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Additive Effect of *tolC* and *rfa* Mutations on the Hydrophobic Barrier of the Outer Membrane of *Escherichia coli* K-12

JOE A. FRALICK[1]* AND LISA L. BURNS-KELIHER[2]

Department of Microbiology and Immunology, Texas Tech University Health Sciences Center, Lubbock, Texas 79430,[1] and Department of Biology, Washington University, St. Louis, Missouri 63130[2]

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Studies using *tolC* mutant derivatives of deep rough (*rfa*) mutants indicate that *tolC* and *rfa* mutations have an additive effect with respect to their sensitivity to hydrophobic agents, suggesting that they are not acting through a mutual mechanism to alter the permeability of the outer membrane.

Enteric bacteria are usually refractory to environmental agents such as bile salts, detergents, fatty acids, and hydrophobic antibiotics (15, 17–19). This is partially due to the presence of an outer membrane, which, unlike most biological membranes, acts as a barrier to the passage of hydrophobic and amphipathic molecules (2, 8, 15, 18). This specialized function is reflected in its unique lipid composition and molecular architecture. The distribution of its lipids is highly asymmetric. A unique glycolipid, lipopolysaccharide (LPS), is found exclusively in the outer leaflet of the outer membrane, while phospholipids occupy the inner leaflet of this bilayer (14). Unlike phospholipids, monolayers of LPS are relatively impermeable to hydrophobic compounds, and it has been argued that this low permeability is due, in part, to the strong lateral interactions between adjacent LPS molecules (2, 18, 26), resulting in a highly ordered LPS monolayer surrounding the cell. As might be expected, some mutations which affect LPS structure also result in an alteration in the permeability barrier of the outer membrane (7, 18, 22). These mutations map in genes whose products are involved in the biosynthesis and assembly of the inner core region of LPS (*rfa* mutants) and are phenotypically known as "deep rough" mutants. The deep rough phenotype includes hypersensitivity to hydrophobic agents such as sodium lauryl sulfate (SDS) and novobiocin; resistance to an LPS-specific phage, U3; and decreased elaboration of the porin proteins OmpF and OmpC (1, 18, 22). Other mutations which do not map in the *rfa* locus but which affect the sensitivity to hydrophobic agents have also been described (24, 30). One such mutation maps to *tolC*, a gene whose product is a minor outer membrane protein which plays a major role in outer membrane function (12). TolC$^-$ mutants are exquisitely sensitive to hydrophobic agents, including detergents, dyes, and hydrophobic antibiotics (31); are defective in the import of colicin E1 (30, 31) and the export of hemolysin (28, 29) and colicin V (5); are resistant to an LPS-specific phage, U3 (1); down regulate OmpF (11, 13); and are defective in the segregation-partitioning of chromosomes (6). Whether these phenotypes are the result of a single underlying TolC function or reflect the complexity of TolC activity remains to be determined.

TolC has recently been implicated in the maturation-assembly of LPS in the outer membrane (21, 22). It has been convincingly shown by Schnaitman and his colleagues that mutations resulting in defects of the heptose region of the inner core of LPS (*rfaCDEF*) and mutations which affect the attachment of a phosphoryl substituent to the heptose I (Hep I) moiety of LPS (*rfaP*) exhibit a deep rough phenotype (1). Furthermore, it has been hypothesized that the phosphoryl substituent on Hep I is originally pyrophosphorylethanolamine (PPEA), whose ethanolamine moiety serves as a blocking group to limit cross-bridging of the LPS during its translocation and assembly in the outer membrane (1, 22). According to this hypothesis, the PPEA is then modified to phosphate (P) by the removal of phosphorylethanolamine (PEA) by TolC, which permits ion bridging between the phosphate groups of adjacent LPS molecules (22). Supporting evidence for this hypothesis comes from unpublished preliminary analysis of LPS by gel filtration which indicates that the Hep I of the *tolC* mutant LPS may have only PPEA while Hep I of the *tolC*$^+$ LPS has both phosphate and PPEA groups (22).

The assumption, therefore, is that both deep rough mutants, which lack the HepI-P, and *tolC* mutants, whose HepI-P is blocked by a PEA group, act through a common mechanism in the alteration of the hydrophobic barrier of the outer membrane (22). Such a supposition would predict that *tolC* and deep rough (*rfa*) mutations should not show additive effects with respect to sensitivities to hydrophobic agents. However, past studies in our laboratory have suggested that this may not be the case. Using an isogenic series of well-characterized *rfa* mutants (Fig. 1), which express the deep rough phenotype, we examined the effect of the addition of a *tolC* insertion mutation on their sensitivity to hydrophobic agents. The results of our experiments are given in Table 1. It can be seen that a *tolC*::Tn*10* derivative of D21, the parent of the *rfa* mutants, was more sensitive to hydrophobic agents than were the deep rough mutants which were missing either heptose phosphorylation (D21e7) or the entire heptose region of the LPS inner core (D21f2) (4, 20).

Furthermore, we observed an additive effect with respect to the sensitivity to hydrophobic agents by the addition of the *tolC*::Tn*10* mutation to the *rfa* mutants. In other words, strains carrying *tolC*::Tn*10* in combination with deep rough *rfa* mutations were more sensitive to deoxycholate, SDS, and novobiocin than were either the *tolC*::Tn*10* or the *rfa* strains by themselves. However, their sensitivity to a hydrophilic antibiotic, kanamycin, was essentially identical, indicating that the effect seen was not a general disruption of the outer membrane. In an attempt to better quantify the sensitivity to hydrophobic agents, we determined the MIC of SDS for D21f2, D21*tolC*::Tn*10*, and D21f2*tolC*::Tn*10* strains. The results are given in Table 2. It can be seen that the D21f2*tolC*::Tn*10* derivative is approximately threefold more sensitive to

* Corresponding author. Phone: (806) 743-2555. Fax: (806) 743-2334.

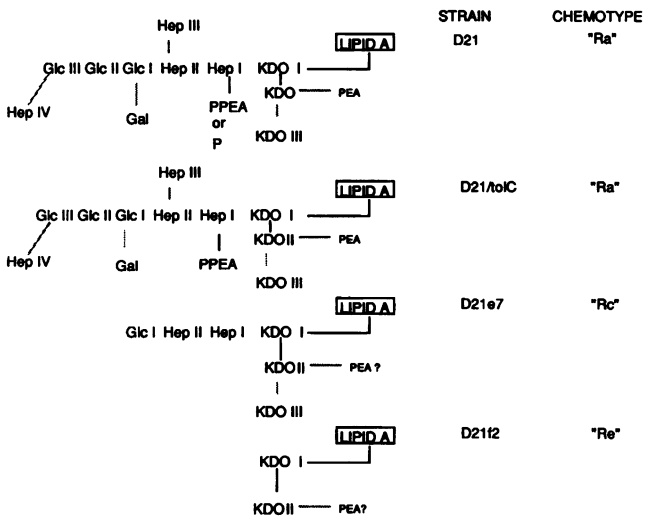

FIG. 1. Schematic representation of the tentative chemical structure of the LPS core produced by the D21 series used in this study. "Ra" through "Re" refer to equivalent chemotypes of the mutant LPS as determined by the chemical analysis of Boman and Monner (4) using the model of Austin et al. for LPS structure (1). Characterization of LPS from the *tolC*::Tn*10* derivative of D21 (D21*tolC*::Tn*10*) is based on the hypothesis that TolC removes the PPEA from Hep I (22). Partial substituents are indicated by dashed lines, and substituents whose sites of attachment are not well documented are not shown. Abbreviations are as follows: Hep, L-glycerol-D-mannoheptose; KDO, 2-keto-3-deoxyoctulosonic acid; Glc, D-glucose; Gal, D-galactose; P, phosphate; PPEA, pyrophosphorylethanolamine. D21 (CGSC no. 5158) is K-12 F$^-$ *proA23 lac-28 trp-30 his-51 rpsL173 tsx-81 ampC*. D21e7 (CGSC no. 5157) is an *rfa-1* derivative of D21 missing galactose and heptose-bound phosphate from its LPS; D21f2 (CGSC no. 5162) is a heptoseless derivative of D21e7 (4, 20). The *tolC* derivatives of the D21 strains were obtained by T4GT7 transduction (32) of the *tolC210*::Tn*10* insertion mutation (23).

SDS than is the D21*tolC*::Tn*10* strain, which, in turn, is approximately sixfold more sensitive than is the heptoseless mutant (D21f2). Although one might argue that the difference in sensitivity to hydrophobic agents between *tolC* and *rfa* mutants is due to the presence of a PPEA substitution on Hep I (i.e., *tolC* mutant LPS would disrupt LPS-LPS associations more than would the loss of the heptose phosphate), it is difficult to use the same logic to explain why we find an enhanced sensitivity to hydrophobic agents in the *tolC* mutant

TABLE 1. Sensitivity to hydrophobic agents[a]

Strain	NOV	SDS	DOC	KAN
D21	15	0	0	40
D21e7	20	17	10	40
D21f2	22	19	17	40
D21/*tolC*	28	30	26	40
D21e7/*tolC*	37	33	30	40
D21f2/*tolC*	42	41	40	41

[a] Sterile blank paper discs (BBL Microbiology Systems) were placed on a lawn of the indicated strain (approximately 10^6 cells/ml) which had been spread on an L agar plate. A total of 30 μl of novobiocin (NOV; 20 mg/ml), SDS (10% [wt/vol]), deoxycholate (DOC; 5% [wt/vol]), and kanamycin (KAN; 50 mg/ml) was pipetted onto separate discs. The L agar plate was allowed to incubate at 37°C for 24 h, and the diameter of the zone of inhibition was measured (millimeters). The average of three separate experiments rounded off to the nearest whole number is given.

TABLE 2. MIC of SDS

Strain	MIC[a]	Ratio[b]
D21	>10,000[c]	
		>88
D21f2	113	
		6.6
D21/TolC$^-$	17	
		2.8
D21f2/TolC$^-$	6	

[a] The MIC of SDS was determined by serial dilution of SDS in L broth. The bacterial inoculum was approximately 10^5 cells/ml. The MIC was determined as the concentration of SDS, in micrograms per milliliter (final concentration), which prevented growth after 18 to 24 h. The values given are the averages of three separate experiments rounded off to the nearest whole number.

[b] The ratios of the MICs of D21/D21f2, D21f2/D21*tolC*::Tn*10*, and D21*tolC*::Tn*10*/D21f2*tolC*::Tn*10* are given.

[c] D21 grew in the presence of 10 mg of SDS per ml.

derivative of the heptoseless mutant. It appears that *rfa* and *tolC* mutations exhibit an additive effect with respect to the sensitivity to hydrophobic agents and, therefore, are not acting through the same mechanism to alter the hydrophobicity of the outer membrane.

It has been shown that deep rough mutants do not incorporate normal levels of proteins into their outer membrane, resulting in a compensatory increase of phospholipid (15, 17) and a decreased outer membrane density (2, 18, 19). Hence, in deep rough mutants both the phospholipid content and the structure of LPS are altered and it is difficult to know which is more responsible for the increase in permeability to hydrophobic agents. More than likely, it is both the increase in phospholipid content and the decrease in strong lateral LPS-LPS interactions which permit the formation of patches of glycerophospholipid bilayers in the outer membrane through which hydrophobic solutes can pass (25, 27). The *tolC* mutants, however, show no dramatic decrease in outer membrane protein content or decrease in outer membrane density (1). Whether or not the outer membranes of *tolC* mutants contain increased levels of phospholipid or phospholipid bilayer domains has not been determined. A possible explanation of our results is that *tolC* does not act on the Hep I-PPEA moiety of LPS, but rather on some other aspect of LPS maturation which affects outer membrane integrity and results in an added increase in permeability in deep rough mutants. One possibility would be that the LPS cross-bridging involves more than a single bridge between adjacent LPS molecules. A good prospect would be 2-keto-3-deoxyoctulosonic acid II. If TolC was involved in the removal of phosphoethanolamine from the PPEA of 2-keto-3-deoxyoctulosonic acid II and if a second gene product, a pyrophosphatase, removes the PEA from the Hep I-PPEA, then one might expect to see an additive effect in deep rough-*tolC* mutants. However, with the discovery that prokaryotes possess systems that pump hydrophobic compounds out of the cell (9, 16), we cannot exclude the possibility that the susceptibility to various hydrophobic agents in *tolC* mutants is due to the inactivation of an active extrusion pump. Hence, an alternative explanation, and one we favor, is that *tolC* mutations do not affect the outer membrane permeability barrier to hydrophobic agents per se but affect their removal from the cell. In other words, while deep rough *rfa* mutants affect the influx of hydrophobic compounds, *tolC* mutants affect their efflux. For instance, the *acrA* mutation of *Escherichia coli* K-12, which was previously thought to increase outer membrane permeability to various agents, including dyes and detergents, was shown to inactivate a multidrug efflux complex,

AcrAE (10, 16). So far, most endogenous multidrug resistance systems found in gram-negative bacteria have been composed of an efflux transporter, located in the cytoplasmic membrane, and an accessory protein which is thought to bridge the cytoplasmic transporter with an outer membrane channel so that the drugs can be extruded directly into the surrounding medium rather than into the periplasm (16). It has been suggested that TolC may form such a channel in light of its role in hemolysin and colicin V export (5, 28) and in colicin E1 import (31) and a recent report indicating that TolC may exist in the outer membrane as an oligomeric pore (3). Furthermore, it is of interest to note that increased expression of drug efflux systems is often accompanied by the induction of the antisense *micF* RNA and concomitant repression of OmpF porin synthesis (16), presumably to decrease the permeability of the outer membrane. The synthesis of *micF* antisense RNA has also been shown to be increased in *tolC* mutants (11), which is what one might expect if the hydrophobic agents being translocated can also act as inducers of the efflux system.

In summary, it appears that the definitive answer to the mechanism(s) by which *tolC* mutants alter the cells' sensitivity to hydrophobic agents remains to be resolved. However, our findings indicate that *tolC* and deep rough *rfa* mutants are not acting through a mutual mechanism to alter the permeability of the outer membrane. The slow influx of hydrophobic agents through the low-permeability outer membrane of *E. coli* makes an efflux system an especially potent mechanism for maintaining resistance to many noxious hydrophobic agents common to their habitat, and the *tolC* mutant phenotype is consistent with TolC being a component of such a system.

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