**A Membrane-located Glycosyltransferase Complex Required for Biosynthesis of the D-Galactan I Lipopolysaccharide O Antigen in Klebsiella pneumoniae**

Veronica Kos and Chris Whitfield

From the Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

D-Galactan I is a polysaccharide with the disaccharide repeat unit structure \( [\text{3-}\beta-D-\text{Galp}(1\rightarrow3)-\alpha-D-\text{Galp}(1\rightarrow) ] \). This glyccan represents the lipopolysaccharide O antigen found in many Gram-negative bacteria, including several *Klebsiella pneumoniae* O serotypes. The polysaccharide is synthesized in the cytoplasm prior to its export via an ATP-binding cassette transporter. Sequence analysis predicts three galactosyltransferases in the D-galactan I genetic locus. They are WbbO (belonging to glycosyltransferase (GT) family 4), WbbM (GT-family 8), and WbbN (GT-family 2). The WbbO and WbbM proteins are each predicted to contain two domains, with the GT modules located toward their C termini. The N-terminal domains of WbbO and WbbM exhibit no similarity to proteins with known function. In *vivo* complementation assays suggest that all three glycosyltransferases are required for D-galactan I biosynthesis. Using a bacterial two-hybrid system and confirmatory co-purification strategies, evidence is provided for protein-protein interactions among the glycosyltransferases, creating a membrane-located enzyme complex dedicated to D-galactan I biosynthesis.

The O-PS polymer known as D-galactan I contains the disaccharide repeat unit structure: \( [\text{3-}\beta-D-\text{Galp}(1\rightarrow3)-\alpha-D-\text{Galp}(1\rightarrow) ] \) (Fig. 1) and is found in the LPS of several Gram-negative bacteria, including some *Klebsiella pneumoniae* O serotypes. It is the sole O-PS in *K. pneumoniae* O2a (2). In other serotypes, D-galactan I can be modified by O-acetylation (3, 4) or capped by an additional polymeric domain with a different repeat unit structure (5–7). Each of these modifications confers a unique O-serospecificity. Similar structures are found in other bacterial genera, including *Serratia* (8, 9), and the genetic loci for D-galactan I biosynthesis are conserved in organization and content (4).

There are two fundamentally different pathways by which most O-PSs are assembled: the Wzy-dependent pathway and the ATP-binding cassette (ABC) transporter-dependent pathway (1). D-Galactan I provides an example of ABC transporter-dependent biosynthesis. In the Enterobacteriaceae (and many other bacteria), the ABC transporter-dependent pathways begin with the synthesis of the polymer repeat units on an acceptor consisting of undecaprenol diphospho-N-acetylgalcosamine (und-PP-GlCNAC). The acceptor is synthesized by the UDP-N-acetylgalcosamine:Und-P GlCNAC-1-P transferase, WeCa (10–13). This requirement has been established for D-galactan-I (14). O-PS is elongated on this acceptor at the cytoplasmic face of the inner membrane by dedicated O-antigen-specific glycosyltransferases. The polymer is elongated by sequential glycosyltransfer to the non-reducing terminus. Chain extension in the ABC transporter-dependent pathways is then terminated by one of two remarkably different strategies. In a model exemplified by the polysialic acid O-PSs from *Escherichia coli* O8 and O9a, a residue not found in the repeat unit structure is added to the non-reducing terminus and this serves as an export signal (15–17). The completed O-PS structure is then exported across the membrane by the ABC transporter, which is composed of two Wzm (transmembrane domain) polypeptides, and two Wzt (nucleotide-binding domain) polypeptides. In *E. coli* O8/O9a, Wzt contains an additional carbohydrate-binding module that recognizes the precise export signal on the nascent polymer (16, 17). In contrast, in the biosynthesis of D-galactan I in *K. pneumoniae* O2a, chain termination is determined by an interaction between the transporter and the glycosyltransferases (or their product) in a manner that is not yet determined (18). There is no identifiable export signal on the polymer and, unlike the O8/O9a situation, the D-galactan I Wzt protein has no specificity for a particular polysaccharide structure. Regardless of the assembly pathway,
the completed O-PS is then ligated to lipid-A core at the periplasmic face of the membrane by the O-PS ligase. The mature LPS molecules are then shuttled to the outer membrane by the Lpt pathway (19).

Biosynthesis and export of the O-PS are predicted to require strict coordination of initiation, elongation, and export to maintain the specific O-PS chain length. Current models for O-PS biosynthesis invoke a coordinated multienzyme complex but experimental evidence for such complexes is generally limited. The goal of this study was to examine potential protein-protein interactions among enzymes required for D-galactan I biosynthesis. Bioinformatic analysis indicates that the *K. pneumoniae* D-galactan I biosynthesis gene cluster encodes three predicted galactosyltransferases, designated WbbM, WbbN, and WbbO, with confirmatory experimental evidence being available for WbbO and WbbM (20). The cluster also encodes the UDP-galactopyranose mutase (Glf) enzyme responsible for production of the UDP-Gal precursor (21) (see Fig. 1). Using a bacterial two-hybrid system and co-purification, interactions between the glycosyltransferases involved in D-galactan I biosynthesis have been confirmed, suggesting that they do indeed form a multienzyme complex.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**—The bacterial strains and plasmids used in this study are described in Table 1. Bacteria were grown at 37 or 30 °C in Luria-Bertani (LB) medium (22). Glucose (0.05–0.4% w/v), galactose (0.1% w/v), arabinose (0.002–0.2% w/v), or isopropyl 1-thio-β-D-galactopyranoside (0.5–1.0 mM) were added as required. The antibiotics ampicillin (100 μg/ml), chloramphenicol (34 μg/ml), kanamycin (50 μg/ml), and trimethoprim (100 μg/ml) were added where appropriate. For bacterial two-hybrid analyses, bromochloroindolyl galactopyranoside (X-gal; 40 μg/ml) was added to the medium.

**DNA Methods**—DNAzol reagent (Invitrogen) was used to purify chromosomal DNA. DNA fragments were PCR-amplified using *Pwo* polymerase (Roche Applied Science) with custom oligonucleotide primers (Sigma). Where appropriate, the primers included restriction sites to facilitate cloning (supplemental Table S1). Mutagenesis was carried out using complementary oligonucleotides designed to incorporate the desired base changes (supplemental Table S1). The procedure was based upon the QuikChange method (Stratagene). Mutagenesis of the desired base pairs was confirmed by sequencing (Guelph Molecular Super Center). DNA fragments from PCR or restriction enzyme digests were purified from agarose gels using the GeneElute gel extraction kit (Sigma). Plasmid DNA was purified using the GeneElute plasmid purification kit (Sigma). Restriction enzyme digests and DNA ligation reactions were performed according to the manufacturer’s instructions.

**Separation of Cellular Fractions**— Cultures were grown in 250 ml of LB medium at 37 °C. Genes encoding proteins of interest were cloned behind arabinose-inducible promoters in pBAD vectors (23). Protein expression was induced at mid-exponential growth phase (*A*₆₀₀ = 0.6) by the addition of 0.02% arabinose and the cultures were grown for an additional 2 h. Cells were collected by centrifugation and resuspended in 25 ml of buffer A (50 mM HEPES, 100 mM NaCl, pH 7.5), prior to lysis by passage through a French pressure cell. A cleared cell-free lysate was obtained by centrifugation at 4,000 × g for 10 min. This was followed by a second centrifugation step at 12,000 × g for 15 min. Centrifugation of the lysate at 100,000 × g for 60 min separated the membrane fraction (P100) from the soluble fraction (S100).

**SDS-PAGE and Western Immunoblot Analysis of Proteins**—Proteins were analyzed by SDS-PAGE using 12% resolving gels and visualized by Simply Blue stain (Invitrogen). Samples containing FLAG-α-, polyhistidine-, or glutathione S-transferase (GST)-tagged proteins were transferred to nitrocellulose membranes (PerkinElmer Life Science). Immunoblots were probed with a 1:2,000 dilution of anti-FLAG antibody (Sigma), anti-His₅ antibody (Qiagen), or anti-GST antibody (Novagen). Nitrocellulose membranes were then washed three times for 5 min in phosphate-buffered saline. This was followed by incubation for 1 h in a 1:3,000 dilution of horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories). Detection was carried out according to instructions supplied with the Western Lightning reagent (PerkinElmer).

**Preparation of WbbM-specific Antibody—** His₁₀-WbbM protein was purified from *E. coli* Top10 containing plasmid pWQ52 and protein expression was induced as described above using 0.2% arabinose. The cell pellet was then stored at −20 °C until required. Cell pellets were resuspended in buffer B (50 mM sodium phosphate, 500 mM NaCl, pH 7.5) containing the dissolved protease inhibitor tablets (Roche) and lysed by sonication. The lysate was cleared by centrifugation steps at 4,000 × g for 10 min and 12,000 × g for 15 min. The soluble fraction was then collected by further centrifugation at 100,000 × g for 1 h and His₁₀-WbbM was purified using His-Select Nickel Affinity Gel (Sigma). After sequential washes with buffer B and buffer C containing 10 mM imidazole and 25 mM imidazole, the protein was eluted from the column in buffer B containing 250 mM imidazole. The eluate containing His₁₀-WbbM protein was exchanged into buffer C (25 mM Tris-HCl, 100 mM NaCl, pH 7.5), using a PD-10 column (GE Healthcare) and concentrated using a Microcon YM-10 column (Millipore). Purified protein was mixed with Freund’s Incomplete Adjuvant (Sigma) in a 1:1 ratio prior to inoculation into New Zealand White rabbits at the Central Animal Facility at the University of Guelph. The antibody preparation was partially purified over a DEAE Affi-Gel Blue column (Bio-Rad) and nonspecific antibody was then removed by adsorption against whole nitrocellulose membrane-immobilized cell lysate of *E. coli* Top10. For Western blot analysis, anti-WbbM was used at a 1:2,000 dilution and detected with a 1:3,000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories).

**Co-purification Strategies to Detect Interacting Proteins**— Combinations of the putative galactosyltransferases bearing various epitope and purification tags were coexpressed in *E. coli* Top10. Protein expression was induced by the addition of 0.2% arabinose or 1 mM isopropyl 1-thio-β-D-galactopyranoside as described above. Cell pellets were collected and stored at
−20 °C overnight. Pellets were resuspended in 25 ml of buffer D (20 mM sodium phosphate buffer, 750 mM NaCl, 10% (v/v) glycerol, pH 7.5) and lysed by sonication. Triton X-100 (0.1% final concentration) was added to the lysate to release the typically membrane-associated glycosyltransferases into the soluble fraction. After centrifugation at 12,000 × g for 15 min, the supernatant was collected and mixed with the appropriate affinity resin. Mixing was performed for either 1 h at 4 °C for TALON metal affinity His-select resin (BD Biosciences), or overnight at 4 °C for GST affinity resin (Novagen). After binding, the resin was collected by centrifugation for 5 min at 800 × g and 10 volumes of buffer D containing 0.1% Triton X-100 was added and mixed at room temperature for 10 min. This wash step was repeated before the resin was loaded into a column. The relevant protein was then eluted using buffer D containing either 300 mM imidazole or 10 mM reduced glutathione. Eluates were collected by removing the resin in a final centrifugation step at 800 × g. Samples were collected and analyzed by SDS-PAGE and Western immunoblotting.

**Bacterial Two-hybrid Analysis**—The D-galactan I-biosynthesis enzymes were fused to the catalytic domains of adenylate cyclase from *Bordetella pertussis* via cloning into the BTH vec-

### Table 1: Bacterial strains and plasmids

| Strain/plasmid | Description or genotype | Ref. or source |
|---------------|-------------------------|---------------|
| BTH101       | F−, cya-854, recA1, endA1, gyrA96, thi-1, hsdR17, spoT1, rfbD1, glvIV44(AS); NalR | 24 |
| CWA286       | K-12, lac, trp, hsdR17, thy-1, mcrA, TOP100, recA1, araD139, ara-ara-leu7679, galK, galE, repSl(Sr'), endA1 | 21 |
| TOP10        | F−, merA, Δ(mrr-hudRM-scr-BC), 680, lacZAM15, Aac674, deoR, nupG, recA1, araD139, [ara-ara-leu7679, galK, galE, repSl(Sr'), endA1 | Invitrogen |
| pBAD24       | L-arabinose-inducible plasmid; ApR | 23 |
| pBAD33       | L-arabinose-inducible plasmid; CmR | 23 |
| pBAD322Tp    | L-arabinose-inducible plasmid; TpR | 49 |
| pGEXAT-3     | Cloning vector containing GST; ApR | Amersham Biosciences |
| pKT25        | Bacterial two-hybrid vector, encodes the T25 fragment of adenylate cyclase from *B. pertussis* S5 to a multiple cloning site | 24 |
| pUT18C       | Bacterial two-hybrid vector, encodes the T18 fragment of adenylate cyclase from *B. pertussis* S5 to a multiple cloning site | 24 |
| pKT25-zip    | pKT25 encoding the CyaA T25 fragment fused to the leucine zipper of the yeast GCN4 protein | 24 |
| pUT18C-zip   | pUT18C encoding the CyaA T18 fragment fused to the leucine zipper of the yeast GCN4 protein | 24 |
| pUT18-zip    | pUT18 encoding the CyaA T18 fragment fused to the leucine zipper of the yeast GCN4 protein | This study |
| pACYC184     | Cloning vector; CmR, TcR | 50 |
| pWQ288       | pACYC184 derivative containing a BamHI/Smal fragment encoding the D-galactan I cluster (wzzn, wezl, wbbm, glf, wbbn, wbbO) | 18 |
| pWQ289       | pACYC184 derivative containing a BamHI/Smal fragment encoding the D-galactan I biosynthesis genes (wbbm, glf, wbbn, and wbbO); CmR | 18 |
| pWQ290       | pBAD24 derivative containing wzzn-wezl; Ncol, Xbal | 18 |
| pWQ294       | pBAD24 derivative containing a CmR cassette | 16 |
| pWQ516       | pACYC184 derivative containing a BamHI/Smal fragment D-galactan I biosynthesis cluster with a wbb0 deletion | This study |
| pWQ517       | pWQ288 derivative containing a BamHI/Smal fragment D-galactan I biosynthesis cluster with a wbbM deletion | This study |
| pWQ548       | pWQ288 derivative containing a BamHI/Smal fragment D-galactan I biosynthesis cluster with a frameshift mutation in wbbn which results in a truncated version of WbbN (only the first 79 amino acids are expressed) | This study |
| pWQ520       | pBAD24 derivative encoding His-WbbO; ApR | This study |
| pWQ522       | pBAD24 derivative encoding His-WbbM; ApR | This study |
| pWQ523       | pBAD24 derivative encoding His-WbbN; ApR | This study |
| pWQ524       | pBAD24 derivative encoding WbbM; ApR | This study |
| pWQ525       | pBAD33 derivative encoding WbbM; CmR | This study |
| pWQ526       | pWQ284 derivative encoding FLAG-Wbbn; CmR | This study |
| pWQ527       | pBAD322Tp derivative encoding FLAG-WbbN; TpR | This study |
| pWQ528       | pBAD24 derivative encoding His-WbbO; residues 1–172; ApR | This study |
| pWQ529       | pBAD24 derivative encoding His-WbbO; residues 131–377; ApR | This study |
| pWQ530       | pBAD24 derivative encoding WbbnM-His-WbbO; residues 1–265; ApR | This study |
| pWQ531       | pBAD24 derivative encoding His-WbbM; residues 219–634; ApR | This study |
| pWQ532       | pBAD322Tp derivative encoding His-WbbM; residues 219–634; TpR | This study |
| pWQ542       | pGEXAT-3 containing WbbO fusion; ApR | This study |
| pWQ533       | pKT25 derivative encoding T25-WbbO; KmR | This study |
| pWQ534       | pKT25 derivative encoding T25-WbbM; KmR | This study |
| pWQ535       | pKT25 derivative encoding T25-WbbN; KmR | This study |
| pWQ536       | pKT25 derivative encoding T25-WbbO; KmR | This study |
| pWQ537       | pUT18C derivative encoding T18-WbbO; ApR | This study |
| pWQ519       | pUT18 derivative encoding WbbnO-T18; TpR | This study |
| pWQ539       | pUT18C derivative encoding T18-WbbO; ApR | This study |
| pWQ540       | pUT18C derivative encoding T18-WbbN; ApR | This study |
| pWQ541       | pUT18C derivative encoding WbbnT18; ApR | This study |
| pWQ543       | pUT18 derivative encoding WbbnT18; ApR | This study |
| pWQ544       | pUT18 derivative encoding WbbnT18; ApR | This study |
| pWQ545       | pUT18 derivative encoding WbbM-T18; ApR | This study |
| pWQ549       | pUT18 derivative encoding WbbnT18; ApR | This study |
| pWQ550       | pKT25 derivative encoding WbbO; KmR | This study |
| pWQ551       | pUT18C derivative encoding WbbO-T18; ApR | This study |
Organization of Glycosyltransferases in \( \delta \)-Galactan I Biosynthesis

RESULTS

Localization and Domain Analysis of WbbO—The first dedicated step in \( \delta \)-galactan I biosynthesis is catalyzed by WbbO. From both \textit{in vivo} and \textit{in vitro} experiments, WbbO is proposed overnight in antibiotic-supplemented LB broth. Protein expression was induced by the addition of arabinose. The \textit{galE}:\textit{::Tn10} insertion in CWG286 makes \( \delta \)-galactan I production conditional on addition of galactose to the growth medium (18). After arabinose (varying concentrations) and galactose (0.1%) were added to the medium, cultures were inoculated and then grown for \( \sim 2.5 \) h. Immunofluorescence microscopy of intact cells was performed as described previously, using \( \delta \)-galactan I-specific rabbit polyclonal antibodies (18). Detection was performed using rhodamine red-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories Inc.). Visualization was carried out using a Zeiss Axiostar 200 microscope using a \( \times 100 \) objective lens, and the images were processed using Openlab software (Improvision).

LPS samples were prepared using the whole cell lysate method of Hitchcock and Brown (25). Samples were separated using 15% polyacrylamide gels and visualized by silver staining, or by immunoblot analysis after transfer to nitrocellulose membrane (PerkinElmer Life Sciences). Immunoblots were probed with absorbed polyclonal rabbit anti-\( \delta \)-galactan I antibody, as previously described (18). The secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit antibody (Jackson Immunoresearch Laboratories).

Computer Analysis—Secondary structure predictions of protein sequences were performed using the programs Prof, Predictprotein, PSIPred, and Jpred (26–29). A domain linker prediction for WbbO is illustrated in Figure 1B, using a support vector machine (SVM) was used to determine ideal locations to make domain constructs of the protein.

FIGURE 2. Secondary structure prediction and localization of His\(_{10}\)-WbbO. A, sequence and predicted secondary structure of WbbO outlining the \( \beta \)-sheets (black arrows) and \( \alpha \)-helices (black boxes). The GT-4 domain (residues 131–377) is identified, as is a putative linker region that may separate two independent domains (WbbO\(^{A}\) and WbbO\(^{C}\)). B, cellular localization of the His\(_{10}\)-WbbO protein (pWQ282) and its two domains, His\(_{10}\)-WbbO\(^{A}\) (pWQ282) and His\(_{10}\)-WbbO\(^{C}\) (pWQ529), expressed in \( E. \) coli CWG286. Cell-free lysates (S12) were separated into soluble (S100) and membrane-containing (P100) fractions and the His\(_{10}\)-tagged proteins were detected using mouse anti-His\(_{10}\) monoclonal antibody. His\(_{10}\)-WbbO\(^{A}\) could not be detected using anti-His\(_{10}\) antibodies, so the SDS-PAGE Simply Blue-stained gel showing the complete localization is illustrated. The \( \textit{arrow} \) indicates a band present in the whole cell lysate and subsequent fractions, which is found only in cells containing pWQ529; the band corresponds to the predicted size of His\(_{10}\)-WbbO\(^{C}\) (29.0 kDa). Sample loading was standardized to the original cell suspension (prior to lysis), allowing direct visual assessment of the cellular distribution of His\(_{10}\)-WbbO.
to add the first two sugars (Galp-Galp) to the und-PP-GlcNAc acceptor (14, 20), forming the adaptor region of the O-PS (Fig. 1). Based on its sequence, WbbO belongs to the CAZy glycosyltransferase family 4 (GT-4) (30). The predicted WbbO polypeptide contains 377 residues (42.6 kDa) and SDS-PAGE of His10-WbbO reveals a polypeptide of the expected size (Fig. 2). In E. coli cells, the majority of the overexpressed WbbO protein is located in the membrane (P100) fraction (Fig. 2). WbbO is not predicted to have any membrane-spanning regions (according to DAS, HHMTOP 2.0; TMpred, TopPred II). The protein sequence predicts one short hydrophobic segment (residues 108–120). Although this is not sufficient to span the membrane, this region could potentially intercalate into the membrane (31). WbbO does contain a number of additional helices (particularly the segment between residues 326 and 342), which contain hydrophobic residues and could also participate in its interaction with the membrane; there is precedent for this in other glycosyltransferases (32, 33).

The predicted WbbO protein sequence was analyzed using a domain linker prediction program using a SVM. The results identified a putative linker, suggesting that WbbO may contain two potential independently folded domains, with the galactosyltransferase activity predicted to be contained within the C-terminal domain (residues 170–365; WbbOC) (Fig. 2). The two putative domains were cloned separately into pBAD vectors. Although the majority of His10-WbbO was confined to the membrane fraction, consistent with its predicted structural features, the separated polypeptide domains were found in both the soluble and membrane fractions with the majority in the soluble fraction. These results suggest that membrane association of full-length WbbO is directed by regions throughout the protein and not solely by the predicted N-terminal hydrophobic region (residues 108–120).

Mutant complementation experiments established that full-length His10-WbbO could restore D-galactan I biosynthesis in E. coli CWG286 cells harboring the ΔwbbO construct. This was evident in SDS-PAGE, which revealed long-chain “smooth” LPS (S-LPS) molecules missing in the mutant (Fig. 3). The addition of higher levels of arabinose inducer yielded a S-LPS profile with similar size distribution and staining intensity to the parent. When expressed alone, the galactosyltransferase-containing WbbOC domain (plasmid pWQ529) was sufficient to complement the biosynthesis defect in the ΔwbbO mutant, at least in an overexpression situation. Based on the silver-stained gel, the efficiency of complementation was less than that observed with the full-length WbbO protein, particularly with lower amounts of arabinose, but the size distribution was comparable at higher levels of arabinose. Given the nature of the system, it is difficult to monitor expression of properly folded and localized enzyme, so the significance (if any) of the reduced efficiency is impossible to interpret. In all cases, the identity of the S-LPS was verified by immunoblotting with anti-D-galactan I antibodies (data not shown). Immunofluorescence was used to verify that the S-LPS was present on the cell surface (Fig. 3B). The predicted N-terminal domain (WbbON) could not complement the ΔwbbO mutation as expected (data not shown). Therefore, when expressed from a plasmid, WbbO does not require the N terminus for galactosyltransferase function and the reported α-galactopyranosyl- and β-galactofuranosyltransferase activity (14, 20) of the enzyme is confined to the C-terminal domain of WbbO.

**Localization and Domain Analysis of WbbM**—WbbM is classified in GT family 8 and in vitro data shows that it adds galactose residues to elongate the product of WbbO (20). However, the precise structure of the residue(s) added has not been established. WbbM is the largest galactosyltransferase involved in
### Organization of Glycosyltransferases in D-Galactan I Biosynthesis

#### Analysis of the Putative Galactosyltransferase WbbN—

BLAST analysis identifies WbbN as a third putative galactosyltransferase. WbbN belongs to GT-2, one of the largest families that synthesize O-glycosylated enzymes (34, 35). Unlike WbbO and WbbM, WbbN (290 residues, 33.2 kDa) is predicted to contain a single domain (Fig. 6). An N-terminal FLAG epitope-tagged protein (FLAG-WbbN) migrated in SDS-PAGE consistent with its expected size (34.2 kDa) (Fig. 6). This protein was localized predominantly to the membrane fraction.

Previous in vitro work suggested that WbbO and WbbM could together form a galactose-containing polymer (20). Although the structure (and size) of the product was not established, these results raised questions about whether WbbN participates in D-galactan I biosynthesis. To address this question, the requirement for WbbN was assessed in vivo. Plasmid pWQ548 is a derivative of pWQ288 that contains a frameshift mutation in wbbN. This is predicted to result in a truncated peptide terminating at residue 79 and therefore lacking a large portion of the GT-2 domain. This mutant is unable to produce S-LPS containing D-galactan I (Fig. 7). Introduction of plasmid pWQ527 (carrying wbbN) restored D-galactan I biosynthesis and S-LPS production. The results suggest that WbbN is indeed a galactosyltransferase and furthermore, that this enzyme is required for the synthesis of the D-galactan I polymer.

The wbbN mutant cells were pleomorphic (Fig. 7). In the absence of an ABC transporter, the four D-galactan I biosynthesis genes (in pWQ289) direct the formation of intracellular polymer (18). When a DwbN version of the pWQ289 construct was examined, the cells lacked D-galactan I but had a normal cell size and shape (data not shown). Transport defects in the E. coli O9a O-PS assembly system also give rise to pleomorphic cells, which lose viability (15, 17). Although the underlying mechanisms may differ, both instances could reflect either some toxicity resulting from accumulation of D-galactan I intermediates, or depletion of the cellular pool of the essential und-P carrier, which participates in peptidoglycan biosynthesis (36).

### Interaction Network within the D-Galactan I Biosynthetic Machinery

It has been assumed that the glycosyltransferases that synthesize O-PS polymer form a membrane-associated multienzyme complex but data to support this contention has been lacking for all but a very few systems. Potential binary interactions between all of the proteins involved in D-galactan I synthesis were therefore examined using both co-purification methods and a bacterial two-hybrid approach (24).

For the bacterial two-hybrid assay, the T25 and T18 fragments of the catalytic domain of the B. pertussis adenylate cyclase were fused to full-length copies of each of the D-galactan I proteins. One potential advantage of this system over other two-hybrid approaches is that it does not require the interac-

---

**FIGURE 4. Secondary structure predictions and localization of His<sub>10</sub>-WbbM.**

*Panel A:* Sequence and predicted secondary structure of WbbM outlining β-sheets (black arrows) and α-helices (black boxes). The GT-8 region (residues 284–549) is identified, as is a putative linked region that may separate two independent domains (WbbMN and WbbMC). *Panel B:* Localization of His<sub>10</sub>-WbbM (expressed from pWQ522 in E. coli) and His<sub>10</sub>-WbbM<sup>+</sup> (pWQ530), and His<sub>10</sub>-WbbM<sup>+</sup> (pWQ531). Cell-free lysates (S100) were separated into soluble (S100) and membrane-containing (P100) fractions and the His-tagged proteins were detected in Western immunoblots using mouse anti-His<sub>5</sub> monoclonal antibody followed by goat anti-mouse horseradish peroxidase-conjugated antibody. Loading was standardized to the original cell suspension (prior to lysis), allowing direct visual assessment of the cellular distribution of His<sub>10</sub>-WbbM.
tion partners to be associated with the chromosomal DNA for the interaction to be reported. It is therefore more suited for the study of membrane (or membrane-associated) proteins (24). All of the N-terminal fusion constructs (pKT25 and pUT18C) were functional, based on complementation (restoration of D-galactan I expression) in the respective deletion mutants (data not shown).

A positive interaction was considered valid when the β-galactosidase activity measured was at least two times that of the background levels measured for the vector controls (Table 2). Using these criteria, strong homotypic interactions were observed in WbbO and WbbM, but not in WbbN. These results suggest that WbbO and WbbM may form dimers (or higher order multimers) in the final complex. WbbM, WbbN, and WbbO all gave positive interaction signals with one another. Importantly, reciprocal interactions were detected when the various bait and prey combinations were reversed. The strongest inter-enzyme interaction occurred between WbbO and WbbM (Table 2).

As a further control, we investigated a second set of constructs where the T18 fragment was located at the C terminus of the galactosyltransferases and the interactions with the T25 N-terminal-tagged constructs tested (Table 2). Of these, only the WbbO-T18 construct was inactive in mutant complementation analyses (data not shown). In most cases, the results obtained were qualitatively consistent with the N-terminal-tagged derivatives. The exceptions were negative results for C-terminal-tagged WbbO-WbbN and WbbM-WbbN pairs. In each case, a positive result was obtained from the reciprocal C-terminal-tagged pair, suggesting that steric hindrance may be a factor in certain combinations. As might be expected, the absolute β-galactosidase activities varied.

To support this data, pairwise combinations of galactosyltrans-
Ferases were assessed for interaction by a co-purification strategy. One or both partners were epitope-tagged for detection and purification of the interacting proteins. Examination of proteins present in the bound fractions from the affinity columns (E1, E2) indicated that transferases WbbM, WbbN, and WbbO all interact with one another (Fig. 8). Thus the same result for protein-protein interactions was obtained by two radically different experimental strategies.

Attempts were made to investigate the roles of the different domains of WbbO in protein-protein interactions, using the two-hybrid approach (Table 2). The outcome of these experiments was more complex. Some evidence was found for the interaction of WbbON with full-length WbbO, WbbM, and WbbN. In contrast, the WbbOC domain was unable to mediate any interactions sufficient for a positive result in the two-hybrid system. The simplest explanation is that WbbO contains one

| TABLE 2 |

In vivo interactions between the putative galactosyltransferases involved in α-galactan I synthesis

| N-terminal T25 | N- or C-terminal T18 derivative | β-Galactosidase activity Miller units |
|----------------|---------------------------------|-------------------------------------|
|                | Leucine zipper                   | Leucine zipper                      |
| Positive control |                                 | 700 ± 70                            | 880 ± 200 |
| Negative control |                                 | 190 ± 40                            | 160 ± 30 |
| Homotypic interactions |                                 | WbbO WbbO                          | 1010 ± 20 | 1360 ± 130 |
|                        | WbbM WbbM                          | 1940 ± 200                          | 1480 ± 80 |
|                        | WbbN WbbN                          | 100 ± 30                            | 170 ± 10 |
| Heterotypic interactions |                                 | WbbO WbbN                          | 880 ± 50  | 140 ± 10 |
|                        | WbbM WbbM                          | 1560 ± 250                          | 1420 ± 70 |
|                        | WbbN WbbN                          | 1010 ± 20                           | 540 ± 140 |
|                        | WbbM WbbO                          | 1160 ± 20                           | 920 ± 70 |
|                        | WbbN WbbO                          | 1640 ± 40                           | 1700 ± 70 |
|                        | WbbM WbbN                          | 1270 ± 110                          | 130 ± 20 |
|                        | WbbN WbbO                          | 180 ± 30                            | 510 ± 90 |
|                        | WbbO WbbO                          | 760 ± 20                            | 120 ± 10 |
|                        | WbbO WbbO                          | 960 ± 110                           | 800 ± 50 |
|                        | WbbM WbbO                          | 790 ± 90                            | 550 ± 50 |
|                        | WbbO WbbO                          | 450 ± 140                           | 620 ± 110 |
|                        | WbbN WbbO                          | 990 ± 90                            | 1100 ± 280 |
|                        | WbbO WbbO                          | 230 ± 60                            | 120 ± 20 |
|                        | WbbM WbbN                          | 150 ± 30                            | 180 ± 20 |
|                        | WbbM WbbM                          | 110 ± 40                            | 100 ± 30 |
|                        | WbbM WbbO                          | 90 ± 30                             | 160 ± 40 |
|                        | WbbN WbbN                          | 110 ± 20                            | 160 ± 30 |
|                        | WbbN WbbO                          | 140 ± 20                            | 110 ± 30 |

* Data were obtained from triplicate samples each done in triplicate experiments.
In the biosynthesis of D-galactan I, WbbO, a GT-4 enzyme commits und-PP-GlcNAc to D-galactan I synthesis. It is responsible for forming the adaptor region (14, 20). Its activity is followed by WbbM (20), implicating this GT-8 enzyme as an α1,3-Galp-transferase. If correct, the GT-2 WbbN enzyme presumably completes the synthesis by providing the α1,3-Galp-transferase activity. However, there is some inconsistency between the limited in vitro available data and the essential role of WbbN unequivocally established in these in vivo studies. We are unable to explain the basis for the different results. The precise catalytic activities for these enzymes can only be established by examining each purified enzyme with the appropriate synthetic acceptor, reagents that are not currently available. However, such information is not essential for the current investigation.

Although none of the three galactosyltransferases are integral membrane proteins, all target independently to the membrane. This potentially puts those enzymes in proximity to the und-PP-GlcNAc acceptor and the polymer export apparatus.

The galactosyltransferases all interact with one another based on two independent experimental strategies: two-hybrid and co-purification methods. In the case of WbbO and WbbM a functional complex may contain more than one copy as these proteins form homotypic interactions. Interestingly, these interactions do not extend to Glf, the only dedicated protein among the membrane-associated complex of galactosyltransferases involved in D-galactan I biosynthesis.

In the biosynthesis of D-galactan I, WbbO, a GT-4 enzyme commits und-PP-GlcNAc to D-galactan I synthesis. It is responsible for forming the adaptor region (14, 20). Its activity is followed by WbbM (20), implicating this GT-8 enzyme as an α1,3-Galp-transferase. If correct, the GT-2 WbbN enzyme presumably completes the synthesis by providing the α1,3-Galp-transferase activity. However, there is some inconsistency between the limited in vitro available data and the essential role of WbbN unequivocally established in these in vivo studies. We are unable to explain the basis for the different results. The precise catalytic activities for these enzymes can only be established by examining each purified enzyme with the appropriate synthetic acceptor, reagents that are not currently available. However, such information is not essential for the current investigation.

Although none of the three galactosyltransferases are integral membrane proteins, all target independently to the membrane. This potentially puts those enzymes in proximity to the und-PP-GlcNAc acceptor and the polymer export apparatus.

The galactosyltransferases all interact with one another based on two independent experimental strategies: two-hybrid and co-purification methods. In the case of WbbO and WbbM a functional complex may contain more than one copy as these proteins form homotypic interactions. Interestingly, these interactions do not extend to Glf, the only dedicated protein involved in producing the UDP-linked precursors. No interactions were detected between Glf and WbbO, WbbM, and WbbN in two-hybrid experiments (data not shown). Logically, such interactions would not be essential given that the Glf product, UDP-Galf, is soluble and may diffuse to the site of synthesis.

WbbO has been shown to have two separable domains, with only WbbO being essential for the glycosyltransferase activity. However, the activity of this construct was low relative to the full-length protein and it was only detected in the presence of high amounts of arabinose inducer, presumably reflecting substantial overexpression. Consistent with the activity, a significant amount of WbbO targets to the membrane independent of WbbON. WbbON apparently participates in interactions between WbbO and the other galactosyltransferases. It is tempting to speculate that this identifies a clear separation in domain functions. However, we cannot currently rule out addi-
tional interactions mediated by WbbO. Although all of the two-hybrid experiments involving WbbO gave negative results, the key fusion-protein constructs were functionally inactive and must be interpreted with caution.

WbbM, also has two separable domains but, unlike WbbO, both domains are essential for its participation in \( \alpha \)-galactan I biosynthesis. The CAZy GT-8 family has a relatively strong foundation of structure-function data. WbbM contains the characteristic fold and motif within the C-terminal domain. In contrast, WbbMN has no motif that would identify a catalytic activity. Interestingly, the two-domain organization and conserved N-terminal domain sequence is found in other putative GT-8 glycosyltransferases. A BLAST search identifies putative glycosyltransferases from *Bifidobacterium longum* sp. *infantis* ATCC15697 (ACJ53443.1; 32% identity 50% similarity), *Lactococcus lactis* subsp. *crenoris* SK11 (ABJ72535.1; 33% identity 54% similarity), *Campylobacter jejuni* (CAI38725.1; 29% identity, 48% similarity), and *Burkholderia multivorans* (EED99048.1; 34% identity, 52% similarity). Conservation of the N-terminal domain is consistent with its essential role in the biosynthesis of the two WbbM domains reconstitutes WbbM activity. Its function warrants further investigation given the surprising observation that expression of the two WbbM domains reconstitutes WbbM glycosyltransferase activity of WbbM. Its function warrants further investigation given the surprising observation that expression of the two WbbM domains reconstitutes WbbM (albeit with low efficiency). WbbMN is not required for membrane association of WbbMC. Unfortunately, no active fusion proteins were obtained to give insight into its possible role in protein-protein interactions.

WbbN was initially assigned as a galactosyltransferase based on its Rossmann-like fold, characteristic of the GT-A fold (34, 39). The frameshift mutation of wbbN in the \( \alpha \)-galactan I cluster eliminated formation of \( \alpha \)-galactan I, a result entirely consistent with WbbN being a galactosyltransferase involved in the overall chain extension process. In this family of GT enzymes, a DXD motif binds a catalytically important divalent metal ion (40, 41). Site-directed changes of the identified DXD motif (in WbbN this is DDD) to AAA eliminate its activity as expected (data not shown).

There is growing evidence supporting multienzyme complexes in the biosynthesis of cell-surface polymers. Protein interactions have been examined by a two-hybrid approach in the group 2 capsule system of *E. coli* K1, which also involves export of the polymer via an ABC transporter (42). Several of the biosynthesis proteins were found to interact. In the related *E. coli* K5 system, recent studies of glycosyltransferases involved in the synthesis of the K5 capsular polysaccharide found that protein-protein interactions were essential for activity. The glucuronyltransferase activity of KfiC is dependent on physical association with the N-acetylgalcosaminyltransferase, KfiA (43). These proteins may be part of a larger trans-envelope complex (44, 45). Interactions have also been observed between glycosyltransferases involved in wall teichoic synthesis in *Bacillus subtilis* 168 (46).

In the only other O-PS biosynthesis system where interactions between the components have been investigated to date, *E. coli* O9a, three glycosyltransferases are required for O-PS biosynthesis. Two of these target to the membrane independently, whereas the third is absolutely dependent on interaction with a membrane-bound chain terminating protein (47). In the O9a system, there are no detectable inter-glycosyltransferase interactions. Comparison with the *K. pneumoniae* \( \alpha \)-galactan I system suggests that membrane association is a conserved theme, as might be expected, but the manner by which this is achieved and the extent of protein-protein interactions differs significantly between systems. Notably, the O9a and \( \alpha \)-galactan I-assembly systems also couple biosynthesis, chain termination, and export in different ways and this may be reflected in the surprisingly different interaction strategies (16–18). The next challenge for this research field will be to provide a structural context to describe the molecular architecture of model multi-enzyme complexes. The characterization of these prototype systems represents the essential first step.

Acknowledgments—We thank D. E. Taylor and E. Vinm for providing vector plasmids. We also thank C. Bouman for construction of pUT18-zip.

REFERENCES

1. Raetz, C. R., and Whitfield, C. (2002) *Annu. Rev. Biochem.* 71, 635–700
2. Whitfield, C., Perry, M. B., MacLean, L. L., and Yu, S. H. (1992) *J. Bacteriol.* 174, 4913–4919
3. Kelly, R. F., Severn, W. B., Richards, J. C., Perry, M. B., MacLean, L. L., Tomás, J. M., Merino, S., and Whitfield, C. (1993) *Mol. Microbiol.* 10, 615–625
4. Kelly, R. F., and Whitfield, C. (1996) *J. Bacteriol.* 178, 5205–5214
5. Kol, O., Wieruszewski, J. M., Strecker, G., Fournet, B., Zalisz, R., and Smets, P. (1992) *Carbohydr. Res.* 236, 339–344
6. Kol, O., Wieruszewski, J. M., Strecker, G., Montreuil, J., Fournet, B., Zalisz, R., and Smets, P. (1991) *Carbohydr. Res.* 217, 117–125
7. Whitfield, C., Richards, J. C., Perry, M. B., Clarke, B. R., and MacLean, L. L. (1991) *J. Bacteriol.* 173, 1420–1431
8. Szabo, M., Bronner, D., and Whitfield, C. (1995) *J. Bacteriol.* 177, 1544–1553
9. Oxley, D., and Wilkinson, S. G. (1989) *Carbohydr. Res.* 193, 241–248
10. Alexander, D. C., and Valvano, M. A. (1994) *J. Bacteriol.* 176, 7079–7084
11. Meier-Dieter, U., Barr, K., Starman, R., Hatch, L., and Rick, P. D. (1992) *J. Biol. Chem.* 267, 746–753
12. Rick, P. D., Hubbard, G. L., and Barr, K. (1994) *J. Bacteriol.* 176, 2877–2884
13. Jann, K., Goldemann, G., Weisgerber, C., Wolf-Ullisch, C., and Kane-gasak, I. (1982) *Eur. J. Biochem.* 127, 157–164
14. Clarke, B. R., Bronner, D., Keenleyside, W. J., Severn, W. B., Richards, J. C., and Whitfield, C. (1995) *J. Bacteriol.* 177, 5411–5418
15. Clarke, B. R., Cuthbertson, L., and Whitfield, C. (2004) *J. Biol. Chem.* 279, 35709–35718
16. Cuthbertson, L., Kimber, M. S., and Whitfield, C. (2007) *Proc. Natl. Acad. Sci. U.S.A.* 104, 19529–19534
17. Cuthbertson, L., Powers, J., and Whitfield, C. (2005) *Proc. Natl. Acad. Sci. U.S.A.* 102, 30301–30309
18. Cuthbertson, L., Kimber, M. S., and Whitfield, C. (2007) *Proc. Natl. Acad. Sci. U.S.A.* 104, 19529–19534
19. Cuthbertson, L., Powers, J., and Whitfield, C. (2005) *Proc. Natl. Acad. Sci. U.S.A.* 102, 30301–30309
20. Cuthbertson, L., Kimber, M. S., and Whitfield, C. (2007) *Proc. Natl. Acad. Sci. U.S.A.* 104, 19529–19534
21. Cuthbertson, L., Kimber, M. S., and Whitfield, C. (2007) *Proc. Natl. Acad. Sci. U.S.A.* 104, 19529–19534
22. Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor, Cold Spring Harbor, NY
23. Guzman, L. M., Belin, D., Carson, M. J., and Beckwith, J. (1995) *J. Bacteriol.* 177, 4121–4130
24. Karimova, G., Pidoux, J., Ullmann, A., and Ladant, D. (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95, 5752–5756
25. Hitchcock, P. J., and Brown, T. M. (1983) *J. Bacteriol.* 154, 269–277
Organization of Glycosyltransferases in D-Galactan I Biosynthesis

26. Ouali, M., and King, R. D. (2000) *Protein Sci.* **9**, 1162–1176
27. Rost, B., Yachdav, G., and Liu, J. (2004) *Nucleic Acids Res.* **32**, W321–326
28. Bryson, K., McGuffin, L. J., Marsden, R. L., Ward, J. J., Sodhi, J. S., and Jones, D. T. (2005) *Nucleic Acids Res.* **33**, W36–38
29. Cole, C., Barber, J. D., and Barton, G. J. (2008) *Nucleic Acids Res.* **36**, W197–201
30. Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009) *Nucleic Acids Res.* **37**, D233–238
31. von Heijne, G. (2006) *Nat. Rev. Mol. Cell Biol.* **7**, 909–918
32. Brockhausen, I., Hu, B., Liu, B., Lau, K., Szarek, W. A., Wang, L., and Feng, L. (2008) *J. Bacteriol.* **190**, 4922–4932
33. Leipold, M. D., Kaniuk, N. A., and Whitfield, C. (2007) *J. Biol. Chem.* **282**, 1257–1264
34. Breton, C., Snajdrova, L., Jeanneau, C., Koca, J., and Imberty, A. (2006) *Glycobiology* **16**, 29R–37R
35. Lairson, L. L., Henrissat, B., Davies, G. J., and Withers, S. G. (2008) *Annu. Rev. Biochem.* **77**, 521–555
36. van Heijenoort, J. (2007) *Microbiol. Mol. Biol. Rev.* **71**, 620–635
37. Zhang, L., al-Hendy, A., Toivanen, P., and Skurnik, M. (1993) *Mol. Microbiol.* **9**, 309–321
38. Strother, U. H., Karageorgos, L. E., Morona, R., and Manning, P. A. (1992) *Proc. Natl. Acad. U.S.A.* **89**, 2566–2570
39. Lairson, L. L., Chiu, C. P., Ly, H. D., He, S., Wakarchuk, W. W., Strynadka, N. C., and Withers, S. G. (2004) *J. Biol. Chem.* **279**, 28339–28344
40. Breton, C., Bettler, E., Joziassie, D. H., Geremia, R. A., and Imberty, A. (1998) *J. Biochem.* **123**, 1000–1009
41. Wiggins, C. A., and Munro, S. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7945–7950
42. Steenbergen, S. M., and Vimr, E. R. (2008) *Mol. Microbiol.* **68**, 1252–1267
43. Sugiru, N., Baba, Y., Kawaguchi, Y., Iwatani, T., Suzuki, K., Kusakabe, T., Yamagishi, K., Kimata, K., Kakuta, Y., and Watanabe, H. (2010) *J. Biol. Chem.* **285**, 1597–1606
44. Rigg, G. P., Barrett, B., and Roberts, I. S. (1998) *Microbiology* **144**, 2905–2914
45. McNulty, C., Thompson, J., Barrett, B., Lord, L., Andersen, C., and Roberts, I. S. (2006) *Mol. Microbiol.* **59**, 907–922
46. Formstone, A., Carballido-López, R., Noirot, P., Errington, J., and Scheffers, D. J. (2008) *J. Bacteriol.* **190**, 1812–1821
47. Clarke, B. R., Greenfield, L. K., Bouwman, C., and Whitfield, C. (2009) *J. Biol. Chem.* **284**, 30662–30672
48. Peng, H. L., Fu, T. F., Liu, S. F., and Chang, H. Y. (1992) *J. Biochem.* **112**, 604–608
49. Cronan, J. E. (2006) *Plasmid* **55**, 152–157
50. Chang, A. C., and Cohen, S. N. (1978) *J. Bacteriol.* **134**, 1141–1156