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 & Bennett, 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
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**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 thermostable, in contrast to the thermostability of the inducible enzyme. Wertheimer & 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 (Igarishi 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*::MudXKm 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 BstI and BstWI (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 pKT254-Ω-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::TnlO from a strain provided by C. Turlan (CNRS, 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 λKSR5 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 ApaI fragment of pBAD18 (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, with thermostable DNA polymerase Pfu (Stratagene) and 25 cycles of 94 °C for 30 s, 56 °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 pMLM120 (Kan'). A derivative conferring trimethoprim resistance, pMLM145, was made by inserting a HimCL–Eco47III fragment from pGB2 (Churchward et al., 1984) containing the aadA gene into the Eco47III site of pMLM120.

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 pR5551 (Simons et al., 1987) to give pDAG123 and 124. The PCR fragments were cleaved with BstI 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 for 24-30 h at 0°C. For production of CadA, 100 ml F-MES medium supplemented with lysine (0.5%) was inoculated into a 100 ml screw-cap bottle and grown in L broth (Lennox, 1958) 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).

**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 NaN₃. 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 CH₂COONa pH 6.0, 1 mM EDTA, 0.1 M 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 80000 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 1 M NaCl in 0.05 M potassium phosphate pH 6.5 and transferred to 1.5 ml Eppendorf tubes. Samples were kept chilled throughout. After recentrifugation, the cells were resuspended in 200 µl 20 mM potassium phosphate at pH 5.6 or 7.6, depending on whether CadA or Ldc was to be measured.}

**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>Bird et al. (1972)</td>
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<td>GNB8385K</td>
<td>MC4100 cadB::MudXKm</td>
<td>Auger et al. (1989) / Silhavy et al. (1984)</td>
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<td>JC7623</td>
<td>AB1157 recB2 recC22 sbcB15</td>
<td>J.-P. Bouche et al. (1971)</td>
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<td>CB0129 cadB Ldc::Omega; from JC7623 recombinant</td>
<td>This work</td>
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<td>ML9 cadB::MudXKm; from GNB8385K</td>
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<tr>
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<td>rep_gbci01, bla⁺; Kan' lacZYA</td>
<td>Simons et al. (1987)</td>
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<td>pMESIS11</td>
<td>ldc chromosomal fragment in pGB2 (see Fig. 1a)</td>
<td>S. Pichoff &amp; J.-P. Bouche et al. (1987)</td>
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<tr>
<td>pKT254Ω-Δp</td>
<td>rep_cad, bla⁺</td>
<td>Fellay et al. (1987)</td>
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<tr>
<td>pFUS2</td>
<td>rep_gbci01, kan' araC-para</td>
<td>This work</td>
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<tr>
<td>pMLM120</td>
<td>pFUS2::luc⁺</td>
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</tr>
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<td>pMLM145</td>
<td>pMLM120::aadA⁺</td>
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<td>pRSS51::luc⁺ promoter region (-46 to +105)</td>
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* CNRS, Toulouse.
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), and 754.09°C for 15 min, then mixed with 120 μl of a 10 mM solution) was added, and the mixtures were incubated at 40°C for 4 min before rechilling, then extracted with 1 ml toluene 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 (~0.9 μg protein) in Ldc buffer was added instead of CHCl₃-treated cells. Lysine decarboxylase activity was determined as the difference in A₅₉₀ 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 × A₅₉₀ 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 equal volume of ‘ethanol-phenol’ (Sugino et al., 1972) for extraction of RNA by the hot phenol method (Alba et al., 1981). RNA was quantified by absorption at 260 nm. Ten or twenty micrograms of total RNA were fractionated by

Fig. 1. E. coli ldc gene locus, and comparison of lysine decarboxylase sequences. (a) The cad and ldc loci. The MadX insertion allele of cadB used in this work is shown; it causes a CadA⁻ phenotype by polarity (Auger et al., 1989). The ldo locus is shown as the insert carried by pMES111. The restriction sites shown were used to construct the Δldc 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 D49445) with those of E. coli CadA (CAD: U00096), Salmonella typhimurium Ldc (STY: U37109) 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 study.
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 patch 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-budding site, was inserted downstream from the inducible arabinose promoter (araBAD) 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.
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Fig. 3. 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 (cad8::lacZ ΔldcC); ●, DLT814(pMLM145); ○, DLT814(pMLM145) without added arabinose. The dotted line shows a composite growth curve; all strains grew at the same rate.

plasmid. Taken together these results are strong evidence that ldc encodes a polypeptide with lysine decarboxylase activity.

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.

Fig. 4. Properties of Ldc and CadA from cells grown aerobically in F-HEPES and anaerobically in F-MES respectively. (a) pH profile of lysine decarboxylase activity: CadA in cell-free extract prepared from DLT813 (●), presented as percentage of maximum specific activity (61.9 units mg⁻¹); Ldc in cell-free extract from ML10(pMLM120 (○); 52.1 units mg⁻¹) and whole cells (△; 209 units per OD₆₀₀ unit). (b) Activity of lysine decarboxylase in cell-free extract as a function of temperature: symbols as above; maxima CadA 67.6 units mg⁻¹, Ldc 49.4 units mg⁻¹. The Ldc data have adjusted for thermosensitivity above 37 °C on the assumption that the effective enzyme concentration is midway between that added and that remaining after 15 min as determined in (c). (c) Inactivation of lysine decarboxylase by incubation for 15 min at the temperatures on the abscissa, prior to chilling and determination of lysine decarboxylase by the standard assay. Symbols as above; maxima CadA 66.3 units mg⁻¹, Ldc 41.3 units mg⁻¹.
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 OD, 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 idc gene dosage is indicated by + +. ND, Not determined.

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<th>cad</th>
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<th>F-MES</th>
<th>F-HEPES</th>
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<td></td>
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**Control of expression**

Inspection of the sequence upstream of idc 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 idc 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

Fig. 5. Detection of idc mRNA by Northern analysis. Total RNA was isolated from cells grown in minimal medium to OD, in the case of DLT814/pMLM145 the medium was supplemented with arabinose (0.4%) for the last hour (~ 0.9 generations) of growth. Twenty微克grams (10 µg) of each RNA preparation was subjected to formaldehyde-agarose gel electrophoresis, followed by transfer to membrane and hybridization with 32P-labelled idcC 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 idc is transcribed, we performed a Northern analysis of RNA extracted from cells of various strains growing exponentially in...
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).

ldc 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 lower-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
promoter, normally repressed, of which only a weaker, promoters, one weak and immune to repression, the deca
decarboxylases, that any second lysine decarboxylase in way to finding conditions in which
It has been supposed, by analogy with other amino acid competition by multiple cloned copies less effective. In other polyamines (Hafner et al. 1979) may point the
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 Aldc cad double mutants suggest that a constitutive lysine decarboxylase might not exist in this organism.

**NOTE ADDED IN PROOF**


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REFERENCES


