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Effects of Multicopy LeuO on the Expression of the Acid-Inducible Lysine Decarboxylase Gene in *Escherichia coli*

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We previously reported that mutations in *hns*, the structural gene for the histone-like protein H-NS, cause derepressed expression of *cadA*, which encodes the acid-inducible lysine decarboxylase at noninducing pH (pH 8.0). This study reports the characterization of a plasmid isolated from an *Escherichia coli* library that suppresses the effect of an *hns* mutation on *cadA* expression. A previously sequenced open reading frame, *leuO*, proves to be the gene that causes the *hns*-complementing phenotype. The mechanism for this phenotype appears to be overexpression of *leuO* from a multicopy plasmid, which drastically reduces production of CadC, the essential activator for *cadA* induction. These results show an in vivo regulatory phenotype for *leuO*, consistent with its proposed protein sequence.

Escherichia coli belongs to the class of neutrophiles that can grow in the pH range of 5 to 9. Although our understanding of the mechanisms used to maintain an internal pH of 7.6 to 7.8 is incomplete, it has been suggested that the actions of amino acid decarboxylases and deaminases contribute to the efforts of *E. coli* to return the pH to neutrality (3, 6).

Biodegradative lysine decarboxylase, encoded by *cadA* at 93.7 min on the *E. coli* chromosome (1, 6), is one of the amino acid decarboxylases induced under acidic growth conditions. Besides moderate acidity (pH 6), maximal expression of *cadA* also requires rich media, anaerobiosis, and an excess amount of substrate (i.e., lysine [10]). The nucleotide sequence of the *cad* operon in *E. coli* K-12 has previously been determined and revealed another gene, *cadB*, located promoter proximal to *cadA* (6). The CadB protein sequence suggests that it functions as a lysine-cadaverine antiporter (7). Expression of the *cadBA* operon under inducing conditions has an absolute requirement for an activator protein, CadC, whose structural gene (*cadC*) is located about 300 bp upstream from the *cadBA* promoter (16). Previous site-directed mutagenesis and *lac* fusion studies localized a site essential for acid pH activation to a 66-bp region from −97 to −161 bp upstream of the *cadBA* transcription start site (7). Experiments have revealed that the activity of CadC is regulated by medium pH and that the production of CadC protein is low level at all times (16). The amino terminus of CadC protein has significant homology to the DNA binding domain of a number of activators and is overall most similar to ToxR of *Vibrio cholerae* (16). Another component of *cadBA* regulation, the lysine-responsive regulatory gene (*cadR*), mapped at 48 min on the *E. coli* chromosome. Mutations in this gene were found to exempt the *cad* operon from the requirement for lysine to achieve acid pH induction (1, 9). The function of the protein encoded by *cadR* (*lysP*) as a lysine-specific transporter has been demonstrated, and the expression of *cadR* has been shown to be induced by the same conditions that induce *cadBA* expression (13). The exact regulatory role of CadR in *cadBA* expression is not clear, though it has been shown that overproduction of CadR prevents *cadBA* induction, indicating a negative function for CadR, apparently operating through CadC (8).

Like many otherwise unrelated stress-induced genes, the *cad* operon is derepressed by mutations in *hns*, the structural gene for the histone-like protein H-NS (11). A group of plasmids that complement the *hns* mutant phenotype for *cadBA* expression have been isolated from an *E. coli* library, and most of them carry an *hns*$^+$ gene (11). Among other *hns*-complementing plasmids, we have characterized plasmid p37T7 and found that a previously sequenced gene, *leuO*, is responsible for the observed phenotype. Assays of β-galactosidase expression from a *cadC*::*lac* fusion and quantitation of *cadC* mRNA production suggest that LeuO protein probably acts by repressing production of the essential activator CadC at the transcriptional level.

Plasmid p37T7: isolation and characterization. In efforts to further study the mechanism of derepression of *cad* in *hns* mutants, the expression of *cadC* in *hns*$^+$ and *hns*::Tn*10* strains was studied by using the plasmid pPH1856 (Table 1). There was little difference in the expression of *cadC* by *hns*$^+$ and *hns* mutant strains at either pH 8.0 or pH 5.5 (data not shown). These results indicate that the *hns* effect is not mediated through altering levels of activator CadC.

p37T7 was isolated from an *E. coli* library (16) as a plasmid that complemented the *cadA* derepression phenotype of one of the *hns* mutants, GNB837 (Table 1). Transformed into a *cadA*::Mu d*lac hns* mutant (GNB837 or GNB88), this plasmid was able to reduce the level of β-galactosidase expression. The *cadA* repression effect of plasmid p37T7 seemed to be specific for *hns* mutants, though; when it was transformed into the parent strain, it did not reduce β-galactosidase activity (Table 2). Thus, the observed phenotype of p37T7 is to repress *cadA* expression in an *hns* mutant background. Its lack of activity in GNB7145K (*adi*::*lac*) *hns* strains, which contain acid-induced *lac* fusions, also indicates specificity of action (data not shown).

Southern hybridization experiments with an *hns*-containing fragment as the probe showed that p37T7 does not contain an *hns* gene. Using the eight restriction enzymes employed by Kohara et al. to construct the *E. coli* physical map (5), we constructed a preliminary restriction map of the insert on p37T7, which showed no similarity to that of the *hns* region (11). Plaque lift screening of the miniset of the ordered phage library provided by Yuji Kohara (5) with ^{32}P-labeled p37T7 DNA showed a strong positive signal with phage 6C1 that

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
Strains		
MC4100	F⁻ *araD139* (*argF-lac*)*U169 rpsL150 relA flb-5301 ptsF25 deoC1*	2
GNB8385K	MC4100 *cadBA*::Mu dI1734 (Km^r *lac*)	1
GNB820	GNB8385K *hns-20*::Tn*5* (Cm^r)	This study
GNB88	GNB8385K *hns-8*::Tn*10* (Tc^r)	11
GNB837	GNB8385K *hns-37*::Tn*10* (Tc^r)	11
Plasmids		
pBR322	Ap^r Tc^r	15
pUC19	Ap^r	New England Biolabs
p37T7	pBR322 with 7.2-kb insert that contains *leuA*$^+$ *leuO*$^+$ *ilvIH*$^+$	This study
pPH1856	2-kb fragment that contains *cadC* promoter and 5′ coding sequence cloned into promoter fusion vector pRS415, Ap^r	16
pXS720	pBR322 with 4.1-kb *Hin*dIII insert, *leuA*$^+$ *leuO*$^+$ *ilvI*$^+$	This study
pXS6 and pXS10	pUC19 with 3.1-kb *Hin*dIII-*Kpn*I insert, *leuA*$^+$ *leuO*$^+$	This study
pXS6A	pBR322 Tc^r Ap^s with 2.4-kb *Ase*I insert, *leuO*$^+$	This study
pCADC$^+$	PCR-amplified *cadC* cloned into the *Eco*RI site of pEMBL8$^+$	7

maps at 1.8 to 1.9 min on the *E. coli* chromosome. The restriction map of the p37T7 insert aligned well with those of phage clones (Fig. 1). A Southern hybridization experiment with restriction enzyme-digested p37T7 and phage 6C1 DNAs with [α-^{32}P]dATP-labeled p37T7 DNA as the probe confirmed that p37T7 indeed contained a fragment located at 1.8 to 1.9 min on the *E. coli* chromosome (data not shown).

Identification of *leuO* as the gene responsible for the phenotype of p37T7. Serial subcloning of plasmid p37T7 was carried out to identify the gene that caused the observed *hns*-complementing phenotype. Strain GNB88 formed tiny red colonies on MacConkey lactose plates; its p37T7 transformants were distinguishably large, pink, and very mucoid, so we used this strain as the host for identification of functional subclones. In Fig. 1, the results obtained with several subclones are presented. Plasmids pXS10 and pXS6, which contained the 3.1-kb *Hin*dIII-*Kpn*I fragment with intact *leuO* and *leuA* genes, had stronger phenotypes than that of the parent plasmid, p37T7. A smaller subclone, pXS6A, was obtained by cloning a 2.4-kb *Ase*I fragment from pXS6 into the *Ase*I site of pBR322 (Fig. 1). pXS6A exhibited exceptionally strong reduction of expression, presumably because of more efficient expression of the functional *trans* factor from this construct. Since the *Ase*I site resides in the β-lactamase (Ap^r) gene on pBR322, pXS6A is Tc^r Ap^s. This plasmid is useful for cotransformation with another Ap^r plasmid (see below).

The deduced amino acid sequence for LeuO showed high homology to LysR family proteins (4), all of which have a helix-turn-helix motif at an aligned position and most of which are known activator proteins. While the DNA sequence of *leuO* is known, no biochemical property or genetic role of the gene product has been reported. From the available sequence information, a unique *Eco*NI site was noted on pXS10, residing in the middle of the *leuO* coding region. Thus, we were able to inactivate *leuO* on pXS10 by blunting the ends generated by *Eco*NI digestion and recircularization. The resulting plasmid, pXS10N, whose structure was confirmed by restriction enzyme digestion and sequence analysis, no longer complemented the effect of an *hns* mutation on *cad* expression (Table 2). Therefore, loss of the *hns*-complementing phenotype correlated with disruption of the *leuO* coding region, proving that *leuO* was the gene responsible for this observed phenotype.

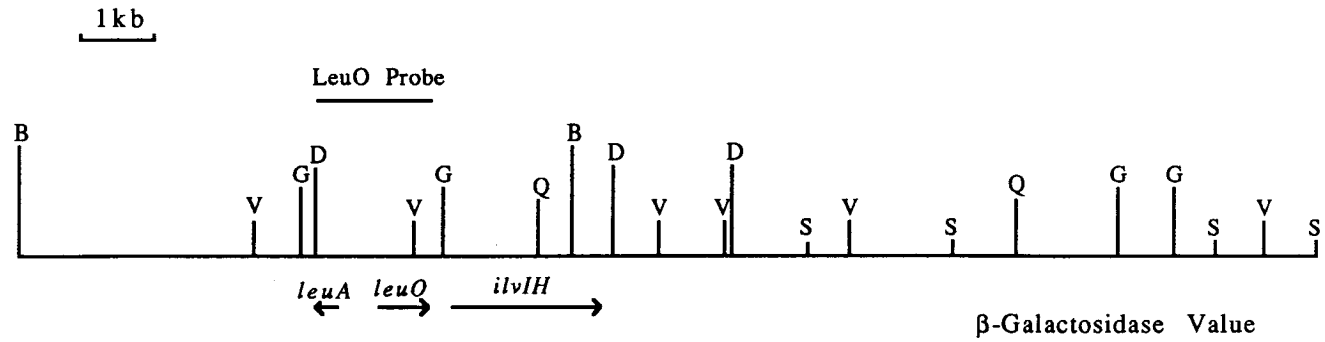
Effects of a *leuO*$^+$ plasmid on β-galactosidase expression from a *cadC*::*lac* fusion. Since activator CadC had already been identified as indispensable for *cadA* promoter activity (16), we studied the effects of excess LeuO protein on the level of *cadC* expression. pPH1856 is a *cadC*::*lac* protein fusion construct which carries an ~2-kb *Eco*RV fragment that contains ~840 bp of sequence upstream of the *cadC* start codon and ~1,160 bp of *cadC* coding sequences on vector pRS415 (16). This plasmid was transformed into a *lac* mutant host, MC4100, and the *cadC* expression levels from this plasmid were monitored by β-galactosidase assays. β-Galactosidase activities at growth pHs of 8.0 and 5.5 (~2,000 Miller units) were similar, indicating that the level of CadC protein production is not dramatically regulated by growth pH, which confirms previous studies (16). When Tc^r *leuO*$^+$ plasmid pXS6A was cotransformed into MC4100 with pPH1856, however, there was an approximate 8- to 10-fold decrease in β-galactosidase expression from the

TABLE 2. Phenotypes of plasmid p37T7 and its derivatives

Plasmid (genotype)	β-Galactosidase activity[a]					
	GNB8385K (*cadBA*::*lac*)		GNB837 (*cadBA*::*lac hns-8*)		GNB88 (*cadBA*::*lac hns-8*)	
	pH 8.0	pH 5.5	pH 8.0	pH 5.5	pH 8.0	pH 5.5
None	150 ± 18	2,920 ± 160	1,900 ± 47	4,820 ± 300	2,920 ± 100	5,210 ± 340
pBR322	130 ± 15	3,310 ± 450	1,270 ± 130	4,870 ± 470	2,450 ± 160	4,590 ± 210
pUC19	140 ± 26	2,900 ± 310	1,350 ± 45	4,950 ± 200	2,200 ± 190	5,380 ± 70
p37T7 (*leuO*$^+$)	200 ± 15	3,800 ± 230	40 ± 6	220 ± 16	140 ± 20	350 ± 20
pXS720 (*leuO*$^+$)	177 ± 35	5,020 ± 400	13 ± 1	74 ± 17	46 ± 6	180 ± 30
pXS6 (*leuO*$^+$)	207 ± 24	2,630 ± 180	44 ± 8	790 ± 36	35 ± 6	160 ± 11
pXS6A (*leuO*$^+$)[b]	1				1.2	9
pXS10 (*leuO*$^+$)	31 ± 6	2,990 ± 230	40 ± 3	390 ± 10	40 ± 8	514 ± 27
pXS10N (LeuO$^-$/*Eco*NI)	230 ± 30	3,920 ± 590	1,160 ± 70	4,810 ± 150	2,560 ± 320	5,650 ± 240

[a] At least three independent colonies were assayed for each construct, and data are averages ± standard deviations. Assays were conducted as previously described (11).

[b] Plasmid pXS6A was assayed in strain GNB820 rather than GNB88. GNB820 exhibits approximately the same β-galactosidase values as GNB88.



		β-Galactosidase Value	
		pH 8.0	pH 5.5
p37T7 (pBR322)		140	350
pXS720 (pBR322)		46	180
pXS6 (pUC19)		35	160
pXS10 (pUC19)		40	510
pXS10N (pUC19)	A/T	2560	5650
pXS6A (pBR322 Tc^r)		1.2	9
	pBR322	2450	4590
	pUC19	2200	5380
	None	2920	5210

FIG. 1. Alignment of inserts on p37T7 and its derivatives with the restriction map of the *E. coli* chromosomal region carried on phage 6C1. The vectors for various plasmid constructs are shown in parentheses. Plasmids pXS6 and pXS10 carry the same insert fragment but may have different copies of polylinker sequences at cloning junctures. pXS10N is a derivative of pXS10 with a single AT base pair insertion. Also shown are the locations and transcriptional directions of three genes that have been sequenced, *leuA* (17), *ilvIH* (12), and *leuO* (4). The β-galactosidase values for GNB88 (*cadBA*::*lac hns-8*) transformants (GNB820 in the case of pXS6A) with the indicated plasmids are on the right. Cells were grown and assayed as previously described (11). GNB88 cells transformed with pBR322, pUC19, or no plasmid (None) were included as controls. The LeuO probe used for Northern (RNA) hybridization (Fig. 2B) is presented as a thin line above the map. Abbreviations: B, *Bam*HI; D, *Hin*dIII; G, *Bgl*I; Q, *Kpn*I; S, *Pst*I; V, *Pvu*II.

cadC::*lac* fusion (data not shown). A similar effect was also observed when these two plasmids were introduced into an *hns* mutant strain. Since both plasmids pXS6A and pPH1856 contain ColE1 origins but differ in drug resistance, their relative amounts may vary from culture to culture. To at least partially account for this effect, β-lactamase measurements were conducted on cultures. Averages from eight cultures indicated that the β-lactamase level had not dropped proportionally (less than a twofold change), although the standard deviation was larger than in single plasmid cultures. This experiment thus suggested that a significant decrease in *cadC* expression occurred upon *leuO* overexpression.

Effects of excess LeuO on *cadC* mRNA. Because CadC protein exists at very low levels in cells, we studied *cadC* expression from a pEMBL8$^+$-derived plasmid, pCADC$^+$, which carries the *cadC* gene and upstream sequence (7). pCADC$^+$ (Ap^r) was cotransformed with pXS6A (*leuO*$^+$ Tc^r Ap^s) into MC4100, and both plasmids were maintained by selection with both antibiotics. When these two plasmids were introduced into either GNB8385K (*cad*::*lac*) or GNB820 (*cad*::*lac hns-20*::Tn*5* [Cm^r]), the β-galactosidase assay values at pH 5.5 or pH 8.0 were the same as those obtained when there was only pXS6A in either strain at the corresponding pH (data not shown). This result is consistent with the results of our study of the repressive effects of excess LeuO on *cadC* expression described above. When both pXS6A and pCADC$^+$ plasmids were present in cells, the amount of LeuO produced from pXS6A was enough to efficiently deprive the *cadA* promoter of CadC.

Total RNA was prepared from cultures grown in a similar manner. Strains were inoculated in 5 ml of Luria broth with appropriate antibiotics and grown at 37°C overnight with agitation. Plasmid DNA was purified from an aliquot of each culture and analyzed to show that each plasmid was maintained. At the same time, 0.4 ml of overnight culture was transferred into 10 ml of lysine-supplemented modified Falkow medium, buffered to pH 8.0 (1) and supplemented with appropriate antibiotic(s), and grown as previously described (11)

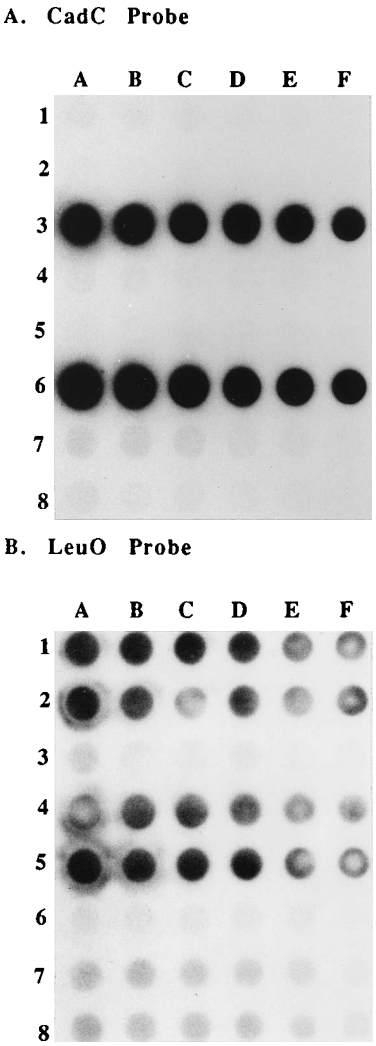


FIG. 2. Northern hybridization of total RNA dot blots. Filter blots were obtained with a 96-well filtration manifold apparatus (Schleicher & Schuell, Inc.). Six quantities of RNA (20, 15, 10, 5, 3, and 1 μg for columns A to F, respectively) were applied from each sample. (A) Nick-translated, [α-^{32}P]dATP-labeled 1.9-kb *Eco*RI fragment from pCADC$^+$ was used as the probe (CadC probe). (B) Nick-translated 2.4-kb *Ase*I fragment of pXS6A was used as the probe (LeuO probe). The RNA concentrations and A_{260}/A_{280} ratios were approximately the same for all cultures. The β-galactosidase value for each of these cultures was as follows: sample 1 (GNB8385K/pXS6A), <1; sample 2 (GNB8385K/pXS6A + pCADC$^+$), 15; sample 3 (GNB8385K/pCADC$^+$), 3,200; sample 4 (GNB820/pXS6A), 1.2; sample 5 (GNB820/pXS6A + pCADC$^+$), 1.2; sample 6 (GNB820/pCADC$^+$), 2,650; sample 7 (GNB8385K/p37T7), 140; and sample 8 (GNB820/p37T7), 540.

until the A_{600} of the culture reached 0.4 to 0.6. An aliquot of the culture was subjected to β-galactosidase assay as previously described (11), and the values are given in the legend to Fig. 2. The remainder of the culture was collected by centrifugation, and total RNA was extracted as described by Summers (14). RNA samples were treated and applied onto a Hybond-N$^+$ membrane (Amersham) with a filtration manifold apparatus (Schleicher & Schuell, Inc.) as recommended by the manufacturers. Two parallel blots were hybridized with either the [α-^{32}P]dATP-labeled 2.4-kb *Eco*RI fragment from pCADC$^+$ (CadC probe) or the labeled 2.4-kb *Ase*I fragment from pXS6A (LeuO probe). The hybridization results are shown in Fig. 2. In Fig. 2A (CadC probed), dots of samples 2 (GNB 8385K/pXS6A + pCADC$^+$) and 5 (GNB820/pXS6A + pCADC$^+$) showed considerably lower intensities than those of samples 3 (GNB8385K/pCADC$^+$) and 6 (GNB820/pCADC$^+$), respectively, indicating a drastic reduction in mRNA production from the *cadC* gene when *leuO* is present on a multicopy plasmid. In Fig. 2B (LeuO probed), samples 2 (GNB8385K/pXS6A + pCADC$^+$) and 5 (GNB820/pXS6A + pCADC$^+$) showed intensities of hybridization similar to those of samples 1 (GNB8385K/pXS6A) and 4 (GNB820/pXS6A), respectively, indicating that the presence of *cadC* on a multicopy plasmid does not have any observed effect on *leuO* mRNA production. The significantly lower intensities of samples 7 (GNB8385K/p37T7) and 8 (GNB820/p37T7) compared with those of samples 1 and 4, respectively, indicate that at the transcriptional level, *leuO* is expressed much more efficiently from the smaller plasmid, pXS6A, than from p37T7. Controls showed that a consistent amount of mRNA was applied for each sample and that the intensities correlated with RNA amount.

In conclusion, the results shown in Fig. 2A suggest that excess LeuO protein (from the *leuO* gene on a multicopy plasmid) drastically represses *cadC* gene expression at the transcriptional level. Since *leuO* appears to be homologous to the *lysR* family of genes that code for helix-turn-helix DNA-binding proteins, it is likely that LeuO directly binds to *cadC* promoter sequences to reduce *cadC* gene expression. If this hypothesis is true, then the affinity of LeuO to *cadC* promoter is probably low because LeuO needs to be in excess in order to show the *cad*-repressing effect. The small effect observed in *hns*$^+$ strains also indicates that LeuO may be inhibited by H-NS or that *leuO* is not expressed as well in *hns*$^+$ strains. The results do show a regulatory phenotype associated with the LysR-like LeuO protein; however, its normal role in cells is still unclear.

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