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Posttranscriptional Activation of the Transcriptional Activator Rob by Dipyridyl in *Escherichia coli*

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The transcriptional activator Rob consists of an N-terminal domain (NTD) of 120 amino acids responsible for DNA binding and promoter activation and a C-terminal domain (CTD) of 169 amino acids of unknown function. Although several thousand molecules of Rob are normally present per *Escherichia coli* cell, they activate promoters of the *rob* regulon poorly. We report here that in cells treated with either 2,2′- or 4,4′-dipyridyl (the latter is not a metal chelator), Rob-mediated transcription of various *rob* regulon promoters was increased substantially. A small, growth-phase-dependent effect of dipyridyl on the *rob* promoter was observed. However, dipyridyl enhanced Rob's activity even when *rob* was regulated by a heterologous (*lac*) promoter showing that the action of dipyridyl is mainly posttranscriptional. Mutants lacking from 30 to 166 of the C-terminal amino acids of Rob had basal levels of activity similar to that of wild-type cells, but dipyridyl treatment did not enhance this activity. Thus, the CTD is not an inhibitor of Rob but is required for activation of Rob by dipyridyl. In contrast to its relatively low activity in vivo, Rob binding to cognate DNA and activation of transcription in vitro is similar to that of MarA, which has a homologous NTD but no CTD. In vitro nuclear magnetic resonance studies demonstrated that 2,2′-dipyridyl binds to Rob but not to the CTD-truncated Rob or to MarA, suggesting that the effect of dipyridyl on Rob is direct. Thus, it appears that Rob can be converted from a low activity state to a high-activity state by a CTD-mediated mechanism in vivo or by purification in vitro.

Rob is an abundant 289-amino-acid protein originally discovered by virtue of its binding to DNA containing the "right side" of the origin of replication (*oriC*) in *Escherichia coli* (28). Subsequently, it was found that Rob, which has an N-terminal domain (NTD) of 120 amino acids that is highly homologous to the small transcriptional activators MarA and SoxS, is also a transcriptional activator when overexpressed and has DNA-binding, bending, and promoter specificities in vitro that are similar to those of MarA and SoxS (3, 11, 16). For convenience, the dozen or more promoters activated by these proteins are collectively referred to here as the *mar*/*sox*/*rob* regulon even though there are considerable differences among these activators in their abilities to activate particular promoters (see references 3 and 20 and references therein). In spite of these differences, the overexpression of MarA, SoxS, or Rob confers resistance to multiple antibiotics, superoxides, and organic solvents (1, 2, 3, 23, 35).

While the regulation of *marA* and *soxS* is well understood (1, 7), little is known about how *rob* is regulated. Transcription of a *rob*::*lacZ* reporter gene was found to increase severalfold during growth from early log phase to stationary phase, and this was partly dependent on *rpoS* (12). A similar *rpoS* dependency was observed for glucose-limited or phosphate-limited growth in which *rob*::*lacZ* transcription increased ~5-fold (12). Western blot analysis indicated ca. 10,000 molecules of Rob (also called CbpB) per log-phase cell (comparable to the 5,000 molecules per cell estimated from protein purification in reference 28) and a higher concentration of Rob in the smaller stationary-phase cell (30). Thus, Rob is a highly abundant DNA-binding protein throughout the growth cycle.

In spite of this, basal levels of Rob do not seem to be effective in stimulating transcription. Null mutants of *rob* appear to have a normal phenotype under a variety of different growth conditions (12, 28). However, they are somewhat more sensitive to *n*-hexane than the wild type, presumably because they express lower levels of the *mar*/*sox*/*rob* regulon *acrAB*-encoded efflux pump than do wild-type cells (35). Recently, small reductions in transcription from the *mar*/*sox*/*rob* regulon promoters *inaA*, *mar*, and *micF* were found in *rob* null mutants, but other regulon promoters were not affected (20, 34). This indicates that, despite the very high basal amounts of Rob per cell, it is not an effective transcriptional activator. However, when *rob* is overexpressed from a strong promoter on a multicopy plasmid, many of the regulon genes are activated (3). Furthermore, on a molar basis, purified Rob activates the transcription of many regulon promoters in vitro about as well as MarA and half as well as SoxS (11).

The structure of a Rob:*micF* cocrystal shows that the DNA-binding NTD of Rob is very similar to that of MarA, whereas the C-terminal domain (CTD) resembles GalT (13, 24). The NTD is sufficient for DNA binding and transcriptional activation, but the CTD function is not known (3). The Rob CTD is also related by amino acid sequence to the CTDs of other AraC-type regulators, Caf1R and AfrR (3). Interestingly, the crystal structures show that Rob binds the DNA by inserting only one recognition helix into the major groove, whereas MarA inserts two helices into adjacent sections of the major groove (13, 24).

To explore the question of whether Rob activity in vivo can

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TABLE 1. List of selected strains used[a]

Strain, plasmid, or phage	Parent strain	Relevant genotype and/or characteristics	Source or reference
Bacterial strains			
DJ901	GC4468	*soxS*::*kan*	25
M542	GC4468	λ*RS45*:*rob2*::*lacZ kan*	This study
M564	RK5173	*inaA*::*lacZ*	This study
M565	RK12466	*inaA*::*lacZ*	This study
M597	N8461	pTA108	This study
M598	N8461	pTA108:*marA*	This study
M599	N8461	pTA108:*soxS*	This study
M600	N8461	pTA108:*rob*	This study
M794	BL21 (λDE3)	pET15b:*rob*	This study
M808	N8461	pTA108:*rob*Δ(370–867)	This study
M848	N8461	pTA108:*rob*Δ(778–867)	This study
M851	N8461	pTA108:*rob*Δ(643–867)	This study
M853	N8461	pTA108:*rob*Δ(529–867)	This study
M854	N8461	pTA108:*rob*Δ(451–867)	This study
M871	N7840	*mar*Δ *sox-8*::*cat*	32; this study
N7840	GC4468	*mar*Δ Cam^s	25
N7962	N7840	*inaA*::*lacZ*	25
N7969	DJ901	*soxS*::*kan mar*Δ *inaA*::*lacZ*	25
N8461	GC4468	*mar*Δ *sox-8*::*cat rob*::*kan*	This study
N8695	N7840	*mar*::*lacZ*	20
N9082	N7840	*fpr*::*lacZ*	20
N9083	N7840	*fumC*::*lacZ*	20
N9084	N7840	*micF*::*lacZ*	20
N9085	N7840	*nfo*::*lacZ*	20
N9086	N7840	*sodA*::*lacZ*	20
N9087	N7840	*zwf*::*lacZ*	20
RK5173	MC4100		9
RK12466	MC4100	*tonB*::*kan*	9
Plasmids and phage			
pRS551		pBR322 derivative; AmpR	27
pTA108		pSC101 derivative; *lac* promoter; AmpR	31
λRS45		λimm21; KanR	27
P1 cat clr-100		Used for transductions	25

[a] See Materials and Methods for additional strains used.

be increased, the effects of various chemicals on a regulon reporter *inaA*::*lacZ* transcriptional fusion in a *mar sox rob*$^+$ strain were assayed. 2,2′-Dipyridyl and 4,4′-dipyridyl were found to increase the expression of *inaA* >6-fold, primarily by a posttranslational enhancement of Rob activity which requires the CTD of Rob.

MATERIALS AND METHODS

Bacterial strains, growth, and assay of β-galactosidase. All strains were derivatives of *Escherichia coli* K-12 (Table 1) (see reference 25 for parental strains and genetic methods). Overnight cultures of bacteria, grown in Luria-Bertani (LB) broth (pH 7.5) at 37°C were diluted at least 1,000-fold into fresh medium and cultivated with shaking at 200 rpm until their absorbance at 600 nm (A_{600}) reached ~0.07. Samples (0.75 ml) were diluted with equal volumes of prewarmed LB broth containing the tester compound and then aerated for 1 h. The cultures were chilled and assayed for β-galactosidase by using the $CHCl_3$-sodium dodecyl sulfate method. The specific activity is expressed in Miller units as described previously (21). To measure the kinetics of *inaA* activation, 10 ml of log-phase cells was added to 10 ml of prewarmed and aerated LB broth with dipyridyl (Sigma Chemical Co., St. Louis, Mo.) in a 250-ml flask, and 0.75-ml samples were removed at the indicated intervals to 0.75 ml of iced Z buffer (21) and assayed for β-galactosidase. When necessary, the cells were pelleted by centrifugation and resuspended in Z buffer prior to assay. All assays were performed at least twice in duplicate and agreed to within 15%.

Dipyridyl-insensitive *rob* mutants were selected by spreading 200 μl of an overnight culture of strain M600 on LB plates supplemented with 6 mM 4,4′-dipyridyl and incubating the plates at 37°C for 3 to 5 days. Cells that had lost the plasmid (AmpS) or had mutations in the NTD of Rob made pink colonies, whereas those with mutations in the CTD of Rob made red colonies on MacConkey-lactose plates (Difco, Detroit, Mich.) after overnight incubation at 37°C.

DNA manipulations. A *rob* promoter::*lacZ* transcriptional fusion was constructed by amplifying *rob* from the chromosome of N7969 by PCR with the primers 813 (5′-CCATTTTTATGAATTCCACGAGCAATTAGTTCGTCACG G-3′; the *Eco*RI site is underlined) and 814 (CCTTGGATCCAGATTAAAAG GTCGCGAATAATGCCGGCCTGATCC; the *Bam*HI site is underlined), respectively. The 250-bp fragment was digested with *Eco*RI and *Bam*HI and cloned in similarly cut plasmid pRS551 (27), thereby fusing the 192-bp upstream of the *rob* initiation codon and the first 38 bp of the coding sequence to *lacZ*. This transcriptional fusion, called *rob2*::*lacZ*, was transferred to λRS45, and single-copy lysogens were isolated (27).

Plasmid pTA108 is an AmpR, low-copy-number plasmid derived from pSC101 and contains the *lac* operator-promoter region of plasmid pUC8 (31). Derivatives of pTA108 with the *lac* promoter controlling the *marA*, *soxS*, or *rob* structural genes were constructed as follows. The *marA* and *soxS* coding sequences were amplified by PCR from the pRGM9817-based plasmids, pRGM9818 (*marA*) and pJLR70 (*soxS*) (20), with primer 864 (GAAGCTTAACTATGCGG CATCAGAGCACGGATCC; the *Hin*dIII site is underlined) and either primer 865 (AGGAATTCGATGTCCAGACGCAATACTGACGC; the *Eco*RI site is underlined) or primer 866 (AGGAATTCCCATCAGAAAATTATTCAGGAT CTTATCGCATGG), respectively. These fragments were digested with *Eco*RI and *Hin*dIII and ligated to similarly cut plasmid pTA108. The *rob* coding sequence was amplified from the pRGM9817:*rob* plasmid, pRGMM489 (see below), with primers 867 (ACCAATTGTCAGGCCGGCATTATTCGCGACC; the underlined *Mfe*I site precedes the sixth base pair of the *rob* coding sequence) and primer 864 (see above). The fragment was digested with *Mfe*I and *Hin*dIII and ligated to *Eco*RI- and *Hin*dIII-cut plasmid pTA108, resulting in plasmid pRGMM649 (and replacement of the pTA108 *Eco*RI site GAATTC with GAATTG). Accordingly, transcription and translation from the *lac* promoter of

pRGMM649 results in a modified Rob protein with the first five amino acids derived from *lacZ* (Met-Ile-Thr-Asn-Cys) substituted for the first two amino acids of Rob (Met-Asp). The *lac* promoter in the pTA108 plasmids is not repressed in these strains since they have no *lacI* gene. However, the promoter is not very active in the early logarithmic phase or in cells grown in LB broth supplemented with 0.4% glucose due to the absence of cyclic AMP (data not shown).

pRGMM489 plasmid (pRGM9817:*rob*) was constructed by amplification of the *rob* coding sequence from strain N7969 by PCR with primers 821 (AACATATGGATCAGGCCGGCATTATTCGCGACC; the *Nde*I site [underlined] includes the first ATG codon of *rob*) and 822 (AAGGATCCTTAACGACGGATCGGAATCAGCAGTTCACAGCG; the *Bam*HI site adjacent to ochre codon is underlined). The fragment was cut with *Nde*I and *Bam*HI and ligated to similarly cut and phosphatase-treated plasmid pRGM9817. For overexpression and purification of Rob, pRGMM489 was cut with *Nde*I and *Bam*HI and ligated to similarly cut and phosphatase-treated plasmid pET15b (Novagen, Madison, Wis.) to make plasmid pRGMM794. Strain M794 is strain BL21(λDE3) transformed with this plasmid.

Deletions of the 3′ end of *rob* were constructed by PCR amplification of pRGMM489 with, as the 5′ primer in each case, primer 871 (GGCTTTACACTTTATGCTTCCGGCTCG) corresponding to bp 145 to 171 of the pTA108 sequence, which lies 65 bp upstream of the site where *rob* is inserted in pRGMM489. The 3′ primers used to make the CTD-truncated *rob* plasmids in the N8461-derived strains M854, M853, M851, and M848 were, respectively, primers 983 (TTTAAGCTTACTCCAGCGAACAGGAGTAGCTCTGGG; *rob* nucleotides [nt] 450 to 427, underlined), 984 (TTTAAGCTTACGGCGGAATGGTCGGCGCGTTGCCGAG; *rob* nt 528 to 502, underlined), 985 (TTTAAGCTTACCCCGTCAGTACATAGCCATCTGCC; *rob* nt 642 to 618, underlined), and 986 (TTTAAGCTTACTGACCTTTACGGCGCGTCAGG; *rob* nt 777 to 756, underlined). The underlined segments correspond to the new positions of the 3′ ends of the *rob* coding sequence with the TTA creating an ochre codon and the AAGCTT creating a *Hin*dIII site. The resulting fragments of 518, 596, 710, and 845 bp were cut with *Eco*RI and *Hin*dIII to yield fragments of 86, 164, 278, and 413 bp, respectively, and were ligated to similarly cut and phosphatase-treated plasmid pRGMM489, thereby substituting the truncated fragments for the wild-type *rob* sequence downstream of the *Eco*RI site. To make the plasmid present in the N8461 derivative, strain M808, the pTA108:*rob* plasmid was deleted back from the 3′ portion of the *rob* coding sequence to the *Eco*RI site at bp 364 and an ochre codon inserted as follows. Plasmid pRGMM489 was treated with *Eco*RI, *Hin*dIII, and phosphatase and ligated to a linker containing an ochre codon made by annealing oligomers 881 (AATTCTAAGGATCCA) and 882 (AGCTTGGATCCTTAG) that had been treated with polynucleotide kinase. DNA oligomers were prepared by using an ABI DNA/RNA Synthesizer, and the sequences were confirmed for all constructs by using an ABI Prism 310 Genetic Analyzer.

Electrophoretic mobility assays were performed as described previously (18). *Eco*RI-to-*Bam*HI fragments containing the minimal promoters (20) of the *fumC* (−56 to +6), *fpr* (−66 to +3), and *mar* (−72 to +3) promoters were ^{32}P end labeled and used as probes with purified Rob protein at final concentrations of 50, 100, and 200 nM (without or with equal amounts of RNAP) in 50 mM Tris (pH 7.5)–20% glycerol–0.1 M NaCl–1 μg of poly(I-C)/ml with or without 5 mM 2,2′-dipyridyl. The samples were subjected to electrophoresis at 90 V on 6% polyacrylamide gels made up in 0.5× Tris-borate-EDTA with or without 5 mM 2,2′-dipyridyl and in electrolyte of the same composition.

Purification of MarA, Rob, and truncated Rob. MarA, Rob, and truncated Rob (amino acids 124 to 289 absent) were purified as His-tagged proteins from strains N8224, M794, and M796, respectively, as described previously (10, 19), and the His tags were removed with thrombin. The truncated Rob was purified from strain M796 by using the scheme for MarA since it was highly insoluble like MarA. To construct M796, plasmid pRGMM794 was digested with *Eco*RI, *Bam*HI, and calf alkaline phosphatase and purified on a Promega PCR column (Madison, Wis.). The digested plasmid was ligated to a fragment consisting of the two kinase-treated oligonucleotides: AATTCTAAG (TTC regenerates the Phe-123 codon and TAA creates the terminator codon) and GATCCTTAG and cloned into the BL21 *ompT* strain from Stratagene (La Jolla, Calif.).

NMR studies. Uniformly ^{15}N-labeled proteins were produced by growing cells in M9 minimal medium containing ^{15}N-labeled ammonium chloride as the sole source of nitrogen. Purification of the proteins was carried out as for the unlabeled material. Each of the proteins, MarA, Rob, and the 166-residue CTD-truncated Rob were complexed with a 26-bp double-stranded DNA containing the sequence for the *mar*-binding site so that they would be soluble at the required concentrations. The complexes were dialyzed against a buffer containing 20 mM sodium phosphate, 20 μM EDTA, and 0.02% sodium azide (pH 6.5) and then concentrated to ~0.5 mM for the nuclear magnetic resonance (NMR) studies. 2,2′-Dipyridyl was added to each of the samples to a final concentration of 5 mM. ^{1}H-^{15}N HSQC spectra (5) were collected for each of the complexes in the presence or absence of 2,2′-dipyridyl. All NMR data were acquired on Bruker DMX-600 or DMX-500 MHz spectrometers at 35°C. NMR data were processed by using the NMRPipe suite of programs (6).

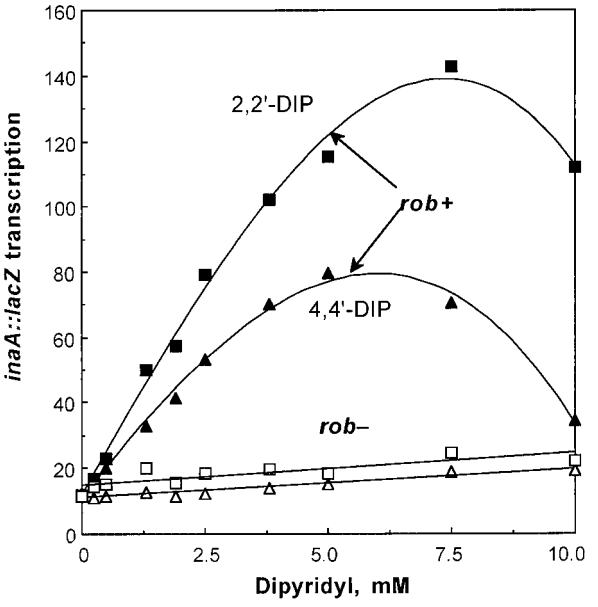


FIG. 1. Effects of different concentrations of dipyridyl on transcriptional activation of *inaA*::*lacZ*. The *mar sox inaA*::*lacZ* strain M871 (solid symbols) and its *rob* mutant derivative N8461 (open symbols) were treated with the indicated concentrations of 2,2′-dipyridyl (squares) or 4,4′-dipyridyl (triangles) for 1 h and assayed for β-galactosidase (Miller units).

RESULTS

Both 2,2′- and 4,4′-dipyridyl activate *inaA* via *rob*. Overexpression of Rob transcriptionally activates a number of genes of the *mar*/*sox*/*rob* regulon including *inaA* (3). To identify compounds that induce the overexpression or activation of Rob, various compounds were tested for their ability to stimulate β-galactosidase synthesis in a *rob*$^{+}$ *mar sox* strain (M871) bearing an *inaA*::*lacZ* transcriptional fusion. The *inaA*::*lacZ* fusion was used as a reporter since it has a low basal level of activity but is highly activated in response to Rob (3, 25, 34). Among these compounds, 2,2′-dipyridyl and 4,4′-dipyridyl increased the levels of β-galactosidase by 19- and 13-fold, respectively (Table 2). 2,2′-Dipyridyl is a potent chelator of iron and has been used at 100 to 200 μM to deplete culture media of iron (see, for example, reference 33). However, the optimal activation of *inaA* was obtained at much higher concentrations than that needed for chelation (Fig. 1). The 4,4′-dipyridyl isomer, which is not a metal chelator, also activated *inaA* but to a lesser extent (Fig. 1). To determine whether *rob* is required for the *inaA* activation, the effects of both 2,2′-dipyridyl and 4,4′-dipyridyl were assayed on strain N8461 (*rob*::*kan mar sox inaA*::*lacZ*), a *rob* mutant version of M871 (Fig. 1). Neither 2,2′-dipyridyl nor 4,4′-dipyridyl substantially activated *inaA* in this strain, showing that Rob is required for the effect. Simi-

TABLE 2. Effects of various compounds on *inaA*::*lacZ* activity in *rob*$^+$ strains[a]

Addition	Concn	β-galactosidase activity (Miller units)	Ratio relative to untreated control[b]
None (M871)[c]		5.0	1.0
Biphenyl + ethanol	194 μM 3%	10.6	2.1
2,2′-Dipyridyl	5 mM	97.7	**19.4**
4,4′-Dipyridyl	5 mM	63.8	**12.7**
2,4-Dinitrophenol	0.5 mM	15.1	**3.0**
2,4-Dinitrophenol	1.0 mM	26.4	**5.3**
Ethanol	3%	6.1	1.2
Ethanol	25%	10.1	2.0
8-Hydroxyquinoline + ethanol	5 mM 25%	7.0	1.4
Naphthalene + ethanol	0.5 mM 0.5%	11.9	2.4
Paraquat	50 μM	10.0	2.0
Pyridine	5 mM	5.6	1.1
Pyridine	10 mM	6.1	1.2
Pyridoxal	5 mM	9.9	2.0
Pyridoxine	5 mM	6.8	1.3
Na salicylate	5 mM	13.4	2.7
None (N7969)[d]		10.8	1.0
Desferroxamine	5 mM	14.9	1.4
2,2′-Dipyridyl	5 mM	84.0	**7.8**
EDDA[f]	5 mM	10.6	1.0
EGTA	5 mM	13.3	1.2
Na salicylate	10 mM	23.9	2.2
Na salicylate	20 mM	42.5	**3.9**
Na salicylate	40 mM	60.2	**5.6**
None (N7969)[d]		6.0	1.0
CCCP	10 μM	5.2	0.9
CCCP	50 μM	6.7	1.1
2,4-Dinitrophenol	0.5 mM	17.1	2.8
2,4-Dinitrophenol	1.0 mM	22.0	**3.7**
EDTA	0.1 mM	6.3	1.1
EDTA	1.0 mM	8.9	1.5
EDTA	10 mM	2.6	0.4
Ferric citrate	7 mM	8.6	1.4
NaN_3	1.0 mM	6.4	1.1
NaN_3	10 mM	6.0	1.0
NaN_3	100 mM	5.1	0.8
Na citrate	7 mM	6.8	1.1
NaF	1.0 mM	6.1	1.0
NaF	10 mM	6.5	1.1
NaF	100 mM	6.9	1.1
None (M564)[e]		33.0	1.0
2,2′-Dipyridyl	5 mM	151.9	**4.6**
$FeSO_4$	Saturated	32.0	1.0
8-Hydroxyquinoline + ethanol	5 mM 25%	13.9	0.4
Nonidet P-40	0.5%	23.0	0.7
Pyridine	5 mM	36.3	1.1
Na acetate	50 mM	19.2	0.6
None (M565)[e]		35.6	1.0
2,2′-Dipyridyl	5 mM	156.6	**4.4**
FeSO4	Saturated	28.4	0.8
8-Hydroxyquinoline + ethanol	5 mM 25%	12.6	0.4
Nonidet P-40	0.5%	17.1	0.5
Pyridine	5 mM	32.9	0.9
Na acetate	50 mM	19.5	0.5

[a] The indicated log-phase *inaA*::*lacZ* cells were incubated with the indicated compounds in LB broth for 1 h at 37°C and assayed for β-galactosidase activity.
[b] Ratios of ≥3.0 are indicated in boldface.
[c] The *mar sox* strain M871 was used.
[d] The *mar sox* strain N7969 was used.
[e] Strains M564 and M565 (*mar*$^+$ *sox*$^+$) have higher basal levels of *inaA*::*lacZ* expression due to *mar* expression (25, 34). In addition, M565 has a *tonB*::*kan* mutation.
[f] EDDA, ethylenediamine-*N*,*N*′-diacetic acid.

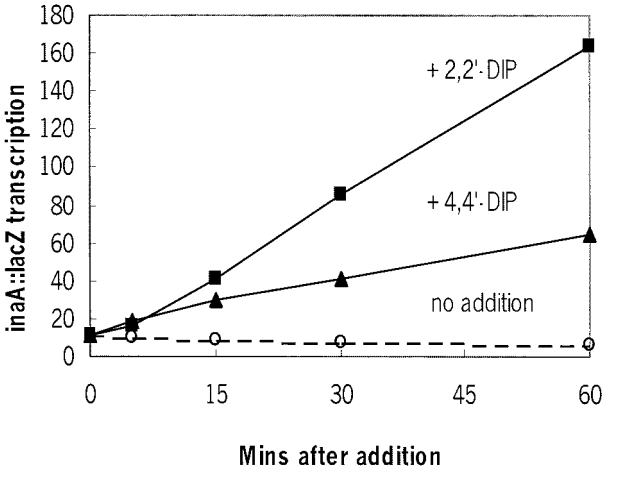


FIG. 2. Kinetics of acccumulation of *inaA*::*lacZ* upon treatment of cells with 0 (○) or 5 mM 2,2′-dipyridyl (■) or 4,4′-dipyridyl (▲). Samples of the *mar sox inaA*::*lacZ* M871 cells treated for the indicated times were rapidly chilled on ice and then assayed for β-galactosidase (Miller units).

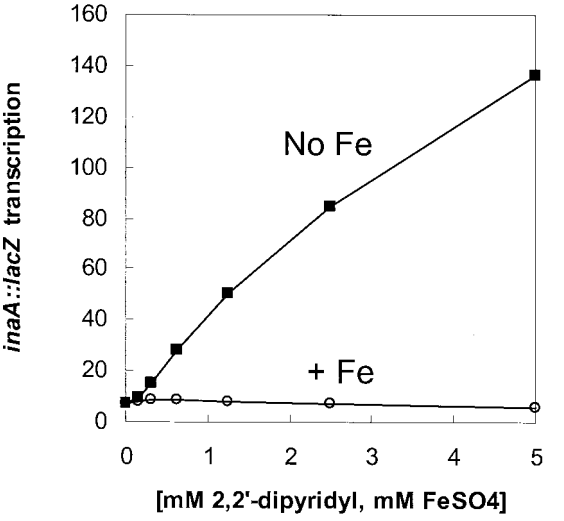


FIG. 3. Effect of Fe on activation of *inaA*::*lacZ* by 2,2′-dipyridyl. The *mar sox rob*$^+$ *inaA*::*lacZ* strain N7969 was treated with the indicated concentrations of 2,2′-dipyridyl (■) or 2,2′-dipyridyl plus equimolar $FeSO_4$ (○) for 1 h in LB broth and assayed for β-galactosidase (Miller units).

larly, *inaA* was not activated by dipyridyl in *rob*::*kan* strains carrying wild-type *marA* and/or *soxS* (data not shown).

The effects of both dipyridyl isomers were fairly rapid (Fig. 2). Within 10 min of addition of 5 mM dipyridyl, *inaA*::*lacZ* activity was elevated and increased linearly for at least 60 min. This increase occurred even though growth was inhibited. The cellular A_{600} value showed that the growth of log-phase cultures treated with 5 mM 2,2′-dipyridyl was severely inhibited: growth slowed within 20 min and increased by <4-fold after overnight incubation. The growth of the 4,4′-dipyridyl-treated cells was less affected: it also slowed within 20 min but increased 20-fold after overnight incubation. Untreated cells grew with a 30-min doubling time and increased 35-fold overnight. *rob* mutants were similarly inhibited by 2,2′- and 4,4′-dipyridyl. Thus, in addition to their effects on Rob activity, both 2,2′- and 4,4′-dipyridyl have *rob*-independent inhibitory effects on growth.

Metal chelation does not activate *inaA*. Since 2,2′-dipyridyl is a powerful chelator of Fe, the possibility was explored that part of the Rob-mediated activation of *inaA* might be due to removal of Fe from the culture. Accordingly, various *rob*$^+$ *inaA*::*lacZ* strains were treated with other chelators, including citrate, EDTA, EDDA, EGTA, and 8-hydroxyquinoline. None were effective in activating *inaA* transcription over the range of concentrations tested (Table 2). Other compounds with some structural resemblance to dipyridyl, such as pyridine, biphenyl, naphthalene, pyridoxal, and pyridoxine had little or no effect on *inaA* expression (Table 2). Inhibitors of cellular energy generation, such as CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), sodium azide, and sodium flouride, were also ineffective, as were a number of other compounds tested. Interestingly, 2,4-dinitrophenol and high concentrations of sodium salicylate, both of which derepress the *mar* operon (4), were somewhat effective in activating *inaA*::*lacZ* in Δ*mar* strains M871 and N7969. *mar*-Independent effects of salicylate have been noted previously (4, 25) and are not further explored here.

TonB is an important component of the Fe uptake systems (for a review, see reference 22). Strain M565, a *mar sox rob inaA*::*lacZ* strain that is defective in Fe uptake because of a *tonB*::*kan* null mutation (15), and its *tonB*$^+$ parent, strain M564, were treated with dipyridyl. No significant effect of the *tonB* mutation was seen (Table 2). Furthermore, neither mutations in *fnr*, *arcA*, or *fur* nor the anaerobic growth of strain N7969 significantly affected the activation of *inaA*::*lacZ* by 2,2′-dipyridyl (data not shown). Finally, the addition of various divalent metals to the cultures did not affect *inaA* transcription (data not shown). Thus, metal chelation is not a relevant aspect of the activation of *inaA* by dipyridyl.

The 2,2′-dipyridyl-iron complex does not activate *inaA*. Nevertheless, since 2,2′-dipyridyl (but not 4,4′-dipyridyl) forms coordination complexes with iron, we asked whether the presence of iron would affect the activity of 2,2′-dipyridyl. Interestingly, the addition of equimolar $FeSO_4$ to 2,2′-dipyridyl completely abolished the activation of *inaA* (Fig. 3). This suggests that, by binding iron, the configuration of 2,2′-dipyridyl is so altered that it is rendered inactive or unable to gain entrance to its cellular target.

To test whether 2,2′- and 4,4′-dipyridyl activate Rob by independent mechanisms, we investigated the effects of the combined isomers on *inaA* transcription. A synergistic effect would suggest that the isomers have independent modes of action. Strain N7969 (*inaA*::*lacZ rob*$^+$) was treated with either a 2.5 mM concentration of both isomers or with a 2.5 mM concentration of only one of the isomers. The β-galactosidase activity in the culture treated with a 2.5 mM concentration of both isomers (97 Miller units) was approximately equal to the total of the separately treated cultures (93 Miller units). This absence of synergy suggests that the two isomers activate Rob via the same mechanism.

Activation of other *mar*/*sox*/*rob* regulon promoters. If the effect of dipyridyl is to increase the expression or activity of

TABLE 3. Activation of *rob* regulon promoters by treatment with dipyridyl[a]

Strain (promoter-*lacZ* fusion)	β-Galactosidase activity (Miller units) with:		Ratio[b]	β-Galactosidase activity (Miller units) with 4,4′-dipyridyl	Ratio[b]
	No added dipyridyl	5 mM 2,2′-dipyridyl			
N9082 (*fpr*)	32	159	4.9	69	2.2
N9083 (*fumC*)	22	417	18.8	248	11.2
N7962 (*inaA*)	9.8	125	12.7	83	8.5
N8695 (*mar*)	344	1,117	3.2	912	2.7
N9084 (*micF*)	39	293	7.5	337	8.6
N9085 (*nfo*)	107	232	2.2	139	1.3
N9086 (*sodA*)	518	1,378	2.7	705	1.4
N9087 (*zwf*)	103	229	2.2	129	1.2

[a] Derivatives of strain N7840 (Δ*mar*) with the indicated single-copy promoter-*lacZ* transcriptional fusions were treated with 5 mM 2,2′- or 4,4′-dipyridyl for 1 h at 37°C and assayed for β-galactosidase.
[b] That is, the ratio of β-galactosidase activities in the cells treated with dipyridyl to that in the untreated cells.

Rob, it should also activate other promoters of the *mar*/*sox*/*rob* regulon, just as the overexpression of Rob on a multicopy plasmid does. This was tested by using appropriate regulon promoter-*lacZ* transcriptional fusions (Table 3). Both 2,2′- and 4,4′-dipyridyl were found to increase the expression of the different regulon promoters, albeit to different extents. The effects were substantial for the *fumC*, *inaA*, and *micF* promoters (8- to 19-fold increases), moderate for *mar* and *fpr*, and modest (2,2′-dipyridyl) or insignificant (4,4′-dipyridyl) for *nfo*, *sodA*, and *zwf*. A similar profile of promoter-specific activation by Rob has been seen when *rob* is overexpressed from a plasmid (in the absence of dipyridyl) (3, 20).

Enhancement of Rob activity by a posttranscriptional mechanism. To determine whether the transcription of *rob* itself is activated by 2,2′-dipyridyl, a strain with a *rob2*::*lacZ* transcriptional fusion was tested. As found previously (12), growth into the stationary phase increased the transcription of *rob* ~3-fold (Table 4). Treatment with 2,2′-dipyridyl in the early log phase reproducibly induced *rob* transcription by 1.5- to 2-fold, but this effect disappeared as cells entered the late log phase. In contrast, the basal level of *inaA* transcription was not affected significantly by growth phase but was increased by treatment with 2,2′-dipyridyl even in stationary phase. This suggested that 2,2′-dipyridyl enhances Rob activity primarily by a nontranscriptional mechanism.

To test this possibility, the *marA*, *soxS*, and *rob* coding sequences were inserted downstream of the *lac* promoter in the low-copy-number plasmid pTA108 (31) so that *lac* and not *rob* provided the promoter and translational signals. These plasmids were introduced into the *rob sox mar* mutant strain N8461, and the effect of 2,2′-dipyridyl on *inaA* transcription was monitored (Table 5). In the absence of 2,2′-dipyridyl, the basal levels of *inaA* found in the low-copy *marA*, *soxS*, or *rob* plasmid-bearing strains were three- to fourfold greater than in the control strain M597. In the presence of 2,2′-dipyridyl, *inaA* expression was further increased 18-fold when the plasmid carried *rob* but not when the plasmid carried *marA* or *soxS*. A similar effect was found when these strains were treated with 4,4′-dipyridyl (data not shown). Thus, the effect of dipyridyl is specific for *rob* and occurs primarily at a posttranscriptional level.

Isolation of Rob CTD mutants that do not respond to dipyridyl treatment. Overexpression of *marA*, *soxS*, or *rob* can be deleterious to the growth of the cell (3; R. G. Martin and J. L. Rosner, unpublished data). We observed that the growth of the pTA108:*rob* strain (M600) was severely inhibited when streaked on LB agar plates containing 6 mM 4,4′-dipyridyl compared to the strain containing only the pTA108 vector (M597) or the single-copy *rob* chromosomal gene (M871). We reasoned that *rob* mutants that were not activated by 4,4′-dipyridyl should survive these conditions. Indeed, faster-growing spontaneous mutants were readily selected on such plates after several days of incubation at 37°C. When purified, three types of dipyridyl-insensitive clones were found: 19 of 37 had lost the pTA108:*rob* plasmid and made pink colonies on MacConkey-lactose plates; 8 of 37 retained the plasmid and made pink colonies on MacConkey-lactose plates but expressed *inaA*::*lacZ* at the very low basal levels typical of strains containing no functional *rob* gene (β-galactosidase activity of *inaA*::*lacZ* of <10 Miller units); and 10 of 37 retained the

TABLE 4. 2,2′-Dipyridyl affects the transcription of *inaA* and *rob* promoters differently depending on the growth phase of the cells[a]

Strain (promoter-*lacZ* fusion)	2,2′-Dipyridyl concn (mM) or ratio[b]	β-Galactosidase activity (Miller units) at various growth phases		
		Early log	Mid log	Stationary
M542 (*rob*)	0	240	469	732
	5	396	507	741
	Ratio	1.7	1.1	1.0
M871 (*inaA*)	0	16	16	20
	5	89	131	119
	Ratio	5.6	8.2	6.0

[a] Cells were grown from small inoculae in LB broth to an A_{600} of 0.07 (early log phase) or 0.5 (mid-log phase) or overnight to an A_{600} of 2.5 (stationary phase). The samples were then diluted with an equal volume of prewarmed 10 mM 2,2′-dipyridyl in LB broth. After aeration for 1 h at 37°C, the β-galactosidase activity was assayed.
[b] That is, the ratio of activities in the cells treated with dipyridyl to that in the untreated cells.

TABLE 5. Effects of 2,2′-dipyridyl on Rob-mediated activation of *inaA*::*lacZ* when Rob is expressed from the heterologous *lac* promoter[a]

Strain	Activator controlled by the *lac* promoter	β-Galactosidase activity (Miller units) with 2,2′-dipyridyl at:		Ratio[b]
		0 mM	5 mM	
M597	None	10	16	1.6
M598	MarA	45	23	0.51
M599	SoxS	30	32	1.1
M600	Rob	33	587	18.0

[a] The *mar sox rob* mutant strains carrying the indicated plasmids were grown to early log phase, treated with 0 or 5 mM 2,2′-dipyridyl for 1 h at 37°C, and assayed for β-galactosidase activity.
[b] That is, the ratio of activities in the cells treated with 2,2′-dipyridyl to that in the untreated cells.

plasmid, made red colonies on MacConkey-lactose plates, and showed slightly higher levels of *inaA* expression (10 to 40 Miller units) but showed no increase in β-galactosidase after treatment with 2,2′-dipyridyl or 4,4′-dipyridyl (ratio of Miller units of treated to untreated cultures of 1.3 ± 0.3). The *rob* genes in three isolates from the latter group were sequenced. Two were found to have the 6-kb transposon Tn*1000* (γδ) (17) inserted in the portion of *rob* that encodes the C-terminal domain: in one (*rob-9*) between the *rob* structural gene from nt 590 to 591 and in the other (*rob-28*) between nt 607 to 608. The third mutant (*rob-13*) contained an in-frame deletion of the *rob* structural gene from nt 560 to 772 and therefore encodes a protein deleted of 71 amino acids within the CTD. Two 1-bp mutants were later identified that have Rob activity but did not respond to dipyridyl: C658T and C709T, which change the Gln-220 and Gln-237 codons, respectively, to UAG amber codons. While we do not have direct evidence that the *rob* mRNA from these amber mutants is normal, it seems unlikely that it is the *rob* mRNA that is responding to the dipyridyl treatment. We tentatively conclude that dipyridyl has a post-translational effect on the Rob CTD.

The role of the CTD was further examined by deletion analysis. The 3′ end of the *rob* coding sequence (bp 867) was deleted back to bp 777, 642, 528, 450, or 369, and a TAA ochre codon was added, thereby creating a new 3′ terminus of translation. These constructs were placed downstream of the *lac* promoter of pTA108 (as in the original pTA108:*rob* plasmid), and the resulting plasmids transformed into the *rob sox mar* *inaA*::*lacZ* strain N8461. The 3′ deletions of *rob* (encoding proteins with C-terminal truncations of 30, 75, 113, 139, and 166 amino acids, respectively) did not significantly lower *inaA*::*lacZ* activity in the absence of dipyridyl compared to strain M600, whose plasmid carries wild-type *rob* (Table 6). Thus, the full-length Rob CTD is not needed for basal Rob activity. Two strains, M851 and M854, had ca. 50% higher basal levels than strain M600. These strains are similar in *inaA*::*lacZ* activity to *rob133*, which encodes the first 123 amino acids of Rob plus 10 amino acids from the vector (3). Thus, while we do not know whether these truncations influence the stability of the protein, it seems likely that the CTD of Rob neither promotes nor inhibits the basal-level activity of Rob to a great extent. Nevertheless, even when the CTD of Rob was truncated by only 30 amino acids, the posttranslational response (measured by *inaA*::*lacZ* activity) to 2,2′- or 4,4′-dipyridyl was abolished. Thus, determinants near the carboxy terminus of Rob are necessary for activation by dipyridyl.

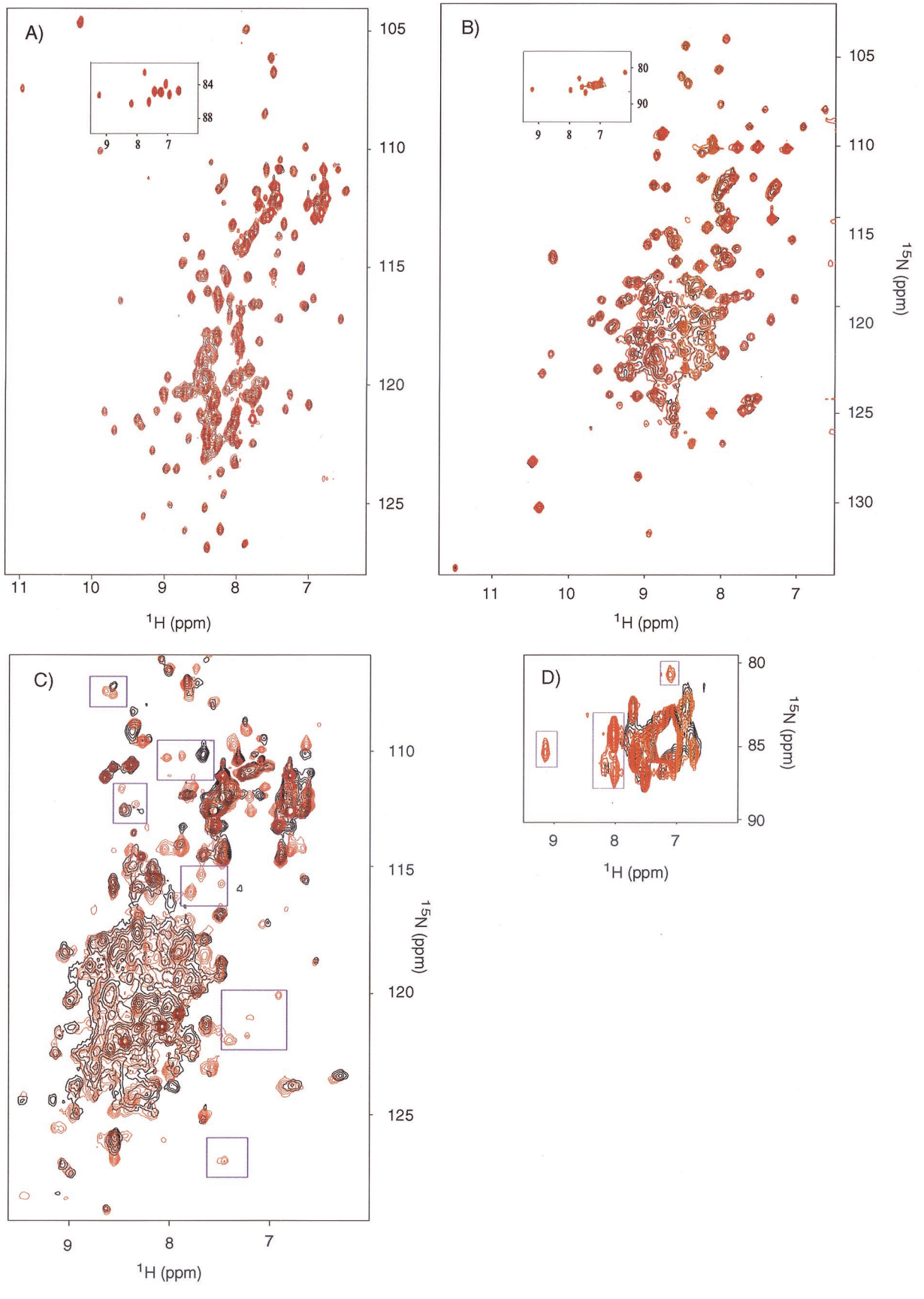
Binding of 2,2′-dipyridyl to Rob. Binding of 2,2′-dipyridyl to Rob was assessed by NMR spectroscopy. The resonance positions for each amide group are indicative of the particular chemical, conformational, or electronic environments of the associated proton and nitrogen nuclei. Slight changes in the environment induced by ligand binding, hydrogen bonding, or conformational changes manifest themselves by differences in chemical shifts and provide a useful tool for mapping binding sites in a protein (8, 26). Uniformly ^{15}N-labeled full-length Rob, CTD-truncated Rob, and MarA were purified and complexed with a *mar* DNA-binding site to maintain the proteins in solution. ^{1}H-^{15}N HSQC spectra for the MarA-*mar* and the CTD-truncated Rob-*mar* complexes in the presence or absence of excess 2,2′-dipyridyl were determined (Fig. 4A and B). No chemical shift differences in the backbone amide, side chain amino, or arginine side chain NεH groups were observed. In contrast, similar spectra for the full-length Rob-*mar* complex (Fig. 4C and D) exhibited several significant shifts in resonance frequencies upon 2,2′-dipyridyl addition, both in the backbone amide and in the side chain region of the spectra. This demonstrates that 2,2′-dipyridyl binds to the Rob-*mar* complex by a mechanism involving the CTD and perturbs the structure of Rob.

Rob binds more tightly to many of its cognate sites in vitro than does MarA or SoxS (13, 19), and yet basal levels of Rob activate the *mar*/*sox*/*rob* regulon promoters to a rather low

TABLE 6. Effects of carboxyl-terminus deletions on activation of Rob by dipyridyl[a]

Strain	*rob* coding sequences present (bp)	β-Galactosidase activity (Miller units):		Ratio[b]	β-Galactosidase activity (Miller units) with 5 mM 4,4′-dipyridyl	Ratio[b]
		In untreated samples	With 5 mM 2,2′-dipyridyl			
M597	None	7.8	18	2.3	13	1.6
M600	1–867(wildtype)	21	392	18.3	149	6.9
M848	1–777	16	19	1.2	21	1.3
M851	1–642	29	23	0.80	25	0.87
M853	1–528	20	15	0.74	18	0.92
M854	1–450	30	32	1.1	19	0.74
M808	1–369	17	18	1.1	24	1.4

[a] The *mar sox rob* mutant strains carrying the indicated plasmids were grown to early log phase, treated with 0 or 5 mM 2,2′- or 4,4′-dipyridyl for 1 h at 37°C, and assayed for β-galactosidase activity.
[b] That is, the ratio of activities in the cells treated with 2,2′- or 4,4′-dipyridyl to that in the untreated cells.


A)
B)
C)
D)
^{15}N (ppm)
^{1}H (ppm)

extent in vivo (3, 20, 34). Importantly, single-round in vitro transcription assays showed that Rob is as active as MarA and half as active as SoxS on a molar basis with respect to the extent of transcriptional activation of six regulon promoters (11). To determine whether 2,2′-dipyridyl enhances the affinity of Rob for its binding sites in the promoters of *mar* regulon genes in vitro or whether it stabilizes Rob-DNA-RNAP ternary complexes, gel retardation assays were performed. The DNA fragments contained the cognate binding sites of the promoters for *fumC*, *mar*, or *fpr*. No significant change in complex formation by Rob with any of these DNAs was seen with 2,2′-dipyridyl at 5 mM (data not shown). Explanations for the lack of a dipyridyl effect in vitro are presented below.

DISCUSSION

Posttranslational activation by dipyridyl. There are about 10,000 molecules of the transcriptional activator Rob in *E. coli*, and yet they have marginal effects on the cell (3, 12, 20, 28, 30, 35). Dramatic activation of the regulon promoters by Rob (e.g., a 10-fold effect on *inaA*::*lacZ* activity) is only achieved by vast overexpression of *rob* with heterologous promoters and multicopy plasmids (3, 14, 23). In contrast, substantial activation of the regulon promoters is achieved by induction of *marRAB* or *soxRS* leading to the production of an estimated 2,300 MarA or 350 SoxS molecules per cell, respectively (19; S. Ishita, K. L. Griffith, and R. E. Wolf, Jr., unpublished data). Thus, Rob has comparatively low activity in vivo. The present study shows that, by treating cells with dipyridyl, this low activity form of Rob is converted to a high-activity form. We tentatively conclude that this is a posttranslational event since (1) it occurs in the absence of the *rob* promoter and translational signals (2); it does not occur in *rob* nonsense mutants, which presumably synthesize otherwise wild-type *rob* mRNA, and (3) it does not occur when the CTD is truncated.

Activation requires the CTD of Rob. In a crystal complex with DNA, the CTD of Rob was seen to lie on top of the NTD and to make no contact with the DNA (13). The smaller MarA and SoxS proteins do not have a CTD but are homologous to Rob's NTD. Therefore, we considered the possibility that the CTD is an inhibitor of the NTD of Rob and that dipyridyl treatment antagonizes the inhibition. If so, truncation of the CTD should relieve the inhibition and increase the activity of Rob to levels seen with dipyridyl treatment. A twofold increase in the activation of the *inaA*::*lacZ* mutant over that of wild type was reported previously for a Rob construct (*rob133*) in which 156 C-terminal amino acids were replaced with 10 amino acids of the vector (3). However, in our systematic study, no increase in *inaA*::*lacZ* expression due to Rob's activity was seen with complete or partial C-terminal truncations of 168, 113, or 30 amino acids, and a modest 1.5-fold increase in activity was seen for Rob with C-terminal truncations of 75 or 139 amino acids (Table 6). In contrast, 2,2′-dipyridyl had an 18-fold effect on *inaA*::*lacZ* expression in a strain encoding the wild-type Rob. Thus, if the Rob CTD inhibits the NTD's activity, it is only to a minor extent. This indicates that the normally low activity of Rob is a function of the NTD. Nevertheless, removing as few as 30 amino acids from the carboxyl terminus of Rob prevented activation by dipyridyl. The simplest interpretation is that dipyridyl treatment reconfigures Rob by an interaction involving the CTD, which then converts the NTD from a low-activity form to a high-activity form in vivo.

Is dipyridyl the direct effector of the Rob activation? 2,2′- and 4,4′-dipyridyl are hydrophobic compounds that are not known to be normal cellular or environmental constituents of *E. coli*. 2,2′-Dipyridyl has long been used to chelate environmental iron, and it has been assumed that little if any of it is taken up by the cells. Thus, the treatment of cells with millimolar concentrations are likely to produce cellular concentrations in the micromolar range or lower. Substantial evidence that the effects studied here are not due to metal chelation has been provided above. Whether the dipyridyls are direct effectors of Rob, whether they are first converted intracellularly into the direct effectors, or whether they stimulate the cell to produce the direct effector is not known. Evidence for a direct interaction of dipyridyl with Rob in vitro comes from NMR studies. 2,2′-Dipyridyl engendered striking changes in the backbone amide and side chain regions of the ^{1}H-^{15}N HSQC spectra with full-length Rob but not with either CTD-truncated Rob or MarA, which has no CTD (Fig. 4). In addition, the kinetics of induction of *inaA* transcription by dipyridyl in vivo are consistent with a rapid activation of Rob (Fig. 2). However, preliminary attempts to demonstrate that 2,2′-dipyridyl enhances either the binding of purified Rob to DNA or the activation of transcription in vitro have been unsuccessful (data not shown).

Three explanations for the lack of activation in vitro may be considered. (i) 2,2′-Dipyridyl is a direct effector of Rob, but the purified Rob used in vitro differs from the low activity form found in vivo. Purified Rob has been found to be as active as MarA and half as active as SoxS in stimulating transcription of six regulon promoters in vitro (11). Thus, the low activity of Rob in vivo may be due to the binding of an inhibitor or to the cellular sequestration of Rob (3). Indeed, immunostaining has shown that Rob proteins are clustered in a few discrete foci in the nucleoid (29). The interaction of dipyridyl with Rob in vivo would reduce its affinity for inhibitor or reverse the sequestration. However, if purification of Rob eliminates the inhibition or sequestration, as seems to be the case (11), dipyridyl would not show an effect in vitro. (ii) 2,2′-Dipyridyl is not a direct effector of Rob, and thus is not effective in vitro. The binding seen in the NMR data would then be ascribed to an interaction with the CTD that is not relevant to the activation mechanism. (iii) A negative in vitro result could mean that the conditions of the assay are not appropriate for detecting the effects of 2,2′-dipyridyl or that an additional factor necessary for activation is

FIG. 4. An overlay of the ^{1}H-^{15}N HSQC spectra of *mar* DNA–^{15}N-labeled MarA complex (A), *mar* DNA–^{15}N-labeled CTD-truncated Rob complex (B), and *mar* DNA–^{15}N-labeled full-length Rob complex (C). The spectra in black and red were acquired in the absence and presence of 5 mM 2,2′-dipyridyl, respectively. The NεH protons of the arginines in the protein-DNA complexes are represented in the insets of panels A and B and in panel D. The blue boxes in panels C and D indicate some of the prominent differences in the spectra due to the presence of 2,2′-dipyridyl.

missing. Analysis of mutants with increased basal level activities of Rob and decreased response to dipyridyl may help distinguish between these possibilities.

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REFERENCES

1. **Alekshun, M. N., and S. B. Levy.** 1997. Regulation of chromosomally mediated multiple antibiotic resistance: the *mar* regulon. Antimicrob. Agents Chemother. **41:**2067–2075.
2. **Aono, R., N. Tsukagoshi, and M. Yamamoto.** 1998. Involvement of outer membrane protein TolC, a possible member of the *mar-sox* regulon, in maintenance and improvement of organic solvent tolerance of *Escherichia coli* K-12. J. Bacteriol. **180:**938–944.
3. **Ariza, R. R., Z. Li, N. Ringstad, and B. Demple.** 1995. Activation of multiple antibiotic resistance and binding of stress-inducible promoters by *Escherichia coli* Rob protein. J. Bacteriol. **177:**1655–1661.
4. **Cohen, S. P., S. B. Levy, J. Foulds, and J. L. Rosner.** 1993. Salicylate induction of antibiotic resistance in *Escherichia coli*: activation of the *mar* operon and a *mar*-independent pathway. J. Bacteriol. **175:**7856–7862.
5. **Clore, G. M., and A. M. Gronenborn.** 1991. Two-, three-, and four-dimensional NMR methods for obtaining larger and more precise three-dimensional structures of proteins in solution. Annu. Rev. Biophys. Chem. **20:**29–63.
6. **Delaglio, F., S. Grzesiek, G. W. Vuister, G. Zhu, J. Pfeifer, and A. Bax.** 1995. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J. Biomol. NMR **6:**277–293.
7. **Demple, B.** 1996. Redox signaling and gene control in the *Escherichia coli soxRS* oxidative stress regulon--a review. Gene **179:**53–57.
8. **Hajduk, P. J., J. Dinges, G. F. Miknis, M. Merlock, T. Middleton, D. J. Kempf, D. A. Egan, K. A. Walter, T. S. Robins, S. B. Shuker, T. F. Holzman, and S. W. Fesik.** 1997. NMR-based discovery of lead inhibitors that block DNA binding of the human papillomavirus E2 protein. J. Med. Chem. **40:**3144–3150.
9. **Heller, K., B. J. Mann, and R. J. Kadner.** 1985. Cloning and expression of the gene for the vitamin B12 receptor protein in the outer membrane of *Escherichia coli*. J. Bacteriol. **161:**896–903.
10. **Jair, K. W., R. G. Martin, J. L. Rosner, N. Fujita, A. Ishihama, and R. E. Wolf, Jr.** 1995. Purification and regulatory properties of MarA protein, a transcriptional activator of *Escherichia coli* multiple antibiotic and superoxide resistance promoters. J. Bacteriol. **177:**7100–7104.
11. **Jair, K. W., X. Yu, K. Skarstad, B. Thöny, N. Fujita, A. Ishihama, and R. E. Wolf, Jr.** 1996. Transcriptional activation of promoters of the superoxide and multiple antibiotic resistance regulons by Rob, a binding protein of the *Escherichia coli* origin of chromosomal replication. J. Bacteriol. **178:**2507–2513.
12. **Kakeda, M., C. Ueguchi, H. Yamada, and T. Mizuno.** 1995. An *Escherichia coli* curved DNA-binding protein whose expression is affected by the stationary phase-specific sigma factor sigma S. Mol. Gen. Genet. **248:**629–634.
13. **Kwon, H. J., M. H. Bennik, B. Demple, and T. Ellenberger.** 2000. Crystal structure of the *Escherichia coli* Rob transcription factor in complex with DNA. Nat. Struct. Biol. **7:**424–430.
14. **Lee, E. H., E. Collatz, I. Podglajen, and L. Gutmann.** 1996. A *rob*-like gene of *Enterobacter cloacae* affecting porin synthesis and susceptibility to multiple antibiotics. Antimicrob. Agents Chemother. **40:**2029–2033.
15. **Larsen, R. A., M. G. Thomas, G. E. Wood, and K. Postle.** 1994. Partial suppression of an *Escherichia coli* TonB transmembrane domain mutation (delta V17) by a missense mutation in ExbB. Mol. Microbiol. **13:**627–640.
16. **Li, Z., and B. Demple.** 1996. Sequence specificity for DNA binding by *Escherichia coli* SoxS and Rob proteins. Mol. Microbiol. **20:**937–945.
17. **Maekawa, T., and E. Ohtsubo.** 1994. Identification of the region that determines the specificity of binding of the transposases encoded by Tn*3* and gamma delta to the terminal inverted repeat sequences. Jpn. J. Genet. **69:**269–285.
18. **Martin, R. G., and J. L. Rosner.** 1995. Binding of purified multiple antibiotic-resistance repressor protein (MarR) to *mar* operator sequences. Proc. Natl. Acad. Sci. USA **92:**5456–5460.
19. **Martin, R. G., W. K. Gillette, N. I. Martin, and J. L. Rosner.** 2002. Complex formation between activator and RNA polymerase as the basis for transcriptional activation by MarA and SoxS in *Escherichia coli*. Mol. Microbiol. **43:**355–370.
20. **Martin, R. G., W. K. Gillette, and J. L. Rosner.** 2000. Promoter discrimination by the related transcriptional activators MarA and SoxS: differential regulation by differential binding. Mol. Microbiol. **35:**623–634.
21. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Moeck, G. S., and J. W. Coulton.** 1998. TonB-dependent iron acquisition: mechanisms of siderophore-mediated active transport. Mol. Microbiol. **28:**675–681.
23. **Nakajima, H., K. Kobayashi, Kobayashi, M., H. Asako, and R. Aono.** 1995. Overexpression of the *rob*A gene increases organic solvent tolerance and multiple antibiotic and heavy metal ion resistance in *Escherichia coli*. Appl. Environ. Microbiol. **61:**2302–2307.
24. **Rhee, S., R. G. Martin, J. L. Rosner, and D. R. Davies.** 1998. A novel DNA-binding motif in MarA: the first structure for an AraC family transcriptional activator. Proc. Natl. Acad. Sci. USA **95:**10413–10418.
25. **Rosner, J. L., and J. L. Slonczewski.** 1994. Dual regulation of *inaA* by the multiple antibiotic resistance (*mar*) and superoxide (*soxRS*) stress response systems of *Escherichia coli*. J. Bacteriol. **176:**6262–6269.
26. **Shuker, S. B., P. J. Hajduk, R. P. Meadows, and S. W. Fesik.** 1996. Discovering high-affinity ligands for proteins: SAR by NMR. Science **274:**1531–1534.
27. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single copy and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
28. **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.
29. **Talukder, A. A., S. Hiraga, and A. Ishihama.** 2000. Two types of localization of the DNA-binding proteins within the *Escherichia coli* nucleoid. Genes Cells **5:**613–626.
30. **Talukder, A. A., A. Iwata, A. Nishimura, S. Ueda, and A. Ishihama.** 1999. Growth-phase-dependent variation in protein composition of the *Escherichia coli* nucleoid. J. Bacteriol. **181:**6361–6370.
31. **Trun, N. J., and T. J. Silhavy.** 1987. Characterization and in vivo cloning of *prlC*, a suppressor of signal sequence mutations in *Escherichia coli* K12. Genetics **116:**513–521.
32. **Tsaneva, I. R., and B. Weiss.** 1990. *soxR*, a locus governing a superoxide response regulon in *Escherichia coli* K-12. J. Bacteriol. **172:**4197–4205.
33. **Tsolis, R. M., A. J. Baumler, I. Stojiljkovic, and F. Heffron.** 1995. Fur regulon of *Salmonella typhimurium*: identification of new iron-regulated genes. J. Bacteriol. **177:**4628–4637.
34. **Van Dyk, T. K., B. L. Ayers, R. W. Morgan, and R. A. Larossa.** 1998. Constricted flux through the branched-chain amino acid biosynthetic enzyme acetolactate synthase triggers elevated expression of genes regulated by *rpoS* and internal acidification. J. Bacteriol. **180:**785–792.
35. **White, D. G., J. D. Goldman, B. Demple, and S. B. Levy.** 1997. Role of the *acrAB* locus in organic solvent tolerance mediated by expression of *marA*. soxS, or *robA* in *Escherichia coli.* J. Bacteriol. **179:**6122–6126.