaryotes and are unrelated to PNPTs.

The WecA protein has not been purified to homogeneity. However, both biochemical information and genetic informa-
tion support the hypothesis that its role involves the transfer of GlcNAc-1-P from UDP-GlcNAc to Und-P to form an Und-P-P-GlcNAc intermediate (37, 46). WecA can also transfer N-acetylgalactosamine-1-phosphate, as it is also essential for the synthesis of O antigens containing N-acetylgalactosamine (55). Furthermore, this enzyme has specificity for Und-P and cannot function with the eukaryotic lipid carrier dolichol phosphate (46). A predicted topological model for WecA suggests that there are 11 transmembrane segments, five cytosolic loops, and five periplasmic loops (5). We have previously identified highly conserved aspartic acids in two predicted cytosolic loops of WecA: Asp90 and Asp91 in cytosolic loop 2 and Asp156 and Asp159 in cytosolic loop 3 (4). Replacement of these residues with other amino acids affected the function of WecA, as demonstrated by the lack of in vitro enzymatic activity or reduced in vitro enzymatic activity of the mutated proteins, which could not mediate O-antigen LPS production in vivo (4). Aspartic acids have nucleophilic side chains, which may be involved in binding divalent metal ions (most commonly Mn$^{2+}$ or Mg$^{2+}$) or in catalysis (23, 47, 59), as shown previously for other glycosyl and prenyl transferases (10, 33, 34, 50). We hypothesized that Asp90, Asp91, Asp156, and Asp159 are important for the catalytic activity of WecA (4). In this study, we characterized in more detail the functional roles of the conserved aspartic acids of WecA. Together, the in vivo activities of parental WecA and mutated WecA (having conservative amino acid replacements) and the steady-state kinetic parameters of these proteins demonstrated that Asp90/Asp91 and Asp156/Asp159 define two distinct functional regions in the protein. A refined topological map of WecA, based on the substituted cysteine accessibility method (8), conclusively demonstrated that Asp90/Asp91 and Asp156/Asp159, as well as a critical histidine in the predicted cytosolic loop 5, His278 (3), are exposed to the cytosolic side of the plasma membrane. We also found that the C terminus of WecA is exposed to the cytosol and provide evidence suggesting that WecA localizes in discrete regions of the bacterial membrane.

**MATERIALS AND METHODS**

**Strains and growth conditions.** The properties of the *E. coli* strains used in this study are described in Table 1. Strain MV501, a *wecA*:Tn10 mutant (1), was used to assess protein expression of all constructs and for in vivo complementation studies. *E. coli* DH5a was used for plasmid maintenance and recovery after mutagenesis. Bacteria were cultured at 37°C in Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/ml), tetracycline (20 μg/ml), and 2% (wt/vol) arabinose, when appropriate. Transformation was performed by either the calcium chloride method or electroporation, as described elsewhere (12, 15). All biochemical reagents were purchased from Sigma (St. Louis, MO), unless indicated otherwise. Restriction endonucleases, T4 DNA ligase, and associated buffers were purchased from Roche Molecular Biochemicals (Illdoral, Quebec, Canada).

**PCR and cloning strategies.** PCRs were carried out with a PTC-200 Peltier thermal cycler (MJ Research Inc, Watertown, MA). The plasmid containing the *wecA*FLAG-ligated gene was constructed by amplification of a 1.2-kb fragment using pAA26 as the template (5) and the sense and antisense primers 5'-GCC ACACTATGAAATTACGTACATTGAGT-3' (Ndel site underlined) and 5'-GGTCCGCGCTTTGTGCTCTTGAGTCCTCC-3' (SacI site underlined), respectively. The amplicon was digested with Ndel and SacI and ligated to Ndel-SacI ends of pBAD-His (Table 1). This plasmid is a modified pAA26 expressing WecAFLAG tagged with His$^6$(WecAFLAG-ligated), also digested with NdeI and SacI, resulting in replacement of the FLAG epitope by the gfp mutant 3 gene from pFV25 (51) with primers having PstI ends. This amplicon was ligated into pAA26 encoding *wecA*FLAG (5), also digested with PstI, resulting in replacement of the DNA sequence encoding the FLAG epitope by the gfp mutant 3 gene. All constructs were verified by DNA sequencing, which was carried out at the DNA Sequencing Facility, Robarts Research Institute (London, Ontario, Canada).

**Membrane preparation.** Bacterial cultures were grown overnight in 5 ml of LB medium, diluted to obtain an initial optical density at 600 nm (OD$_{600}$) of 0.02, and incubated at 37°C for 2 h until the OD$_{600}$ was between 0.8 and 1.0. Cells were then harvested by centrifugation at 10,000 $g$ for 15 min at 4°C, and the clear supernatant was used.

**LPS analysis.** LPS was extracted as described previously (32). Briefly, cells from overnight plate cultures were suspended in a lysis buffer containing proteinase K, which was followed by hot phenol extraction and subsequent extraction of the aqueous phase with ether. LPS was resolved by electrophoresis in 14% polyacrylamide gels using a Tricine-sodium dodecyl sulfate (SDS) system and stained with ampicillin resistance.  

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<th>Strain or plasmid</th>
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$^a$ Tc', tetracycline resistance; *Amp*, ampicillin resistance.

TABLE 1. Characteristics of the bacterial strains and plasmids used in this study
was visualized by silver staining (30). The concentration of LPS was determined by the keto-deoxyoctulosonic (KDO) assay (40).

**Transferase assay and kinetic analysis.** The reaction mixture used for the standard in vitro transferase assay (modified from the mixture used by McGrath and Osborn [35]) contained 40 μg total protein membrane fraction (providing the enzyme and the endogenous Und-P acceptor), 0.2 mM MnCl₂, 1 mM MgCl₂, and 131.4 pmol radiolabeled UDP-N-acetyl[14C]glucosamine (225 mCi/mmol) in 250 μl of buffer (5 mM Tris acetate [pH 8.5]). After incubation at 37°C for 15 min, the lipid-associated material was extracted twice with 150 μl of 1-butanol. The combined 1-butanol extracts were washed once with 300 μl of distilled water. The radioactive count for a 150-μl aliquot of the 1-butanol fraction was determined with a Beckman liquid scintillation counter (Beckman Coulter Canada Inc., Mississauga, Ontario, Canada). Radioactive counts were normalized for the background value. To determine the divalent metal ion requirements, incorporation of UDP-GlcNAc into the lipid fraction was established using various concentrations of Mg²⁺ and Mn²⁺ (0 to 3 mM) with a fixed amount of UDP-GlcNAc (131.4 pmol). The kinetic parameters for UDP-GlcNAc were determined with excess Mg²⁺ or Mn²⁺ and amounts of UDP-GlcNAc ranging from 8.76 to 175.2 pmol. Data were analyzed by nonlinear regression using GraphPad Prism v.4 and the Michaelis-Menten equation. One unit of enzyme activity was defined as 10⁻⁶ pmol GlcNAc incorporated into the lipid fraction per min per mg of total protein. In some experiments, product conversion was examined by thin-layer chromatography (TLC). To do this, 200 μl of the 1-butanol fraction was dried in a microcentrifuge tube and resuspended in 20 μl chloroform-methanol (2:1), and 4-μl portions were spotted on a Whatman silica gel (PE SIL G) plate, which was developed in a solvent containing di-isobutylketone, acetic acid, and water (80:50:10), as described previously (19). After drying and overnight exposure of the plate to a PhosphorImager screen, product formation was detected and quantified with a PhosphorImager (Storm 840; Amersham Biosciences) equipped with Image-Quant software. Purified Und-P and Und-P; and amounts of UDP-GlcNAc were obtained from the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland.

**Preparation of cells and vesicles for labeling with sulphydryl-reactive reagents.** LB medium (500 ml) supplemented with 100 μg/ml ampicillin and 20/μl tetracycline was inoculated with an overnight culture of strain MV501 cells containing the appropriate plasmids. The cells were grown and protein expression was induced with arabinose as described above. At the end of the induction period the culture was split into two 250-ml aliquots for orientation-specific labeling. Bacteria were harvested by centrifugation at 3,300 × g for 15 min, washed twice with 0.1 M sodium phosphate buffer (pH 7.5), and resuspended in 25 ml of buffer. One aliquot was pretreated with 0.5 mM [2-(trimethylammonium)ethyl] methanesulfonate sulfate (MSTET) (Toronto Research Chemicals Inc., Toronto, Ontario, Canada) for 10 min at room temperature, with occasional shaking. The reaction was terminated by addition of 500 μl of 2% (vol/vol) 2-mercaptoethanol in 0.1 M sodium phosphate buffer. Treated cultures were then washed twice with 0.2 ml phosphate buffer and lyzed with 0.2 ml buffer at 15,000 r/min using a French press. The lysates were centrifuged at 39,000 × g for 15 min, and each supernatant was pelleted by centrifugation at 280,000 × g for 30 min. The pellet, containing total membranes, was resuspended in 1 ml of 0.1 M sodium phosphate buffer, and 50 μl was solubilized with 0.5% Triton X-100 in 0.5 M sodium phosphate buffer with 8 M urea (pH 7.8) for 2 h.

**Purification of His-tagged protein.** Solubilized samples were centrifuged at 39,000 × g for 15 min to remove insoluble material. One hundred microliters of covalent-bound chelating Sepharose Fast Flow resin (GE Healthcare), equilibrated with wash buffer (0.1 M sodium phosphate, 300 mM NaCl, 100 mM imidazole, 8 M urea; pH 7.8) was mixed with the supernatant in a microcentrifuge tube. After 1 h of gentle mixing at room temperature, the resin was centrifuged at 1,000 × g for 1 min, the supernatant was aspirated, and the protein-loaded column was washed three times with 1 ml of wash buffer. WecA was eluted by incubating the resin with 100 μl of elution buffer (0.1 M sodium phosphate, 300 mM NaCl, 100 mM imidazole, 8 M urea, 0.5% Triton X-100; pH 7.8) for 15 min.

**Gelectrophoresis and analysis.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE), protein transfer to nitrocellulose membranes, and immunoblotting with the FLAG M2 monoclonal antibody were performed as described previously (4), except that the reacting bands were detected by fluoroscence with an Odyssey infrared imaging system (Li-cor Biosciences, Lincoln, NE) using Alexa Fluor 680 goat anti-mouse secondary antibody (Rockland Immunochemicals, Gilbertsville, PA). Various amounts (0.8 to 3.2 ng) of C-terminally FLAG-tagged bacterial alkaline phosphatase (BAP-FLAG) were loaded on the same gels as standards for quantitative immunoblotting (41). The pixel density of the gel bands was analyzed with ImageJ software (W. S. Rasband, U.S. National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/). Biotinylated proteins were detected by incubation with horseradish peroxidase-linked streptavidin and chemiluminescence using the BM chemiluminescence blotting substrate (Roche Diagnostics), as recommended by the manufacturer.

**Microscopy.** An overnight culture of E. coli DH5α cells containing pLDT29, with previously expressed WecA, was used to control the expression of pBAD promoter (Table 1), diluted in LB medium to obtain an OD₆₀₀ of 0.1, and protein expression was induced with arabinose as described above. After 3 h of induction, the culture was placed on ice for 1 to 2 h to facilitate GFP folding. Similar experiments were performed without arabinose in the growth medium. Bacteria were visualized with no fixation using an Axioscope 2 (Carl Zeiss) microscope with an ×100/1.3 numerical aperture Plan-Neofluor objective and a 50-W mercury arc lamp with a GFP band pass emission filter set (Chroma Technology) with excitation at 470 ± 20 nm and emission at 525 ± 25 nm. Images were digitally processed using the Northern Eclipse imaging analysis software (version 6.0; Empix Imaging, Mississauga, Ontario, Canada).

**RESULTS**

**Functional characterization of WecAFLAG-5-His.** Plasmid pKV1, encoding WecAFLAG-5-His, was constructed to provide WecA with the FLAG epitope tag for immunodetection and a C-terminal five-His sequence for purification by Ni²⁺ affinity chromatography. Plasmid pKV1 was transformed into E. coli MV501 (wecA::Tn10), and membranes isolated from the transformant were analyzed by SDS-PAGE and Western blotting. A 38-kDa polypeptide band (Fig. 1, lane 1), absent in membranes from the MV501(pBAD-His) control (Fig. 1, lane 2), was found in membranes from MV501(pKV1). The observed molecular mass for WecAFLAG-5-His was lower than the predicted mass deduced from its amino acid sequence (44.8 kDa). The anomalous migration of WecA in SDS-PAGE was reported previously (3–5) and was probably due to the hydrophilicity and high pI of this protein (Theoretical pI, 10.01) (20). The bands at higher molecular masses in the blot were likely due to WecAFLAG-5-His oligomers that resulted from the mild denaturation conditions (incubation at 45°C for 30 min in loading sample buffer containing 50 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, and 0.1% bromophenol blue) used to visualize WecA since, as we previously reported (5), incubation at
higher temperatures resulted in a failure to detect WecA. Plasmid pKV1 was sufficient to complement O7 LPS production in MV501 (Fig. 2, lane 2), demonstrating that WecAFLAG-5 His was functional in vivo. Therefore, we concluded that the incorporation of two epitope tags in tandem into the C terminus of WecA resulted in a protein that not only can be targeted to the plasma membrane but also retains functionality compared with untagged WecA.

We next investigated the enzymatic activity of WecAFLAG-5 His. The product of the reaction, Und-P-P-GlcNac, was directly identified using TLC plates. For this experiment, membranes were prepared from E. coli K-12 strain CLM37 (∆wecA) carrying pKV1, and the lipid fraction containing the Und-P-P-GlcNac product was extracted with 1-butanol. The radiolabeled control, UDP-GlcNac, did not migrate beyond the loading point ($R_f$ of 0.167) (Fig. 3, lane 1), but a spot with an $R_f$ of 0.167 was detected in the extract from membranes containing WecAFLAG-5His (Fig. 3, lane 6). In the same buffer system, authentic Und-P and Und-P-P had $R_f$ values of 0.64 and 0.38, respectively (data not shown). Based on these results, we deduced that the spot with an $R_f$ of 0.167 corresponded to Und-P-P-GlcNac. The kinetic parameters of the transfer of radiolabeled GlcNac-1-P to Und-P were also determined. Membranes from MV501(pKV1), providing both the enzyme and the endogenous Und-P acceptor, were incubated with radioactive UDP-GlcNac. The lipid fraction containing the Und-P-P-GlcNac product was extracted with 1-butanol, and the radioactivity in the 1-butanol extract reflected the amount of radiolabeled GlcNac-1-P incorporated into Und-P. Under the standard conditions for this assay (1 mM Mg$^{2+}$ and 40 μg of membrane proteins), we detected a level of product conversion of 120 nmol of GlcNac incorporated into the lipid phase per mg of total membrane protein, corresponding to 8.8 U of enzyme activity. In addition, we estimated the amount of WecA in the membrane fractions by quantitative immunoblotting, using a similar strategy described previously (41). The total amount of WecA in the samples was determined by using C-terminal BAP-FLAG as a standard. An example of this analysis is shown in Fig. 4. The relative intensities of the bands were determined with the program ImageJ, versus the amounts of purified protein loaded on the same gel. The graph is a plot of the relative intensity of the bands in the lanes. The inset shows the blot with the different amounts of BAP-FLAG (left four lanes) and WecAFLAG-5His (right four lanes). The amount of WecAFLAG-5His was deduced from the standard curve.

The transferase assay allowed us to determine the steady-state kinetics of enzyme activity in membranes containing WecAFLAG-5His using various UDP-GlcNac concentrations in the presence of a 10-fold excess of Mg$^{2+}$ (17 mM) or Mn$^{2+}$ (3 mM). These metal ion concentrations represented 10-fold excesses relative to the $K_m$ values calculated with Mg$^{2+}$ and Mn$^{2+}$ (see below). Similar curves were observed for the two metal ions (Fig. 5A). The apparent $K_m$ values for UDP-GlcNac were 0.12 ± 0.06 and 0.19 ± 0.05 μM in the presence...
of Mg\(^{2+}\) and Mn\(^{2+}\), respectively, while the \(V_{\text{max}}\) values were 57 ± 4 and 56.4 ± 3.5 pmol min\(^{-1}\), respectively (Table 2 and data not shown). We investigated in more detail the metal ion requirements for WecA\(_{\text{FLAG-5\timesHis}}\) enzyme activity. No transfer activity was detected in membranes incubated with Ca\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\), Zn\(^{2+}\), or 1 mM EDTA (data not shown), while either Mn\(^{2+}\) or Mg\(^{2+}\) activated the enzyme (Fig. 5B). In the presence of excess UDP-GlcNAc, the apparent \(K_m\) and \(V_{\text{max}}\) values with Mg\(^{2+}\) were 1.7 ± 0.2 mM and 23 ± 1.3 pmol min\(^{-1}\), respectively, whereas the apparent \(K_m\) and \(V_{\text{max}}\) values with Mn\(^{2+}\) were 0.3 ± 0.04 mM and 71.7 ± 2.6 pmol min\(^{-1}\), respectively (Table 3). These results indicate that in vitro and in the presence of excess UDP-GlcNAc, the enzyme is nearly six times more effective with Mn\(^{2+}\) than with Mg\(^{2+}\).

Functional characterization of aspartic acids in predicted cytosolic loops 2 and 3 of WecA\(_{\text{FLAG-5\timesHis}}\). To investigate the function of the highly conserved aspartic acids in predicted cytosolic loops 2 (Asp90 and Asp91) and 3 (Asp156 and Asp159), we constructed derivatives of WecA\(_{\text{FLAG-5\timesHis}}\) with conservative amino acid replacements. In all cases, we generated two derivatives for each position by replacing the aspartic acid with either asparagine or glutamic acid. To rule out the possibility that the amino acid replacements compromised either protein stability or targeting to the plasma membrane, the expression of the WecA\(_{\text{FLAG-5\timesHis}}\) mutant forms was investigated by Western blot analysis using MV501 membranes transformed with plasmids containing the mutated wecA\(_{\text{FLAG-5\timesHis}}\) genes. All mutants with mutations in predicted cytosolic loops 2 and 3 had the same levels of expression as the parental WecA\(_{\text{FLAG-5\timesHis}}\) strain (Fig. 1, lanes 3 to 8, and data not shown), and these results were also consistent with the similar amounts of WecA\(_{\text{FLAG-5\timesHis}}\) and mutant forms in the membrane fractions, as estimated by quantitative immunoblotting.

Kinetic analyses, as described above for the parental WecA\(_{\text{FLAG-5\timesHis}}\) protein, were performed to characterize in more detail the functional defects of each mutant protein. Membranes containing WecA-D156E\(_{\text{FLAG-5\timesHis}}\) and WecA-D156N\(_{\text{FLAG-5\timesHis}}\) did not exhibit any detectable transfer activity (Table 2) and, as expected, did not restore production of O7 antigen in strain MV501 (Fig. 2, lanes 7 and 8). Also, product conversion was not detected by TLC (Fig. 3, lane 3, and data not shown). These results suggest that Asp156 could be required for catalysis, by acting as a nucleophile for cleavage of the pyrophosphate bond of the biphosphate nucleotide sugar substrate. The GlcNAc-1-P transfer activity of membranes containing the D159E and D159N proteins was detectable but drastically reduced. In both cases, the enzyme exhibited a 10-fold increase in the apparent \(K_m\) for UDP-GlcNAc compared to the wild type and 1% relative efficiency (Table 2). These findings are consistent with the TLC results showing that product conversion in reactions with membranes containing WecA-D159N\(_{\text{FLAG-5\timesHis}}\) was barely detectable, with less than 20% of the amount of Und-P-GlcNAc detected for parental WecA\(_{\text{FLAG-5\timesHis}}\) (Fig. 3, lane 5). WecA-D159E\(_{\text{FLAG-5\timesHis}}\) and WecA-D159N\(_{\text{FLAG-5\timesHis}}\) mediated the production of small amounts of one O7 unit attached to the lipid A core (Fig. 2, lanes 9 and 10), confirming that the enzymes were poorly functional in vivo. The kinetic parameters for the Mg\(^{2+}\) and Mn\(^{2+}\) cofactors showed that there were small increases (±2-fold compared to the values for the parental protein) in the apparent \(K_m\) values with both metals for WecA-D159E\(_{\text{FLAG-5\timesHis}}\) and virtually no change for WecA-D159N\(_{\text{FLAG-5\timesHis}}\). However, the small variations in \(K_m\) were associated with drastic reductions (range, 7- to 35-fold) in the \(V_{\text{max}}\) values (Table 3). The ratios of the \(K_m\) values for the proteins with Asp-to-Asn replacements to the

![Figure 5](http://jb.asm.org/)

**FIG. 5.** Transfer of GlcNAc-1-P to Und-P. All assays were carried out at 37°C for 15 min in triplicate. One unit of enzyme activity was defined as 10\(^{-3}\) pmol of GlcNAc incorporated/min/mg protein. (A) UDP-GlcNAc-dependent activity of WecA\(_{\text{FLAG-5\timesHis}}\) in the presence of excess Mg\(^{2+}\) (16.7 mM) and Mn\(^{2+}\) (3.1 mM). (B) Mg\(^{2+}\) and Mn\(^{2+}\)-dependent enzyme activity of WecA\(_{\text{FLAG-5\timesHis}}\). Assays were carried out at 37°C for 15 min with a fixed concentration of UDP-GlcNAc (131.4 pmol), 40 μg of total membranes from an MV501(pKV1) preparation, and different concentrations (0 to 3 mM) of Mg\(^{2+}\) and Mn\(^{2+}\).
Similar variations were observed with Mn\(^{2+}\) for the D90N and D90E forms were 1.2 and 0.6 with Mg\(^{2+}\) and WecA-D91N FLAG-5 actions with excess metal ion cofactors in membranes containing the parental enzyme (Table 3). Lane 1, vector control, pBAD-His; lane 2, WecA FLAG-5 His-Cys, cysteineless version of WecA encoded by pKV1; lane 3, WecAFLAG-His, pKV1; lane 4, vector control, pBAD-His. Each lane contained 4 \(\mu\)g of protein.

The kinetic parameters for the GlcNAc-1-P transfer reactions with excess metal ion cofactors in membranes containing WecA-D90EFLAG-5xHis, WecA-D90NFLAG-5xHis, and WecA-D91NFLAG-5xHis revealed slightly reduced velocities and small increases in the apparent \(K_m\) for UTP-GlcNAc (Table 2). The data suggest that Asp90 and Asp91 do not directly interact with UTP-GlcNAc. Membranes containing WecA-D91EFLAG-5xHis exhibited a sixfold decrease in the apparent \(K_m\) for UTP-GlcNAc compared to the apparent \(K_m\) of the parental enzyme. However, despite the apparently higher enzymatic efficiency of WecA-D91EFLAG-5xHis, none of the replacements at the Asp90 and Asp91 positions resulted in functional proteins, as determined by complementation of the WecA function in strain MV501 (Fig. 2, lanes 3 to 6). Also, the TLC experiment did not detect product conversion with membranes containing the D90N replacement protein (Fig. 3, lane 4). The kinetic parameters for the transfer reaction with Mg\(^{2+}\) and Mn\(^{2+}\) showed that replacement of Asp90 and Asp91 had different effects on the enzyme function. In membranes containing the D90E and D90N forms of WecA, the apparent \(K_m\) values for Mg\(^{2+}\) and Mn\(^{2+}\) increased three- to fivefold compared to the apparent \(K_m\) of the parental enzyme (Table 3). Also, the ratios of the \(K_m\) values for the D90N and D90E forms were 1.2 and 0.6 with Mg\(^{2+}\) and Mn\(^{2+}\), respectively (Table 3), indicating that the enzyme affinity for the metal ion did not vary dramatically regardless of the residue charge, which suggests that Asp90 does not interact with either Mg\(^{2+}\) or Mn\(^{2+}\). In contrast, in membranes containing the D91N and D91E forms of WecA, the apparent \(K_m\) values with Mg\(^{2+}\) increased threefold and decreased threefold, respectively, compared to the apparent \(K_m\) of the parental enzyme (Table 3). Similar variations were observed with Mn\(^{2+}\). The ratios of the \(K_m\) values for the D91N and D91E forms with Mg\(^{2+}\) and Mn\(^{2+}\) were 11.4 and 6.2, respectively (Table 3), indicating that the D91N substitution resulted in reduced affinity for the metal ions and suggesting that Asp91 interacts with both Mg\(^{2+}\) and Mn\(^{2+}\).

The \(V_{max}\) values with both metals were more affected for the D90E and D91E forms than for the forms with Asn substitutions, except for a higher-than-wild-type value for the D90N form (Table 3). This suggests that the larger aliphatic chain of glutamic acid at both positions may interfere with enzymatic activity, possibly by altering the geometry of a putative catalytic site.

**Topological analysis of WecA demonstrates that Asp90/91 and Asp156 are exposed to the cytosol.** The topology of WecA has been predicted only from computer algorithms and was not experimentally assessed. We determined the transmembrane topology of WecA by the substituted cysteine accessibility method. Standard WecAFLAG-His assays were performed with total membrane extracts prepared from E. coli MV501 cells carrying plasmids containing parental or mutant wecA genes. The assays were performed as described in Materials and Methods. \(K_m\) for the protein with an asparagine substitution; \(K_m\) (E), \(K_m\) for the protein with a glutamic acid substitution; NA, not applicable.

<table>
<thead>
<tr>
<th>Parental or mutant WecAFLAG-His</th>
<th>(V_{max}) (pmol/min/mg)</th>
<th>(K_m) (mM)</th>
<th>(K_m)(N)/(K_m)(E) ratio</th>
<th>(V_{max}) (pmol/min/mg)</th>
<th>(K_m) (mM)</th>
<th>(K_m)(N)/(K_m)(E) ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>WecAFLAG-His</td>
<td>22.0 ± 1.3</td>
<td>1.7 ± 0.2</td>
<td>NA</td>
<td>71.7 ± 2.6</td>
<td>0.3 ± 0.04</td>
<td>NA</td>
</tr>
<tr>
<td>D90E</td>
<td>21.3 ± 7</td>
<td>9.3 ± 3.8</td>
<td>1.2</td>
<td>22.3 ± 7.8</td>
<td>1.6 ± 0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>D90N</td>
<td>169.7 ± 43</td>
<td>11.0 ± 3.4</td>
<td>0.94 ± 0.3</td>
<td>77.8 ± 14.3</td>
<td>1.08 ± 0.1</td>
<td>0.6</td>
</tr>
<tr>
<td>D91E</td>
<td>0.6 ± 0.2</td>
<td>0.5 ± 0.3</td>
<td>1.4</td>
<td>0.7 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.6</td>
</tr>
<tr>
<td>D91N</td>
<td>39.4 ± 7</td>
<td>5.7 ± 1.4</td>
<td>0.5</td>
<td>44.3 ± 7</td>
<td>0.5 ± 0.1</td>
<td>0.6</td>
</tr>
<tr>
<td>D159E</td>
<td>3.1 ± 0.4</td>
<td>2.7 ± 0.6</td>
<td>1.4</td>
<td>2.3 ± 0.3</td>
<td>0.6 ± 0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>D159N</td>
<td>0.9 ± 0.4</td>
<td>1.4 ± 1.5</td>
<td>0.5</td>
<td>2.0 ± 0.4</td>
<td>0.3 ± 0.2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* TABLE 3. Comparison of kinetic parameters of Mn\(^{2+}\) and Mg\(^{2+}\) for parental and mutant WecAFLAG-5xHis. The assays were performed as described in Materials and Methods. \(K_m\) values for the parental enzyme (Table 3). Also, the ratios of the \(K_m\) values for parental and mutant WecAFLAG-5xHis revealed slightly reduced velocities and small increases in the apparent \(K_m\) for UTP-GlcNAc (Table 2). The data suggest that Asp90 and Asp91 do not directly interact with UTP-GlcNAc. Membranes containing WecA-D91EFLAG-5xHis exhibited a sixfold decrease in the apparent \(K_m\) for UTP-GlcNAc compared to the apparent \(K_m\) of the parental enzyme. However, despite the apparently higher enzymatic efficiency of WecA-D91EFLAG-5xHis, none of the replacements at the Asp90 and Asp91 positions resulted in functional proteins, as determined by complementation of the WecA function in strain MV501 (Fig. 2, lanes 3 to 6). Also, the TLC experiment did not detect product conversion with membranes containing the D90N replacement protein (Fig. 3, lane 4). The kinetic parameters for the transfer reaction with Mg\(^{2+}\) and Mn\(^{2+}\) showed that replacement of Asp90 and Asp91 had different effects on the enzyme function. In membranes containing the D90E and D90N forms of WecA, the apparent \(K_m\) values for Mg\(^{2+}\) and Mn\(^{2+}\) increased three- to fivefold compared to the apparent \(K_m\) of the parental enzyme (Table 3). Also, the ratios of the \(K_m\) values for the D90N and D90E forms were 1.2 and 0.6 with Mg\(^{2+}\) and Mn\(^{2+}\), respectively (Table 3), indicating that the enzyme affinity for the metal ion did not vary dramatically regardless of the residue charge, which suggests that Asp90 does not interact with either Mg\(^{2+}\) or Mn\(^{2+}\). In contrast, in membranes containing the D91N and D91E forms of WecA, the apparent \(K_m\) values with Mg\(^{2+}\) increased threefold and decreased threefold, respectively, compared to the apparent \(K_m\) of the parental enzyme (Table 3). Similar variations were observed with Mn\(^{2+}\). The ratios of the \(K_m\) values for the D91N and D91E forms with Mg\(^{2+}\) and Mn\(^{2+}\) were 11.4 and 6.2, respectively (Table 3), indicating that the D91N substitution resulted in reduced affinity for the metal ions and suggesting that Asp91 interacts with both Mg\(^{2+}\) and Mn\(^{2+}\). The \(V_{max}\) values with both metals were more affected for the D90E and D91E forms than for the forms with Asn substitutions, except for a higher-than-wild-type value for the D90N form (Table 3). This suggests that the larger aliphatic chain of glutamic acid at both positions may interfere with enzymatic activity, possibly by altering the geometry of a putative catalytic site.

**Topological analysis of WecA demonstrates that Asp90/91 and Asp156 are exposed to the cytosol.** The topology of WecA has been predicted only from computer algorithms and was not experimentally assessed. We determined the transmembrane topology of WecA by the substituted cysteine accessibility method.
method (8). In this method substituted cysteine mutants and a combination of membrane-permeable and membrane-impermeable sulfhydryl-directed chemical labeling reagents are used. WecA contains five cysteine residues located at positions 62, 79, 167, 189, and 337, all of which were replaced by alanine, resulting in cysteineless WecAFLAG-5饲His (encoded by pJL5 [Table 1]), which was further modified by incorporating two additional histidine residues, resulting in WecAFLAG-7饲His (encoded by pJL7). The additional histidine residues facilitated WecA binding in Ni2饲affinity chromatography (data not shown), a critical step for detection of specific sulfhydryl labeling of this protein. The Cys-to-Ala substitutions and the incorporation of the seven-His tag did not interfere with protein stability or targeting to the plasma membrane (Fig. 6A). Also, the cysteineless WecA forms encoded by pJL5 and pJL7 did not differ in the ability to complement O-antigen LPS production in E. coli MV501 compared to the ability of the parental protein encoded by pKV1 (Fig. 6B).

We used pJL7 as a template to construct a library of Cys replacement mutants with mutations at various positions in the WecA protein (Fig. 7). Recombinant plasmids expressing these proteins were transformed into MV501, and each mutant protein was examined by Western blotting to confirm that the Cys replacement did not affect protein stability or targeting to the plasma membrane (Fig. 8). The accessibility of the cysteine residues to the sulfhydryl-reactive reagent biotin maleimide was determined by incubating MV501 with the label, followed by treatment with excess β-mercaptoethanol before lysis. This treatment prevented labeling of any cysteine that became exposed to biotin maleimide during cell fractionation. Biotin maleimide is membrane permeable and can react with thiol groups on either side of the membrane that are next to water molecules since the reaction with an ionized thiol group requires a water molecule as a proton acceptor (8). Therefore, cysteine residues buried in the core of the hydrophobic transmembrane segments are usually not labeled (8). Cys-substituted Asp35, Gly87, Leu89, Asp90, Ser96, Ser128, Met154, Asp156, Gly181, Arg209, Thr239, His278, His316, and Ser362 were accessible to biotin maleimide (Fig. 7 and 8), suggesting they were not located in transmembrane regions (Fig. 7). In contrast, proteins with substitutions in Asp67, Phe143, Ala 106, Ala144, Leu161, His281, Ala287, Tyr314, Glu320, and Tyr321 did not react with the label (Fig. 7 and 8 and data not shown), suggesting that these residues are in close proximity to the inner membrane or are embedded in the membrane bilayer. Cys-substituted Gly87 and Leu89, which were predicted to be in the inner membrane by the computer model (4, 5), were detectable with
Biotin maleimide. Therefore, we extended the boundaries of cytosolic loop 2 to include these residues (Fig. 7).

To determine the sidedness of the mutated residues in WecA, we used MTSET, a charged thiol-specific probe that reacts with sulfhydryl groups under conditions similar to those used for biotin maleimide but cannot penetrate the cytoplasmic membrane due to its positive charge (8). Incubation of bacterial cells expressing the cysteine-substituted WecA derivatives with MTSET prior to treatment with biotin maleimide prevented labeling of periplasmic cysteine residues. The results of these experiments demonstrated that Cys-substituted Gly87, Leu89, Asp90, Ser128, Gly181, Thr239, Tyr314, and His316 did not occur after MTSET pretreatment, suggesting they were exposed to the periplasmic face of the inner membrane. These experiments demonstrated unequivocally that Asp90, Asp91, and Asp156 are exposed to the cytosolic side of the plasma membrane.

The results of cysteine accessibility experiments with the S362C replacement protein suggested that the C-terminal segment of WecA resides in the cytosol. To independently confirm this suggestion, we constructed a C-terminal fusion with GFP, which can fluoresce only if it is present in the cytosol (16). Cells expressing the WecA<sub>GFP</sub> hybrid displayed fluorescence around the cell perimeter with a punctate pattern (Fig. 9), suggesting that WecA was in the membrane with the C terminus facing the cytosol. The punctate pattern was observed only with bacteria cultured with 0.002% (wt/vol) arabinose in the growth medium. In contrast, when a higher concentration of arabinose (0.2%) was added to the medium, the periphery of the bacterial cells was uniformly fluorescently labeled (data not shown), suggesting that the punctate localization of WecA<sub>GFP</sub> was not an artifact resulting from protein overexpression. Therefore, we concluded that WecA localized to discrete regions within the membrane.

We also investigated the effects of the Cys-substituted WecA<sub>FLAG-7</sub>/H<sub>11003</sub> His mutants on O7 antigen expression in vivo using MV501. Replacement of Asp35, Gly87, Asp90, Phe143, Met154, Asp156, and His278 with cysteine precluded complementation of O7 antigen production in MV501. Three of these residues, Asp90, Asp156, and His278, were previously determined to compromise enzyme function (3, 4). Substitution of Asp67, Ala144, and Ala287 partially complemented O7 antigen production, as detected by production of a core band with a single sugar subunit. These results suggested that additional residues, previously not identified, are involved in the WecA function. All the remaining Cys replacements complemented the <i>wecA</i> mutation in MV501, as indicated by the formation of a complete O7 polysaccharide (Fig. 10).

**DISCUSSION**

The detailed mechanism of the transfer reaction of PNPT enzymes is unknown. A double-displacement mechanism has been proposed for MraY, one of the members of this family (18). Double displacement involves the formation of an acyl-phosphoenzyme intermediate due to nucleophilic attack by an active site residue on the sugar nucleotide alpha phosphate, followed by an attack of Und-P that results in...
replacement mutants is indicated by its short designation (see Fig. 7 for the location of each residue in the topological model of WecA).

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tation of O7 LPS production in MV501 cells expressing the member with MTSET. Also, the M154C replacement resulted to biotin maleimide labeling and not protected by pretreatment with biotin. These two residues are part of a conserved NXXNXDG IDGL motif in predicted cytosolic loop 3 of the bacterial members of the family (4, 42). We obtained experimental evidence that at least two residues of this motif, Met154 and Asp156, are located on the cytosolic side of the membrane, since when they are replaced by cysteine, they are accessible to biotin maleimide labeling and not protected by pretreatment with MTSET. Also, the M154C replacement resulted in loss of function, as detected by the lack of complementation of O7 LPS production in MV501 cells expressing the WecAFLAG-7×His, M154C protein, suggesting that this region is important for enzymatic activity.

In a previous study, Asp267 of the E. coli MraY protein was proposed to be the active site nucleophile responsible for cleavage of the pyrophosphate bond of the biphosphate nucleotide sugar substrate (28). Asp217 in E. coli WecA, located in transmembrane segment 8 (Fig. 7), was predicted to be comparable to Asp267 in MraY (28, 42), but it is not clear how a residue buried in the transmembrane region could access the soluble substrate in the cytosol. In contrast, Asp156 in WecA is clearly exposed to the cytosol, and the kinetic data from the analysis of conservative replacements with Asn and Gln demonstrated that there was a drastic reduction in the enzymatic activity. Moreover, Asp156Glu, Asp156Gln, and Asp156Cys replacements resulted in nonfunctional proteins that could not restore O7 antigen production in MV501. Therefore, our results suggest that the Asp156 residue in WecA may be comparable to the Asp267 residue in MraY, although additional experiments are necessary to demonstrate its involvement in attacking the pyrophosphate bond of UDP-GlcNAc.

The replacement of Asp159 with glutamic acid resulted in a reduced level of catalytic enzyme efficiency. Whereas some bacterial WecA homologues and the eukaryotic members of the family (all of which utilize UDP-GlcNAc as a substrate) have an asparagine in the same location as Asp159 (4), the enzymes with the Asp159Asn replacement also had reduced enzymatic activity compared to that of the parental WecA. Bacteria containing the mutant D159N protein produced dramatically reduced levels of O7 antigen. From these data we concluded that Asp159 may be less critical than Asp156 for the function of WecA. Our results also demonstrate that M154C is defective in O7 antigen synthesis. Therefore, the region in cytosolic loop 3 of WecA spanning at least Met154 through Asp159 plays an important role in enzyme function.

Divalent cations, especially Mn$^{2+}$ and Mg$^{2+}$, may be involved in the phosphoryl transfer reaction in different enzymes that catalyze the formation of phosphodiester bonds (27). We show here that WecA can function well with either Mg$^{2+}$ or Mn$^{2+}$, although the kinetic parameters indicated that the enzymatic reaction in vitro is more effective with Mn$^{2+}$. However, it is likely that either divalent cation may be used in vivo since the optimal concentrations of Mn$^{2+}$ and Mg$^{2+}$ for enzyme activity are below the average concentrations of these metals in bacterial cells, 0.8 mM for Mn$^{2+}$ (2) and 4 mM for Mg$^{2+}$ (29).

In previous studies, a DDXD motif that is located in predicted cytosolic loop 2 of bacterial PNPTs (this motif corresponds in WecA to D$_{\text{asp}}$DDXXD$_{\text{Asn}}$) and is also present in the eukaryotic members of the family was proposed to be potentially involved in binding of an Mg$^{2+}$ or Mn$^{2+}$ cofactor. This proposal was based on comparisons with prenyl transferases, especially polyprenyl pyrophosphate synthetases (7, 50). These enzymes contain two DDXD regions that have the carboxylate groups of the aspartic acids, which have been implicated in binding the metal ion, pointing to the active site cleft (50). However, the mechanism of polyprenyl pyrophosphate synthetases is different than the displacement mechanism proposed for PNPTs. Also, PNPTs have only one DDXD motif. Our data obtained in this study indicate that the D$_{\text{asp}}$DDXXD$_{\text{Asn}}$ motif in WecA is not analogous to the corresponding motifs in prenyl transferases. First, the kinetic parameters for Mg$^{2+}$ and Mn$^{2+}$ showed that replacement of Asp90 and replacement of Asp91 had different effects on WecA enzyme function. Our results suggest that Asp91, but not Asp90, interacts with either Mg$^{2+}$ or Mn$^{2+}$. Given that Asp91 is universally conserved in all PNPTs, this residue may be critical for the correct functioning of the enzyme due to its involvement with the metal ion cofactor. Second, we have previously shown that replacement of Asp94 has no effect on WecA function (4), and this residue is much less conserved in the members of the family. Third, we

FIG. 10. Complementation of O7 LPS expression in E. coli MV501 by plasmids encoding the cysteine-substituted derivatives of cysteineless WecAFLAG-7×His, as determined with silver-stained polyacrylamide gels. WecA-Cys is the cysteineless WecA protein encoded by pJL7. Each of the replacement mutants is indicated by its short designation (see Fig. 7 for the location of each residue in the topological model of WecA).
show here that replacement of Gly87 by cysteine (Fig. 6) destroys enzyme function. Since Gly87 is also highly conserved in PNPTs (4), we propose that the functional motif in these enzymes is \(\text{Gly}^{87}\text{XXDD}_{90}\), where Asp91 interacts with the metal ion.

Very little information concerning the topology of PNPTs is available. Using \(\beta\)-lactamase fusions, a topological map was constructed for MraY (9), but most of the topological information for other members of the family has been obtained from computer predictions of the transmembrane topology (3, 6, 28, 42). The substituted cysteine accessibility method employed in this study allowed more precise mapping of the boundaries of cytosolic loops 2 and 3 and unequivocal demonstration of the cytosolic location of Asp90, Asp91, and Asp 156. We also demonstrated that His278, a critical residue in cytosolic loop 5 involved in binding of UDP-GlcNAc (3), is indeed exposed to the cytosol. In contrast, other residues in cytosolic loop 5, such as His281 and Ala286, were not accessible to biotinylation when they were replaced by cysteine, suggesting they are in the membrane bilayer or they form part of a structure inaccessible to biotin maleimide. Moreover, when Asp35 and Arg209, located in cytosolic loops 1 and 4, respectively, were replaced by cysteine, the proteins were resistant to biotinylation in the presence of the membrane-impermeable blocking reagent MTSET. The results suggest that these residues are exposed to the periplasmic side of the membrane, which does not agree with the computer predictions. Also, replacement of Asp35 by Cys was associated with loss of complementation of O7 production in strain MV501. We observed in a previous study that replacement of Asp35 by glycine resulted in reduced WecA enzymatic activity and O-antigen production (4). Further analysis is required to define more precisely the boundaries of cytosolic loops 1, 4, and 5 and the function of Asp35. Also, the substituted cysteine accessibility method made it possible to identify additional residues required for WecA function, which were not previously recognized and which are currently being investigated in more detail in our laboratory.

Transferases for the initiation reaction of the O unit and enterobacterial common antigen are the only enzymes in these systems with multiple transmembrane domains, while the other enzymes involved in extension of the glycans are peripheral membrane proteins (43, 52). Fluorescence microscopy with WecA-DFF not only confirmed a membrane location but also revealed a punctate distribution rather than a homogenous distribution around the cell perimeter. This observation suggests that WecA may be located in discrete regions within the plasma membrane. Early work with Salmonella showed that new O-antigen LPS molecules appear on the cell surface at a limited number of sites (24, 39), and more recent work provided experimental evidence which supports the hypothesis that there are multitransmembrane complexes for assembly of capsular polysaccharides serving as a molecular “scaffold” across the periplasm (13, 36). Therefore, it is tempting to speculate that other proteins involved in O-antigen assembly, which are thought to interact with each other (31), are also localized in similar membrane domains. Experiments to address this hypothesis are under way in our laboratory.