Conserved amino acid residues found in a predicted cytosolic domain of the lipopolysaccharide biosynthetic protein WecA are implicated in the recognition of UDP-*N*-acetylglucosamine

Amal O. Amer¹ and Miguel A. Valvano^{1,2}

Author for correspondence: Miguel A. Valvano. Tel: +1 519 661 3996. Fax: +1 519 661 3499.
e-mail: mvalvano@uwo.ca

Departments of Microbiology and Immunology¹ and Medicine,² The University of Western Ontario, London, Ontario, Canada N6A 5C1

WecA, an integral membrane protein that belongs to a family of polyisoprenyl phosphate *N*-acetylhexosamine-1-phosphate transferases, is required for the biosynthesis of O-specific LPS and enterobacterial common antigen in *Escherichia coli* and other enteric bacteria. WecA functions as an UDP-*N*-acetylglucosamine (GlcNAc):undecaprenyl-phosphate GlcNAc-1-phosphate transferase. A conserved short sequence motif (His-Ile-His-His; HHHH) and a conserved arginine were identified in WecA at positions 279–282 and 265, respectively. This region is located within a predicted cytosolic segment common to all bacterial homologues of WecA. Both HHHH_{279–282} and the Arg₂₆₅ are reminiscent of the HIGH motif (His-Ile-Gly-His) and a nearby upstream lysine, which contribute to the three-dimensional architecture of the nucleotide-binding site among various enzymes displaying nucleotidyltransferase activity. Thus, it was hypothesized that these residues may play a role in the interaction of WecA with UDP-GlcNAc. Replacement of the entire HHHH motif by site-directed mutagenesis produced a protein that, when expressed in the *E. coli* *wecA* mutant MV501, did not complement the synthesis of O7 LPS. Membrane extracts containing the mutated protein failed to transfer UDP-GlcNAc into a lipid-rich fraction and to bind the UDP-GlcNAc analogue tunicamycin. Similar results were obtained by individually replacing the first histidine (H₂₇₉) of the HHHH motif as well as the Arg₂₆₅ residue. The functional importance of these residues is underscored by the high level of conservation of H₂₇₉ and Arg₂₆₅ among bacterial WecA homologues that utilize several different UDP-*N*-acetylhexosamine substrates.

Keywords: undecaprenol, O antigen biosynthesis, membrane protein, transferase, tunicamycin

INTRODUCTION

WecA is an UDP-*N*-acetylglucosamine (GlcNAc):undecaprenyl-phosphate (Und-P) GlcNAc-1-phosphate transferase that belongs to a large family of eukaryotic and prokaryotic polyisoprenyl-phosphate *N*-acetylhexosamine-1-phosphate transferases (Anderson *et al.*, 2000; Dal Nogare & Lehrman, 1988; Lehrman, 1994; Rick & Silver, 1996). The members of this family

catalyse the formation of a phosphodiester bond between a membrane-associated polyisoprenol phosphate molecule and *N*-acetylhexosamine 1-phosphate, which is usually donated by a soluble UDP-*N*-acetylhexosamine precursor. WecA participates in the biosynthesis of O antigen LPS in many enteric bacteria and it is also involved in the biosynthesis of enterobacterial common antigen (Rick & Silver, 1996). Although WecA has not been purified to homogeneity there is sufficient biochemical and genetic information to support its role in the transfer of GlcNAc 1-phosphate from UDP-GlcNAc onto Und-P to form Und-P-P-GlcNAc (Rick &

Abbreviations: GlcNAc, *N*-acetylglucosamine; Und-P, undecaprenyl phosphate.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
<i>E. coli</i> strains		
CLM20	<i>lacZ trpA(sbcB-rfb) upp rel rpsL recA Δ(wecAC)</i>	Feldman <i>et al.</i> (1999)
DH5α	F ⁻ <i>φ80lacZM15 endA recA hsdR(r_K⁻ m_K⁻) supE thi gyrA relA Δ(lacZYA-argF)U169</i>	Laboratory stock
JT4000	<i>Δlac rpsL relA⁺ araD139 flbB Δlon510</i>	S. Gottesman, NIH, Bethesda, USA
MV501	VW187; <i>wecA::Tn10</i> , Tet ^R	Alexander & Valvano (1994)
XL-1 Blue	F ⁻ <i>recA endA gyrA thi-1 hsdR supE relA lac[F' proAB lacI^qΔM15 Tn10(Tet^R)]</i>	Stratagene
Plasmids		
pAA9	1.2 kb fragment containing <i>wecA</i> _{E320} tagged with FLAG sequence	This study
pAA10	pAA12 containing <i>wecA</i> _{MalFTM} with the 54 bp fragment encoding <i>wecA</i> TMXI swapped with the 90 bp fragment encoding the last transmembrane domain of MalF	This study
pAA12	1.4 kb fragment containing tagged <i>wecA</i> cloned in pBluescript II KS+; <i>wecA</i> ⁺ , Amp ^R	Amer & Valvano (2000)
pAA26	1.2 kb <i>EcoRI</i> – <i>PvuII</i> fragment from pAA14 (Amer & Valvano, 2000) containing <i>wecA</i> _{FLAG} ligated into <i>EcoRI</i> and <i>SmaI</i> sites of pBAD24, Amp ^R	This study
pAA33	pAA26 containing <i>wecA</i> _{H11H/GGGG}	This study
pAA51	pAA26 containing <i>wecA</i> _{H279S}	This study
pAA52	pAA26 containing <i>wecA</i> _{R265K}	This study
pBAD24	Cloning vector, Amp ^R	Guzman <i>et al.</i> (1995)
pMF21	1.5 kb PCR amplicon containing <i>wzx</i> _{Eco7} cloned into pEXT21, Spc ^R	Feldman <i>et al.</i> (1999)

Silver, 1996). The product of this reaction serves as an acceptor for the addition of subsequent sugars to complete the biosynthesis of O antigen or enterobacterial common antigen repeating subunits. In the case of the O7 polysaccharide, a model system used in our laboratory, the repeating subunit consists of a backbone of GlcNAc, galactose, mannose and N-acetylvirosamine, and a side chain of rhamnose (L'Vov *et al.*, 1984; Marolda *et al.*, 1999). Once complete, the O7 subunit is translocated to the periplasmic surface of the inner membrane by a process that requires the Wzx protein (Feldman *et al.*, 1999). O7 subunits are polymerized and subsequently ligated to preformed lipid A core oligosaccharide to form the complete O7 LPS molecule (Feldman *et al.*, 1999; Marolda *et al.*, 1999).

Comparisons of amino acid sequences among the members of the polyisoprenyl-phosphate N-acetylhexosamine-1-phosphate transferase family reveal an alternate clustering of hydrophobic and hydrophilic domains, suggesting the presence of multiple transmembrane regions. The topology of two members of this family, the bacterial protein *MraY* that is involved in peptidoglycan synthesis (Bouhss *et al.*, 1999) and the eukaryotic GPT that is involved in protein glycosylation (Dan *et al.*, 1996), has been characterized experimentally. Bacterial and eukaryotic proteins share discrete regions of conserved amino acid sequence, especially those located in hydrophilic segments of the protein that are exposed to the cytosolic face of the plasma membrane or the membrane of the endoplasmic reticulum (Bouhss *et al.*, 1999; Dal Nogare & Lehrman, 1988).

Bacterial homologues also carry a large cytosolic loop that shares very little similarity with the eukaryotic members of the family (Bouhss *et al.*, 1999; Dal Nogare & Lehrman, 1988). It has recently been proposed that this large cytosolic region, ranging from approximately 32 to 43 aa in length, may be important for the recognition of the nucleotide-sugar substrates and may also determine the specificity of such an interaction (Anderson *et al.*, 2000). In a previous study, we have constructed a functional *WecA* derivative carrying a FLAG epitope tag, which was fused to the C-terminal end of the protein (Amer & Valvano, 2000). Using the *WecA*_{FLAG} construct, we have determined the role of aspartic acid residues located in two distinct regions within *WecA*, both of which are predicted to correspond to cytosolic-exposed segments of the protein (A. O. Amer & M. A. Valvano, unpublished). These residues may contribute to the formation of the phosphodiester bond and the interaction with divalent cations that is essential for catalytic activity (A. O. Amer & M. A. Valvano, unpublished). In this study, we have modelled the topology of *WecA* taking into account the available experimental data from the topological analysis of *MraY* (Bouhss *et al.*, 1999), and examined in detail the role of a conserved motif within the predicted large cytosolic loop.

METHODS

Strains and plasmids. The characteristics of the *Escherichia coli* strains and plasmids used in this study are described in Table 1. *E. coli* strains DH5α and XL-1 Blue were used for all

Table 2. Oligonucleotide primers used in this work

The nucleotide sequence encoding the last transmembrane domain in MalF is underlined. Mutagenic oligonucleotides are bold and underlined.

WecA mutant	Primer no.	Sequence (5'–3')
WecA ₃₂₀	62	TCGATGCAATGGAAT
	191	CACTCCGGGACAAAATG
WecA _{MalFTM}	185	GGCGTGCTGGCAGAAATTTCTCATTTTGTCCCGGAG <u>TTTGAAGCGCGCGGGGGTCAGGACTTCGGTCTGGC</u> <u>GGCAGCAATTGCC</u>
	186	GCGCTTAATAAAGCGAGCAACTTTCCAGGCACGCTT <u>CAGTTGACTATCGCCAGCGCACCCACCAGCAGGAA</u> <u>GATCAGCGT</u>
WecA _{HHH/GGGG}	165	CTCGAGTCACTTGTCGTC
	170	ATTACGAATTCGGTGAATTTACTGACAGTG
	237	<u>GGGGGATTGATCATGCGTGCCGGG</u>
	238	<u>ACCTCCCTGACGGTCAGGAGAGAA</u>
WecA _{H279S}	544	CCTGACCGTCAGTCTATTACCATTTGATCATGCGTGCC
	545	GGCACGCATGATCAAATGGTGAATAGACTGACGGTCAGG
WecA _{R265K}	546	GGATATGGTGGCGATTATGTACCGTAAACTGCGTAAAGGC ATGAGC
	547	GCTCATGCCTTTACGCAGTTTACGGTACATAATCGCCACCA TATCC

plasmid manipulations, and *E. coli* JT4000 was used for the expression of WecA_{MalFTM} and WecA_{E320}. Competent cells were prepared for transformation by either the calcium chloride method or electroporation as described elsewhere (Cohen *et al.*, 1972; Dower *et al.*, 1988). Bacteria were cultured in Luria–Bertani (LB) medium supplemented with 100 µg ampicillin ml⁻¹ or 20 µg tetracycline ml⁻¹ and 0.02 % (w/v) arabinose as appropriate. All reagents were from Sigma, unless otherwise indicated.

General methods for plasmid constructions. Mutagenic oligonucleotides are listed in Table 2. PCR reactions were carried out with a Hybaid Ommigene thermocycler (Interscience) and conducted with *Pwo*I DNA polymerase (Roche Diagnostics) unless otherwise indicated. DNA fragments were purified from 0.7 % agarose gels with the QIAquick gel extraction kit (Qiagen) and ligated with T4 DNA ligase (Roche Diagnostics) to the corresponding plasmid vectors. All constructs made in this study were confirmed by sequencing the entire *wecA* gene with an automated sequencer at the DNA Sequencing Facility, Roberts Research Institute, London, Ontario. Plasmid DNA for sequencing was prepared with the High Purity Plasmid Preparation Kit (Qiagen).

Construction of the *wecA*_{E320} gene encoding a FLAG-tagged C-terminal deletion of WecA. pAA26 DNA template encoding the parental *wecA*_{FLAG}, the sense primer 62, incorporating an *Eco*RI site, and the antisense primer 191, encoding the C-terminal part of the *wecA* gene up to the glutamic acid residue at position 320 (E₃₂₀) were used in a PCR reaction. The DNA product was digested with *Eco*RI and *Sma*I, and ligated into the pAA8 vector (Amer & Valvano, 2000), which had also been cleaved with *Eco*RI and *Sma*I. This construct, designated pAA9, resulted in a *wecA* gene lacking the coding region for the last transmembrane domain and the C-terminal tail of the protein (*wecA*_{E320}). Primer 191 was designed in such a way

that the FLAG epitope tag in pAA8 was fused in-frame with *wecA*_{E320}.

Construction of the *wecA*_{MalFTM} gene encoding a FLAG-tagged WecA protein with a swapped last transmembrane domain. pAA12 (Amer & Valvano, 2000) was first linearized by digestion with *Nhe*I, which cuts in the middle of the sequence encoding the last transmembrane domain of WecA. This fragment was used as a template for a PCR reaction with primers 185 (sense) and 186 (antisense). Primer 185 was designed to encode part of the fifth external loop region of the *wecA* gene followed by the sequence encoding the N-terminal half of the last transmembrane domain of the MalF protein (Boyd *et al.*, 1987). Primer 186 encoded part of the *wecA* gene C-terminal tail and the C-terminal half of the MalF last transmembrane domain with the introduction of a *Hinc*II site to facilitate the identification of the new construct. The PCR product was purified, self-ligated and transformed into *E. coli* DH5α, resulting in the isolation of pAA10. This plasmid contained a *wecA* gene encoding a WecA protein in which the last transmembrane domain was replaced by the last transmembrane segment of MalF (WecA_{MalFTM}).

Oligonucleotide-directed mutagenesis of *wecA*. The replacement of the HHH_{279–282} motif by four glycines was conducted in two steps. Primer 165, which anneals to the FLAG sequence, was used in reactions amplifying the C-terminal part of *wecA*, and primer 170, which contains an *Eco*RI cleavage site, was used in reactions amplifying the N-terminal portion of *wecA*. A 0.26 kb C-terminal fragment of *wecA* was first amplified by PCR using antisense primer 165 and sense primer 237, which contains the codons for two glycine residues to substitute His₂₈₁ and His₂₈₂. This fragment was ligated into the *Sma*I site of pBAD24. Then the N-terminal part of *wecA* was amplified using sense primer 170 and antisense primer 238, which contains the codons for the other two glycine residues

replacing His₂₇₉ and Ile₃₈₀ of the HHH motif. The amplified fragment was ligated into the previous construct after digesting it with *Eco*RI and *Sma*I, producing pAA33. This strategy resulted in the reconstruction of a full-length *wecA* gene tagged with the FLAG sequence and encoding four glycine codons in place of the parental HHH codons.

Single His₂₇₉ and Arg₂₆₅ substitutions in the *wecA* gene were introduced by site-directed mutagenesis using QuikChange Site-Directed Mutagenesis Kit from Stratagene as recommended by the manufacturer. Plasmid pAA26 containing the *wecA* gene tagged with the FLAG sequence was used as a template in all reactions. Mutagenized plasmids were transformed into *E. coli* XL-1 Blue as recommended by the supplier. These experiments resulted in plasmids pAA51 (*wecA*_{H279S}) and pAA52 (*wecA*_{R265K}).

Transferase and binding assays. Both pAA26 encoding the parental *wecA*_{FLAG} gene and the other plasmids encoding the mutated *wecA* derivatives were transformed into strain MV501. Membranes were isolated as described by Osborn *et al.* (1972) after induction of the cultures with 0.02% arabinose for 3 h. The reaction mixture for the transferase assay contained the membrane fraction (20 µg total protein) and 96 pmol radiolabelled UDP-N-acetyl-[¹⁴C]glucosamine (225 mCi mmol⁻¹; Amersham Pharmacia Biotech) in 250 µl buffer (5 mM Tris-acetate, pH 8.5, 0.1 mM EDTA and 1 mM MgCl₂). After 30 min incubation at 37 °C, the lipid-associated material was extracted twice with 250 µl 1-butanol. The combined 1-butanol extracts were washed once with 500 µl distilled water. The radioactive counts of the 1-butanol fraction were determined with a Beckman liquid scintillation counter. Radioactive counts were normalized and expressed as a percentage of parental *WecA*_{FLAG} activity at 1 mM MgCl₂ concentration (A. O. Amer & M. A. Valvano, unpublished). An indirect assay that specifically measures the biosynthesis of O7 antigen by assaying the transfer of radiolabelled galactose into Und-P-P-GlcNAc acceptor in the presence of unlabelled UDP-GlcNAc was also used to determine *WecA* activity. The O7 polysaccharide repeating subunit consists of a backbone of GlcNAc, galactose, mannose and N-acetylglucosamine, and a side chain of rhamnose (L'Vov *et al.*, 1984). Since *WecA* mediates the formation of Und-P-P-GlcNAc, the incorporation of radioactive galactose is strictly dependent on the presence of UDP-GlcNAc. This assay was carried out in a similar manner as described above except that the reaction mixture contained 0.5 µCi UDP-[³H]galactose (Amersham) and 0.08 mmol UDP-GlcNAc.

Binding assays were performed as follows. Briefly, 40 µg membranes from MV501 cells transformed with plasmids encoding the various *wecA* mutants were incubated with 45 ng of the inhibitor tunicamycin for 10 min at room temperature. In previous experiments, we had determined that 45 ng tunicamycin was the minimal amount of inhibitor required to inhibit the enzymic activity of 20 µg membranes from MV501(pAA26) cells containing the parental *WecA*. The inhibitory activity of residual tunicamycin, which did not bind to the mutant proteins, was determined by adding 20 µg membranes prepared from MV501(pAA26) in the presence of UDP-N-acetyl-[¹⁴C]glucosamine. Samples were incubated for 30 min at 37 °C and processed with 1-butanol as described above for the transferase assay. Radioactive counts were expressed as a percentage of parental *WecA* activity assayed under the same conditions but in the absence of tunicamycin.

Immunoblot analysis. The same membrane preparations used for transferase and binding assays were subjected to Western

Blot analysis (Amer & Valvano, 2000). Blots were developed using the FLAG M2 mAb as the primary antibody and horseradish peroxidase-linked sheep anti-mouse IgG (Amersham Pharmacia Biotechnology) as the secondary antibody. Detection by chemiluminescence was performed using the Chemiluminescence Blotting Substrate (Roche Diagnostics) as recommended by the manufacturer.

LPS analysis. LPS was extracted and analysed by SDS-PAGE followed by silver staining as previously described (Marolda *et al.*, 1990).

Fractionation of membranes. The location of *WecA* and its mutated derivatives in the plasma membrane was verified by sucrose gradient fractionation of total bacterial membranes (Amer & Valvano, 2000). Fractions were collected and assayed for NADH oxidase activity (Osborn *et al.*, 1972) as a plasma membrane marker, and for the presence of outer membrane porins using SDS-PAGE to detect the outer membrane fractions (Amer & Valvano, 2000). The presence of *WecA* in the fractions was examined by immunoblotting with the anti-FLAG mAb as described above.

Amino acid sequence alignment. BLAST version 2 (Altschul *et al.*, 1997) was used for homology searches in the database of non-redundant sequence. Amino acid sequence alignments of *WecA* homologues were performed using CLUSTAL W (Thompson *et al.*, 1994). The transmembrane helices of *WecA* were predicted from the analysis of its amino acid sequence with TMHMM (Transmembrane Hidden Markov Model; Sonnhammer *et al.*, 1998).

RESULTS AND DISCUSSION

A topological model for *WecA*

Analysis of the *WecA* amino acid sequence reveals alternating hydrophobic and hydrophilic segments that are consistent with a polytopic transmembrane protein structure. We used a hidden Markov model for predicting transmembrane helices within the *WecA* sequence. This model has recently been tested for high accuracy in a standard dataset of 83 membrane proteins whose topologies have been experimentally determined (Sonnhammer *et al.*, 1998). The results suggested the presence of 11 transmembrane segments (Fig. 1a). The prediction also includes topological information on the orientation of the transmembrane helices, which is based on the fact that the positively charged residues arginine and lysine are mainly found in non-transmembrane parts of the protein on the cytoplasmic side (von Heijne, 1986, 1997). The predicted model for the topology of *WecA* was compared with that of the *E. coli* *MraY* protein (Fig. 1b). *MraY* belongs to the same family of proteins as *WecA* and its topology has been confirmed experimentally (Bouhss *et al.*, 1999). Fig. 1 shows a good agreement between the predicted model for *WecA* and the established model for *MraY*, especially in regards to the topological location of non-transmembrane segments with high levels of amino acid sequence conservation, which are orientated towards the cytosolic compartment. We have recently discovered that regions in *WecA* corresponding to cytosolic segments II and III of *MraY* (Fig. 1) contain highly conserved aspartic acid residues (A. O. Amer & M. A. Valvano, unpublished).

Fig. 1. Topological models of *E. coli* WecA and MrayY transferases. (a) WecA model as predicted with TMHMM (Sonnhammer *et al.*, 1998). The boundaries of deletions eliminating the C-terminal tail of WecA ($\Delta 338$) and the last transmembrane segment ($\Delta 320$) are indicated. (b) Topology of MrayY as determined by Bouhss *et al.* (1999). In both models, the non-transmembrane segments that are exposed to the cytosol are indicated with roman numerals. Amino acids within circles denote those residues that are highly conserved within the WecA family (D₉₀-D₉₁ in cytosolic segment II, D₁₅₆ and D₁₅₉ in cytosolic segment III, R₂₆₅ and H1HH₂₇₉₋₂₈₂ in cytosolic segment V). Amino acids F₁₅₂-N₁₅₃-M₁₅₄-V₁₅₅, which together with D₁₅₆ are characteristic of a Walker B motif, are also indicated.

These residues are important for the biological function of WecA, and because of their conservation, they may also play a role in the function of the other bacterial and eukaryotic WecA homologues (A. O. Amer & M. A. Valvano, unpublished).

In contrast to MrayY, WecA has an additional transmembrane helix and a prominent C-terminal segment (Fig. 1). We have previously found that WecA does not require the last 27 C-terminal amino acids to remain functional (A. O. Amer & M. A. Valvano, unpublished), and that a protein derivative containing a C-terminal FLAG epitope tag (WecA_{FLAG}) has the same properties as wild-type WecA (Amer & Valvano, 2000). The dispensable C-terminal region contains highly charged amino acids and has a net positive charge, suggesting a cytosolic location. This region is preceded

by a predicted transmembrane helix containing 18 aa (Fig. 1a). To evaluate the possibility of using a deletion-fusion approach with reporter proteins (Boyd *et al.*, 1993; Prinz & Beckwith, 1994) for providing experimental information on the topology of WecA, we investigated whether the most C-terminal transmembrane helix was required for WecA protein stability. Therefore, we constructed a deleted form of WecA_{FLAG} lacking the C-terminal amino acids located downstream of a glutamic acid at position 320 (WecA_{E320}; Fig. 1a). Since the FLAG epitope still remained fused to WecA_{E320}, the expression of this protein was examined by Western blot with the FLAG-specific mAb M2. Fig. 2 shows that in contrast to the parental WecA_{FLAG} (lane 1), WecA_{E320} was not detected (lane 2). To determine whether the lack of expression was due to the absence of the missing transmembrane

Fig. 2. Expression of WecA_{E320} and WecA_{MalFTM}. Western blot of a polyacrylamide gel containing whole-cell lysates of strain JT4000 transformed with the following plasmids: lane 1, pAA12 (encoding the FLAG-tagged *wecA*); lane 2, pAA10 (encoding the FLAG-tagged *wecA*_{MalFTM}); lane 3, pAA9 (encoding the FLAG-tagged *wecA*_{E320}). The blot was developed using mAb M2 (anti-FLAG). The WecA monomer was identified at ~36 kDa. Polypeptides of higher molecular mass can be detected under the conditions utilized for protein denaturation, which may represent oligomeric forms of WecA (Amer & Valvano, 2000). The position of the following molecular mass standards is shown: myosin (250 kDa), bovine serum albumin (98 kDa), glutamic acid dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa) and myoglobin (30 kDa).

domain, we constructed another WecA_{FLAG} derivative in which aa 321–339 were replaced by aa 476–506 of the MalF protein. These amino acids span the last transmembrane helix of MalF, which has similar hydrophobicity to that of WecA's last transmembrane helix (Boyd *et al.*, 1987). The resulting derivative, WecA_{MalFTM}, was also not detectable by Western blotting (Fig. 2, lane 3). The results of these experiments suggest that the last transmembrane helix of WecA is essential for the stability of the protein, perhaps by interacting with other transmembrane segments or by playing a role in targeting the protein to the plasma membrane. Furthermore, we have previously demonstrated that although WecA can tolerate a substantial deletion of N-terminal amino acids, resulting in the loss of at least three predicted transmembrane helices and still insert in the plasma membrane, the protein is no longer functional (Amer & Valvano, 2000). Thus, a practical outcome of these results is that they precluded us from using a sequential deletion-fusion strategy to experimentally map the topology of WecA, as it has been performed with *MraY* (Bouhss *et al.*, 1999).

Conserved residues in the predicted large cytosolic loop of WecA that are involved in the recognition of UDP-GlcNAc

The topological model of WecA predicts a relatively large (32 aa) non-transmembrane segment exposed to the cytosolic face of the plasma membrane (Fig. 1a). This segment corresponds to a similar region also found in the *MraY* protein (Fig. 1b). Sequence comparisons with additional members of this family indicate that this

region is particularly conserved in the bacterial homologues of WecA (Anderson *et al.*, 2000). Alignment of the amino acid sequences of WecA homologues shows that Arg₂₆₅ and His₂₇₉ (the numbers refer to the position of these amino acids in the *E. coli* K-12 WecA protein) are highly conserved (Fig. 3). Furthermore, in WecA homologues from *E. coli*, *Salmonella enterica* serovar Typhimurium and *Deinococcus radiodurans*, and in the TagO protein from *Bacillus subtilis*, the highly conserved His₂₇₉ is part of a less conserved short sequence motif HHHH (His-Ile-His-His; Fig. 3). This motif bears resemblance to the HIGH (His-Ile-Gly-His) motif identified in class I aminoacyl-tRNA synthetases and also in the superfamily of related nucleotidyltransferases (Bork *et al.*, 1995; Sekine *et al.*, 2001; Venkatachalam *et al.*, 1999). In these enzymes, the HIGH motif constitutes a portion of the three-dimensional structure of the ATP-binding site and is implicated in nucleotide binding as well as in α/β phosphodiesterase activity (Sekine *et al.*, 2001; Venkatachalam *et al.*, 1999). Given that the HIGH motif has a role in nucleotide phosphate binding, it is conceivable that a similar structural motif in WecA may be involved in binding of the nucleotide substrate UDP-N-acetylhexosamine and the release of UMP, a step that would also necessitate phosphodiesterase activity.

To ascertain the role of the HHHH motif in WecA function, the four residues were substituted by glycine, resulting in the WecA_{HHHH/GGGG} mutant (pAA33). Glycine was preferred for the replacements to avoid the use of hydrophobic residues like alanine for amino acid replacements, which could dramatically alter the topology of a non-transmembrane region. Topological predictions on the mutant protein using TMHMM (Transmembrane Hidden Markov Model) showed results identical to wild-type WecA. We also replaced the highly conserved His₂₇₉ residue individually with serine, resulting in WecA_{H279S}. Serine was used for the single replacement because it is considered to be a conservative substitution for histidine (Bordo & Argos, 1991). Plasmids harbouring *wecA*_{FLAG} (pAA26), as well as the mutated *wecA*_{HHHH/GGGG} (pAA33) and *wecA*_{H279S} (pAA51) genes were transformed into *E. coli* MV501. This strain, a derivative of *E. coli* VW187 containing a *wecA*::Tn10 insertion, is unable to form O7-specific LPS (Alexander & Valvano, 1994). Expression of WecA_{FLAG}, WecA_{HHHH/GGGG} and WecA_{H279S} was monitored under induction with 0.02% arabinose. Fig. 4a shows that, in contrast to parental WecA_{FLAG} (lane 1), WecA_{HHHH/GGGG} was unable to restore the formation of the characteristic O7 LPS ladder in MV501 (lane 3). Although WecA_{H279S} also did not restore the characteristic O7 polysaccharide ladder, a reduced level of activity was present as indicated by the formation of LPS containing a single O7 subunit (Fig. 4a, lane 4). To rule out the possibility that the amino acid replacements could have affected either protein stability or targeting to the plasma membrane, the levels of expression of the two constructs were examined by Western blot using the M2 FLAG-specific mAb. WecA_{HHHH/GGGG} and WecA_{H279S} proteins showed comparable levels of ex-

Fig. 3. Alignment of bacterial WecA homologues. WecA homologues were identified from a BLAST search using the *E. coli* WecA protein as a query. Alignments were determined with the program CLUSTAL W. The figure shows a partial alignment of the region spanning the cytosolic segment V and part of the two neighbouring transmembrane domains (indicated with black bars). The boundaries of the transmembrane domains were defined according to the predicted model for WecA (top) and the established model for MraY (bottom). Amino acids presented with a black background denote highly conserved residues across the proteins examined. The sequences from the following organisms were compared (accession numbers are shown in parentheses): WECA_ECOLI, *E. coli* (P24235); WECA_SALTY, *Salmonella typhimurium* strain LT2 (AAF33469.1); WECA_DEIRA, *Deinococcus radiodurans* (B75380); TAGO_BACSU, *Bacillus subtilis* (B69721); WECA_HAEIN, *Haemophilus influenzae* (P45341); RGP_SMUTA, *Streptococcus mutans* (BAA82114.1); OrfDE3_SFA, *S. faecalis* (AAC35916.1); WECA_MYCTU, *Mycobacterium tuberculosis* (Q10606); WECA_MYCLE, *Mycobacterium leprae* (P45830); LIM_SAURE, *Staphylococcus aureus* (A55856); WECA_STRCO, *Streptomyces coelicolor* (T36607); WECA_HPYLO, *Helicobacter pylori* strain J99 (B71801); LIM_SYNEC, *Synechocystis* sp. (S74927); WECA_AEOLI, *Aquifex aeolicus* (H70346); WECA_LPNEU, *Legionella pneumophila* (CAB65202.1); LIM_TMARI, *Thermotoga maritima* (H72238); WBPL_PSAER, *Pseudomonas aeruginosa* serotype O5 (AAC43404.1); TRSF_YENTE, *Yersinia enterocolitica* (S51265); WECA_BFRAG, *Bacteroides fragilis* (AAD40724.1); GPT_CRIGR, *Cricetulus griseus* (P24140); and MRAY_ECOLI, *E. coli* (P15876).

pression to WecA_{FLAG} (Fig. 4b, lanes 2–4). The localization of the mutant proteins in the plasma membrane was examined by sucrose gradient centrifugation. These experiments revealed that both parental and mutant proteins were localized in the fractions containing cytoplasmic membrane components. Indeed, these fractions demonstrated a high level of NADH oxidase activity and they lacked outer membrane porins (data not shown). We concluded that the inability of WecA_{HHHH/GGGG} and WecA_{H279S} to complement O7 LPS synthesis *in vivo* was due to a defect in the enzymic activity of WecA rather than a topological artefact introduced by the amino acid replacements.

The enzymic activity of WecA and the mutant proteins was investigated in more detail using two *in vitro* assays for determining the transfer of UDP-GlcNAc to Und-P. One assay determined the direct incorporation of radiolabelled [¹⁴C]GlcNAc (provided in the reaction as UDP-GlcNAc) into a butanol-extractable fraction,

which contains Und-P-P-GlcNAc intermediates. Previous studies in our laboratory showed that WecA initiates the synthesis of the O7 oligosaccharide subunit by forming Und-P-P-linked GlcNAc. This glycolipid then serves as the acceptor for the galactose, the subsequent sugar of the O7 subunit (Alexander & Valvano, 1994; Marolda *et al.*, 1999). Therefore, in the second assay we determined the UDP-GlcNAc-dependent incorporation of radiolabelled [³H]Gal (provided as UDP-Gal) into the butanol-extractable fraction. This reaction served as a control to rule out the possibility that GlcNAc may have also been added to Und-P-P-N-acetylmuramoyl-pentapeptide, which serves as a peptidoglycan biosynthesis intermediate, by the endogenous activity of the MraY enzyme that is present in the crude membrane extract. Membrane extracts containing endogenous polyprenyl phosphate acceptor were prepared from MV501 transformed with the various plasmids encoding *wecA*_{FLAG} or its mutated derivatives. The results of the direct transferase assay demonstrated

Fig. 4. (a) O7 LPS synthesis in strain MV501 containing the parental *wecA*_{FLAG} and mutated *wecA* genes. LPS samples were obtained from *E. coli* MV501 transformed with the following plasmids: lane 1, pAA26 (encoding *wecA*_{FLAG}); lane 2, pBAD24; lane 3, pAA33 (encoding *wecA*_{H111H/GGGG}); lane 4, pAA51 (encoding *wecA*_{H279S}); and lane 5, pAA52 (encoding *wecA*_{R265K}). LPS was separated by SDS-PAGE and silver stained. Arrows indicate the lipid A core plus one O7 subunit. (b) Expression of mutant WecA protein derivatives. Bacterial cell membranes were prepared from strain MV501 transformed with the following plasmids: pBAD24 (lane 1), pAA26 (lane 2), pAA33 (lane 3), pAA51 (lane 4) and pAA52 (lane 5). The positions of the molecular mass standards are shown: glutamic acid dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa) and carbonic anhydrase (36 kDa) were used.

that membranes from cells expressing WecA_{H111H/GGGG} and WecA_{H279S} had virtually no enzymic activity, since the levels of activity were lower than those observed with membranes from cells carrying the control plasmid pBAD24 (Table 3). The low background in control membranes demonstrated that the incorporation of radioactive sugar into the non-polar butanol fraction was only due to WecA enzyme activity. The indirect assay using radioactive UDP-Gal gave very similar results (data not shown), confirming that GlcNAc 1-phosphate was directly incorporated to Und-P instead of a peptidoglycan biosynthesis intermediate.

We have previously shown that when WecA and the O-antigen translocase Wzx are present a terminal GlcNAc is attached to the *E. coli* lipid A core, which can be detected using digoxigenin-labelled wheat germ agglutinin (Feldman *et al.*, 1999). This assay was used as an independent confirmation of the functionality of the WecA mutants. In this case, *E. coli* strain CLM20 was

utilized, as this strain lacks the genes necessary for the transfer and the translocation of the sugars involved in the biosynthesis of the O-specific LPS (Feldman *et al.*, 1999). CLM20 was co-transformed with pAA33 or pAA51 (expressing WecA_{H111H/GGGG} and WecA_{H279S}, respectively), and pMF21 (encoding the O-antigen translocase Wzx). Fig. 5a, lane 3, shows that no GlcNAc was detected in the LPS core obtained from cells expressing WecA_{H111H/GGGG}. In contrast, a small amount of terminal GlcNAc was detected in the LPS core of cells expressing WecA_{H279S} as compared to the parental WecA_{FLAG} (Fig. 5a, lanes 1 and 4). The amount of lipid A core obtained from these cells was very similar (Fig. 5b), thus ruling out the possibility that these differences are due to a loading artefact. Taken together, these results demonstrate that the failure to form O7 polysaccharide chain *in vivo* correlates with absent or reduced *in vitro* transfer activity of WecA_{H111H/GGGG} and WecA_{H279S}, respectively.

The previous experiments did not distinguish whether the mutations have an effect on the ability of WecA to bind the nucleotide-sugar substrate or on the mechanism of transfer of GlcNAc 1-phosphate to Und-P. To differentiate between these possibilities, we used a binding assay involving the use of the UDP-GlcNAc analogue tunicamycin, which inhibits the activity of the enzyme (Dal Nogare & Lehrman, 1988). We reasoned that mutations in a putative binding region that would prevent the interaction between WecA and UDP-GlcNAc would also prevent the binding of tunicamycin. Membrane preparations containing the mutated WecA forms were treated with minimal amounts of tunicamycin and the presence of residual inhibitor was determined by assaying the activity of the parental WecA_{FLAG}. Table 3 shows that membranes containing WecA_{H111H/GGGG} and WecA_{H279S} displayed a markedly reduced tunicamycin binding as compared to membranes containing WecA_{FLAG}. We conclude from these experiments that the amino acid replacements affected the ability of WecA to bind UDP-GlcNAc, and probably identify critical residues in the protein that may be involved in the recognition of the nucleotide-sugar substrate. Furthermore, the relative conservation of the H111H motif and the high conservation of His₂₇₉ in the other members of the WecA protein family support the idea that these residues play a functional role. The conserved His₂₇₉ is also the first residue of the H111H motif. Interestingly, mutagenesis studies in enzymes with the HIGH motif have shown that the first His of the motif is critical for enzymic activity (Venkatachalam *et al.*, 1999).

Structural analysis of class I tRNA synthetases has revealed another short sequence motif, KMSK (Lys-Met-Ser-Lys), that is involved in the three-dimensional architecture of the ATP-binding site (Sekine *et al.*, 2001). It was suggested that the second lysine of the KMSK motif plays a key role in catalysis, as it interacts with the α -phosphate of the ATP molecule by forming a salt bridge with the phosphate group of the adenylate (Sekine *et al.*, 2001). We suggest that the positive charge at

Table 3. Comparative transfer and binding activities of WecA_{FLAG} and its mutated derivatives

Membrane extract*	WecA protein	Transfer assay†	Binding assay‡
pBAD24	None	10.9 ± 6.3	12.8 ± 0.9
pAA26	WecA _{FLAG}	100	100
pAA33	WecA _{HIHH/GGGG}	7.5 ± 2.5	10.2 ± 1.1
pAA51	WecA _{H279S}	7.9 ± 2.7	15.1 ± 2.4
pAA52	WecA _{R265K}	44.5 ± 18.4	48.3 ± 11.6

* Membrane extracts were prepared from *E. coli* MV510 cells transformed with the indicated plasmids.

† Incorporation of radioactive GlcNAc into a butanol-extractable fraction as described in Methods. The results, expressed as a percentage of the parental WecA_{FLAG} activity at 1 mM MgCl₂ using normalized counts, represent the mean ± SD of three independent experiments.

‡ Incorporation of radioactive GlcNAc into a butanol-extractable fraction in competition with residual tunicamycin as described in Methods. The results, expressed as a percentage of the parental WecA_{FLAG} activity at 1 mM MgCl₂ using normalized counts, represent the mean ± SD of three independent experiments.

Fig. 5. (a) Lectin-Western blot. LPS samples were extracted from *E. coli* strain CLM20(pMF21) transformed with the following plasmids: pAA26, encoding *wecA*_{FLAG} (lane 1); pBAD24 (lane 2); pAA33, encoding *wecA*_{HIHH/GGGG} (lane 3); pAA51, encoding *wecA*_{H279S} (lane 4); and pAA52, encoding *wecA*_{R265K} (lane 5). Western blotting was performed using digoxigenin-labelled wheat-germ agglutinin and horseradish peroxidase labelled anti-digoxigenin antibodies (Feldman *et al.*, 1999). (b) Silver-stained gel of the same samples used in (a).

position 265 could be involved in binding the phosphates of UDP-GlcNAc, similar to the role of the second lysine in KMSK. The proposed function for Arg₂₆₅ is supported by its high level of conservation among WecA homologues, within a region containing a cluster of additional positively charged residues (Fig. 3). A conservative replacement of Arg₂₆₅ with lysine was made to determine whether this residue has any role in WecA function. The mutant protein, WecA_{R265K}, was expressed at comparable levels with respect to WecA_{FLAG} and was localized in the plasma membrane (Fig. 4b, lane 5 and data not shown). WecA_{R265K} showed a partial com-

plementation of O7 polysaccharide biosynthesis *in vivo* (Fig. 4a, lane 5) which correlated with a 44% and 48% reduction in the levels of transferase and binding activities *in vitro*, respectively (Table 3). These results cannot be explained by a topological defect, since the conserved replacement of Arg₂₆₅ would not affect the overall positive charge of the non-transmembrane segment, suggesting that the decreased functionality of the mutant WecA may be due to a defect in substrate binding. We speculate that the replacement of arginine with lysine decreases the affinity of the protein for UDP-GlcNAc. However, further experiments involving purified protein are required to confirm this conclusion.

Concluding remarks

In this study, we have compared the predicted topology of WecA with that of the MraY protein, whose topology has been determined experimentally (Bouhss *et al.*, 1999). Both proteins contain a large cytosolic-exposed non-transmembrane region, which contains conserved amino acid residues, especially Arg₂₆₅ and His₂₇₉. These and additional residues resemble key amino acids found in nucleotidyl transferases. The analysis of WecA mutant proteins containing amino acid replacements of these key residues strongly suggests their involvement in the recognition of UDP-GlcNAc. Since WecA can also complement the biosynthesis of O antigens containing N-acetylgalactosamine, other investigators have suggested that WecA has a loose substrate specificity, which involves the recognition of UDP-N-acetylgalactosamine in addition to UDP-GlcNAc (Amor & Whitfield, 1997; Zhang *et al.*, 1997). Future studies involving purified WecA, currently under way in our laboratory, are needed to clarify its substrate specificity. At any rate, the amino acids identified in this study are also conserved in other WecA homologues such as *Yersinia enterocolitica* WbcO and *Pseudomonas aeruginosa* WbpL, which have predicted specificity for UDP-

N-acetylfucosamine (Skurnik, 1999). Therefore, it is possible that these amino acid residues may have a general role in the function of these proteins, perhaps by contributing to the architecture of a common fold required for the recognition of UDP-N-acetylhexosamines.

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