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CztR, a LysR-Type Transcriptional Regulator Involved in Zinc Homeostasis and Oxidative Stress Defense in *Caulobacter crescentus*[▽]

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***Caulobacter crescentus* is a free-living alphaproteobacterium that has 11 predicted LysR-type transcriptional regulators (LTTRs). Previously, a *C. crescentus* mutant strain with a mini-Tn*5lacZ* transposon inserted into a gene encoding an LTTR was isolated; this mutant was sensitive to cadmium. In this work, a mutant strain with a deletion was obtained, and the role of this LTTR (called CztR here) was evaluated. The transcriptional start site of this gene was determined by primer extension analysis, and its promoter was cloned in front of a *lacZ* reporter gene. β-Galactosidase activity assays, performed with the wild-type and mutant strains, indicated that this gene is 2-fold induced when cells enter stationary phase and that it is negatively autoregulated. Moreover, this regulator is essential for the expression of the divergent *cztA* gene at stationary phase, in minimal medium, and in response to zinc depletion. This gene encodes a hypothetical protein containing 10 predicted transmembrane segments, and its expression pattern suggests that it encodes a putative zinc transporter. The *cztR* strain was also shown to be sensitive to superoxide (generated by paraquat) and to hydrogen peroxide but not to *tert*-butyl hydroperoxide. The expression of *katG* and *ahpC*, but not that of the superoxide dismutase genes, was increased in the *cztR* mutant. A model is proposed to explain how CztR binding to the divergent regulatory regions could activate *cztA* expression and repress its own transcription.**

Some metals, such as iron, cadmium, chromium, lead, nickel, copper, and mercury, produce reactive oxygen species (ROS) in the cell, causing damage to the membranes, proteins, or DNA (35). ROS can be generated endogenously, by the oxidation of components of the respiratory electron chain (22), or by exposure to metals, oxidant agents, or radiation in the presence of oxygen (36). Oxidative stress is caused by the accumulation of ROS or the depletion of antioxidant molecules or enzymes, and the response to oxidative stress aims to prevent, counteract, or repair the damage caused by ROS (36). Exposure to metals and oxidative stress species can trigger a common response, as has been established in *Xanthomonas campestris*. When this bacterium is exposed to low concentrations of cadmium, it triggers cross-protection against the loss of viability caused by H_2O_2 or tBOOH (*tert*-butyl hydroperoxide). This cross-protection results from increases in the levels of antioxidant enzymes, such as alkyl hydroperoxide reductase (Ahp), catalase (KatA), and organic hydroperoxide resistance protein (Ohr) (1).

Zinc is an essential metal that has an important role in the catalytic activity and structural stability of several enzymes and DNA-binding proteins, but it is toxic when in excess. Bacteria need to strictly regulate the levels of this ion, and in Gram-negative bacteria, zinc homeostasis is achieved by controlling uptake and efflux systems that respond to intracellular zinc concentrations (11). In *Escherichia coli*, zinc uptake is performed mainly by ABC transporters, such as the high-affinity system ZnuABC (26), but other systems also contribute, such as the low-affinity zinc-phosphate cotransporter Pit (2) and the broad-spectrum substrate transporter ZupT (9), both of which work when there are medium levels of zinc in the environment. In *E. coli*, *znuABC* is repressed by the transcriptional repressor Zur at high zinc concentrations, and the system is derepressed at low intracellular zinc levels (25). When zinc is present in excess, efflux systems of the resistance-nodulation-cell division (RND) family of CzcABC-like transporters, cation diffusion facilitators (CzcD-like), and P-type ATPases, such as ZntA, are activated (11). In *X. campestris*, Zur both represses genes for zinc uptake and activates genes encoding efflux pumps by binding directly to their regulatory regions (13).

Previous work from our group identified an efflux system of the RND family that is required for the resistance of *Caulobacter crescentus* to high concentrations of cadmium and zinc (4). This operon (*czcABC*) is next to a gene encoding a cation P-type ATPase, and the expression of the *czc* operon is induced by cadmium and zinc. A second cluster of genes encoding an efflux system upregulated under cadmium stress, which could also play a minor role in maintaining zinc homeostasis, was identified by global analysis of gene expression (12).

The *Caulobacter* genome presents 11 putative transcriptional regulators belonging to the LysR family (LysR-type transcriptional regulators [LTTRs]). The IlvR protein was the only LTTR studied so far in *C. crescentus*. IlvR positively regulates the expression of the *ilvD* gene, which is involved in isoleucine/valine biosynthesis, and negatively autoregulates its own expression; however, the coinducer necessary for its activation was not identified (18). Members of this family usually have diverse cellular functions and bind DNA through a conserved

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
Strains		
E. coli		
S17-1λpir	*E. coli* strain for plasmid mobilization	32
DH5α	*E. coli* strain for cloning purposes	10
C. crescentus		
NA1000	Synchronizable derivative of wild-type CB15	6
SP2012	NA1000 Δ*cztR*::Ω*spec*	This study
SP2012 pUJ*cztR*	NA1000 Δ*cztR*::Ω*spec* (pUJ142 *cztR*)	This study
SP2012 pMR*cztR*	NA1000 Δ*cztR*::Ω*spec* (pMR20 *cztR*)	This study
SP2014	NA1000 Δ*cztA*::Ω*spec*	This study
SP2014 pMR*cztA*	NA1000 Δ*cztA*::Ω*spec* (pMR20 *cztA*)	This study
Plasmids		
pGEM-T Easy	Cloning vector; Amp^r	Promega
pNPTS138	Suicide vector containing *oriT* and *sacB*; Kan^r	D. Alley
pHPΩ45	Vector containing a $Spec^r$ cassette	28
pUJ142	Xylose-inducible promoter; Chl^r	21
pRK*lacZ*290	pRK2-derived vector with a promoterless *lacZ* gene; Tet^r	7
pMR20	Broad-host-range, low-copy-number vector; Tet^r	29

N-terminal domain in response to the binding of a coinducer, usually a small molecule. These transcription factors are usually negatively autoregulated and can activate or repress the expression of the divergent gene, although some members of this family act as global regulators (17, 19). Moreover, some regulators can have a dual function; an example is *Bacillus subtilis* GltC, which, depending on the nature of the coinducer, acts as a transcriptional activator or repressor (17, 27).

In this work we characterized a *C. crescentus* LTTR that was previously isolated in a screening of a transposon library for mutants sensitive to cadmium (4). Three independent insertions were obtained in this gene (CC3510), and the mutant strains were shown to be highly sensitive to Cd^{2+} and moderately sensitive to Zn^{2+} and Cu^{2+} (4). Here we have named this gene *cztR*, for *Caulobacter* zinc transport regulator, for reasons that will be discussed throughout this work. We constructed a *cztR*-null mutant strain and demonstrated that it is sensitive to paraquat and to H_2O_2, but not to *tert*-butyl-hydroperoxide. Expression analysis showed that *cztR* is induced at stationary phase and is negatively autoregulated. CztR binds to the regulatory region between *cztR* and the divergent gene, here named *cztA*, and is necessary for *cztA* induction at stationary phase, in minimal medium, and in response to zinc depletion. A *cztA*-null mutant was generated, and phenotypic characterization suggests that it encodes a putative zinc transporter protein necessary for growth under low-nutrient conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this work are described in Table 1. *C. crescentus* was grown in PYE or M2 minimal medium (5) at 30°C with agitation, and media were supplemented with 20 μg/ml nalidixic acid, 1 μg/ml tetracycline, 5 μg/ml kanamycin, 1 μg/ml chloramphenicol, or 20 μg/ml spectinomycin as necessary. *E. coli* strains were grown in LB medium, supplemented with 12.5 μg/ml tetracycline, 50 μg/ml kanamycin, 30 μg/ml chloramphenicol, 50 μg/ml spectinomycin, or 100 μg/ml ampicillin as necessary.

Growth of strains NA1000 and SP2012 in liquid PYE and M2 media was carried out in 10 ml of each medium at 30°C with agitation. Preinocula grown in the same medium were inoculated into fresh medium to obtain an initial optical density at 600 nm (OD_{600}) of 0.1 to 0.2, and growth was monitored by measuring absorbance at 600 nm at several time points. Experiments were performed at least in triplicate.

Construction of *cztR*- and *cztA*-null mutant strains and complementation. Plasmids used in all cloning procedures are listed in Table 1, and primers are listed in Table 2. *cztR* and *cztA* were deleted by allelic replacement of each gene by a spectinomycin resistance cassette obtained from plasmid pHPΩ45. Two fragments containing the regions downstream and upstream of the *cztR* gene (1,160 bp and 620 bp, respectively) were amplified by PCR from genomic DNA with primers Lys1/Lys2 and Lys3/Lys4, and the products were cloned sequentially into the pGEM-T Easy vector. The resultant 1.8-kb SalI/PstI fragment was then cloned into the pNTPS138 suicide vector, and the 2.0-kb Ω*spec* cassette was inserted between the two fragments. The same was done for the *cztA* gene: a 2.0-kb HindIII/SalI fragment resulting from the ligation of two 1.0-kb *cztA*-flanking fragments (amplified with primers Lys7/Lys12 and Lys9/Lys11) was cloned into pNPTS138 and was disrupted by the Ω*spec* cassette. The pNPTΔ*cztR*::Ω*spec* and pNPTΔ*cztA*::Ω*spec* plasmids were then introduced into *C. crescentus* NA1000 by conjugation with *E. coli* S17-1, and Kan^s $Spec^r$ clones generated by double recombination were selected, generating strains SP2012 (Δ*cztR*::Ω*spec*) and SP2014 (Δ*cztA*::Ω*spec*). Gene replacements were confirmed by PCR and Southern blot experiments.

TABLE 2. Primers used in this study

Name	Sequence (5′ → 3′)[a]
Lys1	G**GAATTC**CCGGCCGGGATGACGAGAGG
Lys2	GCGTCACCGGCGAAGATTTGCTGCGC
Lys3	G**GAATTC**CAGATGGTGCGGCGCGG
Lys4	**CTGCAG**CGCGGGTCGTCAAGGCGGCG
Lys5	C**CTGCAG**GCCCGCCGCCTTCAG
Lys6	G**AAGCTT**CCAGATTGGTGCGGCGCG
Lys7	G**AAGCTT**CTATATCGCCCGCGCCTTTGA
Lys8	G**GAATTC**ATGCCGCGCCGCACCAATC
Lys9	**GGAATTC**GAAACGGGTTCGAGGACC
Lys11	CGTCCTCGCAACGTCGACCG
Lys12	C**GGAATTC**GAACGTCCACGAAAATGC
Lys14	C**AAGCTT**GTGAAACTGCGCTGCTCG
Lys15	CG**CTCGAG**GGGACGATCCTACCCTC
AhpC1	CCGAGATCAAACCCTTTACCGCCCAG
AhpC2	CCCACTTGGCCGGGCAGACTTCGCCC

[a] Boldface letters indicate restriction enzyme recognition sites, used for cloning purposes.

Each mutation was complemented by cloning a 1.26-kb *cztR* DNA fragment (amplified with primers Lys5/Lys7) or a 1.2-kb *cztA* DNA fragment (amplified with primers Lys14/Lys15) containing the promoter and coding regions of each gene into the pMR20 plasmid, generating pMR*cztR* and pMR*cztA*, respectively. A second vector containing the *cztR* gene (amplified with primers Lys7/Lys8) under the regulation of a xylose-inducible promoter was constructed by cloning the *cztR* gene into plasmid pUJ142, generating pUJ*cztR*, and expression of the gene was induced by adding 0.2% xylose.

Determination of the *cztR* transcription start site. The transcription start site (TSS) of *cztR* was determined by primer extension assays using a ^{32}P-labeled primer and total RNA from strain NA1000. Total RNA was isolated from exponential-phase cells with the Trizol reagent by following the manufacturer's instructions. Primer Lys3 was end labeled with 30 μCi [γ-^{32}P]ATP using the T4 polynucleotide kinase enzyme (GE Healthcare Life Sciences) and was extended with SuperScript III reverse transcriptase (Invitrogen). The DNA sequencing ladder was obtained by cycle sequencing with the M13 universal primer and the M13 single-stranded DNA (ssDNA) as the template by using the Thermosequenase cycle sequencing kit (USB).

Cloning of the promoter regions and gene expression analysis. The *katG* promoter region was previously cloned into vector pRK*lacZ*290, generating vector pRK*katG* (15). The fragments containing the *cztR* and *cztA* regulatory regions were amplified from the *C. crescentus* chromosome by PCR with primers Lys5 and Lys6 (see Table 2) using *C. crescentus* NA1000 genomic DNA as the template. The amplified fragments were cloned into the pGEM-T vector (Promega), confirmed by DNA sequencing, and cloned into vector pRK*lacZ*290, generating vectors pRK*cztR* and pRK*cztA*. The plasmids were introduced into *C. crescentus* NA1000 and SP2012 by conjugation. Promoter activities were determined by measuring the β-galactosidase activity by the method of Miller (23).

Expression of *cztR* was determined in *C. crescentus* NA1000 and SP2012 during the exponential-growth and stationary phases (24-h and 48-h cultures). Expression of *cztA* in strains NA1000 and SP2012 was assessed in rich (PYE) and minimal (M2) media at the exponential and stationary phases. Expression was also evaluated during exponential phase in M2 growth medium after 1 h in the presence of different metal chelators at the following concentrations: 300 μM and 500 μM EDTA, 100 μM and 300 μM 2,2-dipyridyl (Sigma), and 300 μM and 500 μM EGTA. In order to identify the divalent cation whose scarcity was inducing the gene, an exponentially growing culture of *C. crescentus* NA1000 harboring plasmid pRK*cztA* was divided into several aliquots. To each culture were added 300 μM EDTA and one of the following metals: 10 μM, 25 μM, 50 μM, or 100 μM $ZnCl_2$; 25 μM $CdCl_2$; 60 μM or 80 μM $CuCl_2$; or 100 μM $NiCl_2$. The cultures were incubated for 24 h at 30°C, and expression was measured by β-galactosidase assays.

Electrophoretic mobility shift assays. Electrophoretic mobility shift assays (EMSA) were carried out using total-protein extracts from strains NA1000 and SP2012. Exponential- or stationary-phase cultures were grown in PYE medium, and total-protein extracts were obtained by sonication in lysis buffer (100 mM sodium phosphate [pH 7.0]. 50 mM NaCl, 1 mM EDTA, 10% glycerol) (34). A 370-bp fragment corresponding to the regulatory region was obtained by PCR using primers Lys5 and Lys6 (Table 2). The fragment was end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Invitrogen), and unincorporated nucleotides were removed with a Qiaquick PCR purification kit (Qiagen). A DNA binding assay was performed in a 50-μl reaction volume containing binding buffer (10 mM Tris-HCl [pH 7.5], 120 mM KCl, 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mg/ml bovine serum albumin, 10% glycerol), salmon sperm DNA (0.1 mg/ml) or 1 μg poly(dI-dC) as a competitor, 50 ng of the labeled DNA probe, and 30 μg of the protein extract. In competition assays, 0.2 to 2.5 μg of a cold probe was used to challenge each of the labeled probes. After incubation at 25°C for 20 min, the samples were loaded onto a native 5% polyacrylamide gel and were electrophoresed in 1× Tris-borate EDTA (TBE) buffer for 3 h at 30 mA. Radioactive species were detected by autoradiography.

Semiquantitative RT-PCR. Total RNA was isolated from strains NA1000 and SP2012 from exponential- and stationary-phase cells. Semiquantitative reverse transcription-PCR (RT-PCR) was carried out as previously described (16), with the following modifications: RNA was treated with 0.6 U of RQ1 DNase (Promega) for 30 min at 37°C, followed by phenol extraction and ethanol precipitation. Control reactions without reverse transcriptase were carried out to confirm the absence of DNA in the RNA samples. Reactions were carried out with 1 μg RNA and 10 pmol (each) primers AhpC1 and AhpC2 (Table 2) under the conditions described by the manufacturer. The PCR conditions were as follows: 55°C for 30 min, followed by 94°C for 2 min and 30 cycles of 94°C for 45 s, 55°C for 1 min, and 72°C for 1 min. After completion, the tubes were incubated at 72°C for 5 min, and the fragments were analyzed by electrophoresis in 2% agarose or 8% acrylamide TBE gels. As a positive control, genomic DNA from strain NA1000 was used instead of RNA.

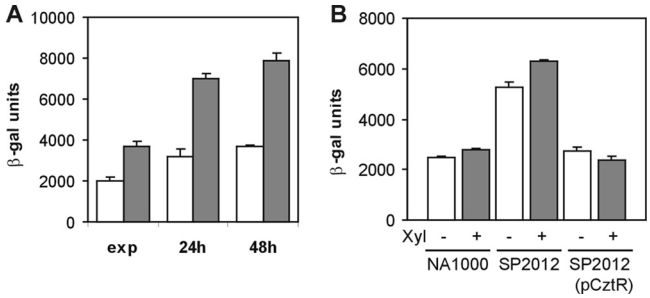

FIG. 1. Autoregulation and expression of *cztR* at different growth phases. (A) The promoter of *cztR* was cloned into plasmid pRK*lacZ*290, generating plasmid pRK*cztR*. Cultures of strain NA1000 (open bars) and strain SP2012 (shaded bars) carrying pRK*cztR* were grown for as long as 48 h, and the promoter activity was determined by measuring β-galactosidase activity at the exponential (exp) and stationary phases (24 h and 48 h). (B) pRK*cztR* was introduced into strains NA1000, SP2012. and SP2012(pUJ*cztR*). β-Galactosidase activity was assayed at late-exponential phase with (+) or without (−) the addition of 0.2% xylose. The results shown are the averages for at least three experiments. Error bars indicate standard deviations.

Oxidative-stress survival tests. Cultures were grown at 30°C with agitation in PYE medium to early-stationary phase (24 h), and 0.5 ml of culture was spread over PYE plates. Resistance to *tert*-butyl hydroperoxide was assessed by measuring the halos of growth inhibition formed by paper discs containing 0.5% (vol/vol) *tert*-butyl hydroperoxide placed on top of the cultures. Tests for survival of hydrogen peroxide and superoxide were performed by adding to 10-ml stationary-phase cultures in PYE medium either paraquat to a final concentration of 10 mM (vol/vol) or H_2O_2 to a final concentration of 5 mM (vol/vol). The cultures were incubated at 30°C with agitation, and at several time points, aliquots were removed, serial dilutions were plated in PYE medium, and plates were incubated at 30°C. Survival was determined as the percentage of CFU relative to the CFU count before the addition of the agent (time zero). The results are averages for two independent experiments performed in duplicate.

RESULTS

CztR is negatively autoregulated and is required for the expression of *cztA*. Three independent mutant strains with insertions in the *cztR* gene were previously isolated from a mini-Tn*5*/*lacZ* transposon library as sensitive to $CdCl_2$ (4). In order to perform expression assays using *lacZ* as a reporter gene, it was necessary to obtain a new *cztR*-null mutant strain devoid of the *lacZ* gene. This was achieved by exchanging the *cztR* gene in wild-type strain NA1000 for a spectinomycin resistance cassette, generating strain SP2012. This strain also presented a Cd- and Zn-sensitive phenotype.

In order to investigate the pattern of expression of *cztR*, a *lacZ* fusion was introduced into strains NA1000 and SP2012, and expression was determined by β-galactosidase activity assays. Expression of *cztR* was also evaluated during growth, and it was observed that the gene is expressed to a higher level in stationary phase, showing approximately 2-fold induction (Fig. 1A) both in NA1000 and in SP2012. It can be observed that the levels of expression in the *cztR* mutant are 2-fold higher than those in the wild type at exponential phase (Fig. 1B). This indicates that CztR negatively regulates its own expression, in agreement with the findings reported for regulators of this family (3, 19). The introduction of the *cztR* gene in a multicopy plasmid under the control of a xylose-inducible promoter in

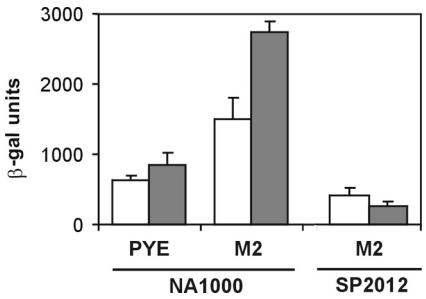

FIG. 2. Growth phase- and medium-dependent expression of *cztA*. *C. crescentus* strains NA1000 and SP2012 carrying plasmid pRK*cztA* were grown in PYE or M2 medium, as indicated, and β-galactosidase activity was determined during the exponential-growth (open bars) and stationary (shaded bars) phases. The results shown are the averages for at least three experiments. Error bars indicate standard deviations.

SP2012(pUJ*cztR*) restores the β-galactosidase activity to levels comparable to those of the wild-type strain, even in the absence of xylose in the medium (Fig. 1B). The leakage of *cztR* transcription observed in the absence of xylose was apparently enough to ensure negative autoregulation.

Transcriptional regulators of the LysR family usually regulate the divergent gene (3, 19). The gene divergent to *cztR*, *cztA*, encodes a putative permease of the SbtA family (31). To test whether *cztA* is regulated by CztR, a *cztA-lacZ* fusion was introduced into strains NA1000 and SP2012. As can be observed in Fig. 2, the gene is induced in stationary-phase NA1000 cells, both in PYE and M2 media, though the levels of expression are 2- to 3-fold higher in M2 medium. The expression of *cztA* was also determined in strain SP2012 in M2 medium, and it was observed that expression was diminished at both growth phases. More importantly, the stationary-phase induction was lost in the *cztR* mutant strain. These results showed that CztR is required for the increase in *cztA* expression that occurs with the shift from rich to minimal medium or upon entry into stationary phase.

CztR binds to the divergent regulatory region. The apparent transcription start site (TSS) of *cztA* was previously determined within a 5-bp interval by tiled microarray analysis (20). In order to characterize the *cis*-regulatory elements controlling the expression of each gene, we determined the TSS of the *cztR* gene by primer extension analysis (Fig. 3A). Two labeled fragments can be observed; the stronger one corresponds to an adenine 68 bp upstream of the ATG, and the weaker to a cytosine 60 bp upstream of the ATG. In this work we considered only the stronger TSS (adenine) in order to determine the *cis*-regulatory elements of the *cztR* promoter.

Based on this and on previous results, the localization of putative *cis*-regulatory elements involved in controlling the expression of each gene could be proposed (Fig. 3B). A putative promoter region can be found upstream of the *cztR* TSS with similarity to the proposed sigma 70 consensus sequence for *C. crescentus* (TTGAC-N_{16}-CCTANA) (20) (Fig. 3C). Located about 68 bp upstream of the *cztA* TSS is a perfect inverted-repeat sequence (CATGATGAG) spaced by an AT-rich 5-bp sequence that could be a putative protein binding site. A consensus binding site that corresponds to a conserved T-N_{11}-A motif within an inverted-repeat sequence has been described for LTTRs (8, 30). The motif found in the *C. crescentus cztR/cztA* divergent region fulfills these requirements, and it is correctly spaced from the *cztA* promoter. This sequence is therefore a good candidate for the CztR binding site, and its position relative to the promoters suggests that CztR

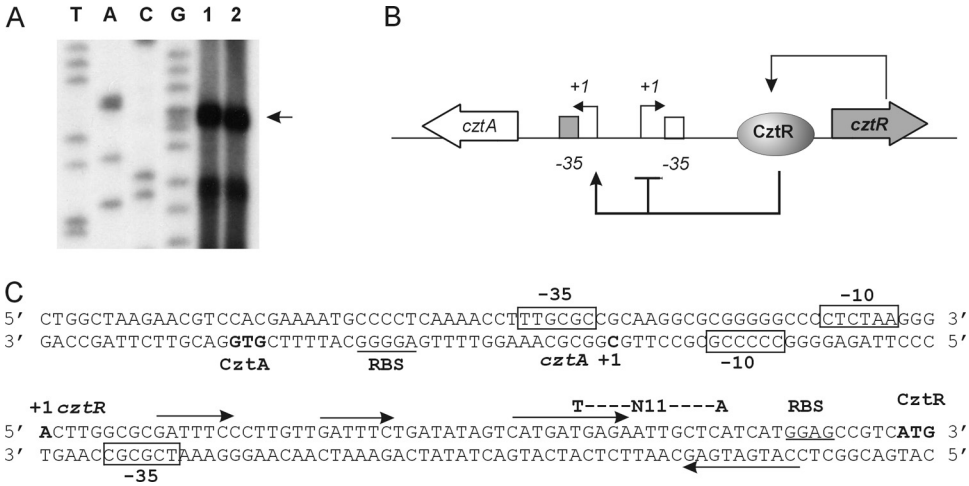

FIG. 3. Determination of athe *cztR* transcriptional start site. (A) Primer extension analysis of the *cztR* gene was carried out using as templates total RNAs from strain NA1000 at the exponential-growth (lane 1) or stationary (lane 2) phase. A ^{32}P-labeled primer hybridizing to *cztR* mRNA was extended with reverse transcriptase. A DNA-sequencing ladder of phage M13mp18 was used as a molecular size marker. The arrow indicates the band corresponding to the main transcription start site observed. (B) Diagram showing the *cztA-cztR* divergent regulatory regions. The −35 regions of each promoter are indicated by boxes and the respective transcription start sites as bent arrows. The CztR protein, binding downstream of its transcription start site, probably represses its own transcription and activates that of *cztA*. (C) DNA sequence of the divergent regulatory region. The −35 and −10 regions of each promoter are boxed, and the respective transcription start sites are marked by "+1" in each strand. Arrows on the same strand pointing in the same direction indicate a direct repeat (GATTTC), and arrows on different strands pointing in opposite directions indicate an inverted repeat. The putative LTTR-binding consensus sequence is indicated above the inverted repeat. The ribosomal binding sites (RBS) of CztR and CztA are underlined.

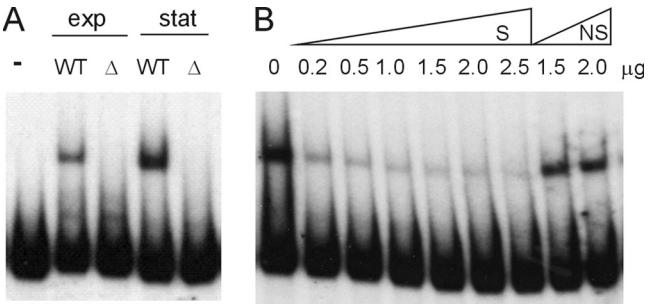

FIG. 4. Assessment of protein binding to the *cztR* regulatory region by gel electrophoresis mobility shift assays. (A) DNA fragments corresponding to the *cztR* promoter were ^{32}P labeled and were then either incubated or not (−) with total-protein extracts from the NA1000 (WT) and SP2012 (Δ) strains at the exponential-growth (exp) and stationary (stat) phases. (B) Competition assays with increasing concentrations of unlabeled fragments of the same region (S) or the *cztR* coding region (NS) as competitors. The concentrations of DNA competitors are given above each lane.

could activate the *cztA* promoter and at the same time repress its own transcription, in agreement with the experimental data.

In order to determine whether CztR binds to the regulatory region, a DNA binding assay was carried out using as a probe a labeled 370-bp DNA fragment containing the divergent promoters. Several attempts at purification of a recombinant His-tagged CztR in *E. coli* were unsuccessful, because we could not obtain the protein in a soluble form (data not shown). In order to circumvent this, we used total-protein extracts from strains NA1000 and SP2012 for the EMSA.

A very distinct retarded band was observed after electrophoresis when the DNA probe was incubated with protein extracts from strain NA1000, but not when it was incubated with protein extracts from SP2012 (Fig. 4A). A retarded band was observed with both exponential- and stationary-phase cell extracts, with an increase in the intensity of the shifted band when the stationary-phase extract was used. However, no retardation was obtained with the SP2012 extracts in either growth phase, indicating that either the CztR protein or a protein whose activity or expression is dependent on the presence of CztR binds to this region. To confirm that the binding activity was specific, a competition assay was carried out using an excess of unlabeled competitor DNA. It can be observed in Fig. 4B that when the same DNA fragment was used, the shifted probe was competed out, while a nonspecific DNA fragment was not able to compete for CztR binding, even at the highest concentrations.

Investigation of the role of CztA. CztA has 10 predicted transmembrane segments, and sequence similarity analysis indicates that it has significant similarity to proteins of the Na^+-dependent bicarbonate transporter (SBT) family. The CztA protein is the only one belonging to this family encoded in the *C. crescentus* NA1000 genome, and our results showed the induction of *cztA* in minimal medium and at stationary phase (Fig. 2), indicating that it is important for growth under low-nutrient conditions, so it could be involved in nutrient transport.

To test this hypothesis, a *cztA*-null mutant strain was generated, and growth in PYE and M2 media was evaluated. Deletion of *cztA* had no effect on growth in PYE medium, but the mutant strain showed a pronounced delay in growth in minimal medium (generation times, 5.1 h for SP2012 and 3.6 h for the wild-type and complemented strains) (Fig. 5A and B), indicating that this gene is important for growth under conditions of low nutrient availability. Complementation with a copy of the *cztA* gene restored growth to levels similar to those of the wild-type strain (Fig. 5B).

All our results suggested that *cztA* may respond to nutrient limitation. To determine which nutrient could be the target of CztA transport, we used a strategy to investigate which nutrient could induce *cztA* expression. Given that a strain with a mutation of the regulator of CztA, CztR, presents a cadmium-sensitive phenotype, we decided to investigate the role of divalent metals in *cztA* expression. Deprivation of metals was achieved by treating the cells with ion chelators with different metal binding specificities: EDTA, 2,2-dipyridyl, and EGTA. The expression of *cztA* was measured in exponentially growing NA1000 cultures in M2 medium that were divided into several aliquots; a different chelator was then added to each aliquot, and after incubation for 1 h, the *cztA* promoter activity was determined. Figure 6A shows that the expression of *cztA* is not altered by the addition of 2,2-dipyridyl or EGTA but is increased about 1.5-fold when EDTA is added to the cells.

EDTA is able to chelate several divalent metal ions with different affinities, so in order to determine which metal's depletion could be inducing *cztA* expression, each individual

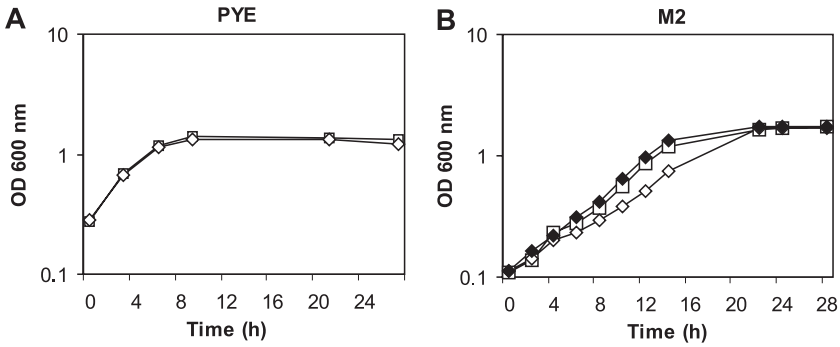

FIG. 5. Growth of NA1000 and SP2014 in rich and minimal media. Growth curves of strains NA1000 (open squares), SP2014 (open diamonds), and SP2014 carrying plasmid pMR*cztA* (filled diamonds) were determined for cultures grown in either PYE (A) or M2 (B) medium. Experiments are described in the text, and the curves shown are representative of the results obtained from at least three experiments.

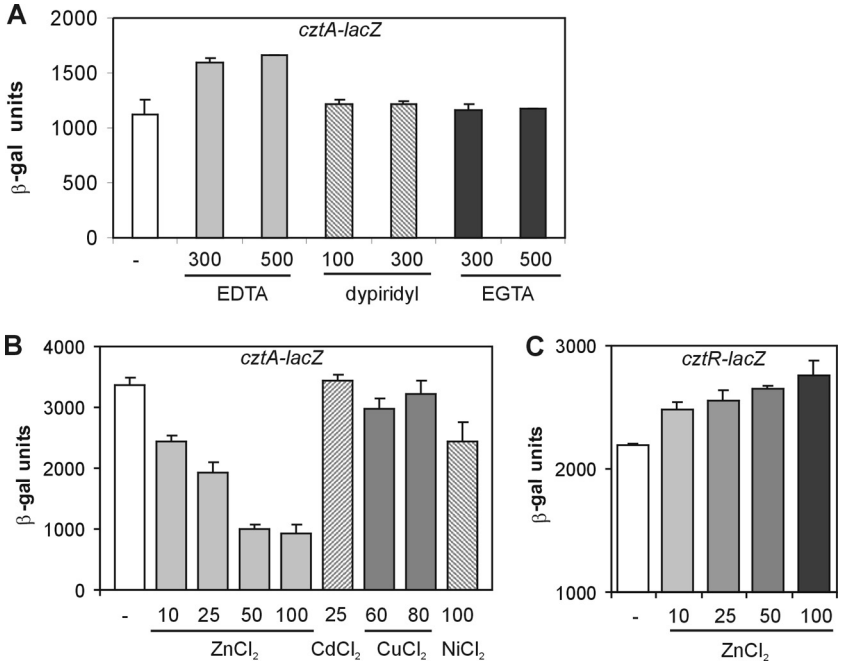

FIG. 6. Effect of metal depletion on *cztA* expression. (A) *C. crescentus* NA1000 carrying plasmid pRK*cztA* was grown up to exponential phase in M2 medium, and cells were incubated in the presence of one of several ion chelators at different micromolar concentrations as indicated. β-Galactosidase activity was determined after 1 h. (B) *cztA* expression was assessed in strain NA1000 cultures incubated simultaneously with 300 μM EDTA and different divalent cations added to aliquots of the culture at the indicated micromolar concentrations. β-Galactosidase activity was determined after 24 h in the presence of the metals and EDTA. (C) *cztR* expression was determined as for panel B. The results shown are the averages for at least three experiments. Error bars indicate standard deviations.

metal was added to the cultures simultaneously with EDTA. As shown in Fig. 6B, the expression of *cztA* in the presence of 10 to 100 μM $ZnCl_2$ is progressively decreased from that in the presence of EDTA alone or with the addition of other cations, such as Cu^{2+}, Ni^{2+}, or Cd^{2+}. These results suggest that *cztA* is induced in response to the depletion of zinc ions from the medium; consequently, the CztA transporter could have a relevant role under conditions of low zinc availability. To test whether this difference in expression was mediated by the presence of the CztR regulator, the expression of the divergent *cztR* gene was measured under the same conditions. As shown in Fig. 6C, the expression of *cztR* increases progressively with the addition of increasing concentrations of $ZnCl_2$ in the presence of EDTA. The results indicate that CztR is able to activate the transcription of *cztA* and to repress its own transcription only when zinc is depleted, which could suggest that zinc ions may affect its binding to the regulatory region.

The results suggest that gene regulation is achieved in response to very low concentrations of zinc ions in the medium. In fact, maximal repression of *cztA* expression is already observed with 50 μM $ZnCl_2$, and the estimated amount of $ZnCl_2$ necessary to inhibit 50% of expression is 30 μM. Further addition of $ZnCl_2$ does not increase inhibition to levels lower than those observed in the exponential-phase culture (compare Fig. 6A and B). We have measured the expression of both *cztR* and *cztA* in stationary phase in the presence of $ZnCl_2$ and the absence of EDTA, but no difference was observed (data not shown), indicating that there is a basal concentration of zinc in the medium that is sufficient to limit *cztA* expression.

The *cztR* mutant is sensitive to oxidative stress. The putative role of CztA in zinc homeostasis and the Cd^{2+}-sensitive phenotype of the *cztR* mutant prompted us to investigate whether CztR and CztA could be important for the oxidative-stress response. The oxidative-stress response of SP2012 was evaluated by testing its resistance to hydrogen peroxide, *tert*-butyl-hydroperoxide, and superoxide (generated intracellularly by the addition of paraquat). Resistance to *tert*-butyl hydroperoxide was determined by measuring the halo of growth inhibition, but no difference in the inhibition halo diameters was observed between SP2012 and NA1000 (data not shown). Resistance to H_2O_2 and superoxide was determined by adding each agent to stationary-phase cultures and removing aliquots for CFU counting. As shown in Fig. 7, strain SP2012 is much more sensitive to paraquat and moderately sensitive to H_2O_2, and this phenotype is complemented by a copy of the gene in *trans*. Taken together, these results indicate that *cztR* is important for resistance to hydrogen peroxide and superoxide, but not for resistance to organic hydroperoxide. We also investigated whether strain SP2014 was sensitive to paraquat, but it showed the same resistance profile as that observed in the wild-type strain (data not shown). The possible involvement of *cztR* in the oxidative-stress response prompted us to investigate whether its expression is altered under oxidative conditions. Exponential-phase cells were incubated with oxidative agents for different times, and expression was determined by β-galactosidase activity assays. However, no difference was observed in the presence of $CdCl_2$, paraquat, or H_2O_2 (data not shown).

Considering the oxidative-stress phenotype of SP2012, we

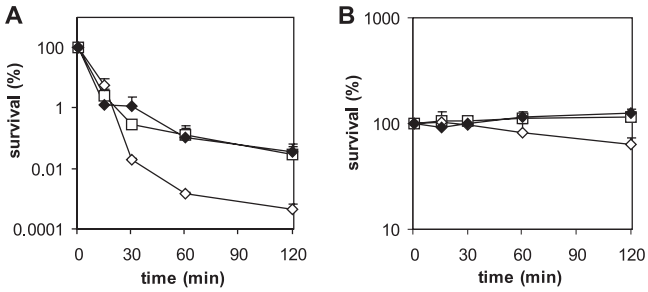

FIG. 7. Survival of strain SP2012 in response to oxidative stress. Stationary-phase cultures of NA1000 (open squares), SP2012 (open diamonds), or SP2012(pMR*cztR*) (filled diamonds) were subjected to oxidative stress, and aliquots were taken at each time point to determine cell viability by CFU counts. An aliquot was taken before the addition of the agents (time zero), and the results were determined relative to the CFU at this time (taken as 100%). (A) Superoxide stress was achieved by the addition of paraquat to stationary-phase cultures at time zero to a final concentration of 10 mM. (B) Hydrogen peroxide was added to stationary-phase cultures at time zero to a final concentration of 5 mM. The results are the averages for two independent experiments. Error bars indicate standard deviations.

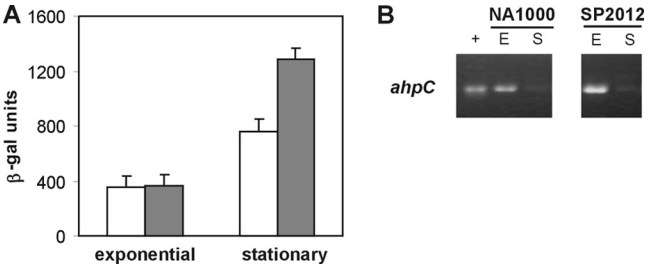

FIG. 8. Expression of oxidative stress response genes. (A) β-Galactosidase activity of the *katG* promoter in strain NA1000 (open bars) and strain SP2012 (shaded bars) was determined during exponential and stationary growth. The results are the averages for two independent experiments. Error bars indicate standard deviations. (B) mRNA levels of the gene encoding the enzyme alkyl hydroperoxide reductase subunit C (*ahpC*) were determined by semiquantitative RT-PCR. The cDNAs were amplified by RT-PCR using as templates the total RNAs of cells from strains NA1000 and SP2012 at the exponential-growth (E) and stationary (S) phases. As a positive control (+), PCR was carried out using NA1000 genomic DNA.

decided to investigate whether the expression of some antioxidative enzymes were affected in the mutant. Expression of the catalase-peroxidase, encoded by the *katG* gene, was determined by β-galactosidase activity assays. We could observe that there was an increase in the transcription of *katG* in SP2012 over that in NA1000 only in stationary phase (Fig. 8A). This result was confirmed by RT-PCR and immunoblotting with an anti-KatG antiserum (data not shown). The transcript levels of the *ahpC* gene, encoding alkyl hydroperoxide reductase subunit C, were measured by semiquantitative RT-PCR. The levels of *ahpC* transcripts at exponential phase were also higher in SP2012 than in NA1000, and no difference was observed at stationary phase, when the gene was much less expressed (Fig. 8B). The expression of *sodA* (Mn superoxide dismutase), *sodB* (Fe superoxide dismutase), and *sodC* (CuZn superoxide dismutase) was also determined by RT-PCR, and the transcript levels of these genes were not evidently different between the two strains (data not shown).

DISCUSSION

The LTTRs constitute the largest family of transcription factors in prokaryotes; they are involved in several cellular functions, such as metabolism, cell division, quorum sensing, virulence, motility, nitrogen fixation, oxidative-stress response, toxin production, attachment, and secretion (17). In this work, we deepened the characterization of the transcriptional regulator CztR, investigating its role in the regulation of the divergent gene *cztA*, which encodes a transporter that is probably involved in zinc homeostasis.

The transcription start site of *cztR* was found to be located near the transcription start site determined for *cztA* (20), indicating an overlap of the promoter regions. A model for LTTR binding proposes that the LTTR binds to a regulatory binding site that contains the consensus sequence T-N_{11}-A (17). In the regulatory region shared by *cztR* and *cztA*, there is an inverted-repeat sequence containing the LTTR consensus T-N_{11}-A, constituting a possible binding site for CztR. Unfortunately, the DNase I footprinting assays to identify the exact sequence of the CztR binding site were not successful.

We showed, by EMSA and gene expression analysis, that CztR negatively regulates its own expression and positively regulates the divergent gene *cztA*. This regulation pattern is in agreement with the classical model for LTTR-dependent transcriptional regulation, which has been well studied in other bacteria (17). For example, in *Neisseria meningitidis*, *mdaB* encodes an NADPH-quinone oxidoreductase and *crgA* encodes a LTTR. The two genes are transcribed from overlapping divergent promoters, and CrgA acts as a repressor of its own transcription and activates the transcription of *mdaB* in the presence of the coinducer MBL (α-methylene-γ-butyrolactone) (14). Although we have not directly determined the need for a coinducer for CztR binding, the induction of *cztA* in response to zinc depletion suggests that the presence of zinc is somehow changing the binding properties of CztR.

In this work, we also began the characterization of the divergently transcribed gene *cztA*, which encodes a membrane protein with 10 transmembrane segments belonging to the SBT family. The prototype of this family is *Synechocystis* SbtA, which plays the main role in HCO_3^- transport and is energized by the primary Na^+ pump NtpJ. It has been suggested that NtpJ establishes an electrochemical gradient of Na^+ across the plasma membrane that could drive the transport of HCO_3^- by SbtA, given that this transport is Na^+ dependent (31). Interestingly, *sbtA* is regulated by the LTTR CcmR in response to inorganic carbon (C_i) limitation (38).

Phylogenetic analyses have suggested that SbtA family members display multiple transport functions (37). The sensitivity of the *cztR* mutant to Cd^{2+}, Zn^{2+}, and Cu^{2+} (4) raised the possibility that CztA could be transporting divalent cations. In agreement with that notion, the *cztA*-null mutant showed a pronounced delay in growth in minimal medium but not in rich medium, and the *cztA* gene showed CztR-dependent induction in minimal medium, at stationary phase, and in the presence of the divalent cation chelator EDTA. This induction was prevented by the presence of an excess of zinc ions, but not of other metals, suggesting that the expression of *cztA* is re-

pressed under Zn^{2+}-rich conditions and induced when Zn^{2+} is depleted, and that the absence of zinc is the signal for *cztA* activation. In *X. campestris* it was verified that genes encoding three Zn^{2+} uptake systems were induced under zinc-deficient conditions and repressed under zinc-rich conditions, while the *czcD* gene, encoding a Zn^{2+} efflux pump, showed an opposite pattern of expression (13). Therefore, the expression profile of *cztA* indicates that it encodes a transporter for zinc uptake and not for efflux. However, we cannot exclude the possibility that CztA transports other ions.

In *E. coli*, the main system for zinc uptake under starvation conditions is the *znuABC* operon, a high-affinity ABC transporter regulated by the repressor Zur, a member of the Fur family (25). Two other proteins, ZupT and Pit, act as cotransporters to aid in zinc uptake when the levels of this metal are not so low (2). In the *C. crescentus* genome, there are at least 45 operons encoding ABC transporters (24), and 1 of these is localized near the gene encoding the predicted transcription regulator Zur. We propose that there is an ABC transporter involved in zinc uptake under rigorous starvation conditions and that CztA could act in zinc transport under moderate starvation conditions, as mentioned for other bacteria.

Three independent *cztR* mutants have been shown to be highly sensitive to Cd^{2+} and also moderately sensitive to Zn^{2+} and Cu^{2+} (4). Since it is well established that cadmium induces oxidative stress in the cell, we investigated the response of SP2012 to oxidative stress. This strain was very sensitive to paraquat and moderately sensitive to H_2O_2 at stationary phase, but no difference was observed in the response to organic hydroperoxide. It was previously shown that *C. crescentus* cells are more resistant to H_2O_2 at stationary phase due to increased catalase-peroxidase (KatG) expression at this phase (33, 39). It was demonstrated that *C. crescentus sodA* was induced in the presence of cadmium, chromate, dichromate, and uranium, while *sodB* was induced only under cadmium stress and *sodC* showed constitutive expression in the presence of all metals (12). While there was increased expression of *katG* in stationary phase and of *ahpC* during exponential growth in strain SP2012, the expression of all three superoxide dismutase genes was unaffected.

The results indicated that CztR is important for the defense against oxidative stress generated by both H_2O_2 and O_2^-, but *cztR* expression is not induced in the presence of H_2O_2 or paraquat. Nevertheless, the increased expression of *katG* and *ahpC* in strain SP2012 could indicate that *cztR* cells are in a state of oxidative stress rather than that CztR is directly involved in the regulation of catalase-peroxidase and alkyl hydroperoxide reductase. One hypothesis to explain these results is that this oxidative-stress state of the *cztR* mutant could result from downregulation of key stress response genes that could be regulated by CztR. Another possibility is that this effect could result from the imbalance in the zinc concentration in the cell, since the CztA transporter would not be fully expressed in this strain. If this is the case, lower levels of zinc would result in a lower efficiency of zinc-containing enzymes, such as Cu-Zn superoxide-dismutase, which would negatively affect the response to stress generated by superoxide. However, the *cztA* mutant strain does not show sensitivity to Zn^{2+}, Cd^{2+}, or oxidative agents, arguing against the latter hypothesis.

In conclusion, the results obtained in this work indicate that the CztR transcriptional regulator negatively regulates its own expression and also positively regulates the divergent gene *cztA*, which encodes a putative transport protein. The activation of *cztA* at stationary phase and in minimal medium indicates that it has a role in responding to low-nutrient conditions, and in fact it is induced by the addition of the metal chelator EDTA. This induction is prevented by the presence of an excess of zinc ions, but not of other metals, suggesting that the signal for *cztA* activation is depletion of zinc. The *cztA* mutant strain is not sensitive to metals or oxidative agents; therefore, the oxidative-stress phenotype displayed by the *cztR* mutant strain cannot be attributed to a defect in *cztA* expression. This suggests that CztR could be necessary for the activation of other genes involved in the *C. crescentus* oxidative-stress response.

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