

Defining a *rob* Regulon in *Escherichia coli* by Using Transposon Mutagenesis

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The Rob protein of *Escherichia coli* is a member of the AraC-XylS family of prokaryotic transcriptional regulators and is expressed constitutively. Deletion of the *rob* gene increases susceptibility to organic solvents, while overexpression of Rob increases tolerance to organic solvents and resistance to a variety of antibiotics and to the superoxide-generating compound phenazine methosulfate. To determine whether constitutive levels of Rob regulate basal gene expression, we performed a MudJ transposon screen in a *rob* deletion mutant containing a plasmid that allows for controlled *rob* gene expression. We identified eight genes and confirmed that seven are transcriptionally activated by normal expression of Rob from the chromosomal *rob* gene (*inaA*, *marR*, *aslB*, *ybaO*, *mdlA*, *yfhD*, and *ybiS*). One gene, *galT*, was repressed by Rob. We also demonstrated by Northern analysis that basal expression of *micF* is significantly higher in wild-type *E. coli* than in a *rob* deletion mutant. Rob binding to the promoter regions of most of these genes was substantiated in electrophoretic mobility shift assays. However, Mu insertions in individual Rob-regulated genes did not affect solvent sensitivity. This phenotype may depend on changes in the expression of several of these Rob-regulated genes or on other genes that were not identified. Rob clearly affects the basal expression of genes with a broad range of functions, including antibiotic resistance, acid adaptation, carbon metabolism, cell wall synthesis, central intermediary metabolism, and transport. The magnitudes of Rob's effects are modest, however, and the protein may thus play a role as a general transcription cofactor.

The Rob protein of *Escherichia coli* is a member of the AraC-XylS family of prokaryotic transcriptional regulators (12). Rob was first identified by its ability to bind the right arm of the origin of chromosomal replication (*oriC*) (30). The N-terminal DNA binding region of Rob (100 residues) is closely related to the *E. coli* SoxS protein, a regulator of the superoxide stress regulon (14, 18), and MarA protein, a regulator of the multiple antibiotic resistance regulon (for a review, see reference 2). The 175-residue C-terminal region of Rob does not have extensive sequence homology to known proteins, and its function has not been established.

Rob is a constitutively expressed protein (estimated up to 5,000 molecules per cell) (30), but its function is unclear. At present, the only phenotype described for an *E. coli rob* null mutant is increased susceptibility to organic solvents (35). Overexpression of Rob, on the other hand, increases the tolerance of *E. coli* to organic solvents (24), a phenotype that may be related to Rob-regulated expression of *acrAB*, which encodes an efflux pump (32, 35). Overexpression of Rob also increases resistance to antibiotics and to the superoxide-generating compound phenazine methosulfate (5, 24, 32). The latter two phenotypes overlap with those associated with increased expression of the well-characterized homologs of Rob, the MarA and SoxS proteins (2, 4).

Normal levels of Rob are proposed to contribute to ~65% of in vivo transcription levels from the *marRAB* promoter (21), which regulates resistance to diverse antibiotics and bacteriocidal agents in *E. coli* (2, 13). Furthermore, in vivo studies (5)

have shown that overexpression of Rob (or its N-terminal domain alone) activates transcription of *sodA* (encoding manganese-containing superoxide dismutase), *fumC* (encoding fumarase C), *inaA* (encoding a weak acid-inducible protein), and *micF* (gene for an antisense RNA repressing the outer membrane porin OmpF). In vitro studies (15) demonstrated Rob-activated transcription of *sodA*, *fumC*, *micF*, *zwf* (encoding glucose-6-phosphate dehydrogenase), *nfo* (encoding DNA repair endonuclease IV), and *fpr* (encoding NADPH-ferredoxin oxidoreductase). However, it is not known whether normal expression of Rob contributes to the in vivo expression of these genes. The Rob homologs MarA and SoxS can activate transcription of a broad range of genes in vivo (reviewed in references 2 and 14), including *sodA*, *fumC*, *micF*, *zwf*, and *inaA*, suggesting broadly overlapping activities of these three regulators. However, the observation that in vivo *zwf* transcription can be activated by MarA and SoxS, but not by Rob (5), shows that control by these regulators may not overlap completely. To gain more insight into these questions, we screened a transposon library in *E. coli* for Rob-regulated insertions, which revealed eight genes under the control of Rob.

MATERIALS AND METHODS

Bacterial strains and media. The *E. coli* strains and plasmids used in this study are listed in Table 1. *E. coli* was cultured in Luria-Bertani (LB) medium or in M9 minimal medium containing 0.01% thiamine and 0.4% glucose (M9 medium) (29) as indicated. Cells were grown at 37°C in a shaking incubator (250 rpm) unless stated otherwise.

Materials and recombinant DNA techniques. Antibiotics, isopropyl-β-D-thiogalactopyranoside (IPTG), and organic solvents were obtained from Sigma Chemical Co. (St. Louis, Mo.). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was obtained from Amersham (Piscataway, N.J.). Oligonucleotides were obtained from Operon Technologies Inc. (Alameda, Calif.). Restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were obtained

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
Strains		
GC4468	F ⁻ $\Delta(lac)U169 rpsL$	33
RA4468	As GC4468 but $\Delta rob::kan$	5
MB4468	As GC4468 but Δrob	This work
N7926	As GC4468 but <i>inaA1::lacZ</i> (Kan ^r) <i>zei-723::Tn10</i> (Tet ^r)	28
MB9701	As RA4468 but <i>inaA1::lacZ</i>	This work
DH5 α	F ⁻ K80d <i>lacZ</i> Δ M15 $\Delta(lacZYA-argF)U169 deoR recA1 endA1 hsdR17$ (r _K ⁻ m _K ⁻) <i>phoA supE44</i> $\lambda^- thi-1 gyrA96 relA1$	Gibco BRL
Plasmids		
pKO3	Temperature-sensitive pSC101 origin of replication; <i>sacB</i> Cm ^r	20
pJP105	ColE1 <i>lacI lacZp::soxS</i> Amp ^r	27
pMB101	ColE1 <i>lacI lacZp::rob</i> Amp ^r	This study
pSRob	926-bp <i>Sall-SacI</i> fragment containing the <i>rob</i> gene inserted into pSE380	5, 30

from New England Biolabs (Cambridge, Mass.). [γ -³²P]ATP and [α -³²P]dATP were obtained from NEN Life Sciences Products (Boston, Mass.).

E. coli genomic DNA was isolated using a QIAGEN-tip kit (Qiagen, Valencia, Calif.). Plasmid DNA preparation, gel extraction of DNA fragments, and purification of DNA amplified by PCR were performed using QIAprep, QIAEX II, and QIAquick kits, respectively (Qiagen). *Taq* polymerase (Promega, Madison, Wis.) or *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) was used as described by the enzyme supplier. After subcloning of the PCR-generated fragments, correct amplification was verified by DNA sequencing (Molecular Biology Core Facility of the Dana Farber Cancer Institute, Boston, Mass.).

Construction of *E. coli* MB4468 and MB9701. The Δrob mutant *E. coli* MB4468 was constructed in a GC4468 (wild-type [wt]) background by using vector pKO3, which contains a thermosensitive replication origin and a counterselection marker (kindly provided by G. M. Church, Harvard Medical School, Boston, Mass.), as described by Link et al. (20). In short, 500-bp fragments encompassing the 5' and 3' flanking regions of the *rob* gene were PCR amplified from *E. coli* GC4468 genomic DNA by using primer set N_o (outside, forward primer) and N_i (inside, reverse primer) and primer set C_o (outside, reverse primer) and C_i (inside, forward primer), respectively (see Table 2). A second crossover PCR was performed with primers N_o and C_o, using the PCR-amplified 3' and 5' flanking regions of *rob* as templates. This yielded a DNA fragment of about 1,000 bp, encompassing 500 bp of the flanking regions of the *rob* gene and a complete in-frame deletion of *rob*. This fragment was digested with *EcoRV* and *Bam*HI, gel purified, and ligated to pKO3 that had been digested with *Sma*I and *Bam*HI. Transformations were performed using *E. coli* strain DH5 α , and cells were plated onto LB plates containing 20 μ g of chloramphenicol/ml and incubated at 30°C. A plasmid with the correct insert (screened by PCR) was subsequently used for transformation of *E. coli* GC4468 (incubation at 30°C). Single Cm^r colonies were picked and cultured at 30°C in 1 ml of LB broth containing chloramphenicol, and samples were plated on LB agar containing chloramphenicol and 5% sucrose and were incubated at 42°C. Screening by PCR and Southern hybridization revealed a positive recombinant lacking the *rob* gene. This Δrob mutant was designated MB4468.

E. coli MB9701 (as RA4468 but *inaA1::lacZ*) was obtained by introducing *inaA1::lacZ* (Kan^r) from N7926 (28) into RA4468 by P1 cotransduction with a linked *zei723::Tn10* (Tet^r) marker and screening for both tetracycline resistance and blue coloration on plates containing X-Gal. P1 transductions were performed by standard methods (22).

Construction of the Rob expression vector pMB101. To construct the Rob expression vector pMB101, allowing for IPTG-regulated Rob expression, the *E. coli rob* gene was PCR amplified from pSRob (5) using primers RobHd3Fwd2 and RobBamrev (see Table 2). The PCR product was digested with *Hind*III and *Bam*HI and was ligated to pJP105 (27) that had been digested with the same enzymes. This Rob expression vector was introduced by transformation into *E. coli* MB4468, and mRNA levels of *rob* were determined by Northern analysis. Plasmid pMB101 was also introduced by transformation into *E. coli* MB9701 (Δrob *inaA1::lacZ*), and β -galactosidase activity was measured after IPTG-induced expression of Rob.

MudJ transposon mutagenesis. The phage MudJ (promoterless *lacZ*; Kan^r) was delivered to the Δrob strain MB4468/pMB101 by lambda phage transduction as described by Bremer et al. (7). Transductants were isolated on LB plates containing kanamycin (50 μ g/ml) and ampicillin (100 μ g/ml). Single colonies were picked and streaked individually onto LB agar plates containing either (i) ampicillin, kanamycin, and X-Gal (70 μ g/ml) or (ii) ampicillin, kanamycin, X-Gal (70 μ g/ml), and IPTG (0.1 mM) to promote expression of *rob* located on plasmid pMB101. Colonies displaying differences in blue coloration between the two plates were selected as candidates to harbor *lacZ* insertions in Rob-regulated loci. Insertional mutants were reverified by single-colony isolation and assayed for β -galactosidase activity in LB broth in the presence or absence of IPTG.

Reproducible differences in the β -galactosidase activities of the mutants confirmed the MudJ insertions at Rob-regulated loci. For each mutant, the MudJ insertion was backcrossed into the wild-type strain GC4468 by P1 transduction (22). The MudJ insertions were then transferred from the GC4468 background into MB4468 (Δrob) by P1 transduction, and each pair of strains was assayed for β -galactosidase activity. Finally, pMB101 was introduced by transformation into the strains containing the MudJ insertions in a strain MB4468 (Δrob) background, and the resulting transformants were assayed for β -galactosidase in the presence and absence of IPTG.

Arbitrary primed PCR to locate MudJ insertions. A first round of PCR was performed on chromosomal DNA of the mutants, using primer Muleft (complementary to the 5' end of phage MudJ) and two different arbitrary primers, Mbarb1 and Mbarb2 (see Table 2). PCRs were carried out in standard PCR buffer, with 0.2 mM deoxynucleoside triphosphates, 1 μ M primers, 5% dimethyl sulfoxide, 1 μ g of template DNA, and 5 U of *Taq* polymerase (Promega) in a total volume of 50 μ l (3 min at 95°C; 11 cycles of 1 min at 95°C, 50 s at 40°C, and 1 min at 72°C; 35 cycles of 1 min at 95°C, 50 s at 55°C, and 1 min at 72°C; followed by 5 min at 72°C). A second round of PCR was performed with primers Mbarb3 and the nested primer Muleftnest (Table 2), using 5 μ l of the product of the first reaction as a template. These second-round PCRs were performed as described above, in a total volume of 100 μ l (3 min at 95°C; 35 rounds of 40 s at 95°C, 50 s at 55°C, and 1 min at 72°C; followed by 5 min at 72°C). The extension products were gel purified and subsequently sequenced, using primer Muleftnest.

β -Galactosidase activities. The β -galactosidase activities of bacterial cultures were determined as described by Miller (22). Cultures were grown overnight in LB broth or M9 glucose medium with shaking at 37°C as indicated, diluted 100-fold, and then grown to an optical density at 600 nm (OD₆₀₀) of approximately 0.5. At that point, cultures were exposed to IPTG (1mM) for 30 or 60 min as indicated or were left untreated. All assays were performed in triplicate.

Organic solvent tolerance assays. Cultures were grown overnight at 30°C in LB broth, subcultured (1/100) in LB broth, and grown for 2.5 h at 30°C. Serial 10-fold dilutions were made in 0.85% NaCl-0.1% (wt/vol) peptone. Aliquots (50 μ l) were plated in duplicate onto LB agar in glass petri dishes and allowed to dry. The plates were overlaid with organic solvents (*n*-hexane, cyclohexane, or a 1:1 mixture of *n*-hexane-cyclohexane) to a depth of 2 to 3 mm and subsequently sealed with silicone rings. After incubation for 24 h at 30°C, colonies were counted, and the CFU per milliliter were calculated.

Northern blot analysis. Cultures were grown overnight in LB medium, diluted 100-fold in 3 ml of LB broth, and grown at 37°C to an OD₆₀₀ of 0.6. At this point, cultures were induced with different concentrations of IPTG for 30 min at 37°C or were left untreated. Total RNA was extracted using an RNeasy kit (Qiagen). The RNA was resuspended in diethyl pyrocarbonate-treated water and was quantified by measuring the A₂₆₀. A total of 2 μ g of RNA was run in a 1% agarose gel containing formaldehyde and was subsequently transferred to Nytran membranes using a Turboblotter setup. The RNA was cross-linked to the membrane by UV irradiation, followed by hybridization at 68°C with ³²P-labeled DNA fragments using Quickhyb solution (Stratagene) and visualization by autoradiography. Probes were generated by standard techniques (29): a 52-mer derived from the *micF* sequence (custom synthesized by Operon Technologies Inc.) was end labeled and used as a *micF* probe; the *rob* probe was obtained by labeling PCR-amplified full-length *rob* (see "Construction of the Rob expression vector pMB101" above) using Klenow fragment.

Purification of Rob-His₆ in *E. coli*. The Rob protein with a C-terminal hexahistidine tag (Rob-His₆) was purified as described by Kwon et al. (17). The purified Rob-His₆ protein was assayed for its DNA binding activity by performing band shift assays with a 263-bp DNA fragment that was PCR amplified from the promoter region of *micF* (see below). Native Rob protein (kindly provided by K. Skarstad, University of Oslo, Oslo, Norway) was used as a control.

TABLE 2. Oligonucleotides used in this study

Primer name	Sequence
AslBdir	5'-AGCGCAGCTTGGTAGCGCAACTGG-3'
AslBrev	5'-CGTTGGAACCTGTTGCAGCATGG-3'
C ₁ primer	5'-GTTATAAATTTGGAGTGTGAAGGTTATTGCGTGCGTTAACGCTGCAGCTCATCTAATGCA-3'
C ₀ primer	5'-CGCGGATCCGCATATCATCAGTAGCGATTAGGACG-3'
Galpromdir	5'-TTAGCACCTCTCCGGCCAAACG-3'
Galpromrev	5'-ACCACCGTAACCAGAACTCTC-3'
Marpromdir	5'-GTAAACAAGGATAAAGTGTCACTC-3'
Marpromrev	5'-ATCTTCTTCTGATTAACCATATGG-3'
Mbarb1	5'-GGCCACTAGTCGACTAGTACNNNNNNNNNGATAT-3'
Mbarb2	5'-GGCCACTAGTCGACTAGTACNNNNNNNNNACGCC-3'
Mbarb3	5'-GGCCACTAGTCGACTAGTAC-3'
MdlAdir	5'-TGTCACGGTGGTACCAGAAATGC-3'
MdlArev	5'-TTAATTGAGCAAATAATCGCACG-3'
MicFdir	5'-GCGGGAGTTATTCTAGTTGCG-3'
MicFrev	5'-GTCCGCAAGTCCATTCTCCCC-3'
Muleft	5'-AATAATCCAATGCCTCCCCG-3'
Muleftnest	5'-ACTACAGGCTTCAAGCCCCA-3'
No primer	5'-AGCTTTGATATCGTTTTTAATACGATCCGACAGTTCAATCG-3'
Ni primer	5'-CACGCAATAACCTTACACTCCAAATTTATAACCATAAAAATATCCTCATCCTTTCAACAA-3'
Randomdir	5'-TCGCTGACGGTGACGGTTGAGGCTTCCGCGGAAGGTATCCACAGACGTTGATACTGG-3'
Randomrev	5'-CCAGTATCAACGTCGTGGATACCTTCCGCGGAAGGCTCAAACCGTCACCGTCAGCGA-3'
RobHd3Fwd2	5'-TGTCGAAAGCTTGTGTTGAAAGGATGAGG-3'
RobBamrev	5'-ATTAGAGGAGCCGACGCGTTAACACGCG-3'
YbaOdir	5'-GTTAGTCAGCGTCGGTAGCGG-3'
YbaOrev	5'-CACGACAATTTATTCTGTGCGG-3'

EMSA. Promoter fragments of the *mar* operon, the *gal* operon, *aslB*, *ybaO*, and *mdlA* were amplified by PCR with *E. coli* GC4468 genomic DNA as the template by using the following respective primer sets (see Table 2): Marpromdir and Marpromrev; Galpromdir and Galpromrev; AslBdir and AslBrev; YbaOdir and YbaOrev; MdlAdir and MdlArev. MicFdir and MicFrev were used to PCR amplify the *micF* promoter region. These PCR-amplified fragments encompassed the (predicted) start of transcription and the 240- to 260-bp regions upstream of the respective genes. The gel-purified PCR fragments were 5' end labeled with [γ -³²P]ATP. DNA binding reaction mixtures (20 μ l) contained 10 mM Tris-HCl (pH 8.0), 75 mM KCl, 10% (vol/vol) glycerol, 1 fmol of a ³²P-labeled DNA fragment, and different amounts of Rob-His₆ protein as indicated. All electrophoretic mobility shift assays (EMSA) were performed using Rob-His₆ protein. In control assays, the binding affinity of Rob-His₆ protein to the *micF* promoter region was compared to that of native Rob protein, and the apparent binding affinity for both proteins was confirmed to be the same. Where indicated, competitor DNA was added in 1:10 or 1:100 molar ratios over the probe DNA. Double-stranded, nonspecific competitor DNA was obtained by annealing 60-mer oligonucleotides containing no similarities to previously reported Rob binding sites (Randomdir and Randomrev [Table 2]). As competitor DNA, we used the double-stranded 35-mer *micF* promoter site 1, containing a known Rob binding site (5'-GTATTTGACAGCACTGAATGTCAAAACAAAACCTT-3', kindly provided by H. J. Kwon, Harvard Medical School, Boston, Mass.). The binding reaction mixtures were incubated at room temperature for 15 min and then subjected to electrophoresis in 6% nondenaturing polyacrylamide gels (0.5 \times Tris-borate-EDTA [TBE]) at 200 V for 2 to 3 h. The gels were dried and visualized by autoradiography.

RESULTS

To evaluate whether Rob controls the expression of proteins in *E. coli*, we compared the total cellular protein of the *E. coli* wt strain GC4468 and Δ *rob* strain RA4468 in a pulse-labeling experiment. We observed at least five protein bands on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel with higher intensities in the wt strain than in the Δ *rob* strain in late-exponential-phase cultures (data not shown), indicating that several genes in *E. coli* are, directly or indirectly, controlled by Rob.

Regulated Rob expression from plasmid pMB101. To identify Rob-regulated genes, a MudJ-*lacZ* transposon library was created in strain MB4468 (Δ *rob*/pMB101). Plasmid pMB101 allows for IPTG-inducible activation of the expression of Rob protein under the control of the *lac* promoter (see Materials

and Methods). To test the transcriptional activation of the *rob* gene from pMB101 in the Δ *rob* strain MB4468, we used Northern analysis to compare *rob* mRNA expressed from pMB101 with the native *rob* mRNA in wt *E. coli* GC4468. The expression of *rob* from pMB101 was observed at IPTG concentrations as low as 0.05 mM, with mRNA levels exceeding those of wt *E. coli* (Fig. 1A). The *rob* message from pMB101 was slightly longer than the wt message, due to the displaced transcriptional start site provided by the *lacZ* promoter. The untransformed Δ *rob* strain showed no detectable *rob*-specific mRNA (Fig. 1A).

To test whether Rob expressed from pMB101 could activate transcription *in vivo*, we measured the transcriptional induction of the *inaA1::lacZ* fusion in *E. coli* MB9701 containing pMB101 at different IPTG concentrations in LB broth (Fig. 1C) and evaluated the blue coloration on LB plates containing different IPTG concentrations after overnight incubation. The visual discrimination between uninduced and induced replicas of *E. coli* MB9701/pMB101 grown on plates was optimal at 0.1 mM IPTG. This concentration of IPTG resulted consistently in a 1.5-fold increase in β -galactosidase activity from the *inaA1::lacZ* fusion (Fig. 1C) and was used for screening for MudJ insertions responsive to Rob.

Isolation of Rob-regulated insertion mutants. To identify genes regulated by Rob, we screened a library of random *lacZ* transcriptional fusions (generated by MudJ) for differential expression in the presence and absence of Rob. After 11,000 MudJ insertion mutants were screened on plates, 109 colonies were further analyzed in liquid medium, and 20 of these showed ≥ 2 -fold differences in β -galactosidase expression in the presence of 1 mM IPTG. To confirm regulation by Rob in a physiologically relevant setting, the MudJ insertions from these mutants were transferred into the wt strain GC4468 and subsequently into the Δ *rob* strain MB4468 by P1 transduction. Plasmid pMB101 was introduced in the Δ *rob* strains to allow controlled Rob expression.

After assays of strains grown in LB broth, MudJ insertions

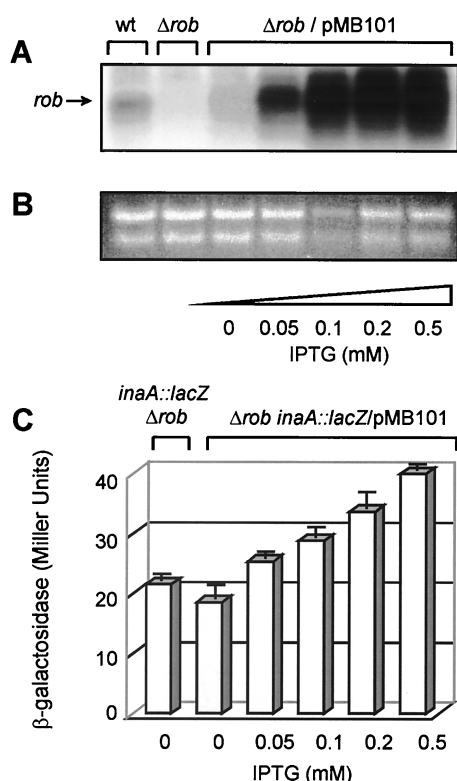


FIG. 1. Regulated *rob* expression. (A) Northern blot of *rob* mRNA. RNA was extracted from cultures of *E. coli* GC4468 (wt), MB4468 (Δrob), or MB4468/pMB101 after exposure to different concentrations of IPTG for 30 min at 37°C. Autoradiograms were exposed for ~8 h. (B) To confirm that equivalent amounts of RNA were loaded, rRNA bands were visualized by ethidium bromide staining. (C) Expression of the *rob*-regulated gene *inaA*. The β -galactosidase activity of *E. coli* MB9701 (Δrob *inaA::lacZ*) containing plasmid pMB101 was quantified as a function of IPTG concentration in the growth medium.

were chosen that showed (i) ≥ 1.5 -fold differences in β -galactosidase activity between the wt and Δrob backgrounds and (ii) ≥ 2 -fold differences in β -galactosidase activity in the Δrob /pMB101 background dependent on IPTG. These criteria were met by 12 MudJ insertions (listed in Table 3). Of these, 11

showed 1.5- to 2.2-fold higher levels of β -galactosidase activity in the wt background than in the Δrob background, indicative of Rob-induced expression. Significantly higher expression of all these insertions was also observed after IPTG induction of Rob from pMB101, ranging from 2.8- to 19.5-fold (Table 3). A single insertion (*mb63*) showed lower β -galactosidase activity in the wt than in the Δrob background (1.5-fold), indicating negative regulation. Rob repression of this fusion in the Δrob /pMB101 background was 2.3-fold (Table 3).

Identities of the Rob-regulated genes. To identify the genetic loci of the 12 Rob-regulated MudJ insertions, the DNA sequences of the junctions between the 5' end of MudJ and the DNA proximal to the fusion junction were determined and compared with the *E. coli* genomic DNA sequence (6). The positions of the MudJ insertions are shown schematically in Fig. 2.

Insertions *mb46* and *mb107* both mapped to *inaA*, a gene that encodes a weak-acid-inducible protein of unknown function (36). The *mb12* insertion mapped to the 3' end of the first gene of the *marRAB* operon, *marR*. The multiple antibiotic resistance (*mar*) locus controls an adaptive response to antibiotics and other environmental stresses (reviewed in reference 2). Both *inaA* and *marRAB* are known Rob-regulated genes (5, 21). The other nine insertions mapped to genes that were not known to be regulated by Rob. The *mb63* insertion was located in the second member of the *galETKM* operon, *galT*, encoding galactose-1-phosphate uridylyltransferase (1). Interestingly, the *galT* insertion was the only Rob-repressed gene identified in this study (Table 3). The *mb38* insertion was located in *aslB*, encoding a potential positive regulator of arylsulfatase (6, 23). Insertions *mb17* and *mb55* were both located in *ybaO*. This gene codes for a predicted protein that has a strong similarity to leucine-responsive regulatory protein (Lrp) from *E. coli* and to Lrp homologs in several other bacteria (25, 34). Insertion *mb83* mapped in *mdlA*, directly downstream of *ybaO*. The *mdlA* gene encodes a protein related to the ATP-binding component of a multiple drug resistance transport system (3, 6). Insertions *mb33*, *mb41*, and *mb48* all mapped to the *yfhD* gene. This gene codes for a predicted protein that has been annotated as a putative periplasmic binding transport protein (6). The *mb108* insertion was located directly upstream of the *ybiS* open reading frame, which has no known function or homolog.

The positions of the insertions downstream from the start

TABLE 3. β -Galactosidase activities of Rob-dependent promoter-*lacZ* fusions in MudJ mutants grown in rich medium^a

Insertion	Insertion site	β -Galactosidase activity (Miller units) in LB broth in the indicated background					
		<i>rob</i> ⁺ (wt)		Expression ratio	Δrob /pMB101		
		<i>rob</i> ⁺ (wt)	Δrob		Uninduced	Induced	Expression ratio
<i>mb46</i>	<i>inaA</i>	14.4 \pm 1.3	7.6 \pm 0.3	1.9	10.3 \pm 0.4	87.7 \pm 1.2	8.5
<i>mb107</i>	<i>inaA</i>	19.9 \pm 1.8	10.1 \pm 0.2	2.0	19.5 \pm 1.0	134 \pm 5	6.9
<i>mb12</i>	<i>marR</i>	111 \pm 6	45.3 \pm 0.6	2.5	112 \pm 3	314 \pm 21	2.8
<i>mb63</i>	<i>galT</i>	516 \pm 16	763 \pm 34	1.5 (-) ^b	628 \pm 28	275 \pm 25	2.3 (-)
<i>mb38</i>	<i>aslB</i>	2.6 \pm 0.4	1.2 \pm 0.4	2.1	2.2 \pm 0.1	33.0 \pm 0.5	14.8
<i>mb17</i>	<i>ybaO</i>	3.4 \pm 0.3	1.5 \pm 0.02	2.2	2.6 \pm 0.4	49.9 \pm 3.1	19.5
<i>mb55</i>	<i>ybaO</i>	1.9 \pm 0.2	1.0 \pm 0.3	1.9	1.2 \pm 0.3	17.9 \pm 1.3	15.2
<i>mb83</i>	<i>mdlA</i>	11.0 \pm 0.9	7.5 \pm 0.3	1.5	12.3 \pm 0.3	38.1 \pm 0.9	3.1
<i>mb33</i>	<i>yfhD</i>	17.5 \pm 1.5	9.5 \pm 0.6	1.8	17.1 \pm 0.3	79.0 \pm 4.9	4.6
<i>mb41</i>	<i>yfhD</i>	11.5 \pm 0.8	6.4 \pm 0.3	1.8	11.4 \pm 0.5	50.8 \pm 2.2	4.5
<i>mb48</i>	<i>yfhD</i>	15.3 \pm 0.1	8.1 \pm 0.2	1.9	15.5 \pm 0.6	53.4 \pm 4.2	3.4
<i>mb108</i>	<i>ybiS</i>	49.2 \pm 2.4	30.3 \pm 1.8	1.6	47.9 \pm 1.6	142 \pm 3	3.0

^a Strains were grown in LB medium (overnight at 37°C), subcultured (1/100), grown to an OD₆₀₀ of 0.5, and incubated for an additional 1 h prior to sampling. Strains carrying pMB101 were grown to an OD₆₀₀ of 0.5, when IPTG (1 mM) was added to the cultures. Incubation was continued for 1 h, and samples were taken. Values represent averages of three different measurements, and standard deviations are indicated.

^b For the *mb63* insertion (*galT*), (-) indicates negative control by Rob.

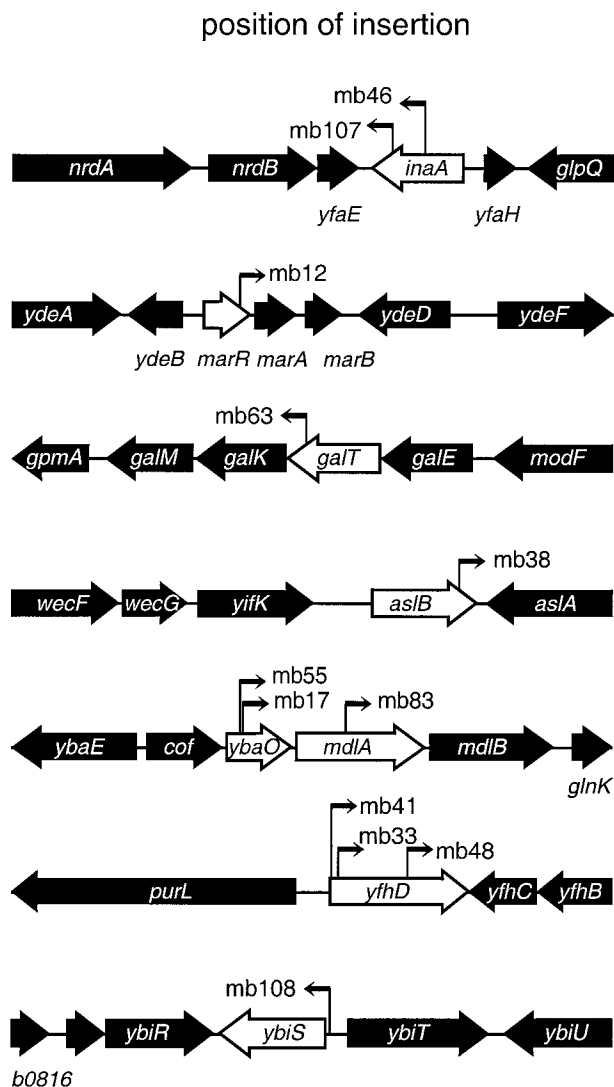


FIG. 2. Locations of insertions of transcriptional fusions of MudJ containing promoterless *lacZ* in Rob-regulated genes identified in this study. Arrows indicate the direction of transcription of genes and of *lacZ*.

codons were as follows: *inaA* (651 bp), insertion *mb46* at bp 572, *mb107* at bp 350; *marR* (378 bp), insertion *mb12* at bp 304; *galT* (1,047 bp), insertion *mb63* at bp 812; *aslB* (1,236 bp), insertion *mb38* at bp 1004; *ybaO* (546 bp), insertion *mb17* at bp 176, *mb55* at bp 174; *mdlA* (1,773 bp), insertion *mb83* at bp 921; *yfhD* (1,419 bp), insertion *mb33* at bp 119, *mb41* at bp 57, *mb48* at bp 891; *ybiS* (921 bp), insertion *mb108* at 31 bp upstream of start codon *ybiS* and 178 bp upstream of start codon *ybiT*.

As indicated above, metabolic labeling of late-exponential cultures of *E. coli* showed at least five protein bands with higher intensities in the wt strain than in the Δrob strain, with M_r s of approximately 30,000, 35,000, 55,000, 60,000, and 115,000 (data not shown). These values were compared with the predicted masses of the proteins encoded by the Rob-regulated genes and operons identified in this study and with those of proteins encoded by genes known to be induced by Rob in vivo (5) or in vitro (15, 32), namely, InaA (25.3 kDa), the *mar* operon products (13.9 kDa [MarR], 15.4 kDa [MarA], and 7.5 kDa [MarB]), the *gal* operon products (37.3 kDa [GalE], 39.6

kDa [GalT], 41.4 kDa [GalK], and 38.2 kDa [GalM]), AslB (46.6 kDa), YbaO (20.9 kDa), the *mdl* operon products (66.0 kDa [MdlA] and 65.2 kDa [MdlB]), YfhD (53.2 kDa), YbiS (33.3 kDa), AcrA (42.2 kDa), AcrB (114 kDa), Fpr (27.8 kDa), FumC (50.5 kDa), Nfo (31.5 kDa), SodA (23.1 kDa), and Zwf (55.7 kDa).

Either the M_r 30,000 or the M_r 35,000 protein band may correspond with the Rob protein, which has a predicted mass of 33.1 kDa, or with proteins encoded by *ybiS* (33.3 kDa), *fpr* (27.8 kDa), or *nfo* (31.5). The band with an M_r of ~55,000 could correspond with proteins encoded by *yfhD* (53.2 kDa) or *zwf* (55.7 kDa); however, previous studies showed no in vivo induction of *zwf* by Rob (5). The band with an M_r of ~60,000 could correspond with MdlAB (66.0 and 65.2 kDa). In this study, we did not identify a Rob-regulated gene encoding a protein with an M_r of ~115,000, but this band might correspond with AcrB (114 kDa). No candidate proteins corresponding to proteins encoded by the *mar* operon, *inaA*, *ybaO*, *fumC*, *sodA*, or *acrA* were observed.

Expression of Rob-dependent genes in minimal medium. We determined the effect of Rob on transcription of the MudJ insertions in minimal medium. The Rob-dependent expression ratios in wt compared with Δrob backgrounds were significantly lower in M9 medium than in LB broth for all fusions except for *ybiS*, dropping below 1.5-fold for the following insertions: *mb107* (*inaA*), *mb63* (*galT*), *mb83* (*mdlA*), *mb33* and *mb48* (*yfhD*), and *mb108* (*ybiS*) (Table 4). Furthermore, the inducibility of the transcriptional *lacZ* fusions in response to Rob induction in the Δrob /pMB101 background was reduced: induction levels of the *inaA*, *aslB*, *ybaO*, *mdlA*, and *yfhD* fusions were, respectively, 2-, 7-, 2.5-, 1.5-, and 2-fold lower in M9 medium than in LB broth (Table 3 versus Table 4). The expression of the fusion directly upstream of *ybiS* remained unchanged in the two different media, while no Rob-mediated repression of the *galT* fusion was observed in M9 medium (Table 4). These results indicated that Rob-dependent effects on gene expression are strongly influenced by the growth medium.

Solvent sensitivity of insertion mutants. Since Rob controls basal resistance to organic solvents (35), the MudJ insertion mutants might have disrupted genes that contribute to this solvent resistance. To test this hypothesis, we determined the sensitivities of *E. coli* GC4468 (wt), MB4468 (Δrob), and the 12 Rob-dependent insertion mutants to *n*-hexane and to a 1:1 mixture of *n*-hexane-cyclohexane. Plating of the strains under *n*-hexane resulted in a 10^4 -fold-decreased survival rate of the Δrob strain compared with that of the unexposed control. *n*-Hexane did not affect the survival of the wt or the insertion mutants. When the strains were exposed to the mixture of *n*-hexane and cyclohexane, an $\sim 10^6$ -fold reduction in colony-forming ability was observed for the Δrob strain, whereas the wt and all the insertion mutants showed only 10^4 -fold reductions in plating efficiency. Thus, disruptions of the individual genes identified in this study may not be sufficient to affect sensitivity to solvents.

Direct binding of Rob to target genes. The regulatory effect of Rob on the various MudJ insertions could be either direct or indirect, through another regulator, such as MarA. We tested this point by using EMSA to assay in vitro binding of Rob to the promoter regions of genes identified in this study. To establish specific binding of Rob protein to the DNA fragments, we used a double-stranded DNA fragment lacking known Rob binding sites (Randomdir plus Randomrev; see Table 2) as a nonspecific competitor DNA and the *micF* promoter (double-stranded 35-mer *micF* promoter site 1; see Materials and Methods) as a specific competitor DNA. In vitro

TABLE 4. β -Galactosidase activities of Rob-dependent promoter-*lacZ* fusions in MudJ mutants grown in minimal medium^a

Insertion	Insertion site	β -Galactosidase activity (Miller units) in M9 minimal medium supplemented with 0.4% glucose in the indicated background					
		<i>rob</i> ⁺ (wt)	Δrob	Expression ratio	$\Delta rob/pMB101$		
					Uninduced	Induced	Expression ratio
<i>mb46</i>	<i>inaA</i>	10.0 \pm 0.3	6.5 \pm 0.2	1.6	7.0 \pm 0.4	27.4 \pm 0.4	3.9
<i>mb107</i>	<i>inaA</i>	12.6 \pm 0.5	9.6 \pm 0.6	1.3	9.6 \pm 1.3	31.6 \pm 1.4	3.3
<i>mb12</i>	<i>marR</i>	ND ^b	ND	ND	ND	ND	ND
<i>mb63</i>	<i>galT</i>	12.3 \pm 1.4	14.9 \pm 0.8	1.2 (-) ^c	14.6 \pm 1.0	12.8 \pm 1.1	0.9
<i>mb38</i>	<i>aslB</i>	2.0 \pm 0.3	1.2 \pm 0.1	1.7	1.7 \pm 0.2	3.2 \pm 0.4	1.9
<i>mb17</i>	<i>ybaO</i>	1.1 \pm 0.1	0.6 \pm 0.05	1.9	0.9 \pm 0.2	7.5 \pm 0.5	7.9
<i>mb55</i>	<i>ybaO</i>	1.0 \pm 0.1	0.4 \pm 0.1	2.2	0.6 \pm 0.1	3.4 \pm 0.8	5.5
<i>mb83</i>	<i>mdlA</i>	5.4 \pm 0.03	4.9 \pm 0.2	1.1	13.7 \pm 0.5	30.8 \pm 1.1	2.2
<i>mb33</i>	<i>yfhD</i>	11.0 \pm 1.5	8.7 \pm 0.4	1.3	9.5 \pm 0.7	24.4 \pm 0.7	2.6
<i>mb41</i>	<i>yfhD</i>	8.5 \pm 2.0	5.3 \pm 0.4	1.6	6.2 \pm 0.7	17.0 \pm 2.7	2.7
<i>mb48</i>	<i>yfhD</i>	9.3 \pm 1.6	6.9 \pm 0.5	1.4	8.5 \pm 1.3	20.8 \pm 0.9	2.5
<i>mb108</i>	<i>ybiS</i>	12.6 \pm 0.8	12.2 \pm 0.3	1.0	43 \pm 2	109 \pm 8	2.5

^a Strains were grown in M9 minimal medium containing 0.4% glucose (overnight at 37°C), subcultured (1/100), grown to an OD₆₀₀ of 0.5, and incubated for an additional 1 h prior to sampling. Strains carrying pMB101 were grown to an OD₆₀₀ of 0.5, and IPTG (1 mM) was added to the cultures. Incubation was continued for 1 h, and samples were taken. Values represent averages of three different measurements, and standard deviations are indicated.

^b ND, not determined.

^c For the *mb63* insertion (*galT*), (-) indicates negative control by Rob.

binding of Rob to the *micF* promoter has been demonstrated previously (5, 19), and Rob activates the transcription of *micF* in vitro (15). Here, the relevance of Rob-dependent expression of *micF* was further established by Northern blotting of *micF* mRNA (Fig. 3), which showed a higher *micF* mRNA level in the wt than in a Δrob mutant.

The concentrations of Rob required to form complexes with the different DNA fragments were determined by assaying Rob-His₆ in a series of EMSA experiments (data not shown). These experiments showed that the fragments with the highest apparent affinity were the *mar* fragment, which produced DNA-protein complexes at Rob-His₆ concentrations of 1.5 to 3 nM, and the *micF* (control) and *ybaO* fragments, both of which showed binding at 3 to 5 nM Rob-His₆. For the remaining fragments, binding was observed at Rob-His₆ concentrations of \geq 9 nM: 9 nM for the *gal* fragment, 13.5 nM for the *aslB* fragment, and 11 nM for the *mdlA* fragment. The ~250-bp sequence directly upstream of the *mdlA* gene has low affinity for Rob. Even though this DNA region contains a putative Rob-binding site, this region is probably not involved in Rob-activated transcription: The *ybaO* and *mdlA* genes are annotated by Blattner et al. (6) as part of a predicted operon and are probably transcribed from a common promoter upstream of *ybaO*.

Results of EMSA of the selected DNA fragments with Rob-His₆ are shown in Fig. 4. Multiple protein-DNA complexes were observed with the *gal*, *aslB*, and *mdlA* probes in our experiments but not with the *mar* and *ybaO* fragments (Fig. 4). It has been shown previously that Rob forms multiple complexes with *micF* and other promoters, probably as a result of the presence of multiple, independent Rob-binding sites (5, 19). The addition of nonspecific competitor DNA in a 100-fold molar excess (Fig. 4, lanes 4) did not eliminate binding of Rob to the different probes (Fig. 4, lanes 2). The strongest effect of the nonspecific competitor was in the *gal* promoter fragment, which nonetheless showed some binding to Rob-His₆ even with a 100-fold excess of nonspecific competitor (Fig. 4B). These results indicate that the observed complexes are not due to nonspecific DNA-protein interactions. When *micF* competitor DNA (35-mer) was present at a 100-fold molar excess, binding to all the fragments was strongly diminished or eliminated (Fig.

4, lanes 5 and 6 versus lanes 2). For the *gal* promoter fragment, the *micF* competitor was much more effective than the non-specific competitor (Fig. 4B). The *mar* fragment showed the most effective resistance to competition by the *micF* fragment: even at a 100-fold excess of this competitor, some residual *mar* promoter-Rob-His₆ complex was still observed (Fig. 4A, lane 6).

DISCUSSION

Rob protein contains a domain strongly related to the SoxS and MarA proteins, which is conserved in the entire AraC-

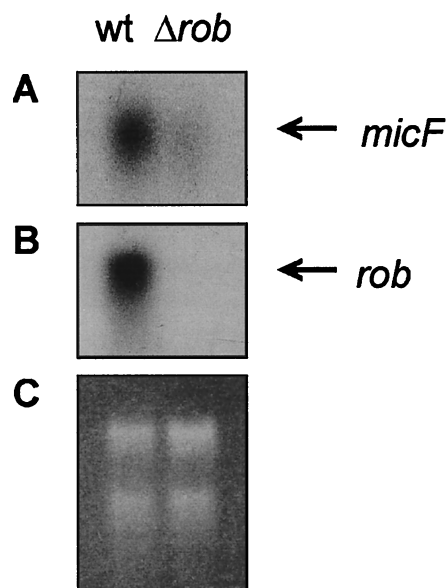


FIG. 3. Expression of *micF* depends on *rob*. Total RNA was extracted from cultures of *E. coli* GC4468 (wt) and MB4468 (Δrob) that were grown in LB medium to an OD₆₀₀ of 0.6 at 37°C. RNA was hybridized with probes specific for *micF* (A) and *rob* (B). Autoradiograms were exposed for ~20 h. To confirm that equivalent amounts of RNA were loaded, rRNA bands were visualized by ethidium bromide staining (C).

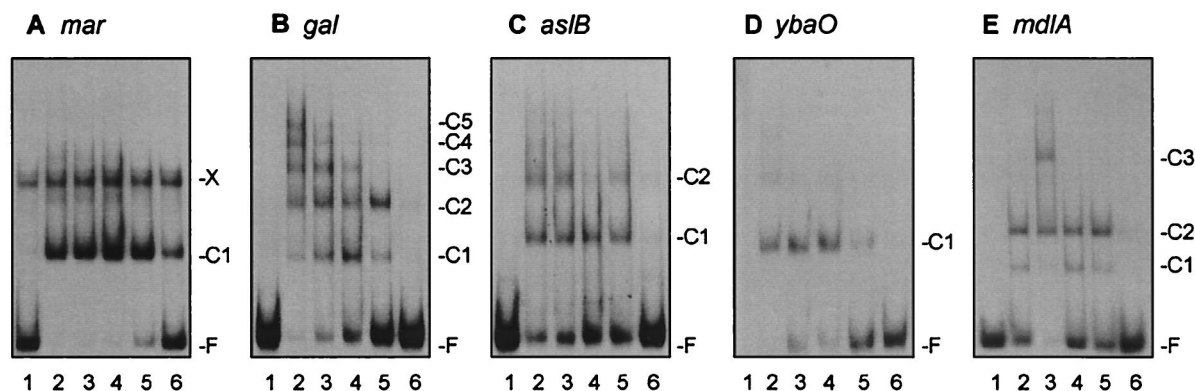


FIG. 4. Binding of Rob-His₆ to the promoter regions of Rob-regulated genes. F, free probe; C1 through C5, DNA-Rob-His₆ complexes; X, contaminating labeled DNA strand. Lanes 1, probe alone (no protein); lanes 2 through 6, Rob-His₆ (see below for amounts) with no competitor (lanes 2), a 10-fold excess of nonspecific competitor (lanes 3), a 100-fold excess of nonspecific competitor (lanes 4), a 10-fold excess of specific competitor (lanes 5), or a 100-fold excess of specific competitor (lanes 6). (A) Rob-His₆ (3 nM) binding to *mar* promoter DNA; (B) Rob-His₆ (9 nM) binding to *gal* promoter DNA; (C) Rob-His₆ (13.5 nM) binding to *aslB* promoter DNA; (D) Rob-His₆ (3 nM) binding to *ybaO* upstream DNA region (free probe of lane 1 ran off gel); (E) Rob-His₆ (11 nM) binding to *mdlA* upstream DNA region.

XylS family of transcriptional regulators (12). Although Rob is expressed constitutively in *E. coli* (30), its biological function has remained obscure. We have now shown that the normal expression of several proteins depends on the *rob* gene. Furthermore, using transposon mutagenesis, we have identified eight genes under Rob control, including six not previously connected to Rob, MarA, or SoxS.

Our analysis revealed that *inaA*, *marRAB*, *aslB*, *ybaO*, *mdlA*, *yfhD*, and *ybiS* were transcriptionally activated by constitutive levels of Rob, while *galT* expression was repressed. Rob contributes significantly to the expression of the *marRAB* operon, which regulates the intrinsic resistance of *E. coli* to various antibiotics, bactericidal agents, and organic solvents (5, 10, 13). Our finding that 60% of *mar* expression depends on the presence of Rob agrees with previous observations that ~65% of *mar* transcription depends on Rob, and that *mar* transcription can be further induced by Rob overexpression (21). Similarly, the demonstrated dependence of *inaA* on Rob is consistent with previously reported transcriptional activation of *inaA* by Rob overexpression (5). Other genes reported to be activated by Rob in vitro (*fpr*, *fumC*, *micF*, *nfo*, *sodA*, and *zwf*) (15) or by Rob overexpression in vivo (*fumC*, *inaA*, *micF*, and *sodA*) (5) were not found in our study; thus, it appears that the mutagenesis and screening procedure was not saturating.

Among functions that could contribute to cellular resistance to environmental agents, Rob enhances expression of *mdlA*, which encodes a multiple-drug-resistance-like ATP-binding component of a transport system (3), and strongly enhances expression of *micF*, the antisense RNA that downregulates the outer membrane porin OmpF. The protein encoded by *yfhD*, predicted to be involved in periplasmic transport (6), could also be involved in cellular resistance. The role of Rob in basal expression of *marRAB*, *mdlA*, and *micF* was further substantiated by in vitro binding assays of Rob to the respective promoter regions of these genes, where Rob showed the highest apparent binding affinity to the *marRAB* and *micF* promoter fragments. It is likely that the increased antibiotic resistance observed after overexpression of Rob is, at least in part, mediated through activation of these genes. The fact that insertions in the individual genes do not affect solvent sensitivity suggests either that multiple genes are involved in this phenotype or that other Rob-regulated genes are important. A good candidate that was not identified in this study is *acrAB*, which encodes an efflux pump that can contribute to Rob-, MarA-, and SoxS-induced organic solvent resistance (32, 35).

Several Rob-regulated genes, such as *marA*, noted above, seem to encode other regulatory proteins. Rob overproduction elicited the strongest transcriptional activation of *ybaO* and *aslB* (15- to 20-fold). The *ybaO* locus encodes a predicted protein of 181 amino acids with strong similarity to *E. coli* leucine-responsive regulatory protein (Lrp; 33% identity over 150 residues). Lrp is a global regulator of various operons (25, 34). The *ybaO*-encoded protein is most closely related to another Lrp homolog: the glutamate uptake regulatory protein (Grp) of *Zymomonas mobilis* (44% identity over 150 residues) (26). The *aslB* gene encodes a putative regulator of the *E. coli* arylsulfatase gene (*aslA*). Homologous systems include *Klebsiella pneumoniae* AtsB (which is involved in posttranslational activation of the arylsulfatase AtsA) (31), *Klebsiella aerogenes* AtsB (controlling arylsulfatase) (23), and ChuR from *Bacteroides thetaiotaomicron* (controlling chondro-6-sulfatase) (8). Rob could thus affect the expression of many more genes indirectly, through its effects on other regulatory proteins.

In this study, *galT* was the only gene repressed by Rob. This gene is part of the *galETKM* operon, encoding galactokinase (*galK*), galactose-1-phosphate uridylyl transferase (*galT*), UDP-galactose 4-epimerase (*galE*), and galactose mutarotase (*galM*). These enzymes are required in galactose metabolism and mediate the conversion of D-galactose to glucose-1-phosphate. Furthermore, these enzymes play an essential role in the production of galactosyl units needed for the biosynthesis of complex carbohydrates in glycoproteins, glycolipids, and the cell wall (1, 11). Transcription of the *gal* operon involves two promoters and operators and can be repressed by GalR (1). For complete repression of both *gal* promoters, DNA looping is reported to be essential (9). We demonstrated that Rob forms multiple complexes with the *gal* promoter in vitro, suggesting the existence of multiple Rob binding sites. The observed Rob-dependent *galT* repression might be related to facilitating the looping of the DNA in conjunction with GalR. Structural aspects of Rob (see below) suggest that this connection merits further investigation.

While normal expression of Rob contributes to the basal expression of the genes identified in this study, it is possible that this group of genes plays a more prominent role in an undiscovered cellular response that activates Rob. We have recently elucidated the crystal structure of Rob complexed with a fragment of the *micF* promoter, which reveals that the C-terminal domain of Rob contains a region that is highly homologous to the ligand binding region of the *galT*-encoded

galactose-1-phosphate uridylyl transferase (17). Thus, it is tempting to speculate that the C-terminal region of Rob is involved in binding an effector molecule that regulates Rob activity. We have not yet identified possible ligands for this domain of Rob.

Rob clearly contributes to the expression of genes with a broad range of functions. The effects on basal expression levels by Rob are modest, but there is a possibility that Rob-induced gene expression might be enhanced upon binding of an effector molecule to the C-terminal region of Rob. Additional effects on the expression of many genes may occur through Rob-mediated activation of other regulatory proteins (MarA, AslB, and YbaO). In this capacity, Rob would have a truly global influence on gene expression in *E. coli*.

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