[Frontiers in Bioscience 8, s452-471, May 1, 2003]

EXPORT OF O-SPECIFIC LIPOPOLYSACCHARIDE

Miguel A. Valvano

Departments of Microbiology and Immunology, and Medicine, University of Western Ontario, London, Ontario N6A 5C1 Canada

TABLE OF CONTENTS

1. ABSTRACT

The O antigen is the most surface-exposed component of the lipopolysaccharide (LPS) molecule and its biogenesis involves several complex mechanisms not completely well understood. All of these mechanisms involve biochemical reactions that occur on the cytoplasmic side of the plasma membrane as well as several different translocation pathways that deliver the nascent O antigens in a glycolipid form to the periplasmic side of the plasma membrane. This article discusses our current understanding of the mechanisms operating in the biogenesis of the O-specific LPS.

2. INTRODUCTION

LPS, a major component of the outer leaflet of outer membranes in Gram-negative bacteria (1), consists of lipid A, core oligosaccharide (OS), and in some microorganisms, O-specific polysaccharide (or O antigen) that is made of repeating OS subunits (1, 2). Lipid A, the most hydrophobic portion of LPS, is embedded in the lipid bilayer of the outer membrane. Lipid A is made of a β-1,6-linked glucosamine disaccharide, which becomes phosphorylated and acylated with a variable number of fatty and hydroxyfatty acid chains (3). The core OS can be subdivided into inner and outer core domains. The outer core usually consists of hexoses and hexosamines while the inner core is, depending on the particular species, composed of one to three residues of 3-deoxy-D-*manno*-octulosonic acid, and two or three residues of L-*glycero*-D-*manno*-heptose (4). LPS plays an important role in maintaining the structural integrity of the bacterial outer membrane by interacting with outer membrane proteins as well as divalent cations (5, 6). Phosphate groups covalently attached to heptose residues in the inner core participate in these ionic interactions, which provide a barrier preventing the passage of hydrophobic substances such as detergents, dyes, and antibiotics across the outer membrane (7, 8).

The biogenesis of LPS is a complex process involving various steps that occur at the plasma membrane followed by the translocation of LPS molecules to the bacterial cell surface. LPS biosynthesis involves a large number of enzyme activities, governed by more than forty genes (2-4, 9). The core OS is assembled on preformed lipid A by sequential glycosyl transfer of monosaccharides, while the O antigen is assembled on undecaprenol-phosphate (Und-P), a polyisoprenoid lipid to which O antigen is linked via a phosphodiester bond (2). These pathways eventually converge by the ligation of the O antigen onto outer core domain of the lipid A-core OS acceptor, with the concomitant release of Und-PP (2-4, 9, 10). Und-P is also required as a lipid intermediate for the biosynthesis of other cell surface structures including peptidoglycan and the enterobacterial common antigen (2, 10, 11).

The genes governing the synthesis of lipid A and substrates for the assembly of the inner core components (*glycero-manno*-heptose and ketodeoxyoctulosonic acid) are scattered throughout the chromosome (2-4, 12-15). For example, genes encoding enzymes involved in the ADP-*glycero-manno*-heptose synthesis pathway such as *gmhA* (12) and *hldE* (14) are located far apart from each other on the chromosome. In marked contrast, the genes encoding functions for the assembly of the outer core OS and the O-specific polysaccharide are clustered (2, 4, 16). Mutations in most core biosynthesis genes (*waa*, formerly *rfa*) (for a discussion on the new LPS genes nomenclature see

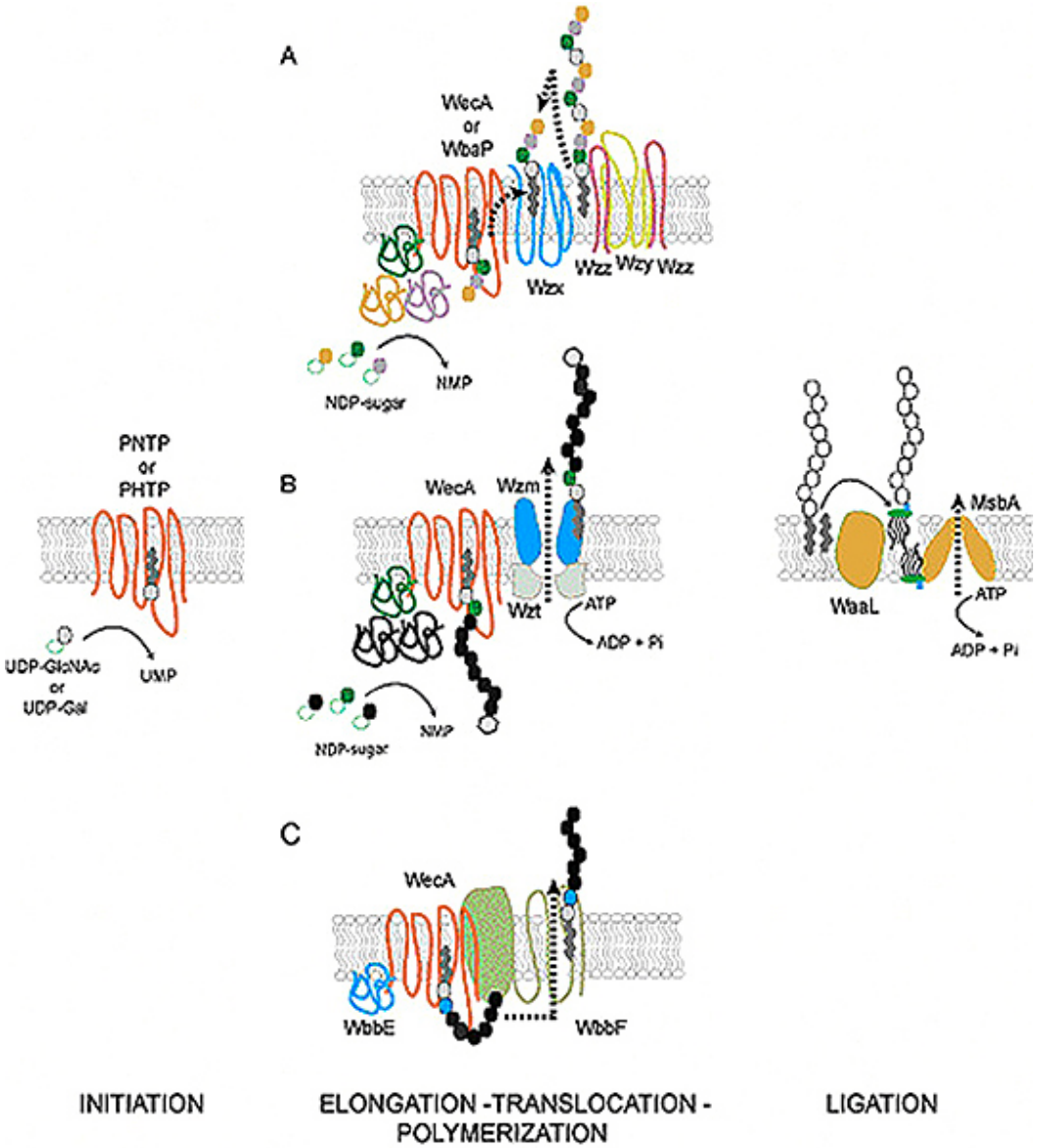

Figure 1. Mechanisms of biogenesis of O-specific LPS. The initiation reaction has conserved features in all mechanisms and involves the formation of an Und-PP-linked sugar, usually an *N*-acetyl hexosamine like GlcNAc or a hexose like Gal (mediated by proteins of the PNTP or PHTP families, respectively). This reaction takes place on the cytosolic face of the plasma membrane where the nucleotide sugar precursors are available to the initiating enzyme. The phoshodiester bond linking the sugar with Und-P is not shown in the figure. Note that number of transmembrane segments depicted in each protein is symbolic, only to indicate their integral membrane nature. The three different intermediate steps in the export of O antigen are depicted. A, denotes the steps involved in the Wzy/Wzx-dependent pathway, where the O repeating subunit is made on the cytosolic side of the plasma membrane and then translocated across the membrane and polymerized at the periplasmic side of the membrane. The glycosyltransferases that complete the formation of the O repeating subunit are depicted a peripheral membrane proteins that mediate the glycosyltransfer of sugar residues using WecA or WbaP as a membrane scaffold. B, denotes the ABC transporter-dependent pathway. In this case the formation and extension of the O-specific polysaccharide occurs in the cytosol and requires the activity of WecA for initiation. The adaptor and chain terminator residues are indicated with different colors and shapes. The polymer is translocated in an ATP-dependent manner by the Wzm/Wzt ABC-2 transporter. C, denotes the synthase-dependent pathway that also requires the activity of WecA for initiation, as well as an adaptor molecule provided by the reaction catalyzed by WbbE. The rest of the chain is extended by the synthase WbbF, which has two domains, one of which is involved in the vectorial secretion of the polymer to the periplasmic side of the plasma membrane. The ligation step is common to all three elongation-translocation-polymerization pathways and involves the formation of a covalent glycosidic bond with a residue of the outer core OS. This step is mediated by WaaL and results in the release of Und-PP. The lipid A-core OS acceptor molecule is synthesized by and independent pathway and translocated to the periplasmic side of the plasma membrane by the MsbA transporter. Transport requires ATP hydrolysis.

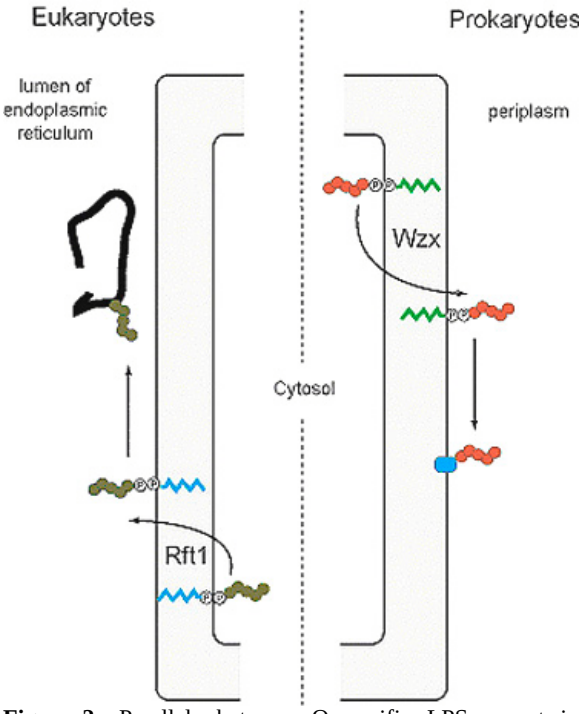

Figure 2. Parallels between O-specific LPS export in bacteria and protein *N*-glycosylation in eukaryotes. Schematic representation of the similarities between the initial steps of protein glycosylation in eukaryotes and the Wzy/Wzx-dependent export pathway for O-specific polysaccharides in bacteria. In both cases the saccharide is assembled at the cytosolic face of the plasma membrane (bacteria) or the ER membrane (eukaryotes). Assembly takes place onto Und-P (bacteria [green]) or Dol-P (eukaryotes [blue]), both of which are polyisoprenol lipids. In the case of bacteria the saccharide is the O repeating subunit while in the case of eukaryotes the saccharide is made of five mannose and two GlcNAc residues. In bacteria, translocation across the membrane involves the Wzx protein (87, 90, 166), while in eukaryotes the translocation of the Dol-PP-$GlcNAc_2Man_5$ unit requires the Rft1 protein (103, 167). Subsequent reactions take place in the bacterial periplasm that result in the polymerization of O antigen subunits and the transfer of the incorporation of the saccharide onto the lipid A-core OS. In eukaryotes, the heptasaccharide moiety is further extended by progressive addition of four other mannose residues and finally capped with three glucose molecules to form Dol-PP-$GlcNAc_2Man_9Glc_3$. This 14-residue oligosaccharide is transferred to appropriate asparagine residues of secretory proteins by the oligosaccharide transferase complex (168, 169). The protein bound oligosaccharide moiety is further modified in the ER and Golgi to yield a mature glycan (for a detailed review see reference 170).

reference 17) lead to rough mutants with an incomplete core, which lacks the site for the attachment of O-polysaccharides. *Escherichia coli* mutants lacking heptose in the LPS display a more dramatic phenotype known as "deep rough". This phenotype is characterized by hypersensitivity to novobiocin, detergents, and bile salts (18), as well as defects in F plasmid conjugation and generalized transduction by the bacteriophage P1 (19-21). All of these defects result from reduced amounts of outer membrane proteins, some of which serve as surface receptors for conjugation and bacteriophage attachment (21-26) or as channel components of efflux systems (27, 28). The impaired stability of the outer membrane in deep rough mutants is associated at least in part with the absence of phosphate groups, since mutations in genes encoding LPS core kinases also show pleiotropic phenotypes similar to those found in heptose-deficient mutants (29, 30).

Mutations in any of the wb* (formerly rfb) genes involved in the synthesis of the O polysaccharide give rise to rough mutants which have a complete core structure (2). In some cases, wb* genes may be plasmid-encoded (31, 32), while in other cases plasmid-mediated functions are required, in addition to chromosomal genes, for the biosynthesis of the O-polysaccharide (33, 34). Typically, wb* gene clusters encode nucleotide sugar synthetases (for biosynthesis of the nucleotide sugar precursors specific to O antigens), and glycosyltransferases (for the sequential and specific addition of sugars that make the O-repeating unit). Additional genes encoding functions involved in the assembly of the O polysaccharide are also present in these clusters, such as wzy (O antigen polymerase) and wzx (putative O antigen flippase; see below) in some systems, and wzm (membrane component of ABC transporter) and wzt (ATP-binding component of ABC transporter) in others (16). In systems containing wzx and wzy, the average size distribution of the O polysaccharide chain is modulated by the product of wzz (35-37), a gene usually located in the proximity of wb* clusters. An O-ligase activity encoded by a gene located within the core OS cluster, waaL (4), is required for the transfer of the O-polysaccharide onto lipid A-core OS.

This article will review the current mechanisms operating in the biogenesis of the O-specific LPS. More comprehensive information on the biosynthesis and assembly of other LPS components and capsular polysaccharides can be found in additional recent reviews (4, 16, 38, 39, 40).

3. BIOGENESIS OF O-SPECIFIC POLYSACCHARIDES

From a mechanistic standpoint, the biogenesis of O-specific polysaccharides can be subdivided in the following stages (Figure 1): (i) the initiation reaction, (ii) the elongation/translocation/polymerization of O repeating subunits, (iii) the ligation reaction to the lipid A-core OS, and (iv) the recycling of the Und-PP polyisoprenoid carrier

3.1. Initiation reaction

The initiation reaction for the biosynthesis of the O antigen subunit occurs at the interface of the plasma membrane where the nucleotide sugar precursors are available from the cytosol. The reaction involves the formation of a phosphodiester bond between a membrane-associated polyprenol phosphate and a cytosolic UDP-sugar with the release of UMP. Depending on the specific microorganism, this reaction is catalyzed by two different

families of proteins. One of these families corresponds to the **p**olyisoprenyl-phosphate **N**-acetylhexosamine-1-**p**hosphate **t**ransferases (PNPT family; 41, 42, 43), comprising proteins that are present both in prokaryotes and in eukaryotes. The other family corresponds to the **p**olyisoprenyl-phosphate **h**exose-1-**p**hosphate **t**ransferases (PHPT family), and its prototype member is WbaP from *Salmonella enterica*. This family of proteins has no known homologues in eukaryotic cells.

3.1.1. Polyisoprenyl-phosphate *N*-acetylhexosamine-1-phosphate transferases

The eukaryotic PNPTs are localized in the membrane of the endoplasmic reticulum (ER). They are all UDP- N-acetylglucosamine (GlcNAc): dolichol-phosphate GlcNAc-1-phosphate transferases that catalyze the first step in N-linked glycoprotein biosynthesis (Figure 2; 43). In contrast, bacterial PNPTs such as WecA, MraY, WbpL, and WbcO utilize different N-acetylhexosamine substrates and they differ in their susceptibility to selective inhibitors (41). Several regions of conserved amino acid sequence can be found in bacterial and eukaryotic members of the PNPT family. It is plausible that all the members of this family utilize a common enzymatic mechanism for the formation of the phosphodiester bond but at the same time they differ in terms of substrate specificity for the nucleotide sugar and the polyisoprenol phosphate.

WecA is a tunicamycin-sensitive UDP-GlcNAc: Und-P GlcNAc-1-phosphate transferase (44, 45). Tunicamycin, a nucleoside antibiotic that is thought to resemble the UDP-GlcNAc-polyisoprenoid lipid reaction intermediate (46, 47), has the ability to inhibit the function of WecA, MraY, and the eukaryotic PNPTs (47, 48). The wecA gene is located within the enterobacterial antigen common antigen gene cluster and serves to initiate both O and enterobacterial common antigen synthesis (11, 44, 49).

Enterobacterial common antigen is a surface glycolipid found on the outer membrane of most enteric bacteria (11). WecA is usually required for the initiation of the biosynthesis of O-specific polysaccharide that have GlcNAc or *N*-acetylgalactosamine (GalNAc) in the O subunit (45, 50-54) and that are translocated across the plasma membrane via the Wzx/Wzy-dependent pathway (see 3.2. below). WecA is also necessary for the initiation of the biosynthesis of O polysaccharides transported by Wzy/Wzx-independent pathways (see 3.2.2. and 3.2.3), such as *E. coli* O8 and O9 (44), *Klebsiella pneumoniae* O1 (55), *Serratia marcescens* O16 (56) and *S. enterica* serovar Borreze O:54 (57). *Pseudomonas aeruginosa*, produces two forms of LPS, designated A-band and B-band LPS. WbpL, is a PNPT member with a seemingly dual substrate translocation reaction, while D156 and D159 form part of the enzyme's catalytic site. Although initial studies predicted that D156 and D159 are required for ionic interactions with metal divalent cations (especially Mg^{2+} or Mn^{2+}) that are essential for phosphoryl transfer reactions (65), a more detailed examination suggests that they are probably interacting directly with the nucleotide UDP or the sugar GlcNAc (K. Vigeant and M. A. Valvano, unpublished data). In addition, WecA has a higher affinity recognition, since it appears to be required for the initiation of B-band LPS synthesis with a FucNAc residue, and A-band synthesis with either a GlcNAc or GalNAc residue (58). The deduced amino acid sequence of WbpL shows high identity to WbcO from *Yersinia enterocolitica* that initiates O antigen synthesis by adding FucNAc to the lipid carrier Und-P (59). The MraY protein catalyzes the formation of Und-P-P-*N*-acetylmuramyl-pentapeptide, the first step in the lipid cycle reactions in biosynthesis of bacterial cell wall peptidoglycan (60). The topology of the *E. coli* as well as the *Staphylococcus aureus* MraY transferase was established experimentally (61) and has served as a template to model the topology of WecA (62). The predicted topology of MraY is also similar to eukaryotic PNPTs (43).

PNPT proteins are all polytopic membrane proteins (41, 43, 61, 63). Limited information is currently available on the structural motifs or critical amino acid residues in these enzymes that may be important for substrate recognition and/or catalysis. It is plausible that regions of the protein extending into the aqueous environment of the cytosolic face of the plasma membrane (or the ER membrane) are involved in the recognition and interaction with the nucleotide sugar substrates. This model is supported by the finding that bacterial and eukaryotic WecA homologues share discrete regions of conserved amino acid sequence located in segments of the protein that are exposed to the cytosolic face of the plasma membrane or the membrane of the endoplasmic reticulum (41, 42, 64). Bacterial homologues also carry a large cytosolic loop containing some conserved residues that may be important for the recognition of the nucleotide sugar substrates (41, 64). In the case of WecA, eleven transmembrane (TM) domains, five external and five internal loops have been predicted using several robust algorithms (Figure 3) (41, 63). While the first three N-terminal TM helices could be deleted without affecting membrane localization of WecA, deletion of the last predicted TM helix of WecA affected protein stability (64). It is possible that TM-XI specifically interacts with another internal TM region to stabilize the protein in the membrane, as the replacement of this region with a similar TM helix from the MalB protein did not correct the stability phenotype (64). Recent studies on WecA characterized conserved aspartic acid residues in the predicted cytoplasmic loops II (D90 and D91) and III (D156 and D 159). WecA derivatives with amino acid replacement at these sites were assayed by *in vivo* complementation of O antigen biosynthesis, as well as by *in vitro* transfer and UDP-GlcNAc binding abilities (62). From these analyses, it was proposed that D90 and D91 are important in forwarding the Und-PP-linked O subunit to the next step in the assembly of the polysaccharide, namely the for Mn^{2+} that Mg^{2+}, with K_M values of 0.6 mM and 3 mM, respectively. These values are in the same range of physiological concentrations of Mn^{2+} and Mg^{2+} in the bacterial cells; thus, it is likely that Mn^{2+} is the preferred ion for enzyme activity. Furthermore, a conserved short sequence motif, His-Ile-His-His (HIHH), and a conserved arginine were identified in WecA at positions 279–282 and 265, respectively (64). This region is located within the cytosolic loop V (Figure 3) that is present in all bacterial

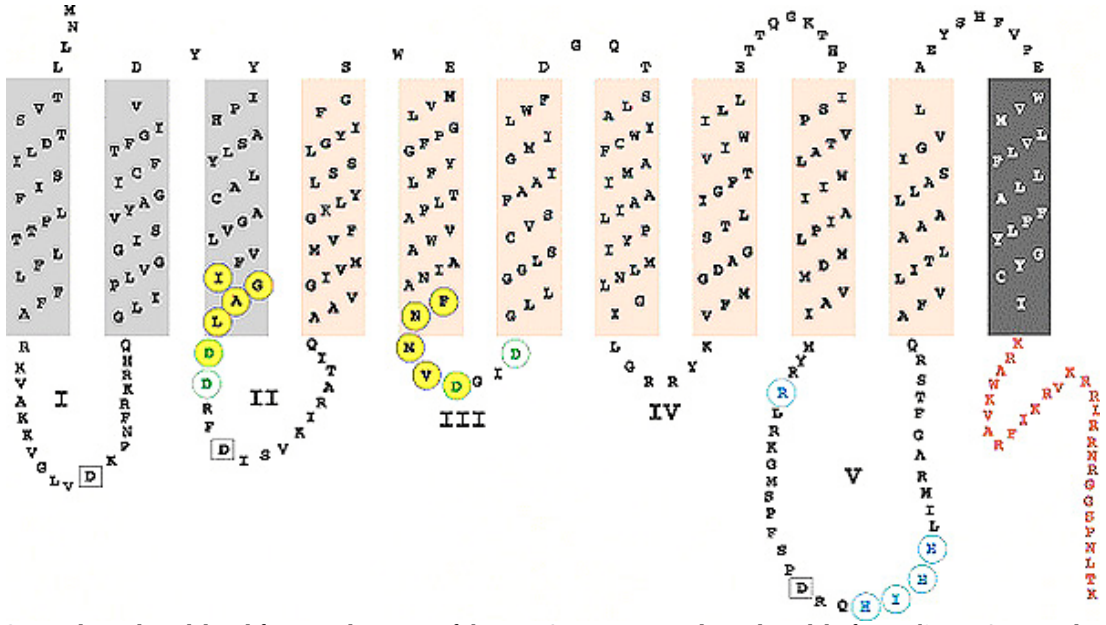

Figure 3. Topological model and functional regions of the WecA protein. Topological model of *E. coli* WecA as predicted with TMHMM (171). The non-transmembrane segments that are exposed to the cytosol are indicated with roman numerals. Amino acids within circles denote those residues that are highly conserved within the WecA family (D90-D91 in cytoloop II, D156 and D159 in cytoloop III, R265 and HIHH279-282 in cytoloop V (64). Amino acids I86-G87-A88-L89 and F152-N153-M154-V155, which together with D90 and D156, respectively, are characteristic of Walker B motifs, are also indicated (yellow). Additional aspartic acids in cytosolic segments, D35, D94, and D276, are indicated within a square, and they are not required for enzymatic activity (62, 64). N-terminal transmembrane segments in grey can be deleted without compromising insertion of WecA in the membrane, although the protein is not functional (63). The last transmembrane domain (dark background) is absolutely required for protein function, possibly by ensuring protein stability in the membrane (64). The C-terminal 29 amino acids (in red) are not required for protein stability or function (64).

homologues of WecA. Both HIHH279–282 and the Arg265 are reminiscent of the His-Ile-Gly-His (HIGH) motif and a nearby upstream lysine, which contribute to the three-dimensional architecture of the nucleotide-binding site among various enzymes displaying nucleotidyltransferase activity (66). Alternatively, the H279 and Arg265 could be required for coordination with Mn^{2+} ions, and could play a role in maintaining the structure of the catalytic site of the enzyme (K. Vigeant and M. A. Valvano, unpublished data). Thus, it was hypothesized that these residues may play a role in the interaction of WecA with UDP-GlcNAc (64). 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.

Despite that there is very strong evidence suggesting that WecA is the enzyme catalyzing the formation of the phosphodiester bond between GlcNAc and undecaprenol, this enzymatic activity has never been directly confirmed since WecA is not available in a pure form. A more detailed characterization of this protein awaits purification of WecA followed by the determination of its tri-dimensional structure.

3.1.2. Polyisoprenyl-phosphate hexose-1-phosphate transferases

WbaP (a member of another family distinct from WecA and its homologues) is responsible for the transfer of galactosyl-1-phosphate from UDP-galactose to Und-P in a reversible reaction, which is the first step of O antigen synthesis in S. enterica (67). The WbaP protein is a large hydrophobic protein, which has a number of potential membrane-spanning domains (67). However, the predicted WbaP topology suggests a different model from that of WecA (M. S. Saldías, C. L. Marolda, I. Contreras, and M. A. Valvano, unpublished data). The protein can be roughly separated into three major structural domains: an N-Terminal region containing a cluster of 4 transmembrane helices, a large central periplasmic loop of approximately 150 amino acids, and a large C-terminal cytosolic tail (Figure 4). The periplasmic region is dispensable since, at least in the Salmonella WbaP, it can be deleted without compromising the function of the protein (M. S. Saldías, C. L. Marolda, I. Contreras, and M. A. Valvano, unpublished data). No homologues to WbaP are found in eukaryotic systems. In contrast, WbaP shows high-level sequence similarity throughout their entire lengths with proteins involved in the synthesis of exopolysaccharides, such as GumD (Xanthomonas campestris), AmsG (Erwinia amylovora), Orf14 (Klebsiella pneumoniae), and WcaJ (Escherichia coli K-12), which are all demonstrated or predicted UDP-glucose:Und-P glucose-1-phophosphate or UDP-galactose:Und-P galactose-1-phosphate transferases (68-71). These proteins all have similar predicted topology to that of WbaP. The level of similarity suggests that the N-terminal portions have similar functions in all of them. The C-terminal half of WbaP shows high-level sequence similarity to ExoY of Rhizobium meliloti (72) as well as with several transferases involved in the formation of group II capsules in Gram positive bacteria.

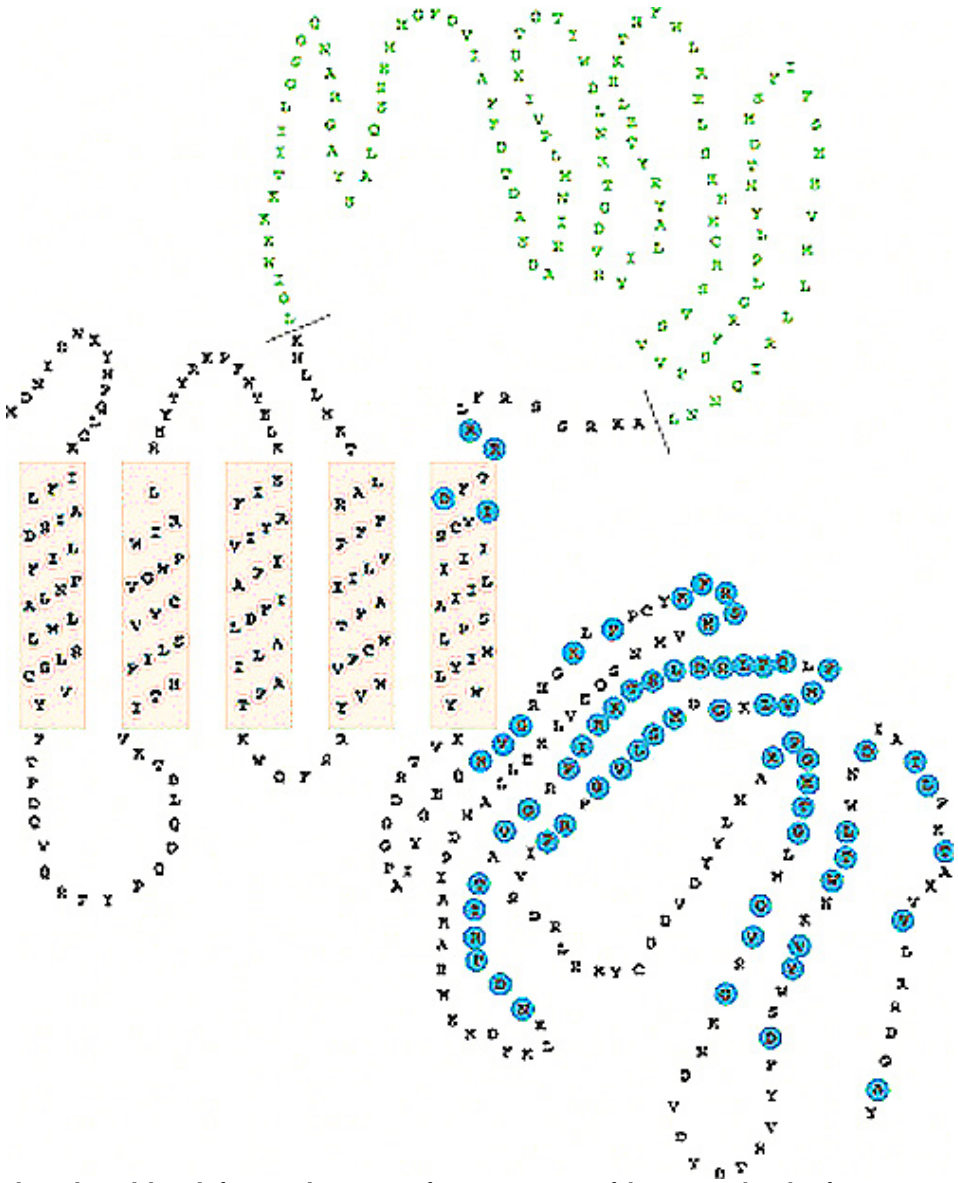

Figure 4. Topological model and functional regions of WbaP. A topological model of *S. enterica* serovar Typhi WbaP was constructed with DAS and TMHMM programs (171, 172). Predicted transmembrane domains are indicated in beige. A large periplasmic loop (green) can be deleted without compromising the function of the protein (M. S. Saldías, C. L. Marolda, I. Contreras, and M. A. Valvano, unpublished data). Amino acids on a blue background denote conserved residues with a large family of glycosyltransferases from Gram-positive and Glam-negative bacteria. The C-terminal portion of the WbaP that is presumably located facing the cytosol, and has the highest level of conservation with the PHPT family carries the galactosyl transferase activity (73).

Wang and Reeves have proposed that the S. enterica serovar Typhimurium WbaP has two functions (73). One function is the actual UDP-galactose:Und-P galactose-1-phosphate transferase reaction that is involved in the first step of O antigen synthesis. This function is primarily attributable to the C-terminal portion of the protein. The other function is manifested at a later step (but prior to the flippase reaction) and involves the release of Und-PP-galactose from WbaP, which is mediated by the N-terminal region rich in transmembrane domains. These conclusions are based on the analysis of wbaP(T) mutations, which occur within the first half of the wbaP gene (73). wbaP(T) mutants retain UDP-galactose:Und-P galactose-1-phosphate transferase activity but they cannot complete the formation of O-specific polysaccharide. This scenario is very similar to that observed with the WecA mutants in residues D90 and D91, which also retain transferase activity in vitro but cannot mediate O antigen synthesis in vivo (62). Altogether, these observations suggest that membrane bound PNPT and PHPT proteins, albeit different in terms of sequence similarity and predicted topology, may provide a membrane scaffold for the additional reactions mediated by loosely membrane-associated glycosyltransferases that complete the formation of the O-specific subunits. In this proposed model, the growing Und-PP-linked subunit would remain associated to PNPT (or PHPT) proteins, prior to further processing involving its translocation across the plasma membrane.

3.2. Elongation/translocation/polymerization

After the initiation reaction is complete, additional sugars must be added to complete the O subunit. These reactions are catalyzed by specific glycosyltransferases, which are either soluble enzymes or associated to the plasma membrane by ionic interactions. Bacterial glycosyltransferases involved in the synthesis of exopolysaccharides have an abundance of positively charged amino acids which result in predicted high pI's and account, at least in part, for the ionic interactions of these proteins with the plasma membrane. Glycosyltransferases are classified in different families (http://afmb.cnrs-mrs.fr/CAZY; for a review see reference 74). The fact that these enzymes are localized in the cytoplasmic compartment is consistent with the notion that the assembly of Und-PP-linked O subunits occurs at the cytosolic face of the plasma membrane. However, the ligation of Und-PP-linked O polysaccharides to lipid A-core OS occurs at the periplasmic face of the plasma membrane (75, 76). As a result, the biogenesis of O polysaccharides requires a process of assembly that includes a mechanism whereby Und-PP-linked saccharides are translocated across the plasma membrane.

At least three different mechanisms for the assembly and translocation of O-specific polysaccharides have been described (Figure 1). One of them involves the synthesis of O repeating subunits by the sequential addition of monosaccharides at the non-reducing end of the molecule, a process that takes place on the cytosolic side of the plasma membrane (10). These subunits are translocated across the plasma membrane, and they subsequently become polymerized by a mechanism involving the successive addition of the reducing end of the growing polymer to the non-reducing end of Und-P-P-linked subunits that is mediated by the Wzy protein. The Und-P-linked polymer is then ligated "en bloc" to the lipid A-core OS by reactions occurring on the periplasmic face of the membrane (75-77). This pathway, also referred to as the *wzy* (polymerase)-dependent pathway, occurs in the synthesis of the majority of O antigens, especially in those made of repeating units of different sugars (heteropolymeric O antigens) (16). The pathway also involves another protein, Wzx, which is a putative flippase, and it is always present in the gene clusters containing the *wzy* gene. Therefore, it would be more appropriate to refer to this pathway as the Wzy/Wzx-dependent pathway.

A second mechanism for O antigen biosynthesis involves the formation of a polymeric O antigen by reactions taking place on the cytosolic face of the plasma membrane, which are mediated by the sequential action of glycosyltransferases elongating the polysaccharide at the non-reducing end (39). The nascent polysaccharide is transported across the plasma membrane by a two-component ATP-binding cassette transporter (16, 78), and subsequently ligated to lipid A-core OS. This pathway has been observed especially in O antigens made of repeating units of the same sugar (homopolymeric O antigens) such as those from *E. coli* O8 and O9 (10) and *Klebsiella pneumoniae* O1 (79), as well as in *E. coli* group 2 and 3 exopolysaccharide capsules (39). Finally, a third mechanism involves a synthase and has only been described to date in *S. enterica* serovar Borreze (34, 57).

A unique aspect in the biogenesis of complex carbohydrate structures in all types of cells is the participation of polyisoprenol lipids. Polyisoprenol-linked sugars are involved in the biosynthesis of eukaryotic glycoproteins, bacterial cell walls, and surface polysaccharides (80, 81). Once assembled, polyisoprenol-linked sugars must cross the lipid bilayer for further processing. In the case of protein glycosylation, a topological model has been proposed involving the transmembrane movement of dolichol-linked sugars across the endoplasmic reticulum membrane (82). A similar model involving transmembrane "flipping" of polyisoprenol-linked sugars has been proposed for the synthesis of bacterial cell wall peptidoglycan, teichoic acids, and enterobacterial common antigen in bacteria as well as *N*-protein glycosylation in eukaryotes (Figure 2). However, how lipid-linked carbohydrates are translocated from one leaflet of the lipid bilayer to the other remains unclear. It has been shown that the unassisted transbilayer movement of polyisoprenol-linked sugars in liposomes is extremely slow (83, 84), suggesting the need for protein-assisted translocation (85, 86).

3.2.1. Wzy/Wzx-dependent pathway

At least three proteins (Wzx, Wzy, and Wzz) are involved in this export pathway but currently, there is no information concerning the manner in which these proteins interact with one another to facilitate the formation of predicted functional complexes. Once the individual Und-PP-linked O subunits are formed, they must be exported to the site of polymerization at the periplasmic face of the plasma membrane (Figure 1). All wzx/wzy-dependent O antigen clusters studied to date contain a gene that encodes a plasma membrane protein designated Wzx that has been postulated as a candidate for the O unit flippase or translocase (87). Based on comparison of predicted hydrophobicity, Wzx proteins were classified within a family of integral membrane proteins with 12 predicted transmembrane helices (88). Wzx proteins share very little amino acid sequence similarity, and their genes have very poor nucleotide sequence homology, to the point that they can be used as genetic markers for distinguishing among specific O antigens (54, 89). In contrast to the Wzy/Wzx-independent O antigens, no obvious ABC transporters have been identified in wzy-dependent systems. The involvement of Wzx proteins in the translocation of Und-PP-linked O subunits was based on experiments using a heterologous O antigen expressing system in S. enterica. In vivo radiolabeling of O antigen precursors in the presence of a wzx mutation suggested the accumulation of Und-PP-linked O subunits with an apparent location on the cytoplasmic face of the plasma membrane (87). However, the small amount of material involved makes definitive localization difficult, since the wzx block only resulted in approximately fifty percent of the radiolabelled material being accumulated on the cytoplasmic face of the plasma membrane. It is possible that Wzx facilitates the transit of Und-PP-linked O-subunits to the periplasmic face of the plasma membrane in a similar manner as a permease, and

with the help of proton motive force as a source of energy. This could explain the absence of typical amino acid motifs in the primary sequence of Wzx that are indicative of ATP or GTP binding sites. However, fractionation experiments using a wzx mutant in E. coli O7 and immunoblot analysis of the cell fractions with O7-specific antibodies, revealed an overall reduction of O7-specific precursors at the plasma membrane instead of an accumulation of precursors as would be predicted for a transporter (C. L. Marolda and M. A. Valvano, unpublished observations). The lack of a definitive biochemical assay to determine whether "flipping" of the Und-PP-linked O-subunits really occurs complicates the conclusively determination of the function of Wzx.

Given that the translocation of Und-PP-linked O-subunits must be a conserved process, the absence of any obvious conserved motifs in the primary amino acid sequences of Wzx proteins is intriguing. One possible explanation for the abundant differences among Wzx proteins could be the requirement for the recognition of specific O subunits, which are highly variable in terms of structure and sugar composition. Recent investigations demonstrated that a complete Und-PP-linked O subunit is not required for "flipping" since a single sugar, GlcNAc, can be incorporated into the lipid A-core OS of *Escherichia coli* K-12 by a process requiring *wecA* and *wzx* (90). Remarkably, Wzx proteins encoded by different O antigen gene clusters that only share a common initiation reaction with the formation of Und-PP-GlcNAc or Und-PP-GalNAc can efficiently complement an *E. coli* K-12(O16) *wzx* mutant (C. L. Marolda and M. A. Valvano, unpublished data, and reference 90). Moreover, poor complementation was observed with the Wzx protein from *S. enterica* serovar Typhimurium, which possess an O antigen subunit whose biosynthesis is initiated with Und-PP-Gal. These studies indicate that the Wzx function is independent of the chemical structure of the saccharide moiety of the O antigen subunit but it requires the recognition of the first Und-PP-linked sugar (C. L. Marolda and M. A. Valvano, unpublished data, and reference 90). Also, Wzx homologues involved in synthesis of exopolysaccharides could not participate in O antigen assembly (90). The reason for this observation is not clear but it is conceivable that Wzx interacts with its cognate export/assembly machinery which may be distinct in the case of exopolysaccharides. These data suggest a model for Wzy/Wzx-dependent O antigen synthesis involving the recognition of Und-P-P-linked sugars by a putative complex made of Wzx translocase and the initiating enzymes WecA or WbaP (C. L. Marolda and M. A. Valvano, unpublished data).

The second component of the Wzy/Wzx pathway is the Wzy protein. This protein is proposed to be involved in the polymerization of Und-PP-linked O subunits at the periplasmic face of the cytoplasmic membrane (Fig 1). The polymerization reaction involves transfer of nascent polymer from its Und-PP carrier to the reducing end of the new Und-PP-linked O subunit (91, 92). Mutants with defects in the *wzy* gene produce LPS consisting of lipid A-core bound to a single O unit (93). The released Und-PP is recycled to the active monophosphoryl form by dephosphorylation (see section 3.4. below), a reaction inhibited by the antibiotic bacitracin (94). Wzy proteins appear to be integral membrane proteins with 11–13 predicted transmembrane domains, and they exhibit little primary sequence similarity (95, 96). In contrast to Wzx, Wzy proteins from different O types are not interchangeable and display specificity for the cognate O subunit or for structures containing the same linkage between O antigen subunits (2, 10). Several Wzy proteins examined with robust computer programs that predict topology appear to possess a relatively large periplasmic loop that may be important in the recognition of the O antigen subunit. This topology has been experimentally demonstrated in at least one Wzy protein (96). However, the enzymatic mechanism of Wzy has not been resolved, in part because of the absence of distinguishing features in Wzy proteins, but also due to difficulties in expressing sufficient amounts for *in vitro* studies, which complicates the identification of catalytic and binding residues in Wzy.

The third component of the Wzy/Wzx-dependent pathway is the Wzz protein. Wzz generates the strain-specific modal distribution of O-polysaccharide chain lengths as reflected in characteristic clusters of bands following gel electrophoresis of LPS samples (97). Several models have been proposed to explain the modality in the polymerization process. Wzz was hypothesized to act as a timing clock, interacting with the Wzy polymerase and modulating its activity between two states that favor either chain elongation or chain termination caused by transfer of the O polymer to the ligase (98). An alternative model implicates Wzz as a molecular chaperone to assemble a complex consisting of Wzy, the WaaL ligase, and Und-PP-linked O-specific polysaccharide (37). The specific modality would then be determined by different kinetics resulting from a Wzz-dependent ratio of Wzy relative to WaaL. More than one *wzz* gene has been observed in some microorganisms like *P. aeruginosa* (99) and *S. flexneri* (100). It is not clear if the presence of additional Wzz activities would have an additive effect in the overall length of the O-polysaccharides or alternatively, they would be differentially required under varying physiological conditions. Wzz proteins are located in the plasma membrane, and all have two transmembrane helices flanking a periplasmic loop with a predicted coiled-coil structure (101). The coiled-coil domains may be important for interactions of Wzz with Wzy, WaaL, or both. However, only Wzz oligomers have been identified by chemical cross-linking experiments (36) while a definitive evidence of cross-linking of Wzz to either Wzy or WaaL is lacking. The function of Wzz proteins appears not to be specific for a given O-repeat subunit structure but the regions of the protein required for Wzz modality are not well defined (36, 102).

The *N*-linked protein glycosylation pathway in eukaryotes has remarkable parallels with the biogenesis of Wzx/Wzy-dependent O-specific polysaccharides (Figure 2). As in bacteria, the process can be divided into similar steps involving the assembly of a lipid linked oligosaccharide (analogous to the O subunit) on the

cytosolic side of the endoplasmic reticulum (ER), the translocation of this molecule across the ER membrane (analogous the flipping reaction mediated by Wzx), and the transfer of the oligosaccharide from its lipid anchor to selected asparagine residues of nascent glycoproteins (analogous to the ligation the reaction of the O-specific polymer with the core-lipid A molecules; 81). The initiation reaction that results in the biosynthesis of Dol-PP-GlcNAc is followed by subsequent reactions on the cytosolic side of the ER membrane that involves the addition of another GlcNAc and three mannose residues. These reactions are mediated by specific glycosyltransferases and result in the formation of a heptasaccharide-lipid linked intermediate, $Man_5GlcNAc_2$-PP-Dol. These reactions are analogous to those taking place on the cytosolic side of the bacterial plasma membrane, which result in the formation of the O antigen subunits as well as other precursor molecules for peptidoglycan and cell surface polysaccharides in general. The translocation of the lipid-linked heptasaccharide intermediate across the ER membrane in *S. cereviseae* is carried out by the recently identified Rft1 protein (103). A mutation in the *rft1* gene is lethal in yeast and its phenotype is associated with a protein glycosylation defect (104).Rft1 is an integral membrane protein with predicted 12 transmembrane domains, which in contrast to other lipid flippases (105), lacks any motifs indicative of ABC-type transporters, and its gene is highly conserved in eukaryotic genomes. Interestingly, an *rft1* gene homologue is absent in *Plasmodium falciparum*, an organism that apparently lacks *N*-linked protein glycosylation (106). The function of Rft1 is analogous to that of bacterial Wzx. Both proteins share similarities in size and hydrophobicity plots, and both also lack identifiable motifs. However, the two families do not share any obvious similarities in their primary amino acid sequences. Whether these two protein families share a similar translocation mechanism or their structural differences reflect the nature of the different substrates it is presently undetermined.

3.2.2. ABC transporter-dependent pathway

In this pathway, the O-specific polysaccharide is completed at the inner face of the cytoplasmic membrane and the export of the polymer to the outer face for ligation requires an ABC (ATP-binding cassette) transporter. One other salient feature of the O polysaccharides formed by this pathway is the participation of a primer Und-PP-GlcNAc intermediate followed by the addition of a sugar adaptor molecule (Figure 1). Although these O polysaccharides are all initiated by the activity of WecA, they differ from Wzy/Wzx O polysaccharides in that the GlcNAc residue transferred to lipid A-core during ligation occurs only once per chain, and thus it is not found within the repeat unit structure itself (44, 107). Next, an O-polysaccharide-specific glycosyltransferase adds an adaptor sugar residue between the und-PP-GlcNAc primer and the repeat subunit domain, and this reaction also occurs only once per chain. Different enzymes are involved in adding the adaptor. In *E. coli* O9, adaptor formation involves the addition of a single mannose residue by WbdC (108). In the other cases, like in some serotypes of *K. pneumoniae* LPS (107), the adaptor is added by the bifunctional galactosyltransferase, WbbO, which also participates in subsequent chain extension reactions (55, 109). The O polysaccharide is assembled in the chain extension phase, by the processive transfer of residues to the nonreducing terminus of the Und-PP-linked acceptor (108-110), which may be mediated by either monofunctional or multifunctional transferases (108, 111, 112).

Following polymerization, the nascent Und-PP-linked O polysaccharide is exported across the plasma membrane by an ABC-2 subfamily of ATP-binding cassette transporters (113). ABC-2 transporters consist of an integral membrane protein, Wzm, with an average of six transmembrane domains, and a hydrophilic protein containing an ATP-binding motif, Wzt. Genes encoding these two components are present within the O-polysaccharide biosynthesis clusters. As with other ABC-transporters involved in transmembrane export, Wzm homologues for O-polysaccharide biosynthesis display very little primary sequence identity, but Wzt homologues are much more highly conserved, especially in the nucleotide-binding region. No structural data are available on the ABC transporters for O polysaccharides and little is known about their organization and exact mode of action. Presumably, these transporters operate in a similar manner as those used for the synthesis of group 2 capsules in *E. coli* and other bacteria (113). The export process, driven by ATP hydrolysis, may involve an ATP-binding-induced conformational change of the Wzt protein, resulting in its insertion into the membrane via an interaction with the Wzm, introducing polymer into the channel. As with other systems, a series of insertion-deinsertion events would then drive the O-specific polymer through the membrane, but this has not been experimentally determined. Although no direct proof is currently available, the substrate of the ABC transporter machinery is presumably the Und-PP-O polysaccharide. This assumption would be consistent with the observation that *E. coli* K-12 can ligate to its lipid A-core polymers assembled and exported by any of the known pathways, all of which result in Und-PP-linked saccharides that are presented to the ligase (see section 3.3. below).

Due to the processive nature of the polymerization, an intriguing aspect of the polymers assembled by the ABC transport dependent pathway resides in their mode of termination. In the case of the Wzy/Wzx-exported O polysaccharides, this process results from the interactions that involve the Wzy and Wzz proteins. Despite that the ABC-transport dependent pathway does not involve a Wzz protein the O-specific polysaccharides formed by this pathway display strain-specific modal distributions (35). A current model favors a competition between "termination-export" and "chain-extension" activities (78). The presence in several O polysaccharides from ABC-transporter-dependent pathways of novel nonreducing terminal residues on the glycan chains, suggest that these residues may play a role as termination signals. For instance, in the polymannan O8 glycans of E. coli (and the K. pneumoniae O3 and O5 counterparts), the chains end in a 3-O-methylmannose residue (C. Whitfield, personal communication and

references 107, 114, 115, 116). In the ABC-transporter-dependent O polysaccharide of B. bronchiseptica a unique 2,3,4-triamino-2,3,4-trideoxy-α-galacturonamide derivative is found at the chain terminus (117, 118). Thus, terminal modifications appear to be a common occurrence in the ABC-transporter dependent systems, and they probably play a functional role by promoting chain termination of the O polysaccharide, perhaps by providing a biochemical signal to initiate the export process.

3.2.3. Synthase-dependent pathway

The plasmid-encoded O:54 antigen of *S. enterica* serovar Borreze is the only known example of a synthase-dependent O polysaccharide (34, 57, 119). The O:54-specific polysaccharide is a homopolymer made of *N*-acetylmannosamine (ManNAc). In a similar fashion to the ABC transport-dependent pathway, the synthesis of the O:54 subunit is initiated by WecA (57) and the first ManNAc residue is transferred to the Und-PP-GlcNAc primer by the nonprocessive ManNAc transferase WbbE (120). The second transferase, WbbF, belongs to the HasA (hyaluronan synthase) family of glycosaminoglycan glycosyltransferases (121), and it is proposed that this enzyme performs the chain-extension steps (Figure 1). Synthases are integral membrane proteins (121, 122), which appear to catalyze a vectorial polymerization reaction by a processive mechanism resulting in the extension of the polysaccharide chain with the simultaneous extrusion of the nascent polymer across the plasma membrane (122). Although the exported the polymer is presumably Und-PP-linked, there is very little information on the exact mechanism of export mediated by WbbF as well as in the process leading to chain termination. The synthase family has other members including the enzymes involved in biosynthesis of cellulose and chitin, hyaluronic acid capsules (121, 122), and the type 3 capsules of *S. pneumoniae* (123, 124). These enzymes are characterized by two conserved domains, one likely involved in the glycosyl transfer reaction and the other implicated in the translocation of the nascent polymer.

3.3. Ligation reaction

The ligation reaction resulting in the completion of the LPS molecule involves the addition of O polysaccharide to the nascent lipid A-core OS (Figure 1). This reaction occurs at the periplasmic face of the cytoplasmic membrane (75), but its mechanism is currently unknown. The *waaL* gene product is currently the only protein presumed to be required for ligation. This gene maps within the *waa* gene cluster that also encodes the other enzymes involved in the biosynthesis and assembly of core OS (4). The WaaL homologues do not have any obvious distinguishing features. Although these proteins are presumably capable of forming glycosidic linkages, they share no similarity with any of the families of glycosyltransferases that utilize sugar nucleotide donors. Collectively, WaaL homologues exhibit low levels of similarity in their amino acid sequences, and they are all predicted to be integral membrane proteins with 8 or more membrane-spanning domains. The membrane location of the *E. coli* K-12 WaaL carrying a C-terminal FLAG-His-6x epitope has recently been confirmed by sucrose gradient fractionation studies followed by immunoblotting with anti-FLAG monoclonal antibodies (C. L. Marolda and M. A. Valvano, unpublished data). *In vitro* assays with well-defined donor and acceptor substrates have not been reported, but the availability of partially purified $WaaL_{FLAG\text{-}His\text{-}6x}$ (C. L. Marolda and M. A. Valvano, unpublished data) will make it possible to devise an appropriate ligation assay. WaaL presumably functions as part of a complex that involves highly specific interactions with the lipid-linked O-polysaccharide intermediates and lipid A-core acceptor. In the case of *E. coli* K-12, WaaL can link to its lipid A-core O polysaccharides with diverse structures, arising from any of the known biosynthesis pathways. The substrates for thus reaction may be Und-PP-linked oligosaccharides, polysaccharides, and even a single Und-PP-GlcNAc (C. L. Marolda and M. A. Valvano, unpublished data, and reference 90). Thus, the lack of discrimination for donor structures suggests that WaaL may recognize the undecaprenol pyrophosphate carrier rather than the saccharide attached to it. The variations in ligase sequences may then reflect specificity for the structure of the lipid A-core OS acceptor. This is supported by genetic and structural evidence demonstrating acceptor specificity that not only involves just the core residue providing the attachment site, but also other proximal residues in the core OS (9, 125).

The acceptor component of the ligation reaction is the lipid A-core oligosaccharide, which is synthesized and assembled by enzymes that are cytoplasmic or associated with the plasma membrane (3, 4, 38). The mechanism by which newly synthesized lipid A-core OS is transported across the plasma membrane has recently been elucidated in *E. coli* and requires the MsbA protein (Figure 1; 38). MsbA is an integral membrane protein required for the export of lipid A and glycerophospholipids (126, 127). MsbA belongs to the ABC superfamily and it is closely related to mammalian multidrug-resistance proteins. Structural analysis of the E. *coli* MsbA revealed that the protein is organized as a homodimer with each subunit containing six transmembrane a-helices and a nucleotide-binding domain (128). All the transmembrane helices are tilted approximately 30° to 40° relative to the plane of the membrane, forming a cone shaped structure with two substantial openings on either side facing the lipid bilayer. This cone shaped structure creates a large chamber facing the cytoplasmic side of the membrane that could easily accommodate a large molecule like lipid A (128). Biochemical studies have demonstrated that depletion of cellular MsbA result in the gradual accumulation of lipid A and glycerophospholipids in the inner membrane (126). Using temperature sensitive *E. coli* point mutant of MsbA, Doerrler *et al.* (127) have shown that loss of MsbA function results in rapid cessation of cell growth, which is associated with the accumulation of lipid A and phospholipids in the inner membrane and the formation of plasma membrane invaginations. The mutant MsbA contains a single amino acid substitution located in its fifth transmembrane span near the opening of the cone that is proximal to the periplasmic side of the plasma membrane. Recent work has also demonstrated *in vitro* that MsbA

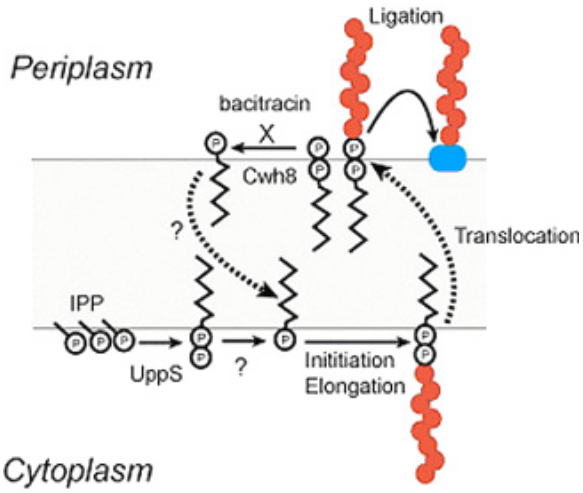

Figure 5. Recycling of Und-PP. Hypothetical model to indicate the recycling of Und-PP after the ligation reaction (or the transfer of the Dol-PP-linked oligosaccharide to the nascent glycoprotein in eukaryotes) (See also Figure 2). Isopentenyl-phosphate units (IPP) are transformed in Und-PP by the activity of the isopentenyl-phosphate synthase UppS. Und-PP is dephosphorylated by an unknown pyrophosphatase and it becomes a substrate for the initiation reaction mediated by PNTP or PHTP proteins. Following processing and translocation of the saccharide moiety, the transfer reaction results in free Und-PP on the periplasmic side of the plasma membrane (or free Dol-PP on the luminal side of the ER membrane). The Cwh8 protein is a membrane bound acid phosphatase from *S. cerevisiae* that has its active site facing the ER lumen and has been shown to be essential for the dephosphorylation of Dol-PP into Dol-P (143). Homologues of these proteins are found in nearly all Gram-negative and Gram-positive bacteria from which genomic sequences are available (M. A. Valvano, unpublished), suggesting that these protein may play a similar role in the dephosphorylation of Und-PP. This biochemical step is inhibited by the antibiotic peptide bacitracin, but direct evidence associated bacitracin with the Cwh8 protein homologues is lacking. Likewise, there is no information on how periplasmic Und-P (or ER luminal Dol-P) are recycled back to the cytosolic face of the membrane.

functions as a lipid activated ATPase and that hexaacylated lipid A is an especially potent activator (129). These results strongly suggest that lipid A is a substrate for the MsbA transporter. Although there is no direct evidence that MsbA can transport lipid A-core OS, the available genetic and biochemical evidence together with the large dimensions of the chamber formed by the MsbA homodimer that could accommodate a large molecules, strongly suggests that this is the case.

3.4. The recycling of Und-PP

The availability of Und-P is a limiting factor in the biosynthesis of O polysaccharides, since this isoprenoid lipid carrier is made in very small amounts, and it is also required for the biosynthesis of cell wall peptidoglycan, teichoic acids, and other carbohydrate polymers. Isopentenyl diphosphate and its isomer dimethylallyl diphosphate are, together, the universal precursors of all isoprenoids (130), and they are synthesized *de novo* by the mevalonate and the 1-*deoxy*-D-xylulose 5-phosphate pathways (131). Und-PP is a precursor for Und-P, and is synthesized by the UppS synthetase from isopentenyl pyrophosphate units (132, 133). To participate in polymer biosynthesis as lipid carrier, newly synthesized Und-PP is dephosphorylated by a membrane bound pyrophosphatase to Und-P (134). Since the UppS protein lacks features indicative on an integral membrane protein these biosynthetic steps probable occur at the cytosolic interface with the plasma membrane. Following initiation and the subsequent processing and translocation reactions, the Und-PP-linked polymers are transferred to the appropriate acceptor on the periplasmic side of the plasma membrane. This process results in the release of Und-PP, which is recycled back to Und-P by a pyrophosphatase (Figure 5). Bacitracin is peptide antibiotic produced by some strains of *Bacillus licheniformis* and *Bacillus subtilis* which, by binding to Und-PP in the presence of divalent cations, prevents the Und-PP dephosphorylation and thus interrupts the recycling of Und-PP to Und-P (135, 136); thus it functions as an inhibitor of cell wall and O antigen biosynthesis. Similarly, colicin M has also an inhibitory effect on O antigen and peptidoglycan biosynthesis by preventing the recycling of Und-PP (137, 138). The biosynthesis of O polysaccharides that utilize the Wzy/Wzz-dependent pathway is severely affected by bacitracin. This may be reflected on the fact that this pathway involves one molecule of Und-PP per O repeating subunit. In contrast, the biosynthesis of ABC transport- and synthase-dependent O antigens requires the participation of only one Und-P molecule per polymer chain. Consequently, Und-PP recycling is not a limiting factor in these cases and the process is more resistant to bacitracin (94). In some Gram-negative bacteria, mutations that block the synthesis of exopolysaccharides also lead to bacitracin resistance, presumably by increasing intracellular levels of a lipid kinase, the product of the *bacA* gene (139, 140). This kinase presumably increases the level of Und-P by phosphorylating isoprenyl alcohol (140), but its precise function has not been conclusively established. The *bacA* gene was first identified in *Escherichia coli* (139) and homologues of the BacA protein are common in Gram-negative and Gram-positive bacteria (141). The enzyme or enzymes targeted by bacitracin, which carry out the Und-PP dephosphorylation reaction, have not been identified. Conceivably, these enzymes must be located in the plasma membrane and they may also have an active site facing the periplasmic space (figure 5).

The recycling of pyrophosphoryl polyisoprenols is not only confined to bacteria. In eukaryotes and also in Archaea, dolichol phosphate (Dol-P) serves as a lipid anchor for the assembly of glycan moieties that are further added to proteins (Figure 2; 80, 81). Following the transfer of the glycan to the glycosylation site of the protein, Dol-PP is released and recycled by dephosphorylation to Dol-P (142, 143). Mutations in the *cwh8* gene, which encodes an ER transmembrane protein with a phosphate binding pocket in *Saccharomyces cerevisiae*, result in a deficiency

in Dol-P-P-linked oligosaccharide intermediate synthesis and protein *N*-glycosylation (144). Overexpression of the Cwh8 protein in a yeast double mutant strain, unable to express the phosphatases Lpp1 and Dpp1, resulted in a dramatic increase in Dol-PP phosphatase activity in microsomal fractions (143). Topological studies using different approaches indicate that Cwh8 is a transmembrane protein with a luminally oriented active site (143). Thus, the specificity, subcellular location, and topological orientation of this novel enzyme are consistent with a role in the re-utilization of the glycosyl carrier lipid for additional rounds of lipid intermediate biosynthesis after its release during protein *N*-glycosylation reactions. Although a similar enzyme has not been directly characterized in bacteria, homologues of Cwh8 are widely conserved in prokaryotes, suggesting they may have a similar function in the recycling of Und-PP. In addition, it is not known how the recycled Und-P from released Und-PP traverses the plasma membrane from its periplasmic face (or from the ER luminal face in the case of Dol-P) to its cytosolic face where it can serve as a substrate for the initiation enzymes.

4. CELL SURFACE LOCALIZATION OF LPS

Virtually no information is available on the mechanism(s) for LPS cell surface localization and remarkably, this has remained as an open question for more than forty years. It has been reported in *Salmonella* that newly synthesized LPS molecules appear on the cell surface at a limited number of sites (145, 146). These observations implicated the participation of structures known as zones of adhesion (or Bayer junctions), which were first described in electron micrographs of *E. coli* (147) and interpreted as regions where the plasma and outer membranes come into apposition. The existence of these structures has been controversial (148, 149), especially given the fact that their appearance depends on the microscopic technique used. However, biochemical fractionation studies isolated differentiated membrane domains from *Escherichia coli* and *Salmonella typhimurium*, including a fraction containing components from both plasma and outer membranes (150), which presumably corresponded to the attachment sites. Pulse-chase experiments revealed that this intermediate fraction contained newly synthesized precursors for LPS, murein and outer membrane proteins (150). Using a similar fractionation method, we have been able to detect WecA in the intermediate fractions containing components from both membranes (L. Tatar and M. A. Valvano, unpublished observations). Therefore, it is possible that the adhesion zones represent areas whereby the translocation machinery for these molecules effectively spans the plasma membrane and creates continuity between a biosynthesis complex and the outer membrane. This continuity has been proposed for the translocation of bacteriophages, vitamins, colicins and ferrisiderophore molecules, which bind to the surface of specific outer membrane protein receptors and are ultimately translocated across the outer membrane with the participation of TonB and TolA proteins (for recent reviews see 151, 152). Also, current models for the assembly and translocation of capsular polysaccharides invoke multiprotein complexes across the periplasm comprising proteins involved in the translocation process (reviewed in 39, 153).

We recently discovered that the TolA protein plays a critical role in the surface expression of O7 LPS by an as yet uncharacterized involvement in the processing of O antigen (154). Indeed, a defect in the expression of TolA was associated with reduced surface expression of O7 LPS, abnormal accumulation of radiolabelled O7 LPS precursors in the plasma membrane, and dramatic effects in growth rate and cell morphology (154). The Tol import system comprises a multi-protein complex involved in the translocation of group A colicins and filamentous bacteriophages (155, 156). The Tol proteins are encoded by a cluster of 7 genes, *orf1-tolQRAB-pal-orf2*, organized into two transcription units (*orf1-tolQRA*, and *tolB-pal-orf2*)(156-158). Extensive studies have resulted in the identification and localization of Tol proteins. TolA, TolQ, and TolR are associated with the plasma membrane (159, 160). TolA has a large periplasmic domain (160, 161), and biochemical studies have shown specific interactions between the central part of TolA and outer membrane porins (162). The recent report of an interaction between TolA and the peptidoglycan-associated lipoprotein Pal, which is anchored to the periplasmic side of the outer membrane (157, 163) demonstrates for the first time a connection between inner membrane and outer membrane Tol components (164). Thus, it is possible that the Tol protein complex, especially TolA-Pal, plays an important role in the biogenesis of the outer membrane, by serving as a conduit for the proper assembly of newly synthesized outer membrane components (including LPS) on the bacterial cell surface. Using a system to reconstitute in *E. coli* K-12(O16) the synthesis of its own O antigen (90, 165), we recently constructed non polar deletions in various *tol-pal* genes and assessed their role in O16-specific LPS biosynthesis. The results indicate that deletion of *pal* has the same effect as the deletion of *tolA*. More importantly, the *tol-pal* block is only manifested in the biogenesis of the Wzy/Wzx-dependent O16 LPS but not in the synthesis of the ABC transporter-dependent *K. pneumoniae* O1 LPS (E. Vines and M. A. Valvano, unpublished data). Therefore, these findings suggest that the *tolA-pal* system is only required for surface expression of Wzx/Wzy-dependent O antigens. Since both Wzx/Wzy- and ABC transporter-dependent O antigens are ligated by WaaL, it is likely that the *tolA* and *pal* mutations are affecting the function of Wzx or the polymerization of the O antigen subunits at the periplasmic face of the plasma membrane.

5. AREAS FOR FUTURE RESEARCH

One of the major challenges for further research is the experimental demonstration and characterization of protein complexes involving the various components that participate in the biogenesis of O polysaccharides. Isolation of these complexes has been difficult in part because expressing membrane proteins like for instance Wzy, Wzx, and WaaL is not trivial. The use of epitope tags has worked in some cases but failed in others (C. L. Marolda, E. Vines, and M. A. Valvano, unpublished observations). The precise

function of Wzx requires devising an assay for the determination of transmembrane flipping. The use of Und-PP-GlcNAc as a single substrate may simplify this assay. Finally, the structural characterization of these proteins at the atomic level would greatly help understanding their function. But in practise this is a long-term approach that will require to successfully enhancing the expression of these proteins for purification. The precise role of polyisoprenoid lipids, their transmembrane movement, and in particular the recycling pathway of Und-PP would also add considerably to our current understanding of the biogenesis of O-specific polysaccharides.

6. ACKNOWLEDGEMENTS

Research in the author's laboratory is supported from grants by the Canadian Institutes of Health Research and the Natural Sciences and Engineering Research Council. The author holds a Canada Research Chair in Infectious Diseases and Microbial Pathogenesis.

7. REFERENCES

1. Nikaido, H: Outer Membrane. In: *Escherichia coli* and *Salmonella*: Cellular and molecular biology. Eds: Neidhardt F C, Curtiss R, Ingraham J L, Lin E C C, Low K B, Magasanik B, Reznikoff W S, Riley M, Schaechter M & Umbarger H E, American Society for Microbiology Press, Washington, D. C. 29-47 (1996)

2. Whitfield, C & M A Valvano: Biosynthesis and expression of cell-surface polysaccharides in gram-negative bacteria. *Adv Microb Physiol* 35: 135-246 (1993)

3. Raetz, C R H: Bacterial lipopolysaccharides: a remarkable family of bioactive molecules. In: *Escherichia coli* and *Salmonella*: cellular and molecular biology. Eds: Neidhardt F C, Curtiss III R, Ingraham J L, Lin E C C, Low K B, Magasanik B, Reznikoff W S, Riley M, Schaechter M & Umbarger H E, American Society for Microbiology Press, Washington, D. C. 1035-1063. (1996)

4. Heinrichs, D E, M A Valvano & C Whitfield: Biosynthesis and genetics of lipopolysaccharide core. In: Endotoxin in health and disease. Eds: Brade H, Morrison D C, Vogel S & Opal S, Marcel Dekker, Inc., New York, N.Y. 305-330 (1999)

5. Hancock, R E W, D N Karunaratne & C Bernegger-Egli: Molecular organization and structural role of outer membrane macromolecules. In: Bacterial cell wall. Vol. 27. Eds: Ghuysen J M & Hackenbeck R, Elsevier Science, Amsterdam, New York. 263-79 (1994)

6. Ferguson, A D, W Welte, E Hofmann, B Lindner, O Holst, J W Coulton & K Diederichs: A conserved structural motif for lipopolysaccharide recognition by procaryotic and eucaryotic proteins. *Structure Fold Des* 8: 585-592. (2000)

7. Nikaido, H & M Vaara: Molecular basis of bacterial outer membrane permeability. *Microbiol Rev* 49: 1-32. (1985)

8. Nikaido, H: Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* 264: 382-8 (1994)

9. Heinrichs, D E, J A Yethon & C Whitfield: Molecular basis for structural diversity in the core regions of the lipopolysaccharides of *Escherichia coli* and *Salmonella enterica*. *Mol Microbiol* 30: 221-232. (1998)

10. Whitfield, C: Biosynthesis of lipopolysaccharide O antigens. *Trends Microbiol* 3: 178-185. (1995)

11. Rick, P D & R P Silver: Enterobacterial common antigen and capsular polysaccharides. In: *Escherichia coli* and *Salmonella*: Cellular and molecular biology. Eds: Neidhardt F C, Curtiss III R, Ingraham J L, Lin E C C, Low K B, Magasanik B, Reznikoff W S, Riley M, Schaechter M & Umbarger H E, American Society for Microbiology Press, Washington, D. C. 104-122 (1996)

12. Brooke, J S & M A Valvano: Biosynthesis of inner core lipopolysaccharide in enteric bacteria identification and characterization of a conserved phosphoheptose isomerase. *J Biol Chem* 271: 3608-3614. (1996)

13. Valvano, M A: Biosynthesis and genetics of ADP-heptose. *J Endotoxin Res* 5: 90-95. (1999)

14. Valvano, M A, C L Marolda, M Bittner, M Glaskin-Clay, T L Simon & J D Klena: The *rfaE* gene from *Escherichia coli* encodes a bifunctional protein involved in the biosynthesis of the lipopolysaccharide core precursor ADP-L-*glycero*-D-*manno*-heptose. *J Bacteriol* 182: 488-497. (2000)

15. Schnaitman, C A & J D Klena: Genetics of lipopolysaccharide biosynthesis in enteric bacteria. *Microbiol Rev* 57: 655-682 (1993)

16. Keenleyside, W J & C Whitfield: Genetics and biosynthesis of lipopolysaccharide O-antigens. In: Endotoxin in Health and Disease. Eds: Brade H, Morrison D C, Vogel S & Opal S, Marcel Dekker, Inc., New York 331-358 (1999)

17. Reeves, P R, M Hobbs, M A Valvano, M Skurnik, C Whitfield, D Coplin, N Kido, J Klena, D Maskell, C R H Raetz & P D Rick: Bacterial polysaccharide synthesis and gene nomenclature. *Trends Microbiol* 4: 495-503 (1996)

18. Tamaki, S, T Sato & M Matsuhashi: Role of lipopolysaccharides in antibiotic resistance and bacteriophage adsorption of *Escherichia coli* K-12. *J Bacteriol* 105: 968-975. (1971)

19. Curtiss, R, J Charamella, D R Stallions & J A Mays: Parental functions during conjugation. *Bacteriol Rev* 32: 320-348. (1968)

20. Havekes, L M, B J J Lugtenberg & W P M Hoekstra: Conjugation deficient *E. coli* K-12 F- mutants with

heptose-less lipopolysaccharide. *Mol Gen Genet* 146: 43-50. (1976)

21. Sherburne, C & D E Taylor: Effect of lipopolysaccharide mutations on recipient ability of *Salmonella typhimurium* for incompatibility group H plasmids. *J Bacteriol* 179: 952-955 (1997)

22. van Alphen, W, B Lugtenberg & W Berendsen: Heptose-deficient mutants of *Escherichia coli* K12 deficient in up to three major outer membrane proteins. *Mol Gen Genet* 147: 263-269 (1976)

23. Vakharia, H & R Misra: A genetic approach for analysing surface-exposed regions of the OmpC protein of *Escherichia coli* K-12. *Mol Microbiol* 19: 881-889 (1996)

24. Verkleij, A J, E J Lugtenberg & P H Ververgaert: Freeze etch morphology of outer membrane mutants of *Escherichia coli* K12. *Biochim Biophys Acta* 426: 581-586. (1976)

25. Bayer, M E, J Koplow & H Goldfine: Alterations in envelope structure of heptose-deficient mutants of *Escherichia coli* as revealed by freeze-etching. *Proc Natl Acad Sci USA* 72: 5145-5149. (1975)

26. Koplow, J & H Goldfine: Alterations in the outer membrane of the cell envelope of heptose-deficient mutants of *Escherichia coli*. *J Bacteriol* 117: 527-543. (1974)

27. Fralick, J A & L L Burns-Keliher: Additive effect of *tolC* and *rfa* mutations on the hydrophobic barrier of the outer membrane of *Escherichia coli* K-12. *J Bacteriol* 176: 6404-6406 (1994)

28. Koronakis, V, E Koronakis, J Li & K Stauffer: Structure of TolC, the outer membrane component of the bacterial type I efflux system, derived from two-dimensional crystals. *Mol Microbiol* 23: 617-626. (1997)

29. Yethon, J A, E Vinogradov, M B Perry & C Whitfield: Mutation of the lipopolysaccharide core glycosyltransferase encoded by waaG destabilizes the outer membrane of Escherichia coli by interfering with core phosphorylation. *J Bacteriol* 182: 5620-5623. (2000)

30. Yethon, J A & C Whitfield: Purification and characterization of WaaP from *Escherichia coli*, a lipopolysaccharide kinase essential for outer membrane stability. *J Biol Chem* 276: 5498-5504. (2001)

31. Riley, L W, L N Junio, L B Libaek & G K Schoolnik: Plasmid-encoded expression of lipopolysaccharide O-antigenic polysaccharide in enteropathogenic *Escherichia coli*. *Infect Immun* 55: 2052-2056 (1987)

32. Kopecko, D J, O Washington & S B Formal: Genetic and physical evidence for plasmid control of *Shigella sonnei* form I cell surface antigen. *Infect Immun* 29: 207-214 (1980)

33. Sturm, S, B Jann, K Jann, P Fortnagel & K N Timmis: Genetic and biochemical analysis of *Shigella dysenteriae* 1 O antigen polysaccharide biosynthesis in *Escherichia coli* K-12: 9 kb plasmid of *S. dysenteriae* 1 determines addition of a galactose residue to the lipopolysaccharide core. *Microb Pathog* 1: 299-306. (1986)

34. Keenleyside, W J & C Whitfield: Lateral transfer of *rfb* genes: a mobilizable ColE1-type plasmid carries the rfbO:54 (O:54 antigen biosynthesis) gene cluster from *Salmonella enterica* serovar Borreze. *J Bacteriol* 177: 5247-5253 (1995)

35. Whitfield, C, P A Amor & R Koplin: Modulation of the surface architecture of gram-negative bacteria by the action of surface polymer:lipid A-core ligase and by determinants of polymer chain length. *Mol Microbiol* 23: 629-638. (1997)

36. Daniels, C & R Morona: Analysis of *Shigella flexneri wzz* (Rol) function by mutagenesis and cross-linking: Wzz is able to oligomerize. *Mol Microbiol* 34: 181-194. (1999)

37. Morona, R, L van den Bosch & P A Manning: Molecular, genetic, and topological characterization of O-antigen chain length regulation in *Shigella flexneri. J Bacteriol* 177: 1059-1068. (1995)

38. Raetz, C R H & C Whitfield: Lipopolysaccharide endotoxins. *Annu Rev Biochem* 71: 635-700. (2002)

39. Whitfield, C & I S Roberts: Structure, assembly and regulation of expression of capsules in *Escherichia coli*. *Mol Microbiol* 31: 1307-1319. (1999)

40. Valvano, M A, P Messner & P Kosma: Novel pathways for biosynthesis of nucleotide-activated glycero-manno-heptose precursors of bacterial glycoproteins and cell surface polysaccharides. *Microbiology* 148: 1979-1989. (2002)

41. Anderson, M S, S S Eveland & N P Price: Conserved cytoplasmic motifs that distinguish sub-groups of the polyprenol phosphate:*N*-acetylhexosamine-1-phosphate transferase family. *FEMS Microbiol Lett* 191: 169-175. (2000)

42. Dal Nogare, A R & M A Lehrman: Conserved sequences in enzymes of the UDP-GlcNAc/MurNAc family are essential in hamster UDP-GlcNAc:dolichol-P GlcNAc-1-P transferase. *Glycobiology* 8: 625-632. (1988)

43. Lehrman, M A: A family of UDP-GlcNAc/MurNAc: polyisoprenol-P GlcNAc/MurNAc-1-P transferases. *Glycobiology* 4: 768-771. (1994)

44. Rick, P D, G L Hubbard & K Barr: Role of the *rfe* gene in the synthesis of the O8 antigen in Escherichia coli K-12. *J Bacteriol* 176: 2877-84 (1994)

45. Alexander, D C & M A Valvano: Role of the *rfe* gene in the biosynthesis of the *Escherichia coli* O7-specific

lipopolysaccharide and other O-specific polysaccharides containing *N*-acetylglucosamine. *J Bacteriol* 176: 7079-7084 (1994)

46. Heifetz, A, R W Keenan & A D Elbein: Mechanism of action of tunicamycin on the UDP-GlcNAc:dolichyl phosphate GlcNAc-1-phosphate transferase. *Biochemistry* 18: 2186–2192. (1979)

47. Brandish, P, K Kimura, M Inukai, R Southgate, J Lonsdale & T Bugg: Modes of action of tunicamycin, liposidomycin B, and mureidomycin A: inhibition of phospho-*N*-acetylmuramyl-pentapeptide translocase from *Escherichia coli*. *Antimicrob Agents Chemother* 40: 1640-1644 (1996)

48. Mahoney, W C & D Duksin: Biological activities of the two major components of tunicamycin. *J Biol Chem* 254: 6572-6576. (1979)

49. Meier-Dieter, U, R Starman, K Barr, H Mayer & P D Rick: Biosynthesis of enterobacterial common antigen in *Escherichia coli*. *J Biol Chem* 265: 13490-13497. (1990)

50. Klena, J D & C A Schnaitman: Function of the *rfb* gene cluster and the *rfe* gene in the synthesis of O antigen by *Shigella dysenteriae* 1. *Mol Microbiol* 9: 393-402 (1993)

51. Yao, Z & M A Valvano: Genetic analysis of the O-specific lipopolysaccharide biosynthesis region (*rfb*) of *Escherichia coli* K-12 W3110: identification of genes that confer group 6 specificity to *Shigella flexneri* serotypes Y and 4a. *J Bacteriol* 176: 4133-4143 (1994)

52. Wang, L, S Huskic, A Cisterne, D Rothemund & P R Reeves: The O-antigen gene cluster of *Escherichia coli* O55:H7 and identification of a new UDP-GlcNAc C4 epimerase gene. *J Bacteriol* 184: 2620-2620. (2002)

53. Zhang, L, J Radziejewska-Lebrecht, D Krajewska-Pietrasik, P Toivanen & M Skurnik: Molecular and chemical characterization of the lipopolysaccharide O-antigen and its role in the virulence of *Yersinia enterocolitica* serotype O:8. *Mol Microbiol* 23: 63-76. (1997)

54. Wang, L & P R Reeves: Organization of the *Escherichia coli* O157 O antigen cluster and identification of its specific genes. *Infect Immun* 66: 3545-3551 (1998)

55. Clarke, B R, D Bronner, W J Keenleyside, W B Severn, J C Richards & C Whitfield: Role of Rfe and RfbF in the initiation of biosynthesis of D-galactan I, the lipopolysaccharide O antigen from *Klebsiella pneumoniae* serotype O1. *J Bacteriol* 177: 5411-5418 (1995)

56. Szabo, M, D Bronner & C Whitfield: Relationships between rfb gene clusters required for biosynthesis of identical D-galactose-containing O antigens in Klebsiella pneumoniae serotype O1 and Serratia marcescens serotype O16. *Journal of Bacteriology* 177: 1544-53 (1995)

57. Keenleyside, W J, M Perry, L Maclean, C Poppe & C Whitfield: A plasmid-encoded rfbO:54 gene cluster is required for biosynthesis of the O:54 antigen in *Salmonella enterica* serovar Borreze. *Mol Microbiol* 11: 437-448. (1994)

58. Rocchetta, H L, L L Burrows, J C Pacan & J S Lam: Three rhamnosyltransferases responsible for assembly of the A-band D- rhamnan polysaccharide in *Pseudomonas aeruginosa*: a fourth transferase, WbpL, is required for the initiation of both A-band and B-band lipopolysaccharide synthesis. *Mol Microbiol* 28: 1103-1119. (1998)

59. Skurnik, M: Molecular genetics of *Yersinia* lipopolysaccharide. In: Genetics of bacterial polysaccharides. Eds: Goldberg J B, CRC Press, Boca Raton, FL. 23-51 (1999)

60. Ikeda, M, M Wachi, H K Jung, F Ishino & M Matsuhashi: The *Escherichia coli mraY* gene encoding UDP-*N*-acetylmuramoyl-pentapeptide: undecaprenyl-phosphate phospho-*N*-acetylmuramoyl-pentapeptide transferase. *J Bacteriol* 173: 1021-1026. (1991)

61. Bouhss, A, D Mengin-Lecreulx, D Le Beller & J Van Heijenoort: Topological analysis of the MraY protein catalysing the first membrane step of peptidoglycan synthesis. *Mol Microbiol* 34: 576-585. (1999)

62. Amer, A O & M A Valvano: Conserved aspartic acids are essential for the enzymatic activity of the WecA protein initiating the biosynthesis of O-specific lipopolysaccharide and enterobacterial common antigen in *Escherichia coli*. *Microbiology* 148: 571-582. (2002)

63. Amer, A O & M A Valvano: The N-terminal region of the *Escherichia coli* WecA (Rfe) protein containing three predicted transmembrane helices is required for function but not for membrane insertion. *J Bacteriol* 182: 498-503. (2000)

64. Amer, A O & M A Valvano: Conserved amino acid residues found in a predicted cytosolic domain of WecA (UDP-*N*-acetyl glucosamine:undecaprenol-phosphate *N*-acetylglucosamine-1-phosphate transferase) are implicated in the recognition of UDP-*N*-acetylglucosamine. *Microbiology* 147: 3015-3025. (2001)

65. Allingham, J S, P A Pribil & D B Haniford: All three residues of the Tn*10* transposase DDE catalytic triad function in divalent metal ion binding. *J Mol Biol* 289: 1195-1206. (1999)

66. Sekine, S, A Shimada, O Nureki, J Cavarelli, D Moras, D G Vassylyev & S Yokoyama: Crucial Role of the HIGH-loop Lysine for the Catalytic Activity of Arginyl-tRNA Synthetase. *J Biol Chem* 276: 3723-3726 (2001)

67. Jiang, X M, B Neal, F Santiago, S J Lee, L K Romana & P R Reeves: Structure and sequence of the rfb (O antigen) gene cluster of *Salmonella serovar* typhimurium (strain LT2) *Mol Microbiol* 5: 695-713. (1991)

68. Arakawa, Y, R Wacharotayankun, T Nagatsula, H Ito, N Kato & M Ohta: Genomic organization of the *Klebsiella pneumoniae cps* region responsible for serotype K2 capsular polysaccharide synthesis in the virulent strain Chedid. *J Bacteriol* 177: 1788–1796. (1995)

69. Bugert, P & K Geider: Molecular analysis of the *ams* operon required for exopolysaccharide synthesis in *Erwinia amylovora*. *Mol Microbiol* 15: 917-933 (1995)

70. Stevenson, G, K Andrianopoulos, M Hobbs & P R Reeves: Organization of the Escherichia coli K-12 gene cluster responsible for production of the extracellular polysaccharide colanic acid. *J Bacteriol* 178: 4885-4893 (1996)

71. Katzen, F, D U Ferreiro, C G Oddo, M V Ielmini, A Becker, A Puhler & L Ielpi: *Xanthomonas campestris* pv. *campestris gum* mutants: effects on xanthan biosynthesis and plant virulence. *J Bacteriol* 180: 1607-1617. (1998)

72. Reuber, T L & G C Walker: Biosynthesis of succinoglycan, a symbiotically important exopolysaccharide of *Rhizobium meliloti*. *Cell* 74: 269-280. (1993)

73. Wang, L, D Liu & P R Reeves: C-terminal half of *Salmonella enterica* WbaP (RfbP) is the galactosyl-1-phosphate transferase domain catalyzing the first step of O-antigen synthesis. *J Bacteriol* 178: 2598-2604. (1996)

74. Campbell, J A, G J Davies, V Bulone & B Henrissat: A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. *Biochem J* 326: 929-939. (1997)

75. McGrath, B C & M J Osborn: Localization of the terminal steps of O-antigen synthesis in *Salmonella typhimurium*. *J Bacteriol* 173: 649-654 (1991)

76. Mulford, C A & M J Osborn: An intermediate step in translocation of lipopolysaccharide to the outer membrane of *Salmonella typhimurium*. *Proc Natl Acad Sci U S A* 80: 1159-1163. (1983)

77. Marino, P A, B C McGrath & M J Osborn: Energy dependence of O-antigen synthesis in *Salmonella typhimurium*. *J Bacteriol* 173: 3128-3133 (1991)

78. Bronner, D, B R Clarke & C Whitfield: Identification of an ATP-binding cassette transport system required for translocation of lipopolysaccharide O-antigen side-chains across the cytoplasmic membrane of *Klebsiella pneumoniae* serotype O1. *Mol Microbiol* 14: 505-519. (1994)

79. Clarke, B R & C Whitfield: Molecular cloning of the rfb region of *Klebsiella pneumoniae* serotype O1:K20: the *rfb* gene cluster is responsible for synthesis of the D-galactan I O polysaccharide. *J Bacteriol* 174: 4614-21 (1992)

80. Bugg, T D & P E Brandish: From peptidoglycan to glycoproteins: common features of lipid-linked oligosaccharide biosynthesis. *FEMS Microbiol Lett* 119: 255-262. (1994)

81. Burda, P & M Aebi: The dolichol pathway of *N*-linked glycosylation. *Biochim Biophys Acta* 1426: 239-257. (1999)

82. Hirschberg, C B & M D Snider: Topography of glycosylation in the rough endoplasmic reticulum and Golgi apparatus. *Annu Rev Biochem* 54: 63-87 (1987)

83. McCloskey, M A & F A Troy: Paramagnetic isoprenoid carrier lipids. 1. Chemical synthesis and incorporation into model membranes. *Biochemistry* 19: 2061-2066 (1980)

84. Hanover, J A & W J Lennarz: The topological orientation of N,N'-diacetylchitobiosylpyrophosphoryldolichol in artificial and natural membranes. *J Biol Chem* 254: 9237-9246 (1978)

85. Rush, J S & C J Waechter: Transmembrane movement of a water-soluble analogue of mannosylphosphoryldolichol is mediated by an endoplasmic reticulum protein. *J Cell Biol* 130: 529-536 (1995)

86. Rush, J S, K van Leyen, O Ouerfelli, B Wolucka & C J Waechter: Transbilayer movement of Glc-P-dolichol and its function as a glucosyl donor: protein-mediated transport of a water-soluble analog into sealed ER vesicles from pig brain. *Glycobiology* 8: 1195-1205. (1998)

87. Liu, D, R A Cole & P R Reeves: An O-antigen processing function for Wzx (RfbX): A promising candidate for O-unit flippase. *J Bacteriol* 178: 2102-2107. (1996)

88. Paulsen, I T, A M Beness & M H Saier: Computer-based analyses of the protein constituents of transport systems catalysing export of complex carbohydrates in bacteria. *Microbiology* 143: 2685-2699 (1997)

89. Marolda, C L, M F Feldman & M A Valvano: Genetic organization of the O7-specific lipopolysaccharide biosynthesis cluster of *Escherichia coli* VW187 (O7:K1) *Microbiology* 145: 2485-2496. (1999)

90. Feldman, M F, C L Marolda, M A Monteiro, M B Perry, A J Parodi & M A Valvano: The activity of a putative polyisoprenol-linked sugar translocase(Wzx) involved in *Escherichia coli* O antigen assembly is independent of the chemical structure of the O repeat. *J Biol Chem* 274: 35129-35138. (1999)

91. Bray, D & P W Robbins: The direction of chain growth in *Salmonella anatum* O-antigen biosynthesis. *Biochem Biophys Res Commun* 28: 334-339. (1967)

92. Robbins, P W, D Bray, B M Dankert & A Wright: Direction of chain growth in polysaccharide synthesis. *Science* 158: 1536-142. (1967)

93. Collins, L V, S Attridge & J Hackett: Mutations at rfc or pmi attenuate Salmonella typhimurium virulence for mice. *Infection & Immunity* 59: 1079-85 (1991)

94. Jann, K & B Jann: Structure and biosynthesis of O antigens. In: Handbook of Endotoxin vol 1: Chemistry of endotoxin. Eds: Rietschel E T, Elsevier Science Publishers, Amsterdam, The Netherlands 138-186 (1984)

95. Morona, R, M Mavris, A Fallarino & P A Manning: Characterization of the *rfc* region of *Shigella flexneri. J Bacteriol* 176: 733-747. (1994)

96. Daniels, C, C Vindurampulle & R Morona: Overexpression and topology of the *Shigella flexneri* O-antigen polymerase (Rfc/Wzy) *Mol Microbiol* 28: 1211-1222. (1998)

97. Batchelor, R A, G E Haraguchi, R A Hull & S I Hull: Regulation by a novel protein of the bimodal distribution of lipopolysaccharide in the outer membrane of *Escherichia coli*. *J Bacteriol* 173: 5699-5704. (1991)

98. Bastin, D A, G Stevenson, P K Brown, A Haase & P R Reeves: Repeat unit polysaccharides of bacteria: a model for polymerization resembling that of ribosomes and fatty acid synthetase, with a novel mechanism for determining chain length. *Mol Microbiol* 7: 725-734. (1993)

99. Rocchetta, H L, L L Burrows & J S Lam: Genetics of O-antigen biosynthesis in *Pseudomonas aeruginosa*. *Microbiol Mol Biol Rev* 63: 523-553. (1999)

100. Stevenson, G, A Kessler & P R Reeves: A plasmid-borne O-antigen chain length determinant and its relationship to other chain length determinants. *FEMS Microbiol Lett* 125: 23-30. (1995)

101. Morona, R, L Van Den Bosch & C Daniels: Evaluation of Wzz/MPA1/MPA2 proteins based on the presence of coiled-coil regions. *Microbiology* 146: 1-4. (2000)

102. Franco, A V, D Liu & P R Reeves: The wzz (cld) protein in *Escherichia coli*: amino acid sequence variation determines O-antigen chain length specificity. *J Bacteriol* 180: 2670-2675. (1998)

103. Helenius, J, D T Ng, C L Marolda, P Walter, M A Valvano & M Aebi: Translocation of lipid-linked oligosaccharides across the ER membrane requires Rft1 protein. *Nature* 415: 447-450. (2002)

104. Ng, D T W, E D Spear & P Walter: The unfolded protein response regulates multiple aspects of secretory and membrane protein biogenesis and endoplasmic reticulum quality control. *J Cell Biol* 150: 77-88 (2000)

105. Sprong, H, P van der Sluijs & G van Meer: How proteins move lipids and lipid move proteins. *Nat Rev Mol Cell Biol* 2: 504-513. (2001)

106. Davidson, E A & D C Gowda: Glycobiology of *Plasmodium falciparum*. *Biochimie* 83: 601-604. (2001)

107. Süsskind, M, L Brade, H Brade & O Holst: Identification of a novel heptoglycan of a1®2-linked D-*glycero*-D-*manno*-heptopyranose. Chemical and antigenic structure of lipopolysaccharides from Klebsiella pneumoniae ssp. pneumoniae rough strain R20 (O1-:K20-) *J Biol Chem* 273: 7006-7017. (1998)

108. Kido, N, V I Torgov, T Sugiyama, K Uchiya, H Sugihara, T Komatsu, N Kato & K Jann: Expression of the O9 polysaccharide of *Escherichia coli*: sequencing of the *E. coli* O9 *rfb* gene cluster, characterization of mannosyl transferases, and evidence for an ATP-binding cassette transport system. *J Bacteriol* 177: 2178-2187. (1995)

109. Guan, S, A J Clarke & C Whitfield: Functional analysis of the galactosyltransferases required for biosynthesis of D-galactan I, a component of the lipopolysaccharide O1 antigen of *Klebsiella pneumoniae*. *J Bacteriol* 183: 3318-3327. (2001)

110. Weisgerber, C & K Jann: Glucosyldiphosphoundecaprenol, the mannose acceptor in the synthesis of the O9 antigen of *Escherichia coli.* Biosynthesis and characterization. *Eur J Biochem* 127: 165-168. (1982)

111. Kido, N & H Kobayashi: A single amino acid substitution in a mannosyltransferase, WbdA, converts the *Escherichia coli* O9 polysaccharide into O9a: generation of a new O-serotype group. *J Bacteriol* 182: 2567-2573. (2000)

112. Kido, N, T Sugiyama, T Yokochi, H Kobayashi & Y Okawa: Synthesis of *Escherichia coli* O9a polysaccharide requires the participation of two domains of WbdA, a mannosyltransferase encoded within the *wb** gene cluster. *Mol Microbiol* 27: 1213-1221. (1998)

113. Reizer, J, A Reizer & M H Saier, Jr.: A new subfamily of bacterial ABC-type transport systems catalyzing export of drugs and carbohydrates. *Protein Sci* 1: 1326-1332. (1992)

114. Jansson, P E, J Lonngren, G Widmalm, K Leontein, K Slettengren, S B Svenson, G Wrangsell, A Dell & P R Tiller: Structural studies of the O-antigen polysaccharides of *Klebsiella* O5 and *Escherichia coli* O8. *Carbohydr Res* 145: 59-66. (1985)

115. Olsthoorn, M M, J Haverkamp & J E Thomas-Oates: Mass spectrometric analysis of *Klebsiella pneumoniae* ssp. pneumoniae rough strain R20 (O1-: K20-) lipopolysaccharide preparations: identification of novel core oligosaccharide components and three 3-*deoxy*-D-

manno-oct-2-ulopyranosonic artifacts. *J Mass Spectrom* 34: 622-36 (1999)

116. Vinogradov, E, E Frirdich, L L MacLean, M B Perry, B O Petersen, J O Duus & C Whitfield: Structures of Lipopolysaccharides from Klebsiella pneumoniae. Elucidation of the structure of the linkage region between core and polysaccharide O chain and identification of the residues at the non-reducing termini of the O chains. *J Biol Chem* 277: 25070-25081. (2002)

117. Vinogradov, E, M S Peppler & M B Perry: The structure of the nonreducing terminal groups in the O-specific polysaccharides from two strains of *Bordetella bronchiseptica*. *Eur J Biochem* 267: 7230-7237. (2000)

118. Preston, A, A G Allen, J Cadisch, R Thomas, K Stevens, C M Churcher, K L Badcock, J Parkhill, B Barrell & D J Maskell: Genetic basis for lipopolysaccharide O-antigen biosynthesis in bordetellae. *Infect Immun* 67: 3763-3767. (1999)

119. Keenleyside, W J & C Whitfield: A novel pathway for O-polysaccharide biosynthesis in *Salmonella enterica* serovar Borreze. *J Biol Chem* 271: 28581-28592. (1996)

120. Keenleyside, W J, A J Clarke & C Whitfield: Identification of residues involved in catalytic activity of the inverting glycosyl transferase WbbE from *Salmonella enterica* serovar borreze. *J Bacteriol* 183: 77-85. (2001)

121. DeAngelis, P L: Microbial glycosaminoglycan glycosyltransferases. *Glycobiology* 12: 9R-16R. (2002)

122. DeAngelis, P L: Hyaluronan synthases: fascinating glycosyltransferases from vertebrates, bacterial pathogens, and algal viruses. *Cell Mol Life Sci* 56: 670-682. (1999)

123. Cartee, R T, W T Forsee, J S Schutzbach & J Yother: Mechanism of type 3 capsular polysaccharide synthesis in *Streptococcus pneumoniae*. *J Biol Chem* 275: 3907-3914. (2000)

124. Forsee, W T, R T Cartee & J Yother: Biosynthesis of type 3 capsular polysaccharide in *Streptococcus pneumoniae.* Enzymatic chain release by an abortive translocation process. *J Biol Chem* 275: 25972-25978. (2000)

125. Klena, J D, R S Ashford & C A Schnaitman: Role of the *Escherichia coli* K-12 *rfa* genes and the *rfp* gene of *Shigella dysenteriae* 1 in generation of lipopolysaccharide core heterogeneity and attachment of O antigen. *J Bacteriol* 174: 7297-7307. (1992)

126. Zhou, Z, K A White, A Polissi, C Georgopoulos & C R Raetz: Function of *Escherichia coli* MsbA, an essential ABC family transporter, in lipid A and phospholipid biosynthesis. *J Biol Chem* 273: 12466-12475. (1998)

127. Doerrler, W T, M C Reedy & C R H Raetz: An *Escherichia coli* mutant defective in lipid export. *J Biol Chem* 276: 11461-11464. (2001)

128. Chang, G & C B Roth: Structure of MsbA from *E. coli*: A Homolog of the multidrug resistance ATP binding cassette (ABC) transporters. *Science* 293: 1793-1800. (2001)

129. Doerrler, W T & C R H Raetz: ATPase activity of the MsbA lipid flippase of *Escherichia coli*. *J Biol Chem* 277: 36697-36705. (2002)

130. Boucher, Y & W Ford Doolittle: The role of lateral gene transfer in the evolution of isoprenoid biosynthesis pathways. *Mol Microbiol* 37: 703-716. (2000)

131. White, R H: Biosynthesis of isoprenoids in bacteria. In: *Escherichia coli* and *Salmonella*: cellular and molecular biology. Eds: Neidhardt F C, Curtiss III R, Ingraham J L, Lin E C C, Low K B, Magasanik B, Reznikoff W S, Riley M, Schaechter M & Umbarger H E, American Society for Microbiology Press, Washington, D. C. 637-641. (1996)

132. Shimizu, N, T Koyama & K Ogura: Molecular cloning, expression, and purification of undecaprenyl diphosphate synthase. No sequence similarity between E- and Z-prenyl diphosphate synthases. *J Biol Chem* 273: 19476-19481. (1998)

133. Apfel, C M, B Takacs, M Fountoulakis, M Stieger & W Keck: Use of genomics to identify bacterial undecaprenyl pyrophosphate synthetase: cloning, expression, and characterization of the essential *uppS* gene. *J Bacteriol* 181: 483-492. (1999)

134. Goldman, R & J L Strominger: Purification and properties of C 55 -isoprenylpyrophosphate phosphatase from *Micrococcus lysodeikticus*. *J Biol Chem* 247: 5116-5122. (1972)

135. Storm, D R & J L Strominger: Complex formation between bacitracin peptides and isoprenyl pyrophosphates. The specificity of lipid-peptide interactions. *J Biol Chem* 248: 3940-3945. (1973)

136. Stone, K J & J L Strominger: Mechanism of action of bacitracin: complexation with metal ion and C 55-isoprenyl pyrophosphate. *Proc Natl Acad Sci U S A* 68: 3223-3227. (1971)

137. Harkness, R E & V Braun: Colicin M inhibits peptidoglycan biosynthesis by interfering with lipid carrier recycling. *J Biol Chem* 264: 6177-6182. (1989)

138. Harkness, R E & V Braun: Inhibition of lipopolysaccharide O-antigen synthesis by colicin M. *J Biol Chem* 264: 14716-22 (1989)

139. Cain, B D, P J Norton, W Eubanks, H S Nick & C M Allen: Amplification of the bacA gene confers bacitracin resistance to *Escherichia coli. J Bacteriol* 175: 3784-3789. (1993)

140. Pollock, T J, L Thorne, M Yamazaki, M J Mikolajczak & R W Armentrout: Mechanism of bacitracin

resistance in gram-negative bacteria that synthesize exopolysaccharides. *J Bacteriol* 176: 6229-6237. (1994)

141. Cao, M & J D Helmann: Regulation of the Bacillus subtilis bcrC bacitracin resistance gene by two extracytoplasmic function s factors. *J Bacteriol* 184: 6123-6129. (2002)

142. Abeijon, C & C B Hirschberg: Topography of glycosylation reactions in the endoplasmic reticulum. *Trends Biochem Sci* 17: 32-36 (1992)

143. Fernandez, F, J S Rush, D A Toke, G Han, J E Quinn, G M Carman, J-Y Choi, D R Voelker, M Aebi & C J Waechter: The *CWH8* gene encodes a dolichyl pyrophosphate phosphatase with a luminally oriented active site in the endoplasmic reticulum of *Saccharomyces cerevisiae*. *J Biol Chem* 276: 41455-41464. (2001)

144. van Berkel, M A, M Rieger, S te Heesen, A F Ram, H van den Ende, M Aebi & F M Klis. *Glycobiology* 9: 243-253 (1999)

145. Kulpa, C F, Jr. & L Leive: Mode of insertion of lipopolysaccharide into the outer membrane of *Escherichia coli*. *J Bacteriol* 126: 467-477. (1976)

146. Muhlradt, P F, J Menzel, J R Golecki & V Speth: Outer membrane of *Salmonella*. Sites of export of newly synthesised lipopolysaccharide on the bacterial surface. *Eur J Biochem* 35: 471-481. (1973)

147. Bayer, M E: Areas of adhesion between wall and membrane of *Escherichia coli*. *J Gen Microbiol* 53: 395-404. (1968)

148. Kellenberger, E: The 'Bayer bridges' confronted with results from improved electron microscopy methods. *Mol Microbiol* 4: 697-705. (1990)

149. Bayer, M E: Zones of membrane adhesion in the cryofixed envelope of *Escherichia coli*. *J Struct Biol* 107: 268-280 (1991)

150. Ishidate, K, E S Creeger, J Zrike, S Deb, B Glauner, T J MacAlister & L I Rothfield: Isolation of differentiated membrane domains from *Escherichia coli* and *Salmonella typhimurium*, including a fraction containing attachment sites between the inner and outer membranes and the murein skeleton of the cell envelope. *J Biol Chem* 261: 428-443. (1986)

151. Braun, V & M Braun: Active transport of iron and siderophore antibiotics. *Curr Opin Microbiol* 5: 194-201. (2002)

152. Lloubes, R, E Cascales, A Walburger, E Bouveret, C Lazdunski, A Bernadac & L Journet: The Tol-Pal proteins of the *Escherichia coli* cell envelope: an energized system required for outer membrane integrity? *Res Microbiol* 152: 523-529. (2001)

153. Bliss, J M & R P Silver: Coating the surface: a model for expression of capsular polysialic acid in *Escherichia coli* K1. *Mol Microbiol* 21: 221-231. (1996)

154. Gaspar, J A, J A Thomas, C L Marolda & M A Valvano: Surface expression of O-specific lipopolysaccharide in *Escherichia coli* requires the function of the TolA protein. *Mol Microbiol* 38: 262-275 (2000)

155. Webster, R E: The *tol* gene products and the import of macromolecules into *Escherichia coli. Mol Microbiol* 5: 1005-1011. (1991)

156. Lazdunski, C J, E Bouveret, A Rigal, L Journet, R Lloubès & H Bénédetti: Colicin Import into *Escherichia coli* Cells. *J Bacteriol* 180: 4993-5002 (1998)

157. Vianney, A, M M Muller, T Clavel, J C Lazzaroni, R Portalier & R E Webster: Characterization of the *tol-pal* region of *Escherichia coli* K-12: Translational control of *tolR* expression by TolQ and identification of a new open reading frame downstream of *pal* encoding a periplasmic protein. *J Bacteriol* 178: 4031-4038. (1996)

158. Lazzaroni, J C, P Germon, M C Ray & A Vianney: The Tol proteins of Escherichia coli and their involvement in the uptake of biomolecules and outer membrane stability. *FEMS Microbiol Lett* 177: 191-197 (1999)

159. Derouiche, R, H Benedetti, J C Lazzaroni, C Lazdunski & R Lloubes: Protein complex within *Escherichia coli* inner membrane. TolA N-terminal domain interacts with TolQ and TolR proteins. *J Biol Chem* 270: 11078-11084. (1995)

160. Guihard, G, P Boulanger, H Benedetti, R Lloubes, M Besnard & L Letellier: Colicin A and the Tol proteins involved in its translocation are preferentially located in the contact sites between the inner and outer membranes of *Escherichia coli* cells. *J Biol Chem* 269: 5874-5880. (1994)

161. Levengood, S K, W F Beyer & R E Webster: TolA: a membrane protein involved in colicin uptake contains an extended helical region. *Proc Natl Acad Sci U S A* 88: 5939-5943. (1991)

162. Derouiche, R, M Gavioli, H Benedetti, A Prilipov, C Lazdunski & R Lloubes: Tol A central domain interacts with *Escherichia coli* porins. *EMBO J* 15: 6408-6415. (1996)

163. Clavel, T, P Germon, A Vianney, R Portalier & J C Lazzaroni: TolB protein of *Escherichia coli* K-12 interacts with the outer membrane peptidoglycan-associated proteins Pal, Lpp and OmpA. *Mol Microbiol* 29: 359-367 (1998)

164. Cascales, E, M Gavioli, J N Sturgis & R Lloubés: Proton motive force drives the interaction of the inner membrane TolA and outer membrane Pal proteins in *Escherichia coli*. *Mol Microbiol* 38: 904-915. (2000)

165. Liu, D & P R Reeves: *Escherichia coli* K12 regains its O antigen. *Microbiology* 140: 49-57. (1994)

166. Bengoechea, J A, E Pinta, T Salminen, C Oertelt, O Holst, J Radziejewska-Lebrecht, Z Piotrowska-Seget, R Venho & M Skurnik: Functional Characterization of Gne (UDP-N-Acetylglucosamine- 4-Epimerase), Wzz (Chain Length Determinant), and Wzy (O-Antigen Polymerase) of *Yersinia enterocolitica* Serotype O:8. *J Bacteriol* 184: 4277-4287. (2002)

167. Helenius, J & M Aebi: Transmembrane movement of dolichol linked carbohydrates during *N*-glycoprotein biosynthesis in the endoplasmic reticulum. *Semin Cell Dev Biol* 13: 171-178. (2002)

168. Knauer, R & L Lehle: The oligosaccharyltransferase complex from yeast. *Biochim Biophys Acta* 1426: 259-273. (1999)

169. Yan, Q & W J Lennarz: Oligosaccharyltransferase: a complex multisubunit enzyme of the endoplasmic reticulum. *Biochem Biophys Res Commun* 266: 684-689. (1999)

170. Parodi, A J: Protein glucosylation and its role in protein folding. *Annu Rev Biochem* 69: 69-93. (2000)

171. Sonnhammer, E L L, G von Heijne & A Krogh: A hidden Markov model for predicting transmembrane helices in protein sequences. In: Proceedings of Sixth International Conference on Intelligent Systems for Molecular Biology. Eds: Glasgow J, Littlejohn T, Major F, Lathrop R, Sankoff D & Sensen C, AAAI Press, Menlo Park, Ca 175-182. (1998)

172. Cserzo, M, E Wallin, I Simon, G von Heijne & A Elofsson: Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the Dense Alignment Surface method. *Prot Eng* 10: 673-676 (1997)

Key Words: O antigen, flippase, translocation, polymerization, ligase, undecaprenol phosphate, ABC transporter, Review

Send correspondence to: Dr Miguel A. Valvano, Department of Microbiology and Immunology, The University of Western Ontario, Dental Sciences Building Room 3000, London, Ontario, N6A 5C1 Canada, Tel: 519-661-3996, Fax: 519-661-3499, E-mail: mvalvano@uwo.ca