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An Excretory Function for the *Escherichia coli* Outer Membrane Pore TolC: Upregulation of *marA* and *soxS* Transcription and Rob Activity Due to Metabolites Accumulated in *tolC* Mutants[▽]

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Efflux pumps function to rid bacteria of xenobiotics, including antibiotics, bile salts, and organic solvents. TolC, which forms an outer membrane channel, is an essential component of several efflux pumps in *Escherichia coli*. We asked whether TolC has a role during growth in the absence of xenobiotics. Because *tolC* transcription is activated by three paralogous activators, MarA, SoxS, and Rob, we examined the regulation of these activators in *tolC* mutants. Using transcriptional fusions, we detected significant upregulation of *marRAB* and *soxS* transcription and Rob protein activity in *tolC* mutants. Three mechanisms could be distinguished: (i) activation of *marRAB* transcription was independent of *marRAB*, *soxR*, and *rob* functions; (ii) activation of *soxS* transcription required SoxR, a sensor of oxidants; and (iii) Rob protein was activated posttranscriptionally. This mechanism is similar to the mechanisms of upregulation of *marRAB*, *soxS*, and Rob by treatment with certain phenolics, superoxides, and bile salts, respectively. The transcription of other *marA*/*soxS*/*rob* regulon promoters, including *tolC* itself, was also elevated in *tolC* mutants. We propose that TolC is involved in the efflux of certain cellular metabolites, not only xenobiotics. As these metabolites accumulate during growth, they trigger the upregulation of MarA, SoxS, and Rob, which in turn upregulate *tolC* and help rid the bacteria of these metabolites, thereby restoring homeostasis.

Bacteria use efflux pumps to export a variety of xenobiotics (37). Some of these pumps have major clinical significance because they export multiple antibiotics (41). Recently, it has been noted that these pumps also export substances, such as bile salts and steroids, which occur in the environment of enteric bacteria (11; for a review, see reference 42).

An essential component of several efflux systems is TolC. TolC forms a multifunctional outer membrane channel (for a review, see reference 23) with roles in colicin uptake and secretion, bacteriophage adsorption, efflux of multiple antibiotics, detergents, dyes, and organic solvents, and export of hemolysin, heat-stable enterotoxin II (61), microcin J25 (9), and enterobactin (6). Export through the TolC channel requires interaction with two other proteins, an inner membrane transporter (e.g., AcrB) and a periplasmic membrane fusion protein (e.g., AcrA) that links the transporter to TolC. By means of this tripartite structure, xenobiotics or cellular products are pumped directly out of the cell from the cytosol or inner membrane. Basal levels of the AcrAB-TolC pump are important in providing the intrinsic resistance of *Escherichia coli* to many xenobiotics. Upregulation of the AcrAB-TolC pump engenders a multiple-antibiotic-resistance phenotype which is clinically significant. However, at least seven other sets of proteins in *E. coli*, such as AcrEF, EmrAB, and MacAB, form similar tripartite pumps with TolC, but they have different substrate specificities. The structures of TolC, AcrB, and AcrA have been solved, and a docking mechanism for AcrAB-TolC has been proposed (12, 32).

tolC and *acrAB* are members of the *marA*/*soxS*/*rob* regulon, which includes over 40 genes that promote resistance to multiple antibiotics, to numerous other xenobiotics, and to superoxides (3, 13, 24, 27, 38; for comprehensive reviews, see articles cited in reference 54). These genes are transcriptionally activated by three paralogous proteins, MarA, SoxS, and Rob, that bind a sequence upstream of the regulon promoter called the *marbox*.

Each of these transcriptional activators is regulated in a distinct manner. MarA and SoxS are transcriptionally regulated. The *marRAB* operon is repressed by MarR and autoactivated by MarA (the role of MarB is unknown). The operon can be derepressed by treating cells with salicylate and related phenolics which decrease the affinity of MarR for its binding sites (1, 28). However, a "*mar*-independent effect" of salicylate that increases the transcription of *marRAB* and of *inaA*, another member of the *marA*/*soxS*/*rob* regulon, has also been described (7, 49). The effect on *marRAB* transcription was found even in strains with combined deletions or null mutations of *marRAB*, *soxRS*, *rob*, and *emrAB* (29), indicating the existence of an additional mechanism for activating the regulon. *soxS* transcription is activated by SoxR after SoxR is activated by exposure to superoxides or nitric oxide (43). Rob is a very abundant and stable protein in *E. coli* (~10,000 molecules per cell) but has very little activity in vivo (2, 19, 46). Its activity is increased posttranslationally by treatment with 2,2′-dipyridyl, 4,4′-dipyridyl, bile salts, or decanoate (45, 46). Thus, each activator is activated in response to different environmental signals.

Upregulation of these transcriptional activators engenders a

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low but significant level of multiple antibiotic, superoxide, and organic solvent resistance. The antibiotic resistance and solvent resistance are due primarily to the AcrAB-TolC pump (4, 13, 55). *tolC* has four known promoters, two of which (*p*3 and *p*4) are activated by MarA, SoxS, and Rob via a single, uniquely configured *marbox* (10, 25, 62). The *acrAB* promoter is also activated by MarA, SoxS, and Rob (24).

Here, we examined the effects of *tolC* on the regulation of MarA, SoxS, and Rob during growth in standard laboratory media. We found elevated levels of transcription of *marA* and *soxS* and elevated activity of the Rob protein in *tolC* efflux mutants. From these findings, we infer that the following homeostatic loop occurs in wild-type bacteria: (i) normal metabolism results in the generation of certain intracellular metabolites that trigger the upregulation of the transcriptional activators MarA, SoxS, and Rob; (ii) these activators, in turn, upregulate *tolC*, increasing the capacity for excretion of the metabolites via TolC; and (iii) the resulting reduction in the concentrations of the trigger metabolites (TMs) restores the basal levels of the activators. In *tolC* mutants, the metabolites are not as effectively excreted, and the activator levels remain elevated.

MATERIALS AND METHODS

Bacterial strains. All strains used in this study are derivatives of *E. coli* K-12. Their construction and relevant genotypes are given in Table 1. Transduction was performed using bacteriophage P1 *clr-100*(Ts) as described previously (47). Donor phage for the *tolC*210::Tn*10-48* mutation (35) (referred to below as *tolC*::Tn*10*) were obtained by thermal induction of a P1 lysogen of strain LBB735. Transcriptional fusions to *lacZ* were made in λRS45 as described previously (25, 51, 62) and were assayed as single-copy prophages.

Culture media and chemicals. LB (Lennox) media contained (per liter) 10 g Bacto tryptone (Difco, Detroit, MI), 5 g Bacto yeast extract, and 5 g NaCl, and the pH was adjusted to 7.5 with NaOH. M9 minimal medium (33) was supplemented with 0.2% glucose, 1 ng/ml thiamine, and, where indicated, 0.2% Vitamin Assay Casamino Acids (Difco, Detroit, MI). The antibiotics used for genetic selection in *tolC*$^+$ and *tolC* strains were ampicillin (100 and 50 μg/ml, respectively), chloramphenicol (25 and 12.5 μg/ml, respectively), tetracycline (15 and 5 μg/ml, respectively), and kanamycin (30 μg/ml). MacConkey-lactose plates (Difco) contained 1% lactose. Since *tolC* efflux mutants do not grow on MacConkey medium (because it contains bile salts and crystal violet), all strains were routinely checked on this medium.

Growth of cells and β-galactosidase assays. Bacteria were grown in two ways, unless otherwise indicated. (i) For assays of cells in early log phase to late stationary phase (quasi-growth curve), overnight cultures in LB broth were diluted 1,000-fold, and then nine serial threefold dilutions were made. After growth for 10 to 12 h at 32°C, the A_{600} of the cultures usually ranged from 0.02 to over 3.0. The cultures were placed on ice and diluted in Z-buffer, and β-galactosidase activity was assayed, as described previously (33). (ii) For assays of cells in early log phase, cells were grown overnight in LB medium at 32°C, diluted 1,000-fold in fresh medium, aerated, grown to an A_{600} of about 0.2, placed on ice, and diluted, and β-galactosidase activity was assayed as described above. For experiments with cells grown in M9 minimal medium, procedures similar to those described above were used, except that the initial dilution was only 100-fold and the cells were grown for longer times at 32°C. To test posttranscriptional activation of *inaA*::*lacZ* fusions by Rob, cells were grown to an A_{600} of about 0.1, diluted twofold into LB medium with 0 or 5 mM (final concentration) 2,2′-dipyridyl (Sigma Chemical, St. Louis, MO), and aerated for 1 h at 32°C, and β-galactosidase activity was assayed. Expression of *tolC* cloned in the NcoI site of pTrc99A plasmids was accomplished by addition of 0.4 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) to LB broth.

Each β-galactosidase assay was carried out in duplicate using the $CHCl_3$-sodium dodecyl sulfate method (33), and all duplicate values were within ±5% of each other. Assays of cells in early log phase were performed at least twice in triplicate. The Kolmogorov-Smirnoff statistic was used to evaluate the probability (P_{KS}) that the (quasi) growth curve data for paired *tolC*$^+$ and *tolC* strains were from the same distribution, i.e., indistinguishable from each other (44).

RESULTS

Elevated transcription of *marRAB* in a *tolC* mutant. We considered the possibility that MarA, SoxS, and/or Rob activities may be elevated in *tolC* mutants because increased transcription of a *marA*/*soxS*/*rob* regulon member, *micF* (8, 18, 48), had been found in *tolC* mutants (35). To determine whether *marRAB* transcription is elevated in *tolC* mutants, we measured the activity of the *marRAB* promoter using appropriate *lacZ* transcriptional fusions in the wild-type and *tolC*::Tn*10* null mutant strains (35). To eliminate possible cross talk between MarA, SoxS, and Rob (30, 31, 50), strains which carry wild-type *marRAB* but have null mutations in *soxS* and *rob* were tested. These strains were diluted in LB broth and grown at 32°C to a range of densities (quasi-growth curve method), and β-galactosidase activity was assayed, as described in Materials and Methods.

Transcription of *marRAB*::*lacZ* was elevated ~2-fold in the *tolC*::Tn*10* mutant (M4188) compared to the wild-type strain (M3954) in log-phase to early-stationary-phase cells (Fig. 1A). *marRAB* expression decreased in later stationary phase (A_{600}, >1.6) for both the wild-type and mutant strains, but the decrease was more pronounced in the *tolC*::Tn*10* mutant. Thus, the increased activity of *micF* observed previously (35) could be due, at least in part, to the increase in the MarA level resulting from the ~2-fold increase in transcription of *marRAB* in *tolC*::Tn*10* mutants.

Activation of *marRAB* transcription in the absence of *marRAB*, *soxS*, and *rob*. *marRAB* transcription can be increased by four distinct mechanisms (54): (i) mutations which prevent MarR repressor synthesis; (ii) treatment with chemicals (e.g., salicylate) which interfere with MarR activity; (iii) transcriptional activation of the promoter by SoxS or Rob binding the *marRAB marbox* (cross talk); and (iv) a "*mar*-independent effect" of salicylate on *marRAB* transcription that has been shown to be independent of *marRAB*, *soxS*, *rob*, and *emrAB* (7, 29). To determine whether any of these mechanisms played a role in the upregulation of *marRAB* that was seen in the *tolC*::Tn*10* mutant, we measured the *marRAB*::*lacZ* activities in isogenic *tolC*$^+$ (M3953) and *tolC*::Tn*10* mutant (M4187) strains, both of which have a *marRAB* deletion in addition to null mutations in *soxS* and *rob* (Fig. 1B). Deletion of *marR* derepressed the levels of *marRAB* transcription about eightfold, as expected. However, the ratio of the β-galactosidase activity of the *tolC* mutant to the β-galactosidase activity of the *tolC*$^+$ strain was similar to ratio found for the *marRAB*$^+$ strains and was maintained even in late stationary phase. This shows that the effect of the *tolC* mutation on *marRAB* transcription is substantially independent of the first three mechanisms described above since it is found even in cells that are defective in MarRAB, SoxS, and Rob. Therefore, the activation of *marRAB* transcription in *tolC* mutants resembles the "*mar*-independent effect" of cells treated with salicylate.

Activation of *soxS* in *tolC*::Tn*10* mutants depends on *soxR*. A *soxS*::*lacZ* transcriptional fusion was used to monitor *soxS* transcription (59). The *soxS* expression in strains with *marRAB* and *rob* null mutations also was ~2-fold higher in the *tolC* mutant (M4183) than in the *tolC*$^+$ strain (M4014) (Fig. 2A). In both strains, the β-galactosidase activities decreased somewhat in stationary-phase cells, but the ratio of *tolC*::Tn*10* activity to

TABLE 1. *E. coli* strains, plasmids, and phages

Strain, plasmid, or phage	Relevant genotype	Reference(s) or source
Strains		
1411	*lacI3 lacZ118 gyrA*[a]	24, 39
AG100	*lac*$^+$	7
AG100AX	AG100 *acrAB*::Tn*903*(*kan*) *acrEF*::*spc*	36
AG100W	AG100 *acrAB*::*spc*	H. Nikaido
BW1041	GC4468 λJW1-*soxS*::*lacZ* Amp^r	59
CE1	*emrAB*::*cat*	11
CGSC5634	*tolC*Δ(EW1b)	57[c]
GC4468	*lac*Δ*4169*	B. Demple
LBB512	*thyA* derivative of the *tolC*$^+$ parent of CGSC5634	J. Fralick
LBB735	MG1655	J. Fralick
LBB801	MG1655 *tolC210*::Tn*10-48*	35[c]
M542	GC4468 (λRS45-*rob2*::*lacZ*)	46
M2561	N7918 *inaA1*::*lacZ tolC210*::Tn*10*[b]	This study
M2562	M542 *tolC210*::Tn*10*	This study
M2581	N8453 λRS45-*inaA*::*lacZ*	This study
M2583	M2581 *tolC210*::Tn*10*	This study
M2605	1411 *gyrA*$^+$ *inaA1*::*lacZ*[a,b]	This study
M2606	SM1411 *gyrA*$^+$ *inaA1*::*lacZ*[a,b]	This study
M2676	N8452 *soxR9*::*cat* λJW1-*soxS*::*lacZ*	This study
M3953	(*mar sad*)Δ*1738 rob*::*kan soxR8*::*cat* λRS45-*marRAB*::*lacZ*	This study
M3954	*rob*::*kan soxR8*::*cat* λRS45-*marRAB*::*lacZ*	This study
M4014	N8452 λJW1-*soxS*::*lacZ* Amp^r	This study
M4110	M2581/pTA108	This study
M4111	M2581/pTA:*marA*	This study
M4112	M2581/pTA:*soxS*	This study
M4113	M2581/pTA:*rob*	This study
M4114	M2583/pTA108	This study
M4115	M2583/pTA:*marA*	This study
M4116	M2583/pTA:*soxS*	This study
M4117	M2583/pTA:*rob*	This study
M4141	M4262 *tolC210*::Tn*10*	This study
M4142	M4263 *tolC210*::Tn*10*	This study
M4143	M4386 *tolC210*::Tn*10*	This study
M4165	M4275 *tolC210*::Tn*10*	This study
M4167	M2581 *pyrE60*~Tn*10*	This study
M4182	M2676 *tolC210*::Tn*10*	This study
M4183	M4014 *tolC210*::Tn*10*	This study
M4187	M3953 *tolC210*::Tn*10*	This study
M4188	M3954 *tolC210*::Tn*10*	This study
M4195	N7918 *emrAB*::*cat*	This study
M4196	N7918 *acrEF*::*spc*	This study
M4197	M2561 *acrEF*::*spc*	This study
M4198	CGSC5634 *inaA1*::*lacZ*[b]	J. Fralick
M4199	LBB512 *inaA1*::*lacZ*[b]	J. Fralick
M4262	GC4468 λRS45-*tolC*(C)::*lacZ*	This study
M4263	GC4468 λRS45-*tolC*(B)::*lacZ*	This study
M4275	GC4468 λRS45-*acrAB*::*lacZ*	This study
M4386	GC4468 λRS45-*tolC*(A)::*lacZ*	This study
M4807	AG100AX *inaA1*::*lacZ*[b]	This study
M4820	AG100W *inaA1*::*lacZ*[b]	This study
M5572	M2561/pTrc99A Amp^r vector	This study
M5573	M2561/pTrc99A::*tolC*$^+$ (NcoI site) Amp^r	This study
M5574	M2561/pTrc99A:*tolC*(*S257P*) (NcoI site) Amp^r	This study
M5575	M2561/pTrc99A:*tolC*(*A360T*) (NcoI site) Amp^r	This study
N7881	AG100 *inaA1*::*lacZ*[b]	This study
N7918	GC4468 *inaA1*::*lacZ*[b]	This study
N8444	(*mar sad*)Δ*1738 soxRS8*::*cat*	26
N8452	(*mar sad*)Δ*1738 rob*::*kan*	26
N8453	N8444 *rob*::*kan*	This study
SM1411	*lacI3 lacZ118 gyrA acrAB*::Tn*903*(*kan*)[a]	24, 39
Plasmids and phages		
pTA108	Low-copy-number cloning vector	46
pTA:*marA*	*marA* cloned in pTA108	46
pTA:*soxS*	*soxS* cloned in pTA108	46
pTA:*rob*	*rob* cloned in pTA108	46
pTrc99A Amp^r	Expression vector (Pharmacia)	R. Misra
pTrc99A::*tolC*$^+$ (NcoI)	*tolC*$^+$ cloned in pTrc99A NcoI site	52
pTrc99A::*tolC*(*S257P*)	*tolC*(*S257P*) cloned in pTrc99A NcoI site	52
pTrc99A:*tolC*(*A360T*)	*tolC*(*A360T*) cloned in pTrc99A NcoI site	52
λRS45	Phage used to isolate promoter::*lacZ* fusions	51
P1 *clr-100*(Ts)	Transducing phage	47

[a] The *gyrA* mutation present in strains 1411 and SM1411 was replaced by the wild-type *gyrA*$^+$ alleles in M2605 and M2606 during the P1-mediated transduction into these strains of *inaA1*::*lacZ*. *gyrA* and *inaA* are about 11 kb apart.

[b] The *inaA1*::*lacZ* fusions have been described previously (56).

[c] Via J. Fralick.

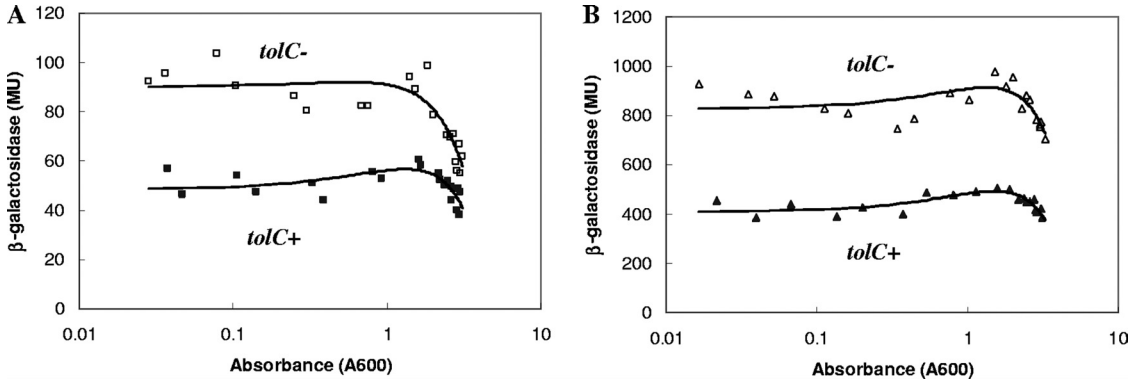

FIG. 1. β-Galactosidase activities of *marRAB*::*lacZ* transcriptional fusions in *tolC*$^+$ and *tolC*::Tn*10* cells. Cells grown in LB broth to different densities were assayed to determine β-galactosidase activities. (A) ■, *marRAB*$^+$ *soxS8*::*cat rob*::*kan* cells (M3954, *tolC*$^+$); □, M4188 (*tolC*). The probability (P_{KS}) that the two sets of data are from the same distribution, computed using the Kolmogorov-Smirnov test, was 1.5×10^{-5}. (B) ▲, *marRAB*Δ *soxS8*::*cat rob*::*kan* cells (M3953, *tolC*$^+$); △, M4187 (*tolC*). $P_{KS} = 3.4 \times 10^{-5}$. Note the different scales used. For this and other figures, MS Excel trend lines (second-order polynomials) were fitted to the data only for help with visualization. MU, Miller units.

tolC$^+$ remained relatively constant. Thus, *soxS* transcription, like *marRAB* transcription, is also upregulated in *tolC* mutants.

Superoxides and nitric oxide activate *soxS* transcription in a two-step manner; they convert SoxR into an active form, which then activates the transcription of *soxS* (43). In addition to the experiments whose results are shown in Fig. 2A, we tested whether transcriptional activation of *soxS* in *tolC*::Tn*10* mutants required functional SoxR. Strains M2676 and M4182 carry a wild-type *soxS* gene but have a *soxR9*::*cat* null mutation (59) in addition to *marRAB* and *rob* null mutations and the *soxS*::*lacZ* fusion. These strains were diluted and grown in LB broth at 32°C, and their β-galactosidase activities were measured (Fig. 2B). As expected, the *soxR9*::*cat tolC*$^+$ strain (M2676) had about one-fifth the *soxS* activity of *soxR*$^+$ *tolC*$^+$ strain M4014 in early log phase due to the absence of SoxR, the activator of *soxS*. Interestingly, the *soxS*::*lacZ* activity increased sharply in later growth phases, an effect not seen in the presence of wild-type SoxR. Nevertheless, strain M2676 and its *tolC*::Tn*10* derivative (M4182) had essentially identical β-galactosidase activities regardless of the growth phase. Thus, *soxR* is necessary for the increased activation of *soxS* transcription seen in the *tolC*::Tn*10* mutant. This suggests that the mechanism of activation of *soxS* in *tolC* mutants is similar to that which occurs when SoxR is activated by superoxides or nitric oxide.

Posttranscriptional activation of Rob in a *tolC* mutant. In contrast to upregulation of *marRAB* and *soxS* transcription in *tolC* mutants, the transcription of *rob* (20) in the *tolC*::Tn*10* mutant (M2562) was similar to that in the wild-type strain (M542) (data not shown). However, since the Rob protein can be activated posttranslationally by treatment with various compounds, including 2,2′-dipyridyl and bile salts (45, 46), we tested the effect of the *tolC*::Tn*10* mutation on the activity of Rob using a strategy previously described (46). Strains with null mutations in *lacI*, *marA*, *soxS*, and *rob* were transformed with a low-copy-number plasmid that carries one of these genes under control of the *lacZYA* promoter in the absence of LacI repressor. We monitored an *inaA*::*lacZ* transcriptional fusion present on λRS74 since the *inaA* promoter is a member of the *marA*/*soxS*/*rob* regulon (49) and is activated by MarA, SoxS, and Rob. However, ectopic expression of the regulators eliminates their transcriptional activation by stress signals. The moderate overexpression of the activators on the plasmids increased the expression of *inaA*::*lacZ* 7- to 14-fold in the

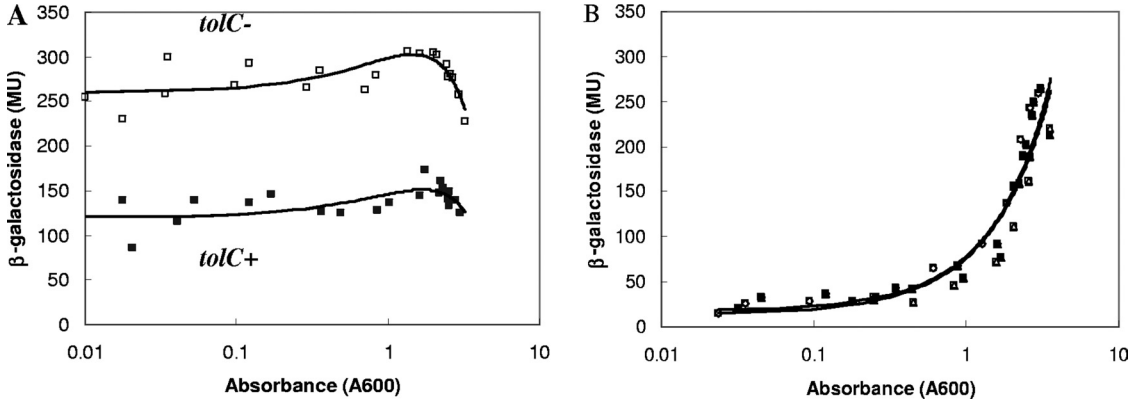

FIG. 2. β-Galactosidase activities of *soxS*::*lacZ* transcriptional fusions in *tolC*$^+$ and *tolC*::Tn*10* cells. (A) ■, *soxR*$^+$ *marRAB*Δ *rob*::*kan* cells (M4014, *tolC*$^+$); □, M4183 (*tolC*). $P_{KS} = 3.4 \times 10^{-6}$. (B) ▲, *soxR*9::*cat marRAB*Δ *rob*::*kan* cells (M2676, *tolC*$^+$); △, M4182 (*tolC*). $P_{KS} = 1.0$. MU, Miller units.

TABLE 2. Activation of *inaA*::*lacZ* in *tolC* mutants[a]

Strain		Activator controlled by *lac* promoter	No treatment with 2,2′-dipyridyl			Treatment with 5 mM 2,2′-dipyridyl		
			β-Galactosidase activity (MU)		Ratio[b]	β-Galactosidase activity (MU)		Ratio[b]
tolC$^+$	*tolC*		*tolC*$^+$ strain	*tolC* strain		*tolC*$^+$ strain	*tolC* strain	
M4110	M4114	None	6.1 (0.42)[c]	11 (1.6)	1.8	12 (0.6)	16 (1.8)	1.3
M4111	M4115	MarA	85 (15)	84 (17)	1.0	63 (17)	53 (7.4)	0.84
M4112	M4116	SoxS	80 (24)	81 (20)	1.0	72 (18)	61 (9.2)	0.85
M4113	M4117	Rob	42 (10)	209 (58)	5.0	675 (139)	919 (154)	1.4

[a] Strains were grown in LB broth at 32°C to an A_{600} of 0.1, diluted twofold into LB broth with or without 2,2′-dipyridyl, and aerated for 1 h at 32°C. Cells were placed on ice, and an assay was performed to determine the β-galactosidase activity, which was expressed in Miller units (MU). All strains have chromosomal *lacIZYA*, *marRAB*, *soxS*, and *rob* null mutations.
[b] Ratio of the activity in the *tolC* mutant to the activity in the *tolC*$^+$ strain.
[c] The standard errors of the means are indicated in parentheses.

tolC$^+$ cells compared to the vector control (Table 2). When *inaA* transcription was assayed in the *tolC*::Tn*10* mutant, a fivefold increase was seen when the *rob* plasmid was present (M4117 compared to M4113) but not when the *marA* (M4115) or *soxS* (M4116) plasmids were present. This effect of the *tolC*::Tn*10* mutation on Rob is posttranscriptional because (i) *rob* expression from the plasmid is under control of the plasmid-borne heterologous *lacZYA* promoter and (ii) the *tolC*::Tn*10* mutation does not affect the transcription of the *lacZYA* promoter (Table 3). Interestingly, a 1.8-fold increase was seen for the *tolC*::Tn*10* vector strain (M4114) compared to the strain with the vector (M4110). Thus, most of the effect is mediated by Rob, but a portion is mediated by the "*mar*-independent" activity previously noted for *inaA* (49).

We also examined the effect of 2,2′-dipyridyl in conjunction with the *tolC*::Tn*10* mutation to see if the effects were additive or multiplicative (Table 2). 2,2′-Dipyridyl had a dramatic effect only on the strains carrying the pTA:*rob* plasmid, as previously observed (46). As expected, 5 mM 2,2′-dipyridyl increased *inaA* expression 16-fold (675/42) in M4113, the *tolC*$^+$ strain with the pTA:*rob* plasmid. However, 2,2′-dipyridyl treatment of the *tolC*::Tn*10* mutant (M4117) with the pTA:*rob* plasmid increased *inaA* expression only about 4-fold (919/209). Assuming that the cellular concentration of 2,2′-dipyridyl is not greater in the wild-type strain than in the *tolC* mutant, the separate effects of 2,2′-dipyridyl treatment and of the *tolC* mutation appear to be additive. This is consistent with the possibility that the *tolC* mutation leads to activation of Rob in a manner similar to the posttranscriptional activation of Rob by 2,2′-dipyridyl.

Elevated expression of *marA*/*soxS*/*rob* regulon promoters in a *tolC* mutant. To determine whether other *marA*/*soxS*/*rob* regulon promoters are upregulated in a *tolC* mutant, we measured the β-galactosidase activities of wild-type and *tolC*::Tn*10* strains carrying various regulon promoter::*lacZ* transcriptional fusions. *tolC* itself has four characterized promoters, *p*1 to *p*4, but only *p*3 and *p*4 are responsive to MarA, SoxS, and Rob (62). *tolC*::*lacZ* promoter fusion A contains all four promoters and showed 1.7-fold-greater β-galactosidase activity in *tolC*::Tn*10* strain M4143 than in parental strain M4386 (Table 3). Similarly, *tolC*::*lacZ* promoter fusion B, which contains only promoters *p*3 and *p*4, had 1.9-fold-greater activity in *tolC*::Tn*10* strain M4142 than in parental strain M4263. However, no increase was found for fusion C (M4141), which has a partially deleted *marbox* upstream of the *p*3 and *p*4 promoters and is not responsive to MarA, SoxS, and Rob (62). Thus, the effect of the *tolC* mutation on promoter transcription is highly specific;

TABLE 3. Activities of promoter::*lacZ* transcriptional fusions in *tolC* mutants[a]

Promoter::*lacZ* fusion	Wild type		Mutant			Mutant/wild-type ratio
	Strain	β-Galactosidase activity (MU)	Strain	Relevant mutation	β-Galactosidase activity (MU)	
tolC(A)[b]	M4386	331 (16)[c]	M4143	*tolC*::Tn*10*	562 (30)	1.7
tolC(B)[b]	M4263	133 (6.5)	M4142	*tolC*::Tn*10*	250 (2.5)	1.9
tolC(C)[b]	M4262	76 (5.6)	M4141	*tolC*::Tn*10*	74 (3.9)	1.0
acrAB	M4275	50 (2.2)	M4165	*tolC*::Tn*10*	128 (3.2)	2.5
inaA	M2581[d]	5.2 (0.17)	M4167[d]	*pyrE*~Tn*10*	6.3 (0.15)	1.2
	M2581[d]	5.2 (0.17)	M2583[d]	*tolC*::Tn*10*	9.3 (0.03)	1.8
inaA1	M4199	43 (1.8)	M4198	*tolC*Δ	77 (0.6)	1.8
lacZYA	LBB735	9.4 (0.14)	LBB801	*tolC*::Tn*10*	8.3 (0.89)	0.88

[a] Cells were grown in LB broth at 32°C to an A_{600} of 0.2 and placed on ice, and an assay was performed to determine the β-galactosidase activity, which was expressed in Miller units (MU).
[b] Fusions A and B contain the *tolC marbox*; fusion C has a 12-bp deletion of the *marbox*. See reference 62 for details.
[c] The standard errors of the means are indicated in parentheses.
[d] The strain has a deletion or null mutation of *marRAB*, *soxS*, and *rob*.

TABLE 4. *acrAB*, *acrEF*, and *emrAB* mutations did not increase *inaA1*::*lacZ* activity

Strain	Relevant mutations	β-Galactosidase activity (MU)	Ratio[a]
N7918	None	16 (0.2)[b]	1.0
M2561	*tolC*::Tn*10*	34 (1.3)	2.1
M4195	*emrAB*::*cat*	17 (0.3)	1.1
M4197	*tolC*::Tn*10*, *emrAB*	34 (0.2)	2.1
M4196	*acrEF*::*spc*	16 (1.2)	1.0
M2605	None	21 (3.1)	1.0
M2606	*acrAB*::Tn*903*	25 (2.2)	1.2
N7881[c]	None	28 (2.4)	1.0
M4807[c]	*acrAB*::Tn*903 acrEF*::*spc*	28 (5.2)	1.0
M4820[c]	*acrAB*::*spc*	33 (2.4)	1.2

[a] Ratio of the β-galactosidase activity (expressed in Miller units [MU]) in the mutant to the β-galactosidase activity in the corresponding wild-type strain.
[b] The standard errors of the means are indicated in parentheses.
[c] The strain was grown in LB broth with 0.2% glucose to repress expression of the chromosomal *lacZ*.

the transcription of *tolC* itself is activated via *p*3 and *p*4 but not when its *marbox* is defective.

Because *acrAB* encodes two components of the AcrAB-TolC pump and is also a member of the *marA*/*soxS*/*rob* regulon, we tested the effect of a *tolC* mutation on *acrAB* transcription. The promoter fusion *acrAB*::*lacZ* had 2.5-fold-higher β-galactosidase activity in the *tolC*::Tn*10* mutant M4165 than in wild-type strain M4275 (Table 3). We also tested the *marA*/*soxS*/*rob* regulon gene *inaA*, whose function is not known. The *inaA*::*lacZ* promoter fusion (49) had 1.8-fold-higher β-galactosidase activity in *tolC*::Tn*10* strain M2583 than in *tolC*$^+$ parental strain M2581. Thus, in the absence of TolC functions, the transcription of several regulon promoters (including *tolC* itself) is upregulated, but not when the *marbox* is defective.

We also tested whether the activating effect of *tolC*::Tn*10* is specific to regulon promoters by examining the β-galactosidase activity of a chromosomal wild-type *lacZYA* promoter. We found no significant difference in activity for *lacZYA* between a wild-type strain (LBB735) and its *tolC*::Tn*10* derivative LBB801 (Table 3). Thus, the effect of *tolC*::Tn*10* is specific for regulon promoters.

The Tn*10* transposon is not responsible for the activation of *inaA*. We tested the possibility that the Tn*10* transposon used here to disrupt *tolC* was responsible for the activator upregulation seen in our experiments since it carries the *tetD* gene, which encodes a paralog of MarA, SoxS, and Rob. Since *tetD* is repressed by TetC, which is also encoded by Tn*10* (40), significant amounts of TetD should not be made in strains with Tn*10* insertions. However, Griffith and coworkers (16) have shown that when *tetD* is cloned and overexpressed on a plasmid in a strain (N8453) in which *marRAB*, *soxS*, and *rob* are deleted, it can activate some *marA*/*soxS*/*rob* regulon promoters. Therefore, we tested whether a Tn*10* insertion linked to (but not disrupting) *pyrE* could activate *inaA*::*lacZ* in a derivative of strain N8453 (Table 3). We found no significant difference in *inaA*::*lacZ* activity between the strain carrying the *pyrE*-linked Tn*10* insertion (M4167) and its parent without the insertion (M2581). A similar result has been obtained for an *ara*::Tn*10* insertion (K. L. Griffith and R. E. Wolf, Jr., personal communication). Furthermore, the expression of *inaA1*::*lacZ* in a strain (M4198) which has a deletion of the 5′ end of *tolC* (*tolC*ΔEW1b) (57) was 1.8-fold greater than that in the *tolC*$^+$ parental strain (M4199). Thus, it is the disruption of *tolC*, not the presence of Tn*10*, that is responsible for the transcriptional activation of *inaA* and, presumably, the other regulon promoters.

Wild-type and receptor-defective *tolC* mutants complement *tolC*::Tn*10* with regard to *inaA* upregulation. TolC has multiple functions. As an outer membrane protein, it serves as a receptor for the adsorption of phage TLS and for internalization of colicin E1. TolC also serves as a pore for export of xenobiotics, proteins, and enterobactin (23). Since the *tolC*::Tn*10* mutation results in complete loss of both outer membrane activities and export activities, we wished to narrow the possibilities of which function is responsible for the upregulation described here. Accordingly, we examined *tolC*(*S275P*) and *tolC*(*A360T*) mutants (52), which are defective as receptors for phage TLS and colicin E1 and have a defect in export of hemolysin but behave normally with regard to the efflux of xenobiotics.

Strain M2561 has a *tolC*::Tn*10* mutation and an *inaA1*::*lacZ* reporter gene and expresses about twice as much β-galactosidase as its wild-type parental strain, strain N7918 (Table 4). We transformed strain M2561 with the pTrc99A vector (M5572) or with the vector carrying wild-type (M5573) or mutant *tolC* under control of the *trc* promoter, whose expression was derepressed by the presence of 0.4 mM IPTG throughout growth. The presence of the plasmid-borne *tolC*$^+$ gene in M5573 reduced the activity of *inaA* ~2-fold (i.e., to normal *tolC*$^+$ levels), whereas the vector alone in strain M5572 did not reduce the activity (Fig. 3). This clearly shows that defective *tolC* is responsible for the upregulation of *inaA1*::*lacZ*. The result was similar when the *tolC* plasmid carried the S257P or A360T mutation (M5574 and M5575, respectively). Thus, these strains behaved like wild-type *tolC* strains with regard to the *inaA* activity even though they are defective in TLS, ColE1, and certain hemolysin functions. This is consistent with the hypothesis that the efflux function of TolC is the critical function that regulates the *inaA* promoter and, by implication, *marA*, *soxS*, and Rob.

Unlike mutations in *tolC*, single null mutations in either *acrAB*, *acrEF*, or *emrAB* do not affect regulon expression. TolC

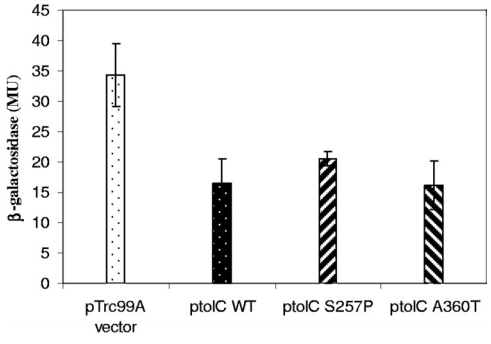

FIG. 3. β-Galactosidase activities of *inaA1*::*lacZ* transcriptional fusions in cells with a chromosomal *tolC*::Tn*10* mutation and plasmids with different *tolC* alleles. Cells grown to early log phase in LB broth in the presence of 0.4 mM IPTG were assayed to determine the β-galactosidase activity (pTrc99A vector, M5572; ptolC WT, M5573; ptolC S257P, M5574; ptolC A360T, M5575). The error bars indicate the standard errors of the means. MU, Miller units.

TABLE 5. Effects of *tolC*::Tn*10* on promoter transcription in minimal medium[a]

Strain		*lacZ* fusion	β-Galactosidase activity (MU)		Ratio[b]
tolC$^+$	*tolC*		*tolC*$^+$ strain	*tolC* strain	
M3954	M4188	*marRAB*	30 (2.8)[c]	57 (12.7)	1.9
M4014	M4183	*soxS*	133 (12.3)	358 (54)	2.7
M4113	M4117	*inaA*::*lacZ*[d]	38 (6.7)	85 (9.2)	2.2

[a] The strains were grown at 32°C to an A_{600} of 0.1 to 0.25 in M9 medium supplemented with 0.2% glucose, 1 ng/ml thiamine, and 0.2% Casamino Acids, and an assay was performed to determine the β-galactosidase activity, which was expressed in Miller units (MU).
[b] Ratio of the activity in the *tolC* mutant to the activity in the wild-type strain.
[c] The standard errors of the means are indicated in parentheses.
[d] As in Table 2, the expression of *inaA*::*lacZ* was used to measure the posttranscriptional activation of Rob.

interacts with at least eight different pairs of cytoplasmic efflux pumps and membrane fusion proteins to form tripartite transporters with different specificities (23). Prominent among these transporters are the multidrug efflux complexes formed in partnership with AcrA and AcrB, with AcrE and AcrF, and with EmrA and EmrB. If the effects of the *tolC* mutation on *marA*, *soxS*, and Rob described here occurred because the *tolC* mutation prevented one of these pumps from functioning, a similar effect on *marRAB*, *soxS*, and Rob should have been seen when the pump or membrane fusion protein alone was defective even though the TolC protein was the wild-type protein. To identify the putative TolC partners, we constructed strains with single or double null mutations in *acrAB*, *acrEF*, and *emrAB* and used *inaA1*::*lacZ* fusions to monitor the effects. However, no upregulation of *inaA* transcription attributable to the individual pumps was seen (Table 4). The finding that *acrAB* is not involved in the upregulation is particularly surprising since *acrAB* is also activated by MarA, SoxS, and Rob and is the major xenobiotic efflux pump in *E. coli*. In similar experiments, we have examined *inaA1*::*lacZ* expression in strains with single null (*kan*) mutations in *tolC*, *acrA*, *acrB*, *acrE*, *acrF*, *emrA*, and *emrB* derived from the KEIO Collection (5). With the exception of the *tolC* strain, which showed three- to fourfold-greater activity than the controls, none of the mutants showed significantly elevated activity (data not shown). Preliminary tests of 32 other mutants from the KEIO Collection that are thought to encode efflux functions have not revealed elevated *inaA1*::*lacZ* activities. Thus, we have not identified the relevant efflux pump that partners with TolC. Alternatively, there may be several TolC-associated pumps that must all be made defective before their roles in upregulation of MarA, SoxS, and Rob can be observed.

***marRAB* and *soxS* transcriptional effects and Rob posttranscriptional effects in minimal medium.** Since LB broth is a rich but poorly defined medium, it may contain trace amounts of xenobiotics that could accumulate inside a *tolC* mutant and upregulate *marRAB*, *soxS*, or Rob. Therefore, we examined the growth of wild-type and *tolC*::Tn*10* strains in minimal M9 salts medium containing glucose, thiamine, and Casamino Acids (Table 5). The *tolC*::Tn*10* mutants grew more slowly than the wild-type parents in this minimal medium (data not shown). *marRAB* transcription and *soxS* transcription were increased about twofold in the the *tolC* mutants, as observed for cells grown in LB medium (Fig. 1 and 2). However, Rob activity was increased only twofold, which was significantly less than the fivefold observed in LB medium. We also tested *inaA*::*lacZ* expression in wild-type and *tolC*::Tn*10* cells grown in M9 minimal medium supplemented only with glucose and thiamine (Fig. 4). Clearly, Casamino Acids are not required for the upregulation seen in *tolC*::Tn*10* mutants. Thus, it is unlikely that xenobiotics in the culture medium are responsible for the upregulation of *marRAB*, *soxS*, and Rob in the *tolC* mutants.

DISCUSSION

TM hypothesis. TolC constitutes an outer membrane channel that functions in the export of multiple xenobiotics, enterobactin, peptides, and proteins and in the binding and uptake of colicins and binding of bacteriophage in *E. coli* and other gram-negative bacteria (23). Misra and Reeves (35) observed that in *tolC* mutants *micF* transcription was elevated. We now offer the following explanation for why *micF* expression is elevated in *tolC* mutants: the levels of MarA, SoxS, and transcriptionally active Rob are increased in *tolC* mutants and these proteins transcriptionally activate *micF*, a member of the *marA*/*soxS*/*rob* regulon. Indeed, other members of the regulon, *inaA*, *acrAB*, and *tolC* itself are also upregulated (Table 3). Furthermore, a *tolC* promoter (C) lacking a portion of the *marbox* and the chromosomal *lacZYA* promoter (not a regulon member) are not upregulated, showing that the upregulation that we have found in *tolC* mutants is specific for the *marA*/*soxS*/*rob* regulon.

The most likely explanation for the increased activities of the *marRAB* and *soxS* promoters and of the Rob protein in *tolC* mutants is that some products of normal cellular metabolism are not exported as rapidly from *tolC* mutants as from wild-type cells and therefore accumulate. We propose that these metabolites interact, directly or indirectly, with the *marRAB* promoter, with SoxR, and with the Rob protein. It seems unlikely that a substance present in the medium is responsible since *marA*, *soxS*, and Rob are upregulated even in *tolC*::Tn*10* cells grown in a chemically defined mineral salts medium with

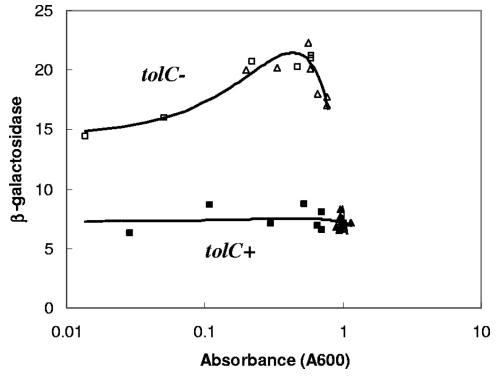

FIG. 4. Effect of *tolC*::Tn*10* on β-galactosidase activities of *inaA1*::*lacZ* transcriptional fusions in cells grown to various densities in M9 minimal medium supplemented with 0.2% glucose and 1 ng/ml thiamine. The results of two experiments (triangles and squares) are combined (filled symbols, N7918 [*tolC*$^+$]; open symbols, M2561 [*tolC*::Tn*10*]). $P_{KS} = 2.2 \times 10^{-6}$.

only glucose and thiamine added (Fig. 4). Furthermore, the critical TolC function with respect to *inaA* upregulation is probably efflux and not outer membrane disruption. Ectopic expression of plasmid-borne *tolC*, including mutations that affect outer membrane properties such as colicin internalization and phage adsorption [*tolC*(*S257P*) and *tolC*(*A360T*)], prevented (complemented) the upregulation of MarA, SoxS, or Rob seen in *tolC* mutants (Fig. 3).

For discussion, we refer to these accumulated substances that stimulate the upregulation as TMs. What is their nature? It is intriguing that the TMs have three distinct modes of action. (i) Like salicylate, TMs transcriptionally activate the *marRAB* promoter even in the absence of MarRAB, SoxS, and Rob functions (Fig. 1). Unlike salicylate, there was no evidence that the TMs interact with MarR. (ii) Like superoxides and other triggers of *soxS* transcription, transcriptional activation of *soxS* by TMs is mediated by SoxR (Fig. 2). (iii) Like bile salts, decanoate, and 2,2′- and 4,4′-dipyridyl, TMs posttranscriptionally activate the Rob protein, suggesting that there is a direct interaction with Rob (Table 2).

Does one metabolite do all this? An overlap between compounds that can activate *marRAB* (by derepression) and *soxS* (via SoxR) has previously been noted (34), and we have observed that high millimolar concentrations of salicylate can activate *soxS* via SoxR (unpublished data). There is also an overlap between compounds that activate *soxS* transcription and Rob protein (15; our unpublished data). Thus, it is possible that a single compound or class of compounds activates *marRAB*, *soxS*, and Rob, but no such compound has been described yet. Therefore, there could be a number of trigger metabolites. A comparison of the metabolomes of *tolC* mutant and wild-type cells should help identify the TMs.

What are the TM pumps? We assume that TolC exports the TMs in conjunction with other components. Generally, TolC seems to interact with an inner membrane-located pump and a membrane fusion protein to form a tripartite complex which extrudes xenobiotics and other molecules into the medium. Among the eight known efflux pump systems that depend upon TolC for function are AcrAB, AcrEF, and EmrAB. However, strains with sets of genes encoding these pumps individually deleted did not show higher activation of *inaA1*::*lacZ* transcription, indicating that significant amounts of TMs were not present in these mutants. This suggests either that some other pump interacts with TolC for efflux of the TMs or that a combination of several pumps is involved in their efflux.

It seems reasonable to suppose that high levels of the TMs are toxic, yet *tolC* mutants grow at rates comparable to those of wild-type strains when they are cultivated in rich media. Thus, there could be a TolC-independent system that exports or detoxifies the TMs. The ability of such a TolC-independent pump or detoxifier to rid the cell of TMs would be expected to be suboptimal; otherwise, we would not have detected the effect of TMs in *tolC* mutants. Since strains which have null mutations in *tolC*, *marRAB*, *soxRS*, and *rob* grow very well in rich media, this putative TolC-independent activity might not be regulated by MarA, SoxS, or Rob. It may be possible to identify this activity by isolating chromosomal fragments with activity that is sufficiently elevated that the upregulation observed in *tolC* mutants is negated.

The present results suggest that the MarA, SoxS, and Rob systems are tuned to detect cellular metabolites, not only xenobiotics like salicylate and bile salts. The buildup of these metabolites may then signal the need to excrete them or detoxify them (Fig. 5). In this way, TolC and TMs may regulate each other in wild-type cells; excess TMs would activate *marA*, *soxS*, and Rob, which would then increase *tolC* expression and increase the export of TMs. Other pumps, now known to export xenobiotics, may export other cellular metabolites. If so, it may be that the efflux of xenobiotics evolved from pumps that originally were dedicated to the export of cellular metabolites.

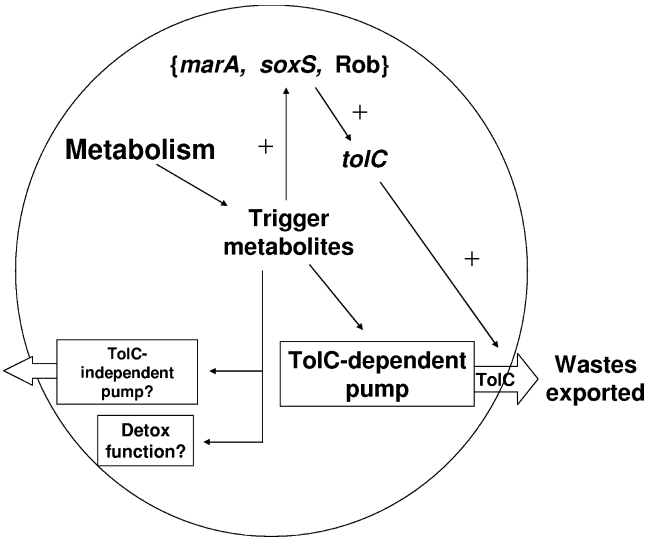

FIG. 5. Hypothetical components of a waste disposal system in *E. coli* (for simplicity, only the outer membrane is indicated). Metabolism generates TMs in the cell that are disposed of via an unspecified TolC-dependent pump. If the TolC pump is defective for efflux, TMs accumulate and trigger the activation (+) of *marRAB*, *soxS*, and Rob, which then activate the *marA-soxS-rob* regulon promoters, including the *tolC* promoters *p*3 and *p*4. Since *tolC* mutants are viable, a suboptimal TolC-independent pump or other detoxification function may also be present.

Export of metabolites. Helling and coworkers (17) have come to similar conclusions. These authors found that 10% of transposon-generated mutants selected for resistance to low levels of nalidixic acid had mutations in amino acid or adenine biosynthetic genes (*icdA*, *metE*, *icdA*, *purB*). The increased resistance was accompanied by an increase in *acrAB* transcription and was dependent on wild-type alleles of *soxS* plus either *marA* or *rob*. Helling and coworkers proposed that the mutational blockage of certain biosynthetic pathways results in accumulation of particular intermediates, which then activate the SoxS and MarA or Rob systems. In effect, this is a "toxic waste disposal" system (17). Our conclusions differ from the conclusions of Helling et al. in one respect. Helling et al. concluded that *acrAB* and *tolC* are required to expel the accumulated metabolic intermediate. Since we found that *acrAB* null mutations did not elevate the expression of an *inaA1*::*lacZ* fusion, we concluded that *acrAB* is not essential for efflux of TMs. This discrepancy is readily accounted for by the fact that Helling and coworkers (17) used a nalidixic acid resistance assay as their end point assay. In the *acrAB* mutants, as in *tolC* mutants, nalidixic acid resistance is so compromised that it cannot be used to monitor the presence of a separate pump.

It has been commonly assumed that when metabolites are overproduced by bacteria and other organisms, they simply leak out of the cells. The "feeding" to neighboring cells of biosynthetic intermediates accumulated in certain mutants is often observed, but how the intermediates get out of the cell has not been explored. Kawamura-Sato et al. (21) have shown that AcrEF is important for indole excretion. Franke et al. (14) have shown that YfiK plays a significant role in the excretion of cysteine-cystine, and Yamada et al. (60) have shown that a number of other pumps, including some pumps known to be active in multidrug efflux, also are involved in cystine excretion. TolC, but not AcrAB, has been shown to also have a role in this process (58). Importantly, the *aaeAB* genes encode an efflux system which exports *p*-hydroxybenzoate (pHBA), an intermediate in ubiquinone synthesis, and which protects the cell from exogenous pHBA and a few related compounds (53). The system is regulated by AaeR and is inducible by pHBA and salicylate. Van Dyk and coworkers suggested that AaeAB might provide a "metabolic relief valve" for excess pHBA. This is very similar to our conclusions. However, null mutations in *aaeA* and *aaeB* did not upregulate *inaA1*::*lacZ* fusions (data not shown).

Downstream of *tolC* are three genes, *ygiA*, *ygiB* and *ygiC*, which may be part of the *tolC* operon (22). If the *tolC* mutations which we used in this study are polar on the downstream *ygiABC* genes, it may be that the latter genes are responsible for the regulatory effects that we have described. We tested this by asking whether a plasmid carrying the *ygiABC* genes (kindly provided by L. Thomason and D. Court) can complement the *tolC*::Tn*10* mutant with regard to *inaA1*::*lacZ* activation. No complementation was observed, indicating that the *ygiABC* genes are not involved in the upregulation of *inaA* (data not shown). Furthermore, we have seen that cloned wild-type *tolC* alone complemented the upregulation due to a *tolC*::Tn*10* chromosomal mutation (Fig. 3). Thus, it is unlikely that the *ygiABC* genes play a role in the *tolC* effect on activator regulation.

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