

# CadC, the Transcriptional Regulatory Protein of the Cadmium Resistance System of *Staphylococcus aureus* Plasmid pI258

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**The CadC protein from the *cadA* cadmium resistance operon of *Staphylococcus aureus* plasmid pI258 regulates transcription of this system in vitro. The CadC protein was overproduced in *Escherichia coli* cells and partially purified. Gel shift assays of the proposed *cadA* operator/promoter region DNA showed specific association with the CadC protein. Control arsenic resistance operator/promoter DNA from the same plasmid was not shifted by the CadC protein. Cd<sup>2+</sup>, Bi<sup>3+</sup>, and Pb<sup>2+</sup> caused the release of CadC from DNA in gel retardation assays. DNase I footprinting measurements showed that the CadC protein specifically associated with and protected a region of operator/promoter DNA from nucleotide positions -7 to +14 relative to the start point of mRNA synthesis. Runoff transcription assays with the operator/promoter region of DNA (plus the first 69 nucleotides of the *cadC* gene) and purified *E. coli* RNA polymerase gave an mRNA product of the predicted size. Added CadC protein inhibited transcription in vitro.**

The cadmium resistance system of *Staphylococcus aureus* plasmid pI258 was identified by Novick and Roth (9) and subsequently mapped by deletion analysis (8). The *cadA* system results in reduced net uptake of Cd<sup>2+</sup> because of increased energy-dependent efflux of the toxic cation (22). Cloning and DNA sequence analysis identified two genes called *cadC* and *cadA* (10). The *cadA* gene product is a P-type ATPase, a member of the large family of related cation transport ATPases found in plant and animal cells as well as in microbes (16, 17). The CadA ATPase protein itself is sufficient for resistance and decreased cadmium uptake in *Bacillus subtilis* cells (10) and for ATP-dependent Cd<sup>2+</sup> uptake by inside-out subcellular membranes (21), equivalent to efflux by intact cells. The CadA ATPase can be labelled with <sup>32</sup>P from [<sup>32</sup>P]ATP (20), the characteristic of P-type ATPases that distinguishes them from other classes of membrane transport ATPases.

While the properties of CadA satisfactorily explain cadmium resistance, the role of *cadC* and the nature of the *trans*-acting component of the carefully regulated CadA system (3, 25) remain unclear. The *cadC* gene is involved in full cadmium resistance; *cadC* can be provided in *trans* and need not be present on the same plasmid as *cadA* (26). The inducible nature of transcription was established, and the initiation site for *cad* mRNA synthesis was identified by reverse transcriptase primer extension (25) to a position 28 nucleotides upstream of the ATG start codon of the *cadC* gene (Fig. 1). Operon-length mRNA of 2.6 kb, including both *cadC* and *cadA*, was identified by Northern blot DNA-RNA hybridization (25). Canonical -10 and -35 *Escherichia coli*-like RNA polymerase recognition sites (Fig. 1) were identified in the sequence (25). A 7-nucleotide inverted repeat segment, including the first nucleotides transcribed into mRNA, was proposed as the operator site for binding of a then-elusive *trans*-acting DNA-binding regulatory protein (25).

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Cation-inducible gene function was studied by measurements of Cd<sup>2+</sup> efflux by whole cells (25) and subcellular membranes (21) and through induction of the reporter genes β-lactamase (25) and luciferase (3). However, the presumed *trans*-acting regulatory protein was not identified (25). Because of the need for *cadC* in gene regulation (21) and the sequence homologies (1, 26) among the predicted CadC protein product, the ArsR regulatory protein (12, 14, 15, 23) for the arsenic resistance system, and the SmtB regulatory protein for cyanobacterial metallothionein synthesis (6), we undertook a direct test of the hypothesis that CadC is the regulatory protein for *cadA* operon transcription. By in vitro assays with CadC protein and the appropriate operator/promoter DNA, CadC has been shown to be a DNA-binding, negatively acting regulatory protein.

## MATERIALS AND METHODS

**Abbreviations.** DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**Bacterial strains and plasmids.** *E. coli* HB101 (13) and plasmid pTrc99A (Pharmacia-LKB, Piscataway, N.J.) were used.

**PCR cloning of the *cadC* gene and overproduction of the CadC protein.** The *cadC* gene was isolated from M13 phage containing the *cadA* operon (10) by PCR-assisted cloning with primers GCGCGCTCATGAAAAAGAAAGATAC (corresponding to the first 17 nucleotides of the *cadC* gene [nucleotides 704 to 720], with GCGCGCTC added to the 5' end to create a *Bsp*HI restriction site TCATGA) and CCCCGGATCCAAGCTTCAGACATTGACCTTCAC (corresponding to the last 18 nucleotides of *cadC* [positions 1072 to 1089 of the published sequence] [10], with CCCCGGATCCAAGCT added to the 5' end of the primer to generate *Bam*HI [GGATCC] and *Hind*III [AAGCTT] restriction sites). The PCR product was digested with endonucleases *Bsp*HI and *Hind*III and cloned into the *trc* expression vector plasmid pTrc99A (digested with *Nco*I [CCATGG] and *Bam*HI). *Bsp*HI and *Nco*I produce complementary single-stranded tetranucleotides. The recombinant plasmid (pSB100) containing the staphylococcal *cadC* gene under the control of the *E. coli trc* promoter of plasmid pTrc99A was transformed into *E. coli* HB101.

*E. coli* HB101(pSB100) was grown overnight in Luria-Bertani medium (13) containing 100 μg of ampicillin per ml at 37°C and diluted 200-fold. After growth to a turbidity of about 60 Klett units, cells were induced with 1 mM IPTG and grown for 3 h. Cells were harvested by centrifugation, washed twice with Tris-HCl buffer (20 mM Tris-HCl [pH 7.5], 5 mM β-mercaptoethanol). Cells from 1 liter of culture were suspended in 20 ml of Tris-EDTA buffer (13) and disrupted by sonication (20 s each time, with cooling on ice, until turbidity was completely deduced). Cell debris was removed by centrifugation at 20,000 rpm in an SS34 rotor in a Sorvall refrigerated centrifuge. The CadC protein present in this crude protein preparation was purified on a heparin-agarose column (2.6 by 10 cm) (type I, H5263; Sigma Chemical Company, St. Louis, Mo.) run on fluid pressure

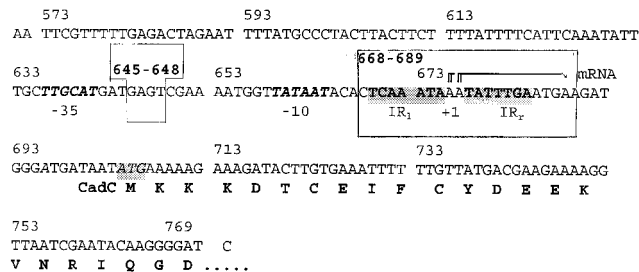


FIG. 1. DNA sequence with proposed regulatory sites of the 203-nucleotide *cadA* fragment used for gel retardation and *in vitro* transcription assays. The initial and final positions are numbered (as in reference 10), with the addition of AA at the 5' end and C at the 3' end, from PCR cloning. The postulated -35 and -10 RNA polymerase binding sites are in boldface italics; the inverted repeats (IR<sub>1</sub> and IR<sub>2</sub>) are in boldface and hatched. The +1 and +2 starting sites for *in vivo* mRNA synthesis (25) are shown, as is the predicted amino acid sequence of the first 23 amino acids of the first gene product of the operon, CadC. The DNA sequences protected by CadC in DNase I footprinting experiments (Fig. 6) are boxed.

liquid chromatography (FPLC) (Pharmacia Biotech) and eluted with TMGB buffer (50 mM Tris-HCl [pH 7.6], 5 mM MgCl<sub>2</sub>, 5 mM β-mercaptoethanol, 100 g of glycerol per liter) on a gradient from 0.1 to 1 M NaCl at a rate of 5 ml/min. Four-milliliter fractions were collected, and protein was measured by Bio-Rad protein reagent assay at 595 nm with a spectrophotometer. Samples (5 μl from each FPLC fraction) were subjected to 0.1% SDS-18% PAGE analysis as described in Results. The protein from fractions containing CadC protein was concentrated by ultrafiltration through an Amicon YM3 filter to 8 mg/ml and stored at -80°C prior to use.

Purified ArsR protein from the regulatory gene of the arsenic resistance operon of plasmid p1258 (4, 18) was used as a negative control for the specificity of CadC binding to DNA and was provided by Y. Chen (1a).

**PCR cloning of the 203-nucleotide fragment that includes the regulatory-region DNA and the start of the *cadC* gene.** The 200-nucleotide region from positions 573 through 772 of the published sequence (10) shown in Fig. 1 was amplified as a 203-nucleotide fragment, with AA at the 5' end and C at the 3' end added, by PCR with 25-mer oligomers AAGAATTCTGTTTGGAGACTAGATAAT (with an *EcoRI* site at the 5' end) and TTGGATCCCCCTTGATTTCGATTAAC (with a *BamHI* site at the 5' end). After the PCR product was digested with restriction endonuclease *EcoRI* or *EcoRI* and *BamHI*, the PCR product was purified by agarose gel electrophoresis. This fragment was isolated by electroelution, extracted with phenol to remove proteins, and precipitated with ethanol. The purified PCR product was used directly as the substrate for <sup>32</sup>P labelling with [α-<sup>32</sup>P]dATP and Klenow DNA polymerase and then for protein-DNA association assays (see below).

**Chemicals and enzymes.** Buffer ingredients and medium components were purchased from Mallinckrodt (St. Louis, Mo.) and Sigma Chemical Company (St. Louis, Mo.). Restriction endonucleases and other enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.) and New England Biolabs (Beverly, Mass.). *E. coli* RNA polymerase was purchased from Pharmacia Biotech. [α-<sup>32</sup>P]dATP and [α-<sup>32</sup>P]UTP (3,000 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, Ill.).

**Gel retardation assay.** The 203-nucleotide *cad* operator/promoter DNA fragment, prepared as described above, was labelled at both ends by using [α-<sup>32</sup>P]dATP, a mixture of deoxynucleoside triphosphates, and DNA polymerase (Klenow fragment). Protein binding was measured by gel mobility shift assay (2, 12, 24). CadC protein with added cations (as indicated) was added to buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM Na<sub>2</sub>-EDTA, 0.1% Nonidet P-40, 50 mM KCl, 0.3 mM DTT, 4% (vol/vol) glycerol, 50 μg of poly(dI-dC) (Boehringer GmbH, Mannheim, Germany) per ml, and 50 μg of bovine serum albumin per ml and incubated for 10 min at room temperature. Then <sup>32</sup>P-labelled proposed *cad* operator/promoter DNA was added (for a total volume of 20 μl), and incubation continued for 15 min before samples were subjected to gel electrophoresis on 5% polyacrylamide gels.

**DNase I footprinting assay.** DNase I footprinting assays were carried out as described by Wu and Rosen (24) in a buffer containing 10 mM Tris-HCl (pH 7.6), 0.1 M KCl, 10% glycerol, 0.2 mM Na<sub>2</sub>-EDTA, 0.2 mM DTT, 50 μg of bovine serum albumin per ml, 50 μg of poly(dI-dC) per ml, <sup>32</sup>P-labelled *cad* operator DNA, and CadC protein (as indicated) in a total volume of 20 μl. The 203-nucleotide *cad* DNA fragment, prepared as described above, was digested with *EcoRI* endonuclease and labelled specifically at the *EcoRI* end with [α-<sup>32</sup>P]dATP and DNA polymerase (Klenow fragment). After 15 min at room temperature, 0.04 U of DNase I (Boehringer GmbH) was added in 30 μl of buffer containing 25 mM Tris-HCl (pH 7.6), 50 mM KCl, 5 mM MgCl<sub>2</sub>, 1.7 mM CaCl<sub>2</sub>, 83 μg of bovine serum albumin per ml, and 0.2 mM DTT. After 45 s at 25°C, the reaction was terminated by adding 10 μg of tRNA in 50 μl of 20 mM

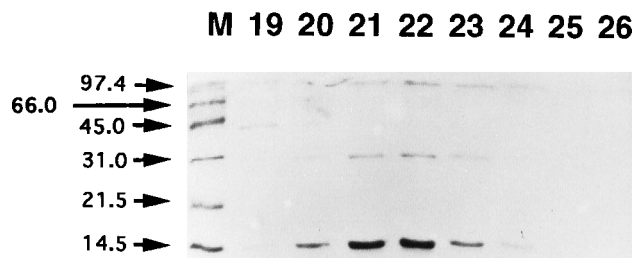


FIG. 2. Partial purification of the CadC protein. SDS-PAGE analysis of heparin-agarose column chromatography of CadC protein stained with Coomassie blue. Lane M, molecular mass standards (Bio-Rad low-range SDS-PAGE protein standards; in kilodaltons); lanes 19 through 26, samples from column fractions 19 to 26, respectively.

Na<sub>2</sub>-EDTA solution. Proteins were extracted with phenol-chloroform, and DNA from the aqueous layer was precipitated with ethanol and redissolved in 7 μl of formamide dye solution (0.05% xylene cyanol, 0.05% bromophenol blue, and 1 mM Na<sub>2</sub>-EDTA [pH 8.0] in deionized formamide). After being denatured by heating at 75°C for 5 min, DNA was analyzed on a sequencing gel.

**Runoff *in vitro* transcription.** Assays were adapted from reference 27 and the Promega transcription kit instructions with RNA polymerase (*E. coli*) from Pharmacia Biotech. Unless specified, *cad* operator/promoter DNA was added to polymerase buffer containing CadC protein (and cations as indicated). RNA polymerase was added, and after 15 min at 38°C, the reaction was started by adding 4 μl of a mixture containing (per reaction) 2 μCi of [α-<sup>32</sup>P]UTP, 0.17 mM nonradioactive UTP, and 2.5 mM (each) ATP, CTP, and GTP. After incubation at 38°C for 20 min and phenol-chloroform extraction, nucleic acids in the aqueous layer were precipitated with ethanol, dissolved, and loaded and electrophoresed on a 4% polyacrylamide sequencing gel.

## RESULTS

**Overproduction of the CadC protein.** The *cadC* gene of *S. aureus* pl258 was cloned into an *E. coli* expression vector plasmid (see Materials and Methods) for overproduction of the CadC protein. A protein band of appropriate size (13.5 kDa) was seen by SDS-PAGE gel analysis after induction by IPTG (data not shown). The protein was found primarily in the soluble fraction after sonication, and inclusion bodies of denatured CadC protein were not made under these conditions. After partial purification by heparin-agarose column chromatography, the CadC protein was found in fractions 20 to 23 (Fig. 2). CadC was estimated to be approximately 70% pure by scanning photographs of the Coomassie blue-stained protein gel (Fig. 2). The protein from fractions 19 through 23 was concentrated by ultrafiltration (see Materials and Methods) and rechromatographed on gel filtration (Sephadex G-75; Sigma Chemical Company) or cation-exchange (S Sepharose, S-1264; Sigma Chemical Company) columns. Purification was not achieved by these chromatographic steps (data not shown), and so protein from heparin-agarose gel chromatography was used without further purification in the experiments discussed below. Efforts to purify CadC to homogeneity continue (7a). Until protein-DNA association assays are confirmed with purified CadC protein, the possibility remains that some of the results reported here are due to contaminating *E. coli* proteins in CadC preparations.

**CadC protein-DNA association measured by gel retardation assay.** As shown in Fig. 3, binding of the CadC protein to the proposed operator/promoter region of *cad* operon DNA resulted in a shift of *cad* DNA to two more slowly migrating positions during gel electrophoresis. In subsequent experiments, the titration of operator/promoter DNA with CadC protein was quantitated with an AMBIS radioactivity scanner (Fig. 4 and 5). Both retarded forms became measurable at CadC protein levels greater than 1 nM (Fig. 4). (Calculations were based on the estimated 70% purity of the CadC protein

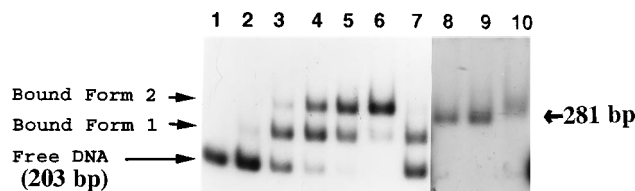


FIG. 3. Gel retardation of operator/promoter DNA by CadC protein. Lane 1, 0.37 nM (1 ng)  $^{32}\text{P}$ -labelled 203-bp *cadA* operator/promoter DNA; lanes 2 through 6, *cadA* DNA plus 1, 10, 26, 52, or 105 nM CadC protein, respectively; lane 7, same as lane 6 with 105 nM CadC protein and 76 nM (200 ng) non-radioactive *cadA* operator/promoter DNA. Lanes 8 through 10, four-times-longer exposure of the same gel with 281-bp *ars* operator/promoter DNA. Lane 8, 0.27 nM (1 ng) pI258 *ars* operator/promoter DNA; lane 9, *ars* DNA plus 105 nM CadC protein; lane 10, *ars* DNA plus 150 nM ArsR protein.

and on the assumption that all protein was added as an active 13,500-Da monomer.) Retarded bands of forms 1 and 2 appeared concurrently, and there was no concentration range of CadC protein that formed only bound form 1 (Fig. 4). At lower ratios of CadC to DNA, form 1 predominated (Fig. 3, and Fig. 4); at higher ratios of CadC to DNA, form 2 was more abundant. A half-binding constant of 5 nM CadC for this association was estimated with 0.37 nM DNA present (Fig. 4). An important control for the specificity of gel retardation was that the addition of 200-fold excess non-radioactive operator/promoter DNA (Fig. 3, lane 7) resulted in displacement of protein from 60% of the radioactive DNA. With a different operator/promoter DNA (from the *ars* operon of plasmid pI258 [4]), the addition of CadC protein did not result in a significant shift in mobility of DNA on the gel (Fig. 3, lane 9).

With specific gel retardation of the *cad* operator/promoter DNA by CadC protein determined, the effects of added cations on the release of CadC from DNA were then measured. The addition of high concentrations of  $\text{Cd}^{2+}$ , above 50  $\mu\text{M}$ , caused a decrease in the amount of retarded form 2 and an increase in the amount of retarded form 1 (Fig. 5A). Free unretarded DNA also appeared as increasing levels of  $\text{Cd}^{2+}$  were added. However, even with 5 mM  $\text{Cd}^{2+}$  in vitro, only half of the DNA was free form (Fig. 5A). It is possible that the CadC protein is not released from DNA by the addition of cations. The inability to obtain complete release of regulatory proteins from DNA in vitro at cation concentrations expected to be function-

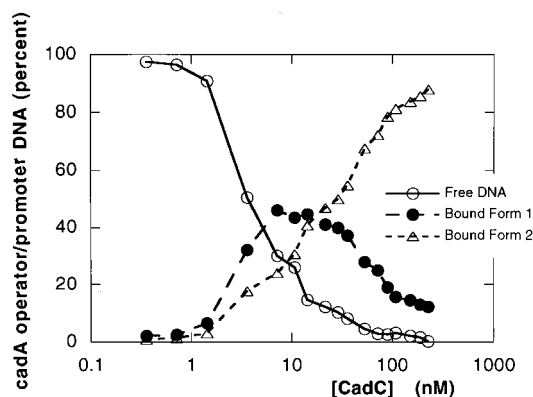


FIG. 4. Quantitation of gel retardation of *cadA* DNA by CadC protein. A series of samples (as in Fig. 3), each containing 0.37 nM *cadA* DNA plus increasing amounts of CadC protein, were subjected to gel retardation electrophoresis, and the dried gel was analyzed on an AMBIS radioactivity scanner. The amounts of free DNA, the intermediate retarded form 1, and the slower retarded form 2 are indicated.

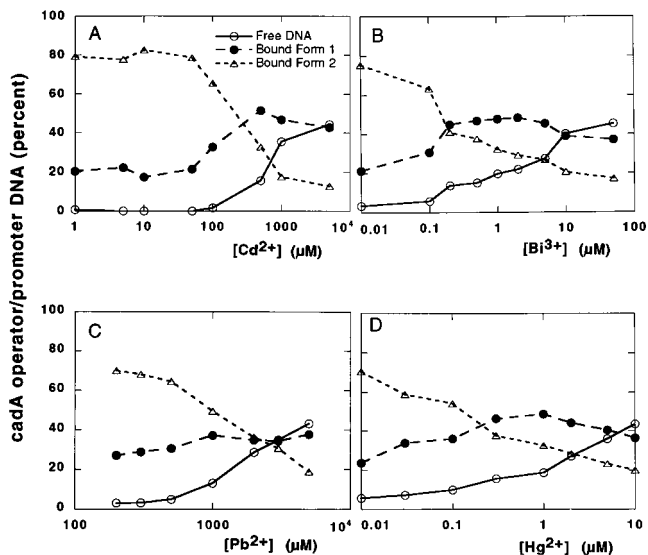


FIG. 5. Effects of cations on CadC association with *cadA* operator/promoter DNA. Conditions were the same as those for Fig. 4, with 0.37 nM *cadA* DNA and 105 nM CadC protein. (A)  $\text{CdCl}_2$  added (from 1  $\mu\text{M}$  to 5 mM); (B)  $\text{Bi}^{3+}$  (tartrate) added (from 10 nM to 50  $\mu\text{M}$ ); (C)  $\text{Pb}^{2+}$  (acetate) added (from 200  $\mu\text{M}$  to 5 mM); (D)  $\text{HgCl}_2$  added (from 10 nM to 50  $\mu\text{M}$ ).

ing in vivo has been found with other systems; for example, high levels of arsenite are required to partially displace the ArsR regulatory protein from *ars* operon DNA (12, 24). The buffer used in gel retardation experiments includes 1 mM EDTA and 0.3 mM DTT, chelators of  $\text{Cd}^{2+}$ . In the absence of DTT, the CadC protein did not bind to this operator/promoter DNA (data not shown). The CadC protein did not bind to *cad* operator/promoter DNA in experiments with the alternative gel retardation assay buffer of Mukhopadhyay et al. (7), which was designed for mercury-binding regulatory proteins and contains cysteine and salmon sperm DNA (data not shown).

Alternative cations that function as inducers in vivo (3, 25) were more effective in vitro than  $\text{Cd}^{2+}$  was. Increasing  $\text{Bi}^{3+}$  concentrations resulted in a loss of retarded form 2 (50% loss with about 0.1  $\mu\text{M}$   $\text{Bi}^{3+}$  [Fig. 5B]) and an increase in the amount of protein-free DNA.  $\text{Pb}^{2+}$  was less effective in this gel retardation release assay (50% loss with about 1 mM  $\text{Pb}^{2+}$  [Fig. 5C]) than  $\text{Bi}^{3+}$  was, and  $\text{Hg}^{2+}$ , which does not function in vivo (3), was about as effective in these assays as  $\text{Bi}^{3+}$  was (50% loss with about 0.2  $\mu\text{M}$   $\text{Hg}^{2+}$  [Fig. 5D]). Up to millimolar concentrations,  $\text{Co}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Mn}^{2+}$  had no effect under the same conditions (data not shown).

**CadC DNA binding site.** DNase I footprinting assays with radioactive *cad* operator/promoter DNA showed a protected region approximately from nucleotides 669 to 689 (according to the numbering system of Nucifora et al. [10]), corresponding to positions -7 to +14 (Fig. 1 and 6) relative to the in vivo initiation site for mRNA synthesis (25). A second location for protection by CadC protein occurred approximately at positions 645 to 648 (Fig. 6), in the region predicted for RNA polymerase binding (Fig. 1). Both regions became protected at about the same concentration of CadC protein (Fig. 6). Current experiments do not allow the determination of whether binding to the transcription initiation site corresponds to bound form 1 of gel retardation experiments and whether additional upstream binding results in bound form 2 (Fig. 3). Experiments with mutant and shortened operator/promoter DNAs will resolve this question.



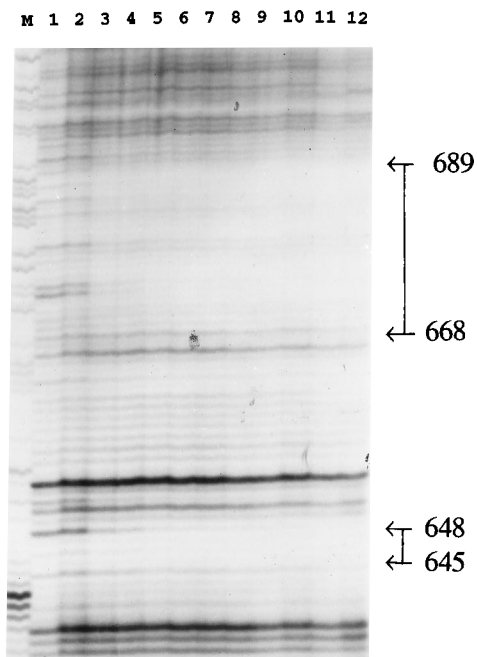


FIG. 6. Footprinting of CadC binding region by DNase I. Lane M, dideoxy-A sequencing ladder (nucleotide size markers). Lanes 1 through 12, 3.7 nM (25 ng/50  $\mu$ l) *cadA* operator DNA with 0, 21, 52, 104, 160, 210, 320, 520, 1,040, 1,550, 2,070, or 3,110 nM CadC protein, respectively, digested with 0.04 U of DNase I for 45 s at 25°C. Brackets with numbers indicate the approximate DNA positions protected by CadC protein, with base pairs numbered as in Fig. 1.

**Runoff in vitro transcription.** With purified *cadC* operator/promoter DNA and RNA polymerase from *E. coli*, in vitro transcription and incorporation of  $^{32}$ P-labelled ribonucleotides into mRNA (Fig. 7, lane 2) occurred from about the position previously determined in vivo (25). The addition of CadC protein completely repressed in vitro transcription (Fig. 7, lane 3). The addition of 10  $\mu$ M Cd $^{2+}$  did not relieve CadC repression of in vitro transcription (Fig. 7; compare lanes 4 and 5), and Cd $^{2+}$  itself was inhibitory for overall transcription (Fig. 4, compare lanes 2 and 4). The addition of RNA polymerase before the addition of CadC protein allowed transcription to

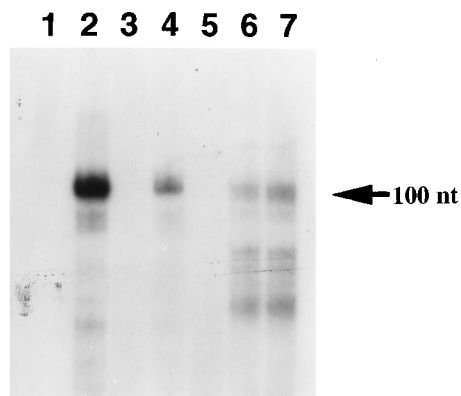


FIG. 7. Runoff in vitro transcription repressed by CadC protein. Lane 1, no template *cadA* operator/promoter DNA; lanes 2 through 7, 0.16  $\mu$ M (0.42  $\mu$ g/20  $\mu$ l) *cadA* operator DNA. Lanes 3, 5, and 7, 41  $\mu$ M CadC protein; lanes 4, 5, and 6, 10  $\mu$ M Cd $^{2+}$  was added. In lanes 6 and 7, 5 U of RNA polymerase was added 10 min before the addition of CadC protein and the radioactive reaction mixture.

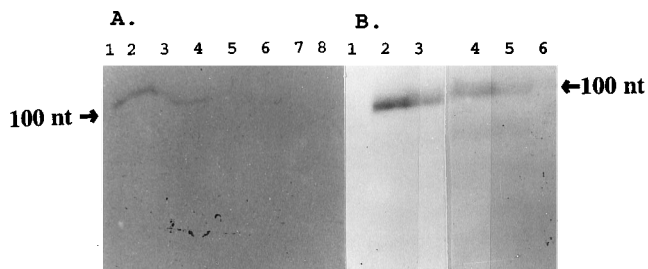


FIG. 8. Runoff in vitro transcription. Effects of increasing concentrations of CadC protein (A) and cations (B). (A) Template *cadA* operator/promoter DNA (60 nM; 0.16  $\mu$ g) was added along with 0, 104, 207, 414, 830, 1,660, 3,320, and 6,630 nM CadC protein (lanes 1 through 8, respectively). (B) *cadA* operator/promoter DNA with 0 (lane 2) or 1.2  $\mu$ M CadC protein (lanes 1 and 3 through 6) and 0 (lanes 1 and 2), 25, 250, 500, or 1,000  $\mu$ M Cd $^{2+}$  (lanes 3 through 6).

proceed, although not at the level for DNA without CadC protein (Fig. 7, lanes 6 and 7).

In runoff transcription assays, gradually increasing CadC concentrations resulted in greater inhibition of mRNA synthesis (Fig. 8A), with a CadC/DNA ratio of about 4:1 (mol/mol) (Fig. 8A, lane 3). Specificity was shown in that purified ArsR repressor protein had no effect (data not shown). When increasing concentrations of Cd $^{2+}$  were added to runoff transcription experiments, 25  $\mu$ M Cd $^{2+}$  protected against CadC inhibition of transcription. At concentrations above 250  $\mu$ M, Cd $^{2+}$  inhibited transcription. In similar experiments, 0.1 to 5  $\mu$ M bismuth tartrate prevented CadC repression of transcription (data not shown).

## DISCUSSION

This is the first report of in vitro regulation of the *cadA* operon of *S. aureus* plasmid pI258, and it provides direct evidence that the CadC protein (10, 25, 26) is a *trans*-acting regulatory protein and functions as a repressor of transcription (Fig. 7). The CadC protein bound specifically to the previously proposed *cad* operator DNA and was released by the addition of inducers Cd $^{2+}$ , Pb $^{2+}$ , and Bi $^{3+}$  (Fig. 5 and 8B). In DNase I footprinting experiments (Fig. 6), CadC bound to DNA protected the predicted (25) inverted repeat operator region and an additional upstream region marked in Fig. 1. The protection afforded by CadC from DNase I attack was also relieved by added Pb $^{2+}$  and Bi $^{3+}$  (data not shown). CadC protein repressed in vitro transcription from the *cad* promoter (Fig. 7 and 8).

CadC is a member of a newly proposed family of DNA-binding regulatory proteins, predicted on the basis of sequence homologies (1, 15) to share functional characteristics. The other members of this family are ArsR (the regulatory protein for the arsenic resistance system [4, 12, 15, 23]) and SmtB (the regulatory protein for cyanobacterial metallothionein synthesis [6]). Bairoch (1) suggests that a helix-turn-helix motif is a recognizable candidate for the DNA binding region. With work just beginning on the interaction of CadC with operator/promoter DNA, we look forward to experimental clarifications of the different actions of these somewhat similar heavy metal cation-responding *trans*-acting DNA-binding regulatory proteins.

The natures of the two forms of CadC protein associated with *cad* operator DNA (in gel retardation experiments) and the two regions of DNA protected in DNase I footprinting assays are also left for future experiments. However, it is tempting to hypothesize that CadC sits as a dimer on the

inverted repeat region shown in Fig. 1, forming retarded intermediate complex 1. Then the addition of a second dimer of CadC (to form a tetramer bound to DNA) might result in retarded form 2, with the additional protected region approximately from base pair 645 to base pair 648 (which occurs 20 bp, or two turns of the DNA helix, further upstream). We have not been able to separate these steps as a function of added CadC concentration (Fig. 4 and 6). A mutational approach, such as disrupting the 7-bp inverted repeat, extending the intrayad distance, or removing the upstream sequence from positions 645 to 648, will clarify the nature of this protein-DNA interaction.

CadC is expected to respond to very low intracellular concentrations of the toxic cation, as MerR responds to submicromolar mercury concentrations (11, 19) both in vivo and in vitro. CadC (Fig. 5) and ArsR (12, 24) in vitro respond poorly to added effector ions. The basis for this unexpected poor response is unknown. It may be that  $Cd^{2+}$  does not cause complete dissociation of CadC from operator DNA (Fig. 5) but only an association sufficiently reduced so that RNA polymerase can proceed or displace CadC (Fig. 8B).

Previous studies showed that expression of the cadmium resistance system is tightly regulated (3, 5, 20, 25, 26), but the *trans*-acting regulatory protein was not identified. The vector plasmid used by Yoon et al. (25, 26) contained an unrecognized copy of the *cadC* gene, which precluded determination of the role of the *cadC* gene in reporter gene expression experiments (25).

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#### REFERENCES

- Bairoch, A. 1993. A possible mechanism for metal-ion induced DNA-protein dissociation in a family of prokaryotic transcriptional regulators. *Nucleic Acids Res.* **21**:2515.
- Chen, Y. Personal communication.
- Chu, L., D. Mukhopadhyay, H. Yu, K.-S. Kim, and T. K. Misra. 1992. Regulation of the *Staphylococcus aureus* plasmid pI258 mercury resistance operon. *J. Bacteriol.* **174**:7044-7047.
- Corbisier, P., G. Nuyts, G. Ji, M. Mergeay, and S. Silver. 1993. *luxAB* gene fusions with the arsenic and cadmium resistance operons of *Staphylococcus aureus* plasmid pI258. *FEMS Microbiol. Lett.* **110**:231-238.
- Ji, G., and S. Silver. 1992. Regulation and expression of the arsenic resistance operon from *Staphylococcus aureus* plasmid pI258. *J. Bacteriol.* **174**:3684-3694.
- Lebrun, M., A. Audurier, and P. Cossart. 1994. Plasmid-borne cadmium resistance genes in *Listeria monocytogenes* are similar to *cadA* and *cadC* of *Staphylococcus aureus* and are induced by cadmium. *J. Bacteriol.* **176**:3040-3048.
- Morby, A. P., J. S. Turner, J. W. Huckle, and N. J. Robinson. 1993. SmtB is a metal-dependent repressor of the cyanobacterial metallothionein gene *smtA*: identification of a Zn inhibited DNA-protein complex. *Nucleic Acids Res.* **21**:921-925.
- Mukhopadhyay, D., H. Yu, G. Nucifora, and T. K. Misra. 1991. Purification and functional characterization of MerD. A coregulator of the mercury resistance operon in gram-negative bacteria. *J. Biol. Chem.* **266**:18538-18542.
- Muyldermans, G. (Free University of Brussels). Personal communication.
- Novick, R. P., E. Murphy, T. J. Gryczan, E. Baron, and I. Edelman. 1979. Penicillinase plasmids of *Staphylococcus aureus*: restriction-deletion maps. *Plasmid* **2**:109-129.
- Novick, R. P., and C. Roth. 1968. Plasmid-linked resistance to inorganic salts in *Staphylococcus aureus*. *J. Bacteriol.* **95**:1335-1342.
- Nucifora, G., L. Chu, T. K. Misra, and S. Silver. 1989. Cadmium resistance from *Staphylococcus aureus* plasmid pI258 *cadA* gene results from a cadmium-efflux ATPase. *Proc. Natl. Acad. Sci. USA* **86**:3544-3548.
- O'Halloran, T. V. 1993. Transition metals in control of gene expression. *Science* **261**:715-725.
- Rosenstein, R., K. Nikoleit, and F. Götz. 1994. Binding of ArsR, the repressor of the *Staphylococcus xyloso* (pSX267) arsenic resistance operon to a sequence with dyad symmetry within the *ars* promoter. *Mol. Gen. Genet.* **242**:566-572.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- San Francisco, M. J. D., C. L. Hope, J. B. Owolabi, L. S. Tisa, and B. P. Rosen. 1990. Identification of the metalloregulatory element of the plasmid-encoded arsenical resistance operon. *Nucleic Acids Res.* **18**:619-624.
- Shi, W. P., J. H. Wu, and B. P. Rosen. 1994. Identification of a putative metal binding site in a new family of metalloregulatory proteins. *J. Biol. Chem.* **269**:19826-19829.
- Silver, S., G. Nucifora, L. Chu, and T. K. Misra. 1989. Bacterial resistance ATPases: primary pumps for exporting toxic cations and anions. *Trends Biochem. Sci.* **14**:76-80.
- Silver, S., G. Nucifora, and L. T. Phung. 1993. Human Menkes X-chromosome disease and the staphylococcal cadmium-resistance ATPase: a remarkable similarity in protein sequences. *Mol. Microbiol.* **10**:7-12.
- Silver, S., and M. Walderhaug. 1992. Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. *Microbiol. Rev.* **56**:195-228.
- Summers, A. O. 1992. Untwist and shout: a heavy metal-responsive transcriptional regulator. *J. Bacteriol.* **174**:3097-3101.
- Tsai, K.-J., and A. L. Linet. 1993. Formation of a phosphorylated enzyme intermediate by the *cadA*  $Cd^{2+}$ -ATPase. *Arch. Biochem. Biophys.* **305**:267-270.
- Tsai, K.-J., K. P. Yoon, and A. R. Lynn. 1992. ATP-dependent cadmium transport by the *cadA* cadmium resistance determinant in everted membrane vesicles of *Bacillus subtilis*. *J. Bacteriol.* **174**:116-121.
- Tynecka, Z., Z. Gos, and J. Zajac. 1981. Energy-dependent efflux of cadmium coded by a plasmid resistance determinant in *Staphylococcus aureus*. *J. Bacteriol.* **147**:313-319.
- Wu, J. H., and B. P. Rosen. 1991. Regulation of the *ars* operon: the *arsR* gene product is a negative regulatory protein. *Mol. Microbiol.* **5**:1331-1336.
- Wu, J. H., and B. P. Rosen. 1993. Metalloregulated expression of the *ars* operon. *J. Biol. Chem.* **268**:52-58.
- Yoon, K. P., T. K. Misra, and S. Silver. 1991. Regulation of the *cadA* cadmium resistance determinant of *Staphylococcus aureus* plasmid pI258. *J. Bacteriol.* **173**:7643-7649.
- Yoon, K. P., and S. Silver. 1991. A second gene in the *Staphylococcus aureus* *cadA* cadmium resistance determinant of plasmid pI258. *J. Bacteriol.* **173**:7636-7642.
- Yu, H., D. Mukhopadhyay, and T. K. Misra. 1994. Purification and characterization of a novel organometallic receptor protein regulating the expression of the broad spectrum mercury-resistant operon of plasmid pDU1358. *J. Biol. Chem.* **269**:15697-15702.