

Role of the *mar-sox-rob* Regulon in Regulating Outer Membrane Porin Expression^{∇†}

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Multiple factors control the expression of the outer membrane porins OmpF and OmpC in *Escherichia coli*. In this work, we investigated the role of the *mar-sox-rob* regulon in regulating outer membrane porin expression in response to salicylate. We provide both genetic and physiological evidence that MarA and Rob can independently activate *micF* transcription in response to salicylate, leading to reduced OmpF expression. MarA was also found to repress OmpF expression through a MicF-independent pathway. In the case of OmpC, we found that its transcription was moderately increased in response to salicylate. However, this increase was independent of MarA and Rob. Finally, we found that the reduction in OmpF expression in a *tolC* mutant is due primarily to Rob. Collectively, this work further clarifies the coordinated role of MarA and Rob in regulating the expression of the outer membrane porins.

Gram-negative bacteria can limit the uptake of membrane-impermeable antimicrobial compounds by modulating the composition of pores in their outer membranes (44). In *Escherichia coli* and closely related organisms, this alteration is accomplished in part by changing the relative expression of two porins, OmpF and OmpC (17, 44, 46). Both OmpF and OmpC form structurally similar outer membrane pores comprising trimers of 16-stranded β -barrels (2, 13). However, the two have different substrate specificities and diffusion rates (2, 13). The OmpF porin, which forms the larger pore, permits the diffusion of molecules at comparatively faster rates than OmpC (13).

The OmpF/OmpC ratio is primarily regulated at the transcriptional level by the EnvZ-OmpR two-component system in response to changes in the osmolarity of the growth medium (20, 26, 53, 55, 56). Numerous other factors also converge to transcriptionally regulate the differential expression of *ompF* and *ompC* in response to environmental conditions such as temperature and nutrient limitation (17, 46). Apart from transcriptional regulation, OmpF and OmpC are also translationally regulated through the action of two small regulatory RNAs (sRNA), MicF and MicC. These sRNA molecules, when expressed, are known to bind to the 5' untranslated regions (5'-UTR) of *ompF* and *ompC* mRNAs and stop translation by preventing the ribosome from binding (7, 39). Although MicC is known to interact with the *ompC* mRNA, its expression is cryptic and has not been observed to substantially influence OmpC expression (7). MicF, on the other hand, is known to be a significant regulator of OmpF expression under certain environmental conditions, and the regulation of its expression has been extensively investigated (1, 14, 18, 49).

MicF expression is regulated by multiple factors. These include not only OmpR but also global transcription factors such as H-NS, Lrp, and IHF (16, 19, 27). In addition, MicF expression is regulated by three homologous AraC/XylS family transcription factors—MarA, SoxS, and Rob—when *E. coli* exhibits the porin-dependent antibiotic resistance phenotype (22, 29, 31, 34). These three transcription factors are the master regulators of the extensive *mar-sox-rob* regulon, involved in intrinsic multidrug resistance in enteric gammaproteobacteria (34). The regulation of MarA and SoxS expression is chiefly mediated at the level of transcription by the MarR repressor and SoxR redox sensor/activator, respectively (11, 42, 58). Rob, on the other hand, is expressed constitutively and regulated posttranslationally by a “sequestration-dispersion” mechanism (23, 29, 48, 50). Together, these three regulators control the expression of a number of downstream, overlapping target genes involved in intrinsic multidrug resistance.

In this work, we investigated the regulation of OmpF and OmpC expression by the *mar-sox-rob* regulon in response to the canonical inducer salicylate. We first provide both genetic and physiological evidence that the reductions in the levels of OmpF during salicylate exposure are through the parallel action of MarA and Rob in increasing *micF* transcription. We also demonstrate that MarA regulates *ompF* translation through a MicF-independent pathway. Finally, we found that the reduced levels of OmpF expression (and correlated increases in OmpC expression) in the absence of the TolC outer membrane efflux pore are primarily due to the action of Rob activating *micF* transcription.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. All strains used in this work are listed in Table 1. Luria-Bertani liquid and solid media (10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl) were used for routine bacterial culture and genetic manipulation. Experiments were conducted in medium A (7 g/liter nutrient broth, 1 g/liter yeast extract, 2 g/liter glycerol, 3.7 g/liter K_2HPO_4 , 1.3 g/liter KH_2PO_4) (28, 36). All bacterial cultures were grown at 37°C except for strains containing plasmids pKD46, pINT-ts, and pCP20, which were grown at 30°C. Antibiotics were used at the following concentrations: ampicillin, 100 μ g/ml; kanamycin, 20 μ g/ml; and chloramphenicol, 20 μ g/ml. For some experiments,

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Genotype or relevant characteristics ^a	Source and/or reference ^b
Strains		
MG1655	F ⁻ λ ⁻ <i>ilvG rph-1</i>	CGSC 7740
BW25141	F ⁻ λ ⁻ Δ(<i>araB-araD</i>)567 Δ <i>lacZ</i> 4787(:: <i>rrnB-3</i>) Δ(<i>phoB-phoR</i>)580 <i>galU</i> 95 Δ <i>uidA</i> 3:: <i>pir</i> ⁺ <i>recA1</i> Δ <i>endA</i> 9::FRT ⁻ <i>rph-1</i> Δ(<i>rhaB-rhaD</i>)568 <i>hsdR</i> 514	CGSC 7635
MDG147	MG1655 Φ(<i>ompF</i> ⁺ - <i>yfp</i> ⁺)30 Φ(<i>ompC</i> ⁺ - <i>cfp</i> ⁺)31	M. D. Goulian, 4
CR701	Δ <i>tolC</i> :: <i>cat</i>	10
CR702	Δ <i>tolC</i> ::FRT	10
CR713	<i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp oriR6K</i>]	
CR714	<i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp(hyb) oriR6K</i>]	
CR715	<i>attλ</i> ::[<i>kan micF'</i> - <i>yfp oriR6K</i>]	
CR716	Δ <i>marRAB</i> :: <i>kan</i> (1617077–1618231)	
CR717	Δ <i>soxRS</i> :: <i>kan</i> (4275083–4275950)	
CR718	Δ <i>rob</i> :: <i>cat</i> (4632554–4633393)	
CR719	Δ <i>micF</i> :: <i>kan</i> (2311106–2311203)	
CR720	Δ <i>ompR</i> :: <i>cat</i> (3533885–3534603)	
CR721	Δ <i>marRAB</i> ::FRT	
CR722	Δ <i>soxRS</i> ::FRT	
CR723	Δ <i>rob</i> ::FRT	
CR724	Δ <i>micF</i> ::FRT	
CR725	Δ <i>marRAB</i> ::FRT Δ <i>soxRS</i> ::FRT	
CR726	Δ <i>marRAB</i> ::FRT Δ <i>rob</i> ::FRT	
CR727	Δ <i>soxRS</i> ::FRT Δ <i>rob</i> ::FRT	
CR728	Δ <i>marRAB</i> ::FRT Δ <i>soxRS</i> ::FRT Δ <i>rob</i> ::FRT	
CR729	Δ <i>micF</i> ::FRT Δ <i>marRAB</i> ::FRT	
CR730	Δ <i>micF</i> ::FRT Δ <i>rob</i> ::FRT	
CR731	Δ <i>micF</i> ::FRT Δ <i>marRAB</i> ::FRT Δ <i>rob</i> ::FRT	
CR732	Δ <i>tolC</i> ::FRT Δ <i>marRAB</i> ::FRT	
CR733	Δ <i>tolC</i> ::FRT Δ <i>soxRS</i> ::FRT	
CR734	Δ <i>tolC</i> ::FRT Δ <i>rob</i> ::FRT	
CR735	Δ <i>tolC</i> ::FRT Δ <i>marRAB</i> ::FRT Δ <i>soxRS</i> ::FRT Δ <i>rob</i> ::FRT	
CR736	Δ <i>tolC</i> ::FRT Δ <i>micF</i> ::FRT	
CR737	Δ <i>marRAB</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp oriR6K</i>]	
CR738	Δ <i>soxRS</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp oriR6K</i>]	
CR739	Δ <i>rob</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp oriR6K</i>]	
CR740	Δ <i>micF</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp oriR6K</i>]	
CR741	Δ <i>marRAB</i> ::FRT Δ <i>soxRS</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp oriR6K</i>]	
CR742	Δ <i>marRAB</i> ::FRT Δ <i>rob</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp oriR6K</i>]	
CR743	Δ <i>soxRS</i> ::FRT Δ <i>rob</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp oriR6K</i>]	
CR744	Δ <i>marRAB</i> ::FRT Δ <i>soxRS</i> ::FRT Δ <i>rob</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp oriR6K</i>]	
CR745	Δ <i>micF</i> ::FRT Δ <i>marRAB</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp oriR6K</i>]	
CR746	Δ <i>micF</i> ::FRT Δ <i>rob</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp oriR6K</i>]	
CR747	Δ <i>micF</i> ::FRT Δ <i>marRAB</i> ::FRT Δ <i>rob</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp oriR6K</i>]	
CR748	Δ <i>tolC</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp oriR6K</i>]	
CR749	Δ <i>tolC</i> ::FRT Δ <i>marRAB</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp oriR6K</i>]	
CR750	Δ <i>tolC</i> ::FRT Δ <i>soxRS</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp oriR6K</i>]	
CR751	Δ <i>tolC</i> ::FRT Δ <i>rob</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp oriR6K</i>]	
CR752	Δ <i>tolC</i> ::FRT Δ <i>marRAB</i> ::FRT Δ <i>soxRS</i> ::FRT Δ <i>rob</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp oriR6K</i>]	
CR753	Δ <i>tolC</i> ::FRT Δ <i>micF</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp oriR6K</i>]	
CR754	Δ <i>marRAB</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp(hyb) oriR6K</i>]	
CR755	Δ <i>soxRS</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp(hyb) oriR6K</i>]	
CR756	Δ <i>rob</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp(hyb) oriR6K</i>]	
CR757	Δ <i>micF</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp(hyb) oriR6K</i>]	
CR758	Δ <i>marRAB</i> ::FRT Δ <i>soxRS</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp(hyb) oriR6K</i>]	
CR759	Δ <i>marRAB</i> ::FRT Δ <i>rob</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp(hyb) oriR6K</i>]	
CR760	Δ <i>soxRS</i> ::FRT Δ <i>rob</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp(hyb) oriR6K</i>]	
CR761	Δ <i>marRAB</i> ::FRT Δ <i>soxRS</i> ::FRT Δ <i>rob</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp(hyb) oriR6K</i>]	
CR762	Δ <i>micF</i> ::FRT Δ <i>marRAB</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp(hyb) oriR6K</i>]	
CR763	Δ <i>micF</i> ::FRT Δ <i>rob</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp(hyb) oriR6K</i>]	
CR764	Δ <i>micF</i> ::FRT Δ <i>marRAB</i> ::FRT Δ <i>rob</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp(hyb) oriR6K</i>]	
CR765	Δ <i>tolC</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp(hyb) oriR6K</i>]	
CR766	Δ <i>tolC</i> ::FRT Δ <i>marRAB</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp(hyb) oriR6K</i>]	
CR767	Δ <i>tolC</i> ::FRT Δ <i>soxRS</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp(hyb) oriR6K</i>]	
CR768	Δ <i>tolC</i> ::FRT Δ <i>rob</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp(hyb) oriR6K</i>]	
CR769	Δ <i>tolC</i> ::FRT Δ <i>marRAB</i> ::FRT Δ <i>soxRS</i> ::FRT Δ <i>rob</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp(hyb) oriR6K</i>]	
CR770	Δ <i>tolC</i> ::FRT Δ <i>micF</i> ::FRT <i>attλ</i> ::[<i>kan ompF'</i> - <i>yfp(hyb) oriR6K</i>]	
CR771	Δ <i>marRAB</i> ::FRT <i>attλ</i> ::[<i>kan micF'</i> - <i>yfp oriR6K</i>]	
CR772	Δ <i>soxRS</i> ::FRT <i>attλ</i> ::[<i>kan micF'</i> - <i>yfp oriR6K</i>]	

Continued on following page

TABLE 1—Continued

Strain or plasmid	Genotype or relevant characteristics ^a	Source and/or reference ^b
CR773	$\Delta rob::FRT att\lambda::[kan micF'-yfp oriR6K]$	
CR774	$\Delta micF::FRT att\lambda::[kan micF'-yfp oriR6K]$	
CR775	$\Delta marRAB::FRT \Delta soxRS::FRT att\lambda::[kan micF'-yfp oriR6K]$	
CR776	$\Delta marRAB::FRT \Delta rob::FRT att\lambda::[kan micF'-yfp oriR6K]$	
CR777	$\Delta soxRS::FRT \Delta rob::FRT att\lambda::[kan micF'-yfp oriR6K]$	
CR778	$\Delta marRAB::FRT \Delta soxRS::FRT \Delta rob::FRT att\lambda::[kan micF'-yfp oriR6K]$	
CR779	$\Delta micF::FRT \Delta marRAB::FRT att\lambda::[kan micF'-yfp oriR6K]$	
CR780	$\Delta micF::FRT \Delta rob::FRT att\lambda::[kan micF'-yfp oriR6K]$	
CR781	$\Delta micF::FRT \Delta marRAB::FRT \Delta rob::FRT att\lambda::[kan micF'-yfp oriR6K]$	
CR782	$\Delta tolC::FRT att\lambda::[kan micF'-yfp oriR6K]$	
CR783	$\Delta tolC::FRT \Delta marRAB::FRT att\lambda::[kan micF'-yfp oriR6K]$	
CR784	$\Delta tolC::FRT \Delta soxRS::FRT att\lambda::[kan micF'-yfp oriR6K]$	
CR785	$\Delta tolC::FRT \Delta rob::FRT att\lambda::[kan micF'-yfp oriR6K]$	
CR786	$\Delta tolC::FRT \Delta marRAB::FRT \Delta soxRS::FRT \Delta rob::FRT att\lambda::[kan micF'-yfp oriR6K]$	
CR787	$\Delta tolC::FRT \Delta micF::FRT att\lambda::[kan micF'-yfp oriR6K]$	
CR788	MDG147 $\Delta marRAB::kan$	
CR789	MDG147 $\Delta rob::cat$	
CR790	MDG147 $\Delta marRAB::kan \Delta rob::cat$	
Plasmids		
pKD46	<i>bla</i> P _{BAD} <i>gam bet exo</i> pSC101 <i>ori(ts)</i>	15
pCP20	<i>bla cat cI857</i> $\lambda P_{R'}$ - <i>flp</i> pSC101 <i>ori(ts)</i>	8
pKD3	<i>bla rgnB FRT cat FRT oriR6K</i>	15
pKD4	<i>bla rgnB FRT aph FRT oriR6K</i>	15
pKD13	<i>bla rgnB FRT aph FRT oriR6K</i>	15
pBAD30	<i>bla araC</i> P _{BAD} p15A <i>ori</i>	24
pMarA	pBAD30:: <i>RBS-marA</i>	
pRob	pBAD30:: <i>RBS-rob</i>	
pVenus	<i>kan MCS yfp(venus) t0 att\lambda oriR6K</i>	52
pVenus-ompF	<i>kan MCS ompF'-yfp(venus) t0 att\lambda oriR6K</i>	
pVenus-FY	<i>kan MCS ompF'-yfp(venus)(hyb) t0 att\lambda oriR6K</i>	
pVenus-micF	<i>kan MCS micF'-yfp(venus) t0 att\lambda oriR6K</i>	

^a All strains are isogenic derivatives of *E. coli* K-12 strain MG1655. Numbers in parentheses indicate deletion endpoints as determined using the MG1655 genome sequence.

^b All strains and plasmids are from this work unless otherwise noted.

^c FRT, FLP recombination target.

arabinose was supplied at 0.1% (wt/vol) and sodium salicylate (Sigma-Aldrich, St. Louis, MO) was introduced into growth medium at 5 mM.

Strain and plasmid construction. All strains used in this work are isogenic derivatives of the sequenced *E. coli* K-12 strain MG1655. The generalized transducing phage P1vir was used in all genetic crosses according to standard methods (57). Targeted gene deletions and subsequent marker removal were made using the λ Red recombinase method of Datsenko and Wanner (15). Site-specific integrations were made using the λ Int/CRIM method of Haldimann and Wanner (25).

Deletion cassettes were generated with the plasmid templates pKD3, pKD4, and pKD13 using standardized priming sites (15). The $\Delta marRAB$, $\Delta soxRS$, Δrob , $\Delta micF$, and $\Delta ompR$ deletion cassettes were generated by PCR using the primer pairs 5'-CTT GAA CCG ATT TAG CAA AAC GTG GCA TCG GTC AAT TCA TTG TAG GCT GGA GCT GCT TCG-3' and 5'-GGG AAG TTA ATA AGC CCC GAG ATG TCG GGG CCA GAA CAA ACA TAT GAA TAT CCT CCT TAG-3', 5'-AGC AAT TAC CCG GCG GGG AGT TAA CGC GCG GGC AAT AAA ATG TAG GCT GGA GCT GCT TCG-3' and 5'-ACC GGA AAA CAA ACT AAA GCG CCC TTG TGG CGC TTT AGT TCA TAT GAA TAT CCT CCT TAG-3', 5'-CTC CCG CTT TGG CAT CTT CTG CCG GGT AGT ATC GCT CAA TTG TAG GCT GGA GCT GCT TCG-3' and 5'-CTC TAC TAA GAA AAA AAC ACT GAA TGC TAA AAC AGC AAA ACA TAT GAA TAT CCT TCG-3', 5'-TGT CAA AAC AAA ACC TTC ACT CGC AAC TAG AAT AAC TCC CAT TCC GGG GAT CCG TCG ACC-3' and 5'-AGT TTT TCT GTG GTA GCA CAG AAT AAT GAA AAG TGT GTA ATG TAG GCT GGA GCT GCT TCG-3', and 5'-GCT TAC AAA TTG TTG CGA ACC TTT GGG AGT ACA AAC AAT GTG TAG GCT GGA GCT GCT TCG-3' and 5'-TAC GGG CAA ATG AAC TTC GTG GCG AGA AGC GCA ATC GCC TCA TAT GAA TAT CCT CCT TAG-3', respectively. All cassettes were transformed into MG1655 cells expressing λ Red recombinase from the pKD46 helper plasmid. Deletions were verified by PCR using primers in the

antibiotic resistance marker and sites adjacent on the host chromosome. All deletions were subsequently transduced into a clean MG1655 background prior to antibiotic cassette removal using the FLP recombinase expressing pCP20 helper plasmid.

Single-copy transcriptional and translational fusions were constructed in *trans* using the pVenus integration vector (52). Transcriptional fusions to the P_{ompF} and P_{micF} promoters were made by PCR amplifying the promoter regions of the *ompF* and *micF* genes using primers 5'-ATA GGT ACC ACG TGC TGG ACG AGC GTA TG-3' and 5'-ATA GAA TTC AGC AGG GAC GAT CAC TGC-3' and 5'-ATA GGT ACC ACC TGA GTT TCA CCT TTG AA-3' and 5'-ATA GAA TTC TGC GAG GCA TCC GGT TGA AA-3', respectively. Following amplification, the PCR products were digested with KpnI and EcoRI (sequences underlined) and ligated into the corresponding restriction sites of pVenus to produce pVenus-ompF and pVenus-micF. The translational fusion of *ompF* to *yfp* [*ompF'-yfp(hyb)*] was produced in two steps. First, a fragment containing the promoter region and the first 39 bases of *ompF* was generated by PCR using primers 5'-ATA GGT ACC ACG TGC TGG ACG AGC GTA TG-3' and 5'-CAG TGA AAA GTT CTT CTC TTT CAG TAG CAG GGA CGA TCA CTG C-3'. The resulting product also contained an overhang complementary to 25 base pairs after the first 6 bases of *yfp(venus)*. Second, the *ompF'-yfp'* fragment was used to amplify the entire *yfp(venus)* and *t0* terminator region from pVenus using the reverse primer 5'-CTC GCA ATC CAG TGC AAA-3'. The *ompF'-yfp(hyb)* fragment was then cloned into the KpnI (sequence underlined) and NheI sites of pVenus to produce pVenus-FY. The pVenus derivatives described above were then integrated into the phage λ attachment site in MG1655 cells expressing λ Int from the pINT-ts helper plasmid. Single-copy integrations were verified by PCR using primers described by Haldimann and Wanner (25). Resulting single-copy fusions were transduced back into a clean MG1655 background.

Complementation vectors for expression of MarA and Rob were constructed

using the medium-copy-number, arabinose-inducible pBAD30 plasmid (24). The *marA* and *rob* genes were PCR amplified using forward and reverse primers 5'-ATA GAA TTC TTT ATA AGG AGG AAA AAC ATA TGA CGA TGT CCA GAC GC-3' and 5'-ATA TCT AGA CTA GCT GTT GTA ATG ATT TAA TGG A-3' and 5'-ATA GAG CTC TTT ATA AGG AGG AAA AAC ATA TGG ATC AGG CCG GCA TTA T-3' and 5'-ATA GGT ACC TTA ACG ACG GAT CGG AAT CA-3', respectively. The *marA* and *rob* products both contain strong, synthetic ribosome binding sites (RBS) to ensure high-level translation. Resulting PCR products were treated with EcoRI and XbaI (sequences underlined) for *marA* and SacI and KpnI (sequences underlined) for *rob*. The digested products were then ligated into the corresponding restriction sites downstream of the P_{BAD} promoter in pBAD30 to produce the plasmids pMarA and pRob.

Fluorescence-based promoter activity assays. Cells were grown overnight in medium A to saturation and subcultured 1:200 in fresh medium with or without 5 mM salicylate. For experiments, 0.5 ml was dispensed to individual wells of microtiter plates with 96 deep, square wells (VWR; 82006-448). Plates were sealed with Breath-Easy membranes (Diversified Biotech) to reduce evaporation, placed on a high-speed, microplate shaker (VWR), and shaken at 1,000 rpm and 37°C.

To measure fluorescence and optical density (OD), 250 μ l of culture was transferred from the deep-well plates to black, clear-bottomed Costar 96-well microtiter plates. Fluorescence (excitation/emission λ , 515/530 nm) and OD (600 nm) were measured using a Tecan Safire2 microplate reader. Fluorescence measurements are reported as the relative fluorescence normalized to the optical density of the sample to correct for differences in cell density. All experimental data presented are the averages and standard deviations of four replicate samples.

Small-scale envelope preparation and SDS-PAGE. Envelope fractions were prepared as described by Slauch and Silhavy with minor modifications (56). Briefly, cells were grown overnight in medium A and subcultured 1:200 in 10 ml of fresh medium with or without 5 mM salicylate. Cultures were grown to mid-log phase (OD = 0.4 to 0.5). Sample volumes were normalized to the lowest optical density to allow for comparison of outer membrane protein quantities across strain backgrounds. Normalized cultures were then pelleted at $3,800 \times g$. The pellet was washed once in 30 mM Tris-HCl (pH 8.1) and repelleted. Cell pellets were then resuspended in 30 mM Tris-HCl-20% sucrose buffer, followed by the addition of 10 μ l of 20-mg/ml lysozyme-0.1 mM EDTA (pH 7.3) and incubated on ice for 30 min. Following lysozyme treatment, 3 ml of 3 mM EDTA (pH 7.3) was added and the resulting extract was disrupted with a single 20-s pulse using a microtip sonicator (Fisher Scientific). A 1.5-ml fraction of the extract was then centrifuged at $16,000 \times g$ for 60 min. Envelope fractions were collected as centrifuged precipitate and resuspended in 40 μ l of Laemmli SDS sample buffer (30). Samples were boiled at 100°C for 5 min prior to SDS-PAGE display. Finally, 10- μ l aliquots were displayed on 10% acrylamide-6 M urea-1% SDS gels at 150 V for 80 min.

RESULTS

Salicylate decreases the expression of OmpF and increases the expression of OmpC. Previous reports have shown that exposure to salicylate decreases the amount of OmpF in the outer membrane (12, 47, 49, 54). Although these reports all observe a reduction in OmpF, discrepancies exist regarding changes in the expression of OmpC. To determine the effects of salicylate on OmpF/OmpC expression, we harvested insoluble membrane fractions from cells grown in a rich, low-osmolarity medium (28) in the presence or absence of 5 mM salicylate (Fig. 1). Under these conditions, we observed a decrease in the levels of OmpF and an increase in the levels of OmpC in the outer membrane.

Most likely, these discrepancies between our work and those in the literature regarding OmpC are due to differences in the strains employed. In our experiments, we used strain MG1655. In those involving strain MC4100, salicylate was found to decrease OmpC despite increases in *ompC* transcription (49). However, in derivatives of strain AG100, no changes in OmpC expression were observed (12).

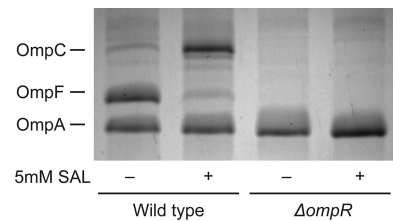


FIG. 1. The levels of OmpC, OmpF, and OmpA in the outer membrane in the presence or absence of salicylate. Cells were grown overnight in medium A and subcultured 1:200 in fresh medium A in the presence or absence of 5 mM salicylate (SAL). OmpC, OmpF, and OmpA protein bands are indicated. Strains used in this experiment were MG1655 and CR720.

We additionally explored the effects of OmpR on regulating OmpF/OmpC expression under salicylate exposure (Fig. 1). Consistent with the observations of Rosner and coworkers (49), both OmpF and OmpC are not expressed in the absence of OmpR, irrespective of whether salicylate is present or not.

MarA and Rob are functionally redundant regulators of MicF and OmpF expression. The MicF sRNA is known to repress the expression of OmpF. The transcription of *micF*, in turn, is activated by MarA, SoxS, and Rob. Of the three, only MarA expression is known to be directly responsive to salicylate (12, 33, 35). However, Cohen and coworkers found that the reduction of OmpF expression in response to salicylate was not solely dependent on increased expression of MarA (12). Based on this observation, we hypothesized that Rob may also be involved. Specifically, we have observed that Rob is indirectly activated by salicylate, independent of either MarA or MarR (9). To test this hypothesis, we measured the expression from single-copy transcriptional fusions of *ompF* and *micF* and a translational fusion of OmpF to the fast-folding yellow fluorescent protein (YFP) variant Venus (41). We performed these experiments in a series of genetic backgrounds where the *marRAB*, *soxRS*, and *rob* regulatory components of the *mar-sox-rob* network were systematically deleted. In addition, we tested the expression of these fusions in a strain lacking *micF*. Consistent with our hypothesis, we found that both MarA and Rob work in parallel to decrease OmpF expression in the presence of 5 mM salicylate (Fig. 2A and 3). We also found that MarA and Rob both increase *ompF* transcription (Fig. 2B). No change, however, was observed upon loss of SoxS under identical conditions. Specifically, we observed a 2.5-fold increase in OmpF expression, as determined using translational fusions to Venus upon loss of either MarA ($\Delta marRAB$) or Rob (Δrob) (Fig. 2A). Likewise, we observed a 20% reduction in *ompF* transcription upon loss of either transcription factor (Fig. 2B). Moreover, their contributions were additive with respect to both OmpF expression and *ompF* transcription. The increases in OmpF expression upon loss of either factor were also reflected by the changes in *micF* transcription, where we found that the loss of MarA or Rob decreased transcription 1.4- or 2.1-fold, respectively (Fig. 2C). In mutants lacking both MarA and Rob, we found that OmpF expression was increased greater than 6-fold, with correlated decreases in *micF* transcription of greater than 26-fold.

We also measured OmpF expression in mutants lacking *micF* in the presence of 5 mM salicylate. Given the current

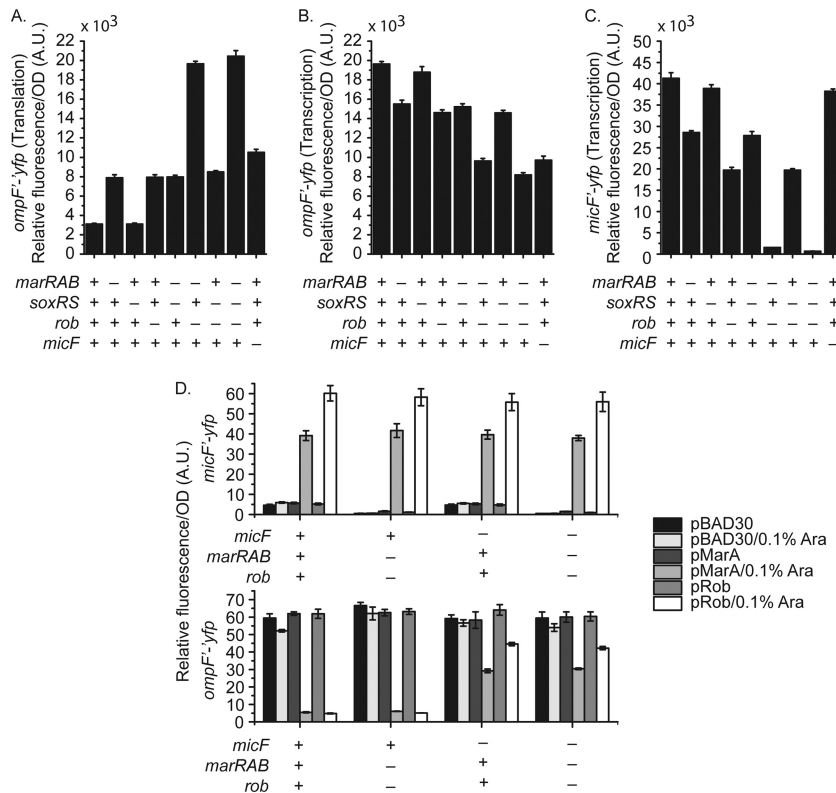


FIG. 2. Full repression of *ompF* translation during salicylate exposure requires both MarA and Rob. (A) Levels of *ompF'-yfp* translation. (B) Transcriptional activity of the P_{ompF} promoter. (C) Transcriptional activity of the P_{micF} promoter. Cells were grown overnight in medium A and subcultured 1:200 in medium A containing 5 mM salicylate for 4 h prior to fluorescence and optical density measurements. Presence or absence of genes is denoted by + or -, respectively. Strains used in this experiment were CR713 to CR715, CR737 to CR744, CR754 to CR761, and CR771 to CR778. (D) Transcriptional activity of P_{micF} and levels of *ompF'-yfp* translation during ectopic complementation of MarA and Rob in the presence and absence of MicF. Cells were grown in medium A overnight and subcultured 1:200 in fresh medium A with and without 0.1% arabinose. Strains used in this experiment are CR715, CR774, CR776, CR781, CR714, CR757, CR759, and CR764.

regulatory model, disruptions in *micF* should result in levels of OmpF translation comparable to those observed in a *marRAB rob* double mutant or *marRAB soxRS rob* triple mutant. Surprisingly, we found that the double and triple mutants exhibited 2-fold-higher levels of OmpF expression than the *micF* mutant (Fig. 2A). These results demonstrate that MarA and Rob do not regulate *ompF* translation in response to salicylate solely through a MicF-dependent pathway. This conclusion is further supported by the phenotypic observation that OmpF

levels in the presence of 5 mM salicylate are higher in mutants lacking both MarA and Rob than in mutants lacking MicF alone (Fig. 3).

Interestingly, we found that *ompF* transcription was reduced in a *micF* mutant in the presence of salicylate (Fig. 2B), opposite to what we see at the levels of translation and protein expression. Consistent with these results, the changes in *ompF* transcription observed in a *marRAB rob* double mutant and a *marRAB soxRS rob* triple mutant were nearly identical to those in a *micF* mutant. These results are surprising, as Cohen and coworkers previously observed that increased *micF* transcription decreases *ompF* transcription (12). One possible explanation is that MicF stabilizes the mRNA of our OmpF transcriptional fusion. Regardless, we suspect that the effect we observe is not physiologically significant.

The experiments described above were performed in the presence of 5 mM salicylate. As a control, we also performed identical experiments in the absence of salicylate. In this case, we found that both OmpF expression and *ompF* transcription were mostly unchanged in the different mutant backgrounds (see Fig. S1 in the supplemental material). The difference was no greater than 10% in the case of the translational fusions and 20% in the case of the transcriptional fusions. Likely, *micF* expression is too weak and OmpF expression too high for there

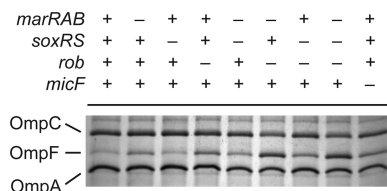


FIG. 3. Both MarA and Rob are required to fully repress OmpF expression in the outer membrane during salicylate exposure. Presence or absence of genes is indicated by + and -, respectively. Cells were grown overnight in medium A and subcultured 1:200 in fresh medium A containing 5 mM salicylate. Cultures were grown to mid-logarithmic phase prior to envelope extraction. Envelope fractions were displayed on 10% acrylamide-6 M urea-1% SDS gels and stained with Coomassie R250. Strains used were MG1655 and CR721 to CR728.

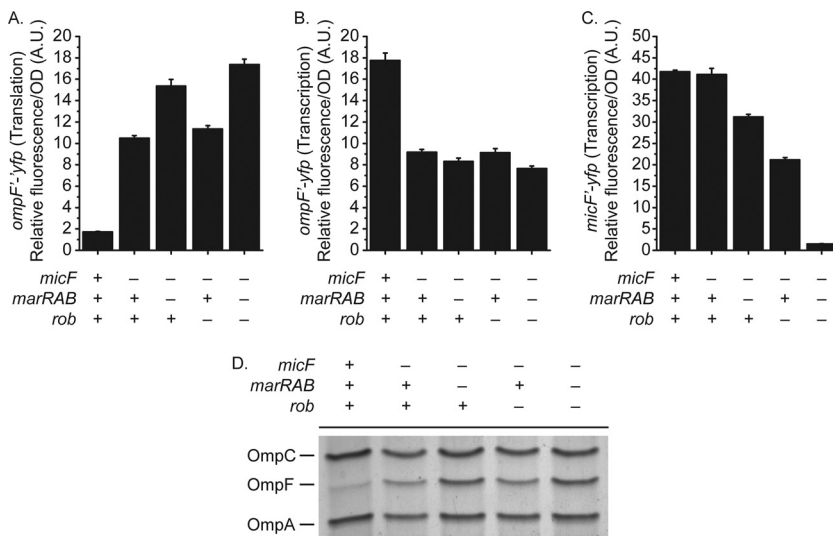


FIG. 4. MarA functions through MicF-dependent and MicF-independent pathways to reduce the levels of OmpF during salicylate exposure. (A) Levels of *ompF'-yfp* translation (strains CR757 and CR762 to CR764). (B) Transcriptional activity of the P_{ompF} promoter (strains CR740 and CR745 to CR747). (C) Transcriptional activity of the P_{micF} promoter (strains CR774 and CR779 to CR781). (D) Levels of OmpC, OmpF, and OmpA in the envelope fraction displayed on a 10% acrylamide-6 M urea-1% SDS gel (strains MG1655, CR724, and CR729 to CR731). Cells were grown in medium A overnight and subcultured 1:200 in fresh medium A with 5 mM salicylate. Cultures were grown for 4 h prior to fluorescence and optical density measurements or to mid-log phase prior to envelope extraction.

to be any change in the absence of salicylate. Indeed, *micF* expression is significantly reduced in the absence of salicylate. One interesting observation, though, is that OmpF expression is almost 2-fold higher in the absence of salicylate in wild-type cells than in mutants in the presence of salicylate. These results suggest that salicylate also represses OmpF expression through an alternate mechanism.

We also tested whether MarA and Rob could independently repress OmpF expression when ectopically expressed from an arabinose-inducible promoter on a plasmid in the absence of salicylate (Fig. 2D). To account for different background levels of OmpF expression, we also performed these experiments in the presence of 200 mM NaCl (see Tables S1 and S2 in the supplemental material). In genetic backgrounds containing *micF*, we found that the ectopic expression of MarA or Rob led to a 10-fold increase in *micF* expression irrespective of whether NaCl was present or not. This 10-fold increase correlates well with the corresponding 10-fold decrease in OmpF expression that we also observed. In strains lacking *micF*, the ectopic expression of MarA led to an approximately 50% reduction in OmpF expression whereas the ectopic expression of Rob led to an approximately 30% reduction in OmpF expression. While the level of repression is significantly reduced, these results suggest, as discussed below, that MarA and possibly Rob can repress OmpF expression independent of MicF.

MarA regulates OmpF expression through a MicF-independent pathway. We observed that the ectopic expression of MarA or Rob could reduce OmpF expression in a *micF* mutant (Fig. 2D), suggesting that the two may function through a MicF-independent pathway. To further explore this putative mechanism, we constructed *marRAB* and *rob* mutants in otherwise *micF* null genetic backgrounds and monitored OmpF expression and *ompF* and *micF* transcription in the presence of salicylate.

We found that loss of Rob had no effect on OmpF expression in the absence of MicF (Fig. 4A and D), indicating that it functions upstream of MicF. In the case of MarA, however, we found that deleting it could affect OmpF translation in the absence of MicF. Specifically, we observed a 50% increase in OmpF expression upon loss of MarA in an otherwise *micF* background (Fig. 4A and D), indicating that MarA represses OmpF translation independent of MicF. Lastly, we found that the decrease in *micF* transcription upon loss of MarA or Rob is independent of MicF (Fig. 4C).

We also found that the decrease in *ompF* transcription upon loss of MarA or Rob is due to MicF (Fig. 4B). In the absence of MicF, MarA or Rob had no effect on *ompF* transcription. This epistasis indicates that both MarA and Rob function upstream of MicF with regard to *ompF* transcription. It also suggests that MicF activates *ompF* transcription, though this is likely an artifact of our transcriptional fusions as discussed above.

Increases in *ompC* transcription are independent of MarA or Rob. Previous results have shown increases in *ompC* transcription during salicylate exposure (49). Despite the close proximity of the MarA/SoxS/Rob binding site in the divergently arranged P_{micF} promoter, the role of MarA and Rob in mediating this increase in *ompC* transcription has not been previously explored. To determine whether the salicylate-induced increase in *ompC* transcription is MarA or Rob dependent, we monitored *ompC* transcription in mutants lacking *marRAB* or *rob* using a cyan fluorescing protein (CFP) gene fused downstream of the *ompC* coding region. This fusion has previously been shown to have minimal effects on OmpC expression and to provide an accurate measure of *ompC* transcription (3, 4). Consistent with the previous findings, we observed modest increases in *ompC* transcription in the presence of 5 mM salicylate (Fig. 5). However, we found that this in-

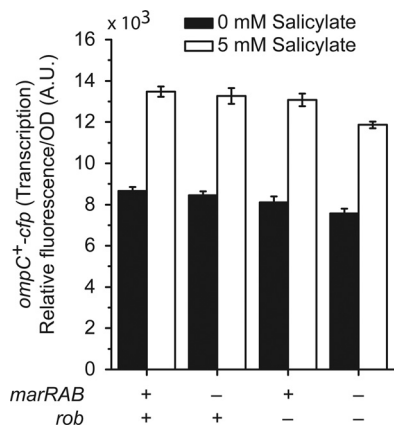


FIG. 5. Increases in *ompC* transcription are independent of MarA and Rob. Cells were grown overnight in medium A and subcultured 1:200 in fresh medium A with or without 5 mM salicylate. Cultures were grown for 4 h prior to fluorescence and optical density measurements. Strains used in this experiment were MDG147 and CR788 to CR790.

crease is independent of MarA and Rob. These results indicate that MarA and Rob do not regulate *ompC* transcription in response to salicylate.

The reduction in OmpF expression in *tolC* mutants is due to Rob. *E. coli* mutants lacking TolC are known to have altered outer membrane porin compositions. Specifically, the expression of OmpF in the outer membrane is significantly reduced regardless of medium osmolarity (40). Misra and Reeves previously showed that the reduction in OmpF expression in a *tolC* mutant was due to MicF (38). However, they did not determine what caused *micF* transcription to increase upon loss of TolC. Recent data from Rosner and Martin suggest that the increase in *micF* transcription in *tolC* mutants is due to the upregulation of MarA, SoxS, and Rob (51). Based on these findings, we wished to determine which of the *mar-sox-rob* systems contribute to the decreased expression of OmpF observed in a *tolC* mutant. Specifically, we studied the effects of MarA, SoxS, Rob, and MicF on the expression of transcriptional and translational fusions described previously.

In the absence of *tolC*, we observed that OmpF expression

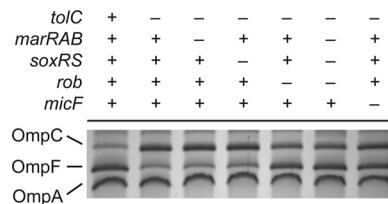


FIG. 7. MicF-dependent reduction of OmpF expression in *tolC* mutants is a result of Rob activation of *micF* gene expression. Cells were grown overnight in medium A and subcultured 1:200 in fresh medium A containing 5 mM salicylate. Cultures were grown to mid-logarithmic phase prior to envelope extraction. Envelope fractions were displayed on 10% acrylamide-6 M urea-1% SDS gels and stained with Coomassie R250. Strains used were MG1655, CR702, and CR732 to CR736.

was decreased and *micF* transcription was increased, consistent with previous findings (Fig. 6A and C). Introducing *marRAB*, *soxRS*, and *rob* deletions into the *tolC* mutant background indicated that Rob is the primary, though not sole, factor increasing *micF* transcription and, as a consequence, decreasing OmpF expression (Fig. 6A and C). Examining OmpF expression in the outer membranes of these mutants also supports this conclusion (Fig. 7). Collectively, these data indicate that Rob is the primary regulator involved in increased MicF expression in *tolC* mutants. The role of MarA and SoxS in this instance appears to be minor.

We also found that *ompF* transcription was increased in *tolC* mutants, though this increase was *rob* and *micF* dependent (Fig. 6B). Specifically, we can attribute the increase in *ompF* transcription to increased MicF expression through Rob. As we have mentioned, increased MicF expression leads to increased *ompF* transcription, though this effect is likely an artifact of our transcriptional reporter.

Though Rob is the key factor regulating MicF expression in a *tolC* mutant, these findings do not directly indicate the source of Rob activation. Whether this is caused by increased intracellular metabolites or perturbation of other elements of cellular physiology is still unknown (10). Interestingly, MarA is upregulated in a *tolC* mutant but does not affect MicF expression (51).

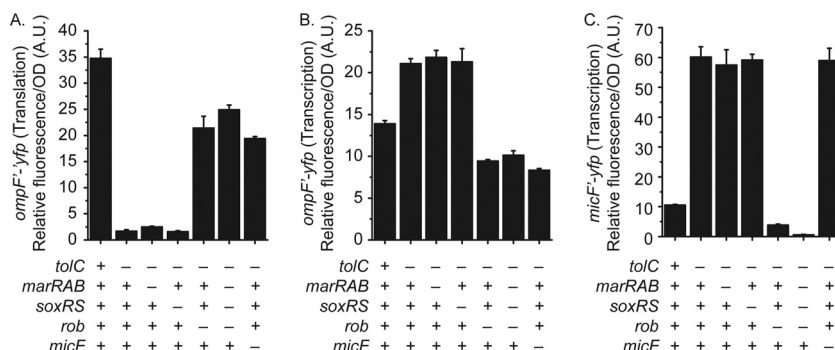


FIG. 6. Reduction in *ompF* translation by MicF in *tolC* mutants is a result of Rob-dependent activation of *micF* gene expression. (A) Levels of *ompF'*-*yfp* translation. (B) Transcriptional activity of the P_{ompF} promoter. (C) Transcriptional activity of the P_{micF} promoter. Cells were grown overnight in medium A and subcultured 1:200 in medium A containing 5 mM salicylate for 4 h prior to fluorescence and optical density measurements. Strains used in this experiment were CR713 to CR715, CR748 to CR753, CR765 to CR769, and CR782 to CR787.

DISCUSSION

In this work, we investigated the role of the *mar-sox-rob* regulon in regulating outer porin expression, where the focus was on the MicF-dependent regulation of OmpF expression upon salicylate exposure. We found that MarA and Rob can independently activate *micF* transcription in response to salicylate, leading to reduced OmpF expression. MarA was also found to repress OmpF expression through a MicF-independent pathway. In the case of OmpC, salicylate increased its transcription though this effect was independent of MarA and Rob. Finally, we were able to show that the reduction in OmpF expression in a *tolC* mutant is due primarily to Rob.

A key finding of this study was that MarA is not the sole factor regulating MicF expression in response to salicylate. Rob is also capable of activating MicF expression in response to salicylate. Both function in parallel regulatory pathways, where their effects on OmpF expression are additive. Previous studies have, in fact, shown that the salicylate-induced reduction in OmpF expression is not solely due to MarA and that some other factor is involved (12). The surprising finding here was that Rob is one of the factors. While salicylate is known to induce MarA expression through the derepression of MarR and is often taken as the canonical inducer for MarA, salicylate has not previously been shown to directly activate Rob to the best of our knowledge. The known activating ligands for Rob are bile salts, fatty acids, and 2,2'-dipyridyl (48, 50). Whether salicylate directly binds and activates Rob, however, is unknown.

We have found that MarA also activates a MicF-independent pathway to reduce OmpF expression. Rob can too but only when ectopically expressed from a plasmid, a result not surprising given the common binding targets for both regulators (5, 29, 34). These observations suggest that MarA utilizes both MicF and the MicF-independent pathway simultaneously to achieve levels of OmpF reduction similar to that which Rob accomplishes through MicF alone. Through the combined action of these factors, the parallel MarA and Rob-dependent pathways may serve to ensure OmpF reduction in the presence of a variety of toxic chemicals.

How MarA is able to work through a MicF-independent pathway to inhibit translation of *ompF* mRNA is unknown. As this regulation occurs at the level of *ompF* translation, MarA likely regulates an additional sRNA not detected by previous microarray analyses. Currently, the only well-characterized sRNA regulator of *ompF* mRNA translation is MicF. Although our data suggest the possibility of an additional sRNA regulator, they do not discount the possibility of MarA-regulated factors that may work to destabilize the *ompF* mRNA. Future implementation of sRNA detection strategies during salicylate exposure will help to differentiate between these possibilities.

OmpF expression is decreased in the absence of the outer membrane efflux pore TolC (40). Misra and Reeves previously demonstrated that this reduction in OmpF expression is due to MicF (37, 38). In the present study, we showed that the reduction in OmpF expression in the absence of TolC is due primarily to Rob. These results are consistent with recent observations made by Rosner and Martin, who have also shown that MarA and SoxS expression and Rob activation are elevated approximately 2-fold in *tolC* null mutants (51). The increase in *mar-sox-rob* regulon activation has been attributed in part to

the elevated intracellular levels of intermediary metabolites that serve as inducers for these three systems (10, 51). What was surprising was that the *tolC* effect could be almost solely ascribed to Rob even though the MarA expression is also increased under these conditions.

Although this work has more clearly defined roles for MarA and Rob in regulating OmpF expression in response to salicylate, there are still a number of unresolved issues. For one, we found that *ompC* transcription is increased during salicylate exposure in a MarA/Rob-independent manner. As the *mar-sox-rob* regulon is extensive, it may be possible that additional downstream elements cause indirect changes in the expression of *ompC*. Alternatively, parallel regulators responsive to salicylate, such as EmrR (MprA), may instead regulate *ompC* transcription (32). Another point of interest may be the convergence of additional two-component systems at the *ompF* promoter that may be stimulated by salicylate and other extracellular toxins. A number of systems, such as the CpxAR and RstAB two-component systems, have been shown to directly and indirectly change the activity of the *ompF* promoter (4, 21, 43). Additionally, salicylate may stimulate other extracytoplasmic stress systems. However, minimal overlap exists between extracytoplasmic stress and salicylate transcriptional responses based on genome-wide microarray data (6, 45). Whether MarA and Rob serve an auxiliary role in changing the activity of these systems or EnvZ-OmpR activation remains to be seen.

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