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Activation of Multiple Antibiotic Resistance and Binding of Stress-Inducible Promoters by *Escherichia coli* Rob Protein

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Multiple antibiotic resistance in *Escherichia coli* can be mediated by induction of the SoxS or MarA protein, triggered by oxygen radicals (in the *soxRS* regulon) or certain antibiotics (in the *marRAB* regulon), respectively. These small proteins (SoxS, 107 residues; MarA, 127 residues) are homologous to the C terminus of the XylS-AraC family of proteins and are more closely related to a ~100-residue segment in the N terminus of Rob protein, which binds the right arm of the replication origin, *oriC*. We investigated whether the SoxS-MarA homology in Rob might extend to the regulation of some of the same inducible genes. Overexpression of Rob indeed conferred multiple antibiotic resistance similar to that known for SoxS and MarA (against chloramphenicol, tetracycline, nalidixic acid, and puromycin), as well as resistance to the superoxide-generating compound phenazine methosulfate. The Rob-induced antibiotic resistance depended only partially on the *micF* antisense RNA that down-regulates the OmpF outer membrane porin to limit antibiotic uptake. Similar antibiotic resistance was conferred by expression of a Rob fragment containing only the N-terminal 123 residues that constitute the SoxS-MarA homology. Both intact Rob and the N-terminal fragment activated expression of stress genes (*inaA*, *fumC*, *sodA*) but with a pattern distinct from that found for SoxS and MarA. Purified Rob protein bound a DNA fragment containing the *micF* promoter (50% bound at $\sim 10^{-9}$ M Rob) as strongly as it did *oriC*, and it bound more weakly to DNA containing the *sodA*, *nfo*, or *zwf* promoter (50% bound at 10^{-8} to 10^{-7} M). Rob formed multiple DNA-protein complexes with these fragments, as seen previously for SoxS. These data point to a DNA-binding gene activator module used in different protein contexts.

Multiple antibiotic resistance in bacteria is an increasing problem in medical microbiology but is still poorly understood at the molecular level (6, 32). Bacterial resistance mechanisms that involve drug inactivation (12) and alteration of the drug target (41) are usually specific for a single class of antibiotics or even an individual compound. More-general mechanisms confer low-level antibiotic resistance that may be important in the development and dissemination of highly resistant strains (12, 32). In this second type of resistance, accumulation of the antibiotic within the cell may be diminished by reduced uptake or by increased efflux from the cell (33). These low-level broad resistances are usually mediated by chromosomal mutations (15, 19, 23, 39), but few are well characterized either as regulatory systems or for the cellular mechanisms involved.

Probably the best-characterized locus for multiple antibiotic resistance is the *mar* complex in *Escherichia coli* (15, 16). Mutations at *mar* confer resistance to several unrelated antibiotics and have been associated with an active efflux of tetracycline (15) and chloramphenicol (29). In addition, *mar* mutants have significantly reduced levels of the outer membrane porin OmpF, which probably diminish drug influx (9, 10). The *mar* locus is organized as an operon of three genes: *marR*, *marA*, and *marB* (8). Transcription of *marRAB* is inducible and is controlled negatively by the MarR protein (4, 20). The MarA protein is evidently a transcriptional regulator whose activity in the cell increases when its concentration is increased (4, 14, 20).

Some of the genes controlled by *marRAB* are also members of the redox-responsive *soxRS* regulon (4, 17), which also confers multiple antibiotic resistance when activated (7, 18). Antibiotic resistance in both cases depends in part on the elevated synthesis of *micF* RNA (7, 10), an antisense transcript that destabilizes the *ompF* mRNA (3, 31). Oxidative stress genes that are transcriptionally activated by both the *marRAB* and *soxRS* systems include *sodA* (encoding a superoxide dismutase), *zwf* (encoding glucose-6-phosphate dehydrogenase [G6PD]), *fumC* (encoding a heat-stable fumarase), *soi-17*/*-19*, and the genes encoding two other oxidative stress proteins of unknown function (4, 17, 18, 26, 28). This regulatory overlap may have a physical basis embodied in the strong homology between the SoxS protein, the direct activator of *soxRS* regulon genes (2, 27, 34), and MarA (8). Like MarA, the activity of SoxS seems to be controlled by regulation of its synthesis, which results from transcriptional regulation governed by the redox-sensing SoxR protein (2, 21, 35, 45).

SoxS and MarA, along with the Tn*10*-encoded TetD protein of unknown function, are related to the XylS-AraC family of transcription regulators (13) but correspond only to the C-terminal ~100-residue domain of this family (Fig. 1). This carboxyl-terminal segment is implicated in DNA binding and may be sufficient for transcriptional activation in some members of the XylS-AraC family (13).

Recently, a protein named Rob, which binds the right arm of the *E. coli* replication origin *oriC*, was found to be related to SoxS and MarA (40). However, in Rob the 100-residue domain homologous to SoxS and MarA is located at the N terminus of the protein; the C-terminal 175-residue segment of Rob is not related to XylS-AraC family members (40) (Fig. 1). The function of Rob is unknown.

Rob, MarA, and SoxS contain similarly positioned helix-turn-helix motifs (Fig. 1) that might mediate specific DNA binding. In Rob and MarA, the second predicted helices are identical and differ from that of SoxS by only one residue (8, 40). Since SoxS and MarA seem able to activate some of the same genes in vitro, we tested whether intact Rob or its iso-

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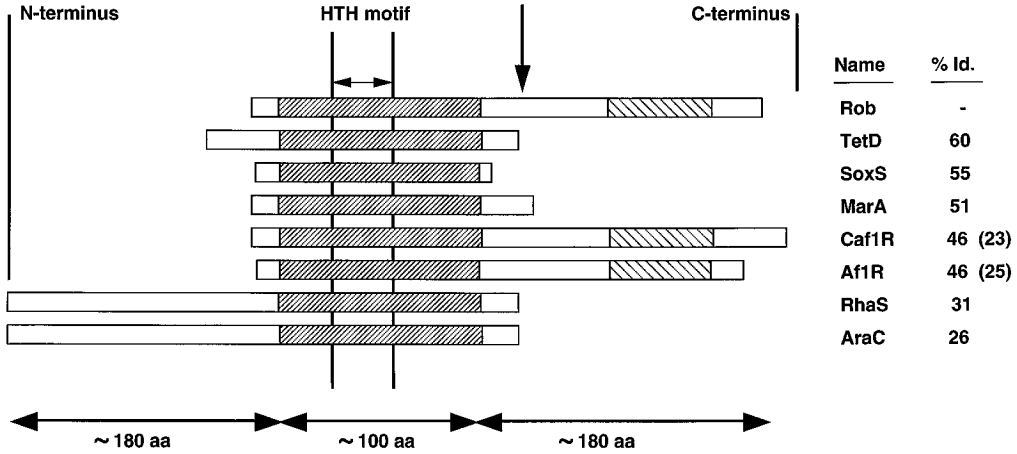

FIG. 1. Schematic alignment of Rob protein sequence with some members of the XylS-AraC family of bacterial regulators. The percent identity (% Id.) of Rob was calculated from the BLAST algorithm (1). The hatched area indicates the core segment of sequence similarity among these different proteins. The hatched area on the right indicates a 50-residue homology shared among Rob, Caf1R, and Af1R (percent identity shown in parentheses). The vertical arrow points to the C-terminal end of the truncated Rob133 protein (see Table 1). aa, amino acids; HTH, helix-turn-helix.

lated N-terminal 133-residue domain could activate phenotypic antibiotic resistance or specific genes for antibiotic resistance or oxidative stress.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *E. coli* strains and plasmids used in this study are listed in Table 1. Permanent bacterial stocks were stored frozen at −80°C in 20% glycerol, and working stocks were maintained on Luria-Bertani (LB) agar (30) at 4°C for up to 2 weeks. Cells were grown at 37°C with shaking at 150 rpm in LB broth (30) in flasks with a 10- to 20-fold-larger volume than the culture, unless otherwise indicated.

Antibiotic susceptibility. The sensitivity of various strains to antibiotics was determined by measuring bacterial growth on gradient plates prepared as described previously (4, 11), with each plate containing 60 ml of LB agar (30 ml in each of the top and bottom layers) and the medium supplemented with 0.1 mg of ampicillin per ml and, where indicated, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Strains carrying the different plasmids were inoculated into LB broth with 100 μg of ampicillin per ml (LB-ampicillin) and incubated for ~16 h. The saturated cultures were diluted 100-fold into fresh LB-ampicillin and incubated for ~1 h to reach an optical density at 600 nm of ~0.2. Each culture was then split into two equal volumes, and IPTG was added to one of these to a final concentration of 1 mM. After further incubation for ~2 h, aliquots of cultures with and without IPTG were plated (2, 17, 18) on gradient plates with and without 1 mM IPTG, respectively. Confluent growth along the gradient was scored after 18 to 24 h at 37°C.

β-Galactosidase activity assays. Overnight cultures in LB-ampicillin were diluted 100-fold into fresh LB-ampicillin and incubated for ~45 min to an optical density at 600 nm of ~0.2. Each culture was then split into two equal volumes, and IPTG was added (final concentration, 1 mM) to one. After further incubation for ~1 h, β-galactosidase activity in sodium dodecyl sulfate-$CHCl_3$-treated cells was determined as described by Miller (30). The optical density at 600 nm was used as a measure of the cell density.

Cell extracts and enzyme assays. Overnight cultures in LB-ampicillin were diluted 100-fold into 100 ml of fresh LB-ampicillin and incubated to reach an optical density at 600 nm of 0.3 to 0.4. Each culture was then split in two equal volumes of 50 ml, IPTG was added to one to a final concentration of 1 mM, and the incubation was continued at 37°C for 45 min. The cells were then chilled, harvested by centrifugation at 5,000 × *g* for 15 min at 4°C, washed with 50 ml of ice-cold M9 salts (30), and frozen as pellets at −80°C. Cell pellets were thawed for 1 to 3 h on ice, suspended in buffer containing 50 mM Tris-HCl (pH 7.5) and 0.2 M NaCl, and disrupted for 3 min at 4°C with glass beads in a Mini-bead beater (Biospec Products, Bartleville, Okla.). Cell debris was removed by centrifugation at 10,000 × *g* for 45 min at 4°C, and the cleared supernatants were collected and stored on ice for immediate assay or stored frozen at −80°C. Protein concentrations were estimated by the method of Bradford (5). G6PD activity was monitored by measuring NADPH production at 340 nm (24). Fumarase C activity was determined as described by Hill and Bradshaw (22), as modified by Liochev and Fridovich (28).

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
Strains		
GC4468	F⁻ Δ(*lac*)*U169 rpsL*	18, 42
DJ901	As GC4468 but Δ*soxRS901* Tn*10*Km^r	18
JHC1098	As GC4468 but Δ*soxRS901* Tn*10*Km^r Δ*marRAB*	17
JHC1112	As DJ901 but Δ*micF*	7
W3110	λ⁻, IN (*rrnD-rrnE*)*1*	40
WTB35-17	As W3110 but *rob*::*kan*	K. Skarstad
RA4468	As GC4468 but *rob*::*kan*	This work
TN1799-1	As DJ901 but Φ(*sodA*::*lacZ*)*29*	T. Nunoshiba
N7940	As DJ901 but *inaA*::*lacZ*	36
Plasmids		
pBluescript	Vector	Stratagene
pBSoriC	*Eco*RI-*Pst*I fragment containing *oriC* inserted into pBluescript	40
pBT35-13	1.7-kb *Sal*I-*Bam*HI fragment containing *rob* inserted in pING-1	40
pSE380	*trc* promoter expression vector, *lacI*^{q} Ap^r	2
pSRob	926-bp *Sal*I-*Sac*I fragment containing entire *rob* gene from pBT35-13 inserted into pSE380	This work
pSRob133	423-bp *Eco*RI-*Eco*RI fragment from pSRob inserted into pSE380	This work
pSXS	432-bp *Eco*RI-*Hin*dIII PCR fragment containing the entire *soxS* gene inserted into pSE380	2

TABLE 2. Independence of Rob-mediated multiple antibiotic resistance from *soxRS* and *marRAB*

Plasmid	Addition of IPTG	Growth (% of gradient) of[a]:							
		(Δ*soxRS*)[b]					(Δ*soxRS* Δ*marRAB*)[b]		
		Nal	PMS	Cm	Tc	Pur	Nal	PMS	Tc
pSE380 (vector)	−	21	41	10	21	67	15	21	21
	+	22	44	9	26	69	15	19	22
pSRob	−	13	40	13	21	73	29	33	28
	+	100	69	100	83	100	92	55	86
pSXS	−	44	99	19	44	100	59	90	32

[a] The measurements were repeated at least twice, with similar results; values from a representative experiment are shown. Amounts (weight per plate [see Materials and Methods]) of the antibiotics were as follows: nalidixic acid (Nal), 400 μg; phenazine methosulfate (PMS), 1,225 μg; chloramphenicol (Cm), 900 μg; tetracycline (Tc), 300 μg; puromycin (Pur), 10 mg.

[b] Strains (see Table 1): Δ*soxRS*, DJ901; Δ*soxRS* Δ*marRAB*, JHC1098.

DNA-binding assays. DNA probes consisting of fragments of the *zwf*, *sodA*, *micF*, and *nfo* promoters were obtained by PCR with the following primers: *micF*, 5′-CCCGGTACTTAAGCCAG-3′ and 5′-GCGGGAAGTTATTCTAGT TGCG-3′; *zwf*, 5′-TCAGTGTCAGATTTTTACCC-3′ and 5′-CCCGGTACTT AAGCCAG-3′; *nfo*, 5′-GAGGATCCGCGTCAGCGC-3′ and 5′-GCGAGGAC TCCTGTTAAACCC-3′; and *sodA*, 5′-ATGAGTGTAAAAATCGTGCTGT CG-3′ and 5′-TCTCCAGTATTGTCGGGCG-3′ (27). The templates were pzwf for the *zwf* fragment (37), pDT1.5 for *sodA* (42), pWB21 for *nfo* (11), and pmicB21 for *micF* (7). The sizes of the PCR products were 263 bp for *micF*, 177 bp for *zwf*, 234 bp for *sodA*, and 196 bp for *nfo*, and they contained sequences from the respective transcription initiation sites to upstream endpoints (27). PCR products were quantified by gel electrophoresis and ethidium bromide staining before being labeled. Individual bands were extracted from a 2% agarose gel onto DEAE paper, which was subsequently washed in high-salt solution, and the DNA was precipitated overnight in ethanol at −20°C (38). Precipitated DNA was centrifuged at 14,000 rpm in an Eppendorf microcentrifuge, dried, and resuspended in 10 μl of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA (TE) solution. Fragments were 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs). The fragments were then purified over Sephadex G-75 in 10 mM Tris-HCl–0.1 M NaCl–0.1 mM EDTA. Dilutions of the probe were made in TE to place 2 fmol in the binding-reaction mixtures (final concentration, 0.1 nM). A 20-bp duplex oligonucleotide corresponding to bp −33 to −52 of the *micF* promoter was generated by chemical synthesis.

Purified Rob protein (40), isolated from an overproducing strain to give a final purity of >95% (39a), was graciously provided by K. Skarstad, Institute for Cancer Research, Oslo, Norway. A labeled probe and the indicated amount of Rob protein were incubated for 10 min at room temperature in binding buffer (10 mM Tris-HCl [pH 7.5], 0.2 mM EDTA, 75 mM KCl, 1 mM dithiothreitol, 10% glycerol) and subjected to electrophoresis at 5°C on a 5% polyacrylamide gel (a 10% polyacrylamide gel for the 20-bp fragment) at 170 V for 2 to 3 h. For competition assays, the appropriate unlabeled competitor DNA was added to the binding-reaction mixtures prior to Rob addition. After autoradiography, the DNA-containing bands were quantified by densitometry with a Sun Microsystems SPARC station and a BioImage software package (Millipore Corp.). Unlabeled DNA fragments were obtained by linearizing plasmid pBSoriC, containing the *oriC* sequence (40), with *Bgl*II and/or pBluescript with *Cla*I (New England Biolabs).

RESULTS

Multiple antibiotic resistance by overproduction of Rob protein. An expression plasmid for Rob protein was constructed by placing the *rob* gene behind the IPTG-inducible *trc* promoter in plasmid pSE380, yielding pSRob (see Materials and Methods). While the presence of pSRob per se did not strongly affect the antibiotic resistance of wild-type *E. coli*, the induction of Rob expression with IPTG noticeably increased cellular resistance to multiple antibiotics (nalidixic acid, chloramphenicol, tetracycline, and puromycin) and to a redox-cycling agent (phenazine methosulfate) (Table 2; data not shown). This increased resistance was independent of both the *soxRS* and the *marRAB* regulons, since it was observed in both Δ*soxRS* and Δ*marRAB* strains (Table 2). Moreover, even the basal Rob expression from pSRob conferred significant antibiotic resistance to the Δ*soxRS* Δ*marRAB* double mutant (Table 2). An even stronger antibiotic resistance was conferred by basal SoxS expression from pSXS (Table 2). Loss of viability in strains bearing pSXS in media containing IPTG (2) prevented determination of antibiotic resistance under those conditions.

Multiple antibiotic resistance controlled by *soxRS* (7) and *marRAB* (10) is partially dependent on induction of the *micF* antisense RNA and the resulting destabilization of the *ompF* mRNA (3). A similar role for *micF* was also observed for the Rob-dependent antibiotic resistances. Thus, bacterial resistance to nalidixic acid and phenazine methosulfate was diminished but not eliminated in a Δ*micF* strain bearing pSRob, under both noninduced and IPTG-induced conditions (Fig. 2).

Activation of gene expression by Rob. Since Rob overproduction provided resistance phenotypes overlapping those conferred by SoxS (2, 18) and MarA (4, 16, 17), we tested the ability of Rob to activate expression of genes controlled by the other two regulators. Two enzymatic activities were assessed: G6PD, encoded by *zwf* (2, 24), and fumarase C, the stable fumarase encoded by *fumC* (4, 28). G6PD activity was not significantly affected even after IPTG induction of Rob (Table 3). In contrast, fumarase C activity increased slightly with basal Rob expression and up to fourfold over that of the wild-type bacteria after IPTG treatment of the pSRob-bearing strain (Table 3). The presence of pSXS strongly increased both activities, even in the absence of IPTG (Table 3).

Effects of Rob on gene expression were also explored with *lac* reporter genes linked to the promoter of *sodA*, the gene encoding managanese-containing superoxide dismutase (4, 18, 42), and to the coding region of *inaA*, which encodes a protein of unknown function (43) controlled by MarA and SoxS (36). Maximum Rob expression (after induction by IPTG) increased the expression of *sodA*::*lacZ* only ca. twofold (Fig. 3). In contrast, the *inaA*::*lacZ* fusion showed up to 10-fold-enhanced expression in response to high-level Rob expression (Fig. 3). SoxS expression increased the expression of both fusions even more markedly (Fig. 3).

An N-terminal fragment of Rob sufficient for multiple antibiotic resistance and gene activation. The homology to SoxS and MarA is restricted to the N-terminal domain of Rob (40), which suggests that a common regulatory activity might reside in this region of the protein. We tested this hypothesis by constructing a plasmid (pSRob133) encoding a truncated Rob protein with only the 123 N-terminal residues and 10 additional amino acids encoded by the vector (Rob133 [Table 1]). In a *rob*::*kan* strain, the Rob133 fragment provided antibiotic resistances equivalent to that of full-length Rob (Fig. 4). However, the *rob*::*kan* mutation itself had no effect on antibiotic resistance under our conditions (data not shown). Rob133 also activated expression of the *sodA*::*lacZ* and *inaA*::*lacZ* fusions, the latter even more effectively than for activation by wild-type Rob (20- versus 10-fold [Fig. 3]).

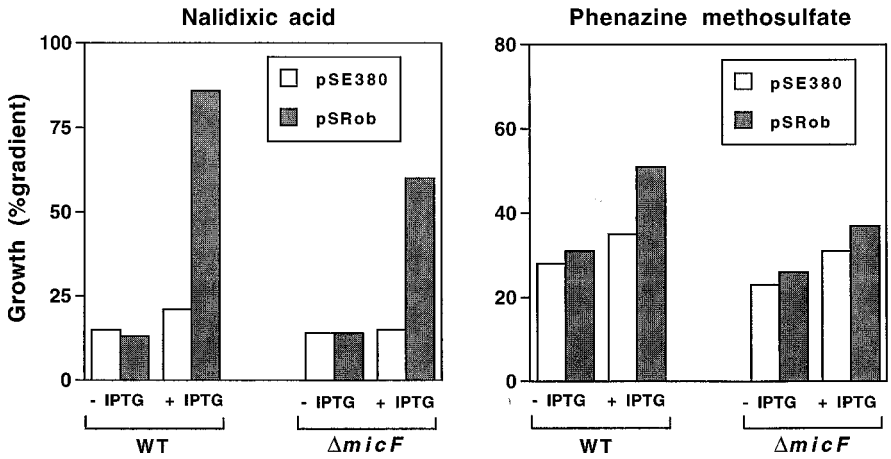

FIG. 2. Dependence of Rob-induced antibiotic resistance on *micF*. The sensitivities of strains DJ901 (Δ*soxRS*) and JHC1112 (Δ*soxRS* Δ*micF*) bearing plasmid pSE380 or pSRob were scored in gradient plates under noninduced (−IPTG) and induced (+IPTG) conditions (see Materials and Methods). The entire experiment was repeated at least three times with independent transformants; values from a representative experiment are shown. Amounts (weight added per plate [see Materials and Methods]) of the antibiotics were as follows: nalidixic acid 400 μg; phenazine methosulfate, 1,225 μg. WT, wild-type phenotype in relation to *micF*.

Rob protein binding to promoters of oxidative stress genes. The gene activation observed in cells expressing high levels of Rob suggested that the protein might interact specifically with the corresponding transcription regulatory sequences. Therefore, we performed a series of gel mobility shift assays to test for in vitro binding of Rob protein to the promoters of oxidative stress or antibiotic resistance genes. Purified Rob protein (40) formed multiple complexes in a concentration-dependent fashion with DNA fragments corresponding to both the *micF* (Fig. 5A) and the *zwf* (Fig. 5B) promoter regions. These multiple complexes may be due to multiple binding sites on the fragment for Rob protein or to multimerization of the protein, and they are reminiscent of DNA binding by the homologous SoxS protein (27). Quantification of the binding showed that 50% of the *zwf* fragment was bound by 20 nM Rob, whereas 1 nM Rob was able to exert the same effect on the *micF* fragment. If Rob exists as a monomer in solution, the dissociation constants for *micF* and *zwf* may be estimated as $\sim 10^{-9}$ and $\geq 2 \times 10^{-8}$ M, respectively.

A sequence overlapping the −35 region of *micF* is protected from DNase I digestion by SoxS protein (27). A 20-bp synthetic duplex oligonucleotide corresponding to this protected region was used in gel mobility shift assays with Rob protein, which revealed only a single complex with half-maximal binding at 1 to 6 nM (data not shown). The formation of a single complex of Rob with this fragment indicates that prior binding of Rob to a single site is not sufficient to generate multiple complexes as seen with larger DNA fragments (Fig. 5).

The relative affinity of Rob for different DNA fragments was assessed in competition experiments. In experiments with the labeled *micF* probe, a slight reduction in the amount of Rob-*micF* complex was observed with equimolar amounts of the *oriC*-containing plasmid pBSoriC (Fig. 6), while a 10-fold molar excess of the *oriC* plasmid nearly eliminated the labeled complex (Fig. 6). The plasmid vector lacking *oriC* was without effect at up to a 100-fold excess (data not shown). The *sodA* and *nfo* fragments were less effective competitors and required a 100-fold molar excess over the *micF* concentration to reduce the amount of complex detected (Fig. 6). The *zwf* fragment was the least effective competitor, with residual Rob-*micF* complex still apparent at a 100-fold excess of the unlabeled fragment (Fig. 6). Taken together, the foregoing data suggest the following order of affinities for Rob: *micF*, *oriC* ($K_d \sim 10^{-9}$ M) > *sodA*, *nfo* > *zwf* ($K_d \sim 10^{-7}$ M).

DISCUSSION

We have described a new situation conferring multiple antibiotic resistance in *E. coli*: overexpression of a putative transcriptional regulator encoded by the *rob* gene. This induction is independent of *soxRS* and *marRAB*, two regulatory loci also previously known to confer resistance to many antibiotics, but shares with those systems a partial dependence on the *micF* gene, which encodes an antisense regulator of OmpF expression. In all three cases, other cellular functions are mobilized to play significant roles in overall antibiotic resistance. Such functions probably include one or more efflux pumps, since active export of tetracycline (15) and chloramphenicol (29) has been described in *mar*-constitutive strains. Despite overlapping regulation by SoxS, MarA, and Rob, the three systems exhibit differential control in terms of both the strength of their effects on individual genes and activation of some promoters uniquely (e.g., *zwf* by SoxS or MarA but not by Rob). These differences probably account for the patterns of resistance toward different drugs mediated by the three systems.

A physical basis for differential gene activation by Rob can

TABLE 3. Enzymatic activities increased by Rob expression

Plasmid	Addition of IPTG	G6PD activity (U/mg of protein)[a]	Fold increase[b]	Fumarase C activity (U/mg of protein)[a]	Fold increase[b]
pSE380 (vector)	−	0.18 ± 0.01		4.7 ± 0.1	
	+	0.19 ± 0.01	1.0	4.4 ± 0.1	0.9
pSRob	−	0.20 ± 0.01	1.1	6.8 ± 0.5	1.4
	+	0.21 ± 0.04	1.2	20 ± 4.3	4.3
pSXS	−	0.66 ± 0.01	3.7	96 ± 2	20
	+	1.56 ± 0.15	8.6	203 ± 21	43

[a] Extracts were made from GC4468 cells containing the indicated plasmid and grown in LB-ampicillin. Values are the mean ± standard error of two determinations from two independent transformants. The entire experiment was repeated at least twice; a representative result is shown.

[b] Increase relative to the value for pSE380 without IPTG.

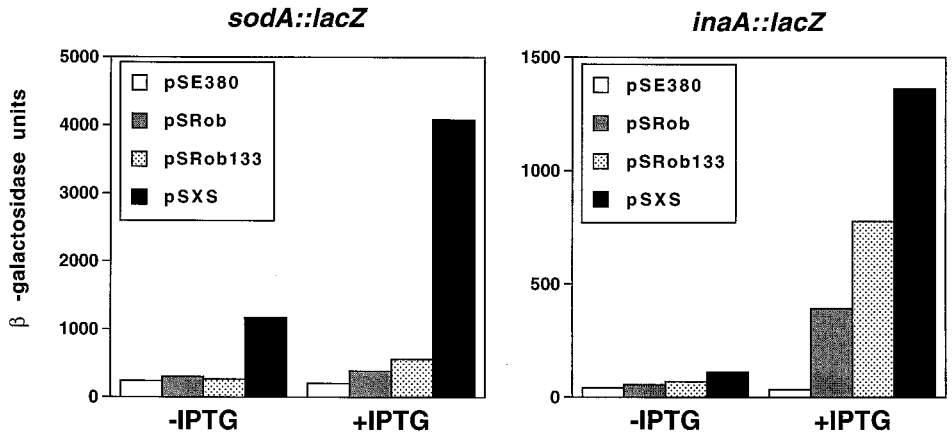

FIG. 3. Effect of the overexpression of Rob and Rob133 on β-galactosidase activity from *lac* fusions to the *sodA* and *inaA* genes. Strains TN1799-1 (Δ*soxRS sodA*::*lacZ*) and N7940 (Δ*soxRS inaA*::*lacZ*) bearing different plasmids (Table 1) were assayed for β-galactosidase activity under noninduced (−IPTG) and induced (+IPTG) conditions (see Materials and Methods). Values are the mean of two independent transformants. The range of determinations (100% × difference between values divided by the mean) was, for *sodA*::*lacZ* (left to right), 4, 5, 4, 37, 1, 12, 3, and 4% and, for *inaA*::*lacZ* (left to right), 5, 6, 11, 5, 10, 16, 14, and 10%. The entire experiment was repeated at least twice; values from a representative experiment are shown.

be suggested from a comparison of its reported binding site in *oriC* (40) with regions near the *micF*, *fumC*, *sodA*, *zwf*, and *nfo* promoters. Matches to the 21-bp *oriC* site of ~50% were found in all five cases. However, only for *micF*, *fumC* and *sodA* were these putative binding sites situated immediately 5′ to or slightly overlapping the respective −35 elements. The best-matched upstream sites in *zwf* (which is not Rob inducible) and *nfo* (not tested for inducibility) were 7 and 21 bp, respectively, 5′ to the corresponding −35 boxes. Thus, even when bound to the *zwf* promoter, Rob may not be correctly oriented to activate RNA polymerase. This analysis also predicts that *nfo* is not inducible by Rob.

The effects of Rob were generally weaker than those of SoxS for either antibiotic resistance or gene activation. Perhaps the cellular activity of Rob is modulated in response to some environmental signal(s). Such a signal could stimulate synthesis of the Rob protein, as occurs for SoxS (2, 34, 45) and MarA (4, 20), but wild-type *E. coli* contains an estimated 5,000 Rob molecules per cell during normal growth (40). Thus, if Rob activity is regulated, such control could involve posttranslational modification or binding of some cofactor.

Two other putative transcription factors have a Rob-like structure (Fig. 1): Caf1-R, which controls capsule synthesis in *Yersinia pestis* (25), and Af1-R, which regulates Af/R1 pilin synthesis in *E. coli* (44). In Rob, the N terminus harbors its DNA-binding and transcription activities (as in Rob133). For control, an activating signal for Rob may be mediated by the C-terminal segment, which is unrelated to SoxS and MarA (Fig. 1). In the XylS-AraC family, transcriptional activity of the SoxS-MarA-homologous segment at the C terminus (Fig. 1) is modulated by the attached N-terminal segment (13).

The regulatory effects of Rob protein are evidently mediated by its direct interaction with the promoters of a group of its target genes. Rob DNA binding has some features similar to binding by SoxS (27). Thus, both SoxS and Rob form multiple DNA-protein complexes with the *micF* and *zwf* promoters, perhaps by interacting with multiple sites in each. The estimated K_d of SoxS and Rob for these fragments ranges from 10^{-9} to 10^{-7} M (27; also see above). Since the affinity of Rob

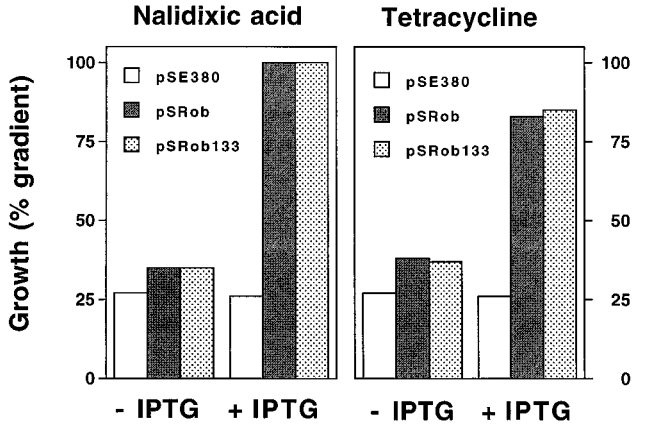

FIG. 4. Effect of overexpression of Rob133 on antibiotic resistance. The sensitivity of strain RA4468 (*rob*::*kan*) was scored in gradient plates under noninduced (−IPTG) and induced (+IPTG) conditions (see Materials and Methods). The entire experiment was repeated at least three times with independent transformants; values from a representative experiment are shown. Amounts (weight added per plate [see Materials and Methods]) of the antibiotics were as follows: nalidixic acid, 400 μg; tetracycline, 300 μg.

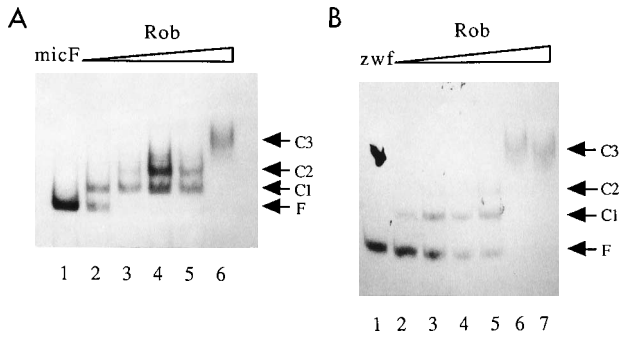

FIG. 5. Binding of oxidative stress promoter regions by purified Rob protein. Products of standard DNA-binding assays were analyzed by gel electrophoresis (see Materials and Methods). F, free probe; C1, C2, and C3, Rob-DNA complexes. (A) Rob binding of the *micF* promoter. The following amounts of Rob protein were used: lane 1, none; lane 2, 0.5 ng; lane 3, 1 ng; lane 4, 2 ng; lane 5, 4 ng; and lane 6, 8 ng. (B) Rob binding of the *zwf* promoter. The following amounts of Rob protein were used: lane 1, none; lane 2, 4 ng; lane 3, 8 ng; lane 4, 12 ng; lane 5, 16 ng; lane 6, 20 ng; and lane 7, 30 ng.

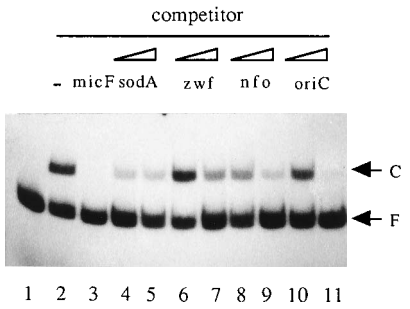

FIG. 6. Competition assay of Rob-binding sites. A labeled *micF* probe (F) was mixed with the indicated unlabeled competitor DNA and incubated without (lane 1) or with (lanes 2 to 11) 0.8 ng of purified Rob protein; this was followed by electrophoresis to separate the Rob-*micF* complex (C). Competitor DNA was used as follows: lanes 1 and 2, none; lanes 3, 4, 6, 8, and 11, a 10-fold molar excess of the indicated competitor; lanes 5, 7, and 9, a 100-fold molar excess of competitor; lane 10, an equimolar amount of *oriC* DNA.

for *oriC* is similar to that for the *micF*, it is unclear whether the interaction of Rob with *oriC* reflects the major function of this protein in the cell. In this regard, the *rob*::*kan* mutation had no apparent effect on DNA replication (39a, 40).

A rough calculation estimates that the intracellular concentration of Rob protein (40) is several micromolar, 2 to 3 orders of magnitude above its observed K_d for binding *oriC* and the *micF*, *sodA*, and *nfo* promoters. If the constitutive expression of Rob is high enough to saturate some or all SoxS- and MarA-inducible promoters, how can the SoxS or MarA proteins exert significant transcriptional activation? One possibility is that Rob is normally inactive in DNA binding or sequestered from these promoters, leaving them accessible to other proteins. Alternatively, if these promoters are always occupied by Rob, the protein might be displaced before transcriptional activation by SoxS or MarA can occur.

The protein sequence motif shared among SoxS, MarA, and the N-terminal region of Rob could be an independently functioning module. If this module is viewed as a DNA-binding transcription-activating domain, it may have been duplicated several times and placed under the control of distinct mechanisms, either through transcriptional regulation or by attachment of various protein segments. It is noteworthy how frequently a multiple antibiotic resistance phenotype has been associated with the overproduction of this activator domain, and we must now understand in molecular terms how these systems are integrated or conflict at various promoters.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Amábile-Cuevas, C. F., and B. Demple.** 1991. Molecular characterization of the *soxRS* genes of *Escherichia coli*: two genes control a superoxide stress regulon. Nucleic Acids Res. **19:**4479–4484.
3. **Andersen, J., and N. Delihas.** 1990. *micF* RNA binds to the 5′ end of *ompF* RNA and to a protein from *Escherichia coli*. Biochemistry **29:**9249–9256.
4. **Ariza, R. R., S. P. Cohen, N. Bachhawat, S. B. Levy, and B. Demple.** 1994. Repressor mutations in the *marRAB* operon that activate oxidative stress genes and multiple antibiotic resistance in *Escherichia coli*. J. Bacteriol. **176:**143–148.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Chin, G. J., and J. Marx.** 1994. Resistance to antibiotics. Science **264:**359.
7. **Chou, J. H., J. T. Greenberg, and B. Demple.** 1993. Posttranscriptional repression of *Escherichia coli* OmpF protein in response to redox stress: positive control of the *micF* antisense RNA by the *soxRS* locus. J. Bacteriol. **175:**1026–1031.
8. **Cohen, S. P., H. Hächler, and S. B. Levy.** 1993. Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. J. Bacteriol. **175:**1484–1492.
9. **Cohen, S. P., L. M. McMurry, D. C. Hooper, J. S. Wolfson, and S. B. Levy.** 1989. Cross-resistance to fluoroquinolones in multiple-antibiotic-resistant (Mar) *Escherichia coli* selected by tetracycline or chloramphenicol: decreased drug accumulation associated with membrane changes in addition to OmpF reduction. Antimicrob. Agents Chemother. **33:**1318–1325.
10. **Cohen, S. P., L. M. McMurry, and S. B. Levy.** 1988. *marA* locus causes decreased expression of OmpF porin in multiple-antibiotic-resistant (Mar) mutants of *Escherichia coli*. J. Bacteriol. **170:**5416–5422.
11. **Cunningham, R. P., S. Saporito, S. G. Spitzer, and B. Weiss.** 1986. Endonuclease IV (*nfo*) mutant of *Escherichia coli*. J. Bacteriol. **168:**1120–1127.
12. **Davies, J.** 1994. Inactivation of antibiotics and the dissemination of resistance genes. Science **264:**375–382.
13. **Gallegos, M. T., C. Michan, and J. L. Ramos.** 1993. The XylS/Arac family of regulators. Nucleic Acids Res. **21:**807–810.
14. **Gambino, L., S. J. Gracheck, and P. F. Miller.** 1993. Overexpression of the MarA positive regulator is sufficient to confer multiple antibiotic resistance in *Escherichia coli*. J. Bacteriol. **175:**2888–2894.
15. **George, A. M., and S. B. Levy.** 1983. Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: involvement of a non-plasmid-encoded efflux of tetracycline. J. Bacteriol. **155:**531–540.
16. **George, A. M., and S. B. Levy.** 1983. Gene in the major cotransduction gap of the *Escherichia coli* K-12 linkage map required for the expression of chromosomal resistance to tetracycline and other antibiotics. J. Bacteriol. **155:**541–548.
17. **Greenberg, J. T., J. H. Chou, P. Monach, and B. Demple.** 1991. Activation of oxidative stress genes by mutations at the *soxQ*/*cfxB*/*marA* locus of *Escherichia coli*. J. Bacteriol. **173:**4433–4439.
18. **Greenberg, J. T., P. Monach, J. H. Chou, P. D. Josephy, and B. Demple.** 1990. Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **87:**6181–6185.
19. **Gutmann, L., R. Williamson, N. Moreau, M. Kitzis, E. Collatz, J. F. Acar, and F. W. Goldstein.** 1985. Cross-resistance to nalidixic acid, trimethoprim and chloramphenicol associated with alterations in outer membrane proteins of *Klebsiella*, *Enterobacter*, and *Serratia*. J. Infect. Dis. **151:**501–507.
20. **Hächler, H., S. P. Cohen, and S. B. Levy.** 1991. *marA*, a regulated locus which controls expression of chromosomal multiple antibiotic resistance in *Escherichia coli*. J. Bacteriol. **173:**5532–5538.
21. **Hidalgo, E., and B. Demple.** 1994. An iron-sulfur center essential for transcriptional activation by the redox-sensing SoxR protein. EMBO J. **13:**138–146.
22. **Hill, R. L., and R. H. Bradshaw.** 1969. Fumarase. Methods Enzymol. **13:**91–99.
23. **Hooper, D. C., J. S. Wolfson, K. S. Souza, E. Y. Ng, G. L. McHugh, and M. N. Swartz.** 1989. Mechanisms of quinolone resistance in *Escherichia coli*: characterization of *nfxB* and *cfxB*, two mutant resistance loci decreasing norfloxacin accumulation. Antimicrob. Agents Chemother. **33:**283–290.
24. **Kao, S. M., and H. M. Hassan.** 1985. Biochemical characterization of a paraquat-tolerant mutant of *Escherichia coli*. J. Biol. Chem. **260:**10478–10481.
25. **Karlyshev, A. V., E. E. Galyov, V. M. Abramov, and V. P. Zavyalov.** 1992. Caf1-R gene and its role in the regulation of capsule formation of *Yersina pestis*. FEBS Lett. **305:**37–40.
26. **Kogoma, T., S. B. Farr, K. M. Joyce, and D. O. Natvig.** 1988. Isolation of gene fusions (*soi*::*lacZ*) inducible by oxidative stress in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **85:**4799–4803.
27. **Li, Z. Y., and B. Demple.** 1994. SoxS, an activator of superoxide stress genes in *Escherichia coli*: purification and interaction with DNA. J. Biol. Chem. **269:**18371–18377.
28. **Liochev, S. I., and I. Fridovich.** 1992. Fumarase C, the stable fumarase of *Escherichia coli*, is controlled by the *soxRS* regulon. Proc. Natl. Acad. Sci. USA **89:**5892–5896.
29. **McMurry, L. M., A. M. George, and S. B. Levy.** 1994. Active efflux of chloramphenicol in susceptible *Escherichia coli* strains and in multiple-antibiotic-resistant (*mar*) mutants. Antimicrob. Agents Chemother. **38:**542–546.
30. **Miller, J. H.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Mizuno, T., M.-Y. Chou, and M. Inouye.** 1984. A unique mechanism regulating gene expression: translational inhibition by a complementary RNA

transcript (micRNA). Proc. Natl. Acad. Sci. USA **81:**1966–1970.
32. **Neu, H. C.** 1992. The crisis in antibiotic resistance. Science **257:**1064–1073.
33. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets—permeability barriers and active efflux. Science **264:**382–388.
34. **Nunoshiba, T., E. Hidalgo, C. F. Amábile-Cuevas, and B. Demple.** 1992. Two-stage control of an oxidative stress regulon: the *Escherichia coli* SoxR protein triggers redox-inducible expression of the *soxS* regulatory gene. J. Bacteriol. **174:**6054–6060.
35. **Nunoshiba, T., E. Hidalgo, Z. Y. Li, and B. Demple.** 1993. Negative autoregulation by the *Escherichia coli* SoxS protein—a dampening mechanism for the *soxRS* redox stress response. J. Bacteriol. **175:**7492–7494.
36. **Rosner, J. L., and J. L. Slonczewski.** Dual regulation of *inaA* by the multiple antibiotic resistance (*mar*) and superoxide (*soxRS*) stress response systems of *Escherichia coli*. J. Bacteriol. **176:**6262–6269.
37. **Rowley, D., and R. E. Wolf, Jr.** 1991. Molecular characterization of the *Escherichia coli* K-12 *zwf* gene encoding glucose 6-phosphate dehydrogenase. J. Bacteriol. **173:**968–977.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Sanders, C. C., W. E. Sanders, R. V. Goering, and V. Wener.** 1984. Selection of multiple antibiotic resistance by quinolones, β-lactams, and aminoglycosides with special references to cross-resistance between unrelated drug classes. Antimicrob. Agents Chemother. **26:**797–801.
39a. **Skarstad, K.** Personal communication.
40. **Skarstad, K., B. Thöny, D. S. Hwang, and A. Kornberg.** 1993. A novel binding-protein of the origin of the *Escherichia coli* chromosome. J. Biol. Chem. **268:**5365–5370.
41. **Spratt, B. G.** 1994. Resistance to antibiotics mediated by target alterations. Science **264:**388–393.
42. **Touati, D.** 1983. Cloning and mapping of the manganese superoxide dismutase gene (*sodA*) of *Escherichia coli* K-12. J. Bacteriol. **155:**1078–1087.
43. **White, S., F. E. Tuttle, D. Blankenhorn, D. C. Dosch, and J. L. Slonczewski.** 1992. pH dependence and gene structure of *inaA* in *Escherichia coli*. J. Bacteriol. **174:**1537–1543.
44. **Wolf, M. K., and E. C. Boedeker.** 1990. Cloning of the genes for AF/R1 pili from rabbit enteroadherent *Escherichia coli* RDEC-1 and DNA sequence of the major structural subunit. Infect. Immun. **58:**1124–1128.
45. **Wu, J., and B. Weiss.** 1992. Two-stage induction of the *soxRS* (superoxide response) regulon of *Escherichia coli*. J. Bacteriol. **174:**3915–3920.