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Evidence that TolC Is Required for Functioning of the Mar/AcrAB Efflux Pump of *Escherichia coli*

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A study examining the influence of TolC on AcrA, AcrR, and MarR1 mutants indicates that functional TolC is required for the operation of the AcrAB efflux system and for the expression of the Mar phenotype. That the effect of TolC on the AcrAB pump is not regulatory in nature is shown by studies measuring the influence of a *tolC*::Tn*10* insertion mutation on the expression of an *acrA*::*lacZ* reporter fusion. These results are compatible with the hypothesis that TolC is a component of the AcrAB efflux complex.

Enteric bacteria have evolved to survive in an environment rich with pernicious agents, such as bile salts, detergents, and fatty acids. Protection against these inhibitors is due, in part, to the selective permeability barrier of an outer membrane, which serves as the first line of defense against the rapid intrusion of lipophilic compounds (2, 11, 24, 25, 27). However, it does not exclude them entirely (30), and the intracellular concentrations of these agents are determined by the net balance between their influx, through the outer membrane, and their removal, via an efflux pump (13, 17, 25). Hence, a mutation affecting either of these processes could dramatically alter the cell's sensitivity to hydrophobic agents.

One mutation which renders its host highly susceptible to hydrophobic inhibitors maps to *tolC* (23, 37). The product of this gene is a minor outer membrane protein (20) which has a major impact on outer membrane function(s) in *Escherichia coli*. The TolC mutant phenotype is highly pleiotropic, resulting in hypersensitivity to hydrophobic agents (23, 37), defects in the import and the export of specific proteins (4, 8, 12, 29, 35, 36), alterations in the regulation of porin proteins (19, 21), and defects in chromosome partitioning (9). However, whether these phenotypes are the result of a single TolC function or reflect the complexity of TolC activity remains to be elucidated.

It has been suggested that TolC mutations may alter the permeability of the outer membrane to hydrophobic agents through alterations in the structure of lipopolysaccharide (LPS) (31, 32). This permeability barrier is thought to be the consequence of a highly ordered monolayer of LPS which exclusively makes up the lipid portion of the outer leaflet of this asymmetric membrane (6, 22). Unlike phospholipids, monolayers of LPS are relatively impermeable to hydrophobic compounds, and it has been suggested that this low level of permeability is due, in large part, to strong lateral interactions between adjacent LPS moieties via ion-phosphate bridges (3, 27, 33). In support of this conclusion, it has been demonstrated 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 moiety of LPS (*rfaP*) lead to a "deep rough" phenotype, which includes hypersensitivity to hydrophobic agents (10, 27, 32). Because of the similarity between deep rough and TolC phenotypes and because of preliminary results which indicated that the heptose I phosphate of TolC LPS may be blocked by a phosphorylethanolamine group (32), it has been suggested that the hypersusceptibility seen in TolC mutants is the result of the inability of TolC LPS to form a highly ordered LPS monolayer (32). There are, however, significant differences between the TolC mutant and deep rough (*rfa*) phenotypes with respect to outer membrane protein content (1) and sensitivities to hydrophobic compounds (5) as well as with respect to the export and import of specific proteins (4, 8, 12, 35, 36). Furthermore, we have recently demonstrated, using isogenic *tolC* and *rfa* mutants, that *tolC* and *rfa* mutations have an additive effect with respect to sensitivity to hydrophobic agents (5), suggesting that they do not act through a mutual mechanism to alter the permeability function of the outer membrane.

An alternative explanation for the susceptibility of TolC mutants to lipophilic molecules is that this important outer membrane protein may play a role in the functioning of an efflux pump involved in the removal of noxious hydrophobic compounds (5). So far, most endogenous multiple-drug-resistance efflux 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, from which they could rapidly reenter the cell (13, 25, 34). However, the outer membrane channels for several important efflux systems have not yet been identified.

One efflux pump which removes a wide spectrum of hydrophobic agents from *E. coli* is the AcrAB multiple-drug-resistance system (13, 15, 25). The model of the AcrAB pump is based on the structure of the hemolysin transporter HlyBD (34). The putative accessory proteins, AcrA and HlyD, are homologous (17), and each may encode a periplasmic lipoprotein whose amino terminus is anchored to the inner membrane (15). The transporter proteins, AcrB and HlyB, are also homologous, and each contains a 12-α-helix transmembrane domain structure (17). In light of TolC's role in hemolysin export (35), its susceptibility to hydrophobic compounds (23, 37), and a report indicating that it may exist in the outer membrane as an oligomeric pore (3), it has been hypothesized that this outer membrane protein may serve as a channel for the AcrAB efflux system (13, 17).

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To investigate the involvement of TolC in the functioning of

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TABLE 1. Sensitivities of AcrA, TolC, and AcrA TolC mutants to hydrophobic agents

Strain	Relevant genotype	Sensitivity (zone diam [mm])[a] to:				MIC (μg/ml)[b] of:	
		ACR	NOV	DOC	SDS	SDS	NOV
W4573	*acrA*$^+$ *tolC*$^+$	17	18	0	0	>1,000[c]	128
W4573 *tolC*::Tn*10*	*acrA*$^+$ *tolC*	27	30	23	30	8	1
N43	*acrA tolC*$^+$	27	26	16	25	28	31
N43 *tolC*::Tn*10*	*acrA tolC*	27	30	23	30	8	1

[a] Sterile blank discs (1/4-in. [0.64-cm] diameter; BBL) were placed on a lawn of the indicated strain (approximately 10^6 cells per ml), which had been spread on an L agar plate. A total of 30 μl of a 25-mg/ml solution of ACR, a 20-mg/ml solution of NOV, a 5% (wt/vol) solution of DOC, or a 10% (wt/vol) solution of SDS was pipetted onto each disc. The plates were allowed to incubate overnight at 37°C, and the diameters of the zones of inhibition were measured. The averages of three separate experiments, rounded off to the nearest whole numbers, are given.

[b] The MICs of SDS and NOV were determined by serial dilutions in L broth. The bacterial inoculum was approximately 10^5 cells per ml. The MIC was determined as the concentration which prevented growth after 18 to 24 h. The values are the averages of three separate experiments, rounded off to the nearest whole numbers.

[c] WA4573 grew in the presence of 10 mg of SDS per ml.

TABLE 2. Sensitivities of AcrR, MarR, and TolC mutants to hydrophobic agents

Strain	Relevant genotype	Sensitivity (zone diam [mm])[a] to:			
		ACR	NOV	DOC	SDS
WM4680	*acrR*$^+$ *marR*$^+$ *tolC*$^+$	22	17	0	0
W4680 *tolC*	*acrR*$^+$ *marR*$^+$ *tolC*	29	35	25	30
WZM124	*acrR marR*$^+$ *tolC*$^+$	19	15	0	0
WZM124 *tolC*	*acrR marR*$^+$ *tolC*	30	34	25	30
AG100	*acrR*$^+$ *marR*$^+$ *tolC*$^+$	20	15	0	0
AG100 *tolC*	*acrR*$^+$ *marR*$^+$ *tolC*	30	35	25	30
AG102	*acrR*$^+$ *marR1 tolC*$^+$	18	12	0	0
AG102 *tolC*	*acrR*$^+$ *marR1 tolC*	30	35	25	30
HN899	*acrR marR tolC*$^+$	15	10	0	0
HN889 *tolC*	*acrR marR tolC*	29	35	25	30

[a] Methods are as described for Table 1.

the AcrAB efflux pump, *tolC*::Tn*10* derivatives of the *acrA* mutant, N43, and of its parent, W4573 (15), were constructed by T4GT7 transduction (38), and the sensitivities of these isogenic strains to the hydrophobic agents acriflavine (ACR), deoxycholate (DOC), sodium dodecyl sulfate (SDS), and novobiocin (NOV) were determined. The results are given in Table 1. As can be seen from these data, there is no additive effect of the *tolC* and *acrA* mutations with respect to sensitivity to the agents tested (i.e., W4573 *tolC*::Tn*10* and N43 *tolC*::Tn*10* strains showed the same sensitivities). These results suggest that *tolC* and *acrA* mutations act through a common mechanism in rendering their hosts hypersensitive to hydrophobic inhibitors.

From the data presented in Table 1, it can be seen that the *tolC*::Tn*10* derivatives of the AcrA mutant, N43, and its parent, W4573, are more susceptible than is N43. These results could be explained if the *acrA* mutation of N43 was leaky, or if TolC was required for the functioning of a second efflux system involved in the removal of hydrophobic molecules. To distinguish between these alternatives, the effect of a *tolC* mutation on two other *acrAB* mutants—JZM222, an *acrAB*Δ strain, and HN818, an *acrB*::Tn*903* strain—was examined. Transducing phage T4GT7 (38) was used to move a *tolC*::Tn*10* mutation into JZM222 and HN818 and their parent strains (kindly provided by D. Ma and H. Nikaido), and the sensitivities of these strains to the hydrophobic compounds listed in Table 1 were examined. It was found that the *tolC*::Tn*10* derivatives of these AcrAB$^-$ strains showed the same sensitivity to hydrophobic agents as did a *tolC*::Tn*10* derivative of their parents and that the TolC$^-$ strains were more sensitive than either of the AcrAB$^-$ mutants (JZM222 and HN818) (data not shown). These results suggest that the increased sensitivity of the TolC$^-$ derivatives seen in Table 1 is not strain related and that TolC may be required for the functioning of a second efflux system. Interestingly, Ma et al. found that *acrA* mutants can extrude significant amounts of ACR (15), implying that *E. coli* may have other multidrug efflux pumps with overlapping specificities. Currently there are three other AcrAB homologs: AcrEF (formally EnvCD), OrfAB, and AcrD (17). It will be important to determine if TolC is involved with any of these multiple-drug-resistance systems.

It has recently been shown that both the transcription of the *acrAB* operon and the resistance to hydrophobic agents are elevated in MarR (multiple-antibiotic-resistance) and AcrR mutants (14, 28). Hence, if TolC acts through the AcrAB efflux system, then one would predict that the increased drug resistance seen in AcrR and MarR mutants would be negated by a *tolC* mutation. If, on the other hand, the AcrAB efflux system is independent of TolC, then an increase in the expression of *acrAB* should diminish the susceptibility of a TolC mutant to such agents. To test this hypothesis a *tolC*::Tn*10* insertion mutation was transduced, via phage T4GT7 (38), into an *acrR*::Tn*903* mutant (HN818), a *marR1* mutant (AG102) (7), and an *acrR*::Tn*903 marR1* double mutant (HN899) (28) and their parents (HN817, AG100, and W4860) (kindly provided by Hiroshi Nikaido and Stuart Levy), and these strains were examined for their sensitivities to amphipathic agents. As can be seen from Table 2, the *tolC*::Tn*10* insertion mutation completely eliminated the elevated resistance to hydrophobic inhibitors seen in strains carrying *acrR*, *marR1*, and *acrR marR1* mutations. These results further support the conclusion that TolC acts through the AcrAB efflux system in determining the susceptibility of *E. coli* to hydrophobic inhibitors.

Mutations in *tolC* are known to elevate the transcription of *micF* antisense RNA, resulting in the concomitant reduction of OmpF (19). To determine if TolC has a regulatory effect on the expression of the *acrAB* operon, the influence of a *tolC* mutation on the expression of an *acrA*::*lacZ* fusion was examined. For these experiments a *tolC*::Tn*10* insertion mutation was transduced, with transducing phage T4GT7 (38), into a strain carrying either a single-copy (pNN602-K) or a multicopy (pDC602) plasmid harboring the same *acrA*::*lacZ* gene fusion construct (14) (both plasmids were the generous gifts of Hiroshi Nikaido). The expression of these reporters was measured during early exponential growth (i.e., at an optical density at 600 nm of less than 0.3), since it has been shown that the *acrAB* operon is up-regulated in stationary-phase cells (14). The results are given in Table 3. It can be seen from these results that the expression of the *acrAB*::*lacZ* fusion in the TolC$^-$ strains is as great as it is in the TolC$^+$ strains, suggesting that the inhibitory effect of a *tolC* mutation on the AcrAB efflux pump is not due to the down-regulation of the *acrAB* operon.

In summary, we can draw four conclusions from this study. (i) The mechanism by which *tolC* mutants become hypersensitive to hydrophobic agents is due, at least in part, to the inactivation of the *acrAB* multiple-drug-resistance efflux system. (ii) TolC may also influence another efflux system(s), besides AcrAB, or some other aspect of hypersensitivity to hydrophobic inhibitors. (iii) A functional TolC is required for

TABLE 3. Expression of *acrA*::*lacZ* fusion in a TolC mutant

Strain	Relevant genotype[a]	LacZ sp act[b]
HN882	*lacZ tolC*$^+$/pNN602-K	4.1
HN882 *tolC*	*lacZ tolC*::Tn*10*/pNN602	4.2
LBB 1302	*lacZ*Δ *tolC*$^+$/pDC602	67.7
LBB 1302 *tolC*	*lacZ*Δ *tolC*::Tn*10*/pDC602	73.4

[a] The plasmid pNN602-K is a kanamycin-resistant derivative of pNN602 (16) which carries the *acrA*::*lacZ* fusion as a single-copy reporter vector. The plasmid pDC602 (16) carries the *acrA*::*lacZ* fusion on a multicopy vector.

[b] Transcription of the *acrA*::*lacZ* fusion was assayed during mid-log-phase growth (A_{600} of 0.3 or less) in L broth medium. Units of LacZ specific activity were determined as described by Miller, by using the chloroform modification (18). Averages of three separate experiments, rounded off to the nearest tenth of a whole number, are given.

the expression of the Mar phenotype, presumably because Mar acts through the AcrAB efflux system (28). (iv) The TolC inactivation of the AcrAB pump does not appear to be regulatory in nature. While these results are compatible with the hypothesis that TolC is a component of the AcrAB efflux machinery, there are other possible explanations and definitive proof will have to await the demonstration of a physical association between TolC and components of the AcrAB complex.

Tina VanDyk found results similar to those presented in Table 1, using a variety of hydrophobic environmental contaminants as inhibitors. I thank her for so openly sharing her results. I also thank Barbara Bachmann and Mary Berlyn for the W4573 and N43 strains, Hiroshi Nikaido and Zack Ma for the *acrA*, *acrAB*, *acrR*, and *acrR marR1* mutants and plasmids pNN602-K and pDC602, and Stuart Levy for the MarR1 strain. Finally, I thank Abdul Hamood for the critical reading of the manuscript.

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