Identification of Elements Involved in Transcriptional Regulation of the Escherichia coli cad Operon by External pH

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Expression of the lysine decarboxylase gene (cadA) of Escherichia coli is induced upon external acidification. To dissect the molecular mechanisms responsible for this regulation, we analyzed a 4.2-kbp region upstream from cadA. DNA sequencing revealed two long open reading frames upstream of and on the same strand as cadA. One of these, cadB, is 444 codons long and is situated immediately upstream of cadA. Transcriptional fusions between fragments upstream of cadA and lacZ, Northern (RNA) hybridization, primer extension, and site-directed mutagenesis experiments defined a promoter, Pcad, upstream of cadB that was responsible for pH-regulated expression of cadA. Upstream of Pcad is an open reading frame, cadC, consisting of 512 codons. The predicted amino terminal region of the cadC gene product (CadC) resembles the carboxy-terminal domain of prokaryotic transcriptional activators involved in environmental sensing. Tn10 insertions within or immediately upstream of cadC abolished Pcad activity, suggesting that cadC encodes a positive transcription factor. Expression of plasmid-borne cadC in the Tn10 mutants restored Pcad activity, while introduction of a plasmid expressing truncated CadC resulted in the inability to complement. The presence of Pcad on a multicopy plasmid was found to lower expression arising from chromosomal Pcad, suggesting that a positive-acting factor is limiting. Our data suggest that cadA, cadB, and the acid-inducible Pcad comprise, at least in part, the cad operon which is under control of the cadC product.

External pH has been implicated as a signal in a growing number of genetic responses in enteric bacteria. In a number of cases, acid-induced gene expression functions to decrease the acidity of bacterial products in an acidic environment, thus enhancing growth at low pH. For example, expression of genes encoding lysine decarboxylase (cadA) and arginine decarboxylase (adi) in Escherichia coli is dramatically increased at low external pH (2). These decarboxylases remove acid groups, resulting in less external acidification from metabolic processes. Similar effects may exist for deaminases at high external pH (11). Lactate dehydrogenase (encoded by ldh) activity is increased during fermentation, resulting in enhanced production of neutral end products (18). Induction of decarboxylases (2), lactate dehydrogenase (11), antiG and the hyd locus in Salmonella typhimurium (1) is enhanced during anaerobic growth, a condition that produces organic acids. In Vibrio cholerae, activity of the transcriptional regulator ToxR is, in part, regulated by changes in external pH (19).

The mechanism(s) employed by bacteria to sense and respond to external pH have not been elucidated. However, since the internal pH of E. coli remains relatively constant over a wide range of external pH (for reviews, see references 6 and 26) a transmembrane communication system coupled to factors involved in gene expression must exist.

Mud-lacZ fusions were used to isolate loci that respond transcriptionally to an external pH change, including the acid-inducible exa-I locus (30), the pH-dependent inaI locus responsive to membrane-permeable weak acids (30), and the alkaline-inducible alx locus (4). Expression of β-galactosidase in the exa-I Mud fusion strain was maximal at an external pH around 5.8 and repressed above pH 6.8 (30). We report here the physical and genetic characterization of the region upstream of the Mud fusion in the exa-I strain, JLS8602. The exa-I allele was found to be a Mud insertion within the cadA gene, which encodes lysine decarboxylase. Similar fusions to cadA have been described and shown to be induced by low external pH (2). The promoter responsible for pH-regulated expression and two previously unidentified open reading frames, one of which encodes a product required for expression of lacZ in the exa-I strain, were identified. A working model is presented to explain how a change in external pH affects expression from the cadA promoter.

MATERIALS AND METHODS

Strains, growth conditions, and media. E. coli, phages, and plasmids used are shown in Table 1. LB (Luria–Bertani), per liter, was 10 g of Bacto Tryptone, 10 g of sodium chloride, and 5 g of yeast extract. LB-7.6 medium was LB buffered at pH 7.6 with a final concentration of 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS), and LB-5.8 was LB buffered at pH 5.8 with a final concentration of 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) (30). λ and P1 lysates were grown in LB plus 5 mM CaCl2. The following antibiotics and concentrations were used: ampicillin sodium salt, 50 μg/ml; kanamycin sulfate, 50 μg/ml; chloramphenicol, 34 μg/ml; and tetracycline, 10 μg/ml. Antibiotics were present in all media in which cultures containing plasmids were grown. Cells harboring plasmids containing fragments from the pH region were grown and maintained on LB-7.6 with the appropriate antibiotic.

DNA biochemistry. Plasmid DNA was isolated as described previously (5) and in some instances purified by
cesium chloride-ethidium bromide gradients (16). λ and chromosomal DNA were isolated as described previously (28). All ligations were performed overnight at 16°C in ligation buffer as described previously (16). Purified plasmid DNAs as well as ligation mixtures were introduced into *E. coli* made competent as described previously (16). Oligonucleotides were supplied by N. Hatzenbuher (The Upjohn Co., Kalamazoo, Mich.). DNA sequencing was done on double-stranded templates with the Sequenase 2.0 kit (U.S. Biochemicals, Cleveland, Ohio). Both strands of the sequence shown in Fig. 2 as well as all junctions from subclones were determined from plasmids containing fragments from λ5G7 and λ21H11. Southern blots and hybridizations were done by transferring the DNA to nitrocellulose filters as described previously (16), prehybridizing for 1 h at 65°C in a solution consisting of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt’s solution, 100 μg of denatured salmon sperm DNA per ml and 0.1% sodium dodecyl sulfate (SDS) and hybridizing at 65°C overnight in a solution consisting of 5× SSC, 1× Denhardt’s solution, 100 μg of denatured salmon sperm DNA per ml, and 0.1% SDS plus denatured labeled DNA probe, (see reference 16 for description, preparation, and use of SSC and Denhardt’s solution). Filters were washed (three 15-min washes) in a solution consisting of 0.15 M NaCl, 0.03 M Tris-HCl (pH 8.0), 1 mM EDTA, and 0.1% SDS at 65°C, air dried, and exposed to X-ray film. Site-directed mutagenesis was done as described previously (17) using plasmid pH33

### TABLE 1. Bacteria, phages, and plasmids

<table>
<thead>
<tr>
<th>Strain, phage, or plasmid</th>
<th>Genotype and/or relevant characteristics*</th>
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<td>F− In(rrnD-rrnE)</td>
<td>F. Neidhardt; 13 Stratagene Inc.</td>
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<td>XL-1 Blue</td>
<td>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac F− proAB lacI2</td>
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<td></td>
<td>ZAM15 Tn10</td>
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<td>JLS821</td>
<td>F− araD139 Δ ara-leu7697 Δ(proAB-argF-lacPOZYA)XIII1 rspl Nalr</td>
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<td>JLS821 exa-1::Mud11734</td>
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<td>λEMBL containing Sau3A junction fragment from JLS8602</td>
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<td>R. Simons (29)</td>
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<tr>
<td>pBS2138</td>
<td>[pBS] As pPH33, with −35 mutation</td>
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* Plasmid descriptions are indicated by the following convention: [vector] insert endpoints [insert source]. Restriction sites correspond to the sequence shown in Fig. 2. Sites destroyed during cloning are marked with asterisks. In some cases, there were intermediate plasmids between the original source and the plasmid indicated. For reasons of clarity, these intermediates are not listed. Plasmid pH1647 contains sequences beyond nucleotide number 1 shown in Fig. 2.
as the template and oligonucleotide 5'CTTTTTTTCGTG
AGTGGTCTATGCAAGTTTTT
TTG to create the −10 AA-to-
GG change and 5’ACCTAACTTTTTGTTTTTTTT
TTG to create the −35 AGGA-to-TCTT alteration. Following mutagenesis and transformation, colonies were
selected on plates containing LB plus ampicillin sodium salt,
.lifted onto nitrocellulose, and hybridized to the appropriate
mutagenic oligonucleotide labeled at its 5’ end with
32P]ATP. Prehybridizations were performed at 65°C in
a solution consisting of 5 × Denhardt’s solution and 0.5% SDS
for 1 h. Hybridizations were performed overnight at 40°C in
a solution consisting of 5 × SSC, 1 × Denhardt’s solution,
0.5% SDS, and labeled oligonucleotide (1 μg; 106 cpm/μg).
After the filters were washed (three 30-min washes) in 2× SSC-0.5%
SDS at 65°C, dried and exposed to X-ray film. Plasmids from
the strongly hybridizing colonies were analyzed and
shown by DNA sequence analysis to contain the desired
−10 (pBS2133) and −35 (pBS2138) mutations.

β-Galactosidase assays. For data shown in Tables 2, 3, and
4 and Fig. 3 and 6, the following procedures were used. Cells
from an overnight LB-7.6 plate were resuspended in 1 ml of
LB-7.6 to an optical density at 550 nm between 1.5 and 2.0.
One hundred microliters of resuspended cells was used to
inoculate 10 ml of LB-7.6 in 50-ml Belco culture flask and
then incubated at 37°C in a New Brunswick model GT2C
water bath set at 200 rpm. When the culture reached an
optical density at 550 nm of around 0.1, 5 ml were removed
and centrifuged, and these cells were resuspended in 5 ml of
LB-5.8 and then incubated in parallel with the remaining
LB-7.6 culture for 8 h. Samples (0.1 or 0.01 ml) of the culture
were used in the whole-cell β-galactosidase assay as
described previously (22). For oxygen-limiting conditions (Ta-
bles 3) 5 ml of cells were placed into screw cap tubes (20 by
125 mm) in a stationary 37°C water bath for 3 h. Tubes were
gently shaken every 30 min to prevent cells from settling.

RNA analysis. RNA was isolated from log-phase cultures
grown in either LB-7.6 or LB-5.8, denatured, and electropho-
ersed as previously described (25) and then transferred to
nitrocellulose filters. Filters were prehybridized for 1 h at
65°C in a solution consisting of 5 × SSC, 5 × Denhardt’s,
0.5% SDS, 10 μg of denatured salmon sperm DNA per ml,
and 50% formamide. The filters were hybridized overnight at
42°C in prehybridization solution containing the radiolabeled
probe (6 × 107 cpm of pH20 and 3 × 106 cpm of pH32),
washed (three 15-min washes) in 1 × SSC–0.1% SDS at 65°C,
dried, and exposed to X-ray film. Probes used were
32P]dCTP-labeled antisense RNA molecules prepared with
T3 polymerase, as suggested by the manufacturer (Strata-
gen, La Jolla, Calif.). The plasmids used to generate the
probes were pH32 (linearized with BamHI) and pH20
(linearized with EcoRI). Plasmid pH32 is PBS containing the
EcoRV 2183EcoRV 254A fragment. Plasmid pH20 is PBS
containing the EcoRV 2183EcoRV 254A fragment. Primer ex-
tension experiments were carried out using essentially the
reagents and protocol for first-strand cDNA synthesis
supplied in the cDNA Synthesis System Plus kit (1a) from
Amer sham Inc., Arlington Heights, Ill., with the following
exceptions: (i) 5 μg of RNA and end-labeled oligonucleotide
were coprecipitated with ethanol and resuspended in 25 μl of
distilled water before addition of the remaining components,
(ii) 5 μl of 20 U of avian myeloblastosis virus reverse
transcriptase (Seikagaku America Inc., St. Petersburg, Flor-
da) per ml was used, (ii) following synthesis, the reaction
mixture was phenol extracted, ethanol precipitated, and
resuspended in 10 μl of DNA sequencing loading dye. The
products were electrophoresed on an 8% sequencing gel
along with a sequencing ladder generated by using the same
oligonucleotides and plasmid pH33. The oligonucleotides
used are described in Results.

Tn10 mutagenesis. JLS8602 was infected with λ1098 as
described previously (34). Tet’ colonies were screened on
LB-7.6 and LB-5.8 plates containing tetracycline (10 μg/ml)
and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-
Gal) (40 μg/ml). Colonies showing altered phenotypes (dif-
terent intensity of blue than JLS8602 on X-Gal plates)
were purified on the same medium in which they exhibited their
altered phenotype. Phase P1vir was grown in the mutants
and used to transduce JLS8602 to Tet’ to verify that the
phenotypes were like those of the original mutant. Two of
these transductants (E2088 and E2110) from two independent
mutants were analyzed further.

Nucleotide sequence accession number. The nucleotide
sequence data reported in this study has been assigned
GenBank accession number M67452.

RESULTS

Mapping and cloning of the exa-l fusion junction from
JLS8602. Two approaches were taken to genetically and
physically localize the exa-l locus (Murd transcription fusion)
responsible for acid-inducible lacZ expression in JLS8602.
The first approach was based on the observation that induc-
tion of lacZ in this strain resembled that of a strain contain-
ing a cadA-lacZ fusion (min.93.5) (2), suggesting that the
Murd insertion was in the same operon as cadA. The cadA
gen encodes lysine decarboxylase and has been shown to be
regulated, in part, by external pH. Genetic mapping using
Hfr and phage P1vir-mediated crosses demonstrated that the
Murd insertion in JLS8602 was linked to cadA (data not
shown). The second approach was to isolate a lambda clone
that carried a fusion fragment between the chromosome and
the Murd insertion and determine whether the clone con-
tained DNA linked to cadA. A library of Sau3A partial
digestion fragments from JLS8602 was constructed in
λEMBL3, and a phage carrying lacZ was isolated by its
ability to form blue plaques on a lawn of Lac+ cells (JLS821)
with X-Gal in the top agar. Restriction analysis of DNA from
this phage, called LA1-4, showed that it carried a 15-kbp
insert with 6 kbp from the chromosome flanking the end of
the Murd insertion (Fig. 1).

A 3.7-kbp Sau3A fragment from LA1-4 that mapped within
the chromosomal insert and upstream of lacZ was isolated,
nick translated, and hybridized to DNA from lambda clones
that contained DNA derived from the min 93 region of the
chromosome (14). This fragment hybridized to DNA from

FIG. 1. Physical maps of recombinant λ derivatives carrying
DNA from min.93.5 of the chromosome. Thick black lines represent
chromosomal inserts. The thin black lines represent the phage arms.
The stippled region represents the Murd portion of the chromosomal
insert from JLS8602. RA and LA refer to the right and left arms of
the λ cloning vectors, respectively. S and E, SaI and EcoRI
restriction sites, respectively. Not all of these sites are shown.
VOL. W3110 and clones. The X21H11, and fragment shown that the Mud insertion lies within from not XSG7 and X21H11 that this EcoRI missing fragment plus Sall the clones shown reading only and extends nucleotide 4106 and would The lacZYA, shown sequence pH2190, and activity. resulting Three other were needed for showed up simultaneous levels of cadA and from the Mud insertion lies within cadA.

DNA sequence analysis. The DNA sequence of the 3.7-kbp SalI fragment plus 1.3 kbp of an adjoining SalI-EcoRI fragment shown in Fig. 1 was determined and is shown in Fig. 2. The sequence begins at the SalI site shown at the left in the clones shown in Fig. 1. Computer analysis revealed the presence of three long open reading frames on one strand and only small ones on the opposite strand. The three long open reading frames are shown in Fig. 2. The first open reading frame, which we have named cadC (see below), extends from a methionine codon at nucleotide 872 to nucleotide 2407 and would be predicted to encode a 512-amino-acid protein of 57.7 kDa. The second open reading frame extends from a methionine codon at nucleotide 2775 to nucleotide 4106 and would be predicted to encode a 444-amino-acid protein of 46.7 kDa. This open reading frame has also been identified by G. Bennett and colleagues who have proposed calling it cadB (3). The third open reading frame extends from a methionine codon at nucleotide 4189 past the sequence shown and corresponds to the cadA gene (3).

Localization of transcriptional elements by lacZ fusions. The promoter probe plasmids pRS415 and pRSS28 (differing only in the orientation of the cloning sites upstream from lacZYA) were used to localize DNA fragments with promoter activity. Various fragments were cloned upstream from lacZYA, introduced into the Lac− strain JLS821 and those strains assayed for β-galactosidase after growth at pH 5.8 and 7.6 (Fig. 3). Three plasmids, pPH2146, pPH2190, and pPH1647, produced substantial levels of β-galactosidase at pH 5.8, while plasmids pPH1856 and pPH1832 showed no induction at pH 5.8. These results suggested that sequences upstream of EcoRV 2579 were required for expression of cadA and that those upstream of the Csp45154 site were needed for maximal expression in these plasmids. Three other plasmids, pPH2189, pPH2191, and pPH2089, had smaller, but reproducible, levels of low pH-induced expression.

Northern (RNA) hybridizations. The transcription pattern of the cadC-cadB region was examined by Northern hybridization. RNA isolated from JLS8602 and JLS821 grown at pH 7.6 and 5.8 was blotted and hybridized to two antisense RNA probes made from pBS derivatives containing fragment EcoRV 2018-EcoRV 2579 to produce PH20 or fragment EcoRV 2579-PstI 3751 to produce PH32. Each of these fragments were oriented such that probes synthesized in vitro with the T3 promoter would be antisense to mRNA transcribing toward cadA. Following hybridization, the blots were stained and visually checked to ensure that approximately equal levels of rRNA were present in each lane. The results of the hybridization are shown in Fig. 4. This data suggests that transcription of an acid-induced RNA begins downstream (or very close) to the EcoRV 2579 site between cadC and cadB.

Primer extension. Consideration of the location of cadB and results of the Northern hybridizations suggested that the pH-inducible promoter was in the intercistronic region between cadC and cadB. Primer extension experiments using three oligonucleotides were carried out to test this hypothesis. Oligonucleotide 1 corresponds to nucleotides 2615 to 2635 and did not produce any detectable extension product (not shown). Oligonucleotide 2 corresponds to nucleotides 2758 to 2778 and produced an extension product ending at an A:T bp at nucleotide 2700 with template RNA from cells grown at pH 5.8 but not pH 7.6 (Fig. 5). Oligonucleotide 3 corresponds to nucleotides 2883 to 2903 and also produced a pH 5.8-specific extension product ending around nucleotide 2700 (not shown). These results suggest that differential transcription initiating around nucleotide 2700 is, at least in part, responsible for the observed pH-regulated expression of lacZ in JLS8602.

Examination of the sequence upstream from nucleotide 2700 revealed the presence of a hexanucleotide centered 10 bp away (5'TTAATCT that is similar to the consensus hexanucleotide 5'TATAAT) found in E. coli σ70-dependent promoters (12). The most highly conserved bases among this class of promoters, the first, second and sixth nucleotides, are identical to those found in Pcad. It is more difficult to assign on the basis of inspection a hexanucleotide 35 bp upstream from the start site that matches the consensus for σ70-dependent promoters (5'TTAGCA, with 17 bp most common between the −10 and −35 hexanucleotides) (12). The best sequence match to the consensus is 5'TTAGGA. However, the spacing between this and the −10 sequence is 19 bp.

To determine whether the −10 and −35 sequences are important for Pcad function, the following mutations were introduced by site-directed mutagenesis: (i) the −10 sequence was changed from 5'TTAATCT to 5'TGGTCT and (ii) the −35 sequence was changed from 5'TTAGGA to 5'TTTCCT. The mutations were introduced into the 1.1-kbp PvuII fragment and placed upstream of lacZ in pRS415. Table 2 shows that both mutations resulted in the loss of pH-induced lacZ expression. Although the wild-type plasmid, pH2089, produced a 3- to 4-fold induction (compared with pH2146, which produced a 40-fold induction), we feel that this induction is physiologically significant, since a Tn10 insertion that abolished cadA expression in the chromosome (cadCl::Tn10, see below) also abolished pH induction from pH2089 (Table 2).

Taken together, the results of the Northern hybridizations, primer extension experiment, and site-directed mutagenesis led us to conclude that the sequence upstream from nucleotide 2700 defines a promoter that is responsible for pH-regulated expression of cadA and cadB. We define this promoter as Pcad.

Isolation of Tn10 mutants that alter regulation of Pcad. Strain JLS8602 was mutagenized with Tn10 and mutants that showed altered expression, as evidenced by differences in intensity of blue on LB-X-Gal plates at pH 5.8 were obtained. Two mutants that produced white colonies at pH 5.8 were analyzed further, since they represented insertion mutants that were not inducible by low pH. The Tn10 insertions were transduced from the original isolates to JLS8602 (resulting in strains E2088 and E2110) and were found to confer the same phenotypes. Genetic mapping of the Tn10's in E2088 and E2110 revealed that they were linked to the Mud insertion in cadA (around 50% of the Tet'
transductants resulting from crosses between E2088 or E2110 and JLS821 were Kan'). Southern hybridization of chromosomal DNA showed that the Tn10 insertion in E2088 was localized to the SspI692-ClaI1031 fragment and that of E2110 was found to be in the ClaI1031-EcoRI1101 fragment (data not shown). The mutations were given the allele numbers cadCl::Tn10 and cadCZ::Tn10 corresponding to the mutations in E2088 and E2110, respectively. The levels of β-galactosidase in these mutants (E2088 and E2110) when grown in liquid culture are shown in Table 3. Since these insertions were well upstream of Pcad, it suggested that an element upstream of Pcad was required for induction. This result was consistent with those from the promoter probe cloning experiments in which only plasmids carrying Pcad plus a large upstream region gave maximal pH inductions (Fig. 3). A model explaining these results is presented in the discussion.

The cadC gene product, CadC, acts in trans to activate Pcad.

The results described above suggested that an upstream genetic element was required for Pcad induction. This element could either encode a trans-acting factor or be a site needed for optimal promoter activity (enhancer). A likely candidate for a trans-acting factor would be the putative cadC gene product. The different possibilities were distinguished by testing for the ability of the upstream region to complement the cadCl::Tn10 allele in trans. Plasmid pH2200 contains an SspI692-SspI1244 fragment extending from 177 bp upstream to 33 bp downstream of the cadC open reading frame inserted downstream of the lac promoter in plasmid pSR-Plac. Plasmid pSR-Plac also contains transcription termination signals on each side of the lac promoter. Plasmid pH2249 differs from pH2200 by the presence of a frameshift mutation at the Clai site at nucleotide 1031 (53 amino acids from the amino terminus of CadC). This mutation was introduced by utilizing the Klenow fragment of DNA polymerase I and nucleotides to fill in the Clai site and performing intramolecular ligation.

Plasmids pH2200 and pH2249 were introduced into E2229 and E2230, both of which contained the Pcad-lacZ chromosomal fusion from JLS8602 and an F' containing lacI (encoding lac repressor). In addition, E2230 contained the cadCl::Tn10 allele. These strains, with and without pH2200 and pH2249, were assayed for β-galactosidase after growth at pH 5.8 and 7.6 (Table 4). Several conclusions were drawn from this data. First, plasmid pH2200 encodes a product that functions in trans to activate Pcad. Second, that product is most likely a protein, since introduction of a frameshift mutation within the cad region destroyed its ability to complement. Third, since the presence of pH2200 increased expression in the wild-type background, CadC must normally be limiting for Pcad activation. Although this data is for strains grown without the Plac inducer, isopropyl-β-D-thiogalactopyranoside (IPTG), its addition had no substantial effect on expression levels detected from the chromosomal Pcad-lacZ fusion (data not shown). Thus, the 177 bp between the SspI692 and the start of the cadC coding region may contain a promoter capable of expressing cadC under these conditions. At present, we cannot explain the difference in β-galactosidase values shown for strains E2229 and E2230 carrying pH2249 at pH 7.6 (72 versus 4 units). The only difference in these
FIG. 4. Northern blot of RNA isolated from cells grown at pH 5.8 and 7.6. (A) Location of fragments cloned into pBS to generate single-stranded antisense RNA probes. (B) The matrix above the gel indicates which probe was used, at what pH the cells were grown, and source of the RNA. The lane marked M contains RNA markers used as standards. To the left of the gels are the sizes (in kilobases) and locations of the markers and 16S and 23S RNA, as determined by staining the filter following hybridization. Following electrophoresis, the filter was cut in half to generate the two filters shown.

strains is the presence of a wild-type copy of cadC in E2229. One explanation is that the truncated version of CadC from pPH2249 may be partially active when present with wild-type CadC. This and other hypotheses remain to be tested.

FIG. 5. Primer extension analysis. The two leftmost lanes contain RNA isolated from JLS8602 grown at pH 5.8 and 7.6 that was extended with an antisense primer corresponding to nucleotides 2758 to 2778. The right lanes contain a DNA sequencing ladder generated by using the same oligonucleotide and plasmid pPH33. The asterisk to the right of the gel indicates the endpoint of the extended product. The arrow indicates the location of the extended product.

### TABLE 2. Effects of -10 and -35 changes on Pcad activity

<table>
<thead>
<tr>
<th>Strain</th>
<th>Aerobic β-Galactosidase activity</th>
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<tr>
<td></td>
<td>pH 7.6</td>
</tr>
<tr>
<td>JLS821/pRS415</td>
<td>19</td>
</tr>
<tr>
<td>JLS821/pPH2089 (wild type)</td>
<td>312</td>
</tr>
<tr>
<td>JLS821/pPH2134 (-10 mutation)</td>
<td>292</td>
</tr>
<tr>
<td>JLS821/pPH2143 (-35 mutation)</td>
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</tr>
<tr>
<td>JLS821 cadC1::Tn10/pPH2089</td>
<td>210</td>
</tr>
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</table>

* Cells were grown and assayed as described in Materials and Methods.

### TABLE 3. Effects of Tn10 mutations on Pcad activity during aerobic and oxygen-limiting growth

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<tr>
<td>JLS8602</td>
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</tr>
<tr>
<td>JLS8602 cadC1::Tn10</td>
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</tr>
<tr>
<td>JLS8602 cadC2::Tn10</td>
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</tbody>
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* Reported in Miller units.
TABLE 4. Complementation by the cadC plasmid, pPH2200<sup>a</sup>

<table>
<thead>
<tr>
<th>Strain</th>
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<td>E2229/pPH2200</td>
<td>151</td>
<td>1,963</td>
<td></td>
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<tr>
<td>E2229/pPH2249</td>
<td>72</td>
<td>597</td>
<td></td>
</tr>
<tr>
<td>E2230</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>E2230/pPH2200</td>
<td>188</td>
<td>1,740</td>
<td></td>
</tr>
<tr>
<td>E2230/pPH2249</td>
<td>4</td>
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</table>

<sup>a</sup> Cells were grown and assayed as described in Materials and Methods.

<sup>b</sup> Reported in Miller units.

Sequences upstream of Pcad interact with a factor necessary for its activation. Plasmid pPH33 is a PBS derivative containing a 1.1-kbp PvuII fragment flanking Pcad. This fragment is in an orientation such that Pcad and Plac from the vector direct transcription in opposite directions. Introduction of pPH33 into JLS8602 completely abolished expression from the chromosomal cadA-lacZ fusion at pH 5.8, suggesting that the plasmid is titrating out a factor necessary for Pcad activity (Fig. 6). Additional plasmids containing portions of this 1.1-kbp region were constructed and tested for their ability to compete. Figure 6 shows that an RsaI fragment containing the entire intergenic region between cadC and cadB also inhibited expression, but not to the same degree as pPH33. Splitting either the PvuII or RsaI fragment at the EcoRV site 121 bp upstream from the transcription start site, however, diminished the effect, suggesting that a site overlapping the EcoRV site titrates out a positive activator of Pcad. None of these plasmids affected expression from the chromosomal cadA-lacZ fusion at pH 7.6 (data not shown). Although the limiting factor being titrated in these experiments has not yet been identified, one candidate would be CadC.

Computer-assisted analysis of CadC. The amino acid sequence of CadC was compared with sequences in the NBRF protein data base. A region of about 100 amino acids at the amino terminus of CadC was found to be similar to the carboxy termini of several bacterial proteins known to be transcriptional activators (31). Included in this class, among others, is PhoP from Bacillus subtilis (27), PhoB (15), OmpR (8) from E. coli, and VirG from Agrobacterium tumefaciens (20). This region of OmpR has been shown to be both sufficient and necessary for the specific interaction between OmpR and the ompC and ompF promoters (24, 32). Another protein containing this DNA binding domain is the ToxR transcriptional activator from V. cholerae (23). Unlike the other members of this class, however, the DNA binding...


The Mud insertion defining the exa-1 locus in strain JLS8602 was found to be in the gene encoding lysine decarboxylase (cadA). This gene has previously been shown to be regulated at the transcriptional level by external acid pH as well as by the presence of lysine and oxygen (2). Recently, the complete DNA sequence of cadA has been determined (3). Regulation of cadA expression provides a potentially useful model for understanding signalling and transcriptional mechanisms in bacteria that are influenced by external pH. The approach described in this work was to genetically and physically identify elements that influence cadA expression by cloning the region upstream from cadA, determining its DNA sequence, and examining its role in cadA transcription. Isolation of Tn10 insertion mutants with altered expression of a cadA-lacZ transcriptional fusion were also identified. Our interpretation of the data presented here supports the following conclusions. (i) A promoter, which we have named Pcad, is responsible for pH-regulated expression of cadA. (ii) There is an open reading frame situated between Pcad and cadA called cadB. (iii) Upstream of Pcad is an open reading frame, cadC, which encodes a protein necessary for pH-induced expression of Pcad. (iv) A region upstream of the Pcad start site interacts with a factor necessary for Pcad induction. (v) CadC is limiting in the cell for Pcad induction.

Plasmids pPH2189, pPH2191, and pPH2089 all contain Pcad-lacZ fusions but only gave modest (two- to threefold) inductions of lacZ at pH 5.8 (Fig. 3). This observation is somewhat inconsistent with our interpretation of the Northern hybridization and primer extension data which physically located the Pcad start site between cadC and cadB. We hypothesize that CadC levels were limiting in cells carrying a single copy of cadC such that multiple copies of the Pcad-lacZ fusion were not fully induced. Cells that carried both cadC and Pcad on a plasmid would be predicted to have higher levels of CadC and thus a more pronounced induction of Pcad. The exception to this was the result with plasmid pPH2189. This plasmid contains a complete copy of cadC, yet the level of expression resembled those lacking cadC. Since the endpoint of the fragment in pPH2189 is just 18 bp upstream of the cadC start codon (at the Csp451 site), we presume that this plasmid does not express cadC and that an element(s) upstream of the Csp451 site (a promoter?) is required for cadC expression. If CadC expression is required for Pcad activation, one would expect detection of a cadC-specific mRNA. Upon longer exposure of the Northern blots using the EcoRV probe shown in Fig. 4, we did observe weak hybridization; however, we do not know whether this was cadC specific or due to some cross-reactivity with other mRNAs.

The mechanism by which CadC functions to activate Pcad is not clear; however the observation that its amino terminus is similar to the carboxyl DNA binding domain of a group of transcriptional activators (OmpR-like proteins) suggests that a CadC-DNA interaction is involved (24, 31, 32). The OmpR class of proteins comprise a subclass of the response elements of bacterial two-component regulatory systems responsible for the cell's ability to respond to several different environmental stimuli (31). Comprising the second component are the membrane-bound proteins which are capable of both sensing the external environment and interacting with the response element to effect a change inside the cell. The ToxR protein of V. cholerae, which is involved in the regulation of several genes associated with the infectious process, is also a member of this class and has been implicated in the ability of the organism to sense several features of its environment including osmolality, pH, amino acids, and temperature (21). ToxR, however, differs from the OmpR class of response proteins in at least two fundamental ways: it is membrane bound, and like CadC, the conserved DNA binding domain is at the amino terminus (23).

The similarity in organization between CadC and ToxR leads to speculation about whether functional similarities between the two exist. Specifically, is CadC a DNA-binding protein? Does CadC, like ToxR, have both cytoplasmic and periplasmic domains? Although a direct interaction between CadC and Pcad has not been demonstrated, the genetic evidence presented here along with the similarity between CadC and other DNA-binding proteins strongly suggests that such an interaction is responsible for pH-induced transcriptional response. The physical location of CadC has not been determined; however, computer analysis of the CadC primary structure predicts a 25-amino-acid sequence (residues 155 through 180) that resembles membrane-spanning regions of other proteins (9) (data not shown). This putative transmembrane segment aligns with the predicted transmembrane segment from ToxR (Fig. 7) (23).

The similarity between CadC and other transcriptional activators, the requirement of CadC for Pcad function, the prediction of a transmembrane segment, and the analogy with ToxR supports the following outline of a working model as to how transcription from Pcad is pH regulated. The basic premise of the model is that like ToxR, the amino domain of CadC is a DNA binding domain and resides in the cytoplasm with one or more domains localized in the outer face of the cytoplasmic membrane or periplasmic space. This outer domain senses, either directly or indirectly, fluctuations in periplasmic pH resulting in an unspecified alteration of CadC. Such a change would result in a signal being transmitted to the cytoplasmic DNA binding domain, which in turn would interact with either Pcad directly or another DNA target controlling the expression of a required factor. It should be emphasized that the basic features, of this model, namely, the cellular location of CadC and the ability to bind DNA, have not yet been experimentally tested.

Implicit in this model is the notion that the activity, as opposed to the expression, of CadC is altered at different pHs. Since the promoter for cadC has not been rigorously identified, this hypothesis also awaits direct testing. However, two lines of evidence shed light on this issue. First, the fact that pPH2200 expressed cadC independently of IPTG addition suggests that there is a promoter between the Ssp1850 site and the start of cadC. Second, a fragment containing this region was inserted upstream of lacZ and found to contain promoter activity that was not induced at low pH (pPH1856 [Fig. 3]). In fact, activity was slightly higher with this plasmid at pH 7.6 than at pH 5.8. Together, this suggests that transcription of cadC is not induced by low external pH.

The hypothesis that CadC is limiting in the cell, even at pH 5.8, was invoked to explain differences in the induced levels of Pcad-lacZ plasmids shown in Fig. 3. Another piece of evidence supporting this notion comes from the data presented in Table 4, in which expression of cadC from pPH2200 was shown to increase expression from the chromosomal cadA-lacZ fusion. The observation that the Pcad
promoter region, when on a multicopy plasmid (but not fused to lacZ), titrates out a factor necessary for expression of the cadA-lacZ chromosomal fusion (Fig. 6) also suggests that a positive element is limiting, although we do not yet know whether CadC is the limiting factor. The competition experiment suggests that the EcoRV<sub>2579</sub> site is part of the site involved in recognizing a limiting factor. G. Bennett’s laboratory has carried out similar experiments and obtained the same result (3). The finding that the 1.1-kbp PvuII fragment competes more effectively than the 0.4-kbp Rsal fragment suggests that either the context that the site is in has an effect on the ability to recognize the putative factor or that sequences beyond the Rsal sites are directly involved in the interaction. We do not have additional data to support either one of these hypotheses.

The cadB gene and its putative gene product, CadB, have been examined by G. Bennett and colleagues. They proposed that the protein is involved in the exchange of lysine and cadaverine between the cytoplasm and external environment (3).

Induction of decarboxylase genes, including cadA, by low external pH is further enhanced under anaerobic conditions. For cadA, this can be due to either a promoter other than Pcad being utilized during anaerobiosis or a higher level of induction of Pcad. Although the data here does not distinguish between these two possibilities, it is clear from Table 3 that cadC is required for expression under oxygen-limiting conditions. In addition, we observed using indicator broth under anaerobic conditions that the cadC1::Tn10 and cadC2::Tn10 alleles in JLS821 resulted in a lysine decarboxylase-deficient phenotype. Physical localization of the 5′ ends of mRNA produced under anaerobic conditions is required to determine whether Pcad is utilized under these conditions.

Our data suggests that cadA is cotranscribed with cadB and that cadC is transcribed separately. We suggest that the cadB, cadA, and Pcad elements define, at least in part, an operon to be referred to as the cad operon. The size of the operon is unknown, since the region downstream of cadA has not been thoroughly analyzed. A heat shock gene coding for a lysine synthetase, lysU, maps downstream from cadA (7, 33) but whether it is also transcribed by Pcad is not known. The isolation of cadC1::Tn10 and cadC2::Tn10 mutations should allow one to determine whether lysU expression is affected by Pcad activity.

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