Expression of the second lysine decarboxylase gene of *Escherichia coli*

Marc Lemonnier and David Lane

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Certain amino acids are substrates for two decarboxylase enzymes in *Escherichia coli*, one inducible by anaerobic growth at low pH and the other constitutive. In the case of lysine, an inducible decarboxylase (CadA) has been extensively characterized, but evidence for the existence of a second lysine decarboxylase is fragmentary and uncertain. This paper confirms that a second lysine decarboxylase is encoded by a locus (*Idc*) previously suggested to be a lysine decarboxylase gene on the basis of sequence comparisons. Overexpression of the cloned gene provided sufficient quantities of enzyme in cell-free extracts for preliminary examination of the properties of the *Idc* gene product, Ldc. The enzyme is active over a broad range of pH with an optimum at 7.6, much higher than that of CadA, about 5.5. The temperature optimum for both enzymes is similar, at about 52 °C, but Ldc is more readily inactivated by heat than CadA. Expression of *Idc* from its own promoter was very weak for cells growing in a variety of media, although a low level of lysine decarboxylase was present in cells that carried the *Idc* region on an oligo-copy plasmid when these were grown in minimal-glucose medium. Northern analysis of RNA extracted from such cells revealed a transcript whose length corresponded to that of the *Idc* gene, suggesting that *Idc* is normally transcribed from a promoter immediately upstream. However, most of the *Idc* mRNA was shorter, indicating degradation or premature termination. The *Idc* upstream sequence promoted transcription of a *lacZ* gene to which it was fused. Introduction of the upstream sequence as an insert in a multicopy vector increased transcription of the resident *lacZ* fusion. The low level of expression in single copy, the emergence of expression when the gene is present at moderate copy number, and the derepression by the upstream sequence *in trans* imply that this second lysine decarboxylase gene may not be constitutive but subject to specific repression by a factor which remains to be identified.

**Keywords**: lysine decarboxylase, gene expression

**INTRODUCTION**

Of the several amino acid decarboxylases known to be present in *Escherichia coli*, those that attack the basic amino acids, arginine, ornithine and lysine, are the most significant physiologically and the best studied. The enzymes fall into two groups. One consists of decarboxylases, termed degradative, whose production is induced by low oxygen tension, high acidity and high concentrations of the respective amino acids. Their activity leads to the synthesis and excretion of polyamines and CO₂, which restore the external pH (Meng & Bennet, 1992; Watson *et al.*, 1992) and maintain the required CO₂ level of the medium (Boeker & Snell, 1972; Takayama *et al.*, 1994). The enzymes of the other group are produced constitutively at low levels in normal culture conditions. They are called biosynthetic because their role appears to be the catalysis of the first steps in the synthesis of putrescine and spermidine, polyamines needed for optimal ribosome function and growth rate (Tabor & Tabor, 1985). Thus, in general, each of the basic amino acids is a substrate for two decarboxylases. Inducible and constitutive decarboxylases specific for arginine and ornithine have been purified and characterized, although inducible ornithine decarboxylase seems to be absent from certain...
E. coli strains, including K-12 (Applebaum et al., 1977). Inducible lysine decarboxylase has also been purified and extensively analysed (Sabo et al., 1974). The gene encoding it, cadA, is the second of a two-cistron operon whose expression is positively regulated by the product of an upstream gene, cadC (Auger et al., 1989; Watson et al., 1992; see Fig. 1a) and negatively regulated by the product of the distant cadR gene (Popkin & Maas, 1980; Tabor et al., 1980). Acid induction is mediated by the CadC regulator (Watson et al., 1992). However, in E. coli growing in minimal medium at neutral pH the operon is effectively silenced, since the decarboxylation product of lysine, cadaverine, is not detected (Hafner et al., 1979).

On the other hand, evidence for the existence of a constitutive decarboxylase specific for lysine is equivocal. Mutants deprived of putrescine and spermidine make detectable quantities of cadaverine when grown under conditions which do not induce the cad operon (Hafner et al., 1979). Goldemberg (1980) reported that a small proportion of the lysine decarboxylase in wild-type E. coli was thermolabile, in contrast to the thermostability of the inducible enzyme. Werheimer & Leifer (1983) detected lysine decarboxylase activity in crude extracts of cells grown in non-inducing conditions which, unlike the inducible enzyme, was inhibited by putrescine and spermidine. However, a subsequent report (Igarashi et al., 1986) showed that a lysine decarboxylase that copurified with ornithine decarboxylase protein was also inhibited by putrescine and spermidine, and that it was absent from cells of an ornithine decarboxylase mutant, raising the possibility that relaxed substrate specificity of ornithine decarboxylase was responsible for the lysine decarboxylase activity previously seen. In its turn, this conclusion seems at odds with the failure of purified ornithine decarboxylase to use lysine as a substrate (Applebaum et al., 1977).

In the course of an unrelated project, to be reported elsewhere, we observed that mini-F plasmid maintenance could be disrupted by overexpression of a cloned gene fragment (see Fig. 1a). The sequence of this fragment revealed that it originated within a putative gene whose sequence shows marked similarity to that of the inducible lysine decarboxylase gene, cadA. This homology had previously been noted by Yamamoto et al. (1995), who dubbed the sequence ldc ("probably constitutive lysine decarboxylase"). For reasons that will become clear, constitutive expression of this sequence remains an unsettled issue, and throughout this paper we shall refer to it as ldc. The existence of the ldc open reading frame at a chromosomal location (4-7 min) distinct from those of known decarboxylase genes suggested that, after all, E. coli might contain another lysine decarboxylase. We report here that the ldc sequence does indeed encode a protein with lysine decarboxylase activity. After submitting this article we became aware of a very recent report describing the identification and purification of this enzyme (Kikuchi et al., 1997). The results of both papers are fully consistent. In addition, we provide more detail on certain aspects of enzyme activity as well a first analysis of ldc transcription and ldc promoter activity.

**METHODS**

**Construction of bacterial strains and plasmids.** The strains used are listed in Table 1. Mutations were transferred between strains by transduction with P1vir. The cadB::MudK Km mutation was provided, as strain GNB6385K, by David Watson (Rice University, Houston). The ldc::Ωbla mutation was constructed on a plasmid, then substituted for ldc* on the chromosome by homologous recombination, as follows. The bulk of the ldc gene was removed by cutting pMESJE11 with BaII and BsrWI (see Fig. 1a), and the remaining plasmid DNA was end-repaired using Klenow polymerase and ligated to a 2 kb Smal omega fragment containing bla obtained from pKT254α-Ap (Fellay et al., 1987). The chromosomal DNA was excised from the resulting plasmid with Eco47III and SphI (see Fig. 1a) and used to transform JC7623. Transformants resistant to ampicillin (25 μg ml−1) were tested for allele replacement by Southern hybridization using ldc and bla probe DNAs and by PCR amplification of bla DNA using ldc-flanking sequence oligonucleotides 05L and 05R (see below). Strains allowing derepression of the vector ara promoter without metabolism of arabinose were made by transduction of Δ(ara−leu)=7696 linked to zac3501::Tn10 from a strain provided by C. Turlan (CRNS, Toulouse). Strains carrying single-copy transcriptional fusions of lacZ to ldc upstream sequences were made as described by Simons et al. (1987), by selection for Kan' mono-lysogens after infection with λK45 lysates of strains carrying pDAG123 and pDAG124 (see below).

The pFUS2 expression vector was made by ligating a fragment containing the kanamycin-resistance gene of Tn5 and the rep region of pMB1 to the large Apal fragment of plBAD18 (Guzman et al., 1995) containing Para and araC (details available from the authors on request).

The ldc expression plasmid was made by: (i) amplifying ldc DNA, using 10 ng CB0129 chromosomal DNA as template, oligonucleotides 05L (5'-AAGGAGAGGATTTCAAGGG-G) and 05R (5'-TAACAGCACGTTACTCGCCCG), DNA polymerase Pfu (Stratagene) and 25 cycles of 94 °C for 30 s, 60 °C for 1 min, 72 °C for 2.5 min followed by 72 °C for 10 min; (ii) purification of the product by agarose gel electrophoresis and extraction (Qiagen kit), followed by end-repair with Klenow DNA polymerase and deoxynucleotides and insertion into the Smal site of pFUS2 with T4 DNA ligase to form pMML120 (Kan'). A derivative conferring spectinomycin resistance, pMML145, was made by inserting a HindIII–Eco47III fragment from pBG2 (Churchward et al., 1984) containing the adaA gene into the Eco47III site of pMML120.

Plasmids carrying the ldc upstream sequences were made by inserting the PCR products shown in Fig. 6 (made as above, using oligonucleotide primers LDC1, 2 and 3) into pUC9 (Vieira & Messing, 1982) to give pDAG121 and 122, and into the transcriptional fusion vector pRSS51 (Simons et al., 1987) to give pDAG123 and 124. The PCR fragments were cleaved with BaII and EcoRI, and the vectors cleaved with BamHI (end-repaired with Klenow fragment) and EcoRI, prior to ligation.

**DNA manipulations and other procedures.** Restriction enzyme digestion, Klenow DNA polymerase reactions and ligation with T4 DNA ligase were carried out using conditions recommended by the supplier, New England Biolabs. Standard
were diluted 100-fold into the same medium and grown with production of Ldc, overnight cultures in F-HEPES medium supplemented with lysine (40 μM)
from the components of decarboxylase medium base (Difco) at 0.1 M and supplemented with thymine (50 μg ml−1) and stored at -20 °C. 

Cell-free extracts. Cultures grown in F-MES (100 ml) or F-HEPES (40 ml) were chilled in the presence of 50 mM NaCl, 50 mM Tris/HCl pH 8.0, 5 mM EDTA, 1 mM NaNO₃. The cells were centrifuged, washed with cold 0.5 M NaCl, 0.05 M potassium phosphate pH 6.0, and resuspended in 1 ml Ldc buffer (0.1 M CH₃COONa pH 6.0, 1 mM EDTA, 0.1 mM pyridoxal phosphate, 10 mM 2-mercaptoethanol, 10%, w/v, glycerol). Lysozyme was added to 0.1 mg ml⁻¹ and the mixture was incubated at 0 °C for 30 min. The cells were broken by sonication and the lysate centrifuged at 80 000 g for 30 min; 0.5 ml of the supernatant was retained as the cell-free extract and stored at -20 °C.

Lysine decarboxylase assay. The method described by Phan et al. (1982) was adapted for use with intact cells and modified to allow the assay to be carried out in Eppendorf tubes. For measuring lysine decarboxylase activity in cells, culture samples of 1-5 ml were centrifuged and the cells were resuspended in 1 ml Ldc buffer (0.1 M CH₃COONa pH 6.0, 1 mM EDTA, 0.1 mM pyridoxal phosphate, 10 mM 2-mercaptoethanol, 10%, w/v, glycerol). Lysozyme was added to 0.1 mg ml⁻¹ and the mixture was incubated at 0 °C for 30 min. The cells were broken by sonication and the lysate centrifuged at 80 000 g for 30 min; 0.5 ml of the supernatant was retained as the cell-free extract and stored at -20 °C.

Lysine decarboxylase activity was determined on samples of exponential-phase culture grown for about five generations to about 10⁵ cells ml⁻¹, and assayed as described by Pardee et al. (1959) with minor modifications (Lane et al., 1994); specific activities were calculated as Miller units (Miller, 1972).


tables: Table 1. Bacterial strains and plasmids

All strains are derivatives of E. coli K-12. The MudXKm insertion in cadB is polar on cadA, and hence strains carrying this mutation are phenotypically CadA⁻ (Auger et al., 1989).

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<td>W1485 F' thi leu thyA deoB supE cad⁺ lde⁺</td>
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<td>GNB8385K</td>
<td>MC1000 cadB::MudXKm</td>
<td>Auger et al. (1989)/</td>
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<tr>
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<td>Silhavy et al. (1984)</td>
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<td>rep₂pUC121; addA⁺</td>
<td>Vieira &amp; Messing (1982)</td>
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<td>pRS551</td>
<td>rep₂pBR322; blα⁺</td>
<td>Simons et al. (1987)</td>
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<td>pMESJ11</td>
<td>ldc chromosomal fragment in pGB2 (see Fig. 1a)</td>
<td>S. Pichoff &amp; J.-P. Bouché*</td>
</tr>
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<td>rep₂CVD; blα⁺</td>
<td>Fellay et al. (1987)</td>
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<td>rep₂pBR322; kan⁺ araC-para</td>
<td>This work</td>
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<td>pMLM120::addA⁺</td>
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<td>pDAG124</td>
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</tbody>
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* CNRS, Toulouse.

procedures were used for transformation of calcium-treated cells, agarose gel electrophoresis and SDS-PAGE (Maniatis et al., 1982, and references therein). In vivo β-galactosidase activity was determined on samples of exponential-phase cultures grown for about five generations to about 3 x 10⁸ cells ml⁻¹, and assayed as described by Pardee et al. (1959) with minor modifications (Lane et al., 1994); specific activities were calculated as Miller units (Miller, 1972).

Media and growth conditions. For routine work, bacteria were grown in L broth (Lennox, 1955) at 37 °C. Antibiotics were added as appropriate: kanamycin 50 μg ml⁻¹, spectinomycin 100 μg ml⁻¹, ampicillin 25 μg ml⁻¹. Cultures for lysine decarboxylase assay or extraction were grown in F broth (modified Falkow medium; Falkow, 1958) made up from the components of decarboxylase medium base (Difco) -5 g peptone, 3 g yeast extract and 1 g b-glucose in 1 litre H₂O - buffered with either MES (pH 5.2) or HEPES (pH 7.6) at 0.1 M and supplemented with thymine (40 μg ml⁻¹). For production of Ldc, overnight cultures in F-HEPES medium were diluted 100-fold into the same medium and grown with aeration to OD₆₀₀ 0.2-0.3; arabinose was then added to 0.5%, and incubation was continued for 1.5-2 h before harvesting. For production of CadA, 100 ml F-MES medium supplemented with lysine (0.5%) in a 100 ml screw-cap bottle were inoculated with freshly grown L agar colonies and incubated for 24-30 h at 37 °C. Minimal medium was M9 salts (Adams, 1959) supplemented with thiamin (1 μg ml⁻¹), glucose or glycerol (0.4%), leucine (50 μg ml⁻¹) and thymine (40 μg ml⁻¹); culture conditions were as for F-HEPES cultures (above).
measured. One drop of CHCl₃ was added and the suspension briefly vortexed and returned to ice; unless used immediately for assay, 150 μl of suspension was transferred to a fresh tube to limit exposure to CHCl₃. The assay was carried out by adding 5–50 μl of suspension to prewarmed 5 mM lysine, 0.1 mM pyridoxal phosphate, 16 mM potassium phosphate (pH 5.6 or 7.6) in a final volume of 120 μl; a parallel mixture without lysine was also prepared, to control for the level of endogenous polyamines, since these react in the assay as cadaverine. The mixtures were incubated at 37 °C for 15 min, then mixed with 120 μl 1 M Na₂CO₃ and placed on ice. 2,4,6-Trinitrobenzenesulphonate (120 μl of a 10 mM solution) was added, and the mixtures were incubated at 40 °C for 4 min before chilling, then extracted with 1 ml tolune by vortexing for 20 s, and centrifuged for 1–2 min. Assay of cell-free extracts was identical, except that usually 5 μl of a 20-fold dilution (∼0.93 μg protein) in Ldc buffer was added instead of CHCl₃-treated cells. Lysine decarboxylase activity was determined as the difference in A₅₅₀ between the sample incubated with lysine and that incubated without. Specific activities were calculated as nmol cadaverine produced per min (units) per mg total protein (cell-free extracts) or as 1000 × A₅₅₀ per min (units) per OD₆₀₀ unit (whole cells). Following addition of cell or extract samples to the assay mixture the synthesis of cadaverine remains linear for about 20 min before slowing to a plateau value (Fig. 2a, b). In the experiments described here, reactions were halted at 15 min. Reaction rate was proportional to cell or extract concentration over at least a 10-fold range (Fig. 2c).

**Northern hybridization.** Culture samples were added to an extraction of RNA by the hot phenol method (Aiba et al., 1981). RNA was quantified by absorption at 260 nm. Ten or twenty micrograms of total RNA were fractionated by gel electrophoresis, transferred to a membrane, and Northern hybridization analysis was carried out using a 0.8-kb DNA fragment from the cad locus of *E. coli* as a probe (Aiba et al., 1981). The restriction sites shown were used to construct the MudX locus is shown as the insert carried by pMES111. The MudX clone used in this work is shown; it causes a CadA- phenotype by polarity (Auger et al., 1989). The cad locus is shown as the insert carried by pMES111. The restriction sites shown were used to construct the CadA- mutant (see Methods). (b) Dendrogram showing relatedness of LdcS derived by the Clustal alignment program (PC Gene) and based on the amino acid sequences in the GenBank database shown below. (c) Alignment of amino acid sequence of *E. coli* Ldc (ECO: GenBank accession number U37109) with those of *E. coli* CadA (CAD: 00096), *Salmonella typhimurium* Ldc (STY: X03774) and *Hafnia alvei* Ldc (HAL: X03774). Identity in all proteins is shown by dots. Boxed residues indicate the pyridoxal phosphate binding motif. The underlined sequence is that confirmed (as nucleotide sequence) in this laboratory: it represents the peptide that interferes with mini-F maintenance.

**Fig. 1.** *E. coli* cad gene locus, and comparison of lysine decarboxylase sequences. (a) The cad and *E. coli* loci. The MudX insertion allele of cadB used in this work is shown; it causes a CadA- phenotype by polarity (Auger et al., 1989). The cad locus is shown as the insert carried by pMES111. The restriction sites shown were used to construct the CadA- mutant (see Methods). (b) Dendrogram showing relatedness of LdcS derived by the Clustal alignment program (PC Gene) and based on the amino acid sequences in the GenBank database shown below. (c) Alignment of amino acid sequence of *E. coli* Ldc (ECO: GenBank accession number U37109) with those of *E. coli* CadA (CAD: 00096), *Salmonella typhimurium* Ldc (STY: X03774) and *Hafnia alvei* Ldc (HAL: X03774). Identity in all proteins is shown by dots. Boxed residues indicate the pyridoxal phosphate binding motif. The underlined sequence is that confirmed (as nucleotide sequence) in this laboratory: it represents the peptide that interferes with mini-F maintenance.
electrophoresis in a formaldehyde-agarose gel (Brown, 1994) and transferred to Positive Membrane (Appligene). The membrane was incubated in 5 ml 50% formamide, 0.75 M NaCl, 0.075 M trisodium citrate, 0.05 M sodium phosphate pH 6.8, 0.1% SDS, 0.1 mg ml⁻¹ sonicated and denatured calf-thymus DNA at 42 °C for 1.5 h. Ten nanograms of ldc PCR product (see above) labelled with ³²P by nick-translation (~10⁶ c.p.m. ng⁻¹) was heat-denatured and added, and incubation at 42 °C was continued for 8 h. After washing at 50 °C (final wash solution 0.03 M NaCl, 0.05% SDS), the filter was exposed to a Fuji phosphor-imager screen.

RESULTS

Sequence relatedness of lysine decarboxylases

The similarity of the translated ldc sequence to that of cadA and the lysine decarboxylase genes of other Gram-negative bacteria is clearly evident from the aligned sequences shown in Fig. 1(c). The alignment reveals 61% amino acid identity among the four polypeptides, with a further 30% of the residues representing replacement by amino acids of similar properties. This strong homology is distributed fairly evenly throughout the polypeptide sequences, although significant divergence is localized near the N- and C-termini and within the stretch of residues 624-640. The dendrogram based on this alignment (Fig. 1b) indicates that E. coli Ldc is the most distantly related within this group, and shows more amino acid sequence similarity to the lysine decarboxylase of Hafnia alvei than to its sister lysine decarboxylase in E. coli, CadA. Closer examination confirms this view: for example, of the 179 positions where the three other proteins all differ from E. coli Ldc, the identical amino acid substitution is found in 108 (60.3%) cases. Nevertheless the homology is close enough to justify the suggestion that the ldc gene encodes a lysine decarboxylase: further inspection shows that homology with other lysine decarboxylases is certainly stronger than that with decarboxylases for other amino acids (35 and 12% identity with E. coli biodegradative and biosynthetic arginine decarboxylases respectively, and 15% with biosynthetic ornithine decarboxylase). In particular, only three of 15 residues in the pyridoxal phosphate binding motif characteristic of amino acid decarboxylases show variation among the four proteins, and in all cases this involves conservative substitution of hydrophobic amino acid residues.

Lysine decarboxylase activity of the ldc gene product

The ldc gene, together with its putative ribosome-binding site, was inserted downstream from the inducible araBAD promoter (Para) in the vector, pFUS2. Addition of arabinose to cultures of DLT814 carrying the resulting ldc expression plasmid (pMLM120) induced the production of a single polypeptide not found in cells carrying the vector, as shown by Coomassie blue staining of total cell protein after SDS-PAGE (Fig. 3a). The polypeptide migrated as a species of 80 kDa, the size expected on the basis of the ldc sequence.

To enable rapid measurement of lysine decarboxylase activity, we developed a permeabilized cell assay which circumvents the preparation of cell-free extracts (see Methods). The essentially identical pH profiles of the enzyme in permeabilized cells and cell-free extracts (Fig. 4a) indicate that the general properties of the enzyme are similar in both assay conditions. This method was used to measure lysine decarboxylase in cells sampled from arabinose-induced cultures of DLT814 carrying either pMLM145 or pFUS2 (Fig. 3b). The steep rise in lysine decarboxylase specific activity seen in cells carrying pMLM145 was absent from cells carrying the vector. Lysine decarboxylase was also detected in uninduced pMLM145-containing cells at a low but significant level; although a corresponding polypeptide band was not detected, this basal enzyme level presumably reflects incomplete repression of Para on the ldc expression
Induced expression of the ldc gene. (a) Coomassie-blue-stained polyacrylamide gel (12.5%) of total proteins obtained 1 h after addition of arabinose (at 0.1%) to L broth cultures of ML9 cells carrying pFUS2 (lane 2) or pMLM120 (lane 3). Lane 1, protein molecular mass standards. The arrowhead points to the band migrating as a peptide of about 80 kDa. (b) Lysine decarboxylase activity in induced cultures. Arabinose was added at 1% final concentration to cells growing exponentially in L-broth. Samples were removed at intervals for lysine decarboxylase assay of whole cells. ▲, DLT812 (cad’ ldcC); ■, DLT814 (cadB::lacZ ΔldcC); ●, DLT814pPMLM145; ○, DLT814pPMLM145 without added arabinose. The dotted line shows a composite growth curve; all strains grew at the same rate.

Properties of Ldc

An examination of Ldc activity in cell-free extracts revealed differences between the enzyme and CadA. Both enzymes are active over a broad pH range, but whereas CadA activity is optimal at about pH 5.6 and virtually eliminated at pH 8.0, optimum Ldc activity is centred around pH 7.6 (Fig. 4a). These data also show that the enzyme responds identically to pH whether in cell-free extracts or in cells. For both enzymes, assay at various temperatures indicated an optimum for activity of about 52 °C (Fig. 4b): CadA activity remains high at assay temperatures up to 67 °C, but Ldc activity is inhibited as temperature increases. A test of the thermoresistance of Ldc (Fig. 4c) showed that whereas CadA remained stable at temperatures up to 70 °C, Ldc was progressively inactivated by incubation above 37 °C.

Expression of ldc

To observe and measure expression of ldc in its normal chromosomal context, we constructed mutants lacking ldc or unable to express cadA, or both. The mutants were then grown in various media and conditions, and samples of the cultures assayed for lysine decarboxylase (Table 2). As expected, strains carrying the wild-type cad operon (DLT812, 813) contained high levels of lysine decarboxylase when grown under inducing conditions but negligible amounts when grown at neutral pH with oxygenation. In contrast, the cad mutant strains contained very little or no lysine decarboxylase under any of the growth conditions tested here (Table 2). Only when the ldc region was present in an oligo-copy plasmid (pMESJEl1) was lysine decarboxylase detected, at about 2.5 units, barely above the limit of significance for the assay used. Notably, this low level of activity was present in cells grown in minimal glucose medium but not in cells grown in rich medium or in minimal medium with glycerol in place of glucose. The addition of lysine to the medium did not alter this low level of activity. These results suggested either that ldc is normally not expressed, or that its product is rapidly inactivated.
Table 2. Lysine decarboxylase activity of mutant strains grown in various media, based on assays of permeabilized whole cells

Data for DLT812 and 813 are single determinations. For the other strains figures are the means from two or three independent experiments; all values fell within the range ±0.9 units per OD₆₀₀ unit. Negative values reflect experimental uncertainty of measurement at very low or zero levels of enzyme, and result from subtraction of background due to endogenous polyamines. The pMESJE11 plasmid is oligo-copy, and the corresponding increase in ldc gene dosage is indicated by + +. ND, Not determined.

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<tr>
<th>Strain</th>
<th>cad</th>
<th>ldc</th>
<th>F-MES +0.5% lysine; aerobic</th>
<th>F-HEPES +0.5% lysine; aerobic</th>
<th>F-HEPES -lysine; aerobic</th>
<th>M9-glucose +0.5% lysine; aerobic</th>
<th>M9-glucose -lysine; aerobic</th>
<th>M9-glycerol -lysine; aerobic</th>
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<td>+</td>
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<td>-1.3</td>
</tr>
<tr>
<td>DLT815</td>
<td>-</td>
<td>+</td>
<td>0.8</td>
<td>-1.2</td>
<td>-0.4</td>
<td>0.2</td>
<td>0.0</td>
<td>0.6</td>
</tr>
<tr>
<td>DLT814/pMESJE11</td>
<td>-</td>
<td>+</td>
<td>1.8</td>
<td>-0.6</td>
<td>0.4</td>
<td>2.9</td>
<td>2.3</td>
<td>-1.6</td>
</tr>
</tbody>
</table>

Fig. 5. Detection of ldc mRNA by Northern analysis. Total RNA was isolated from cells grown in minimal medium to OD₆₀₀ 0.6-0.8; in the case of DLT814/pMLM145 the medium was supplemented with arabinose (0.4%) for the last hour (~0.9 generations) of growth. Twenty micrograms (10 μg for DLT814/pMLM145) of each RNA preparation was subjected to formaldehyde-agarose gel electrophoresis, followed by transfer to membrane and hybridization with ³²P-labelled ldcC DNA. The numbers on the right indicate the positions of ³²P-labelled '1 kb' markers (Gibco-BRL) electrophoresed in parallel.

To determine more directly whether ldc is transcribed, we performed a Northern analysis of RNA extracted from cells of various strains growing exponentially in M9-glucose. The results, shown in Fig. 5, confirm those obtained by measurement of lysine decarboxylase activity. ldc mRNA was not found in either the wild-type or the mutant strains, using conditions which we estimate would have detected two transcripts per cell. Hybridization and washing conditions were sufficiently stringent to prevent extensive cross-hybridization with cadA mRNA. However ldc mRNA was present in cells containing the ldc⁺ plasmid, pMESJE11. Two species were seen, a minor one of about 2.2 kb, corresponding to the full length of the ldc open reading frame, and a more abundant one of about 1.4 kb. The same species were present in RNA isolated from DLT814/pMLM145 cells to which arabinose had been added (Fig. 5, right-hand lane), suggesting that the signals for initiating and terminating transcription of the ldc gene are disposed similarly, relative to ldc, to those in the Para expression plasmid. It appears that although the transcription and translation signals are in place to allow expression of ldc, the level of expression under any of the growth conditions we have tested is too low to make a significant contribution to lysine decarboxylase activity.

Control of expression

Inspection of the sequence upstream of ldc reveals three sets of paired hexamers with enough similarity to the consensus −35 and −10 promoter motifs to be considered plausible promoters. Overlapping the first of the candidate −35 sequences is an inverted repeat which might serve as a repressor-binding site. To test the roles of these sequence elements in ldc expression we isolated two fragments, one containing the entire region and the other only the third candidate promoter, and linked them to the lacZ gene to create transcriptional fusions (Fig. 6). These fusions were then integrated in single copy at the chromosomal attL site, and the concentrations of β-galactosidase in exponentially growing cells were measured. The fragments were also introduced on a high-copy-number vector into the fusion. 757
strains, to test whether derepression of lacZ might provide evidence for the existence of a repressor.

The data in Fig. 6 show that indeed the ldc upstream sequence promotes lacZ transcription, and that the entire sequence does so nearly three times more frequently than that containing only the third potential promoter. Both promoter regions at high copy number in trans cause a twofold increase in promoter activity of the extended upstream region, whereas neither affect the promoter in the short fragment. One simple interpretation of these data is that there are two ldc promoters, the upstream one relatively strong but normally repressed and the downstream one weak and immune to repression. The observation that the short upstream fragment in trans derepresses expression as efficiently as the longer one does not support the proposal that the inverted repeat acts as a repressor binding site.

**DISCUSSION**

The results presented here validate earlier suggestions that *E. coli* possesses a second lysine decarboxylase. Goldemberg (1980) reported that in cells mutant for the inducible lysine decarboxylase, or in uninduced wild-type cells, small amounts of lysine decarboxylase were still present, and that 30% of this activity was destroyed by incubation at 60 °C. Wertheimer & Leifer (1983) also observed that uninduced cells had a residual activity, which differed from the inducible lysine decarboxylase in being sensitive to inhibition by putrescine and spermidine. They were unable, however, to confirm the temperature sensitivity found by Goldemberg. We find that the lysine decarboxylase activity produced by overexpression of the cloned ldc gene shows 40% loss of activity after exposure to 60 °C (Fig. 4), in close accord with Goldemberg's data. Owing to the nature of the assay used here, which detects all polyamines, we could not test polyamine inhibition of Ldc in a straightforward manner; however, it appears likely that the activity studied by Wertheimer & Leifer (1983) was actually due to ornithine decarboxylase (Igarishi et al., 1986).

*lacZ* in its normal chromosomal location was expressed at levels indistinguishable from background in all of the growth conditions used in our experiments (Table 2). Kikuchi et al. (1997) obtained the same result. The possibility that this is due to an amino acid other than lysine being the major substrate was ruled out by showing that Ldc appears to be as avid as CadA in attacking lysine. Ldc produced by arabinose induction of pMLM145-containing cells constitutes 5% of total cell protein (Fig. 3a), or 6% of cell-free extract protein; from its specific activity of about 60 units (mg protein)⁻¹, we estimate that the specific activity of the pure protein would be about 1000 units mg⁻¹, comparable to that reported for the inducible lysine decarboxylase (1018 units mg⁻¹) by Sabo et al. (1974). Kikuchi et al. (1997) reported that neither ornithine nor arginine was a substrate for the enzyme.

Our inability to detect ldc mRNA in ldc⁺ cells (Fig. 5) suggests strongly that either weak transcription or low mRNA stability (or both) is the cause of low lysine decarboxylase levels. Only when the ldc gene dosage was raised about sixfold by insertion into an oligo-copy plasmid was expression of ldc seen, both as enzyme activity and as mRNA. In this case, the largest transcript revealed by the Northern analysis was of just the length needed for expression of ldc (2138 bp), suggesting that ldc mRNA is initiated immediately upstream of the gene rather than being part of a polycistronic transcript that includes the dnaE and accA genes (see Fig. 1). However this transcript was a minor species, and much low-molecular-mass RNA, notably an abundant species of about 1.4 kb, was seen. This RNA presumably results from processing of the 2.2 kb species, or possibly from premature termination; its preponderance in RNA transcribed from *Para* indicates that it is unlikely to arise from initiation at an internal promoter.

Despite the paucity of ldc mRNA, the ldc upstream region is not devoid of promoter activity. Cells containing a chromosomally located *Pldc*:lacZ transcriptional fusion contained 88 Miller units of β-galactosidase (Fig. 6). On the basis of equivalent data reported by Raleigh & Kleckner (1986) for the activity of wild-type and mutant versions of the weak IS10 transposase promoter fused to lacZ, we estimate that this specific activity corresponds to about 150 LacZ monomers per cell. This level of expression of the ldc gene would allow the assembly of 15 molecules of the Ldc decamer, thought to be the active form of the enzyme (Kikuchi et al., 1997). Whether this concentration permits detection of enzyme activity in the assays of Kikuchi et al. (1997) and ourselves is unknown, but we suggest that even this low level of enzyme might not be reached owing to the high sensitivity of ldc mRNA to ribonuclease attack or to premature termination, as witnessed by the relatively abundant low-molecular-mass mRNA species discussed above. From this point of view the detection of ldc mRNA, as well as Ldc enzyme activity, in cells carrying ldc at the approximately sixfold higher copy number of the pSC101 vector would be due simply to a higher ratio of mRNA to RNase or termination factors, which allows the establishment of a small ldc mRNA pool. Clearly, experiments such as pulse-chase analysis are needed to distinguish instability from premature termination and to test whether these factors play a significant role in the control of Ldc availability.

Expression of the plasmid-borne ldc is also compatible with the notion that ldc is regulated by a specific repressor which is present in limiting quantities and is thus subject to titration by additional copies of the ldc control region. Our attempt to test this possibility indeed suggested that a repressor capable of being titrated by excess copies of the ldc promoter region in trans is involved in regulating ldc expression (Fig. 6). The results indicated also that the inverted repeat sequence just ahead of the putative promoter(s) is not necessary for the derepression observed: this element
may serve to terminate transcription of the upstream accA gene, as suggested by Kikuchi et al. (1997). If the derepression indeed reflects repressor titration, the shorter promoter sequence must contain the repressor binding site. Transcription from the shortened Pldc was about 30–40% as frequent as that from the longer one but was not significantly derepressed by multiple copies of Pldc in trans. This observation could be explained in several ways. For example there may be two distinct promoters, one weak and immune to repression, the other stronger but repressed; there might be a single promoter, normally repressed, of which only a weaker, repressor-immune version remains in the shorter fragment; or repressor might bind more strongly to the short fragment, decreasing promoter activity and rendering competition by multiple cloned copies less effective. In any case the putative repressor remains to be identified. One candidate for the repressor gene is represented by a mutation, distant from the ldc locus, reported by Kikuchi et al. (1997) to result in a major increase in Ldc activity, although in view of the results presented here a role for this locus in mRNA degradation must be considered equally likely. More generally, the observation that cadaverine is produced by cells depleted of other polyamines (Hafner et al., 1979) may point the way to finding conditions in which ldc is derepressed.

It has been supposed, by analogy with other amino acid decarboxylases, that any second lysine decarboxylase in E. coli would be constitutively expressed. The possibility that ldc is subject to regulation by a specific repressor and the lack of detectable lysine decarboxylase in the Δldc cad double mutants suggest that a constitutive lysine decarboxylase might not exist in this organism.

NOTE ADDED IN PROOF


ACKNOWLEDGEMENTS

We acknowledge the contribution of Violette Morales, who participated in the initial screening for mini-F destabilizing clones. We also thank David Watson, Josette Patte, Joachim Frey and Jean-Pierre Bouché for strains, and Claudine Rouquette for valuable technical assistance. This work was supported by grants from l’Action Concertée Coordonnée-Sciences du Vivant 1, La Région Midi-Pyrénées (nos 9300294 and 9407531) and l’Association pour la Recherche sur le Cancer (no. 2400). M.L. was supported by studentships from the Ministère de l’Education Nationale de l’Enseignement Supérieur et de la Recherche and the Association pour la Recherche sur le Cancer.
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Received 30 July 1997; revised 26 September 1997; accepted 31 October 1997.