JOURNAL OF BACTERIOLOGY, Oct. 2000, p. 5620–5623 Vol. 182, No. 19
0021-9193/00/$04.00+0

Mutation of the Lipopolysaccharide Core Glycosyltransferase Encoded by *waaG* Destabilizes the Outer Membrane of *Escherichia coli* by Interfering with Core Phosphorylation

JEREMY A. YETHON,[1] EVGENY VINOGRADOV,[2] MALCOLM B. PERRY,[2] AND CHRIS WHITFIELD[1]*

Canadian Bacterial Diseases Network and Department of Microbiology, University of Guelph, Guelph, Ontario N1G 2W1,[1] and Canadian Bacterial Diseases Network and Institute for Biological Sciences, National Research Council, Ottawa, Ontario K1A 0R6,[2] Canada

Received 27 March 2000/Accepted 17 July 2000

In *Escherichia coli*, phosphoryl substituents in the lipopolysaccharide core region are essential for outer membrane stability. Mutation of the core glucosyltransferase encoded by *waaG* (formerly *rfaG*) resulted in lipopolysaccharide truncated immediately after the inner core heptose residues, which serve as the sites for phosphorylation. Surprisingly, mutation of *waaG* also destabilized the outer membrane. Structural analyses of *waaG* mutant lipopolysaccharide showed that the cause for this phenotype was a decrease in core phosphorylation, an unexpected side effect of the *waaG* mutation.

The outer membrane of gram-negative bacteria is a barrier to many antibiotics and host defense factors (14). This barrier function is due mainly to structural features of the lipopolysaccharide (LPS) molecules that make up the outer leaflet of the outer membrane bilayer. In *Escherichia coli*, the LPS molecule is conceptually divided into three parts (Fig. 1). Lipid A, the hydrophobic membrane anchor, is responsible for the endotoxic properties of LPS. Attached to lipid A is the core region, a phosphorylated oligosaccharide composed of 10 to 15 sugar molecules, which serves as the attachment site for the O antigen. Finally, the O antigen is a structurally variable polysaccharide made up of repeating oligomeric units. A critical feature of *E. coli* LPS is the presence of phosphoryl substituents on the heptose residues of the LPS core (Fig. 1). These phosphate-containing substituents are essential to membrane stability, likely because their negative charge allows neighboring LPS molecules to be cross-linked by divalent cations (9, 14).

The genes involved in LPS core phosphorylation have recently been characterized; the *waaP* gene product transfers phosphate to HepI, while *waaY* encodes the phosphotransferase that acts upon HepII (Fig. 1) (19). Of note, WaaY cannot function until after WaaP does, so that mutation of *waaP* alone is enough to completely eliminate core heptose phosphorylation. The *waaP* and *waaY* genes are located in the middle operon of the three operons in the *waa* (formerly *rfa*) locus on the chromosome; these operons encode all of the glycosyltransferases necessary for core elongation (4). Precise functions have been assigned to these glycosyltransferases by determining the core structures resulting from defined nonpolar insertions (Fig. 1) (3, 19).

In this study, the set of defined mutants used to determine core glycosyltransferase function was subjected to antimicrobial sensitivity testing with sodium dodecyl sulfate (SDS) and novobiocin as a measure of outer membrane stability. As expected, the mutants defective in core phosphorylation showed an increased susceptibility to these compounds. However, a surprising observation from this screening was that the defined *E. coli waaG* mutant (with LPS truncated after HepII [Fig. 1]) also appeared to have a compromised outer membrane barrier function. Therefore, the underlying mechanism for the *waaG* phenotype was investigated by a variety of structural methods.

Bacterial strains. *E. coli* F470 is the prototype strain for studies of the R1-type core oligosaccharide (11), and the structure of its complete core is known (6, 16). The R1 prototype was used in these and prior studies because it is the most prevalent among clinical and natural isolates of *E. coli* (1). Derivatives CWG303 (F470 with a *waaG*::*aacC1* insertion), CWG308 (F470 with a *waaO*::*aacC1* insertion), CWG296 (F470 with a *waaP*::*aacC1* insertion), CWG297 (F470 with a *waaQ*::*aacC1* insertion), and CWG312 (F470 with a *waaY*::*aacC1* insertion) have been described previously (3, 19). The *aacC1* cassette contains no transcriptional stop sites, and the absence of translational polarity was verified by complementation to the wild-type phenotype with the appropriate gene in *trans*.

Novobiocin and SDS sensitivity testing. To determine the MICs of novobiocin and SDS for each core mutant, twofold serial dilutions of novobiocin (from 200 to 1.6 μg/ml) and SDS (from 200 to 0.1 mg/ml) were made in 5-ml tubes of Luria-Bertani broth. Each series of tubes was then inoculated from an overnight culture and incubated with shaking at 37°C. Growth was scored as positive if the culture was visibly turbid (i.e., optical density at 600 nm of >0.2) after 8 h. Trials were performed in triplicate and repeated on two separate occasions.

The MIC data are summarized in Table 1. Strains with core mutations distal to *waaO* are not listed because they are phenotypically identical to the wild type. As shown in Table 1, mutation of *waaY* (strain CWG312, lacking the phosphate group on HepII) caused only a slight increase in susceptibility to both compounds, as did mutation of the HepIII transferase encoded by *waaQ* (strain CWG297, also lacking the phosphate group on HepII because the presence of HepIII is a prerequisite for the functioning of WaaY) (19). Mutation of *waaP* (strain CWG296, lacking all core phosphate groups) caused a drastic increase in susceptibility to both compounds, as reported previously (19). On the other hand, mutation of *waaG* (strain CWG303, truncated after HepII) caused a slight increase in susceptibility to novobiocin but a major (1,000-fold)

* Corresponding author. Mailing address: Department of Microbiology, College of Biological Science, University of Guelph, Guelph, Ontario N1G 2W1, Canada. Phone: (519) 824-4120, ext. 3478. Fax: (519) 837-1802. E-mail: cwhitfie@uoguelph.ca.

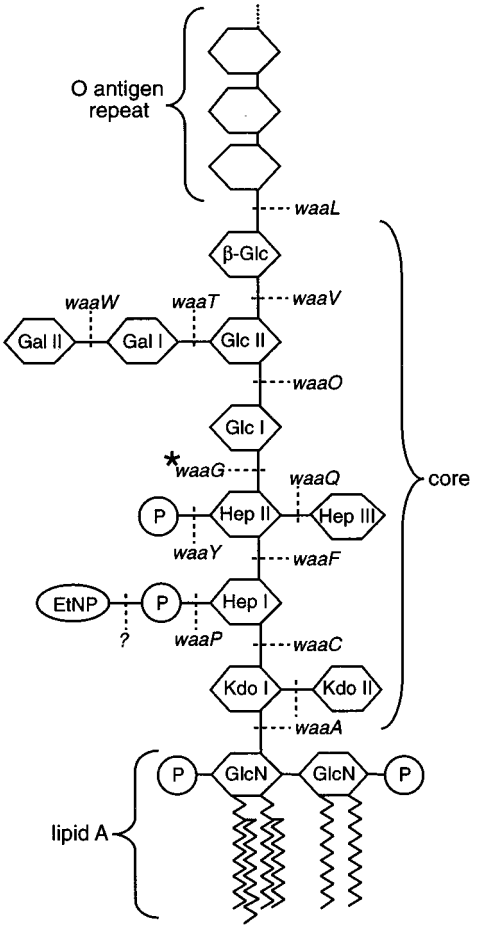


FIG. 1. Structure of the LPS from *E. coli* strains with an R1-type core. Core residues are designated by sugar abbreviation and number to facilitate identification. Abbreviations are as follows: GlcN, D-glucosamine; Hep, L-*glycero*-D-*manno*-heptose; P, phosphate; EtNP, 2-aminoethyl phosphate; Glc, D-glucose; and Gal, D-galactose. All sugars are in the pyranose configuration, and the linkages are α unless otherwise indicated. The assignment of function to genes encoding core glycosyltransferases and phosphotransferases has been reported previously (2, 3, 7, 19). Of particular note, the *waaG* gene product (indicated by an asterisk) is the glucosyltransferase for GlcI.

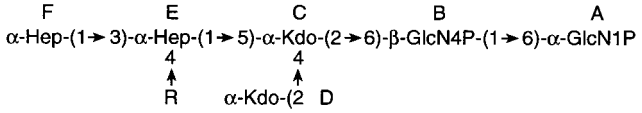
increase in susceptibility to SDS. It should be noted, however, that the change in SDS sensitivity of CWG303 might not be as large as is implied by the decrease in the MIC; the critical micelle concentration of SDS is 7.2 mM (approximately 2 mg/ml), so the concentration of monomeric SDS does not increase

TABLE 1. MICs of SDS and novobiocin for F470 and derivatives[a]

Strain	MIC of:	
	SDS (mg/ml)	Novobiocin (μg/ml)
F470 (parent)	>200	200
CWG312 (*waaY*)	200	100
CWG297 (*waaQ*)	200	100
CWG296 (*waaP*)	0.1	6.3
CWG303 (*waaG*)	0.2	100
CWG308 (*waaO*)	>200	200

[a] Strains with core mutations distal to *waaO* are not listed because they are identical to the wild type.

Deacylated LPS:



Acid-hydrolyzed LPS:

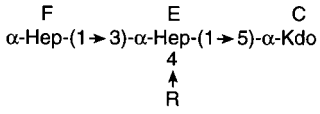


FIG. 2. Deduced structure of the LPS from CWG303 (*waaG*). The two major oligosaccharides observed after complete deacylation of CWG303 LPS differ only in the presence or absence of a phosphate (P) residue on Hep residue E (R = OH in OS-1; R = P in OS-2). The major oligosaccharides after mild acid hydrolysis of CWG303 LPS again differ only by phosphorylation on Hep residue E (R = OH in OS-3; R = P or 2-aminoethyl diphosphate in OS-4). The P substitution on Hep residue E (HepI) occurs in about 40% of LPS molecules, with 30% of these P residues being further modified by PEtN.

linearly beyond this point. To investigate the structural basis for strain CWG303's phenotype, its LPS was purified and analyzed as follows.

LPS purification. LPS was obtained by a modification of the phenol-water cell extraction method (17). Wet cells (300 g, containing approximately 20% dry material) were mixed with 240 ml of phenol and stirred for 30 min at 70°C. Instead of separating the phenol and water phases as described previously, the entire extraction mixture was diluted with 500 ml of water and then dialyzed (against water) for 1 week. LPS was subsequently isolated by centrifugation, with the remainder of the published phenol-water extraction protocol being essentially unchanged.

LPS deacylation, and analysis of resulting oligosaccharides (OS-1 and OS-2). LPS (100 mg) was deacylated and purified as described previously (15). Two major products, OS-1 and OS-2, were observed in a ratio of approximately 3:2 (data not shown). These oligosaccharides were analyzed by nuclear magnetic resonance (NMR) spectroscopy. The two products had the same carbohydrate skeleton, with the only difference being the presence of an additional phosphate group at O-4 of Hep residue E in OS-2 (Fig. 2). The identities of the monosaccharides were established on the basis of chemical shifts and vicinal proton coupling constants, which were in agreement with expected values based on the published structure of the F470 core (6, 16). The sequence of the monomeric components was determined by using nuclear Overhauser effect (NOE) data. NOE correlations between protons B1A6, F1E3, E1C5, E1C7, C3D6, and C3D8 were observed (data not shown), again corresponding to the sequence in Fig. 2. The positions of the phosphate residues were determined by using ^{1}H-^{31}P NMR correlations (data not shown). The electrospray mass spectra of OS-1 and OS-2 were also recorded (using a Micromass Quattro spectrometer). These spectra contained major peaks at *m/z* 1,325.1 and 1,405.1 again in agreement with the structures presented in Fig. 2. (The 80-mass-unit difference is due to the absence or presence of phosphate.)

Mild acid hydrolysis of LPS, and analysis of resulting oligosaccharides (OS-3 and OS-4). LPS (100 mg) was hydrolyzed in 2% acetic acid (which cleaves the acid-labile ketosidic linkage between 3-deoxy-D-*manno*-oct-2-ulosonic acid [Kdo] and lipid A) and purified as described previously (15). Two main products, OS-3 and OS-4, were observed, both being mixtures of several structures differing by Kdo derivatives. Again, the resulting oligosaccharides were analyzed by NMR and electro-

spray mass spectrometry. The NMR spectra of OS-3 and OS-4 were partially interpreted (data not shown) and were found to correspond to the structures presented in Fig. 2. OS-3 and OS-4 also differ in the absence or presence of phosphate at Hep residue E. However, about 30% of the OS-4 product carried an additional 2-aminoethyl phosphate (PEtN) substitution (measured by integration of PEtN H-2 and the sum of both Hep H-1 ^{1}H NMR signals). The mass spectrum of OS-3 contained major peaks at *m/z* 605.3 and 623.2 ($[M-1]^-$ of the products with anhydro-Kdo-ol and Kdo-ol at the end) and several unidentified minor peaks of higher mass. The OS-4 spectrum had a major peak at *m/z* 685.1 and a small peak at *m/z* 703.2 ($[M-1]^-$ of the products with anhydro-Kdo-ol and Kdo-ol at the end), as well as several minor peaks of higher mass, two of which correspond to the product with an additional PEtN residue (at *m/z* 808.0 and 826.2). Taken together, the above data indicate the presence of two main LPS structures for the *waaG* mutant (Fig. 2), differing only by the presence or absence of phosphate on Hep residue E (HepI).

Analysis of outer membrane protein profiles by SDS-PAGE. Supersensitivity to hydrophobic agents is a characteristic often associated with the pleiotropic phenotype termed deep rough, which is exhibited by mutants with severely truncated LPS core regions (i.e., lacking inner-core heptose residues [Fig. 1]). Another characteristic of the deep-rough phenotype is a marked decrease in the protein content of the outer membrane (reviewed in references 4 and 12). Indeed, in the case of deep-rough mutants, a proposed explanation for the increased susceptibility to hydrophobic compounds is that the void left by missing outer membrane proteins is filled by phospholipids, resulting in patches of phospholipid bilayer in the outer membrane (9).

Previous studies done with only partially characterized mutants suggest that the deep-rough phenotype is due mainly to the loss of core phosphoryl substituents (5, 10) and that it can be caused by mutation of the *waaP* gene alone. Therefore, to investigate whether our defined *waaG* and *waaP* mutants exhibited other characteristics of the deep-rough phenotype, such as the outer membrane protein defect, we isolated outer membrane fractions from the parent strain, F470, as well as from our defined mutant strains CWG303 (*waaG*) and CWG296 (*waaP*) and examined their protein profiles by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 3). In brief, equal numbers of cells (standardized by measuring the optical density at 600 nm) from 100-ml overnight cultures were harvested by centrifugation, resuspended in 20 mM Tris buffer (pH 8), and then completely lysed by passage through a French pressure cell. After removal of cell debris (5,000 × *g*, for 10 min), the total membrane fractions were collected by centrifugation (100,000 × *g*, 2 h) and then resuspended in 2% Sarkosyl. The Sarkosyl-insoluble outer membrane fraction was collected by centrifugation, washed a second time in 2% Sarkosyl, and recentrifuged. The resulting pellet was resuspended in 1 ml of 20 mM Tris buffer (pH 8) and analyzed by SDS-PAGE.

On the basis of earlier studies (5, 10), it was expected that CWG296 (*waaP*) would show the outer membrane protein defect, but it was unknown what effect mutation of *waaG* would have in strain CWG303. Surprisingly, the outer membrane protein profiles of F470, CWG296, and CWG303 appeared identical (Fig. 3). Of particular note, the ratio of porins (OmpF and OmpC) to OmpA is constant, which was not the case in earlier work done with partially characterized mutants (5, 10). This discrepancy suggests that these earlier mutants may have carried additional uncharacterized mutations or that the previous results were influenced by differences in genetic backgrounds. Thus, the susceptibility of CWG303 to SDS and

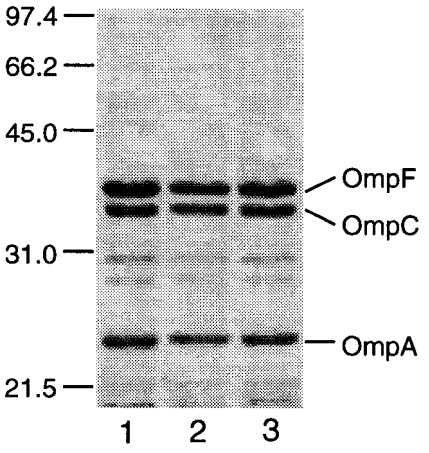


FIG. 3. SDS-PAGE gel (12.5% polyacrylamide) of Sarkosyl-insoluble outer membrane fractions. Lane 1, F470 (parent); lane 2, CWG303 (*waaG*); lane 3, CWG296 (*waaP*). Molecular mass markers (in kilodaltons) are indicated to the left of the gel, and major proteins are labeled on the right.

the susceptibility of CWG296 to SDS and novobiocin cannot be explained simply by the loss of outer membrane proteins and their replacement by phospholipid bilayer patches in the outer membrane. However, these data do not preclude the appearance of phospholipid bilayer patches in the outer membrane as a direct consequence of LPS changes.

Interpretation of the *waaG* phenotype. The structural data presented above show that mutation of *waaG* results in a complete lack of phosphate groups on HepII and in only 40% phosphate substitution at HepI. This corresponds to an 80% total reduction in heptose phosphorylation, given that the modification of HepI and that of HepII are known to be nearly stoichiometric in the wild type (4). The nonstoichiometric modification of HepI phosphate with PEtN appears to be unaffected. The observation that neither CWG303 (*waaG*) nor CWG296 (*waaP*) has an altered outer membrane protein profile (Fig. 3) suggests that the main cause of the observed outer membrane instability lies with this change in LPS core structure and not with gross defects in overall outer membrane organization. Also of note, the differential response of the *waaP* and *waaG* mutants to SDS and novobiocin is somewhat surprising and must reflect the different modes of action of the two test compounds. It is still unknown why an 80% reduction in core phosphorylation drastically affects the MIC of SDS but not that of novobiocin.

Clearly, the level of LPS core phosphorylation plays a crucial role in membrane stability and the biology of the family *Enterobacteriaceae*. Indeed, while *waaP* mutants of *Salmonella enterica* serovar Typhimurium form full-length, O-antigen-capped LPS molecules, the bacteria are avirulent (18). The membrane defect observed in strain CWG303 (*waaG*) was not as severe as that seen with mutation of *waaP* but was more detrimental than mutation of the HepII phosphotransferase encoded by *waaY* (Table 1). However, a *waaO* mutant, with a core only one sugar longer than that of the *waaG* mutant (Fig. 1), had wild-type levels of resistance to the test compounds (Table 1). There is considerable prior data that support the importance of LPS phosphates in maintaining outer membrane stability (reviewed in references 4, 9, 12, and 13).

In hindsight, the effect of the *waaG* mutation on core phosphorylation can be rationalized given the proximity of GlcI to the core heptose phosphorylation sites; the WaaG-catalyzed reaction might be a requirement for WaaP and WaaY sub-

strate specificity. This suggestion fits well with the much earlier observation that the in vitro phosphorylation of LPS (using LPS from an undefined phosphate-deficient mutant and crude lysates from wild-type bacteria) proceeds much faster when GlcI is present in the LPS core acceptor (8). The defined set of core mutants used in this study should now allow the determination of the precise sequence of events in the biosynthesis of the LPS core region, including the exact substrate specificity of each enzyme involved.

This work was supported in part through funding to M.B.P. and C.W. by the Canadian Bacterial Diseases Network (Network of Centres of Excellence). C.W. is a Medical Research Council of Canada Senior Scientist. J.A.Y. is the recipient of a graduate scholarship from the Medical Research Council of Canada.

REFERENCES

1. **Amor, K., D. E. Heinrichs, E. Frirdich, K. Ziebell, R. P. Johnson, and C. Whitfield.** 2000. Distribution of core oligosaccharide types in lipopolysaccharides from *Escherichia coli*. Infect. Immun. **68:**1116–1124.
2. **Brabetz, W., S. Müller-Loennies, O. Holst, and H. Brade.** 1997. Deletion of the heptosyltransferase genes *rfaC* and *rfaF* in *Escherichia coli* K-12 results in an Re-type lipopolysaccharide with a high degree of 2-aminoethanol phosphate substitution. Eur. J. Biochem. **247:**716–724.
3. **Heinrichs, D. E., J. A. Yethon, P. A. Amor, and C. Whitfield.** 1998. The assembly system for the outer core portion of R1- and R4-type lipopolysaccharides of *Escherichia coli*. J. Biol. Chem. **273:**29497–29505.
4. **Heinrichs, D. E., J. A. Yethon, and C. Whitfield.** 1998. Molecular basis for structural diversity in the core regions of the lipopolysaccharides of *Escherichia coli* and *Salmonella enterica*. Mol. Microbiol. **30:**221–232.
5. **Helander, I. M., M. Vaara, S. Sukupolvi, M. Rhen, S. Saarela, U. Zähringer, and P. H. Mäkelä.** 1989. *rfaP* mutants of *Salmonella typhimurium*. Eur. J. Biochem. **185:**541–546.
6. **Holst, O., and H. Brade.** 1992. Chemical structure of the core region of lipopolysaccharides, p. 134–170. *In* D. C. Morrison and J. L. Ryan (ed.), Bacterial endotoxic lipopolysaccharides, vol. I. CRC Press, Boca Raton, Fla.
7. **Kadrmas, J. L., and C. R. Raetz.** 1998. Enzymatic properties of lipopolysaccharide in *Escherichia coli*. Purification and properties of heptosyltransferase I. J. Biol. Chem. **273:**2799–2807.
8. **Mühlradt, P. F.** 1971. Biosynthesis of *Salmonella* lipopolysaccharide. Studies on the transfer of glucose, galactose, and phosphate to the core in a cell free system. Eur. J. Biochem. **18:**20–27.
9. **Nikaido, H., and M. Vaara.** 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49:**1–32.
10. **Parker, C. T., A. W. Kloser, C. A. Schnaitman, M. A. Stein, S. Gottesman, and B. W. Gibson.** 1992. Role of the *rfaG* and *rfaP* genes in determining the lipopolysaccharide core structure and cell surface properties of *Escherichia coli* K-12. J. Bacteriol. **174:**2525–2538.
11. **Schmidt, G., B. Jann, and K. Jann.** 1969. Immunochemistry of R lipopolysaccharides of *Escherichia coli*. Different core regions in the lipopolysaccharides of O group 8. Eur. J. Biochem. **10:**501–510.
12. **Schnaitman, C. A., and J. D. Klena.** 1993. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiol. Rev. **57:**655–682.
13. **Sukupolvi, S., and M. Vaara.** 1989. *Salmonella typhimurium* and *Escherichia coli* mutants with increased outer membrane permeability to hydrophobic compounds. Biochim. Biophys. Acta **988:**377–387.
14. **Vaara, M.** 1992. Agents that increase the permeability of the outer membrane. Microbiol. Rev. **56:**395–411.
15. **Vinogradov, E., and M. B. Perry.** 2000. Structural analysis of the core region of lipopolysaccharides from *Proteus mirabilis* serotypes O6, O48, and O57. Eur. J. Biochem. **267:**2439–2446.
16. **Vinogradov, E. V., K. van der Drift, J. E. Thomas-Oates, S. Meshkov, H. Brade, and O. Holst.** 1999. The structures of the carbohydrate backbones of the lipopolysaccharides from *Escherichia coli* rough mutants F470 (R1 core type) and F576 (R2 core type). Eur. J. Biochem. **261:**629–639.
17. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharide extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. **5:**83–91.
18. **Yethon, J. A., J. S. Gunn, R. K. Ernst, S. I. Miller, L. Laroche, D. Malo, and C. Whitfield.** 2000. *Salmonella enterica* serovar Typhimurium *waaP* mutants show increased susceptibility to polymyxin and loss of virulence in vivo. Infect. Immun. **68:**4485–4491.
19. **Yethon, J. A., D. E. Heinrichs, M. A. Monteiro, M. B. Perry, and C. Whitfield.** 1998. Involvement of *waaY*, *waaQ*, and *waaP* in the modification of *Escherichia coli* lipopolysaccharide and their role in the formation of a stable outer membrane. J. Biol. Chem. **273:**26310–26316.