JOURNAL OF BACTERIOLOGY, Dec. 1991, p. 7643–7649
0021-9193/91/237643-07$02.00/0

Vol. 173, No. 23

Regulation of the *cadA* Cadmium Resistance Determinant of *Staphylococcus aureus* Plasmid pI258

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Received 18 June 1991/Accepted 25 September 1991

Regulation of the *cadA* cadmium and zinc resistance determinant of *Staphylococcus aureus* plasmid pI258 was demonstrated by using gene fusions and direct measurements of transcription. In growth experiments, cells harboring the intact *cadA* operon were induced with different cations and challenged by an inhibitory concentration of $ZnCl_2$, a substrate of the CadA resistance system. Uninduced cells did not grow for 8 h after Zn^{2+} addition, whereas induced cells grew in the presence Zn^{2+}. Cd^{2+} was a strong inducer, and Bi^{3+} and Pb^{2+} also induced well; Co^{2+} and Zn^{2+} were weak inducers. A translational β-lactamase fusion to the *cadA* gene showed the same induction specificity as that seen with growth experiments with the intact *cadA* operon. A short β-lactamase transcriptional fusion to the *cadC* gene also showed the same pattern of induction, establishing that the *cadC* gene was not involved in regulation. In Northern (RNA) blot hybridization experiments, a cadmium-inducible, 2.6-kb, operon-length transcript was detected. Primer extension experiments determined that Cd^{2+}-inducible transcription of the *cadA* operon begins at nucleotides 676 and 677 of the published sequence (G. Nucifora, L. Chu, T. K. Misra, and S. Silver, Proc. Natl. Acad. Sci. USA 86: 3544–3548, 1989).

The *cadA* cadmium resistance determinant of *Staphylococcus aureus* plasmid pI258 contains two open reading frames (8). The protein products of the *cadA* efflux ATPase gene (727 amino acids) and of the shorter *cadC* gene (122 amino acids) have been identified, and the need for both genes for cadmium and zinc resistance has been established in the accompanying paper (24). In the present report, the induction pattern of the *cadA* operon by various toxic heavy metal cations is described. The *cadA* cadmium resistance system was previously thought to function constitutively (8, 12, 20, 22), although this hypothesis was not directly tested. As the results of our new research have shown, the inability to demonstrate regulated control of *cadA* was largely due to the use of Cd^{2+} both as an inducer and as a toxic challenge compound. However, a wide range of experiments described in this report demonstrate the inducibility of the *cadA* system. Most importantly, direct measurements of transcript formation (by Northern [RNA] blot analysis) and reverse transcriptase determination of the transcriptional start point discussed in this report clearly show that the *cadA* operon is inducibly regulated.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. aureus* RN4220 and *Escherichia coli* JM83 and JM101, as well as plasmid pSK265 and the pKPY series of plasmids with *cadA* fragments cloned into pSK265 or pUC19, are described in the accompanying paper (24). The β-lactamase translational fusion vector pWN1819 (21), with a multicloning site immediately upstream from the *blaZ* sequence, and the β-lactamase transcriptional fusion vector pSA3800 (5), with the *blaZ* ribosomal binding site preceded by a triple translational stop sequence, were the gifts of R. P. Novick and were used to construct *cadA-blaZ* fusions. Cell growth on 2XNY medium, the use of the antibiotics ampicillin (100 μg/ml), erythromycin (10 μg/ml), and chloramphenicol (5 μg/ml), and measurements of growth by turbidity were as described in the accompanying report (24). Cell mass as dry weight was calculated from a curve of dry weight versus turbidity (6).

DNA procedures. The manipulation of DNA with enzymes was as described by Sambrook et al. (13). The structure of each fusion was confirmed by nucleotide sequencing by the dideoxynucleotide method (15).

Materials. Cadmium chloride, cobalt dichloride, zinc chloride, manganese chloride, lead chloride, and potassium bismuth tartrate were used as cation salts. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Sigma Chemical Co. (St. Louis, Mo.). ^{35}S-dCTP for sequencing and [α-^{32}P]dCTP to make *cadA*-specific probes with a random primer kit were obtained from Amersham Corp. (Arlington Heights, Ill.). Kodak XAR film was from Eastman Kodak (Rochester, N.Y.). Restriction nuclease enzymes, calf intestinal phosphatase, and T4 DNA ligase came from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), and New England Biolabs, Inc. (Beverly, Mass.), respectively. Lysostaphin was purchased from Bristol-Meyers Pharmaceuticals (Evansville, Ind.). Nitrocefin was purchased from Becton Dickinson Microbiology Systems (Hunt Valley, Md.).

***S. aureus* protoplast transformation and regeneration.** Transformation of *S. aureus* protoplasts was as described in the accompanying paper (24).

Preparation of probe DNA. A *cadA*-specific probe was prepared as follows. Plasmid pKPY21 (with the 3.0-kb intact *cadA* determinant cloned into the multicloning region of a pUC19 derivative) was digested with *Xmn*I and *Hin*dIII, and the products were electrophoresed in a 1% agarose gel. A 1.2-kb *Xmn*I-*Hin*dIII fragment (nucleotides [nt] 1874 to 3043

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of the published sequence [8]) was purified from the gel and used as a template for the random primer kit (3). The [α-^{32}P] dCTP incorporation into the probe was carried out by DNA polymerase I (Klenow fragment).

Northern blot hybridization. Total cellular RNA was prepared by a rapid RNA preparation method (4). *S. aureus* RN4220 cells containing the *cadA* operon on plasmid pKPY2 (24) were grown in 2XNY medium until 50 Klett units of turbidity was reached, and they were induced by the addition of 1.5 μM $CdCl_2$ for 2 to 40 min. To prepare uninduced RNA, $CdCl_2$ was not added. The cultures were harvested at 50 Klett units of turbidity, and the cells were suspended in 1 ml of sucrose buffer containing lysostaphin (1 mg) and held on ice for 15 min. Lysis was brought about by the addition of 1% sodium dodecyl sulfate and 0.25 mg of proteinase K. Lysates were frozen and thawed twice with dry ice-ethanol and warm water (45°C). Loading dye (2 μl of 50% glycerol–200 mM EDTA–0.1% bromophenol blue–0.1% xylene cyanol), 20 μl of deionized formamide, and 4 μl of 10× running buffer (0.2 M morpholinepropanesulfonic acid [MOPS] [pH 7], 50 mM sodium acetate, 1 mM EDTA) were added, and electrophoresis was carried out as described elsewhere (4). Nucleic acids were transferred to nylon membrane filters (Hybond N; Amersham Corp.), and hybridization was carried out (4). The relative intensities of mRNA bands were quantified with an LKB ultrascan XL enhanced laser densitometer.

Preparation of RNA for primer extension. RNA preparation basically followed that described elsewhere (5). Plasmid pKPY500, with a 278-bp *Sst*I fragment (promoter and the 5′ half of *cadC*; see below), was used as the source of cellular RNA. A 100-ml culture of *S. aureus* RN4220 harboring pKPY500 was grown until 50 Klett units of turbidity was reached, induced for 5 min by 1.5 μM $CdCl_2$, and harvested. To prepare the uninduced culture, $CdCl_2$ was not added during the 5-min period. The cells were washed with 1× SMM buffer (1 M sucrose, 40 mM maleic acid, 40 mM $MgCl_2$ [pH 6.5]), centrifuged, and suspended in 10 ml of 1× SMM buffer. Lysostaphin (100 μg/ml) was added, and the culture was kept on ice for 15 to 20 min for protoplast formation. The protoplasts were suspended in 5 M guanidinium thiocyanate and frozen and thawed twice to reduce viscosity.

The total cell lysate was centrifuged through a cesium chloride-EDTA gradient (5) at 35,000 rpm for 20 h at 17°C. The pelleted RNA was dried and dissolved in guanidine-HCl and then precipitated with ethanol at 4°C (13). The drying, dissolving, and precipitation with ethanol were repeated. RNA was precipitated with sodium acetate and ethanol, redissolved, and stored under ethanol at −70°C.

Primer extension experiment. Primer extension experiments (1) were performed by using an M13 universal primer, the 17-mer oligonucleotide 5′deoxy(GTAAAACGACGGC CAGT)3′. For primer extension experiments, a new phage-plasmid pair, M13mKPY500 and pKPY500, containing a 278-bp fragment (nt 533 to 810 of the published sequence) was constructed as described below. Total RNA (20 μg) from *S. aureus* RN4220(pKPY500) was mixed with primer (10 ng) in a total volume of 5 μl. The mixture was heated for 5 min at 65°C and then cooled slowly (over 45 min) to 37°C.

Primer extension reactions were carried out in a total volume of 10 μl containing 100 mM Tris-HCl (pH 8.3)–140 mM KCl–50 mM $MgCl_2$–10 mM dithiothreitol–50 μM each dGTP, dTTP, and dATP–2 μM [α-^{32}P]dCTP–20 U of Moloney murine leukemia virus reverse transcriptase. These components were added to the hybridized nucleic acid mixture and incubated at 37°C for 45 min. Another 20 U of virus reverse transcriptase and 3 μl of a chase solution containing 1.25 mM each dGTP, dCTP, dTTP, and dATP were added, and the mixture was incubated for an additional 45 min at 37°C.

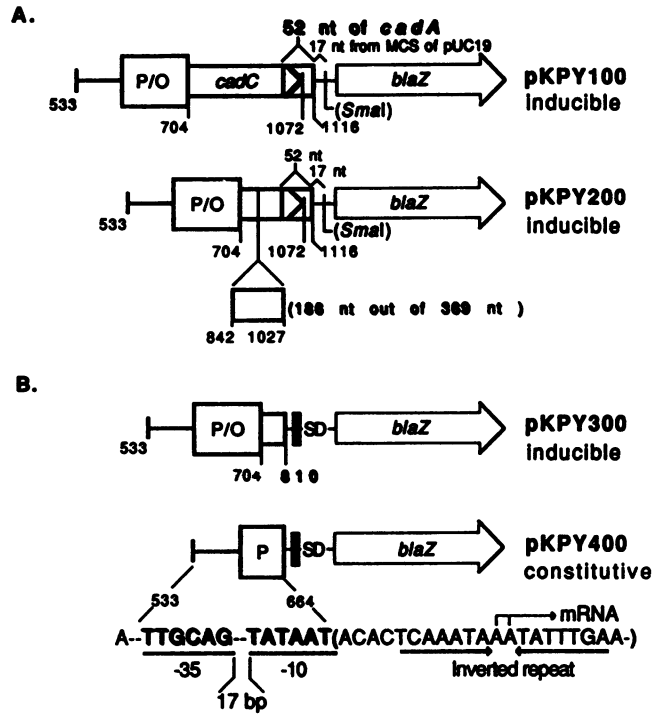

FIG. 1. Structures of the translational and transcriptional *blaZ* fusions used. (A) Translational fusions in plasmids pKPY100 and pKPY200; (B) Transcriptional fusions in plasmids pKPY300 and pKPY400. See Materials and Methods for details. Promoter-operator region (P/O) and *cadC* and *cadA* genes are marked by open bars, with arrows inside indicating the direction of translation. Heavy lines, additional DNA cloned outside of the genes. Arrow at nt 1072, end of the *cadC* gene. The nucleotide numbers are from the published sequence (8). Thick vertical lines in panel B, translational stop codons in all three reading frames; SD, Shine-Dalgarno ribosomal binding site. The box below pKPY200 shows the internally deleted sequence from *cadC*. With plasmid pKPY400, the 3′ end of the cloned promoter sequence is at nt 664.

The reaction was stopped by the addition of an equal volume of 98% (vol/vol) formamide–20 mM EDTA–0.05% xylene cyanol–0.05% bromophenol blue. The samples were boiled for 3 min and electrophoresed in a 6% polyacrylamide–8 M urea sequencing gel. An appropriate sequence ladder starting from precisely the same position was generated by dideoxynucleotide sequencing (15) with the same primer and the appropriate M13 DNA clone (M13mKPY500) and was electrophoresed on the same gel to determine the position of the transcription start site.

Construction of the translational and transcriptional *blaZ* fusions to *cadA* and *cadC* and to a promoter. To construct pKPY100 (*cadA-blaZ* fusion) (Fig. 1), pUC19 was cut with *Eco*RI and *Hin*dIII restriction enzymes, and the ends were blunt-ended and ligated by DNA polymerase I (Klenow fragment) and T4 DNA ligase, respectively. The modified pUC19 (lacking *Hin*dIII and *Eco*RI sites) was named pKPY20. The 3.0-kb *Xba*I fragment containing the intact *cadA* determinant was subcloned into the *Xba*I site of pKPY20, and the resulting plasmid was named pKPY21.

pKPY21 was cut by *Xmn*I, and a 1.35-kb fragment of the digest was purified from an agarose gel, digested by *Nla*IV, and ligated with the *Sma*I-digested pUC19. After transformation, the construct was named pKPY22 (which has the promoter, *cadC*, and first 52 nt of the 5′ end of *cadA* in a 0.6-kb *Xmn*I-*Nla*IV fragment [nt 533 to 1116 from the published sequence [8]).

pKPY22 was cut by *Sca*I and *Hin*cII, and a 1.55-kb fragment was purified and ligated with the *Sma*I-digested *bla* vector pWN1819 (21), transformed into *S. aureus* RN4220 (7), and named pKPY100 (Fig. 1). pKPY100 contains the *cadA* operator-promoter, intact *cadC*, first 52 nt from the 5′ end of *cadA*, and an insertion of a 17-bp *Sma*I-*Hin*cII fragment from the multicloning site of pUC19, between nt 1116 of the published *cadA* sequence and the *Sma*I site of pWN1819, resulting in the correct formation of a translational fusion of *cadA* with *blaZ*.

The translational fusion of pKPY100 was verified by sequencing about 0.3 kb covering the 3′ half of *cadC* and the 52 nt described above, followed by the 17 nt which place the translational fusion in correct register.

pKPY200 (Fig. 1), containing the *cadA-blaZ* translational fusion with an internal deletion of 186 nt within *cadC*, was prepared by isolating the 0.6-kb *Sst*I fragment of pKPY100 (containing the promoter, the intact *cadC* gene, and the translational fusion region) and ligating it with *Sst*I-digested pUC19. The resulting plasmid was named pKPY23. There is only one *Sna*BI site in pKPY23, in the middle of *cadC* (nt 936 of the published sequence). pKPY23 was cut by *Sna*BI and digested by *Bal* 31 nuclease. The ends were blunted by DNA polymerase, self-ligated, cut by *Sst*I, and ligated with the *Sst*I-digested pWN1819. After transformation into strain RN4220, the approximate sizes of the *Bal* 31 deletions were determined by examining *Sst*I-generated fragments on a 4% polyacrylamide gel. A plasmid which contained a 0.4-kb *Sst*I fragment, 200 nt shorter than the original 0.6-kb fragment, was identified and named pKPY200. The precise length of the deleted region was determined by direct sequencing. A 186-bp region between nt 841 and 1028 in the middle of *cadC* (which starts at nt 704 and ends at nt 1072) was deleted.

pKPY300 (Fig. 1), containing the *cadC-blaZ* transcriptional fusion, was prepared by cutting pKPY22 with *Dra*I and ligating with the *Sma*I-digested pSA3800 (5). pSA3800 contains translational stop codons between the multicloning site and the ribosomal binding site of the *blaZ* β-lactamase gene. Translation initiated from an insert, i.e., the *cadC* gene, therefore stops at the in-frame stop codon, and translation of the *blaZ* gene initiates from the *blaZ* ribosomal binding site. The ligation mixture was transformed into RN4220, and the correct plasmid (containing a 0.3-kb *Sst*I fragment; nt 533 to 810 of the published sequence [8]) was identified by restriction enzyme digestion. Direct sequencing verified the construct. pKPY300 contains the *cadA* promoter, an inverted repeat, and the 5′ region of *cadC*.

pKPY400 (Fig. 1), containing the *cadA* operon promoter-*blaZ* transcriptional fusion, was prepared from a 0.6-kb *Eco*RI-*Hin*dIII fragment of pKPY22 that digested with *Fok*I. The ends of digested fragments were blunt-ended by DNA polymerase, digested by *Sst*I, and ligated together with *Sma*I-*Sst*I-digested pUC19. The correct construct containing a 0.15-kb fragment with the *cadA* operon promoter was identified by restriction enzyme digestions, and the plasmid was named pKPY24. Sequencing of the 0.15-kb *Bam*HI fragment showed that it contained nt 533 to 664 of the published sequence (8), encompassing the −35 and −10 sites but not the inverted repeat (Fig. 1; also, see below). The 132-bp *Bam*HI fragment was ligated into the *Bam*HI site of pSA3800 (5) and transformed into strain RN4220, and the plasmid was named pKPY400.

pKPY500 containing the 0.3-kb fragment from nt 533 to nt 810 was prepared from the 0.3-kb *Sst*I fragment of pKPY300, which was subcloned into the *Sst*I site of M13mp18 and named M13mKPY500. M13mKPY500 was digested with *Ava*II and *Bgl*II, blunt-ended with T4 DNA polymerase, and ligated with *Sma*I-digested pSK265. The correct construct was identified by restriction enzyme digestions and named pKPY500.

β-Lactamase assays. β-Lactamase activity was assayed by the nitrocefin method (9, 21). Overnight-grown cells were grown for 2 to 3 h at 37°C in 2XNY broth, diluted 100-fold again, grown until 45 to 50 Klett units of turbidity was reached, and induced by addition of Cd^{2+} (or other cations) for 90 min at 37°C. The cell suspensions were diluted with equal volumes of prewarmed phosphate buffer (pH 6.5) and incubated at 37°C. A 0.1-ml portion of the mixture described above was added to 0.1 ml of a 200-μg/ml prewarmed (37°C) nitrocefin solution. After 5 to 15 min, the reaction was stopped by adding 0.6 ml of ice-cold phosphate buffer (pH 6.5) and placing the mixture on ice. Cells were removed by centrifugation, and the A_{482} of the supernatant fluid was measured.

Efflux assay. *S. aureus* cells for efflux assays were grown, induced (or not induced), and harvested. Cell suspensions (0.3 ml) were incubated with 3 μM $^{109}CdCl_2$ for 5 min at 37°C, and then the cells were kept at 4°C for 40 min to equilibrate the cells with $^{109}CdCl_2$. Loaded cells were diluted 20-fold into 2XNY medium containing 0.15 M sodium acetate (pH 5.5). Samples (0.3 ml) were filtered through 0.45-μm-pore-size nitrocellulose filters (Nuclepore Corp., Pleasanton, Calif.) and rinsed twice with 5 ml of 20 mM $CdCl_2$ in TE buffer (10 mM Tris plus 1 mM EDTA [pH 7.5]). Washed filters were counted in a liquid scintillation spectrophotometer.

RESULTS

Zinc resistance is inducible by Cd^{2+}, Co^{2+}, Pb^{2+}, Zn^{2+}, and Bi^{3+}. *S. aureus* RN4220(pKPY2) cells were grown, induced with increasing amounts of Cd^{2+}, Co^{2+}, Pb^{2+}, Zn^{2+}, and Bi^{3+}, and challenged with an inhibitory concentration of Zn^{2+} (Fig. 2). Uninduced cells stopped growing immediately after the addition of 2 mM Zn^{2+}. Cells induced by optimum levels of Cd^{2+}, Co^{2+}, Pb^{2+}, Zn^{2+}, or Bi^{3+} grew almost as well in the presence of 2 mM Zn^{2+} as in its absence. Optimum inducer levels under these conditions were about 30 μM Cd^{2+} (Fig. 2A), 50 μM Co^{2+} (Fig. 2B), 10 μM Pb^{2+} (Fig. 2C), 2 μM Bi^{3+} (Fig. 2D), and 150 μM Zn^{2+} (Fig. 2E). When *S. aureus* cells harboring pKPY2 were induced with Cd^{2+} and challenged by inhibitory Cd^{2+} concentrations, induced bacteria grew slightly better than uninduced bacteria (data not shown). However, we have been unable to establish clear conditions for Cd^{2+}-inducible Cd^{2+} resistance, such as those shown in Fig. 2 for Zn^{2+} resistance.

Bi^{3+} and Pb^{2+} were strong inducers of the *cadA* system, which confers resistances primarily to Cd^{2+} (6, 24) and to Zn^{2+} (Fig. 2). Bi^{3+} resistance was characterized by somewhat larger and more numerous colonies in Bi^{3+}-containing agar in petri dishes, but this resistance was not distinct enough for quantitation (data not shown). Pb^{2+} resistance

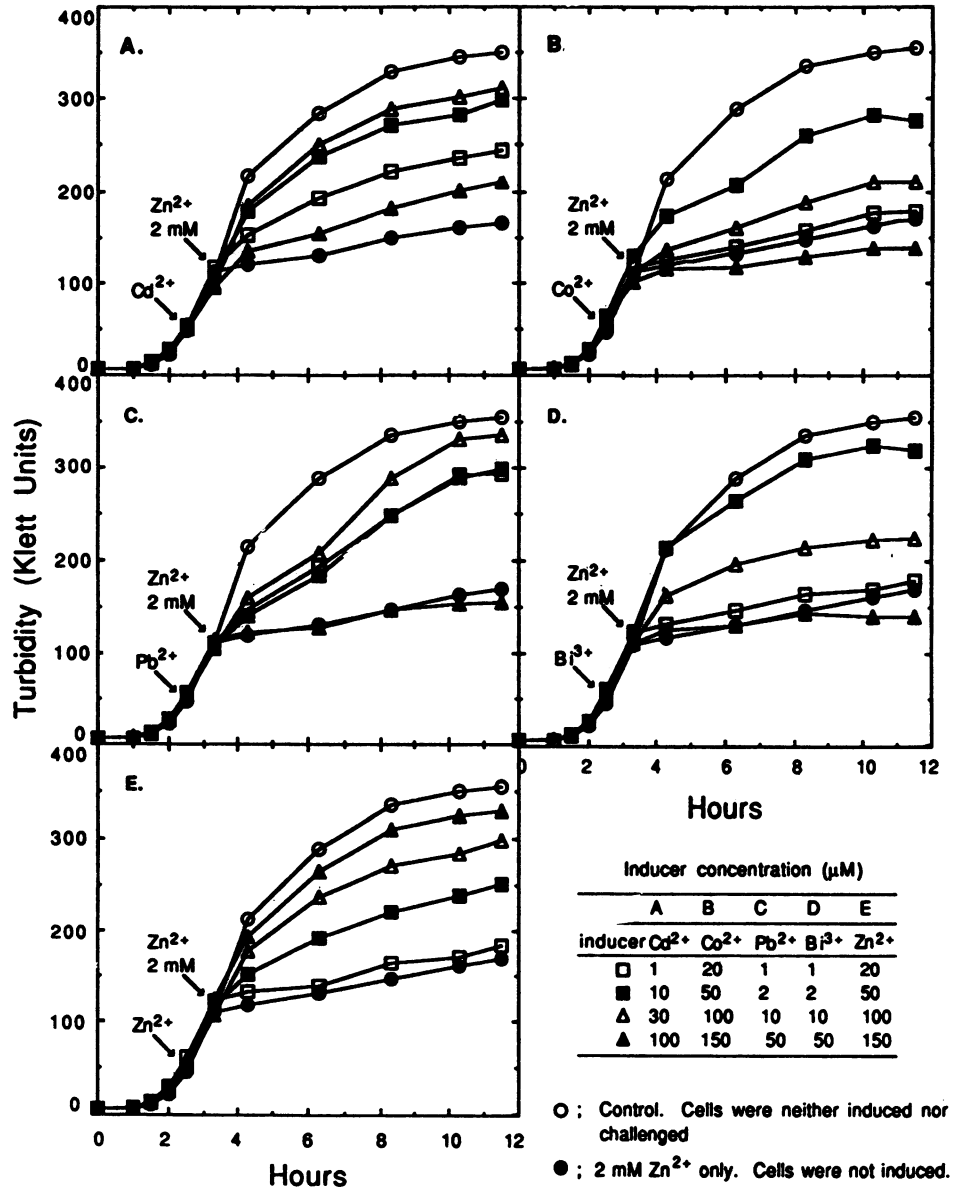

FIG. 2. Induction of zinc resistance by cations. *S. aureus* RN4220(pKPY2), with the intact *cadA* operon, was grown and induced by addition of Cd^{2+}, Zn^{2+}, Co^{2+}, Bi^{3+}, or Pb^{2+} for 45 min and then challenged by addition of 2 mM $ZnCl_2$. Growth was followed by measurements of turbidity.

could not be detected in our experiments (data not shown), although Bi^{3+} and Pb^{2+} resistances were reported to be located in the region of plasmid pI258 between *bla* and *cadA* (6).

Efflux of Cd^{2+}. Cells induced with 2 μM Bi^{3+} were loaded in the presence of 3 μM $^{109}CdCl_2$ and then diluted into the prewarmed medium for efflux. Cadmium efflux occurred only when cells were induced (Fig. 3A). The *cadC* gene was required for Cd^{2+} efflux, but *cadC* could be present either in an intact *cadA* determinant (Fig. 3A) or in *trans* (Fig. 3B) on complementing plasmid pKPY9.

Induction of *cadA-blaZ* fusions. The *blaZ* gene determining staphylococcal β-lactamase was used as a reporter gene in a translational gene fusion. Cd^{2+} was the most efficient inducer (Fig. 4), although higher levels of Cd^{2+} were inhibitory. [Note that strain RN4220(pKPY100) is cadmium sensitive, since it lacks most of the *cadA* operon.] Pb^{2+} and Bi^{3+} also induced *cadA* well. Zn^{2+} and Co^{2+} were poor inducers, but significant and reproducible induction was seen at 100 μM; Mn^{2+} did not induce β-lactamase activity (Fig. 4). The *cadA* system does not confer cobalt resistance to *S. aureus*. The MICs for *S. aureus* RN4220 of Bi^{3+} and Pb^{2+} were around 8 mM, and therefore, the cells were not exposed to inhibitory levels of Bi^{3+} or Pb^{2+} in the experiment represented in Fig. 4. The vector plasmid pWN1819, with no insert, did not produce measurable β-lactamase activity, regardless of addition of heavy metals (data not shown).

The absence of a role of *cadC* in operon regulation was tested with a *cadC-blaZ* transcriptional fusion by using the β-lactamase transcriptional fusion vector pSA3800 (5). The β-lactamase gene was fused to nt 810 of the published

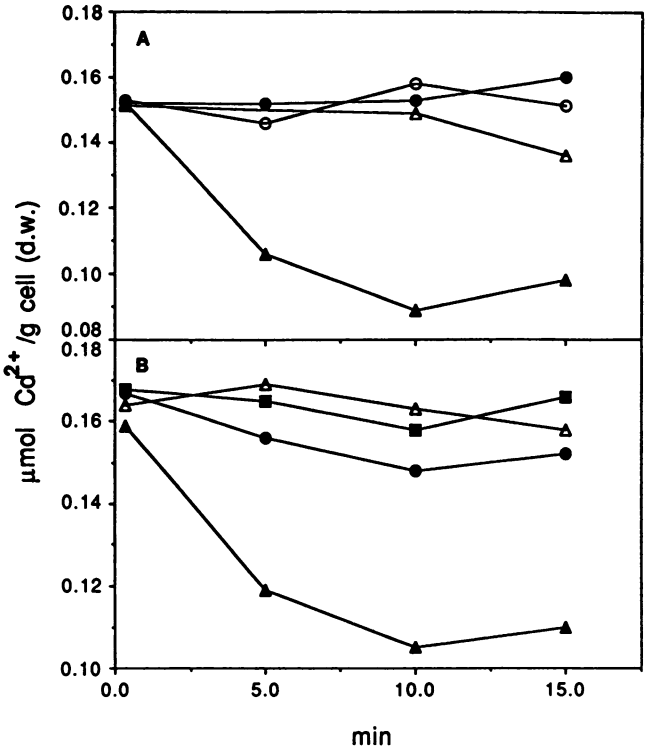

FIG. 3. Inducible cadmium efflux. Induced (2 μM Bi^{3+} for 45 min) and uninduced cultures were prepared as described in Materials and Methods. Cells were loaded in the presence of 3 μM $^{109}CdCl_2$ as described in Materials and Methods and diluted 20-fold. Samples (0.3 ml) were filtered, washed and counted. (A) Symbols: circles, cells with pSK265; triangles, cells with pKPY2. Closed and open symbols, induced and uninduced cells, respectively. (B) Symbols: ▲ and △, induced and uninduced cells with both plasmids pKPY6 and pKPY9, respectively; ●, induced cells with pKPY6; ■, induced cells with pKPY9. d.w., dry weight of cells.

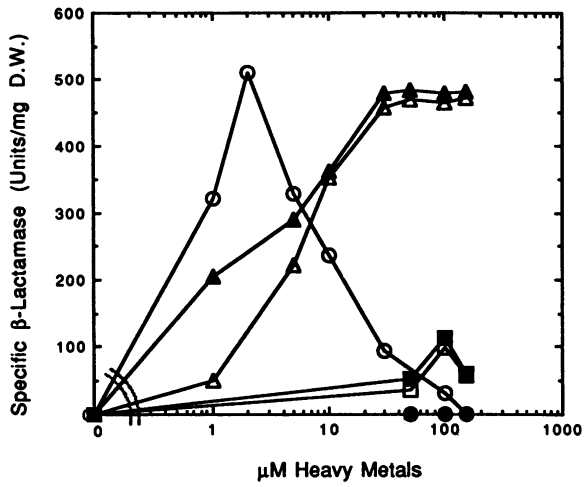

FIG. 4. Induction of the *cadA-blaZ* translational fusion strain. Cells containing plasmid pKPY100 were grown and induced by the addition of indicated levels of Cd^{2+} (○), Mn^{2+} (●), Zn^{2+} (□), Co^{2+} (■), Pb^{2+} (△), or Bi^{3+} (▲) for 1.5 h. The cells were centrifuged, and nitrocefin was added to the cell-free supernatant fluid. Nitrocefin hydrolysis during 5 min was measured as described in Materials and Methods. The specific activity of β-lactamase is given in units per milligram (dry weight [D.W.]) of cells.

sequence (the 107th nt of the *cadC* gene) (Fig. 1). β-Lactamase synthesis by cells with the *cadC-blaZ* fusion plasmid pKPY300 was inducible by Cd^{2+} (Fig. 5).

Effect of a deletion of the inverted repeat in the mRNA initiation region. Since the previously described induction experiment (Fig. 5) showed that CadC was not required for induction by Cd^{2+}, we removed the inverted repeat upstream of the *cadC* gene (Fig. 1). A transcriptional fusion to the −10 sequence (TATAAT) (plasmid pKPY400, as described in Materials and Methods) was constructed. Plasmid pKPY400 has only 132 bp from the *cadA* operon promoter region transcriptionally fused to *blaZ*. Cells with pKPY400 produced β-lactamase constitutively (Fig. 5). The level of β-lactamase was about one third of that found with plasmid pKPY300, a low but significant enzyme level. Constitutive expression of β-lactamase after deletion of the inverted repeat sequence suggests that the inverted repeat region might function specifically in *cadA* operon regulation. However, the deletion in plasmid pKPY400 removed the endogenous mRNA start site (Fig. 1), so the constitutive transcription of the *blaZ* in cells with this plasmid must start elsewhere. The endogenous mRNA was directly determined (see below), but the location of this novel non-*cadA* mRNA start site was not measured.

Northern blot hybridization measurement of induced mRNA synthesis. The size and amount of *cadA*-specific mRNA was directly measured by Northern blot hybridization. Total cellular mRNA was isolated from cells of *S. aureus* RN4220(pKPY2), induced by the addition of 1.5 μM Cd^{2+} from 2 to 40 min before harvesting the cells. RNA was hybridized with a *cadA*-specific probe consisting of nt 1874 to 3043 of the published sequence (8), a region lying entirely within the *cadA* gene. With uninduced cells, the *cadA*-specific RNA band was not visible (Fig. 6, lane 2). An operon-length, 2.6-kb *cadA*-specific RNA band was visible after 2 min of induction (Fig. 6, lane 3). After 5 min of induction, the *cadA*-specific RNA band had reached a peak level.

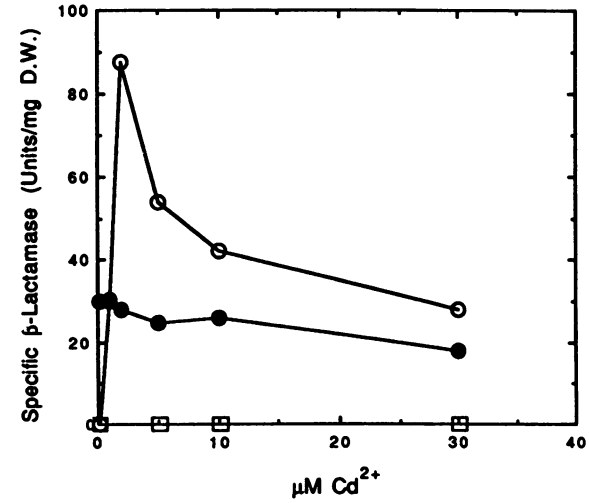

FIG. 5. Induction pattern of transcriptional fusions. Growth, induction, and assay of β-lactamase were as in the experiment described in the legend to Fig. 4. Symbols: ○, pKPY300; ●, pKPY400; □, pSA3800; D.W., dry weight of cells.

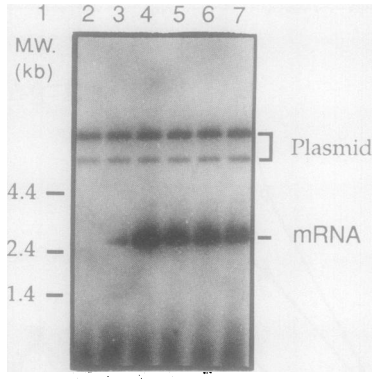

FIG. 6. Northern blot hybridization. Total RNA was isolated from *S. aureus* RN4220(pKPY2) after induction for 2, 5, 10, 20, or 40 min (lanes 3, 4, 5, 6, and 7, respectively) with 1.5 μM Cd^{2+}. Purified total RNA was electrophoresed, blotted, and hybridized with the ^{32}P-labelled *cadA*-specific probe. The upper two visible bands contain plasmid DNAs. The 2.6-kb, operon-length *cadA* transcript is marked (mRNA), and RNA size markers are shown to the left. M.W., molecular weight.

Primer extension experiment. To directly measure the mRNA start site, two new vector constructs were prepared. These contain a 278-bp *Sst*I fragment (nt 533 to 810 of the published sequence) from plasmid pKPY300, subcloned into the *Sst*I site of M13mp18, named M13mKPY500, and used to generate the control sequence ladder. The 278-bp fragment was removed as part of a 0.6-kb *Ava*II-*Bgl*II fragment. The protruding ends were blunt-ended with DNA polymerase I (Klenow fragment), and the fragment was cloned into the *Sma*I site of vector pSK265. This construct (called pKPY500) was transformed into strain RN4220. Total cellular mRNA was prepared from RN4220(pKPY500), and the mRNA start site of the *cadA* operon was identified by reverse transcriptase primer extension. The pair of constructs M13mKPY500 and pKPY500 permitted use of the M13 universal primer for the primer extension experiment (Fig. 7, lanes 5 and 6) and use of the same primer to generate the sequence ladder (Fig. 7, lanes 1 through 4). The mRNA starts at nt 676 and 677 of the published sequence (24) (Fig. 7), in the middle of the inverted repeat. The mRNA-DNA band was present only when the RNA was isolated from cells induced by 1.5 μM Cd^{2+} (Fig. 7).

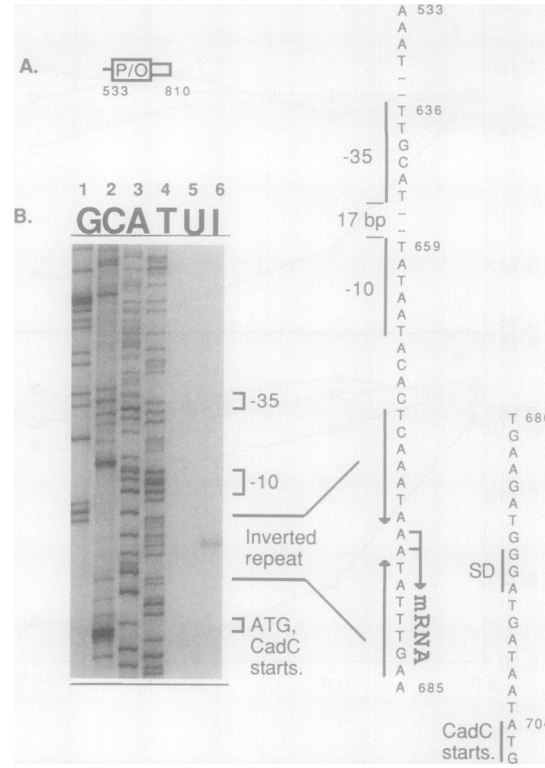

FIG. 7. Primer extension identification of the mRNA start site. (A) Diagram of the 278-bp *cadA* fragment cloned in vector pSK265. (B) Lanes 1 through 4, G, C, A, and T lanes from the dideoxy sequencing ladder of M13mKPY500 with the universal primer; lane 5, reverse transcriptase primer extension results with total RNA isolated from uninduced *S. aureus* RN4220(pKPY500); lane 6, same as lane 5 except that cells were induced with 1.5 μM Cd^{2+} for 5 min at 37°C. On the right is shown the relevant sequence of the DNA strand equivalent to the mRNA sequence, including the −35 and −10 promoter consensus sequences, the 17-bp distance between them, the inverted repeat, and the start site for mRNA synthesis. SD, Shine-Dalgarno ribosomal binding site for the *cadC* gene.

DISCUSSION

The *cadA* operon of *S. aureus* plasmid pI258 is regulated at the transcriptional level. Growth experiments showed that cells that were exposed to low levels of Cd^{2+}, Co^{2+}, Pb^{2+}, Zn^{2+}, or Bi^{3+} and subsequently challenged by an inhibitory concentration of Zn^{2+} were inducibly resistant (Fig. 2). This is the first report of inducibility of the *cadA* operon, which had been recognized previously (8, 17–20). Apparently, the difficulty with earlier studies and with our own recent inability to demonstrate Cd^{2+}-inducible Cd^{2+} resistance (data not shown) is that Cd^{2+} inhibition of growth occurs gradually. Whatever test concentrations of Cd^{2+} are used to inhibit growth will also induce the system prior to full growth inhibition. Efflux experiments demonstrated that the physiological basis for cadmium resistance (that is the efflux of accumulated cadmium from resistant cells) was also inducible (Fig. 3) and that efflux required both the CadC and the CadA proteins (Fig. 3B).

Early reports on the genetics of plasmid pI258 indicated a gene for marginal resistance to bismuth and lead salts mapping between *bla* and *cadA* (6, 16, 19). We found weak bismuth resistance with the *cadA* system of plasmid pI258 in the present study (data not shown). A lead resistance gene was reported to map in this region of the pI258 plasmid (6, 19), but we were unable to detect plasmid-dependent lead resistance in our experiments (data not shown).

The finding that *cadA*-dependent resistance to zinc is inducible raises questions of *cis*-acting and *trans*-acting elements involved in induction. Induction specificity was studied with *blaZ* (β-lactamase) gene translational and transcriptional fusions to either the promoter region or the *cadC* or *cadA* genes (Fig. 1). The pattern of induction of β-lactamase synthesis with the *cadA-blaZ* fusion was similar to the pattern of inducible zinc resistance in the growth experiments (compare Fig. 2 and 4).

The level of induced *cadA* mRNA was measured directly in Northern blot hybridization experiments (Fig. 6). Operon-length, *cadA*-specific mRNA was induced by 2 min or more of exposure to 1.5 μM Cd^{2+}. The approximate size of the mRNA (2.6 kb) was sufficient to encode both the CadC and the CadA proteins. There was no sign of partial operon

transcripts in our Northern blots, such as were found with the arsenite resistance system of an *E. coli* plasmid, in which posttranscriptional mRNA processing (11) accounts for decreased production of the protein product of the middle of three genes (compared with increased production of the protein products of the first and third genes).

trans-acting ArsR and MerR regulatory proteins are encoded by genes in the *ars* operon (11, 14, 23) and *mer* operon (2, 10, 18), respectively. However, a *trans*-acting regulatory protein in the *cadA* operon has not been identified. The *cadC-blaZ* fusion experiment represented in Fig. 5 eliminates such a regulatory role for either known gene in the *cadA* operon. That the hypothesized *cadR* gene must lie outside of the *cadA* operon is also required by the primer extension experiment, which showed that only the operator-promoter region and 107 nt from *cadC* suffice for regulated mRNA synthesis (Fig. 7). We postulate the existence of a *trans*-acting regulatory CadR protein, because transcriptionally regulated systems generally use diffusible proteins to recognize a small activator compound (in the case of *cadA*, the toxic inducer cations). There is no room in the cloned fragments (used in the experiments represented in Fig. 5 and 7) for an additional gene, and no potential open reading frames were found in the cloned fragments in either orientation (8). Only the CadC and CadA proteins were identified with the intact cadmium resistance operon cloned in the T7 bacteriophage expression system in both orientations (24). Inducibility of the *cadA* system was lost only when *blaZ* was transcriptionally fused to a promoter which did not have the inverted repeat marked in Fig. 1 and 7, indicating that the inverted repeat might function as the site of binding of the hypothesized CadR protein. We are left with the tentative regulatory model of an additional regulatory gene (*cadR*) on the chromosome and of the plasmid inverted repeat functioning as an operator.

The present experimental work has furthered our understanding of the *cadA* operon by demonstrating regulation, the range and concentration of inducers (Fig. 2 and 4), the inducible efflux of cadmium (Fig. 3), and the start site and the length of mRNA (Fig. 6 and 7).

ACKNOWLEDGMENTS

We thank R. P. Novick and P. Wang for providing plasmids pWN1819 and pSA3800, and we thank B. P. Rosen and A. Lynn for suggestions and discussions during the course of this work. We also thank J. S. Kornblum for suggestions for the Northern blot and primer extension experiments and J. P. Quinn for nitrocefin.

The experiments reported here were supported by grants from the National Science Foundation, the Electric Power Research Institute, and the National Institutes of Health.

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