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Regulation and Activity of a Zinc Uptake Regulator, Zur, in *Corynebacterium diphtheriae*▽

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Regulation of metal ion homeostasis is essential to bacterial cell survival, and in most species it is controlled by metal-dependent transcriptional regulators. In this study, we describe a *Corynebacterium diphtheriae* ferric uptake regulator-family protein, Zur, that controls expression of genes involved in zinc uptake. By measuring promoter activities and mRNA levels, we demonstrate that Zur represses transcription of three genes (*zrg*, *cmrA*, and *troA*) in zinc-replete conditions. All three of these genes have similarity to genes involved in zinc uptake. Transcription of *zrg* and *cmrA* was also shown to be regulated in response to iron and manganese, respectively, by mechanisms that are independent of Zur. We demonstrate that the activity of the *zur* promoter is slightly decreased under low zinc conditions in a process that is dependent on Zur itself. This regulation of *zur* transcription is distinctive and has not yet been described for any other *zur*. An adjacent gene, predicted to encode a metal-dependent transcriptional regulator in the ArsR/SmtB family, is transcribed from a separate promoter whose activity is unaffected by Zur. A *C. diphtheriae zur* mutant was more sensitive to peroxide stress, which suggests that *zur* has a role in protecting the bacterium from oxidative damage. Our studies provide the first evidence of a zinc specific transcriptional regulator in *C. diphtheriae* and give new insights into the intricate regulatory network responsible for regulating metal ion concentrations in this toxigenic human pathogen.

Metal homeostasis in bacteria is mediated by five families of metal-dependent transcriptional regulators: DtxR, Fur, ArsR/SmtB, MerR, and NikR (35). Proteins within each structurally related family respond to the intracellular levels of essential metals such as manganese, iron, and zinc. The DtxR, Fur, and the less-characterized NikR family proteins predominantly regulate genes required for metal uptake, whereas ArsR/SmtB and MerR family proteins regulate metal efflux (35). Control of metal concentrations inside the bacterial cell is essential for survival and plays a key role in bacterial pathogenesis. For instance, a large number of bacterial toxins are expressed under metal-depleted conditions, and this expression is controlled by metal-dependent transcriptional regulators. In *Corynebacterium diphtheriae*, the etiological agent of the toxin-mediated upper respiratory tract infection diphtheria, DtxR, regulates transcription of the diphtheria toxin gene and multiple other genes involved in iron uptake (18, 38, 40). In addition to DtxR, the *C. diphtheriae* genome encodes a second DtxR-like protein, MntR, which responds to Mn (37, 38), and it is predicted to encode one Fur, three ArsR/SmtB, four MerR, and zero NikR homologs.

Iron-dependent global gene regulation is controlled by DtxR-like proteins in many gram-positive and acid-fast bacterial species, including *C. diphtheriae*, and by Fur-like proteins in many gram-negative bacteria. Although DtxR-like and Fur-like proteins share little sequence homology, they both act as transcriptional repressors when complexed with a cognate metal ion. In metal-depleted conditions, apo-Fur and apo-DtxR (metal-free forms) are unable to bind their target promoters, allowing transcription of the downstream genes (22). The Fur homolog in *C. diphtheriae* is uncharacterized but has similarity to a zinc-dependent regulator found in *Mycobacterium tuberculosis*, Zur (for zinc uptake regulator) (23).

Zinc is essential for cell survival, serving as a cofactor for more than 300 enzymes such as superoxide dismutase and alcohol dehydrogenase. It also functions as a structural scaffold for RNA polymerase, tRNA synthetases, and approximately 40 additional proteins (10, 11, 31, 41). In fact, *Escherichia coli* requires as much zinc as it does iron and calcium for cellular processes (31). In addition, zinc protects sulfhydryl groups from free radicals and inhibits free radical formation by competing with redox-active metals such as iron. Alternatively, high concentrations of zinc can be toxic by blocking thiols and binding of other metals to their cognate active sites within enzymes such as cytochrome *c* oxidase (3, 5, 26).

Unlike iron homeostasis, which has been extensively characterized (1), little research has focused on the uptake and/or storage of other metals, including zinc, by pathogenic bacteria. High-affinity zinc uptake is mediated by several ABC transporters, such as the Ycdhi-yceA and zinc uptake ABC systems (ZnuABC) (16). ZnuA encodes a periplasmic binding protein, ZnuB encodes an integral protein, and ZnuC encodes the ATPase of the transporter. In addition, YciC (CobW) may serve as a low-affinity zinc uptake protein or a metallochaperone (16). The total zinc concentration within bacteria is typically in the millimolar range; however, free unbound intracellular zinc concentrations at femtomolar levels are sufficient to trigger zinc uptake or efflux (31). Zinc uptake is controlled in a zinc-dependent manner by Zur in bacteria such as *E. coli*, *Bacillus subtilis*, and *M. tuberculosis* (14, 23, 34). The importance of zinc homeostasis in bacterial pathogenesis is only

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TABLE 1. Primers used in this study

Primer	Sequence (5′–3′)	Use
InterArsRFor	CCACGACGCTTACAGAGGTCGACGA	Cloning promoter for *arsR*-like gene
InterArsRev	TGATGATGGTCGTGGAGAAGGATCC	Cloning promoter for *arsR*-like gene
InterZurFor	GTTCTCAAGCAGGCACACATCGTCGAC	Cloning promoter for *zur*
InterZurRev	CCCCAACTTCGGGATCCAACGATC	Cloning promoter for *zur*
ArsRSalI	GTGAGATAAAACGTCGACGGAGGTT	Cloning and deletion of *zur*
Zurdelta1	CCCCAACTTCGGGCCCGAACGATC	Cloning and deletion of *zur*
Zurdelta2	CCGAAATCTAGGGCCCCTGCGAAAG	Cloning and deletion of *zur*
ZurSph	CGGCGATTATGTTGGCATGCCC	Cloning and deletion of *zur*
ZrgUpFor	GACGGGAGTCGACTGCGACATTC	Cloning promoter for *zrg*
ZrgUpRev	GCGGAAGATCTGACATGAAGG	Cloning promoter for *zrg*
ArsZurCoFor	AGACATCATTGCGAAAGTCG	Detection of *arsR-zur* cotranscript
ArsZurCoRev	AACGATCAATGGTGCGATTC	Detection of *arsR-zur* cotranscript
KQ-troafor	CGCAATACCATCAATGTTGC	qRT-PCR for *troA*
KQ-troarev	GGTGGTTGCTGGATCGTAGT	qRT-PCR for *troA*
KQ-arsfor	CTCCACGACCATCATCACTG	qRT-PCR for *arsR*-like gene
KQ-arsrev	CGAGTCGAGAGCACTCAAAA	qRT-PCR for *arsR*-like gene
KQ-cmrafor	TGTTGCAAAGTCCAGTGAGC	qRT-PCR for *cmrA*
KQ-cmrarev	ATACCAAAGAACGCCAATGC	qRT-PCR for *cmrA*
KQ-zrgfor	CCAAAGGACACTGCTGGATT	qRT-PCR for *zrg*
KQ-zrgrev	GAGAAGTTGCTGGGCTTGAG	qRT-PCR for *zrg*
KQ-zurfor	TCGTTGGGGTTCTCAAAGAT	qRT-PCR for *zur*
KQ-zurrev	TGATTGCAAGGTTCGGTACA	qRT-PCR for *zur*
KQ-gyrbfor	GGTCTGACCATTACGCTGGT	qRT-PCR for *gyrB*
KQ-gyrbrev	TCTTCTCGCGTTTCTTTGGT	qRT-PCR for *gyrB*

beginning to be characterized, but *Salmonella enterica* serovar Typhimurium mutants lacking Zur or ZnuC have decreased virulence in mice, indicating an essential role for zinc in this model of infection (7).

In the present study, we characterize the only *fur*-like gene in *C. diphtheriae* and demonstrate that it is a zinc-responsive regulator and is therefore more accurately described as *zur* (for zinc uptake regulator). In the presence of zinc, *C. diphtheriae* Zur represses transcription of three genes predicted to be involved in zinc uptake: *cmrA*, whose product is predicted to be anchored to the surface of the cell; *troA* (for transport related operon A), whose product has homology to metal uptake membrane proteins, such as *Streptococcus pneumoniae psaA* (16) and *Treponema pallidum troA* (17); and *zrg* (for zinc-regulated gene), which is similar to *yciC* (14). Interestingly, the transcription of *zur* itself is repressed in zinc-depleted conditions by a process that involves Zur. This observation indicates that Zur may have a role in controlling its own expression by a mechanism that is independent of its characterized activity as a zinc-dependent DNA-binding protein. The present study is the first molecular characterization of a zinc-dependent transcriptional regulator in *C. diphtheriae*, a paradigm for pathogenesis and gene expression in gram-positive and acid-fast organisms.

MATERIALS AND METHODS

Culture media, strains, and growth conditions. *C. diphtheriae* strains NCTC13129 (9), the sequenced isolate of the 1990s outbreak in the former Soviet Union, and C7(β) (2), a toxigenic strain used extensively for experimental work since the 1950s, were cultured in PGT, a casein hydrolysate medium (2). Metal ions were removed from the medium by treating with 10g/liter Chelex-100 (Bio-Rad, Hercules, CA) for 2 h, followed by filter sterilization (42). Where indicated, supplementation with specific metals was done at the following concentrations: 10 μM $FeCl_3$, 10 μM $MnCl_2$, 5 μM $CuSO_4$, and 25 μM $ZnSO_4$. To further chelate zinc remaining in the medium following treatment with Chelex-100, a zinc-specific chelator *N*,*N*,*N*′,*N*′-tetrakis (2-pyridylmethyl)-ethylene diamine (TPEN) was added to a concentration of 20 μM. *E. coli* TE1 (20) was used for cloning, and *E. coli* S17-1 (39), an RP4 mobilizing strain, was used as a donor for the conjugative transfer of plasmids into *C. diphtheriae*. *E. coli* was cultured in Luria-Bertani broth (LB) (25). Kanamycin, spectinomycin, and nalidixic acid were added at concentrations of 20, 100, and 20 μg/ml, respectively.

Deletion of *zur* in *C. diphtheriae* NCTC13129. To construct a deletion within *zur*, the plasmid pK19mobsacB, which contains an origin of replication that functions in *E. coli*, but not *C. diphtheriae*, was utilized (36). The extreme 5′ and 3′ ends of *zur* were amplified by using PCR (the primers are listed in Table 1), and the resulting DNA fragments were digested with SalI/ApaI and ApaI/SphI. The digested PCR fragments were ligated to SalI/SphI-digested pK19mobsacB to construct pK19mobsacBΔ*zur*. This plasmid, which includes a deleted *zur* gene (lacking 369 bp of the 426-bp gene), was transformed into *E. coli* S17-1 and then mated into NCTC13129 (44). Kanamycin-resistant transconjugants were counterselected for resistance to 10% sucrose, indicating the loss of integrated plasmid vector. A deletion of the *zur* gene on the chromosome of NCTC13129 was confirmed by PCR and sequencing, and this strain was named NCTC13129Δ*zur*.

Complementation of NCTC13129Δ*zur*. Full-length wild-type *zur*, along with its native promoter and ribosomal binding site, was amplified from NCTC13129 by using PCR (see primers in Table 1). The resulting fragment was digested with SacI and ligated into pKPIM (30), which had been identically digested to create pKPIM*zur*. Purified pKPIM*zur* was then transformed into S17-1 and transferred to NCTC13129 strains via conjugation. The plasmid pKPIM integrates site specifically at *attB2* in NCTC13129. Incorporation of the plasmid into the chromosome via an *attP* site was confirmed by detecting the recombinant *attL2* site created by integration of the plasmid using PCR (30).

Resistance to killing by hydrogen peroxide. Resistance to killing by hydrogen peroxide, H_2O_2, was assayed as described previously (28). Briefly, in the zone of inhibition assay, we measured the diameters of the zones of inhibition of bacterial growth when 20 μl of 1 M H_2O_2 was applied to 0.6-cm-diameter paper disks in the centers of plates containing heart infusion agar and lawns of various strains of *C. diphtheriae*.

In the percent killing assay, cultures were grown in PGT until the absorbance of the culture measured at 600 nm was between 1 and 2, at which time H_2O_2 was added to the growth medium at a final concentration of 10 mM, and the cultures were then incubated for an additional 10 min. Viable counts from each culture were then determined by plating dilutions of the culture onto heart infusion agar.

Cloning of promoter regions and β-galactosidase assays. PCR was used to amplify ~200 bp of the region upstream of *arsR* (dip1709), *zur* (dip1710), and *zrg* (dip1486) (see primers in Table 1). The resulting fragments were digested with the restriction enzymes BamHI/SalI (*arsR* and *zur*) or SalI/BglII (*zrg*) and then ligated into a similarly digested pSPZ (29), a reporter vector with a promoterless *lacZ* gene. The upstream regions of *cmrA* (dip2325) and *troA* (dip0438) were

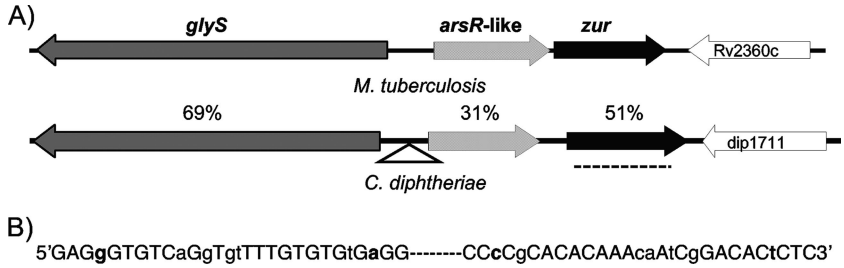


FIG. 1. Genetic arrangement of the C. *diphtheriae* and *M. tuberculosis* zur loci. (A) The dark gray arrow represents *glyS*, a glycyl-tRNA synthetase beta subunit. The gray striped arrow represents an *arsR*-like gene, a putative metal-dependent transcriptional regulator. The black arrow represents zur, a zinc-dependent transcriptional regulator. dip1711 and Rv2360c are genes with unknown function. The insertion site of the IS element in *C. diphtheriae* C7(β) is shown as a triangle, and the region deleted in the Δ*zur* strain is indicated as a dashed line. The percentages of amino acid sequence similarities between genes are indicated between the loci. (B) The sequences of the 28-bp inverted repeats at the ends of the IS element are shown. Bases that are interruptions in the inverted repeat are shown in lowercase, and the rest of the IS element is indicated as a dashed line.

digested and purified from pcmrA-PO (4) and pdip0438 (M. P. Schmitt, unpublished data), respectively, with the restriction enzymes SphI/XmaI and ligated into identically digested pSPZ. The constructs were transformed via electroporation into *C. diphtheriae* NCTC13129 and isogenic Δ*zur* strains. For the β-galactosidase assays, strains were inoculated in PGT containing different metal concentrations and incubated with shaking at 37°C overnight. Twenty micromolar TPEN was added to the medium in zinc depleted conditions. β-Galactosidase assays were performed on overnight cultures as previously described (25, 38). pSPZ*tox* (29) and pSPZ*cmrA* were used as positive controls for iron- and zinc-depleted conditions, respectively. The plasmid pSPZ*mntA* was constructed by cloning the *mntA* promoter containing fragment from pCM*mntA* (pPO3), and it was used as a control for manganese conditions (37).

Quantitative reverse transcriptase PCR (qRT-PCR). RNA was isolated, using the RNApro Blue kit (QBioGene), from NCTC13129, NCTC13129Δ*zur*, and C7(β) grown in zinc-replete and -depleted conditions. DNA was eliminated from RNA samples by using New England Biolab RNase-free DNase as described by the manufacturer. cDNA was synthesized from RNA templates by using the SuperScript III RT (Invitrogen). Negative controls contained RNase-free water substituted for RT. The cDNA generated was quantitated by quantitative PCR using a Bio-Rad iQcycler with the Absolute QPCR SYBR green fluorescein mix (Thermo Scientific). For generation of cDNA and quantitative PCR analysis, gene-specific primers were used (Table 1). Standard curves were constructed from serial dilutions of NCTC13129 genomic DNA. The transcript level of *gyrB* (a gene that is constitutively expressed in the tested conditions [data not shown]) was used as a control for RNA concentration. To determine the relative transcript quantity, the amount of gene-specific transcript was divided by the amount of *gyrB* transcript.

RESULTS

Characterization of the *zur* loci. The genome sequence of *C. diphtheriae* NCTC13129 contains a predicted coding sequence (dip1710) with similarity to genes encoding Fur-like regulators (12). A BLAST search confirmed that dip1710 is the only sequence in the genome with similarity to *fur*-like genes and that the Zur protein of *M. tuberculosis* is its closest characterized homolog. Based on its homology to the *M. tuberculosis* gene and the functional assays described below, we renamed dip1710 as *zur*. The genomic loci that include *zur* in *M. tuberculosis* and *C. diphtheriae* NCTC13129 are similar in structure (Fig. 1A). Upstream of *zur* in both organisms is a gene that encodes a putative metal-dependent transcriptional regulator similar to *arsR*. ArsR family proteins usually repress transcription of genes involved in metal efflux (6). In the *M. tuberculosis* genome, the *zur* gene slightly overlaps the 3′ end of the *arsR*-like gene, and these genes are cotranscribed from a single zinc-dependent promoter located upstream of the *arsR*-like gene (24). In contrast, there is a 179-bp intergenic region between the *arsR*-like gene and *zur* in *C. diphtheriae* (Fig. 1A). An aminoacyl-tRNA synthetase *glyS* and a gene with no identified homologs are directly upstream and downstream of *arsR-zur*, respectively. In the C7(β) strain of *C. diphtheriae*, which has been studied for many years as a model for gene regulation (2, 27, 28, 38), we identified an insertion sequence (IS element) upstream of the *arsR*-like gene. DNA sequencing of the region of the C7(β) genome containing the IS element (GenBank no. FJ470294) revealed that the 1,445-bp element includes a transposase whose coding sequence encompasses 1,350 bp. Inverted repeat sequences of 28 bp were found at the ends of the element (Fig. 1B), and its insertion resulted in the direct target site duplication of 8 bp (TTTCGATC). The IS element is located 78 bp upstream of the ATG codon for the *arsR*-like gene and 240 bp upstream of the GTG start codon of *glyS*. The effect, if any, of this IS element on expression of the genes at the C7(β) *zur* locus is unknown. Other than the insertion of the IS element, the gene organization in the *zur* loci are identical in NCTC13129 and C7(β).

Physiological importance of the Zur protein. To investigate the function of *C. diphtheriae zur*, a nonpolar deletion mutation was constructed in the genome of NCTC13129 using the mobilizable vector pK19mobsacBΔ*zur* (see Materials and Methods and Fig. 1), creating NCTC13129Δ*zur*. To complement this strain, we cloned wild-type *zur* into integration vector pKPIM (30), resulting in pKPIM*zur*. We then compared the growth curves of NCTC13129 to those of NCTC13129Δ*zur* in the presence or absence of zinc, iron, and manganese to determine whether the absence of Zur affects the growth of *C. diphtheriae*. Similar growth curves were observed for the NCTC13129 and NCTC13129Δ*zur* regardless of the presence or absence of 10 μM iron, 10 μM manganese, or 25 μM zinc (data not shown). Zinc concentrations of ≥200 μM inhibited growth of both the NCTC13129 and NCTC13129Δ*zur* strains of *C. diphtheriae* to comparable extents, demonstrating the toxic effect of high zinc concentrations on this organism (data not shown). Also, no differences were observed between NCTC13129 and NCTC13129Δ*zur* in protein profiles or cell morphology, via whole-cell protein lysates and light microscopy, respectively (data not shown). Thus, the absence of Zur had no detectable effect on *C. diphtheriae* in these experiments.

Since zinc has been shown to protect bacteria from oxidative

TABLE 2. Peroxide stress susceptibility of *C. diphtheriae* strains

Strain	Avg ± SD[a]	
	% of wild-type zone of inhibition	% Killing in exposure assay
C7(β)	100	38 ± 11
C7(β)Δ*dtxR*	170 ± 15*	68 ± 11*
NCTC13129/pKPIM	100	24 ± 5
NCTC13129/pKPIM*zur*	87 ± 7	18 ± 15
NCTC13129Δ*zur*/pKPIM	110 ± 8	50 ± 9*
NCTC13129Δ*zur*/pKPIM*zur*	91 ± 14	30 ± 10

[a] Values are given as averages for at least three samples. *, Statistically significant difference from the wild-type parent strain ($P < 0.05$) as determined by a one-way analysis of variance, followed by the Holm-Sidak method.

stress (13), we also tested the ability of NCTC13129 and NCTC13129Δ*zur* to survive challenge with H_2O_2. H_2O_2 susceptibility was determined by using two methods: growth inhibition assays and killing assays. Both C7(β) and C7(β)Δ*dtxR* strains have been assessed for levels of H_2O_2 stress resistance in previous studies and were used as controls (29). The NCTC13129Δ*zur* pKPIM strain showed a slightly larger zone of inhibition (Table 2) and a higher percentage of killing (Table 2) than both NCTC13129 pKPIM and complemented NCTC13129Δ*zur* pKPIM*zur*, indicating that it was more sensitive to H_2O_2 stress than its wild-type parent. Although the *P* value for the difference between the wild-type and Δ*zur* strain zones of inhibition was >0.05 (i.e., 0.08), a trend of higher peroxide sensitivity is observed in the *zur* mutant. In the killing assay, the difference between the Δ*zur* strain and the NCTC13129 pKPIM or NCTC13129Δ*zur* pKPIM*zur* strains had *P* values of <0.05 (0.01 and 0.034, respectively). Therefore, the absence of Zur in NCTC13129 resulted in a slight increase in sensitivity to H_2O_2.

Identification of the *zur* promoter(s). To identify the promoter(s) of *zur*, we cloned the region immediately upstream of *zur* into a β-galactosidase promoter reporter vector that replicates in *C. diphtheriae*, pSPZ (29). Since the promoter of the *M. tuberculosis zur* gene is located upstream of rv2358/*arsR* (Fig. 1, (24), the upstream region of the NCTC13129 *arsR*-like gene was also tested for promoter activity. In contrast to *M. tuberculosis*, *C. diphtheriae* NCTC13129 contains promoters directly upstream of both the *arsR*-like gene and *zur* (Fig. 2). We also investigated whether there was an *arsR*/*zur* cotranscript using RT-PCR with primers that annealed within the intergenic region (Table 1). No *arsR*-*zur* cotranscript was observed but transcripts that contained only the *arsR*-like gene or *zur* were detected (data not shown).

Regulation of *zur* transcription. In many organisms, transcription of metal-dependent transcriptional regulators is regulated in response to the presence of their cognate metal (24). Therefore, we tested the activity of the promoters upstream of the *arsR*-like gene and *zur* in the presence or absence of zinc. The promoter upstream of the *arsR*-like gene showed no significant difference in response to differential zinc conditions or to the presence of Zur (Fig. 2). In contrast, the *zur* promoter had lower activity in zinc depleted versus replete conditions in both the NCTC13129 and NCTC13129Δ*zur* pKPIM*zur* with *P* values <0.05 (<0.001). Strikingly, *zur* promoter activity remained high in the NCTC13129Δ*zur* background irrespective of the presence or absence of zinc, suggesting that Zur has a role in the regulation in its own expression. This phenomenon was confirmed by the observation that the zinc dependence of the *zur* promoter was restored in NCTC13129Δ*zur* pKPIM*zur* (Fig. 2). Both the *arsR*-like gene promoter and *zur* promoter activities were unaltered by the presence or absence of either iron or manganese (data not shown). These data indicate that the *zur* promoter is repressed specifically in zinc-depleted conditions in a Zur-dependent manner.

β-Galactosidase assays determine promoter strength without being impacted by mRNA stability, whereas qRT-PCR, although affected by transcript stability, more precisely quantifies the transcripts present at a single time point. Therefore, we used qRT-PCR to further analyze the *arsR*-like gene and *zur* transcript levels in NCTC13129 and NCTC13129Δ*zur* under zinc-replete and -depleted conditions. The *arsR*-like gene transcripts, although barely detectable, remained constant in NCTC13129 and NCTC13129Δ*zur* regardless of the zinc concentration, confirming the promoter fusion data described above (Fig. 3A and C). In contrast, the *zur* transcript level was 4.5-fold higher in zinc-depleted conditions (Fig. 3B and C), which correlated with the observed promoter activity (Fig. 2). The *zur* transcript was only assayed in NCTC13129 because the majority of the *zur* gene was deleted in NCTC13129Δ*zur*, preventing its detection in this assay. With a weakly transcribed constitutive promoter, the accumulation of β-galactosidase may indicate a higher promoter activity compared to transcript levels at a single time point detected in qRT-PCR data (compare *arsR* in Table 3 and Fig. 2).

To determine whether zinc-dependent regulation of *zur* occurs in other strains of *C. diphtheriae*, transcription of *zur* and the *arsR*-like gene was assayed in the C7(β) strain by using qRT-PCR. The transcript levels of *arsR* were lower in C7(β) than in NCTC13129 but remained unchanged in zinc-replete and -depleted conditions (Table 3). Interestingly, not only was the transcript of *zur* at lower levels in C7(β), the differential regulation observed between zinc-replete and -depleted con-

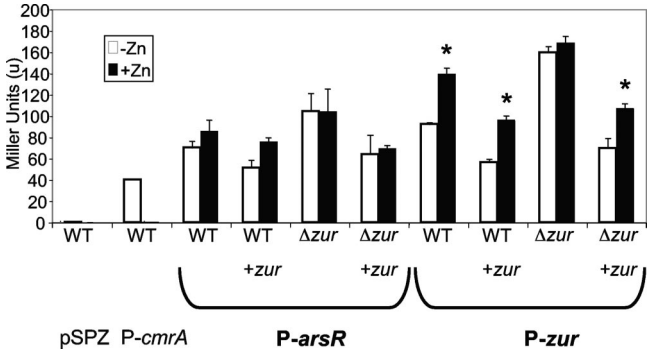
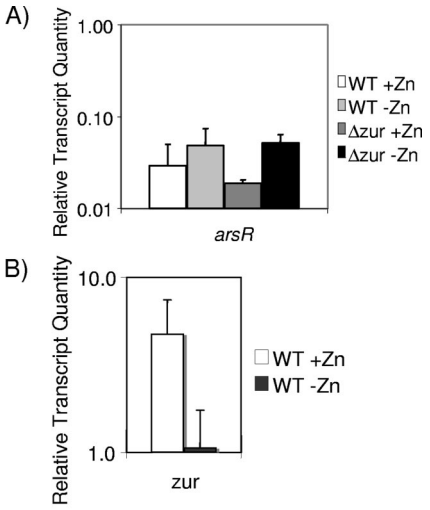


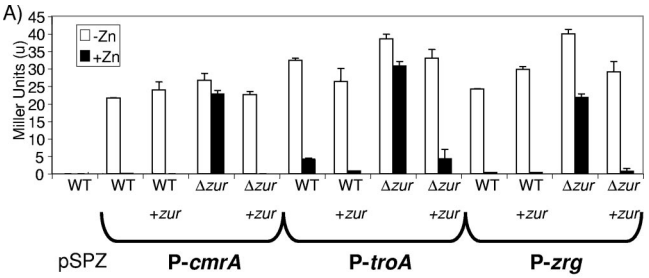
FIG. 2. *arsR* and *zur* promoter activity. NCTC13129 (indicated as wild type [WT]) and NCTC13129Δ*zur* (indicated as Δ*zur*) strains with either vector control, pKPIM, or complementing wild-type *zur*, pKPIM*zur* (shown as +*zur*), were inoculated into zinc-replete (+Zn) or -depleted (–Zn) conditions and tested for *arsR* and *zur* promoter activity. β-Galactosidase activity is expressed in Miller units. The *cmrA* promoter is a positive control for zinc-depleted conditions. The asterisks denote statistical significance (<0.05) between zinc-replete and -depleted conditions, as determined by a one-way analysis of variance, followed by the Holm-Sidak method.



C)

	WT +Zn	WT -Zn	Δ*zur* +Zn	Δ*zur* -Zn
arsR	-	1.7	-1.6	1.8
zur	-	-4.5	NA	NA

FIG. 3. Detection of *arsR* and *zur* transcripts. RNA isolated from NCTC13129 (WT) and NCTC13129Δ*zur* (Δ*zur*) in zinc-replete and -depleted conditions was analyzed for relative transcript levels (normalized to *gyrB* mRNA levels) of *arsR* (A) and *zur* (B). (C) Fold change of these genes from NCTC13129 in zinc-replete conditions.



B)

	+Zn/+Fe	+Zn/-Fe	-Zn/+Fe	-Zn/-Fe
tox	0.3 ± 0.2	52 ± 4	1.8 ± 1	66 ± 6
zrg	0.03 ± 0.04	0.04 + 0.06	34 ± 1	51 ± 3

C)

	+Zn/+Mn	+Zn/-Mn	-Zn/+Mn	-Zn/-Mn
mntA	1.6 ± 0.1	115 ± 3	1.9 ± 0.1	200 ± 11
cmrA	0.45 ± 0.02	0.32 ± 0.4	21 ± 1	120 ± 7

FIG. 4. Activity of Zur-regulated promoters. NCTC13129 (indicated as WT) and NCTC13129Δ*zur* (indicated as Δ*zur*) strains with either vector control, pKPIM, or complementing wild-type *zur*, pKPIM*zur* (shown as +*zur*), inoculated into zinc-replete (+Zn) or -depleted (–Zn) conditions were used to test the activity of promoters (*zrg*, *cmrA*, and *troA*) in the reporter plasmid pSPZ. (A) The β-galactosidase activity was determined under differential conditions. The activity of the *zrg* promoter under iron and zinc stress conditions (B) and activity of the *cmrA* promoter under manganese and zinc stress conditions (C) are also presented. The promoters of *tox*, *mntA*, and *cmrA* genes (cloned into the reporter vector) were used as positive controls for iron-, manganese-, and zinc-depleted conditions, respectively.

ditions was not seen in this strain. Thus, the regulation and accumulation of the *zur* transcript differed in these *C. diphtheriae* strains.

Transcriptional regulation by Zur. We performed a BLAST search of the *C. diphtheriae* genome to identify genes that may be regulated by Zur. Sequence probes that were used for the BLAST search included genes that are known to be regulated by Zur in other bacteria and genes that encode proteins involved in metal transport. This analysis identified a gene we named *zrg* (dip1486), with homology to *cobW*/*yciC* genes from a variety of bacterial species and which encodes a putative low-affinity zinc uptake protein, as well as a gene we named *troA* (dip0438), with sequence similarity to *T. pallidum troA* and to *S. pneumoniae psaA*, that is predicted to encode a component of an ABC-type metal transporter. The *cmrA* gene, which was previously shown to be zinc regulated (4), is predicted to encode a sortase anchored cell wall protein. The mechanism of the zinc-dependent repression of *cmrA* has not previously been investigated. We thus sought to determine whether Zur had a role in regulating transcription of these three genes.

We cloned the upstream regions of *zrg*, *cmrA*, and *troA*, into the promoter reporter vector pSPZ and assayed the activity of each in NCTC13129 and NCTC13129Δ*zur*. All three promoters were repressed in zinc-replete conditions and derepressed in zinc-depleted conditions in NCTC13129 (Fig. 4A). In NCTC13129Δ*zur*, the activity of all three promoters was constitutive in response to zinc. Zinc-dependent promoter activity was restored when *zur* was provided from pKPIM*zur* in NCTC13129Δ*zur*. These data indicate that the activities of these three promoters were controlled by Zur in response to changes in zinc concentration.

We tested each of the promoters for activity in the presence or absence of iron and manganese. When the activity of each promoter was compared under iron-replete or -depleted conditions in the wild-type NCTC13129 strain, no activity was observed. Identical results were obtained when manganese was substituted for iron (Fig. 4B and C, first two columns). This result was not unexpected since the assay medium contained zinc, which represses the expression of all three promoters. Interestingly, we did observe higher levels of activity from the *zrg* promoter under iron-depleted conditions in the NCTC 13129Δ*zur* strain (23 Miller units in iron-replete medium versus 33 Miller units in iron-depleted medium). Similarly, we

TABLE 3. Detection of *arsR* and *zur* transcripts

Gene	Strain	Relative transcript quantity (avg ± SD)[a]		Fold change[b]
		+Zn	–Zn	
arsR	C7(β)	0.01 ± 0.00	0.01 ± 0.00	<2
	NCTC13129	0.03 ± 0.02	0.05 ± 0.03	<2
zur	C7(β)	0.58 ± 0.13	0.56 ± 0.06	<2
	NCTC13129	4.8 ± 2.7	1.1 ± 0.67	4.5

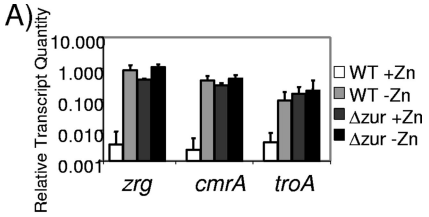
[a] Normalized with *gyrB*. Values are averages for at least three samples.
[b] The fold change is calculated as +Zn/–Zn.

observed higher activity from the *cmrA* promoter in manganese-depleted medium in the NCT13129Δ*zur* strain background (28 Miller units in manganese-replete versus 92 Miller units in manganese-depleted medium). The activity of the promoter for *troA* was unchanged when the medium was depleted of either iron or manganese in the NCTC13129Δ*zur* background. These observations led us to hypothesize that the effects of iron and manganese on the *zrg* and *cmrA* promoters, respectively, might be epistatic to the effects of zinc and Zur.

We next examined the activities of the *zrg* and *cmrA* promoters in medium depleted of both iron and zinc or both manganese and zinc. As shown in Fig. 4B (columns 3 and 4), depletion of both iron and zinc resulted in a 1.5 increase in the activity of the *zrg* promoter compared to the activity in medium depleted only for zinc. As a control we used the well-characterized *tox* promoter, whose activity is responsive only to iron and not to zinc (38). The activity of the *cmrA* promoter increased sixfold when the growth medium was depleted of both zinc and manganese compared to activity in medium depleted only of zinc (Fig. 4C, columns 3 and 4). For this assay, we used the *mntA* promoter as a control for manganese-dependent regulation since it is controlled by MntR in response to manganese (37). Interestingly, the *mntA* promoter showed an increase in activity when the growth medium was depleted of both manganese and zinc (compared to medium lacking only manganese), indicating that MntR-dependent manganese regulation is epistatic to the effect of zinc.

Our observations imply that the *zrg* promoter is controlled first by zinc in a process that requires Zur and secondarily by the presence of iron in a process that is independent of Zur. The *cmrA* promoter is controlled first by zinc and Zur and secondarily by manganese in a Zur-independent mechanism. Clearly, the regulation of the *zrg*, *cmrA*, and *mntA* promoters is more complex than the one-metal, one-regulator models proposed for many metal-dependent promoters.

To confirm that the transcript levels correlated with the promoter activity, we used qRT-PCR to assay *cmrA*, *zrg*, and *troA* transcription in NCTC13129 and NCTC13129Δ*zur* under zinc-replete and -depleted conditions. The transcript levels of all three genes were significantly lower in NCTC13129 under zinc-replete conditions than the levels in NCTC13129 in zinc-depleted conditions and in NCTC13129Δ*zur* in all conditions (Fig. 5A). The fold change was calculated by dividing all transcript levels by the corresponding NCTC13129 wild-type strain transcript level under zinc-replete conditions (Fig. 5B). The largest transcript level fold change was observed for *zrg* with an average of 250. The *cmrA* transcript had an average of a 170-fold change, and the *troA* transcript level had an average fold change of 36. Large differences between the fold changes of *zrg*, *cmrA*, and *troA* transcript levels correlate with the differences in transcripts present under induced conditions (i.e., zinc depleted or lacking Zur), rather than a difference in the repression or uninduced state (i.e., zinc replete and wild-type Zur levels). These data confirm that Zur represses the transcription of *zrg*, *cmrA*, and *troA* in zinc-replete conditions.



B)

	WT +Zn	WT -Zn	Δzur +Zn	Δzur -Zn
zrg	-	246	122	309
cmrA	-	172	122	203
troA	-	23	36	47

FIG. 5. RNA levels of Zur-regulated genes. RNA isolated from NCTC13129 and NCTC13129Δ*zur* under zinc-replete and -depleted conditions was analyzed by using qRT-PCR. (A) Relative level of transcripts (normalized to *gyrB* mRNA levels) of *cmrA*, *zrg*, and *troA*. (B) Fold change of these genes from NCTC13129 under zinc-replete conditions.

DISCUSSION

To regulate metal homeostasis, bacteria use multiple metal-dependent transcriptional regulators, each controlling different regulons in response to different inducers. We describe here the characterization of a zinc-dependent Fur family homolog in *C. diphtheriae*. To our knowledge, Zur, is the first zinc-dependent transcriptional regulator described in *Corynebacteria*. We identified three genes, *cmrA*, *zrg*, and *troA* whose transcription is repressed by Zur. In addition, we demonstrated that the *zur* gene is transcribed from a promoter that is repressed by Zur when zinc is scarce. Our data also indicate that *C. diphtheriae* is capable of integrating signals from different metals to exert exquisite control over the transcription of specific genes.

Although *C. diphtheriae* lacking Zur do not have a growth defect compared to the wild-type, all *C. diphtheriae* strains exhibited slowed growth rates in medium containing concentrations of zinc greater than 200 μM, thus indicating that zinc toxicity occurs in *C. diphtheriae*. Due to uncontrolled uptake of zinc, one might expect the *C. diphtheriae zur* mutant strain to be more sensitive to high concentrations of zinc than its wild-type parent, as is observed for *Xanthomonas campestris zur* mutants (43). This was not the case. In *X. campestris* Zur controls the expression of both zinc uptake and efflux; thus, when Zur is absent, *X. campestris* not only undergoes unregulated uptake of zinc, but it also fails to activate zinc efflux systems (19). Our data suggest that, unlike *X. campestris* Zur, *C. diphtheriae* Zur is not required for the activation of zinc efflux. In addition, given the roles of zinc as an antioxidant and as a cofactor for enzymes such as superoxide dismutase, we tested the ability of a Δ*zur* mutant strain to survive challenge with H_2O_2. The *C. diphtheriae* NCTC13129Δ*zur* strain demonstrated an increased susceptibility to peroxide stress, suggesting that Zur is required for expression of oxidative stress defenses.

Interestingly, in *C. diphtheriae* NCTC13129, *zur* transcript levels are affected by zinc concentrations, but the levels of the adjacent *arsR*-like gene transcript are not. This distinguishes *C. diphtheriae zur* from its homolog in *M. tuberculosis*, where the ArsR-like protein represses the *arsR-zur* cotranscript in response to zinc-depleted conditions (8, 24). In addition, the

repression of *zur* transcription in *C. diphtheriae* NCTC13129 in zinc-depleted conditions is dependent on the presence of Zur. These variations highlight important differences and suggest that Zur may play different roles in the physiology of these two species. The regulation of *C. diphtheriae zur* transcription by zinc and Zur is the first indication of Zur activity in zinc-depleted conditions. However, in other species, Fur proteins have been shown to exhibit uncharacteristic activity, such as functioning as a direct DNA-binding transcriptional activator in *Neisseria meningitidis* and as a repressor in the absence of metal in *Helicobacter pylori* (22). DNA binding experiments are currently in progress to determine whether this effect on the *zur* transcript is a result of direct Zur binding to its promoter.

We observed differences in transcription of *zur* in *C. diphtheriae* strains C7(β) and NCTC13129, demonstrated by both the absence of zinc-dependent *zur* regulation and the lower overall *zur* transcript levels in C7(β). Using qRT-PCR, the Zur-regulated genes *zrg*, *cmrA*, and *troA* were all shown to be regulated by zinc and Zur in C7(β) and, unlike the finding for the *zur* transcript levels, the transcript levels of *zrg*, *cmrA*, and *troA* in C7(β) were all extremely similar in NCTC13129 under identical growth conditions (data not shown). It is unlikely that the zinc stress conditions used were significantly different or that transcription of all genes is generally lower in C7(β) compared to NCTC13129. Although the IS element upstream of *arsR* in C7(β) could result in lower *arsR* transcription, it is unlikely that this IS element is affecting *zur* transcription directly, since *zur* has its own promoter. In addition, we determined the DNA sequence of the region between *arsR* and *zur* in C7(β), and it was identical to that in NCTC13129. There remains the possibility that there are other differences between these strains that account for the loss of zinc-dependent regulation of *zur* transcript in C7(β), including unknown *trans*-acting factors. C7(β) is a lab strain originally isolated in the 1950s, while NCTC13129 is a recent clinical isolate representative of an outbreak that occurred in the late 1990s (2, 9). The differences in *zur* regulation and in the sequence of the loci in these two strains highlight the value of assaying recent clinical isolates and the importance of genomic sequencing of multiple strains of a single bacterial species.

In both strains of *C. diphtheriae* the *zrg* transcript is strongly repressed in high zinc conditions in a Zur-dependent manner. This is also the case for its homologs in *B. subtilis* and *M. tuberculosis* (14, 23, 32). Sequence homology suggests that Zrg is a low-affinity zinc transporter protein, or a metallochaperone, that "passes" zinc ions between transport proteins and enzymes. Our data support the notion that Zrg is a low-affinity zinc transporter protein given that repression of a zinc chaperone under high zinc concentrations would not likely be of value to the cell. The evidence that Zrg is a zinc transporter is supported by observations that the *B. subtilis* Zrg homolog YciC is important for zinc utilization (14, 15). Similar to *zrg*, the transcript of *cmrA* is highly repressed by Zur under zinc-replete conditions in *C. diphtheriae*. Based on this observation and the prediction that *cmrA* encodes a sortase anchored cell wall protein, we hypothesize that CmrA is a surface protein associated with an ABC transporter involved in the uptake of zinc.

The final *C. diphtheriae* gene that we characterized was *troA*, which is repressed by Zur in zinc-replete conditions. In contrast to the observations with *cmrA* and *zrg*, *troA* promoter activity was observed in zinc-replete conditions, albeit at low levels (Fig. 5A). We also observed that induction of *troA* transcription occurs in the presence of higher concentrations of zinc than the transcription of either *zrg* or *cmrA* (data not shown). These observations suggest that the *troA* promoter is released from Zur repression when the concentrations of zinc are sufficient to allow binding of Zur at the *zrg* and *cmrA* promoters. The *troA* gene encodes a membrane protein with homology to *S. pneumoniae* PsaA and is the first gene in a seven gene operon. The genes of this operon are predicted to encode the components of an ABC transporter, a putative surface-anchored sortase protein, and three putative membrane proteins of unknown function. Some of these genes have homology to genes in the ZnuABC metal uptake machinery family. The similar gene cluster in *S. pneumoniae* is thought to be a manganese transporter, but recent evidence suggests that it may transport zinc (16). In addition to its role in metal ion transport, *S. pneumoniae* PsaA also contributes to virulence and oxidative stress resistance (21).

One of the most intriguing findings from our investigation of *C. diphtheriae* Zur-dependent gene regulation is the identification of two genes whose transcription is affected by more than one metal ion. Zur is required for the zinc-dependent primary regulation of both *zrg* and *cmrA*. We demonstrated that the *zrg* promoter is secondarily regulated by the availability of iron and that the *cmrA* promoter is secondarily regulated by the presence of manganese. In both cases, the secondary regulation is independent of Zur, suggesting that a second metal-dependent regulator may be involved. There are examples of bacterial promoters regulated by more than one metal-dependent regulator, including the promoter for rv0282, a gene of unknown function in *M. tuberculosis*, which undergoes dual zinc- and iron-dependent regulation by Zur and IdeR (a DtxR homolog), respectively (23). Similarly, *E. coli mntH*, a homolog of the eukaryotic natural resistance associate macrophage protein (NRAMP), is regulated by iron and manganese by mechanisms that require Fur and MntR (33). Since *zrg* and *cmrA* are secondarily regulated by iron and manganese, respectively, we hypothesize that DtxR, the only iron-dependent transcriptional regulator, and MntR, the only manganese-dependent transcriptional regulator characterized in *C. diphtheriae*, are involved. The binding sites for DtxR and MntR have been characterized (37, 38), and we have searched the regions upstream of *zrg* and *cmrA* for sequences with similarity to these sites. The DNA region with highest similarity (10/19) to the consensus DtxR binding site (TTAGGTTAGGCTAACC TAA) upstream of *zrg* is 74 bp upstream of the start codon (Fig. 6). The binding site for MntR is less well characterized (37), but there are two possible binding motifs for MntR upstream of *cmrA*. A five-out-of-seven match to the direct repeat (TGAACAA) found in the MntR binding site is located 19 bp upstream, and a 19-bp sequence with 63% identity to the MntR inverted repeat is located 3 bp upstream of the *cmrA* start codon (Fig. 6). The roles of MntR and DtxR in controlling transcription of *cmrA* and *zrg* are under active investigation.

The binding sites for *C. diphtheriae* Zur have not been defined, but given its similarity to *M. tuberculosis* Zur, we searched regions upstream of *cmrA*, *zrg*, and *troA* for sequences

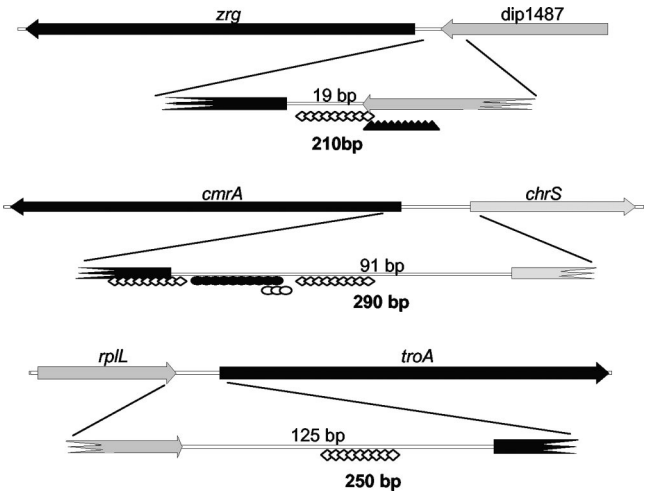


FIG. 6. Regulator binding sites in the upstream regions of *zrg*, *cmrA*, and *troA*. The locations of the putative binding sites for Zur (diamonds), DtxR (triangles), and MntR (black and white circles) are indicated for the *zrg*, *cmrA*, and *troA* promoter regions. The black circles indicate the inverted repeat, and the white circles indicate the direct repeat in the MntR binding site The lengths of the DNA fragment used in the promoter assays (Fig. 5) are shown in boldface below each region, and the distance between adjacent genes is shown in normal typeface above each region. The diagram is not drawn to scale.

similar to the 21 bp at the center of the *M. tuberculosis* Zur binding site (TATTGAAAATNATTTTCAATA) (23). There are two putative Zur binding sites upstream of *cmrA* (Fig. 6). The first site overlaps the start codon (running from +19 to −2) and matches the *M. tuberculosis* consensus site at 16 out of 20 conserved positions. The second site is located 26 bp upstream of the *cmrA* start codon and is a 65% match to the consensus. We identified a single putative Zur binding site located 5 bp upstream of the *zrg* start codon that matches the consensus sequence at 13 of 20 positions (Fig. 6). Finally, upstream (70 bases from the start codon) of *troA* there is a single putative Zur binding site (14 of 20 bases identical). The locations of the putative Zur binding sites upstream of *cmrA*, *zrg*, and *troA* are consistent with the notion that they overlap the promoter sequences in these regions.

In summary, we have characterized zinc-dependent Zur regulation of three genes and the interplay between zinc and other metals in controlling transcription in *C. diphtheriae*. The positions of the putative binding sites for Zur, DtxR, and MntR in the promoter regions of *zrg*, *cmrA*, and *troA* is suggestive that the regulators act at these promoters. DNA-binding assays are in progress to confirm the roles of each regulator. In addition, we characterized the transcription of *zur* and observed that, unlike most other *fur* family genes, *zur* is repressed under low-zinc conditions in a process requiring Zur (in strain NCTC13129). Finally, we observed increased sensitivity of the *C. diphtheriae zur* mutant strain to H_2O_2 stress and, although the exact mechanism of this sensitivity is unknown, this indicates a role for Zur both in metal homeostasis and in protection against host defenses that utilize oxygen radicals to kill pathogenic bacteria.

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