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An O-Antigen Processing Function for Wzx (RfbX): a Promising Candidate for O-Unit Flippase

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O antigen is the major cell surface antigen of gram-negative bacteria, and the genes responsible for its synthesis are located in a single gene cluster. The *wzx* (*rbfX*) gene, which is characteristic of the major class of O-antigen gene clusters, encodes a hydrophobic protein with 12 potential transmembrane segments. We demonstrate that a *wzx* mutant accumulates undecaprenol pyrophosphate-linked O units which appear to be on the cytoplasmic side of the cytoplasmic membrane, suggesting that the *wzx* gene encodes a flippase for O-unit translocation across that membrane.

In enteric bacteria, the lipid component of the outer layer of the cell wall consists of lipopolysaccharide (LPS), which forms a selective barrier between the environment and the cell. An LPS molecule consists of three regions: lipid A, oligosaccharide core, and O-antigen chain. Lipid A, also called endotoxin, is the hydrophobic portion of the molecule which anchors it on the outer membrane. There is little variation found in this part of LPS among different species of enteric bacteria. The core links the lipid A and O antigen, and there are some structural variations in the core within and between species (reviewed by Holst and Brade [4]). The O antigen, consisting of repeat units, usually of three to six sugars, is often the immunodominant surface antigen. The O-antigen structures vary in sugar composition of O units and in linkages between sugars and linkages between O units; these variations determine the O serological specificities (for reviews, see references 5 and 24). Extensive structural variations in O antigens have been found within and between species; for example, there are about 50 serogroups in *Salmonella enterica* and 170 serogroups in *Escherichia coli*.

The mechanisms of LPS biosynthesis have been studied extensively (17, 19, 21, 26). O-antigen synthesis, which is independent of core-lipid A synthesis, starts with assembly of O units by sequential transfer of sugars onto a lipid carrier, undecaprenol phosphate (UndP) (Fig. 1). These reactions occur on the cytoplasmic side of the cytoplasmic membrane. The completed O units are transferred to the periplasmic side of the cytoplasmic membrane and are polymerized from the reducing end by O-antigen polymerase (Wzy) (17). The O-antigen chains are then ligated to the core-lipid A by O-antigen ligase (WaaL), and the complete LPS is translocated to the outer membrane of the bacterial cell. Another mechanism of O-antigen synthesis was established for homopolymer chains (mannan or galactan) of *E. coli* O9 (9), *Klebsiella pneumoniae* O1, and *Serratia marcescens* O16 (32). This mechanism differs from the first in that the O-antigen polymer chains are synthesized entirely by glycosyl transferases on the cytoplasmic side of the cytoplasmic membrane without the involvement of Wzy: the chains are elongated from the nonreducing end, and an ATP-binding cassette (ABC) transporter is required for transport of the complete O polysaccharide across the cytoplasmic membrane (5, 9, 32).

The O-antigen gene cluster encodes the enzymes for biosynthesis of nucleotide sugars and the glycosyl transferases for O-unit assembly. The O-antigen gene clusters of several *S. enterica* serovars have been sequenced and studied in great detail (reviewed by Reeves [23]); those of *E. coli* serovars O9 (6, 9), O16 (strain K-12) (31), O111 (2), Flexneri (*Shigella flexneri*) (15, 16), and Dysenteriae (*Shigella dysenteriae*) (10) and of *Yersinia pseudotuberculosis* serovar IIA (8) have also been sequenced. The variation in the O-antigen gene clusters reflects the structural variation in O antigens. In general, the sugar pathway genes, such as those for dTDP-rhamnose or GDP-mannose, have a high degree of similarity between different O-antigen gene clusters, while the glycosyl transferase genes exhibit great divergence. A gene named *wzx* (previously *rfbX* [Table 1]), which encodes a protein consisting of 12 potential transmembrane segments, is found in all of these O-antigen gene clusters (24) except that of *E. coli* O9. The Wzx proteins from different O-antigen gene clusters are predicted to have structural homology, but they have little similarity even at the amino acid sequence level. It has been proposed that Wzx is responsible for the transfer of O units from the cytoplasmic to the periplasmic side of the cytoplasmic membrane (24). In this study, we provide support for this hypothesis with evidence that a *wzx* mutant accumulates a single O unit linked to the pyrophosphate form of undecaprenol (UndPP) on the cytoplasmic side of the cytoplasmic membrane.

MATERIALS AND METHODS

Media, bacterial strains, and plasmids. Bacteria were grown in L broth (constituents from Difco) with antibiotics added as required. For the pulse experiments, bacteria were grown in medium A (0.1% beef extract [Oxoid], 1% proteose peptone no. 2 [Difco], 0.5% NaCl [pH 7.4]). The final concentrations of antibiotics used were as follows: ampicillin, 25 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; and tetracycline, 16 μg ml^{-1}. Bacterial strains used in this study are listed in Table 1. An explanation of the gene nomenclature used is given in a footnote to Table 1. The plasmids used in this study were pSS37 (10), which carries all of the genes necessary for *E. coli* Dysenteriae 1 O-antigen biosynthesis; B11 (10), which is pSS37 with a Tn*lac* insertion in the *wzy* gene; W25 (10), which is pSS37 with a Tn*lac* insertion in the *wzx* gene; and pMU5236, which carries a *mtr*::*lacZ* fusion and which is isolate 2 in Table 3 of Sarsero and Pittard (30).

waaL wzy double mutant *S. enterica* P9468 was constructed by introduction of a mutated *wzy* gene into *waaL* strain P9322 as follows. Chromosomal DNA of strain LV386, which carries a *wzy*::*aph* fusion, was isolated and sheared by vortexing. The DNA fragments were then transferred into strain P9322 by electroporation, and the kanamycin-resistant recombinant was selected. In a similar manner, P9351 was made by introducing a defective *galE* gene into strain P9029,

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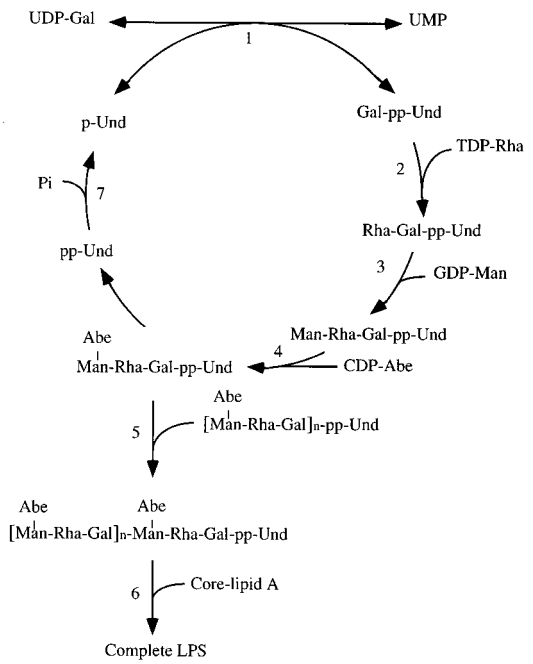

FIG. 1. Biosynthetic pathway of O-antigen of *S. enterica* group B (17). Steps 1 to 4 are the assembly of the O units by transfer of galactose-phosphate (Gal-P), rhamnose (Rha), Man, and abequose (Abe), respectively, onto UndP or oligosaccharide-UndPP. These reactions are catalyzed by glycosyl transferases and occur on the cytoplasmic side of the cytoplasmic membrane. Step 5 is polymerization catalyzed by Wzy, and this reaction occurs on the periplasmic side of the cytoplasmic membrane (17). Step 6 is ligation of the O-antigen chain and core-lipid A, which is catalyzed by WaaL. p-Und, UndP; pp-Und, UndPP.

which has a deletion of the O-antigen gene cluster, the donor strain being TN1117 (29).

Chemicals, enzymes, and antisera. [U-^{14}C]galactose, UDP-[^{14}C]galactose, and GDP-[^{14}C]mannose were purchased from Amersham Corp. UDP-galactose and GDP-mannose were from Sigma. TDP-rhamnose, TDP-[^{14}C]rhamnose, and CDP-[^{14}C]abequose were kindly provided by L. Lindqvist, Department of Clinical Bacteriology, Huddinge Hospital, Karolinska Institute, Huddinge, Sweden. Dysenteriae 1 antiserum (rabbit) was from Murex Diagnostics Ltd. Goat polyclonal anti-rabbit immunoglobulin G antiserum was purchased from Pierce. All other chemicals were purchased from standard commercial sources.

Preparation of everted membrane vesicles. Everted membrane vesicles were prepared by a modified procedure described by Rosen and Tsuchiya (27). Briefly, cells from 100 ml of overnight culture (optical density at 530 nm, 1) were collected by centrifugation and resuspended in 10 ml of phosphate-buffered saline (PBS) with sucrose and NaCl added to final concentrations of 20% (wt/vol) and 2 M, respectively (buffer A). The cells were disrupted by two passages through a French pressure cell at 16,000 lb/in^2. Unbroken cells and cell debris were removed by centrifugation twice at 16,000 rpm for 20 min (Beckman JA20 rotor). The membrane vesicles were pelleted at 35,000 rpm for 1 h (Beckman 70 Ti rotor). The pellet was resuspended in 0.5 ml of Z buffer (18) with 20% (wt/vol) sucrose, 1 M NaCl, and 5 mM EDTA added (buffer B), and the concentration of vesicles was estimated by the optical density at 550 nm. The vesicle suspension was stored at 4°C for up to 1 month.

Purification of cytoplasmic membrane vesicles by sucrose step centrifugation. The purification method used was described by Yamato et al. (36). The membrane vesicles after a 35,000-rpm centrifugation were resuspended in 1 ml of buffer containing 8.5% (wt/vol) sucrose, 3 mM EDTA, and 3 mM Tris (pH 8). The vesicle suspension was loaded in a centrifugation tube on top of 9 ml of 44% (wt/vol) sucrose and was centrifuged at 34,000 rpm for 15 h (Beckman 80 Ti rotor). The cytoplasmic membrane vesicles were collected from the upper layer, and the pellet was the outer membrane fraction. The upper layer was diluted with 2 volumes of 3 mM EDTA, and the membrane vesicles were pelleted at 50,000 rpm for 1.5 h (Beckman 70 Ti rotor). Finally, the inverted cytoplasmic membrane vesicles were resuspended in buffer B.

Preparation of spheroplasts. Spheroplasts were prepared from 1-ml overnight cultures (optical density at 530 nm, 1). The cells were washed with 1 ml of saline and resuspended in 0.2 ml of buffer containing 20% sucrose, 2 M NaCl, and 30 mM Tris (pH 8). Lysozyme and EDTA were added to the cell suspension to final concentrations of 1 mg ml^{-1} and 10 mM, respectively. The cell suspension was then incubated at room temperature for 1 h, and formation of spheroplasts was confirmed by microscopy. Buffer A (1 ml) was added to the spheroplast mixture, and the spheroplasts were then collected by centrifugation and resuspended in 0.1 ml of buffer B.

Enzyme-linked immunosorbent assay (ELISA) for O units exposed on spheroplasts and everted membrane vesicles. Rabbit antiserum to Dysenteriae 1 (2 μl) was added to 0.1 ml of everted vesicle suspension and the mixture was incubated at 37°C for 3 h. The mixture was diluted in 27 ml of buffer A and everted vesicles with bound antibody were collected by centrifugation at 35,000 rpm for 1 h with a 70 Ti rotor to remove unbound antibody. This washing step was repeated to ensure that all unbound antibody was removed. Finally, the vesicle-antibody pellet was resuspended in 0.45 ml of buffer A, and the suspension was loaded in a microtiter plate (50 μl per well) and incubated at 35°C overnight. The liquid in the wells was discarded, and the plate was rinsed three times in distilled water. The wells were blocked with 200 μl of 1% bovine serum albumin (BSA) in PBS at 35°C for 3 h. After being blocked by 1% BSA, the plate was washed three times in distilled water. Horseradish peroxidase-labelled goat polyclonal anti-rabbit Fab (50 μl of a 1-to-3,000 dilution) was added to each well, and the plate was incubated at 35°C for 1 h. After another six washes (200 μl per well) with

TABLE 1. *S. enterica* LT2 strains used in this study

Strain	Characteristics[a]	Source or reference
P9029	*his-388* (deletion from *hisD* to beyond *metG*)	20
TN1117 (P9138)[b]	*zbi-812*::Tn*10 galE*	29
P9298	P9029 carrying plasmids pPR686 and pPR1208 (12)	12
P9299	P9029 carrying plasmids pPR686 and pPR1303 (12)	12
P9320	*hisD27 trpA8 tolC-30 H1-a*, *H2-e,n,x gal-437 xyl-412 wbaP*(*T*) *zbi-812*::Tn*10 galE*	34
P9321	*hisD27 trpA8 tolC-30 H1-a*, *H2-e,n,x gal-437 xyl-412 wbaP*(*T*) *zbi-812*::Tn*10 galE*	34
P9322	*waaL447 gal-851 zbi-812*::Tn*10 galE*	34
P9344	P9029 carrying plasmid pSS37 (10)	This study
P9345	P9029 carrying plasmid W25 (10)	This study
P9348	P9029 carrying plasmid pMU5236 (30)	This study
LV386 (P9350)[b]	*wzy*::*aph*	3
P9351	As P9029, *zbi-812*::Tn*10 galE*	This study
P9352	P9351 carrying plasmid W25 (10)	This study
P9353	P9351 carrying plasmid pSS37 (10)	This study
P9467	P9029 carrying plasmids pPR686 and pPR1563 (13)	13
P9468	As P9322, *wzy*::*aph*	This study
P9469	P9351 carrying plasmid B11 (10)	This study

[a] We are using a revised system of nomenclature for genes involved in bacterial surface polysaccharide synthesis in which *wzx* replaces *rfbX*, *wzy* replaces *rfc*, *waaL* replaces *rfaL*, *wbaP* replaces *rfbP*, and *wbaP*(*T*) replaces *rfbP*(*T*). Further information can be obtained from http://www.angis.su.oz.au/BacPolGenes/welcome.html.

[b] Laboratory stock number is given in parentheses when not the same as the strain name.

wash solution (10 mM Tris, 50 mM NaCl, 1% Tween 20 [pH 7.4]), peroxidase substrate (containing 0.2 g of 3,3′,5,5′-tetramethylbenzidine per liter and 0.01% peroxide [100 μl per well]) was added, and the color reaction was stopped by the addition of 1 M H_2SO_4 (100 μl per well). The plate was read at 450 nm.

Dysenteriae 1 rabbit antiserum (3 μl) was added to 0.1 ml of spheroplast suspension, and the mixture was incubated at 37°C for 3 h. The mixture was then diluted in 50 ml of buffer A, and spheroplast antibody complexes were collected by centrifugation at 20,000 rpm for 30 min and washed again under the same conditions. The remaining steps were performed as described above for vesicle preparation.

In vivo incorporation of [^{14}C]galactose into LPS and intermediates. Bacteria were grown in medium A overnight at 37°C and diluted 1 to 20 in 8 ml of fresh medium A supplemented with L-fucose to a final concentration of 1 mM as a nonmetabolizable inducer (22) and grown to mid-log phase (optical density at 530 nm, 0.4). [^{14}C]galactose (0.3 μM) was added to the culture, which was incubated for a further 10 min. The culture was then chilled on ice, and the labelled cells were collected by centrifugation. The cells were washed with 5 ml of saline, and the cell pellet was resuspended in 200 μl of lysis buffer (14) and then analyzed by sodium dodecyl sulfate-polyacrylamide (12%) gel electrophoresis (SDS-PAGE), or the cells were resuspended in 0.5 ml of 10 mM Tris-HCl (pH 7.5)–10 mM EDTA for butanol extraction.

Extraction of lipid-linked intermediates and their release from the lipid carrier by mild acid treatment. The cell suspensions (0.5 ml) from the experiment described above were extracted twice with an equal volume of butan-1-ol. The two butanol fractions were combined and back-extracted with an equal volume of water. An equal volume of water was added to the butanol fraction, and both butanol and water were evaporated in a rotor drier (from Heto Laboratory Equipment; VR-1). The intermediates were resuspended in 100 μl of water and used for electrophoresis or were resuspended in 0.5 ml of water for mild acid treatment.

The mild acid treatment was performed by addition of an equal volume of phenol to the mixture, which was then heated at 75°C for 15 min. The mixture was cooled to room temperature and then centrifuged to separate the aqueous and phenol phases. The aqueous phase (250 μl) was removed to a fresh tube, and a further 250 μl of water was added to the phenol phase. The two phases were thoroughly mixed by vortexing and separated by centrifugation, and the aqueous phase was collected as described above. The two aqueous phase fractions were combined and extracted three times with ether to remove traces of phenol, before being freeze-dried and resuspended in 100 μl of water. Gel filtration on a Sephadex G-15 column (1.6 by 35 cm) was performed with a 25-μl sample with 10% (wt/wt) CsCl (200 μl). Ammonium acetate (50 mM [pH 7.0]) was used as a packing and elution buffer, and the elution rate was 75 μl/min. Fractions of 0.5 ml were collected, and radioactivity was counted in 4 ml of ASC II (Amersham) in a scintillation counter (LS 6500; Beckman).

Synthesis of oligosaccharide intermediates and polysaccharides. The methods of synthesizing [^{14}CRha]-Gal-decaprenol pyrophosphate (DPP), [^{14}C-Man]-Rha-Gal-DPP, and [^{14}C-Abe]-Man-Rha-Gal-DPP intermediates in vitro were described previously with membrane fractions of P9298, P9299, and P9467, respectively (12, 13). These strains carry two, three, or four transferase genes for assembly of O units from *S. enterica* LT2 on plasmids. The intermediates were extracted by butanol, and the lipid portion of each molecule was removed by phenol treatment as described above. Polysaccharides of *S. enterica* LT2 were extracted from a *waaL* mutant P9322 by the method described by Wang and Reeves (34).

Other methods. The methods for chromosomal and plasmid DNA isolation and transformation were those described by Sambrook et al. (28). SDS-PAGE gels were prepared by the method of Lugtenberg et al. (14), and the conditions of electrophoresis were 100 V for 2 h, followed by 60 V for 16 h. The method for the β-galactosidase assay was as described by Miller (18) with modifications: the reaction mixture contained 0.2 ml of membrane vesicles instead of lysed cells, 0.4 ml of Z buffer (18), and 0.2 ml of *o*-nitrophenyl-β-D-galactoside (4 mg ml^{-1}).

RESULTS AND DISCUSSION

A *wzx* mutant accumulates UndPP carrying a single O unit. Six strains were used in the experiments to study the effects of mutation in *wzx*. Strain P9351 is a *S. enterica* LT2 derivative which is *galE* and lacks the O-antigen gene cluster and will be referred to as the OAg^- strain. Strains P9353, P9352, and P9469 are P9351 carrying the genes for *E. coli* Dysenteriae 1 O-antigen biosynthesis on plasmids pSS37, W25, and B11, respectively. P9353 will be referred to as the OAg^+ strain, and P9352 and P9469, which have mutations in *wzx* and *wzy*, respectively, will be referred to as wzx^- and wzy^- strains, respectively. Strains P9322 and P9468 are *S. enterica* LT2 strains with mutations in the *waaL* and *waaL wzy* genes, respectively, and they will be referred to as $waaL^-$ and waaL-wzy^- strains, respectively. The OAg^+, wzx^-, and wzy^- strains make *E. coli* Dysenteriae O antigen or intermediates, while the $waaL^-$ and waaL-wzy^- strains make *S. enterica* group B intermediates. It would have been preferable to have used mutations in the *wzx* and *wzy* genes of *S. enterica* LT2, but only the latter is available because it has not yet been possible to make the former (25).

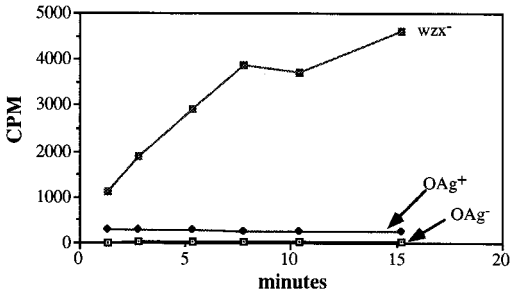

FIG. 2. Accumulation of UndPP-O units in the *wzx* mutant. [^{14}C]galactose and unlabelled galactose were added to the mid-log cultures to final concentrations of 0.3 and 4.5 μM, respectively. Samples (1 ml from each culture) were taken at the time intervals indicated, 2,4-dinitrophenol was added to the samples to a final concentration of 2.5 mM, and then the samples were kept on ice. Butanol extraction of intermediates was performed as described in Materials and Methods.

Cells from OAg^-, wzx^-, wzy^-, OAg^+, $waaL^-$, and waaL-wzy^- strains were labelled with [^{14}C]galactose, and UndPP-based intermediates from the labelled cells were isolated by butanol extraction. The ^{14}C contents of the butanol fractions (25 of 750 μl) were as follows: OAg^-, 45 cpm; wzx^-, 12,196 cpm; wzy^-, 12,846 cpm; OAg^+, 233 cpm; $waaL^-$, 378 cpm; and waaL-wzy^-, 11,051 cpm. The butanol fraction of the OAg^- strain did not contain any ^{14}C-labelled intermediates, indicating that, as expected, the core-lipid A or other polysaccharides containing galactose are not isolated by butanol extraction. For the OAg^+ and $waaL^-$ strains, small amounts of intermediates were found in the butanol fractions. This indicates that most of the intermediates are polymerized rapidly into polysaccharides, which are not extracted by butanol. The accumulation of butanol-extracted intermediates in the waaL-wzy^- strain was expected, because the UndPP-linked O (UndPP-O) unit can be neither polymerized nor ligated to core-lipid A, and hence the UndPP-O unit will accumulate in the cells. wzx^- and wzy^- strains were also found to have accumulated butanol-soluble intermediates. The accumulation of butanol-soluble intermediates over time in the wzx^- strain is shown in Fig. 2.

The labelled butanol-soluble intermediates were concentrated, and butanol was removed by evaporation. The intermediates and the lysed labelled cells were loaded onto an SDS-PAGE gel and electrophoresed. The butanol-soluble intermediates from both wzx^- and waaL-wzy^- strains had the same mobility (Fig. 3, lanes 4 and 8, respectively) and had higher mobility than the core-lipid A (Fig. 3). The butanol-soluble intermediates are likely to be UndPP-O unit(s). The waaL-wzy^- strain is expected to accumulate UndPP with a single attached O unit, because this strain is blocked in both polymerization of O units and the transfer of O units to core-lipid A, and it appears that the wzx^- strain accumulates the same material. The same intermediate accumulates in the *wzy* mutant (Fig. 3, lane 6). In lanes 3, 5, and 7, the whole-cell preparations of the same three strains are shown, and in each one, the core-lipid A is present in addition to the UndPP-O unit. The waaL-wzy^- strain makes the *S. enterica* group B O units (Abe-Man-Rha-Gal) and the wzx^- strain has Dysenteriae O units (Rha-Rha-Gal-GalNAc). However, both have four sugars, and both species of UndPP-O-unit molecules should behave similarly on SDS-PAGE.

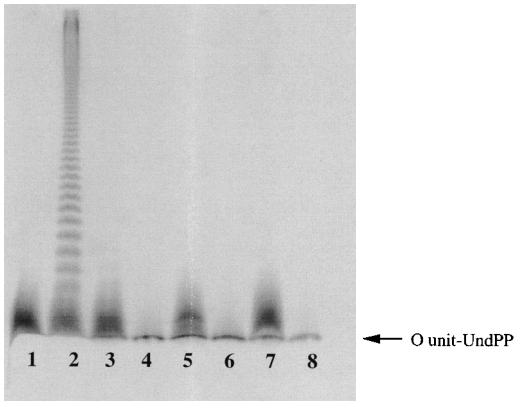

FIG. 3. [^{14}C]galactose-labelled LPS and butanol-soluble intermediates on SDS-PAGE. Lanes 1, 2, 3, 5, and 7 are cell lysates of P9351 (OAg^-), P9353 (OAg^+), P9352 (wzx^-), P9469 (wzy^-), and P9468 (waaL-wzy^-), respectively; lanes 4, 6, and 8 are butanol extracts from P9352, P9469, and P9468, respectively. The gel was vacuum dried, the distribution of radioactivity was quantified with a PhosphorImager (Molecular Dynamics), and the data were analyzed with the Molecular Dynamics ImageQuant computer software package.

The butanol-soluble intermediates were hydrolyzed by phenol (11, 17, 35). The hydrophilic portion of the molecule should be in the aqueous phase, while the lipid portion of the molecule should remain in the phenol phase. The results showed that over 95% of the radioactivity was in the aqueous phase, and only a small percentage remained in the phenol phase. The water-soluble samples from waaL-wzy^-, wzx^-, and wzy^- strains were subjected to gel filtration. One peak was observed in each case (Fig. 4): for all three samples, the peaks were at the position of the tetrasaccharide standard. These results indicate that the butanol-soluble material is indeed the UndPP-O unit.

The wzx^- strain accumulated about 50 times more UndPP-O unit than was present in the OAg^+ strain and had amounts of UndPP-O unit similar to those of the waaL-wzy^- strain. We conclude that in the *wzx* mutant, the UndPP-O unit was synthesized but could not be polymerized or ligated to the core. The only known step required after synthesis of the UndPP-O unit and before polymerization or ligation is translocation of the UndPP-O unit from the cytoplasmic side to the periplasmic side of the cytoplasmic membrane. The *wzx* mutant presumably blocks translocation of the UndPP-O unit across the cytoplasmic membrane and hence accumulates the UndPP-O unit. This result indicates that the *wzx* gene encodes a flippase. The *wzy* mutant was also found to accumulate the UndPP-O unit, which had not been reported previously. It is possible that in the *wzy* mutant, single O units saturate the available core molecules, and the excess UndPP-O units are accumulated.

The *wbaP*(*T*) mutants are a class of mutants with a mutation in *wbaP* which have also been found to accumulate UndPP-O units (34). The *wbaP*(*T*) mutants P9320 and P9321 were also used in the butanol extraction experiments, and the results showed that both *wbaP*(*T*) strains had a very small amount of the UndPP-O unit (P9320, 55 cpm; P9321, 90 cpm) compared with the wzx^- strain (12,196 cpm) under the same conditions. It was previously suggested that the WbaP(T) protein could be involved in flipping of the UndPP-O unit (34); however, this study indicates that the Wzx protein is the better candidate for flippase. More recent work has shown that the WbaP protein consists of two functional domains: a C-terminal domain with galactosyl-1-phosphate transferase function and an N domain in which the *T* mutations are located. It appears that the *wbaP*(*T*) mutants have lost the ability to release UndPP-Gal and that although the other sugars are added to complete UndPP-O unit, it remains associated with WbaP and cannot be further processed (33).

Location of O units in the *wzx* mutant. We have found that the wzx^- strain accumulates the UndPP-O unit. In order to determine the location of the O units, an ELISA method was used to assay the O units on the exposed surface of inverted membrane vesicles and spheroplasts. Inverted membrane vesicles were used to study the cytoplasmic side of the cytoplasmic membrane, and spheroplasts were used to study the periplasmic side. The strains used in these experiments were P9344 (OAg^+) and P9345 (wzx^-), which were P9029 (OAg^-) carrying plasmids pSS37 and W25, respectively; the OAg^- strain was used as a negative control.

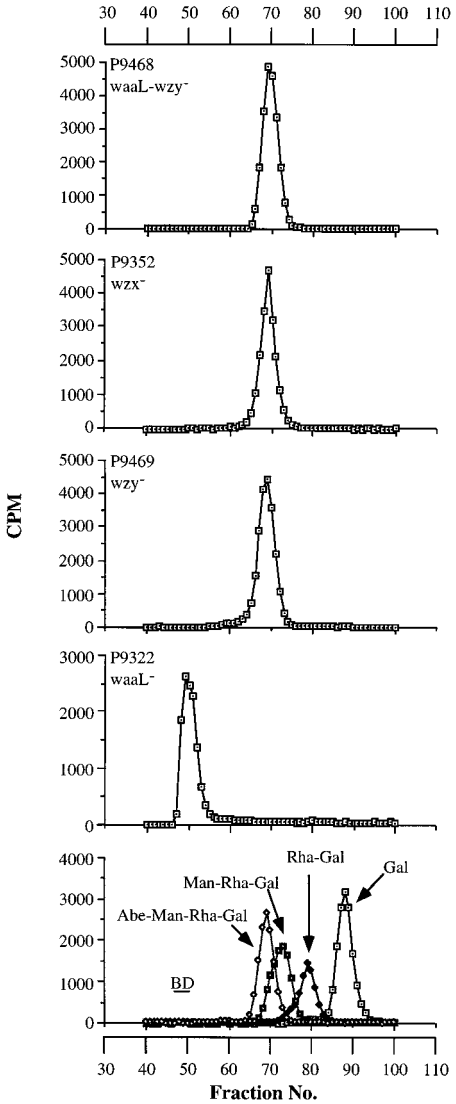

FIG. 4. Gel filtration of free O antigen and intermediates. The methods for labelling and isolation of the O antigen or intermediates are described in Materials and Methods. The O antigen or intermediates were resuspended in 100 μl of water, and 25 μl of sample in 200 μl of 10% CsCl was applied to a Sephadex G-15 column. The elution position of galactose was determined by using [^{14}C]galactose, and the positions of disaccharide, trisaccharide, and tetrasaccharide were determined by using ^{14}C-labelled in vitro-synthesized oligosaccharides (12, 13). BD, blue dextran, which indicates the excluded volume. Other abbreviations are as defined in the legend to Fig. 1.

TABLE 2. ELISA results from inverted membrane vesicles and spheroplasts

Vesicle or spheroplast	OD_{455} (ELISA)[a]		
	OAg^-	wzx^-	OAg^+
Inverted vesicle	0.25	1.32	1.89
Spheroplast	0.18	0.27	2.26

[a] OD_{455}, optical density at 455 nm. The negative control for nonspecific reactions was done by blocking wells with BSA, and the reading is 0.13. All values were averages of eight readings.

The membrane vesicles were prepared as described in Materials and Methods, and their inverted orientation was confirmed by enzyme assay. The *mtr* gene encodes the cytoplasmic membrane protein Mtr permease, responsible for tryptophan-specific transport. An *mtr*::*lacZ* fusion was made in such a way that the LacZ protein is anchored on the cytoplasmic side of the cytoplasmic membrane (30). Strain P9348 was P9029 carrying plasmid pMU5236, which encodes the chimeric protein. β-Galactosidase assays (18), before and after addition of the detergent Tween 20 to a final concentration of 0.5% to lyse vesicles, showed no effect of lysis on β-galactosidase activity (data not shown). These results indicate that most of the membrane vesicles in the preparation were inverted.

Inverted membrane vesicles and spheroplasts were prepared from OAg^-, wzx^-, and OAg^+ strains. The ELISA results for inverted membrane vesicles showed high levels for the wzx^- and OAg^+ strains and a low level for the OAg^- strain (Table 2): the results for the spheroplasts showed a high level only for the OAg^+ strain and low levels for the wzx^- and OAg^- strains (Table 2). These results indicate that in the *wzx* mutant, the O units are located on the cytoplasmic side of the cytoplasmic membrane and that there is very little O unit on the outside. However, the high ELISA reading for inverted membrane vesicles of the OAg^+ strain was not expected, since as shown above, there is very little butanol-extractable intermediate. This result could well be due to contamination by the outer membrane, since the outer membrane fragments were not completely separated from cytoplasmic membrane vesicles in the preparation. The OAg^+ strain is the only one of the strains in Table 2 which proceeds to the synthesis of long-chain O antigen containing multiple O units, which are then transferred to the outer membrane as part of the LPS. The large number of O units present in O antigen means that only minor levels of O-antigen contamination are required in the inverted membrane vesicle preparation to give an abnormally high optical density reading. In order to reduce the effect of outer membrane contamination, the membrane vesicle fractions were further enriched for cytoplasmic membrane vesicles by sucrose step purification (36). After the purification, the ELISA reading from the OAg^+ strain (0.84) was more than halved, while that for the wzx^- strain (1.01) was much less affected. The purification leads to enrichment of the inverted (cytoplasmic) membrane vesicles, but there will also be some loss of this material: the much greater reduction in ELISA reading in the OAg^+ preparation than in the wzx^- preparation showed that the reduction in the OAg^+ strain was not due only to loss of cytoplasmic membrane vesicles during purification, and we conclude that contamination by outer membrane had indeed increased the ELISA reading of the fraction of OAg^+ inverted vesicles, making the data for the OAg^+ strain unreliable.

In the spheroplasts, both the outer membrane and the periplasmic side of the cytoplasmic membrane are exposed, and the ELISA readings resulted from the rabbit antibody binding to O antigen on both membranes. For the wzx^- strain, the results showed that there is little O antigen on the periplasmic side of the cytoplasmic membrane or outer membrane, and for the OAg^+ strain, much of the detected O antigen is believed to be on the outer membrane. Right-side-out cytoplasmic membrane vesicles were made by osmotic lysis of spheroplasts (1, 7) in the hope of obtaining a clearer result for the distribution of readings in the cytoplasmic membrane of an OAg^+ strain. However, the experiment proved to be difficult to do, and the results were not conclusive (data not shown).

In conclusion, a strain carrying a *wzx* mutation of a Dysenteriae 1 O-antigen gene cluster accumulates UndPP-O units on the cytoplasmic side of the cytoplasmic membrane. These results strongly suggest that the *wzx* gene encodes a flippase which is responsible for translocation of O units across the cytoplasmic membrane. Further studies will focus on elucidating the mechanism of O-unit transport.

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REFERENCES

1. **Altendorf, K. H., and L. A. Staehelin.** 1974. Orientation of membrane vesicles from *Escherichia coli* as detected by freeze-cleave electron microscopy. J. Bacteriol. **117:**888–899.
2. **Bastin, D. A., and P. R. Reeves.** 1995. Sequence and analysis of the O antigen gene (*rfb*) cluster of *Escherichia coli* O111. Gene **164:**17–23.
3. **Collins, L. V., S. Attridge, and J. Hackett.** 1991. Mutations at *rfc* or *pmi* attenuate *Salmonella typhimurium* virulence for mice. Infect. Immun. **59:** 1079–1085.
4. **Holst, O., and H. Brade.** 1992. Chemical structure of the core region of lipopolysaccharides, p. 135–170. *In* D. C. Morrison and J. L. Ryan (ed.), Bacterial endotoxic lipopolysaccharides, vol. 1. CRC Press, Boca Raton, Fla.
5. **Jann, K., and B. Jann.** 1984. Structure and biosynthesis of O-antigen, p. 138–186. *In* E. T. Rietschel (ed.), Handbook of endotoxin, vol. 1. Elsevier Scientific Publishers, Amsterdam.
6. **Jayaratne, P., D. Bronner, P. R. MacLachlan, C. Dodgson, N. Kido, and C. Whitfield.** 1994. Cloning and analysis of duplicated *rfbM* and *rfbK* genes involved in the formation of GDP-mannose in *Escherichia coli* O9:K30 and participation of *rfb* genes in the synthesis of the group I K30 capsular polysaccharide. J. Bacteriol. **176:**3126–3139.
7. **Kaback, H. R.** 1971. Bacterial membranes. Methods Enzymol. **22:**89–120.
8. **Kessler, A. C., A. Haase, and P. R. Reeves.** 1993. Molecular analysis of the 3,6-dideoxyhexose pathway genes of *Yersinia pseudotuberculosis* serogroup IIA. J. Bacteriol. **175:**1412–1422.
9. **Kido, N., V. I. Torgov, T. Sugiyama, K. Uchiya, H. Sugihara, T. Komatsu, N. Kato, and K. Jann.** 1995. 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.
10. **Klena, J. D., and C. A. Schnaitman.** 1993. 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.
11. **Lindberg, A. A., T. Holme, C. G. Hellerqvist, and S. Svensson.** 1972. Studies of a Li-hapten isolated from cell-walls of the rough mutant *Salmonella typhimurium* 395 MR10. Acta Pathol. Microbiol. Scand. Sect. B **80:**743–750.
12. **Liu, D., A. M. Haase, L. Lindqvist, A. A. Lindberg, and P. R. Reeves.** 1993. Glycosyl transferases of O-antigen biosynthesis in *S. enterica*: identification and characterization of transferase genes of groups B, C2, and E1. J. Bacteriol. **175:**3408–3413.
13. **Liu, D., L. Lindqvist, and P. R. Reeves.** 1995. Transferases of O-antigen biosynthesis in *Salmonella enterica*: dideoxyhexosyl transferases of groups B and C2 and acetyltransferase of group C2. J. Bacteriol. **177:**4084–4088.
14. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoek, and L. van Alphen.** 1975. Electrophoretic resolution of the major outer membrane proteins of *Escherichia coli* K12 into four bands. FEBS Lett. **58:**254–258.
15. **Macpherson, D. F., P. A. Manning, and R. Morona.** 1994. Characterization of the dTDP-rhamnose biosynthetic genes encoded in the *rfb* locus of *Shigella flexneri*. Mol. Microbiol. **11:**281–292.
16. **Macpherson, D. F., P. A. Manning, and R. Morona.** 1995. Genetic analysis of the *rfbX* gene of *Shigella flexneri*. Gene **155:**9–17.

17. **McGrath, B. C., and M. J. Osborn.** 1991. Localization of the terminal steps of O-antigen synthesis in *Salmonella typhimurium*. J. Bacteriol. **173:**649–654.
18. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Mulford, C. A., and M. J. Osborn.** 1983. A intermediate step in translocation of lipopolysaccharide to outer membrane of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **80:**1159–1163.
20. **Nikaido, H., M. Levinthal, K. Nikaido, and K. Nakane.** 1967. Extended deletions in the histidine-rough-B region of the *Salmonella* chromosome. Proc. Natl. Acad. Sci. USA **57:**1825–1832.
21. **Osborn, M. J., J. E. Gander, and E. Parisi.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*: site of synthesis of lipopolysaccharide. J. Biol. Chem. **247:**3973–3986.
22. **Palva, T., and P. H. Makela.** 1980. Lipopolysaccharide heterogeneity in *Salmonella typhimurium* analysed by sodium dodecylsulfate-polyacrylamide gel electrophoresis. Eur. J. Biochem. **107:**137–143.
23. **Reeves, P. R.** 1993. Evolution of *Salmonella* O antigen variation by interspecific gene transfer on a large scale. Trends Genet. **9:**17–22.
24. **Reeves, P. R.** 1994. Biosynthesis and assembly of lipopolysaccharide, p. 281–314. *In* A. Neuberger and L. L. M. van Deenen (ed.), Bacterial cell wall, new comprehensive biochemistry, vol. 27. Elsevier Science Publishers, New York.
25. **Reeves, P. R., and D. Liu.** Unpublished data.
26. **Robbins, P. W., D. Bray, A. W. Dankert, and A. Wright.** 1967. Direction of chain growth in polysaccharides. Science **158:**1536–1542.
27. **Rosen, B. P., and T. Tsuchiya.** 1979. Preparation of everted membrane vesicles from *Escherichia coli* for the measurement of calcium transport. Methods Enzymol. **56:**233–241.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Sanderson, K. E., and J. R. Roth.** 1983. Linkage map of *Salmonella typhimurium*, edition VI. Microbiol. Rev. **47:**410–453.
30. **Sarsero, J. P., and A. J. Pittard.** 1995. Membrane topology analysis of *Escherichia coli* K-12 Mtr permease by alkaline phosphatase and β-galactosidase fusions. J. Bacteriol. **177:**297–306.
31. **Stevenson, G., B. Neal, D. Liu, M. Hobbs, N. H. Packer, M. Batley, J. W. Redmond, L. Lindquist, and P. Reeves.** 1994. Structure of the O antigen of *Escherichia coli* K-12 and the sequence of its *rfb* gene cluster. J. Bacteriol. **176:**4144–4156.
32. **Szabo, M., D. Bronner, and C. Whitfield.** 1995. 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. J. Bacteriol. **177:**1544–1553.
33. **Wang, L., D. Liu, and P. R. Reeves.** Unpublished data.
34. **Wang, L., and P. R. Reeves.** 1994. Involvement of the galactosyl-1-phosphate transferase encoded by the *Salmonella enterica rfbP* gene in O-antigen subunit processing. J. Bacteriol. **176:**4348–4356.
35. **Wright, A., M. Dandert, and P. W. Robbins.** 1965. Evidence for an intermediate stage in the biosynthesis of the *Salmonella* O-antigen. Proc. Natl. Acad. Sci. USA **54:**235–241.
36. **Yamato, I., Y. Anraku, and K. Hirosawa.** 1975. Cytoplasmic membrane vesicles of *Escherichia coli*. I. A simple method for preparing the cytoplasmic and outer membranes. J. Biochem. **77:**705–718.