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AcrS/EnvR Represses Expression of the *acrAB* Multidrug Efflux Genes in *Escherichia coli*▽

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The *acrS* regulatory gene is located upstream of the *acrEF* multidrug efflux system genes. However, the roles of AcrS in regulation of drug efflux pumps have not been clearly understood. Here we show that AcrS represses other multidrug efflux genes, *acrAB*, which encode a major efflux system in *Escherichia coli*.

One of the important mechanisms underlying resistance to antibiotics involves extrusion of the compounds by drug efflux pumps. Drug efflux pumps are found in a variety of bacterial species, and their expression is often controlled by cognate regulatory proteins (14, 19). In *Escherichia coli*, a major drug efflux system, AcrAB, has a broad substrate range and confers intrinsic drug resistance (12–14). The *acrR* gene is located upstream of *acrA*, and the AcrR protein represses expression of the *acrAB* operon. Deletion or inactivation of *acrR* results in enhanced expression of *acrAB* and increases fluoroquinolone resistance in clinical *E. coli* strains (6, 23).

AcrEF also has a broad substrate range, similar to AcrAB. In contrast to *acrAB*, the expression level of *acrEF* is very low because of global repression by a histonelike protein, H-NS (15, 16). The *acrS* (formerly *envR*) gene is located upstream of *acrE* and encodes a putative repressor (9, 18).

In order to investigate the effects of AcrS and AcrR on the drug susceptibility of *E. coli* cells, the *acrS* or *acrR* gene was cloned into the pTrc99A expression vector. The resulting plasmids were transformed into the W3104 wild-type strain, and then the MICs of toxic compounds for these transformants were determined as described previously (15). When AcrS was

TABLE 1. Susceptibility of *E. coli* repressor-overproducing strains to antibiotics and toxic compounds

Compound	MIC (μg/ml)[a]								
	Wild type strain W3104			Δ*acrAB* mutant			Δ*hns* Δ*acrAB* mutant		
	No repressor	AcrR repressor	AcrS repressor	No repressor	AcrR repressor	AcrS repressor	No repressor	AcrR repressor	AcrS repressor
Chloramphenicol	6.25	3.13	0.78	0.78	0.78	0.78	1.56	0.78	1.56
Tetracycline	6.25	3.13	1.56	1.56	1.56	1.56	1.56	1.56	1.56
Erythromycin	100	100	6.25	6.25	6.25	6.25	25	25	25
Nalidixic acid	3.13	1.56	0.78	0.78	0.78	0.78	1.56	0.78	1.56
Norfloxacin	0.05	0.025	0.025	0.025	0.025	0.025	0.05	0.025	0.05
Novobiocin	1,600	800	25	12.5	12.5	12.5	50	50	50
Acriflavine	200	100	12.5	6.25	12.5	6.25	25	25	25
Crystal violet	25	3.13	0.78	0.78	1.56	0.78	6.25	3.13	3.13
Ethidium	400	200	25	12.5	12.5	12.5	200	200	200
Methylene blue	>1,600	400	12.5	6.25	6.25	6.25	400	400	400
Rhodamine 6G	400	200	3.13	3.13	6.25	3.13	200	200	200
Tetraphenylphosphonium	1,600	800	12.5	12.5	6.25	12.5	50	25	25
Benzalkonium	50	25	3.13	3.13	3.13	3.13	12.5	12.5	6.25
Sodium dodecyl sulfate	>25,600	>25,600	200	50	50	50	200	200	100
Deoxycholate	>40,000	>40,000	2,500	2,500	5,000	2,500	40,000	40,000	20,000

[a] MIC determination experiments were repeated at least three times.

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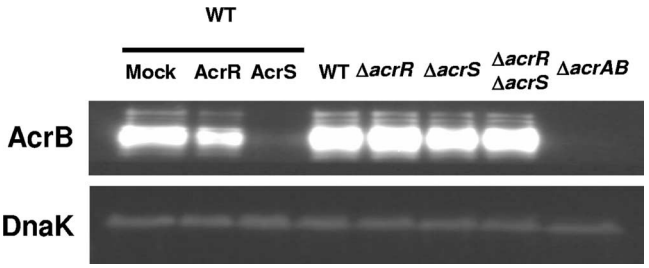

FIG. 1. Detection of AcrB expression in the repressor-overexpressing strain. W3104 (which harbors pTrc99A, pTrc99acrR, and pTrc99acrS), W3104 Δ*acrR*, W3104 Δ*acrS*, W3104 Δ*acrR* Δ*acrS*, and W3104 Δ*acrAB* were grown to an optical density at 600 nm of 0.8 in LB medium containing 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and harvested. AcrB and DnaK (control) in 3 μg of cell lysate protein were analyzed by Western blotting with polyclonal anti-AcrB antibodies for AcrB or a monoclonal anti-DnaK antibody (Calbiochem) and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G for AcrB (Bio-Rad Laboratories, Hercules, CA) or anti-mouse immunoglobulin G for DnaK, and these proteins were detected by using the CDP-Star substrate (GE Healthcare BioScience). WT, wild type.

overexpressed, the intrinsic tolerance of W3104 for several toxic compounds was drastically decreased (Table 1). On the other hand, AcrR overexpression did not affect the MICs, except for eightfold decreases in the crystal violet and methylene blue MICs. The Δ*acrAB* mutant was hypersensitive to various antibiotics, as shown in Table 1. Overexpression of *acrS* or *acrR* did not affect the drug susceptibility of Δ*acrAB*, indicating that the effect of AcrS overexpression on the drug tolerance of the wild-type strain is mediated by AcrAB. We previously reported that deletion of the *hns* gene increases *acrEF* expression and results in an AcrEF-dependent multidrug resistance phenotype in the Δ*acrAB* genetic background (16). The drug susceptibilities of W3104 Δ*hns* Δ*acrAB* were hardly affected even when AcrS and AcrR were overexpressed (Table 1), suggesting that overexpression of neither AcrS nor AcrR suppresses the expression of *acrEF*. We also examined the effect of deletion of *acrR* and/or *acrS*. The deletion mutants were constructed by a gene replacement method using the pKO3 plasmid (8). Neither deletion of *acrR* nor deletion of *acrS* affected the drug susceptibilities, with the exception of susceptibility to novobiocin. Deletion of *acrS* increased the MIC of novobiocin for W3104 (data not shown).

Immunoblotting with anti-AcrB antibody showed that overexpression of *acrS* decreased the level of production of the AcrB protein (Fig. 1). On the other hand, in cells overexpressing *acrR*, the level of production of AcrB was moderately decreased. The transcription level of each transporter gene was also examined by quantitative real-time PCR (as described previously) (3) using an AcrEF-overproducing strain, W3104 Δ*hns*. The presence of the *acrS* expression plasmid decreased the transcriptional level of *acrA* 310-fold, while the decrease was only moderate with *acrR*-expressing plasmids (2.4-fold). On the other hand, the *acrE* transcriptional level was slightly or hardly decreased by *acrS* and *acrR* overexpression (2.8- and 1.3-fold decrease, respectively). These results are consistent with greater potency of AcrS for *acrA* repression than for *acrE* repression. AcrS also represses the expression of *acrA* more efficiently than AcrR does. It is known that *acrAB* expression is also controlled by the global regulators MarA, SoxS, and Rob (4, 5, 10, 21). However, the expression of these regulators was not affected by AcrS and AcrR (data not shown), indicating that the *acrAB* repression by AcrR and AcrS is unlikely to be mediated by MarA, SoxS, or Rob. Thus, AcrS is an effective repressor of *acrAB* but not of *acrEF*. Therefore, the low level of expression of AcrEF is not due to AcrS. Moreover, our observations are consistent with the results of a study that showed that AcrS does not appear to act as a local repressor of *acrEF* in *Salmonella enterica* serovar Typhimurium (17).

To evaluate whether AcrR and AcrS directly regulate *acrAB* expression, a DNase I footprinting analysis was performed. AcrR-His_6 and AcrS-His_6 fusion proteins were purified from *E. coli* crude soluble lysate using nickel affinity resin (GE Healthcare BioScience). The 312-bp DNA fragments including the *acrA* promoter (229 bp of the upstream region and 83 bp of the coding region) were labeled with 6-carboxyfluorescein (6-FAM) fluorophores. The probes (0.45 pmol) were mixed and incubated for 20 min at room temperature with AcrR-His_6 and AcrS-His_6, and then DNase I footprinting analysis was performed using a previously described nonradiochemical capillary electrophoresis method and an ABI PRISM 310 sequencer/genetic analyzer equipped with an ABI PRISM 310 GeneScan (2, 24). Both AcrR and AcrS directly bound to the *acrA* promoter containing the previously predicted 24-bp palindrome sequence (TACATACATT-TATG-AATGTAT GTA) (20). This region was protected from DNase I digestion by adding 4.3 pmol of AcrR or AcrS (Fig. 2). To compare the binding affinity of AcrS with the binding affinity of AcrR, we performed an electrophoretic mobility shift assay. A total of 312 bp, including 229 bp upstream and 83 bp of the coding region, and 276 bp upstream of the start codon were used as *acrA* and *acrD* DNA fragments, respectively. The *acrA* and *acrD* probes (0.15 pmol) were mixed and incubated for 20 min at room temperature with AcrR-His_6 and AcrS-His_6, respectively. Samples were electrophoresed, and SYBR green I (Lonza)-stained DNAs were visualized under blue incident light at 460 nm using an LAS-3000 luminescent image analyzer (Fujifilm). The electrophoretic mobility shift assay revealed that the *acrA* probe was almost completely shifted in the presence of 4.5 pmol AcrS, whereas the shift of the *acrA* probe was not observed at the same concentration of AcrR (Fig. 3). For detection of the shift, 13.5 pmol AcrR was required, indicating that the binding affinity of AcrS for the *acrA* promoter region is higher than that of AcrR. Hence, the differences in the degree of *acrA* repression between AcrS and AcrR can be explained in part by the difference in their binding affinities.

acrS is adjacent to *acrE*, and each gene is divergently transcribed. We examined the relationship between the promoter activities of *acrS* and *acrE*. For construction of reporter plasmids, DNA fragments containing the *acrE* promoter (previously constructed by Hirakawa et al. and Kobayashi et al. [2, 7]) and the *acrS* promoter (400 bp upstream of the start codon) were cloned in front of the β-galactosidase-encoding *lacZ* reporter gene in a single-copy pNN387 vector (1). The resulting plasmids were introduced into endogenous LacZ-negative strains (MC4100 and MC4100 Δ*hns*), and β-galactosidase activity was assayed in cell lysates using *o*-nitrophenyl-β-D-galactopyranoside as the substrate (11). In the wild-type strain, the promoter activity of *acrS* was very low and similar to that of

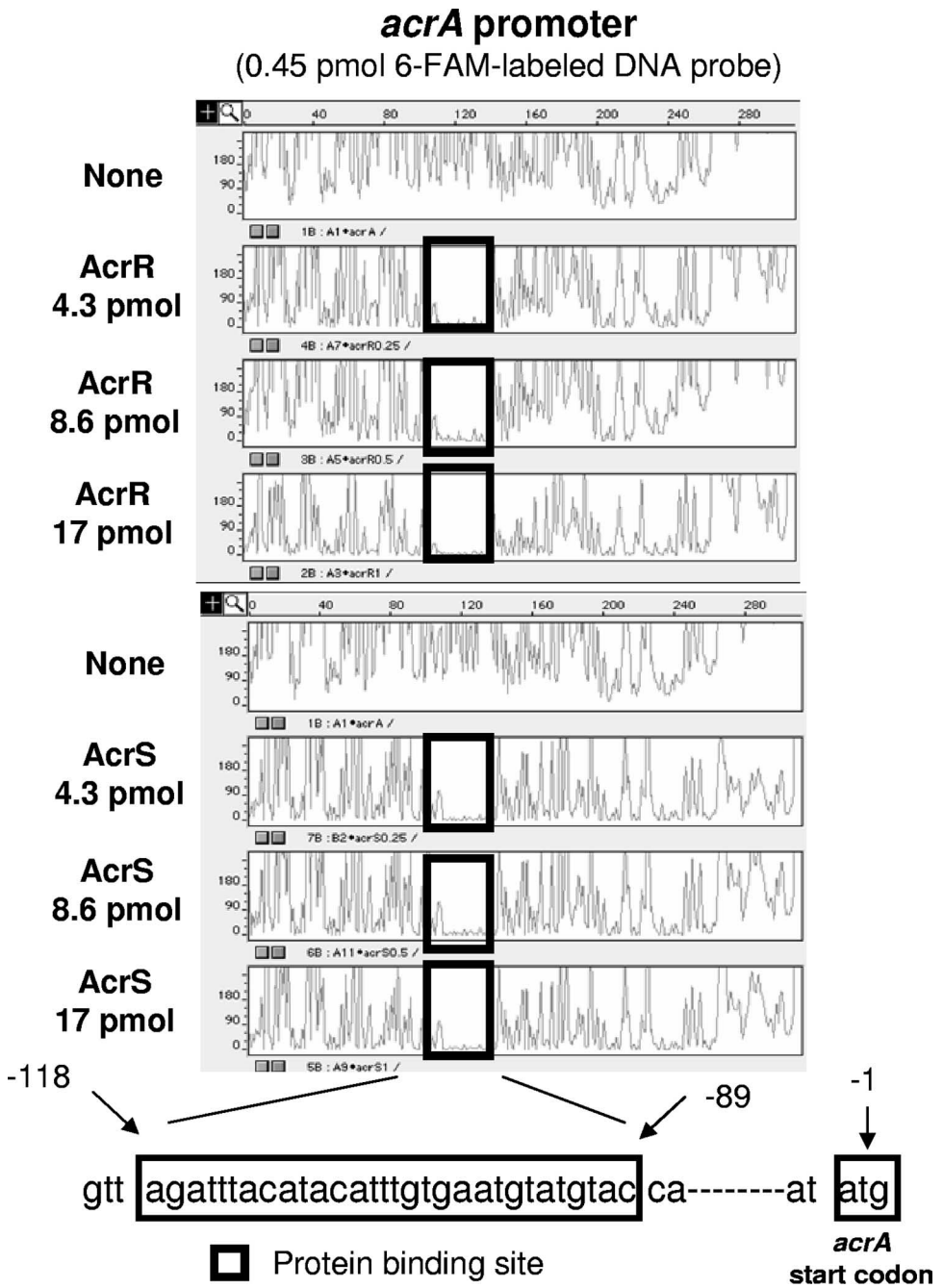

FIG. 2. DNase I footprinting analysis of AcrR or AcrS binding to the *acrA* promoter region. A DNA fragment (0.45 pmol) including the *acrA* promoter region was labeled with 6-FAM at the 5′ end, incubated with AcrR-His_6 or AcrS-His_6 (4.3 to 69 pmol) in a reaction solution containing 20 mM HEPES-Na (pH 7.5) and 1 mM dithiothreitol, and then subjected to DNase I footprinting assays. The fluorescence intensity (ordinate) of 6-FAM-labeled DNA fragments is plotted against the sequence of the fragment (abscissa). Protein-binding sites are enclosed in rectangles.

acrE (less than 1.0 Miller unit). The lack of an effect of *acrS* deletion on AcrAB repression was probably due to the low level of expression of AcrS. When the *hns* gene was deleted, the promoter activity of *acrS* greatly increased, indicating that transcription of *acrS* was stimulated simultaneously with the increase in AcrE expression (11.1 Miller units for *acrE* and 8.4 Miller units for *acrS*).

AcrAB and AcrEF may have common physiological roles and features because they have similar broad substrate spectra and high sequence homology (15). Therefore, when the expression of AcrEF is induced, AcrAB may no longer be required and the production is shut down to prevent excess protein production by AcrS. Thus, we believe that AcrS functions as a switch for the alternative expression of AcrAB and AcrEF. However, what compound and/or condition induces the expression of AcrS remains unknown. The cross-regulation of

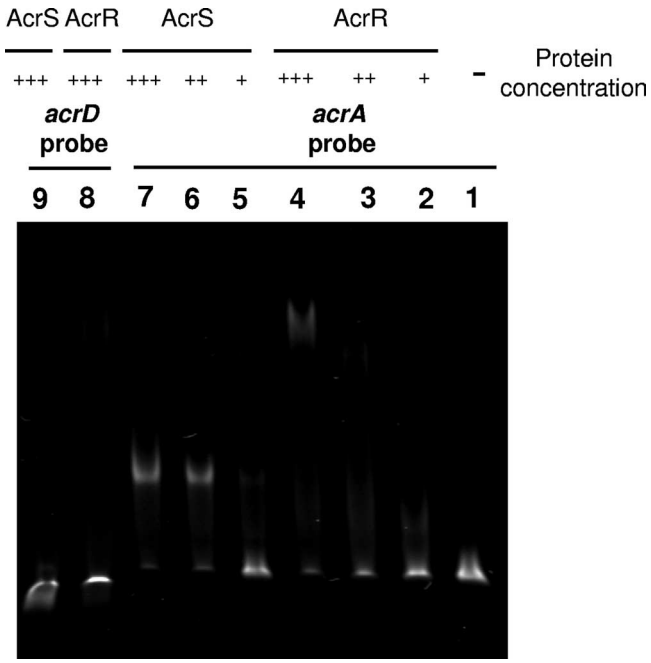

FIG. 3. Electrophoretic mobility shift assay for AcrR and AcrS binding to the *acrA* promoter. DNA fragments (0.15 pmol) including the *acrA* (312 bp) and *acrD* (276 bp; control) promoter regions were incubated without or with various concentrations of AcrR-His_6 and AcrS-His_6 in a reaction solution containing 20 mM HEPES-Na (pH 7.5) and 1 mM dithiothreitol. Lane 1, no repressor; lanes 2, 3, 4, and 8, AcrR protein (lane 2, 1.5 pmol; lane 3, 4.5 pmol; lanes 4 and 8, 13.5 pmol); lanes 5, 6, 7, and 9, AcrS protein (lane 5, 1.5 pmol; lane 6, 4.5 pmol; lanes 7 and 9, 13.5 pmol). Samples were electrophoresed on a 5% nondenaturing polyacrylamide gel.

RND efflux pumps involving their local regulators has been reported for *Pseudomonas putida* (22). In the future we plan to investigate the physiological implications of the switch for alternative expression of AcrAB and AcrEF.

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