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A Wzz (Cld) Protein Determines the Chain Length of K Lipopolysaccharide in *Escherichia coli* O8 and O9 Strains

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The modal distribution of O-antigen chain length is determined by the Wzz (Cld/Rol) protein in those cases in which it has been studied. The system of O-antigen synthesis in *Escherichia coli* serotypes O8 and O9 is different from that reported for most other bacteria, and chain length distribution is thought not to be determined by a Wzz protein. We report the existence in *E. coli* O8 and O9 strains of *wzz* genes which are very similar to and have sequences within the range of variation of those which determine the chain length of typical O antigens. We also find that *wzz* genes previously identified by their effect on O-antigen chain length, when cloned and transferred to O8 and O9 strains, affect the chain length of a capsule-related form of LPS, K_{LPS}. We conclude that in at least some O8 and O9 strains there is a *wzz* gene which controls the chain length of K_{LPS} but has no effect on the O8 or O9 antigen.

The lipopolysaccharide (LPS) of gram-negative bacteria constitutes a major component of the outer membrane. It consists of three different regions: lipid A, core oligosaccharide, and O antigen. Lipid A and core oligosaccharide are synthesized together, while the O antigen is synthesized independently (14).

The O-antigen component of LPS has many repeats of a short oligosaccharide, the O unit. There are two known mechanisms for the biosynthesis of the O antigen. The first mechanism involves the synthesis of the O units on undecaprenyl PP_i ($UndPP_i$) on the inner face of the cytoplasmic membrane, followed by polymerization of the O units by O-antigen polymerase (also known as Wzy and previously known as Rfc [see Table 1]) at the periplasmic face of the inner membrane while they are linked to $UndPP_i$. Polymerization occurs at the reducing end by transfer of the growing chain from $UndPP_i$ to a new repeat unit also on $UndPP_i$ (25, 26). The final step in the synthesis of LPS involves the ligation of the O-antigen polymer to lipid A-core by the O-antigen ligase (23, 32), followed by the translocation of the complete LPS to the outer membrane by an unknown mechanism.

The second mechanism of O-antigen synthesis has been studied as it occurs in *E. coli* O8 and O9 strains, which have O antigens with repeat units of three and five mannose residues, respectively (14). Synthesis of these mannan O antigens takes place entirely at the cytoplasmic side of the inner membrane (15), by sequential addition of mannose residues at its nonreducing end with linkages that give the repeat unit structure (21). The mechanism for the assembly of the mannose polymer involves specific transferases located in the O-antigen gene cluster of *Escherichia coli* O9:K31$^-$ (19), and the mechanism for the translocation of the mannan LPS to the bacterial surface is again not known.

The number of O units per LPS molecule is not constant, but usually the majority of molecules have chain lengths clustered around a modal value. This characteristic O-antigen modal distribution has been shown to be determined by the Wzz (previously Cld [see Table 1]) protein in several O-antigen polymerase-dependent systems (2, 5). The *wzz* gene is located on the chromosome between *gnd* and *his* genes (2, 4). The amino acid sequence showed that the Wzz protein is largely hydrophilic with two potential transmembrane helices, and it was predicted to be an integral membrane protein with the major hydrophilic component in the periplasm (2); this has been confirmed by Tn*phoA* insertion analysis (27). The precise mode of action of the Wzz protein is unknown, but it is thought to interact with Wzy in the periplasm (2) to control the Wzy-mediated polymerization. The O8 and O9 LPS molecules also have a repeat unit O-antigen structure with modal chain length distribution as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (24), but the mechanism for generating the modal distribution with preferred chain length for these O antigens is not known. In this paper, we report the finding that at least some O8 and O9 strains have a *wzz* gene but that its function is probably determination of chain length of a minor form of LPS called K_{LPS} (36).

MATERIALS AND METHODS

Bacterial strains, plasmids, and antisera. The bacterial strains and plasmids used in this study are described in Table 1. Bacteria were grown in L broth (10 g of Bacto Tryptone, 5 g of yeast extract, and 10 g of NaCl per liter of water [pH 7]) supplemented with ampicillin at a concentration of 25 μg/ml when necessary. Antiserum against the O8 antigen was from Denka Seiken Co. Ltd. (Tokyo, Japan). Antisera against the O9, O32, and O104 antigens were from the Institute of Medical and Veterinary Science (IMVS), Adelaide, Australia. The O8 antiserum is a commercial specific serum, the O9 and O104 antisera had been adsorbed at the IMVS with a mixture of other O-antigen types, and the O32 serum which had not been adsorbed by the IMVS was adsorbed with a noncapsulated O8 strain, F492, as described by Gross and Rowe (9).

Enzymes and reagents. Restriction endonucleases and a kit for labelling DNA for Southern blots (Random-Primed Labelling kit) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). *Taq* polymerase for the PCR and sequencing reactions was obtained from Promega (Sydney, Australia). Conditions and buffers used were those recommended by the manufacturers.

LPS analysis. Outer membranes were prepared as described by Achtman et al. (1), with the following minor modifications: cells were grown in 100-ml volumes of Luria-Bertani broth to mid-exponential phase (4×10^8 cells per ml) and resuspended in 10 ml of 10 mM Tris (pH 8.0). The cells were disrupted by being passed twice through a French pressure cell, and unbroken cells were removed

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Antigenic characteristic(s)	Source or reference
Strains		
G7 (M26)	O8:K87[a]:H19	31
Bi 316-42 (M991)	O9:K9[a]:H12	7
RS504 (M990)	O75:K100	IMVS
M92	O111:H$^-$	3
SL 1654	*S. enterica* group B	29
F492	O8:K$^-$	K. Jann
Plasmids		
pPR1351	*wzz*[b] clone of M92	2
pPR1384	*wzz* clone of SL 1654	2
pPR1697	*wzz* clone of G7	This work
pPR1699	*wzz* clone of Bi 316-42	This work

[a] K87 and K9 are type 1B capsules (11).

[b] We are using a revised system of nomenclature for genes involved in bacterial surface polysaccharide synthesis in which *wzz* replaces *cld* and *wzy* replaces *rfc*. Information can be obtained from http://www.angis.su.oz.au/BacPolGenes/welcome.html, including reference to the publication of the revised nomenclature.

by centrifugation (Beckman JA-20 rotor; 7,000 rpm, 10 min, 4°C). The supernatant was centrifuged (Beckman JA-20 rotor; 19,000 rpm, 60 min, 4°C), and the pellet was resuspended in 3.6 ml of a solution containing 1.67% *N*-lauroylsarcosine (Sigma Chemical Co., St. Louis, Mo.) and 11.1 mM Tris (pH 7.6). The insoluble outer membranes were pelleted by centrifugation (Beckman JA-20 rotor; 19,000 rpm, 120 min, 4°C) and resuspended in 200 μl of electrophoresis buffer (16). A 50-μl portion of the sample was boiled for 10 min, digested with 25 μg proteinase K (Sigma) at 65°C for 1 h, and subsequently boiled for 5 min to denature any traces of undigested proteinase K. The samples were loaded on a glycine–SDS–12.5% PAGE gel as described by Lugtenberg et al. (22). Silver staining was performed as described by Hitchcock and Brown (10). Immunodetection was performed as described by Sambrook et al. (35).

DNA methods. The PCR was carried out as described by Saiki et al. (34) with a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). Oligonucleotide primers 465 and 466 were based on the first and last 15 bases of the *wzz* gene sequence of *E. coli* HU1124 (O75) (4) GenBank accession number M89934). Southern hybridizations were performed as described by Sambrook et al. (35) but were modified as follows: the hybridization solution contained 30% formamide and incubation was performed at 65°C (high-stringency conditions). PCR products were purified by using the Wizard PCR purification system (Promega). The *wzz* sequences of *E. coli* G7 and Bi 316-42 were determined by the dye-labelled primer technique using the Perkin-Elmer Cetus thermal cycler and an automated 373A DNA sequencer (Applied Biosystems). Conditions and buffers were those recommended by the manufacturers. DNA and deduced amino acid sequences were determined and analyzed by using the Australian National Genomic Information Service at Sydney University (33). The program GAP in the Genetics Computer Group package (28) was used for comparing sequences.

Nucleotide sequence accession numbers. The nucleotide sequences of the *wzz* genes of *E. coli* G7 and Bi 316-42 have been assigned GenBank accession numbers U39305 and U39306, respectively.

RESULTS

The *wzz* gene of M92 affects LPS chain length distribution in *E. coli* O8 and O9 strains. *E. coli* O8 strains have a modal chain length of 11 to 16 O units, and *E. coli* O9 strains have a modal chain length of approximately 12 to 19 O units (see data in reference 24). During our studies of the *wzz* gene, we transferred plasmids pPR1351 and pPR1384 carrying the *wzz* genes of *E. coli* M92 (O111) and SL 1654 (*Salmonella enterica* LT2), respectively, to *E. coli* O8 and O9 strains G7 and Bi 316-42. Analysis of LPS by SDS-PAGE showed very clearly that the presence of pPR1351 in G7 gives a mode with chain lengths of about 5 to 9 units in addition to the original mode at 11 to 16 units, while the presence of pPR1384 in G7 has no apparent effect on the LPS chain length pattern (Fig. 1). For Bi 316-42, the presence of pPR1351 gives new bands in the same general area as in G7, but the effect of the presence of pPR1384 was less marked (Fig. 1); interpretation was complicated by the

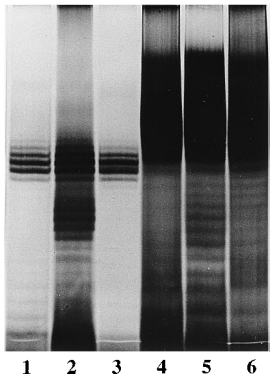

FIG. 1. SDS-PAGE gel showing the effects of the *E. coli* O111 *wzz* gene on *E. coli* serotypes O8 and O9. Lane 1, G7; lane 2, G7 transformed with pPR1351 (Wzz_{Ec_O111}); lane 3, G7 with pPR1384 (Wzz_{Se_LT2}); lane 4, Bi 316-42; lane 5, Bi 316-42 with pPR1351; lane 6, Bi 316-42 with pPR1384. The gel was silver stained.

presence of more material in bands running faster than the major mode in Bi 316-42 than in G7. These results suggested that, contrary to expectation, the *wzz* gene of M92 at least could give a new modal value to the LPS of O8 and O9 strains, implying that the chain length of these O antigens was determined by a resident *wzz* gene.

Hybridization, cloning, and nucleotide sequence analysis of *E. coli* O8 and O9 *wzz* genes. The results presented above were quite unexpected, as *wzz* was not expected to have any effect on O antigens not polymerized in the periplasm from preformed O units. In order to determine the existence of *wzz* genes in O8 and O9 strains, Southern hybridizations using the PCR product of the RS504 (O75) *wzz* gene as a probe with *Eco*RI and *Bam*HI chromosomal double digests were performed. The results showed that the RS504 probe hybridized with bands of approximately 9.2 kb in DNA from G7 and with bands of approximately 12 kb in DNA from Bi 316-42 under high-stringency conditions (Fig. 2), indicating the presence of a *wzz* gene with a sequence similar to that of RS504 (O75). An approximately 980-bp fragment from G7 (O8) and Bi 316-42 (O9) was successfully amplified with primers based on the *wzz* sequence of HU1124 (O75) (4) and cloned in pUC18. The cloned fragments were sequenced in both orientations, revealing high levels of similarity to the *E. coli* HU1124 (O75) and Sfl1 (*E. coli* Flexneri) *wzz* genes (Table 2).

We later learned from C. Whitfield (see the accompanying paper [6]) that his group had undertaken similar work and also found *wzz* genes in O8 and O9 strains but only in those carrying type IB capsules. It so happens that the strains that we used all have type IB capsules (Table 1), and the presence of the *wzz*

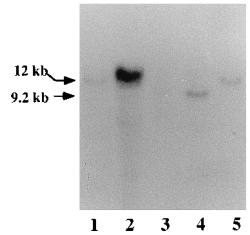

FIG. 2. Southern hybridization of the *wzz* genes in O8 and O9 strains with RS504 (O75) probe. Chromosomal DNA was double digested with *Eco*RI and *Bam*HI. Lane 1, M92 (O111); lane 2, RS504 (O75); lane 3, SL 1654 (*S. enterica* LT2); lane 4, G7 (O8); lane 5, Bi 316-42 (O9).

TABLE 2. Comparison of DNA and amino acid sequence similarities of chain length determinants in *E. coli*

Strain (serotype)	% Identity of determinant with that of:							
	G7		Bi 316-42		M92[a]		Sfl1[b]	
	DNA	Protein	DNA	Protein	DNA	Protein	DNA	Protein
HU1124 (O75)	84.7	89.9	84.9	89.2	66	66.6	95.9	97.2
G7 (O8)			98.5	98.5	65.4	65	83.7	89.3
Bi 316-42 (O9)					65.5	64.7	84	88.7
M92 (O111)							65.4	66.3

[a] The *E. coli* M92 *wzz* gene sequence is that of Bastin et al. (2) (GenBank accession number Z17241).
[b] The strain Sfl1 (*E. coli* Flexneri) *wzz* gene sequence is that of Morona et al. (27) (GenBank accession number X71790).

gene in these strains is consistent with the data of these researchers.

Immunodetection of nonmannan LPS in O8 and O9 strains. We next performed immunoblotting to determine the nature of the material in the various bands observed by SDS-PAGE. We used rabbit anti-*E. coli* O32 and rabbit anti-*E. coli* O104 to detect capsular antigens K87 in strain G7 gels and K9 in strain Bi 316-42 gels, respectively, as the O32 antigen has the same structure as the K87 capsular antigen does (12) and the O104 antigen has the same structure as the K9 capsular antigen does (20).

Anti-O32 serum (Fig. 3C) detected bands with approximately 16 to 24 repeat units in the G7 (O8:K87) preparations, while the O8 antiserum (Fig. 3B) reacted very strongly with the bands in the 11- to 16-repeat-unit range which showed up most strongly with silver staining but also reacted with more slowly moving material. The presence of pPR1351, carrying the chain length determinant of M92 (0111) (Wzz_{Ec_O111}) had no effect on the distribution of bands detected by the O8 antiserum, but the anti-O32 antiserum detected the bands of the lower mode of 5 to 9 units which were not detected at all by the O8 antiserum and also bands running near the original 16- to 24-unit mode (Fig. 3B and C). The bands of material in the five- to nine-unit mode reacted much more strongly than that in the slower moving bands.

Jann et al. (13) have shown that a small proportion of a group IB antigen is present in association with lipid A-core, and this material has been termed K_{LPS} (36). We consider the bands reacting with the anti-O32 serum to be K87 K_{LPS}, as K87 and O32 have identical structures (12) and the capsular material itself is not expected to enter the gel (13). Our results show then that the K87 K_{LPS} in strain G7 has a modal chain length of approximately 16 to 24 units, which is greater than that of the major bands of O8 LPS (approximately 11 to 16 units) but within the range for O8 LPS so that it was masked when silver staining was used; therefore, the K87 K_{LPS} bands were not distinguished as a separate mode by silver staining in the presence of the much greater amount of O8 antigen containing LPS. However, in the presence of the *wzz* gene of M92, there is a new mode of strongly reacting bands which are also detected by silver staining as they are in a region free of O8 LPS (Fig. 3A). It is clear that the *wzz* gene of M92 (O111) affects the K87 K_{LPS} but not O8 LPS.

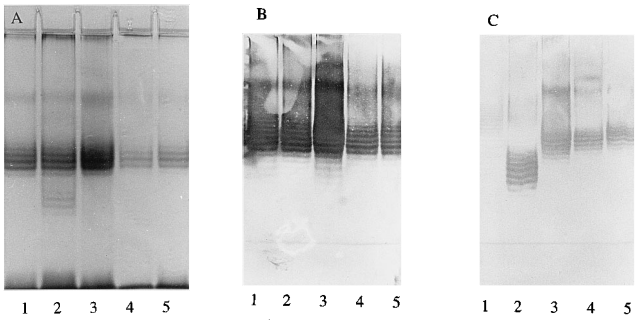

FIG. 3. SDS-PAGE and immunoblotting of *E. coli* O8 LPS and K87 K_{LPS}. Lanes 1, G7; lanes 2, G7 carrying pPR1351 (Wzz_{Ec_O111}); lanes 3, G7 carrying pPR1384 (Wzz_{Se_LT2}); lanes 4, G7 carrying pPR1697 (Wzz_{Ec_K87}); lanes 5, G7 carrying pPR1699 (Wzz_{Ec_K9}). (A) Silver staining; (B) immunoblotting with O8 antiserum; (C) immunoblotting with O32 antiserum.

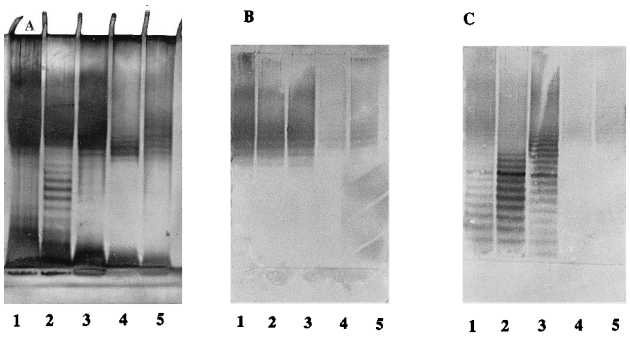

FIG. 4. SDS-PAGE and immunoblotting of *E. coli* O9 LPS and K9 K_{LPS}. Lanes 1, Bi 316-42 (O9); lanes 2, Bi 316-42 (O9) carrying pPR1351 (Wzz_{Ec_O111}); lanes 3, Bi 316-42 (O9) carrying pPR1384 (Wzz_{Se_LT2}); lanes 4, Bi 316-42 (O9) carrying pPR1697 (Wzz_{Ec_K87}); lanes 5, Bi 316-42 (O9) carrying pPR1699 (Wzz_{Ec_K9}). (A) Silver staining; (B) immunoblotting with O9 antiserum; (C) immunoblotting with O104 antiserum.

Immunoblots of G7 carrying pPR1384, which harbors the chain length determanant of *Salmonella esterica* LT2 (Wzz_{Se_LT2}) showed that the modal value of K87 K_{LPS} shifts from 16 to 24 units to 10 to 16 units. This result shows that Wzz_{Se_LT2} also affects the chain length of K87 K_{LPS} rather than that of the O8 antigen (Fig. 3B and C). However, the effect was not detected by silver staining, because both the 10- to 16-O-unit and the 16- to 24-O-unit modes were masked by the higher levels of O8 material banding in the same region when silver staining was used (Fig. 3A).

Plasmids pPR1697 and pPR1699 carrying *wzz* from G7 and Bi 316-42, respectively, were also transferred into G7; the preparations from the resulting strains show that the *wzz* genes from O8 and O9 strains have an effect on K87 K_{LPS} similar to that of Wzz_{Se_LT2} (Fig. 3C). These results show the expression of *wzz* genes from G7 and Bi 316-42 as well as their function as K_{LPS} chain length determinants. Plasmids pPR1384, pPR1697, and pPR1699 also created higher modes not present in the parental strain, as did Wzz_{Se_LT2} (Fig. 3C). Addition of plasmids carrying the *wzz* gene from M92, LT2, G7, or Bi 316-42 leads to amounts of K_{LPS} substantially increased over that present in the parent strain G7.

A similar study of immunoblots of Bi 316-42 and derivatives thereof showed that all the material having higher-level mobility than the major mode of O9 LPS was K9 K_{LPS} (Fig. 4). Bi 316-42 itself has a K_{LPS} modal value at about 16 to 24 units in addition to the shorter-chain-length material. The presence of Wzz_{Ec_O111} gave a mode at about five to nine units. The presence of Wzz_{Se_LT2} gave a mode around 12 to 18 units (Fig. 4C). As for G7, the presence of these cloned *wzz* genes not only led to a change in the K_{LPS} distribution but also increased the overall amount. The presence of the G7 or Bi 316-42 *wzz* genes

in this case had led to a concentration of all K_{LPS} in the 16- to 24-unit mode, with the absence of shorter chain lengths.

DISCUSSION

It is known that group IB capsules can exist in a K_{LPS} form (13, 36). In this study, we have shown that the chain length of K_{LPS} in IB-encapsulated *E. coli* O8 and O9 strains can be affected by Wzz proteins which normally determine the chain length of typical O antigens. We also found a *wzz* gene in these O8 and O9 strains which, when cloned and put into the source strain at a high copy number, modifies the chain length distribution of the K_{LPS}. Similar results are reported in the accompanying paper by Dodgson et al. (6), who also report on IA-encapsulated strains. The role of the *wzz* gene present in O8 and O9 strains G7 and Bi 316-42 is presumably to modulate the chain length of the capsules at least in their K_{LPS} form. Surprisingly, when the *wzz* gene of G7 is transferred back to the parental strain, the original mode is shifted from 16 to 24 O units down to 10 to 16 O units and the amount of K87 K_{LPS} is increased. In previous experiments, the modal value was conserved when the *wzz* genes of *E. coli* O111 and *S. enterica* LT2 were transferred to the parental strains in multicopy plasmids (Franco and Reeves, unpublished data). We believe that the *wzz* genes in G7 and Bi 316-42 regulate the chain lengths of the respective K_{LPS}s, and we have no explanation for the change in modal value when they are present in plasmids.

Both type IA and type IB capsule gene clusters map near *his* (11), as do O-antigen gene clusters. The colanic acid cluster of *E. coli* K-12, which maps upstream of the O-antigen cluster, has been shown to be allelic to those of type IA capsules (18). There are no detailed reports of map position for type IB capsules, but they are not allelic to the colanic acid gene cluster (17, 36) and type IB strains also produce colanic acid (17). It is perhaps significant that type IB capsules occur essentially in strains with O8 and O9 O antigens and that the O8 and O9 gene clusters are not located in the same place as most others but are on the other (downstream) side of the *gnd* gene between *gnd* and *his* (16, 19). Type IB capsular polysaccharides show marked similarities to typical O antigens, being at least in part attached to lipid A-core (13, 30, 36), and as we show in this paper, in that form they have their chain length modulated by the same Wzz protein which modulates the chain length of typical O antigens, suggesting that they are polymerized in the same way. These similarities between type IB capsules and typical O antigens suggest that they may be homologous and that type IB capsule gene clusters map at the typical O-antigen site, which is not otherwise occupied in O8 or O9 strains.

The *wzz* gene sequences of the O8 and O9 serotypes (strains G7 and Bi 316-42), which determine the modal length of K_{LPS}, are within the range of variation found for the previously described *E. coli wzz* genes. The *wzz* genes of G7 and Bi 316-42 are much more similar to those of HU1124 (O75) (4) and Sfl1 (Flexneri O antigen) (27) than any of the four is to that of *E. coli* M92 (Table 2). For the O9:K30 (type 1A) strain studied by the Whitfield group, it is known that there is no *wzz* gene adjacent to *his*, and they have now shown (6) that there is no *wzz* gene present at all in a range of *E. coli* O8 and O9 strains carrying type IA capsules; however, the *wzz* gene in the O8 and O9 type IB strains which do have a *wzz* gene could well be at its usual location.

It seems quite possible that the genes for the O8 and O9 antigens have been added to a typical *E. coli* strain, as the site which they occupy between the *his* and *gnd* genes does not have a comparable gene cluster in other strains, and that when this occurred the original O antigen was maintained but mainly in a capsule form. For G7 or Bi 316-42 we suggest that at some time in the past, the major O-antigen structures were O32 and O104, respectively, but during adaptation to a new niche, the O8 or the O9 antigen was acquired and became the major LPS polysaccharide, with the O32 or O104 antigen converted to a capsule, although with a proportion still present in LPS as K_{LPS}. This hypothesis is supported by the fact that the structures of capsules K87 and K9 are identical to those of O32 and O104, respectively (12, 20).

There seems to be a range of unusual but overlapping situations with the O antigens of some pathogenic *E. coli* strains, from cases such as O111, in which what was first described as O antigen can also occur in capsule form (8), through strains with the atypical O8 or O9 O antigen plus a type IB capsule in what appear to be typical O-antigen structures converted to a capsule form with some also present on LPS as K_{LPS}, to O8 and O9 strains with colanic acid replaced by a type IA capsule and with no structure related to typical O antigens. It should be noted that the O-antigen cluster of *E. coli* O111 strain M92 maps upstream of the *gnd* gene in the same position as do most O-antigen clusters (3) and that its *wzz* gene is in the same location as those of *S. enterica* LT2, *E. coli* O75, and *E. coli* Flexneri.

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