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Transcriptional Activation of an *Escherichia coli* Copper Efflux Regulon by the Chromosomal MerR Homologue, CueR*

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Because copper ions are both essential cofactors and cytotoxic agents, the net accumulation of this element in a cell must be carefully balanced. Depending upon the cellular copper status, copper ions must either be imported or ejected. CopA, the principal copper efflux ATPase in *Escherichia coli*, is induced by elevated copper in the medium, but the copper-sensing regulatory factor is unknown. Inspection of the *copA* promoter reveals signature elements of promoters controlled by metalloregulatory proteins in the MerR family. These same elements are also present upstream of *yacK*, which encodes a putative multi-copper oxidase. Homologues of YacK are found in copper resistance determinants that facilitate copper efflux. Here we show by targeted gene deletion and promoter fusion assays that both *copA* and *yacK* are regulated in a copper-responsive manner by the MerR homologue, *ybbI*. We have designated *ybbI* as *cueR* for the Cu efflux regulator. This represents the first example of a copper-responsive regulon on the *E. coli* chromosome and further extends the roles of MerR family members in prokaryotic stress response.

Escherichia coli, like most microbes, require a minimal level of copper for insertion into metabolic and respiratory enzymes. At the same time, these organisms must maintain intracellular copper below a toxic threshold. In eukaryotes, intracellular copper is controlled by uptake systems, copper chaperones that facilitate specific loading of copper into enzymes, metallothioneins that bind excess copper, efflux systems that remove copper from the cell, and copper-responsive metalloregulatory proteins that coordinate all these mechanisms (1, 2). The chromosomal *cop* system of *Enterococcus hirae*, which contains copper uptake, efflux, and metalloregulatory components, represents one well characterized example of copper homeostasis in prokaryotes (3). Whereas few direct homologues of known copper homeostasis proteins have been found in *E. coli*, copper export systems have been identified, including the plasmid-encoded copper resistance determinants (4).

Numerous attempts have been made to identify the genes involved in controlling copper levels in *E. coli*; however, few of these genes have been directly linked to copper metabolism, transport, or regulation (5, 6). For instance, the *nlpE* (*cutF*) gene, which encodes for a novel lipoprotein, and *dipZ* (*cutA2*), which encodes for a disulfide isomerase, have indirect connections with copper metabolism (6, 7). The recently characterized gene *copA*, which encodes a copper-inducible P-type ATPase (8), represents a central component in *E. coli* copper efflux. The factors responsible for the copper-responsive expression of *E. coli copA* have not been characterized.

Recently, the first copper-responsive regulatory system in the *E. coli* chromosome, a two-component signal transduction system designated Cu-sensing or *cus* locus, was identified (9). The *cusRS* genes form a sensor/regulator pair that activates the adjacent but divergently transcribed gene, *cusC*, and possibly two adjacent downstream genes, *cusBA*, in response to increasing copper. The *cusCBA* genes are homologous to a family of proton-cation antiporter complexes involved in the export of metal ions, xenobiotics, and drugs (10, 11).

We set out to identify the regulator of *E. coli copA* because of its established importance in copper homeostasis. Our analysis of the *copA* promoter showed no CusRS-binding element; however, it did reveal a long spacer between the −10 and −35 elements reminiscent of promoters regulated by the metalloregulatory protein, MerR (12, 13). A search of the *E. coli* chromosome uncovered a second promoter located upstream of the putative multi-copper oxidase *yacK*, which contained similar MerR-like features. Upon searching for a MerR homologue that might regulate these genes, we found the gene *ybbI*. Deletion of *ybbI* resulted in the disruption of copper-responsive regulation at both *yacK* and *copA*, establishing these genes as a copper-inducible regulon. The gene *ybbI* encodes a copper-activated homologue of MerR and was designated *cueR* for Cu efflux regulator. Likewise, the *yacK* gene was renamed *cueO* for Cu efflux oxidase.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Media—All polymerase chain reactions (PCR)[1] described used *E. coli* DH5α chromosomal DNA as the template unless otherwise stated. All plasmids were verified by DNA sequencing, and all DNA manipulations employed standard protocols (14). Strains, phages, and plasmids used in this study are shown in Table I. β-galactosidase assays were conducted in chemically defined medium consisting of 1× A medium (7.6 mM $(NH_4)_2SO_4$, 33 mM KH_2PO_4, 60 mM K_2HPO_4, and 1.7 mM sodium citrate) (15) supplemented with 40 μg/ml of all 20 essential L-amino acids (Sigma), 0.2% glucose, 1 mM $MgSO_4$, and 5×10^{-5}% thiamine. All media components except thiamine and $MgSO_4$ were incubated overnight with 50 g/liter Chelex 100 resin (Bio-Rad) to remove trace metals, mixed and sterile-filtered before use. For construction of the *ybbI* null strain, alternative media were used at various steps in accordance with the published methods (16).

Primer Extension Analysis—Wild-type DH5α and DLG (Δ*cusRS*) (9) were grown in LB to exponential phase. Selected cultures were then induced with 0.5 mM $CuSO_4$ for 60 min. Total RNA was isolated using the Qiagen RNeasy RNA isolation kit. Primer *copA*:PE1 (5′-GGA CAG

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[1] The abbreviation used is: PCR, polymerase chain reaction.

TABLE I
Strains and plasmids

Strain or plasmid	Relevant characteristics or genotype	Ref. or source
Strain		
BW25113	($LacI^q$ $lacZ_{WJ16}$)	16
DLG	DH5α ($\Delta cusRS$)	9
DH5α	($lacZYA$-$argF$)$_{U169}$ *recA1*	15
WOII244A	BW25113 (*cueR*::kan^R)	This study
WOII248B	BW25113 (Δ*cueR*)	This study
WOII260A	WOII248B ϕ(*copA*-*lacZ*)	This study
WOII260B	WOII248B ϕ(*yacK*-*lacZ*)	This study
WOII260C	BW25113 ϕ(PT*copA*-*lacZ*)	This study
WOII260D	DH5α ϕ(−19/−31 *copA*-*lacZ*)	This study
λRS45	Phage for single copy reporter construction	17
Plasmid		
pACYC184	Low copy number cloning vector	New England Biolabs
pACYC184/*cueR*	pACYC184 containing wild-type *cueR*	This study
pKD4	Template for PCR of fragment for insertional mutagenesis of *cueR*	16
pKD46	Vector containing the λ Red genes	16
pCP20	Vector containing the FRT recombinase	16
pUC19	Cloning vector	New England Biolabs
pUC19/*copA*	pUC19 containing the *copA* promoter	This study
pRS551	*lac*-based promoter fusion vector	17
pRS551/*copA*	pRS551 containing the *copA* promoter	This study
pRS551/PT*copA*	pRS551 containing a truncated *copA* promoter	This study
pRS551/−19−31*copA*	pRS551 containing the *copA* promoter with mutations at the −19 and −31 positions	This study
pRS551/*yacK*	pRS551 containing the *yacK* promoter	This study

GCC GTC CAG GGT CAG G-3′) was end-labeled with ^{32}P. 10 μg of total RNA was preheated to 65 °C with *copA*:PE1 for 5 min and chilled on ice. A primer/RNA mixture was added to the reaction buffer with Moloney murine leukemia virus reverse transcriptase (New England Biolabs) and incubated at 42 °C for 60 min. The sequencing of pUC19/*copA* with labeled *copA*:PE1 was carried out with the CircumVent Thermal Cycle Dideoxy DNA sequencing kit (New England Biolabs) as directed by the manufacturer's instructions. Primer extension and sequencing samples were run on an 8 M urea, 8% acrylamide gel and exposed overnight.

Construction of Promoter Fusions to lacZ and Analysis of Promoter Activity—The *copA* promoter was amplified by PCR from genomic DNA using primers *copA*/*Eco*RI (5′-GAT GCG GGA GGG AAT TCC TCA CCC C-3′) and *copA*/*Ava*I (5′-CTG CTC AAC ACC CGG GCG CTG TTC AAG-3′). The PCR fragment was digested with *Eco*RI and *Ava*I and inserted into the *Eco*RI/*Ava*I sites of pUC19 (New England Biolabs) to create pUC19/*copA*. The *Bam*HI/*Eco*RI fragment of pUC19/*copA* was excised and cloned into pRS551 to make a *copA*-*lacZ* promoter fusion. To remove upstream regulatory elements, pUC19/*copA* was used as a template for PCR with primers PT*copA* (5′-CTT TAC GGA CTT GAA TTC GCC TGG TTT ATT-3′) and −47 (New England Biolabs). This fragment was digested and cloned into the *Eco*RI/*Bam*HI sites of pRS551 to create pRS551/PT*copA*. Single copy *copA*-*lacZ* promoter fusion strains were constructed by transduction as described previously (17).

A 307-base pair portion of the upstream region of *yacK* was amplified using primers *yacK*/*Eco*RI (5′-GTT TGC GCA GAC GGA ATT CAG GCT GCC G-3′) and *yacK*/*Bam*HI (5′-GCA AAT ACT GCG CGG ATC CAC AGC G-3′). The fragment was digested with *Eco*RI/*Bam*HI and cloned into the *Eco*RI/*Bam*HI sites of pRS551 to create pRS551/*yacK*. Single copy reporter lysogens were then constructed as described (17).

β-Galactosidase assays were conducted as described previously (18). Briefly, a 1:50 dilution of saturated lysogen culture was used to inoculate the chemically defined medium (see above) containing 40 μg/ml kanamycin. Cultures in the exponential phase were exposed to varying metal concentrations for 60 min followed by assay of β-galactosidase activity.

Disruption of ybbI (cueR)—The gene *ybbI* (hereafter referred to as *cueR*) was disrupted by the insertion of a Kan^R cassette using a protocol developed in the laboratory of B. Wanner and based on the λ Red system as described previously in detail (16). Here a PCR fragment was generated using primers *cueR*(PS1) (5′-GTG CGC AGT ACT TCC TGT ATT ATT GTG GTG GCG GTC GAT GTG TAG GCT GCT TC-3′) and *cueR*(PS2) (5′-GGG ATA ACC CTA CAT ATC CGA GCC GTC TCG TCT TAA TCA CAT ATG AAT ATC CTC CTT AGT-3′) and electroporated into strain BW25113 resulting in insertion into *cueR*. The first 39 nucleotides of *cueR*(PS1) and *cueR*(PS2) correspond to chromosomal sequences flanking *cueR*. The use of these primers with pKD4 as template generates a 1.5-kilobase pair fragment that contains a Kan^R cassette flanked by FRT (FLP recognition target) sites and, on termini, by short stretches of *ybbI* sequence. To construct WOII248B (*cueR*), the Kan^R was removed creating an internal deletion in *cueR* as described previously (16). For complementation experiments, *cueR* was amplified using primers *cueR*(*Eco*RI) (5′-CCT GCG TCA TGG AAT TCA CGC CTC G-3′) and *cueR*(*Bam*HI) (5′-GGT AAT GTT TGG GAT CCT GAC GCT GCT C-3′). The fragment was digested with *Bam*HI and cloned into the *Eco*RV/*Bam*HI sites of pACYC184 (New England Biolabs) to make pACYC184/*cueR*, which was maintained with 30 μg/ml chloramphenicol.

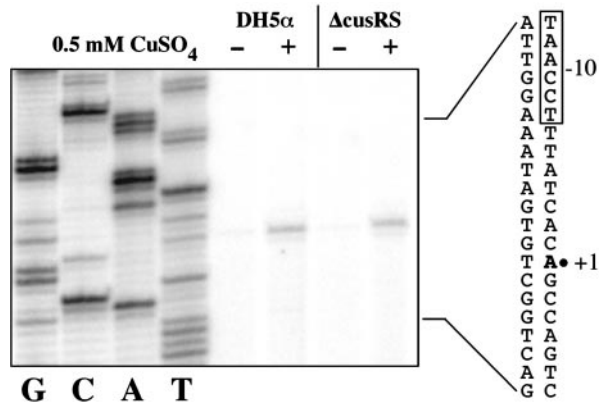

FIG. 1. **Primer extension analysis of the *copA* transcript.** Total RNA, isolated from DH5α and ΔcusRS strain DLG, with and without 0.5 mM $CuSO_4$, was used for primer extension to map the transcriptional start site of *copA* and to determine the transcript levels in the two strains.

CueR Cloning, Purification, and Footprinting—The *cueR* gene was amplified by PCR from genomic DNA using primers CueR N-terminal (5′-GGC GTT GCG CGA CAT ATG AAC ATC AGC G-3′) and CueR C-terminal (5′-CCG AGC CGG ATC CTC TTA ATC ACC CTG C-3′). The fragment was digested with *Nde*I and *Bam*HI and inserted into the overexpression vector pET24a (New England Biolabs), which was then transformed into *E. coli* strain BL21(DE3) (Novagen). The overexpression and purification of CueR is the same as described previously for the ZntR protein (19) without the hydrophobic column step. The molecular mass of CueR (calculated 15,235.0 Da with first Met) was found to be 15,235.4 Da by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (PerSpective Biosystems Voyager-DE). The plasmid pUC19/*copA* (see above) was used for labeling in footprinting assays. DNA labeling and DNase I footprinting procedures are the same as described previously (19).

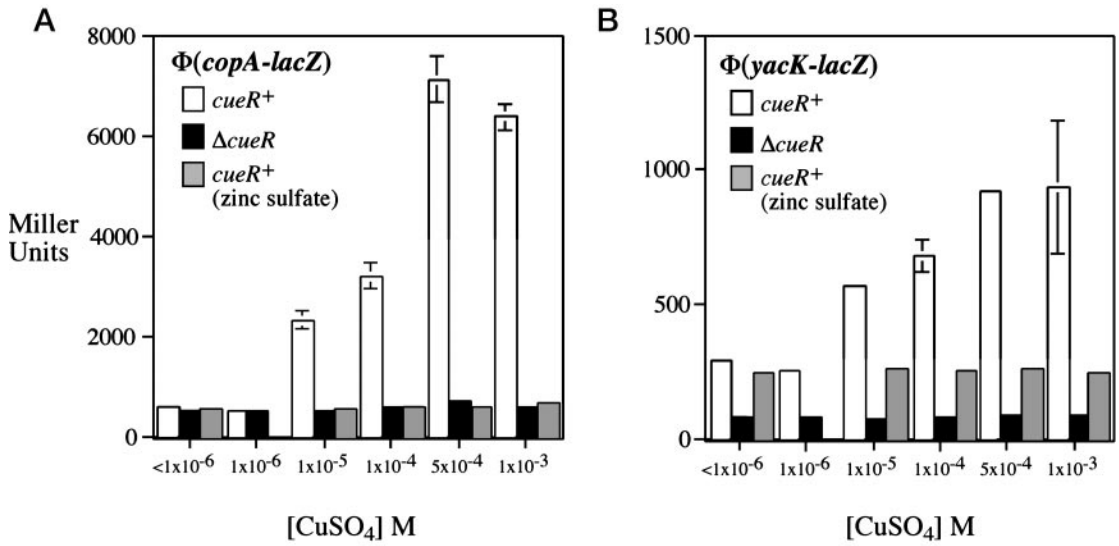

FIG. 2. **Response of *copA* and *yacK* to varying [$CuSO_4$].** *A,* β-galactosidase activity of BW25113 ϕ(*copA-lacZ*) (*open bars*) and WOII248B (Δ*cueR*) ϕ(*copA-lacZ*) (*black bars*) was measured in response to varying [$CuSO_4$]. The response of BW25113 ϕ(*copA-lacZ*) to $ZnSO_4$ (*gray bars*) was also measured (except for 1×10^{-6} M). *B,* β-galactosidase activity of BW25113 ϕ(*yacK-lacZ*) (*open bars*) and WOII248B (Δ*cueR*) ϕ(*yacK-lacZ*) (*black bars*) was measured in response to varying [$CuSO_4$]. The response of BW25113 ϕ(*yacK-lacZ*) to $ZnSO_4$ (*gray bars*) was measured as in *A*.

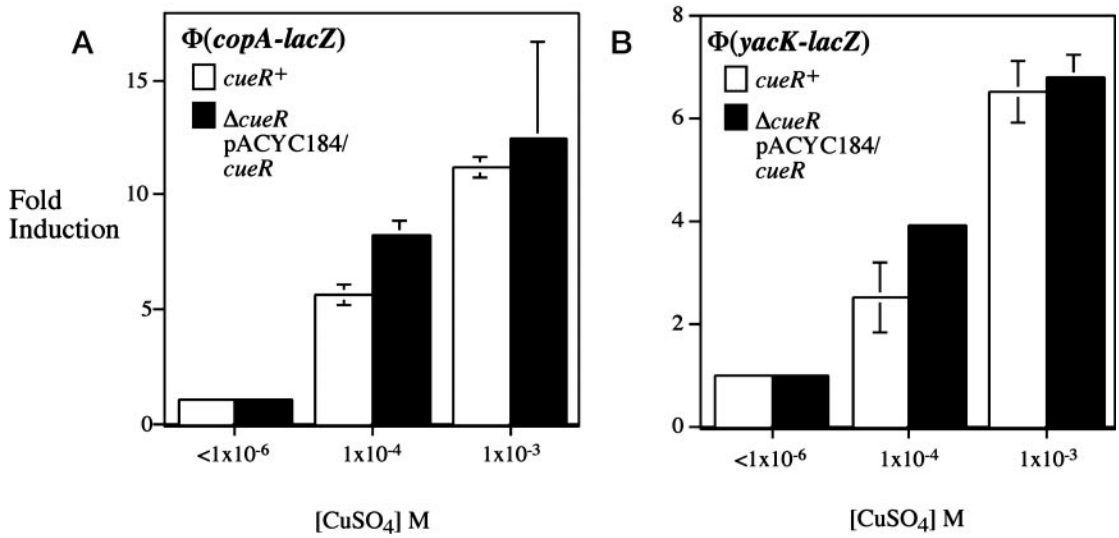

FIG. 3. **Complementation of *cueR* with pACYC184/cueR.** A, WOII248B (Δ*cueR*) ϕ(*copA-lacZ*) was transformed with pACYC184/*cueR*, and β-galactosidase activity was measured in response to varying [$CuSO_4$] (*black bars*). Also shown is the response of parent strain BW25113 ϕ(*copA-lacZ*) to the same copper levels (*open bars*). *B,* WOII248B (Δ*cueR*) ϕ(*yacK-lacZ*) was transformed with pACYC184/*cueR*, and β-galactosidase activity was measured in response to $CuSO_4$ (*black bars*). Also shown is the response of parent strain BW25113 ϕ(*yacK-lacZ*) to the same copper levels (*open bars*).

RESULTS

The copA Promoter Is Copper-responsive but Not CusRS-Regulated—The only previously identified copper-responsive regulatory system on the *E. coli* chromosome is the CusRS two-component system (9). To determine if *copA* is under the regulatory control of CusRS and to map the transcriptional start site for the *copA* promoter, primer extension analysis was conducted using a wild-type strain, DH5α, and a Δ*cusRS* strain, DLG. As the primer extension results show in Fig. 1, *copA* transcription is induced by copper in a manner independent of the CusRS system. The absence of a consensus "copper box" upstream of the −35 element is consistent with a lack of CusRS regulation. This result also establishes the start site of the *copA* promoter (Fig. 1).

To obtain a more routine measure of promoter activity, the *copA* promoter region was cloned, fused to *lacZ*, and transduced as a single copy reporter. The results of the zinc and copper induction profiles are shown in Fig. 2*A*. From these experiments, it is clear that the *copA* promoter is primarily induced by copper because neither zinc nor mercury (data not shown) was able to induce.

copA Is Regulated by CueR, a Member of the MerR Family—The lack of CusRS regulation indicates the existence of an alternative copper-responsive signal transduction system. The unusual spacing seen in the *copA* promoter (Fig. 6*A*) indicates that it may be regulated by a MerR family member. Inspection of the *E. coli* genome sequence revealed an uncharacterized MerR homologue, *ybbI*. To test for *ybbI* regulation of *copA in vivo*, *ybbI* was disrupted on the chromosome by the insertion of a Kan^R cassette using a λ Red-mediated mutagenesis system (16). Strains lacking *ybbI* showed a complete loss of copper induction at the *copA* promoter (Fig. 2*A*). A construct containing *ybbI* carried on the pACYC184 low copy vector was able to complement the Δ*ybbI* phenotype (Fig. 3*A*), confirming that

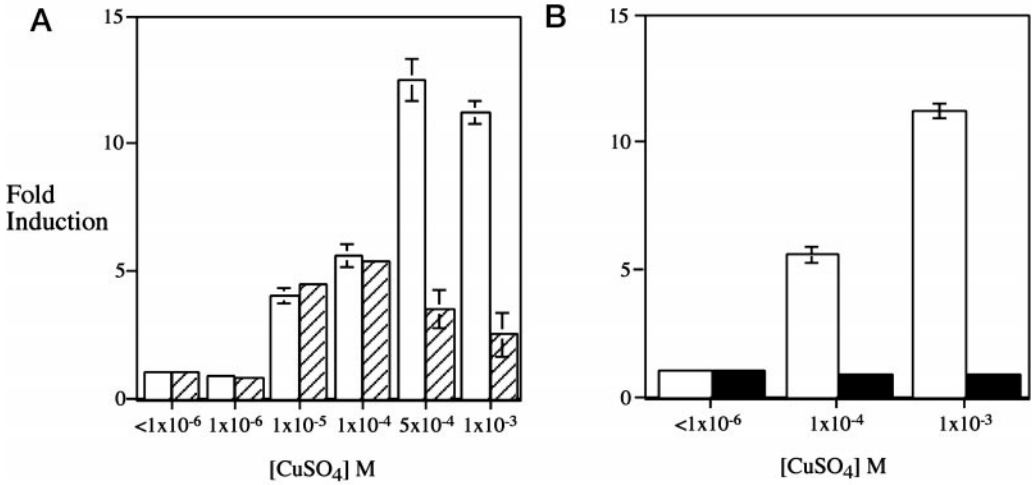

FIG. 4. **Activity of *copA* promoter mutants.** *A,* β-galactosidase activity of BW25113 ϕ(*copA-lacZ*) (*open bars*) and BW25113 ϕ(PT*copA-lacZ*), which lacks the region upstream of the UP element (*hatched bars*), was measured with varying [$CuSO_4$]. *B,* β-galactosidase activity of DH5α ϕ(*copA-lacZ*) (*open bars*) and DH5α ϕ(*copA*−19/−31−*lacZ*), which contains T→A mutation at the −19 position and A→C mutation at the −31 position (*black bars*) was measured with varying [$CuSO_4$].

ybbI (renamed *cueR* for Cu efflux regulator) is the copper-responsive positive activator of *copA*.

Identification of Another Chromosomal Target for CueR—The *copA* promoter (Fig. 6*A*) was used to conduct a Fast A search (UW-GCG) of the *E. coli* chromosome to identify other potential CueR-regulated sites. A palindrome positioned between the −35 and −10 hexamers of the predicted promoter for the gene *yacK* was found. As shown in Fig. 6*A*, the location of the palindrome in relation to the −35 and −10 boxes and the 19-base pair promoter element spacing are identical to the *copA* promoter.

To test whether CueR regulates expression of *yacK in vivo*, the *yacK* promoter was cloned from the chromosome, fused to *lacZ*, and transduced as a single copy reporter. As seen with *copA*, the *yacK* promoter is responsive to copper but not to zinc (Fig. 2*B*). Moreover, copper-responsive regulation of *yacK* depends upon the presence of *cueR*. The *cueR* deletion strain shows a loss of copper activation at the *yacK* promoter (Fig. 2*B*). By providing the *cueR* gene in *trans* on a low copy number plasmid (Fig. 3*B*), copper induction of *yacK* is restored. Sequence analysis of YacK indicates that it is homologous to the multi-copper oxidases PcoA and CopA found in the plasmid-borne copper resistance operons, *pco* from *E. coli* and *cop* from *Pseudomonas syringae* (20, 21). The gene *pcoA* facilitates copper export from the cell by an unknown mechanism (4).

The Copper Response Element of the copA Promoter—The *copA* promoter sequence contains an unusual 19-base pair spacing between the −10 and −35 elements. Both this long spacer and palindromic sequences found within the −10/−35 region (Fig. 6*A*) are similar to those found in promoters regulated by other members of the MerR family of transcriptional regulators, including SoxR and ZntR (12, 13, 19, 22, 23). In addition, the *copA* promoter contains a weak consensus UP element just upstream of the −35 site. Adjacent to that is a consensus binding site for the cell envelope stress response regulator, CpxR (24).

To determine whether copper responsiveness is mediated by the MerR-like region or by other upstream elements, various alterations in the *copA* promoter were made. The entire upstream region beyond the typical UP element region, including the putative CpxR binding site, was removed from the 5′-end of the *copA-lacZ* promoter fusion. Fig. 4*A* demonstrates that copper-responsive activation of *copA* is still observed after the removal of the upstream region. The absence of this region of

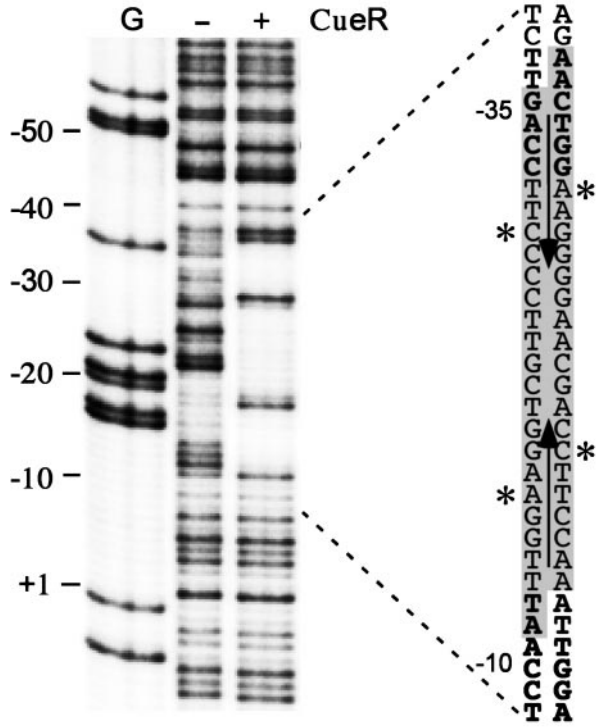

FIG. 5. **CueR DNase I footprinting of the non-template strand of P*copA*.** [CueR] = 65 nM. The area of protection from DNase I is shown as a *shaded box* in the sequence to the right of the gel. The palindromic sequence is shown with the *vertical arrows*. Sites of DNase I hypersensitivity are shown with an *asterisk*. The −10 and −35 elements are shown as *bold letters*.

the promoter, however, resulted in less than maximum induction at high copper concentrations, suggesting that other upstream regulatory elements may work in synergy with CueR to increase the level of transcription under high copper stress. In contrast, two single nucleotide changes in the predicted CueR operator at the −19 and −31 positions abolished copper responsiveness at the *copA* promoter (Fig. 4*B*). These mutations do not affect core promoter elements or the length of the spacer region, but they are important positions in the operators of MerR-like proteins. Finally, to test whether CueR directly interacts with the *copA* promoter, CueR was purified and its

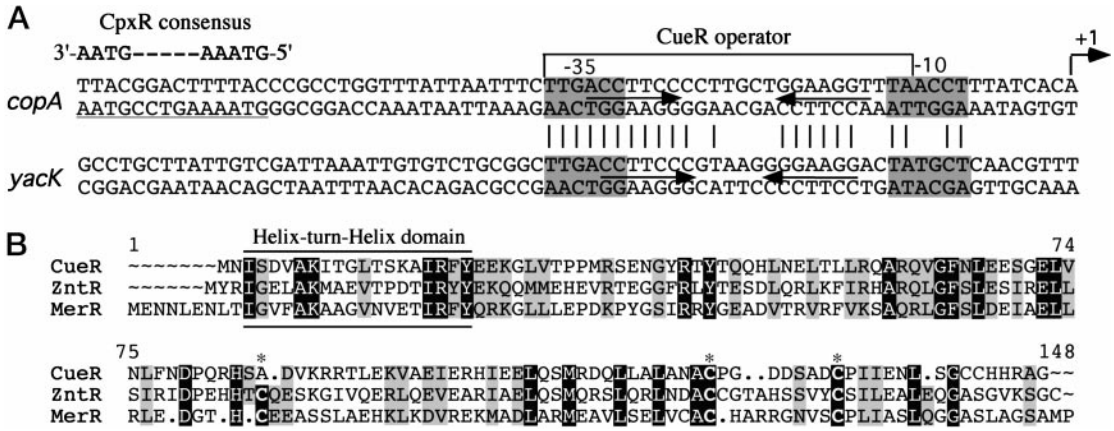

FIG. 6. *A*, promoter sequence comparison between *copA* and *yacK* (*cueO*). Similarities between the two CueR operators (*brackets*) are indicated with *vertical lines*. The palindrome is shown with *arrows,* and the CpxR consensus sequence is *underlined*. *B,* amino acid alignment of ZntR and CueR from *E. coli* and MerR from Tn501. Identical residues are highlighted in *black,* and similar residues are in *gray*. The conserved cysteines are shown with an *asterisk*.

DNase I footprint was determined. Fig. 5 shows that CueR binds between the −35 and −10 elements of the *copA* promoter, confirming that the palindrome is the CueR operator. The size of the footprint, its location within the spacer, and the internal DNase I hypersensitive sites are hallmarks of footprints for MerR family proteins (12, 19, 25). From these results we conclude that CueR exerts positive control at P*copA* and is the primary copper-responsive activator.

DISCUSSION

Our results demonstrate that the chromosomal *E. coli* locus *ybbI* encodes a MerR-like transcriptional activator that regulates at least two promoters in a copper-responsive manner. One of the copper-regulated promoters drives the expression of *copA*, which encodes a copper efflux pump. The other CueR-regulated gene, *yacK*, encodes a putative multi-copper oxidase similar to those observed in the *P. syringae* and *E. coli* plasmid-based copper resistance operons (26). The predicted gene product of *yacK* contains all 12 of the conserved residues that were found to be important for metal binding in the crystal structure of ascorbate oxidase (27). Unlike ascorbate oxidase, YacK has a stretch of about 50 residues with an unusually high methionine content (33% of residues in that region). A similar Met-rich region is observed in the ascorbate oxidase homologues, PcoA and CopA (*P. syringae*), both of which are essential components of their respective copper resistance operons (20). Based on these similarities, *yacK* was renamed *cueO* for Cu efflux oxidase.

All three of the putative oxidases involved in copper detoxification have leader sequences for export and are predicted to be periplasmic proteins. Interestingly, PcoA, CopA (*P. syringae*), and CueO also contain N-terminal twin arginine motifs similar to the consensus SRR*X*FLK (SRRTFLK for PcoA, SRRTFVK for CopA (*P. syringae*), and QRRDFLK for CueO). This sequence targets folded proteins for export via the Tat pathway (28). These similarities suggest that CueO, PcoA, and CopA (*P. syringae*) may play analogous roles in protecting the cell from excess copper; however, the molecular mechanism remains to be elucidated.

Based on the sequence similarities among CueR, ZntR, and MerR (Fig. 6*B*), it is likely that CueR detects cytoplasmic copper stress and activates transcription in response to increasing copper concentrations. CueR consists of binding region and a C-terminal metal binding region, that are also conserved in both ZntR and MerR. CueR does, however, lack a Cys residue that is conserved at approximately position 80 in the ZntR and MerR sequence (Fig. 6*B*). Furthermore, the spacing between two other highly conserved Cys residues is shorter in CueR than in either ZntR or MerR (Fig. 6*B*). These differences in the putative metal binding domain may create a receptor site that allows CueR to distinguish between copper, zinc, and mercury.

Interestingly, we find evidence of a role for potential upstream elements in the regulation of the CopA copper efflux transporter. Removal of the upstream region, which includes a CpxR consensus site, decreases the maximum induction observed at the highest copper concentrations tested. The exact nature of this synergy remains to be elucidated.

These results define the first copper-responsive regulon in *E. coli*. This brings the number of chromosomal, copper-sensing regulatory systems in *E. coli* to two: CusRS, which may sense copper levels in the periplasm, and CueR, which is probably a cytoplasmic sensor. Each system activates transcription of independent copper efflux systems in *E. coli*. The presence of two copper efflux systems highlights the potential toxicity of copper. At present it is not clear if the *copA*/*cueO* and *cusCBA* efflux systems are redundant or if differential advantages can be gained from each system under specific growth or stress conditions. This component of copper signal transduction in bacteria provides a springboard from which to address the molecular mechanisms of copper homeostasis in prokaryotes.

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