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Kinetics of Expression of the *Escherichia coli cad* Operon as a Function of pH and Lysine

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The *Escherichia coli cadBA* genes are regulated at the transcriptional level by external pH and lysine. The membrane-localized CadC protein is required for activation of this operon under inducing conditions, which include acidic external pH, lysine, and oxygen limitation. To better understand the mechanism by which CadC functions, the kinetics of *cadBA* expression as a function of pH and lysine were examined. By primer extension assays, *cadBA* expression was detected within 4 min following exposure of cells to one of the inducing stimuli (low pH or lysine), provided that the cells had first been grown to steady state in the presence of the other inducing stimulus. The induction time was three to four times longer when both inducing stimuli were added simultaneously. *cadBA* expression was shut off within 4 min following a shift from acidic to neutral pH. Treatment of cells with chloramphenicol prevented induction by acidic pH and lysine. Transcription of *lysP* (encodes a lysine transporter) was also examined, since it is a negative regulator of *cadBA* expression in the absence of lysine. *lysP* expression was repressed by lysine but not influenced by pH. Putative transcription start sites for *lysP* and *cadC* were determined. Together, these data suggest that CadC senses the lysine- and pH-induced signals separately and that one of the roles of lysine in inducing *cadBA* may be to repress expression of *lysP*, thus eliminating the repressing effects of LysP.

Degradative lysine and arginine decarboxylases are expressed in *Escherichia coli* under conditions of low external pH in the presence of their respective amino acid substrate (1, 5, 8, 14). The products of these reactions include the decarboxylated amino acids (cadaverine for lysine and agmatine for arginine) and CO_2. Concomitant with the production and excretion of these molecules is an increase in the pHs of the media (due to the consumption of a proton during the decarboxylation reaction). Although the physiological roles of these systems have not been extensively characterized, presumably one or all of the resultant products provide some growth or survival advantage in acidic conditions (2, 4, 5, 9, 12, 18). Investigation of the molecular events that control the expression of the genes encoding these decarboxylases provides the opportunity to understand the natures of the signal transduction pathways that are involved in sensing and responding to changes in external pH.

The *Escherichia coli cadBA* operon and the adjacent *cadC* gene encode lysine decarboxylase (*cadA*), a lysine-cadaverine antiporter (*cadB*), and CadC, a positive regulator of *cadBA* expression (1, 9, 14, 17, 19). Transcription of *cadBA* originates 75 bp upstream of *cadB* from the P*cad* promoter (Fig. 1) (19). Genetic analysis has identified sequences located 125 bp upstream of the P*cad* transcriptional start site that appear to interact with a *trans*-acting factor necessary for P*cad* activation (10, 19). On the basis of the identification of *cadC*, it was proposed that its gene product, CadC, is this required factor. In vivo footprinting experiments and demonstration of a direct interaction between CadC and this upstream region support this hypothesis (10, 21).

cadC encodes a predicted 512-residue inner membrane protein with a single transmembrane segment and a cytoplasmic domain that resembles the DNA-binding domain of OmpR and related transcriptional regulators (3, 19). Since CadC is present at similar levels in cells grown in neutral and acidic external pH media, it appears that CadC's ability to activate P*cad* is altered as a function of pH- and lysine-induced signals (3, 19). Mutants of *cadC* that confer stimulus-independent expression are located in the periplasmic domain, suggesting that a signal is sensed in the periplasm and transmitted across the membrane to the cytoplasmic DNA-binding domain (3). Some of these mutations result in pH-independent *cadBA* expression but do not alter the requirement for lysine, suggesting that separate mechanisms are involved in CadC's ability to sense and respond to the pH- and lysine-induced signals.

Two other genes, *hns* and *lysP* (originally named *cadR*), are required for negatively regulating *cadBA* expression in the absence of the inducing stimuli (11, 13, 15, 17). A transposon insertion in *lysP* abolishes the lysine requirement but does not affect the requirement for low pH (11). Although the mechanism of this LysP-mediated repression is not understood, this result led to the suggestions that LysP inhibits the ability of CadC to activate transcription and that lysine, possibly through an interaction with CadC or LysP, eliminates this inhibition (11).

How CadC senses the pH and lysine signals, how these signals are transmitted across the membrane, which steps in P*cad* activation are regulated by CadC, and what molecular properties define active and inactive CadC have not been determined. To provide a better framework for addressing these issues, we felt it necessary to characterize the transcriptional response in more detail. Specifically, using primer extension to analyze the various mRNAs, we (i) established an in vivo kinetic profile of *cadBA* expression with respect to pH and lysine, (ii) determined the in vivo expression patterns of *cadC* and *lysP* as a function of pH and lysine, and (iii) examined the effect of chloramphenicol on *cadBA* induction.

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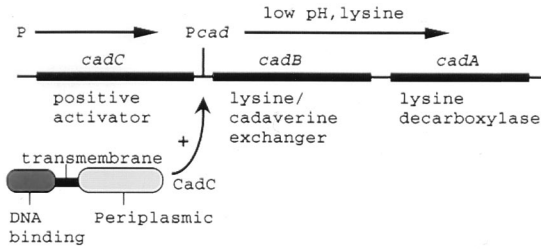

FIG. 1. Schematic of the *cad* genes of *E. coli* and their functions and direction of transcription. The promoter for *cadC* is labeled P and the *cadBA* promoter is labeled P*cad*. The CadC protein is schematically shown and labeled with its three separate domains. Its positive regulatory role in *cadBA* transcription is indicated by the curved arrow.

MATERIALS AND METHODS

Bacterial strains, growth conditions, primers, and plasmids. The *E. coli* K-12 strains used in this work were EP242 [W3110 Δ(*lacIOPZYA*)] and EP243 [(EP242 *exa-1*::Mu-*dI1734*(Kan *lac*)] (11). Plasmid pPH2190 is a pBR322-based plasmid containing *cadCBA* (19). Plasmid pLysP is a pUC10-based plasmid containing *lysP* (16). Primers RG83 (5′ ACCCCAGATAGCAATACCACCGA TACTTGCTAG), ID003 (5′ ACGCCCATTGCGGCTAATTTGGTTTATGG A), and ID007 (5′ AAAGACCTGTACCGATGGAACCGCCAATGG) were used for primer extension analysis of *cadB*, *cadC*, and *lysP* mRNA, respectively. MGR [3-(*N*-morpholino)propanesulfonic acid (MOPS)-glucose-rich medium] was as described previously (11) except that lysine was omitted. MGR-7.6 and MGR-5.8 were made by altering the pH of MGR to pH 7.6 and 5.8, respectively, as previously described (11). Acid induction of a culture grown in MGR-7.6 was achieved by adding prewarmed 1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) to a final concentration of 90 mM. Increasing the pH of a culture growing in MGR-5.8 to pH 7.6 was achieved by adding prewarmed 2 M Trizma Base (Tris) to a final concentration of 130 mM. Cadaverine (free-base form, $C_5H_{14}N_2$) was added to a final concentration of 3 mM and lysine (L-lysine) was added to a final concentration of 10 mM where indicated below. All cultures indicated below as being in steady-state growth had undergone at least five generations under the same conditions (temperature and medium) and were growing exponentially as determined by monitoring their optical densities at 420 nm. Protein synthesis was inhibited by the addition of chloramphenicol to a final concentration of 400 μg/ml. Rifampin was added to a final concentration of 200 μg/ml to block transcription.

RNA isolation. Twenty-milliliter aliquots of growing culture were poured over a frozen slurry (20 ml) of MGR that was missing the amino acids and glucose but was supplemented with 400 μg of chloramphenicol per ml and 100 mM sodium azide. The cells were collected by centrifugation at 6,000 × *g* for 10 min at 4°C, and the pellets were stored at −70°C. RNAs were isolated with TRI reagent (Molecular Research Center, Inc., Cincinnati, Ohio) according to the manufacturer's specifications. RNA pellets were resuspended in diethylpyrocarbonate-treated H_2O and treated with RNase-free DNase I in a reaction buffer of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 50 μg of bovine serum albumin per ml. After incubation at 37°C for 30 min, RNAs were extracted with a solution containing equal volumes of phenol and chloroform and precipitated from the aqueous phase with 2 volumes of ethanol. The RNA pellets were dried and suspended in 20 μl of diethylpyrocarbonate-treated water and quantitated by UV spectroscopy and gel electrophoresis.

Primer extension. Primers were 5′ phosphate end labeled in 10-μl reaction mixtures containing 10 pmol of primer, 20 mM dithiothreitol, 50 μCi of [γ-^{33}P]ATP (4,000 Ci/mM), 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 0.1 mM spermidine, 0.1 mM EDTA, and T4 polynucleotide kinase at 37°C for 30 min. The kinase reaction was stopped by heating at 70°C for 10 min, and the labeled primers were separated from unincorporated nucleotides by chromatography with a Chroma Spin column (Clontech Laboratories, Inc., Palo Alto, Calif.). End-labeled primers were stored at −20°C. Hybridization of labeled primers to RNA was carried out in a final volume of 10 μl consisting of 15 μg of RNA, 0.2 pmol of end-labeled primer, 50 mM KCl, and 25 mM Tris-HCl (pH 8.3). The hybridization mixtures were incubated at 95°C for 1 min and at 55°C for 2 min and then placed on ice for 15 min. To the hybridization mixtures was added 2 μl of Superscript reverse transcriptase (Life Technologies, Gaithersburg, Md.), 2 μl of 0.1 M dithiothreitol, 2 μl of a 10 mM deoxynucleoside triphosphate mixture, and 4 μl of reverse transcriptase buffer, after which the mixtures were incubated sequentially 44°C for 45 min and 65°C for 10 min. The extension products were precipitated by the addition of sodium acetate (pH 5.2; final concentration, 30 mM) and 2 volumes of ethanol and then centrifuged at 10,000 × *g* for 15 min. The pellets were resuspended in 4 μl of loading dye (20 mM EDTA, 0.05% [wt/vol] bromphenol blue, 0.05% [wt/vol] xylene cyanol in formamide). The products were denatured by heating at 95°C for 5 min and separated by denaturing polyacrylamide gel electrophoresis in standard 8% sequencing gels. Following electrophoresis the gels were dried and processed by autoradiography. Sequencing ladders were generated with the same primer used in the corresponding primer extension reaction mixture and pPH2190 and pLysP as templates for *cad* and *lysP* sequences. A Sequenase version 2.0 (Amersham Life Science, Arlington, Ill.) sequencing kit was used according to the manufacturer's directions. All primer extension experiments were performed at least twice from independent cultures done at separate times, and the results presented are representative of the replicates.

RESULTS

Regulation by pH and lysine. Expression of *cadBA* as a function of extracellular lysine and extracellular pH was examined by a primer extension assay. The primer that was employed was complementary to the beginning of the *cadB* mRNA so that the extended products reflected mRNA originating from the *cadBA* promoter (P*cad*). Since formation of the lysine decarboxylation products (CO_2 and cadaverine) is catalyzed by lysine decarboxylase (the product of *cadA*) and since these products have been implicated in regulating *cadBA* transcription (11, 18), the levels of *cadBA* expression were examined in both a *cadA*$^+$ (EP242) and a *cadA* mutant (EP243). EP243 contains a transposon within *cadA* that also results in an operon fusion between *cadA* and a promoterless *lacZ*. The levels of *cadB* mRNA in these two strains grown to steady-state in a pH 5.8 medium containing 10 mM lysine (initial concentration) were examined (Fig. 2A). *cadB* mRNA was detected in EP243 but not in the *cadA*$^+$ strain (EP242) (a faint signal was observed upon a longer exposure of the primer extension gel). Since it has previously been shown that exogenously added cadaverine represses expression of β-galactosidase in a *cadA-lacZ* fusion, even in the presence of lysine, it is possible that the accumulation of cadaverine in the *cadA*$^+$ cells was, at least in part, responsible for the lack of expression (see Discussion for other possibilities). That exogenously added cadaverine prevents *cadBA* transcription (as indicated by the *cadA-lacZ* fusion result) was confirmed by observing a substantial drop in *cadB* mRNA 4 min following the addition of cadaverine (3 mM) to a steady-state culture of EP243 growing at pH 5.8 in the presence of 10 mM lysine (data not shown).

On the basis of the observations described above, the kinetics of *cadBA* expression following a drop in external pH was examined in both strains. Cells were grown to steady state in a pH 7.6 medium containing 10 mM lysine (uninduced), after which MES was added to drop the pH to 5.8. Samples were removed at various times for detection of *cadB* mRNA. First, consider the results with the *cadA*$^+$ strain (Fig. 2B). Expression of *cadB* was transient, being detectable 5 min following the drop in pH and peaking 20 min postinduction. By 60 min postinduction *cadB* mRNA was barely detectable. The same experiment with the *cadA* insertion mutant (EP243) revealed that *cadB* mRNA was detected 4 min postinduction but in contrast to the *cadA*$^+$ strain, was present up to 2 h postinduction (the last sample taken) (Fig. 2C).

The kinetics of induction following lysine addition was determined by growing EP243 to steady state in a pH 5.8 medium lacking lysine, after which lysine was added to 10 mM. As with low pH induction, *cadB* mRNA was detected 4 min postinduction (Fig. 2D). To see what the response time was when cells received both stimuli simultaneously, EP243 was grown to steady state at pH 7.6 in the absence of lysine, after which lysine and MES were added simultaneously to drop the pH to 5.8. As shown in Fig. 2E, *cadB* mRNA was not detectable until 15 min postinduction. Thus, it took longer to activate transcription from P*cad* when the cells received both stimuli simultaneously than when the cells had prior exposure to one stimulus. Although the level of mRNA detected at 60 min appeared to be lower than when the stimuli were added sequentially (Fig.

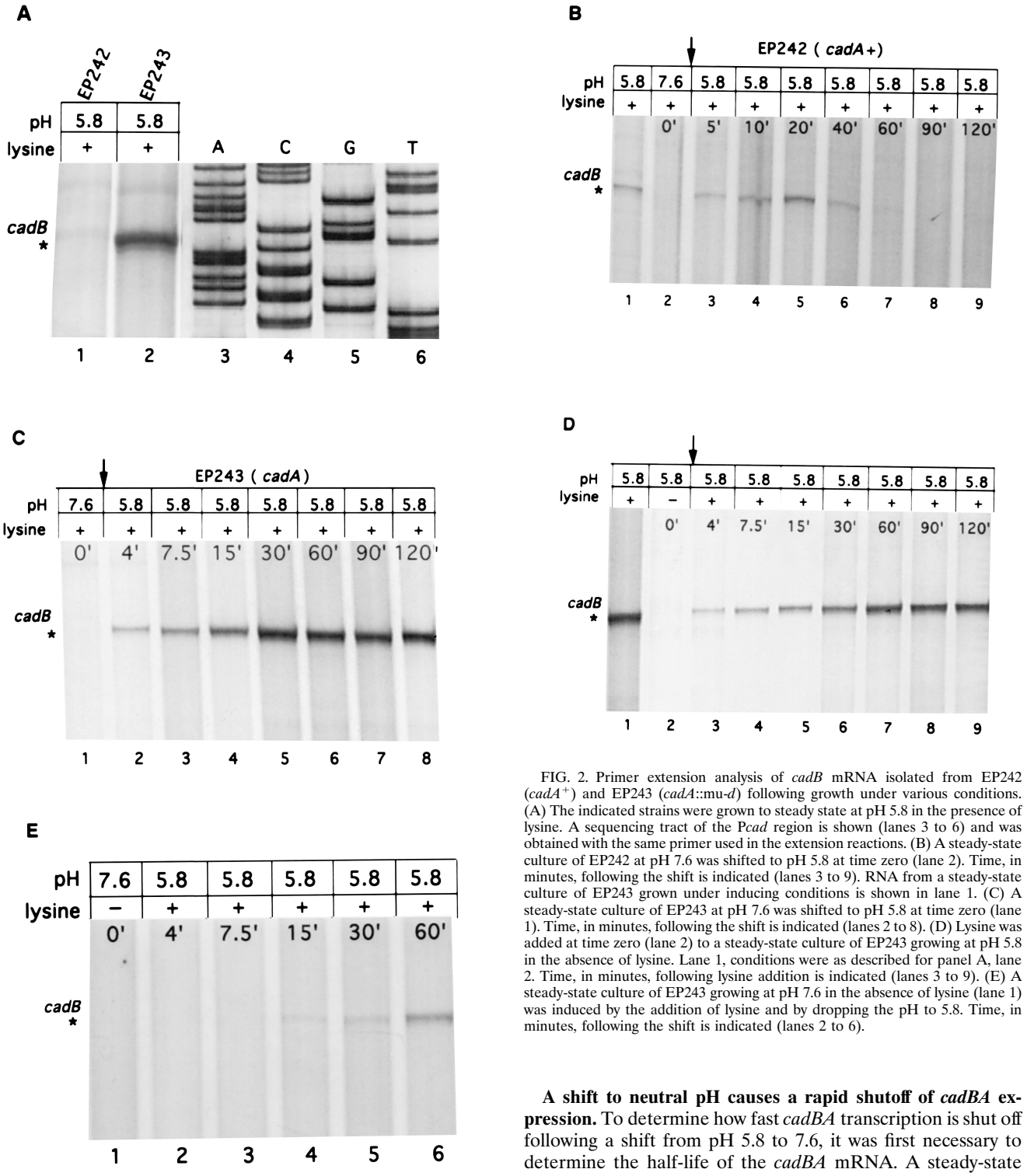

FIG. 2. Primer extension analysis of *cadB* mRNA isolated from EP242 (*cadA*$^+$) and EP243 (*cadA*::mu-*d*) following growth under various conditions. (A) The indicated strains were grown to steady state at pH 5.8 in the presence of lysine. A sequencing tract of the P*cad* region is shown (lanes 3 to 6) and was obtained with the same primer used in the extension reactions. (B) A steady-state culture of EP242 at pH 7.6 was shifted to pH 5.8 at time zero (lane 2). Time, in minutes, following the shift is indicated (lanes 3 to 9). RNA from a steady-state culture of EP243 grown under inducing conditions is shown in lane 1. (C) A steady-state culture of EP243 at pH 7.6 was shifted to pH 5.8 at time zero (lane 1). Time, in minutes, following the shift is indicated (lanes 2 to 8). (D) Lysine was added at time zero (lane 2) to a steady-state culture of EP243 growing at pH 5.8 in the absence of lysine. Lane 1, conditions were as described for panel A, lane 2. Time, in minutes, following lysine addition is indicated (lanes 3 to 9). (E) A steady-state culture of EP243 growing at pH 7.6 in the absence of lysine (lane 1) was induced by the addition of lysine and by dropping the pH to 5.8. Time, in minutes, following the shift is indicated (lanes 2 to 6).

2C and D), we do not feel that our analysis was sufficiently quantitative to conclude that this difference was significant. However, it was clear from independent experiments that at the early time points (4 and 7.5 min) the levels of mRNA were significantly lower in cells that received both stimuli simultaneously.

A shift to neutral pH causes a rapid shutoff of *cadBA* expression. To determine how fast *cadBA* transcription is shut off following a shift from pH 5.8 to 7.6, it was first necessary to determine the half-life of the *cadBA* mRNA. A steady-state culture of EP243 growing in pH 5.8 medium containing lysine was treated with the RNA polymerase inhibitor rifampin. RNA samples were isolated at various times following rifampin addition and analyzed by primer extension. The data shown in Fig. 3A show that the half-life of the *cadB* mRNA is very short (less than 1 min). The kinetics of shutoff of *cadB* transcription following a shift from acidic to neutral pH was examined by growing EP243 to steady state in a pH 5.8 medium containing 10 mM lysine and then shifting the culture to pH 7.6. As shown

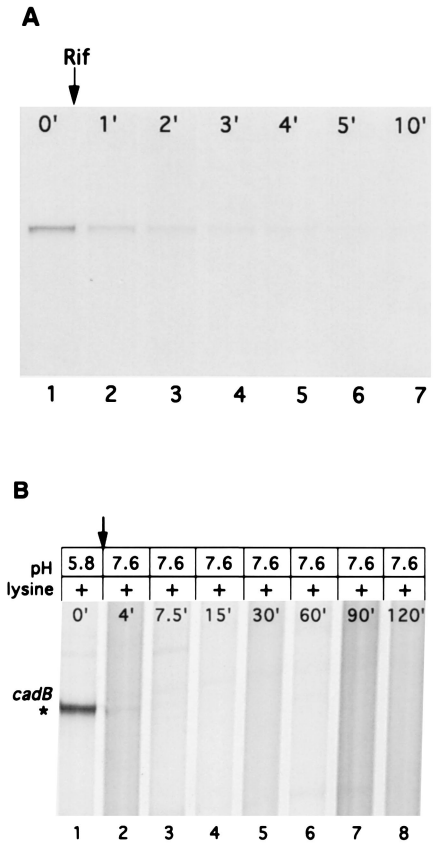

FIG. 3. Primer extension analysis of *cadB* mRNA isolated from EP243 following the addition of rifampin or a shift to pH 7.6. (A) Rifampin (Rif) was added at time zero (lane 1) to a steady-state culture growing at pH 5.8 in the presence of lysine. Samples were removed after rifampin addition at the times indicated (lanes 2 to 7; time is noted in minutes). (B) A steady-state culture at pH 5.8 in the presence of lysine was shifted to pH 7.6 at time zero (lane 1). Samples were removed following the pH shift at the times indicated (lanes 2 to 8).

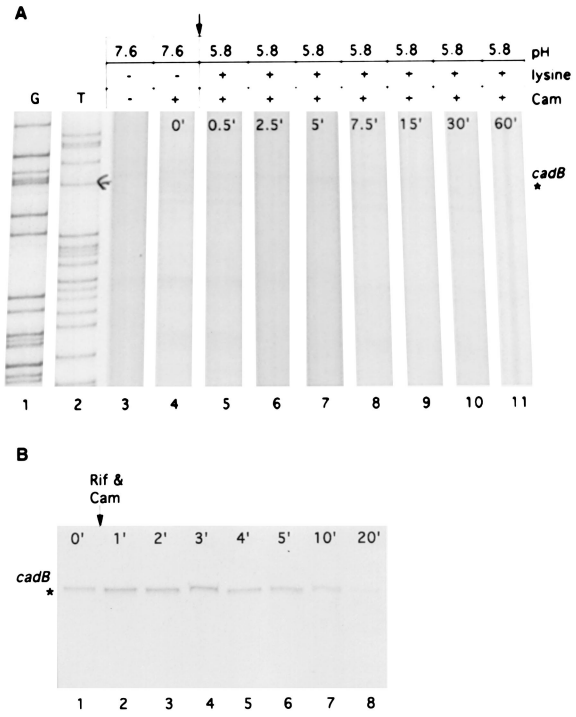

FIG. 4. Primer extension analysis of *cadB* mRNA isolated from EP243 following the addition of chloramphenicol or chloramphenicol and rifampin. (A) Lane 3, a steady-state culture growing at pH 7.6 in the absence of lysine. The culture was incubated with chloramphenicol (Cam) for 5 min (lane 4 is the sample taken after the 5-min chloramphenicol treatment), after which lysine was added and the pH was shifted. Time, in minutes, following lysine addition and the pH shift is indicated (lanes 5 to 11). The sample in lane 1 of Fig. 5B was the positive control for this experiment (i.e., the extension was done at the same time with the same labeled primer and run on the same gel). (B) Rifampin (Rif) and chloramphenicol (Cam) were added at time zero (lane 1) to a steady-state culture growing at pH 5.8 in the presence of lysine. Time, in minutes, following addition of the drugs is indicated (lanes 2 to 8).

in Fig. 3B, *cadB* mRNA was barely detectable 4 min following the increase in pH and was undetectable 7.5 min following the shift.

Addition of chloramphenicol prevents induction. The effect of chloramphenicol on induction by low pH and lysine was examined to determine whether protein synthesis might be required for induction. Chloramphenicol was added to a steady-state culture of EP243 growing in a pH 7.6 medium lacking lysine. Five minutes later lysine was added and the pH was dropped to 5.8. The data in Fig. 4A show that chloramphenicol pretreatment completely abolished the induction of *cadBA*. To rule out that the lack of *cadB* mRNA was due to instability of the mRNA brought about by the chloramphenicol treatment, the *cadB* mRNA half-life was measured following chloramphenicol addition to a culture previously induced for expression. As shown in Fig. 4B, *cadB* mRNA was detected 10 min following the addition of chloramphenicol and rifampin (i.e., chloramphenicol stabilized the *cadB* mRNA). Western blot (immunoblot) analysis of cell extracts prepared from the culture following chloramphenicol addition revealed that CadC was present at all the time points at which samples were taken for the RNA analysis shown in Fig. 4A (data not shown).

***lysP* expression is regulated by lysine but not pH.** The requirement for *lysP* in preventing *cadBA* expression in the absence of lysine has previously been demonstrated (11). However, the mechanisms involved in *lysP*'s action and the role that lysine plays in relieving *lysP*-mediated repression have not been elucidated. To better understand these mechanisms it was of interest to examine the regulation of *lysP* expression. Primer extension analysis of *lysP* mRNA was carried out with RNAs isolated from EP242 and EP243 grown to steady state in pH 5.8 and 7.6 media, each in the absence or presence of 10 mM lysine. The data in Fig. 5A demonstrate that *lysP* expression is negatively regulated by lysine but is not affected by pH. Since levels of expression of *lysP* were identical in the two strains, it appears that the products of lysine decarboxylation were not involved in the observed lysine regulation. If *lysP* repression and *cadBA* induction are causally linked, the kinetics of *lysP* repression, following lysine addition, might be expected to mirror the kinetics of *cadB* induction. As shown in Fig. 5B, the level of *lysP* mRNA was dramatically reduced within 4 min following lysine addition.

On the basis of the primer extension data, a putative *lysP* transcription start site (+1) was identified 28 bp upstream of the *lysP* initiation codon (Fig. 6). Inspection revealed a 6-bp sequence centered about 10 bp upstream from the +1 position (TACAAT) that has 5 nucleotides that are identical to the 6-bp consensus −10 region (TATAAT) of sigma 70-dependent *E. coli* promoters. A 6-bp sequence (TTGCCA) with strong similarity to the sigma 70 −35 consensus sequence (TTGACA) is centered 25 bp upstream from +1, with a spacing between this hexamer and the −10 hexamer of only 9 bp. This −25 hexamer

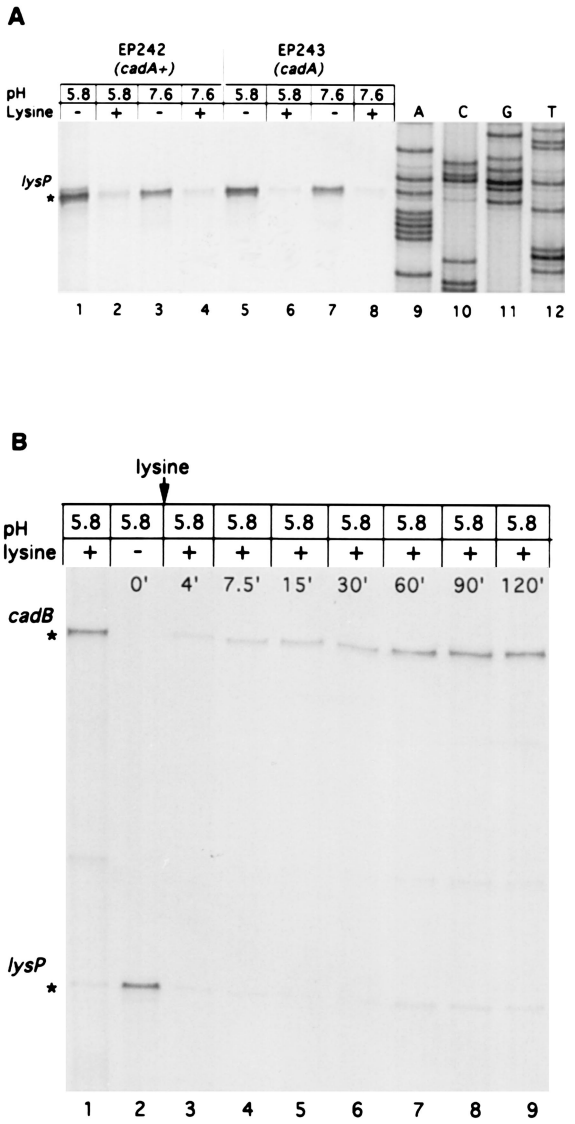

FIG. 5. Primer extension analysis of *lysP* and *cadB* mRNAs. (A) RNAs were isolated from steady-state cultures of EP242 and EP243 grown at the indicated pHs in the presence or absence of lysine and extended with a *lysP*-specific primer. A *lysP* sequencing tract in which the same primer was used is shown (lanes 9 to 12). (B) The same RNA samples that were used in Fig. 2C (lanes 1 to 8) were extended simultaneously with *cadB* and *lysP* primers (lanes 2 to 9). Lane 1 is RNA from a steady-state culture of EP243 grown at pH 5.8 in the presence of lysine and extended with *cadB* and *lysP* primers.

is embedded within an 11-bp sequence (GATTGCCAATC) that displays hyphenated-dyad symmetry. The roles of these sequences in transcription of *lysP* have not been investigated.

Transcription of *cadC* is constitutive. Previous genetic studies and Western blot analysis suggested that the level of CadC is not greatly affected by pH or lysine (3, 19). To examine transcription of *cadC* and more importantly, to localize the transcription start site, primer extension analysis of *cadC* mRNA was carried out with a *cadC*-specific primer. As expected, the levels of *cadC* mRNA did not significantly vary as a function of pH or lysine (Fig. 7). The putative transcription start site was identified 29 bp upstream of the *cadC* start codon (Fig. 6). Inspection revealed a sequence (AATTAT) centered 10 bp upstream of the +1 site that is similar to the consensus −10 sequence (TATAAT) of *E. coli* sigma 70-dependent promoters. The sequence centered 35 bp upstream of +1 (TAT AGA) matched the consensus sigma 70 −35 hexamer in three positions.

DISCUSSION

The work described here addresses several questions concerning the pH- and lysine-mediated signaling mechanism by which CadC, a membrane-localized transcriptional activator, regulates expression of *cadBA*. By primer extension assays to follow the kinetics of *cadBA* and *lysP* expression as a function of pH and lysine, it was found that (i) *cadBA* transcription was activated within 4 min following exposure to one stimulus (low pH or lysine), provided that the cells had been growing in the presence of the other stimulus; (ii) induction of *cadBA* took three to four times longer if the cells received both stimuli simultaneously; (iii) *cadBA* expression was shut off within 4 min following an increase in pH; (iv) induction kinetics of *cadBA* were similar in a *cadA*$^+$ and *cadA* mutant; however, expression in the latter was transient, peaking 20 min postinduction; (v) chloramphenicol treatment blocked induction, suggesting that translation is required; and (vi) expression of *lysP*, a negative regulator of *cadBA* in the absence of lysine, was repressed in the presence of lysine but was unaffected by pH, while as previously shown with *lacZ* fusions, expression of *cadC* was constitutive.

Two general models can be considered for how CadC becomes activated as a result of the cell's exposure to the two stimuli (low pH and lysine). By whatever specific mechanism, these models assume that the conformation or physical state of CadC is ultimately changed as a function of pH and lysine. The first model states that lysine and low pH, or signals generated as a function of these two stimuli, act on CadC in a sequential manner. The activity of one of the signals would be regulated by pH, while the activity of the other would be regulated by lysine. The distinguishing feature of this model is that there is an obligatory order in which the stimuli are processed. For example, CadC would first have to be acted upon by a pH-generated signal prior to receiving the lysine-generated signal. In contrast, the second model states that the signals are independently (nonsequentially) sensed by CadC. Consider the simplest version of the latter model, in which pH and lysine directly alter the conformation of CadC. In this scenario, CadC would be altered by pH regardless of the presence of lysine and lysine would alter CadC regardless of the pH. Examination of the kinetics of induction when the stimuli were added sequentially in the two different orders (low pH and then lysine; lysine and then low pH) and when they were added simultaneously allowed us to experimentally address these two models. If the first model (sequential) is correct we would have expected that exposure to one of the stimuli prior to exposure to the second stimulus would have resulted in an induction time similar to what was observed when both stimuli were added simultaneously (about 15 min). In fact, prior exposure to either stimulus resulted in rapid induction upon introduction of the second stimulus. Thus, we favor the second model (nonsequential) in which the ability of CadC to respond to low pH and lysine is due to mechanistically independent processes. This model is consistent with genetic experiments which showed that mutations in *cadC* that eliminate the requirement for lysine do not alter the requirement for low pH (3).

It was observed that expression of *cadBA* was transient in *cadA*$^+$ cells (EP242) but not in the *cadA* mutant (EP243) (Fig. 2A). Three possible mechanisms (not necessarily mutually ex-

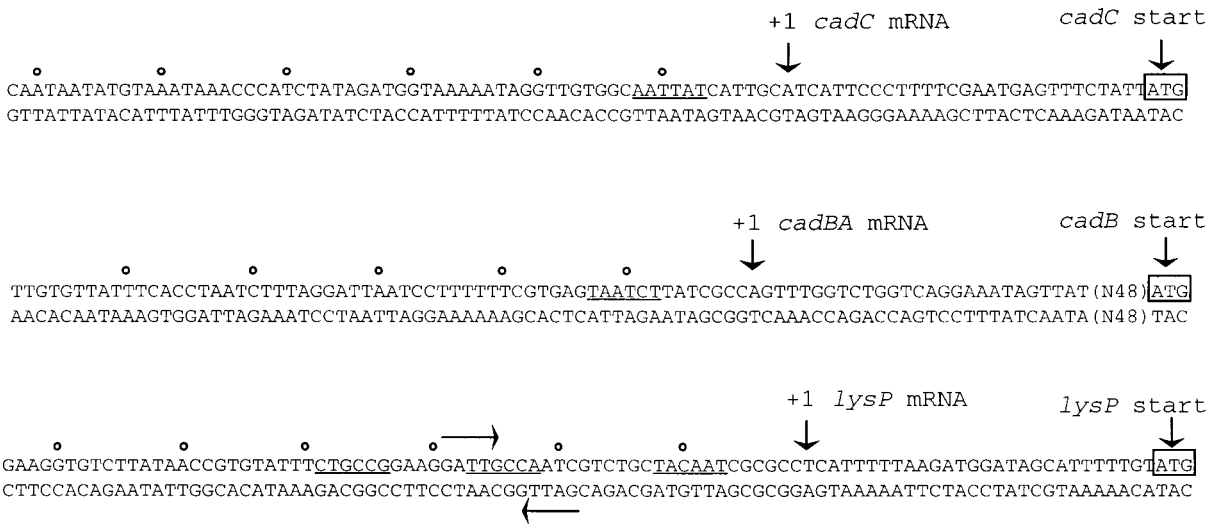

FIG. 6. Sequences of the regions upstream of the *cadC*, *cadB*, and *lysP* initiation codons. The transcription start sites, as determined by primer extension analysis, are indicated (+1). The initiation codons are boxed. Sequences similar to the −10 and −35 consensus sequences recognized by the *E. coli* sigma 70 RNA polymerase are underlined. The arrows represent a repeated sequence. *cadB*, *cadC*, and *lysP* DNA sequences have been previously published (16, 19). The N37 designation preceding the *cadB* start codon indicates 37 bp not shown.

clusive) can be considered to explain this result: (i) there is feedback inhibition by one of the decarboxylation products (cadaverine or CO_2), (ii) lysine decarboxylase plays a regulatory role separate from its enzymatic role, and (iii) there is a decrease in the lysine concentration below what is required for *cadBA* expression. It has been shown that exogenously supplied cadaverine, even in the presence of excess lysine, prevents *cadBA* expression (11). On the basis of this observation, the following argument supports the hypothesis that cadaverine accumulation contributes to the shutoff of expression. The culture of $cadA^+$ cells contained 10 mM lysine at time zero (assuming that the level of lysine was not dramatically reduced prior to the pH drop). Following induction by the pH shift, and assuming a stoichiometry of lysine disappearance and cadaverine appearance of 1:1, at some point the culture was 9 mM lysine and 1 mM cadaverine. We have previously shown the lack of expression of *cadA-lacZ* in the presence of 10 mM lysine and 1.3 mM cadaverine (11). Moreover, 5 mM lysine, in the absence of cadaverine, is sufficient to induce *cadA-lacZ* (although not to the same extent as 10 mM [data not shown]). Taken together it is likely that cadaverine accumulates to a level sufficient to inhibit expression well before the level of lysine has dropped below the threshold level needed for expression. Implicit in this argument are the assumptions that exogenously added and excreted cadaverine behave similarly with respect to *cadBA* operon expression and that cadaverine accumulates in the medium following *cadBA* induction. The latter has been demonstrated (9).

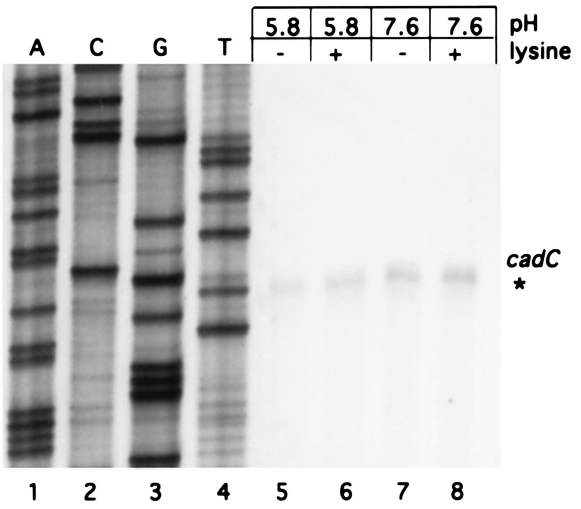

FIG. 7. Primer extension analysis of *cadC* mRNA isolated from steady-state cultures of EP243 grown at the indicated pHs in the presence or absence of lysine. Lanes 1 to 4 contain a sequencing tract of the region upstream of *cadC* generated with the same primer that was used in the primer extension reactions.

Previous work showed that mutations in *lysP* result in lysine-independent expression from P*cad*, demonstrating that in the absence of lysine, *lysP* is a negative regulator of *cadBA* expression (11, 13, 17). This raises two separate but related mechanistic questions. How does LysP repress expression in the absence of lysine, and how does lysine addition overcome the repression? The experiments described in this paper do not address the former question, although we have previously proposed that the repression may be due to a direct CadC-LysP interaction (3, 11). The genetic evidence leading to this hypothesis is based on the phenotypes associated with various *cadC* and *lysP* alleles and is summarized as follows. Several point mutations in the periplasmic and transmembrane coding domains of *cadC* were isolated and shown to confer the same phenotype as a *lysP* null allele (i.e., lysine-independent expression of *cadBA*). In addition, overexpression of wild-type *lysP* resulted in the inability to express *cadBA* in a $cadC^+$ background. The *cadC* mutations that confer lysine-independent expression were also able to overcome the block imposed by *lysP* overexpression. Recently, we identified a point mutation in *lysP* (*lysP696*) that results in the inability to express *cadBA* even in the presence of lysine (the same phenotype as *lysP* overexpression) (20). The *cadC* mutations that confer lysine independence were also able to overcome the effect of *lysP696*. Taken together, these data suggest that at a minimum, LysP negatively regulates *cadBA* expression at a step intimately associated with the action of CadC. However, these results do

not prove that there is a physical interaction between the two proteins. An alternative hypothesis is that LysP directly represses P*cad* (e.g., through a DNA-protein interaction). If this is the case, one would propose that the *lysP696* allele and overexpression of *lysP* results in increased binding of LysP to a putative operator. One would then argue that the *cadC* mutations suppress this hyperrepression by interfering with the LysP-DNA interaction. Understanding the mechanism by which LysP mediates its repressive effects awaits experimentation that directly addresses the issue of what molecules in the P*cad* activation pathway interact directly with LysP.

Regardless of the mechanism by which LysP prevents *cadBA* expression in the absence of lysine, the experiments described here shed light on the possible role of lysine in induction. The two mechanisms we consider are that lysine interferes with either the action of LysP or its level. It is attractive to suggest that lysine affects the action of LysP, since this hypothesis does not require invoking a new interaction (i.e., LysP obviously interacts with lysine). However, the data in Fig. 5 clearly show that the mechanism could also involve lysine-mediated repression of *lysP* at the transcriptional level. If this is the sole mechanism, it follows that the molecules of LysP produced prior to induction do not prevent P*cad* activation following lysine addition. Uncoupling *lysP* expression from the presence of lysine (e.g., expression of *lysP* is induced by a promoter that is regulated by something other than lysine) would allow one to test directly whether this is the primary role of lysine. These data also open up a new line of investigation since they reveal a regulatory mechanism (transcriptional control of *lysP*) that is controlled by lysine. The participants involved in this regulation, in terms of a possible repressor, activator, operator, etc., remain to be identified.

A role of translation in expression of *cadBA* is suggested by the observation that chloramphenicol prevents induction (Fig. 4). An obvious explanation for this result is that there is a requirement for de novo synthesis of one or more proteins. Although we do not have any evidence showing which protein(s) is needed, a requirement for nascent CadC can be rationalized in the context of two hypotheses. First, CadC might not be capable of switching from an inactive to an active state. If it is synthesized during noninducing conditions, it may be permanently inactive. Thus, blocking translation prior to induction would prevent P*cad* activation. The second hypothesis is that CadC is most active when synthesized in the immediate vicinity of its target promoter. *cadC* is located immediately upstream of P*cad*, and if CadC is inserted into the membrane as it is being transcribed, as one would expect during coupled transcription-translation, it would localize the *cad* operon near the membrane. Nascent CadC might then be favorably positioned to interact with the promoter. Conversely, "old" CadC that was released from the ribosome at some earlier time may not be in the vicinity of the target promoter. Precedent exists for membrane proteins localizing DNA sequences near the membrane as the protein is being translated and inserted into the membrane, as has been noted previously (6, 7). There are other hypotheses that could explain the effect of chloramphenicol. There could be another protein (in addition to CadC) that is specifically required for *cadBA* expression that needs to be synthesized at the time of induction. However, no such candidate protein has been identified. Another possibility is that the lack of *cadBA* expression is due to some secondary effect of cessation of global protein synthesis. We do not have any data that argue for or against this hypothesis. Finally, it is formally possible that chloramphenicol exerts an unknown effect independent of its role in translation inhibition. All of these explanations are intriguing. Regardless of which is correct, distinguishing them experimentally should provide new information concerning the mechanism of P*cad* induction.

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