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# The Two Forms of Lysine Decarboxylase; Kinetics and Effect of Expression in Relation to Acid Tolerance Response in *E. coli*

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**ABSTRACT**: Lysine decarboxylase has gained importance recently due to its involvement in acid tolerance response in some pathogenic bacteria. Two forms of the enzyme exist. One, CadA, is part of an operon and is induced by changes in external pH. The other form, Ldc, is constitutively expressed. The differences between the two enzymes have not been understood fully. CadA has been studied more extensively whereas Ldc has not received much attention. The enzymes Ldc and CadA were purified individually using Ni-affinity chromatography from over expressing clones and it was found that their Km for lysine were 0.84mM and 0.27mM respectively. Their velocities, Vmax, were 27.21nmol cadaverine/min/µg of enzyme, and 8.148nmol cadaverine/min/µg of enzyme respectively. Epsilon aminocaproic acid (EACA), benserazide, carbidopa and voveran were added to the enzyme. Voveran inhibited only the inducible form of the enzyme. The assay of activity of each of these enzymes during growth of *E.coli* suggests that the two forms of the enzyme might be expressed at various phases of growth. This might be the probable reason for the presence of two enzymes which catalyse essentially the same reaction.

**Abbreviations:** CadA-inducible lysine decarboxylase, LdcC-constitutive lysine decarboxylase, SDS-PAGE- Sodium dodecyl sulphate Polyacryl amide gel electrophoresis, EACA- Epsilon amino caproic acid.

## Introduction

Lysine decarboxylase has been studied since the 1940s. It was used initially to assay the presence of lysine (Gale and Epps, 1940). The importance of lysine decarboxylase in acid tolerance response was discovered much later (Slonczewski *et al*., 1987). In bacteria and plants, two different genes code for lysine decarboxylase (Goldemberg, 1980; Kikuchi *et al*., 1998; Lemonnier and Lane, 1998). One, *cadA*, is part of an operon and is induced by changes in external pH, excess of lysine and low levels of oxygen (Neely and Olson, 1996). The cad locus is part of a "pathoadaptive" mutation which confers pathogenesis (Torres AG,2009). The cadA-mutated strains constructed show weaker tolerance to acidic conditions than the wild-type strain (Alvarez-Ordóñez A *et al*.,2008, Chattopadhyay MK et al., 2009). The other form, *ldc*, is constitutively expressed, and is independent of pH changes (Kikuchi *et al*., 1998; Lemonnier and Lane, 1998).

Vienozinskiene, *et al.,* (1985) reported the kinetics of lysine decarboxylase from *E. coli*. The kinetics of the enzyme from *Bacterium cadaveris,* was reported by Phan *et al*. (1982). In both these cases, the enzymes were only partially purified, and the sample was considered to contain only the inducible form. Thus, these reports have not considered possible differences in the kinetics between the constitutive and inducible forms. The constitutively expressed Ldc was partially purified and characterized by Lemmonier and Lane (1998), and they reported that the two forms differ in their thermostability. The kinetic parameters of the constitutive form have not been reported so far. This paper presents the kinetic parameters of the two forms of the enzyme purified individually from an over expressed clone, by affinity chromatography.

The importance of CadA in acid tolerance response is well known (Fritz G *et al*.,2009). The reason for existence of two forms of the enzyme for the same function has not received much attention. The role played by the constitutive form is unknown. It was intriguing to know if both forms of the enzyme need to be expressed. The effect of absence of a form of lysine decarboxylase on growth of *E. coli*, was therefore studied using knock-out strains. We obtained knock out strains of both CadA and Ldc from ASKA library (A Complete Set of *E. coli* ORF Archives, Genobase, Research and Education Centre for Genetic Information, Nara Institute for Science and Technology, Japan). In these, the expression of the chromosomal copy of the gene has been disrupted using a kanamycin resistance cassette. These strains were grown at acidic pH and the effect of expression of just one form of the enzyme on growth was studied.

## Materials and Methods

### Bacterial strains

*E.coli* K-12 W3110 strains with the vector pCA24N containing the inserts *cadA* or *ldcC* with Histidine tags, were procured from ASKA library (A Complete Set of *E. coli* ORF Archives, Genobase, Research and Education Centre for Genetic Information, Nara Institute for Science and Technology, Japan). The strains used are: a) *E.coli* BL21, expresses both *ldc* and *cadA* under the control of their natural promoters; b) *E.coli* Ldc, overexpresses the constitutive enzyme from a plasmid. The genome copies of both *ldc* and *cadA* are intact; c) *E.coli* LdcKO, expresses only the inducible form of the enzyme, the constitutive form of the enzyme is disrupted; d) *E.coli* CadA, overexpresses the inducible form of the enzyme. The genome copies of both *ldc* and *cadA* are intact; e) *E.coli* CadAKO, expresses only the constitutive enzyme, the inducible form is disrupted.

### Protein expression and purification

From an overnight grown culture of *E.coli* clone obtained from Genobase, a 100 ml culture was set up. The culture was induced after the O.D of the broth at 500 nm was 1, with 0.1 mM IPTG. The culture was grown after induction for 3 hours. The cells were sonicated in lysis buffer containing 50 mM Tris pH 8.5, 5 mM βME, 1 mM pyridoxal phosphate and 1 mM PMSF. Sonication was done for 20 minutes at power settings of 5. The undisrupted material and other particulate matter were removed by centrifugation at 16000 g (Heraeus Labofuge 400R, Germany). The supernatant was then loaded on a Ni-NTA Agarose column (Qiagen, USA) after equilibration with a buffer containing 50 mM Tris pH 8.5, 100 mM KCl, 5 mM̲βME, 10% glycerol and 20 mM imidazole. The column was then washed twice with the wash buffer (20 mM Tris pH 8.5, 1 mM KCl, 5 mM̲βME, 10% glycerol). The protein was eluted with elution buffer (20 mM Tris pH 8.5, 100 mM KCl, 5 mM̲βME, 10% glycerol and 100 mM imidazole), the fractions were checked on a SDS-PAGE gel and those with the protein were pooled and concentrated using A PM-10 concentrator (Amicon, Beverly, MA). The enzyme activity was assayed based on the method of Phan and Lenhoff, 1982

### Kinetics

10 µg/ml lysine decarboxylase in 0.5 M sodium acetate buffer pH 5.5 was mixed with 0.5 ml of varying concentrations of lysine (1mM to 10mM) in 0.5 M sodium acetate buffer pH 5.5 containing 1 nM pyridoxal phosphate. After incubating for 20 min at 37° C, the reaction was stopped by adding 1 ml of 1 M potassium carbonate. 1 ml of 1.02 X $10^{-2}$ M 2,4,6-trinitro benzene sulphonic acid was added to each sample and incubated for 5 min at 40° C. 2 ml of toluene was added and vortexed for 20 sec. The tubes were centrifuged at 2000 rpm for 5 min at room temperature. The absorbance of toluene layers was read at 340 nm against a blank prepared from

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the toluene extracts where potassium carbonate was added before the enzyme. The Michaelis-Menten constants were obtained from Line weaver Burk plots.

**Inhibition**

5 mM of each of the inhibitors (EACA, Carbidopa, Benserazide, Sigma-Aldrich, USA; Voveran, Novartis, India) were prepared in sterile distilled water. To study the reversibility of inhibition, the inhibitor was added to 10 µg of protein in sodium acetate buffer pH 5.5, and incubated for 1 day. One aliquot of this was stored at 4° C. Another was dialysed into 1000 volumes of sodium acetate buffer pH 5.5 for 24 hours (at which the enzyme is active). The aliquots were then assayed for the activity of lysine decarboxylase. If the inhibitor bound to the enzyme reversibly, it would be dialysed out into the buffer and the enzyme would regain its activity. If the enzyme did not regain its activity after dialysis, it may be an irreversible inhibitor. To study the effect of inhibitor on kinetics of the enzyme, the inhibitor was added along with the substrate during the biochemical assay.

**Thermostability**

The reaction mixture containing 5 mM lysine, 0.5 M sodium acetate buffer at pH 5.5, purified lysine decarboxylase and 1 nM pyridoxal phosphate, was incubated at 37, 50, 60, 70, 80 and 90° C. The specific activity was estimated at each temperature.

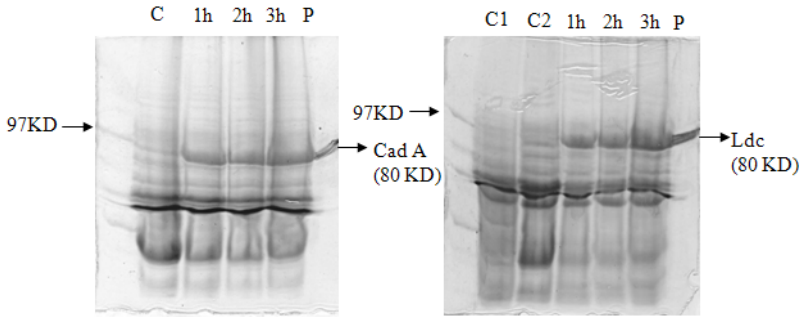
**Growth of *E. coli***

The growth curve of *E. coli* strains was drawn by checking the optical density of the bacterial culture at 500 nm. The bacteria were grown in minimal medium adjusted to various pH values and incubated at 37° C under anaerobic conditions. The organism was grown for a maximum of 10 hours for each experiment.

## Results

Amino acid decarboxylases are enzymes that are expressed in response to acid stress. Two forms of the enzymes exist in cells: one is constitutively expressed and this form does not respond to sudden changes in pH. The other is the inducible form, which is over expressed in response to pH changes. The fact that lysine decarboxylase is no exception to this generalisation was known only in 1980 (Goldemberg, 1980, Lemmonier and Lane, 1998). It was assumed till then that only the inducible form of lysine decarboxylase, namely CadA was present in nature. The experiments such as kinetics, have therefore only accounted for the inducible form of the enzyme. It is possible that the parameters were affected by the other form of the protein. We present here the biochemical characterization of the enzymes after purification individually. The purity of the proteins was checked on a SDS-PAGE (figure.1). The protein preparation used are assumed to be fairly homogenous since these were purified from an over expressing clone, using affinity chromatography.

Figure 1 The expression of *cadA* (a) and *ldc* (b) were checked after every hour of induction. It was observed that after 3 hours of induction, sufficient quantities of protein were expressed. 0.1mM IPTG was used in case of *ldc* and 0.05mM IPTG was used in case of *cadA*. Lanes C, C1 and C2 are uninduced controls and lanes 1h, 2h and 3h indicate the protein profile 1 hour, 2hour and 3 hours after induction respectively. Lane P shows the purified protein



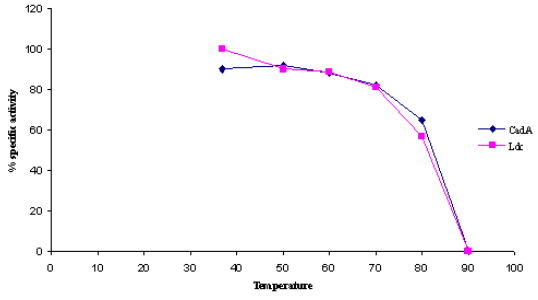

We also present the results of the effect of expression of one form of the enzyme on growth of *E. coli*. This was done to understand if the bacterium needs both forms of the enzyme. The crystallographic structure of the inducible form of the enzyme was published recently(Alexopoulos E, 2010) however the biochemical investigations of the two forms of lysine decarboxylase have not been done so far.

**Thermostability of CadA and Ldc**

Sabo *et al.*, 1974a, described the inducible form of lysine decarboxylase, CadA as a thermostable enzyme. Lemonnier and Lane, (1998), partially purified the consitutively expressed form, Ldc. The purification was based on size exclusion methods which might fail to distinguish between the two forms of the protein, since both have the same molecular weight. They have reported that Ldc retains less than 50% of its activity when incubated at temperatures higher than 60° C, though CadA retained 60% activity till about 80° C. However, their experiments cannot rule out the possibility of the existence of both the proteins in the reaction mixtures. The proteins used in the experiments described here were affinity purified after over expression from a clone, to ensure that only one form of the protein is present

Thus contrary to previous reports (Sabo *et al.*, 1974a; Vienozinskiene *et al.*, 1985; Lemonnier and Lane, 1998), Figure 2 indicates that both the inducible (CadA) and the constitutive (Ldc) forms of the enzyme may be described as thermostable. CadA shows 65% activity to a temperature of 80° C, while Ldc shows 56% activity at temperature of 80° C.

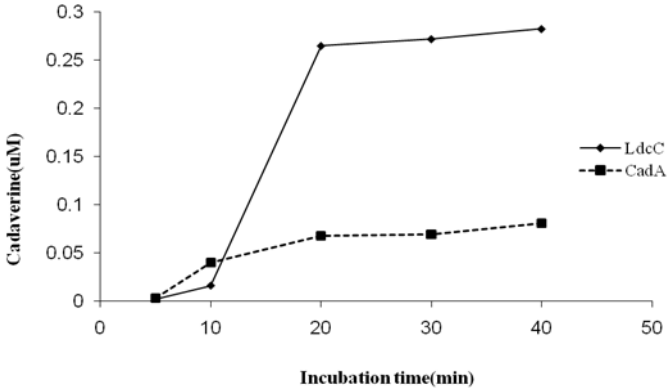
Figure 2 Thermostability of lysine decarboxylase. Both the constitutive and inducible forms of the enzyme are active up to 70°C



**Effect of incubation time on activity of CadA and Ldc**

Figure 3 shows that after 20 minutes of incubation, both forms of the enzyme have reached their peak activity levels. This is similar to the observation of Phan and Lenhoff (1982). They studied the kinetics of lysine decarboxylase from *Bacterium cadaveris*, using crude bacterial lysates as the source of enzyme, and reported that incubation for more than 20 minutes does not result in any significant increase in the product, cadaverine. Figure 3 also shows that the amount of cadaverine produced by Ldc is more than that by CadA.

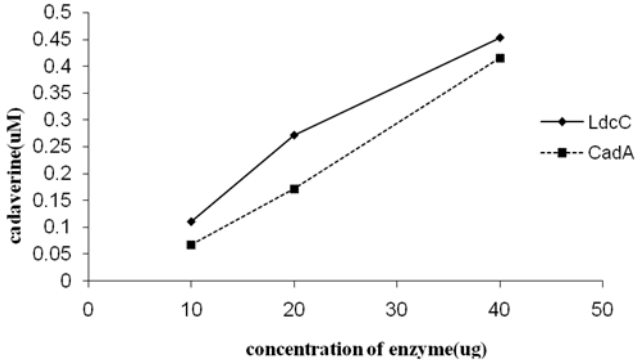
Figure 3 The effect of incubation time on activity of CadA and Ldc




**Effect of enzyme concentration on activity of CadA and Ldc**

When the substrate availability is not a limiting factor, formation of the product is directly dependent on the enzyme concentration. Figure 4 shows that increase in concentration of both CadA and Ldc results in an increase in product formation. Again the figure shows that Ldc produces more cadaverine than CadA.

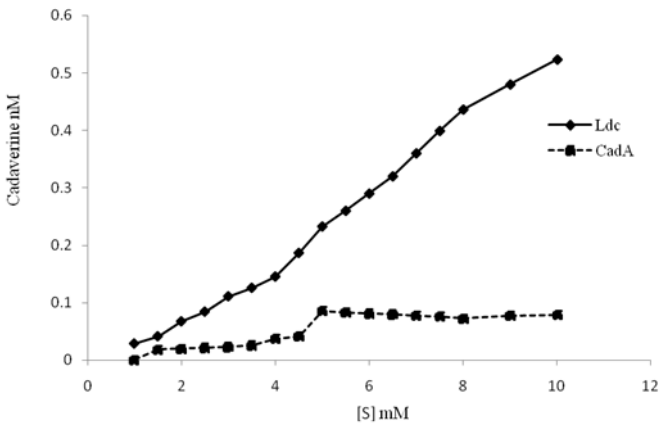
Figure 4 Effect of varying enzyme concentration on activity of lysine decarboxylase. Increase in enzyme concentration results in increase of product formation




**Effect of substrate concentration on activity of CadA and Ldc**

Figure 5 shows the effect of substrate concentration on the activity of the two enzymes. As mentioned earlier, the activity was assayed by the amount of cadaverine released. This cadaverine-based assay is reportedly more reliable than the pH-stat technique that measures changes in the pH (Phan and Lenhoff, 1982). This is because the presence of cadaverine is only possible as a consequence of lysine decarboxylase activity, whereas changes in the pH of the reaction mixture could be brought about by other metabolic pathways (Booth, 1985). Figure 5 shows that CadA is inhibited at lysine concentration higher than 5 mM, while Ldc remained active even at concentrations of substrate as high as 10 mM.

Figure 5 Effect of substrate concentration on activity of lysine decarboxylase. Ldc does not show substrate inhibition upto 10 mM Lysine whereas CadA is inhibited by 6 mM lysine



**Native enzyme kinetics of CadA and Ldc**

The kinetics of an enzyme is expressed by two parameters - the velocity of the reaction $V_{max}$, and the affinity towards the substrate $K_m$. The substrate concentration for calculating kinetic parameters was 5mM, so that both proteins could be compared. It can be recalled from figure 5 that substrate concentrations are tolerated differently by the two forms. Table 1 gives the values of these two parameters for CadA as well as for Ldc. These values were calculated from Line Weaver Burk plots.

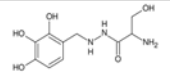
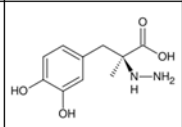
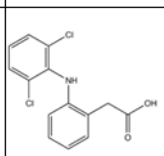
Table 1 The values of kinetic parameters $K_m$ and $V_{max}$ obtained in the present studies as compared to values reported earlier

| Enzyme | Source of data | Assay method | $K_m$ (mM) | $V_{max}$ (nmol product / min / µg of enzyme) |
|---|---|---|---|---|
| Ldc | Present study | Based on cadaverine estimation | 0.8 | 27.2 |
| CadA | Present study | Based on cadaverine estimation | 0.3 | 8.1 |
| Lysine decarboxylase (partially purified) of *Bacterium cadaveris* | Phan and Lenhoff, 1982 | Based on cadaverine estimation | 1.8 | 1.5 |
| Lysine decarboxylase (partially purified) from *E.coli* | Vienozinskiene *et al.*, 1985 | Based on change in pH of the reaction mixture | 2 | 1.5 |
| Equimolar mixture of CadA and Ldc | Present study | Based on cadaverine estimation | 1.4 | 2.9 |

**Kinetics of Inhibition of CadA and Ldc**

As discussed already, lysine decarboxylase is known to perform crucial roles in acid tolerance response in a number of organisms (Merrell and Camilli, 1999; Foster and Hall, 1990; Portillo *et al.*, 1993; Benjamin and Datta, 1995; Lin *et al.*, 1995). The absence of lysine decarboxylase from the animal kingdom (Boeker and Snell, 1972; Sabo *et al.*, 1974a) makes it an attractive drug target. In fact, lysine decarboxylase inhibitors have been shown to be effective as drugs in the case of periodontal diseases (Levine *et al.*, 2001; United States patent no. 6187296, 2001). Here we study the influence four different inhibitors (Table. 2) have on the kinetics of lysine decarboxylase. Benserazide has been reported to be an inhibitor of enzymes that carry out decarboxylation (Da Prada *et al.*, 1987). Cardidopa is an amino acid decarboxylase inhibitor (Da Prada *et al.*, 1987). Epsilon amino caproic acid has been reported to be a competitive inhibitor of lysine decarboxylase (Sabo *et al.*, 1974a and b). Anti-inflammatory drugs have also been reported to inhibit lysine decarboxylase (Bruni *et al.*,1984).

Table 2 Inhibitors of lysine decarboxylase used

| S.No | Inhibitor | Chemical structure | IUPAC names | Molecular weight (g/mol) | Information available |
|---|---|---|---|---|---|
| 1 | Epsilon amino caproic acid |  | 6-aminohexanoic acid | 131.173 | a reported competitive inhibitor (Sabo *et al.*, 1974a) |
| 2 | Benserazide |  | 2-amino-3-hydroxy-N'-[(2,3,4-trihydroxyphenyl) methyl]propane hydrazide | 293.71 | a general amino acid decarboxylase inhibitor (Da Prada *et al.*, 1987) |
| 3 | Carbidopa |  | (2*S*)-3-(3,4-dihydroxyphenyl) -2-hydrazinyl-2-methyl-propanoic acid | 226.229 | general decarboxylase inhibitor (Durso *et al.*, 2000) |
| 4 | Voveran (diclofenac) |  | 2-[2-(2,6-dichlorophenyl) aminophenyl] ethanoic acid | 296.148 | anti-inflammatory drug (reported to be an inhibitor). (Bruni *et al.*, 1984) |

The optimum substrate concentration for activity of lysine decarboxylase is 5 mM. Therefore 5 mM of each of the 4 inhibitors was used in the assay. The parameters $K_m'$ and $V_{max}'$ in the presence of the inhibitors was determined as explained in the Methods section. The values obtained are given in Table 3.

Table 3 The kinetic parameters $K_m$ and $V_{max}$ of Ldc and CadA estimated in the presence and absence of inhibitors are summarised in this table

| S.No | Inhibitor | Enzyme | $K_I$ | $K_m'$ (mM) | $V_{max}'$ (nmol/μg/min) | Inhibition mode |
|---|---|---|---|---|---|---|
| 1 | EACA | CadA | 23.0 | 0.3 | 6.8 | Non-competitive, irreversible |
| | | LdcC | 0.7 | 6.8 | 4.8 | Mixed, irreversible |
| 2 | Benserazide | CadA | 1.0 | 1.6 | 1.9 | Mixed, irreversible |
| | | LdcC | 4.5 | 1.6 | 15.1 | Mixed. Irreversible |
| 3 | Carbidopa | CadA | 15.0 | 0.3 | 6.2 | Non-competitive, reversible |
| | | LdcC | 3.5 | 2 | 1.3 | Mixed, reversible |
| 4 | Voveran | CadA | 2.0 | | 2.9 | Mixed, irreversible |
| | | LdcC | | | | No inhibition |
| 5 | No inhibitor | CadA | | 0.3 | 8.1 | Positive control |
| | | LdcC | | 0.8 | 27.2 | Positive control |

***Epsilon amino caproic acid (EACA)***

This compound has been reported to be a competitive inhibitor of lysine decarboxylase (Sabo *et al.*, 1974a). The compound is widely used as an antifibrinolytic agent. This activity is achieved by the ability of EACA to inhibit streptokinase (Nilsson, 1980). It is also used as a protease inhibitor (Burden *et al.*, 1979). EACA has also been reported to inhibit various proteins in the complement pathway (Vallota, 1978). The $K_m'$ values, the Dixon plot and the Lineweaver-Burk plots (Tables 3, Figures 6a and b) for this inhibitor show that it is not a competitive inhibitor. The inhibition of CadA is by the non-competitive mode. In the case of Ldc, it is a mixed inhibitor. This is contrary to what has been reported by Sabo *et al.*, (1974). The velocity of the inhibited enzyme is less than that of the native enzyme. But the extent of inhibition in Ldc is more pronounced than in CadA. In the case of CadA, the velocity is 79% as compared to the uninhibited enzyme (8.1 as against 6.8 nmol/μg/min). The velocity of Ldc is reduced to 17% (from 27.2 to 4.8 nmol/μg/min). Thus, this inhibitor is more effective in inhibiting Ldc than CadA. The inhibition is irreversible, since dialysis of the reaction mixture for 24 hours against sodium acetate buffer pH 5.5 did not reverse the inhibition.

Figure 6a Dixon plots showing the Ki values obtained upon inhibition of Ldc and CadA by 5mM Epsilon amino caproic acid (EACA)

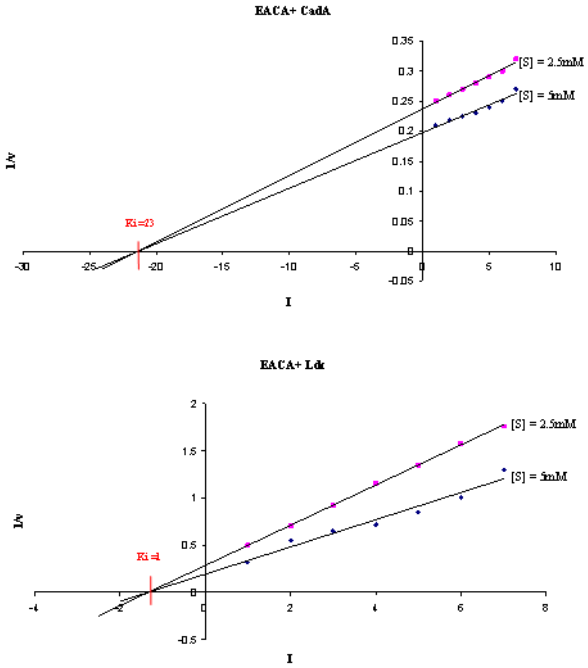
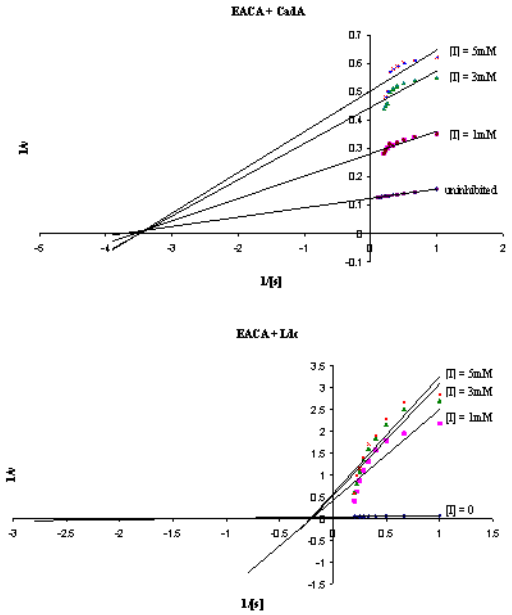


Figure 6b Lineweaver Burk plot showing that EACA is a non-competitive inhibitor of both CadA and mixed type inhibitor of Ldc




***Benserazide***

Benserazide is known to inhibit decarboxylase enzymes in general, though the mode by which it inhibits lysine decarboxylase has not been reported. It has been reported to be the most potent drug for inhibiting amino acid decarboxylases. (Da Prada *et al.*, 1987).

The present results (Table 3, Figures 7a and b) show that the mode of inhibition by Benserazide is the same in the case of both CadA andLdc.

Figure 7a Dixon plots showing the Ki values obtained upon inhibition of Ldc and CadA by 5mM Benserazide

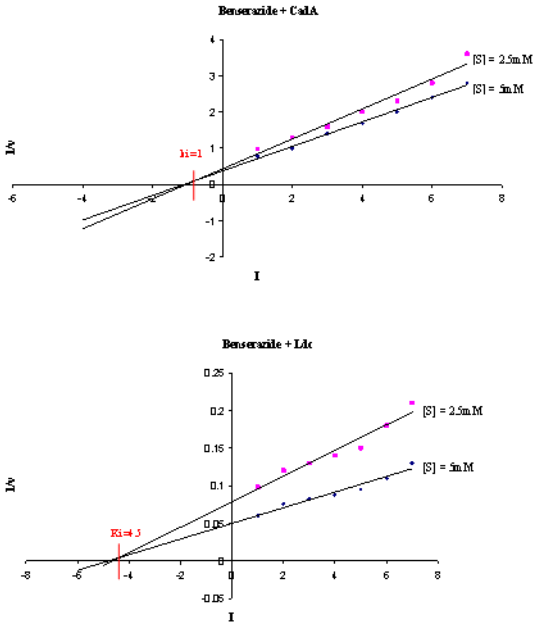
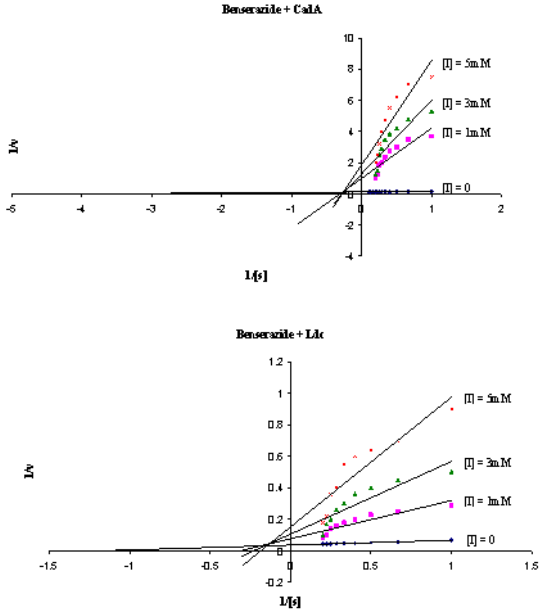


Figure 7b Lineweaver Burk plot showing that benserazide is a mixed type inhibitor of both CadA and Ldc




***Carbidopa***

Carbidopa has been reported to be a competitive inhibitor of aromatic l-amino acid decarboxylases. It is similar to benserazide in its clinical applications, though Benserazide is a more potent inhibitor than Carbidopa (Durso *et al.*, 2000). Table 3, and Figures 8a and b show that this inhibitor is very similar to EACA, both in its mode of inhibition and its effect on Ldc. Inhibition by Carbidopa can be completely reversed by dialysis for 10 hours against the buffer containing all other components in the reaction mixture except the inhibitor

Figure 8a Dixon plots showing the Ki values obtained upon inhibition of Ldc and CadA by 5mM Benserazide

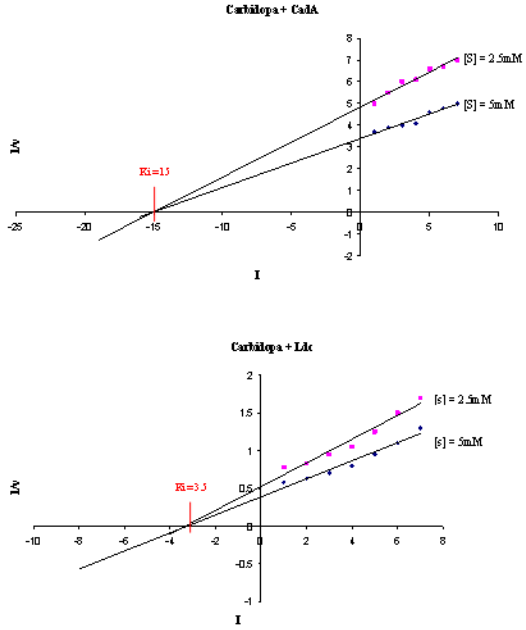
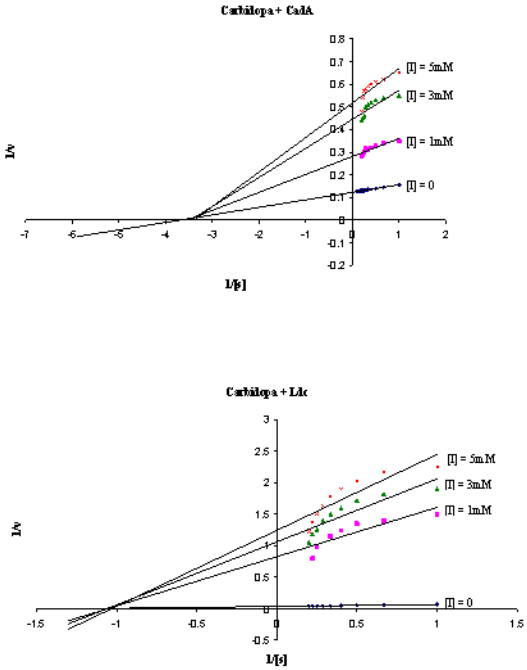


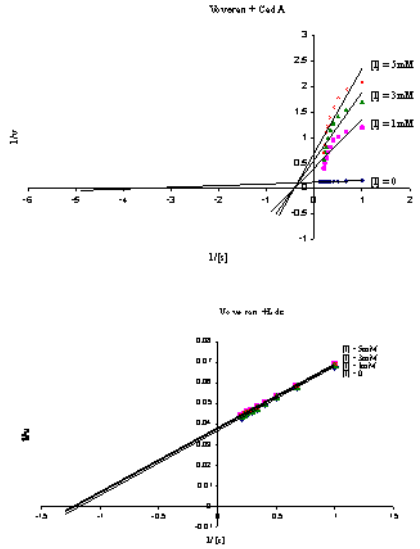
Figure 8b Lineweaver Burk plot showing that Carbidopa is a non-competitive inhibitor of CadA and it inhibits Ldc as a mixed inhibitor




***Voveran***

It has been reported that anti-inflammatory drugs inhibit amino acid decarboxylases (Bruni *et al.*, 1984). Voveran is the trade name of diclofenac. Diclofenac is a competitive inhibitor of cyclooxygenase enzyme (Chen *et al.*, 2002). As may be seen from Table 3, and Figure 9 Voveran inhibits only CadA, and not Ldc.

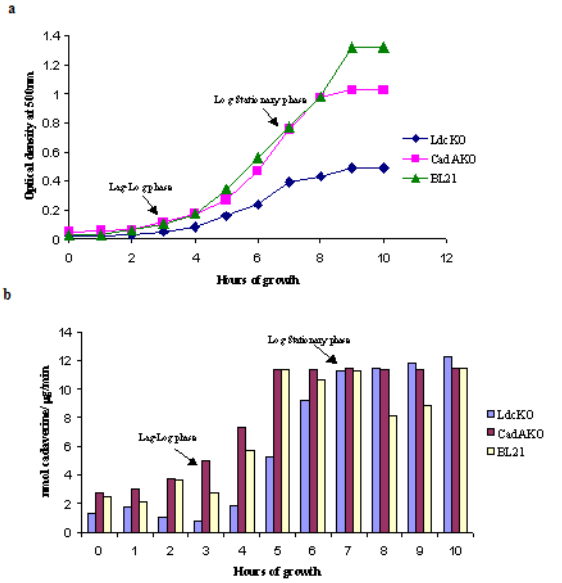
Figure 9 Lineweaver Burk plot showing that Voveran inhibits CadA as through Mixed mode. The constitutive form of the enzyme was not inhibited by voveran and it can be seent from the graph that the kinetic parameters do not change




**Growth curve of *E. coli* strains in minimal medium pH 5.5**

The *E. coli* strains BL21, LdcKO and CadAKO were grown in the minimal medium at pH 5.5 for 10 hours. Figure 10a shows the growth curve obtained.

Figure 10 a) Growth curve of three strains of *E.coli* grown for 10 hours in minimal medium pH 5.5. The absence of the gene encoding even one form of lysine decarboxylase results in retardation of growth. The knock out strain lacking the constitutive form of lysine decarboxylase grows more slowly than the other two strains. b) Activity of lysine decarboxylase taken from *E. coli* grown at minimal medium pH 5.5 for 10 hours at hourly intervals. The amount of cadaverine produced increases as the bacteria enter the stationary phase (after 7 hours of growth) in the case of LdcKO. The amount of cadaverine released in the case of CadAKO increases during the log phase, but reaches a plateau during stationary phase. In the case of BL21, both the mid log phase and stationary phase show increase in cadaverine released, since it has both forms of the enzyme active




It is known that amino acid decarboxylases are expressed to levels that contribute to 2% of the total protein in the cell (Shi *et al.*, 1996). In *Salmonella typhimurium*, it has been reported that *cadA* is expressed to high levels in the stationary phase as a response to acidification (Foster and Hall, 1991). In order to understand if the reason for the slowing down of the rate of growth of LdcKO strain could be connected to the expression of *cadA*, lysine decarboxylase activity was measured for these strains as they grew.

**Acid tolerance response and Acid stress response**

Two responses have been described in acidic pH management in bacteria. One is the stress response and the other is the adaptive tolerance response. Acid stress response is triggered when an organism is suddenly exposed to acidic pH, after being grown at neutral pH. In tolerance response the cells are gradually adapted to increasingly acidic pH values and therefore the proteins expressed in this case are different from those expressed in stress response (Foster and Hall, 1990; Merrell and Camilli, 1999; Warnecke and Gill, 2005). In *Vibrio cholerae*, lysine decarboxylase has been shown to be involved in stress response as well as adaptive tolerance response.

**Adaptive acid tolerance**

*E. coli* BL21 was grown in pH 7.0 over night and used as innoculum. The bacteria grown at pH 7.0 were first transferred to minimal medium pH 6.5 and grown for 11 hours at 37° C under anaerobic conditions. Aliquots of this culture were taken at three phases, namely, 0 hour (this is the aliquot taken immediately after transferring the bacterial innoculum), mid log phase (this was taken after 4 hours of growth) and stationary phase (taken after 10 hours of growth), for measuring the optical density and for assay of activity of lysine decarboxylase. An aliquot from the mid log phase (i.e., after growing the culture for 3 hours) was inoculated into minimal medium pH 5.5 under sterile conditions and the culture grown for 11 hours at 37° C under anaerobic conditions. Thus the bacteria were gradually adapted to increasingly acidic pH values.

**Acid stress**

*cadA* is produced in response to acid stress in case of *Vibrio cholerae* (Merrell and Camilli, 1999). In this organism, cells transferred to pH 4.5 from pH 7.5 fail to survive when *cadA* is knocked out. To understand the response of *E.coli* BL21 to acid stress, the bacteria were initially grown in minimal medium pH 7.0 at 37° C under anaerobic conditions overnight. This culture was used to inoculate minimal media adjusted to pH 7.0 and pH 4.5. The cultures were incubated for 10 hours at 37° C under anaerobic conditions. Growth of bacteria was measured in terms of optical density at 500 nm. Aliquots of the culture were taken at mid log phase of growth (after 4 hours of growth) and at stationary phase (after 10 hours), for measuring growth and for assay of activity of lysine decarboxylase

## Discussion

There are reports in the literature that pairs of isoenzymes show differences in their kinetic parameters and efficiency. For example, in the case of isoenzymes of human aldehyde dehydrogenase it has been found that enzymes located in the liver are more efficient than those found in the erythrocytes and placental tissue. Also these isoenzymes are reported to interact by different modes with inhibitors (Zorzano and Herrera, 1990). It is therefore not surprising to find that CadA and Ldc, which are expressed from genes located in loci situated far from each other, show differences in the kinetic characteristics. Bioinformatics studies done on the two forms of the enzyme suggest that they might have evolved independently. (Data not shown).

The biochemical characterization of the two forms of lysine decarboxylase is presented here. Contradictory to previous experiments it was observed that both the enzymes were thermostable. (Figure.3).

**Effect of incubation time on activity of the enzymes**

It was found that LdcC produced more cadaverine than CadA. (Figure.4). Amino acid decarboxylases are synthesized to levels of 2% of the total protein of the cell, under conditions of low pH, high substrate availability and oxygen depletion (Shi *et al.*, 1993). Since the expression of the inducible form CadA responds to external pH stimuli (Slonczweski *et al.*, 1987; Watson *et al.*, 1992), it is assumed that this is produced in larger amounts than the constitutively expressed form Ldc. If intracellular CadA is present in greater quantities than Ldc, it may be expected that the amount of cadaverine produced by CadA is also larger. The above results are contrary to these expectations.

**The effect of substrate concentration on activity of lysine decarboxylase**

Vienozinskiene *et al.,* (1985) reported that lysine decarboxylase from *E. coli* did not show substrate inhibition i.e. an increase in the lysine concentration did not inhibit the decarboxylase activity of the enzyme. It was found to be active even at substrate concentrations as high as 25 mM. The enzyme from *Bacterium cadaveris* however, was inhibited by 6 mM lysine (Phan and Lenhoff, 1982). It is again noteworthy to mention that these experiments did not use individually purified forms of lysine decarboxylase.

**Native enzyme kinetics of CadA and Ldc**

Table 1 shows the values of kinetic parameters. The table also shows parameters measured by Phan and Lenhoff (1982) and by Vienozinskiene *et al.*, (1985). The last row of the table gives the values for these parameters estimated using an equimolar mixture of purified CadA and Ldc. These results indicate the following. a) CadA binds more strongly to lysine than Ldc. b) However; the velocity of the reaction is greater for Ldc than for CadA. Therefore, the inducible enzyme is more efficient in recognising and binding to the substrate than the constitutive enzyme; the situation is the other way around for the reaction rate. The values of $K_m$ and $V_{max}$ reported for lysine decarboxylase by other workers (Phan and Lenhoff, 1982; Vienozinskiene *et al.*, 1985) indicate that the partially purified form of the enzyme (or possibly a mixture of the two forms of the enzyme) is less efficient than each of the purified enzymes acting alone. The possibility that the enzyme used by other workers in their assays is in fact a mixture of the two forms is supported by the fact that an equimolar mixture of the two purified forms produced

parameter values close to those reported by the others (Table 1). These results also indicate that the two forms of the enzyme may antagonise each other. Such behaviour has been noticed in the case of the fungus *Aspergillus japonicus*, where antagonism between two groups of enzymes, pectin esterase and pectin lyase has been observed (Semanova *et al.*, 2003).

To summarise the above, CadA binds to lysine with greater affinity than Ldc. However the velocity of Ldc is greater than CadA. These differences in the kinetic parameters help us understand the differences in the behaviour of the two forms of the enzyme in the experiments carried out in our laboratory (Figures 1, 2, 3 and 4). A hypothesis can be proposed based on this difference in kinetic parameters, to explain the possible biological roles of two forms of the enzymes and the probable reason behind having two forms of the enzyme that carry out the same reaction. The increased affinity of CadA for lysine suggests that the extracellular lysine could be scavenged as an immediate response and slowly converted to cadaverine, and released in order to regulate the pH. If the cell had only CadA, it would have bound to lysine with so much affinity that it would have been unavailable for cellular functions. On the other hand, if the constitutive form alone were present, the extracellular pH would have been altered too drastically, since the velocity of the enzyme is so high. The equimolar mixture has lower velocity and lesser affinity than the individual enzymes.

The proposed interactions between the two proteins are difficult to probe through gel electrophoresis experiments owing to the large native molecular weight of the two proteins (about 8,00,000 Daltons). The native enzyme is present as a decamer formed by 10 monomers each of 80 KDa. The three-dimensional structure of the two proteins might throw more light on their activity.

**Kinetics of Inhibition of CadA and Ldc**

***Epsilon amino caproic acid***

Tables 3, Figures 6a and b show the inhibition by EACA. It was observed that the inhibition was different for the two forms. In case of CadA it was a non competitive inhibitor and in the case of LdcC it was a mixed inhibitor.

***Benserazide***

Table 3, Figures 7a and b show that the mode of inhibition by Benserazide is the same in the case of both CadA and Ldc. However, the values of kinetic parameters (Table 3) suggest that benserazide inhibits CadA more effectively. The velocity of CadA is reduced to 22% (from 8.1 to 9 nmol/µg/min) of the native enzyme, whereas the velocity of Ldc is reduced by 55% (from 27.2 to 15.1 nmol/µg/min).

***Carbidopa***

Carbidopa has been reported to be a competitive inhibitor of aromatic l-amino acid decarboxylases. Table 3, and Figures 8a and b show that this inhibitor is very similar to EACA, both in its mode of inhibition and its effect on Ldc. It is interesting to note that the two compounds Carbidopa and Benserazide, which are used for in the same clinical applications (Durso *et al.*, 2000) interact with lysine decarboxylase by different modes.

*Voveran*

Table 3, Figure 9 shows that this drug distinguishes between the two forms of lysine decarboxylase more effectively than the other three inhibitors. As may be seen from Table 3, and Figure 9 Voveran inhibits only CadA, and not Ldc.

**Growth curve of *E. coli* strains in minimal medium pH 5.5**

Figure 10a shows the growth curve obtained. The figure shows that all three *E. coli* strains reach the log phase of growth after 3 hours. The stationary phase is reached after 8 hours of growth. The absence of the gene encoding even one form of lysine decarboxylase results in retardation of growth. The strain *E. coli* LdcKO that expresses only the inducible form of the enzyme grows more slowly compared to the other two strains. It is known that the inducible form *cadA* is expressed in response to acidic pH (Gale and Epps, 1944). It is therefore surprising to note that the strain in which the inducible form of the enzyme is knocked out (i.e., CadAKO) grows more rapidly when compared to the LdcKO strain.

Figure 10b shows that in the case of LdcKO the activity of lysine decarboxylase increases as the bacteria enter the stationary phase (after 7 hours of growth). The amount of cadaverine released in case of CadAKO increases during the log phase but reaches a plateau during stationary phase. This means that the inducible form of the enzyme is produced during the stationary phase. It is known (Goldemberg, 1980) that the constitutive form of the enzyme does not respond to changes in pH. It may therefore be possible that the increase in activity during the log phase in the CadAKO strain represents consumption of lysine in the medium. In case of BL21, it can be seen that both the mid log phase and stationary phase show increase in cadaverine released, since it has both forms of the enzyme active. Thus our experiments suggest that *cadA* is over expressed during the stationary phase of growth (Foster and Hall, 1991) of *E.coli.* It may be noted that the expression of *ldc* increases in the log phase and attains a plateau during the stationary phase. This indicates that expression of *ldc* may not be responding to external acidification as suggested by Goldemberg (1980).

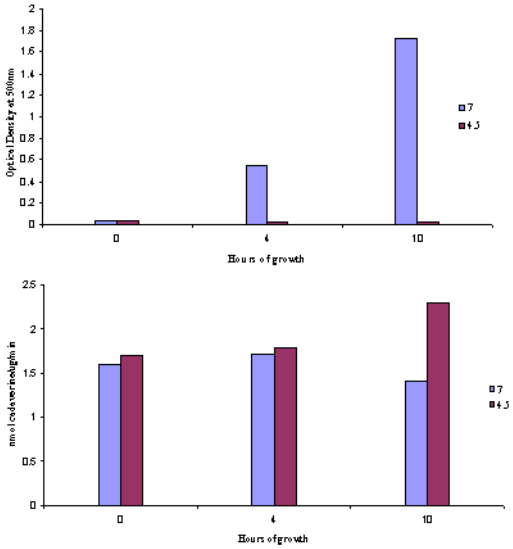
**Acid tolerance response and Acid stress response**

The deletion of *cadA* results in cell death (Merrell and Camilli, 1999). In *Salmonella typhimurium*, *cadA* is expressed in acid tolerance response (Foster and Hall, 1991). In *E.coli*, it is not known if *cadA* is expressed in response to acid stress or during acid tolerance.

***Adaptive acid tolerance***

Figure 11a shows the growth of *E. coli* cells adapted to acidic pH values. It may be observed that the bacteria thrive when grown in pH 7.0. The rate of growth becomes slower as the pH of the medium becomes more acidic. Figure 11b shows that activity of lysine decarboxylase begins to increase as soon as the cells are transferred to even media that are mildly acidic. The activity of lysine decarboxylase does not increase considerably at pH 7. Cells grown at pH 5.5 show an increase in the amount of cadaverine produced when compared to the cells grown at pH 7 and 6.5.

Figure11 a) Growth of *E.coli* BL21 under acid stress. It can be observed that there was no increase in the optical density of the culture when transferred from pH 7 to pH 4.5. b) Activity of lysine decarboxylase in *E.coli* BL21 subjected to acid stress

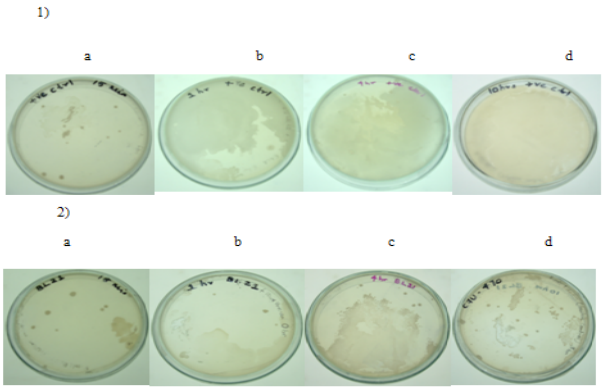



***Acid stress***

Figure 12a shows the growth in terms of optical density of *E. coli* BL21 subjected to acid stress. It may be observed that the bacteria grow rapidly at pH 7.0. The bacteria transferred to medium of pH 4.5 do not show any increase in optical density. Optical density is a measure of transmittance and absorbance of a given solution. The contribution of both live cells and dead cells to the optical density would be alike. Therefore lack of increase in optical density cannot be attributed to death of cells. The viability of cells grown under these stress conditions was therefore checked as follows. Aliquots of the culture were taken after 4 hours of growth and after 10 hours of growth. The culture was serially diluted and plated on LB agar medium. The number of colonies was counted (Figure 12). It was found that after 4 hours of growth, the number of colonies was 3000, whereas after 10 hours the number of colonies was only 500. It is therefore clear that the cells die when exposed to acid stress.

The activity of lysine decarboxylase was also measured in these cells. It was found that the activity increased. Figure 12b shows that the amount of cadaverine produced in cells grown at pH 4.5 after 10 hours of growth is slightly greater than after 4 hours. Moreover, as estimated from plating after serial dilution (Figure 12), the number of cells after 4 hours of growth is $3x10^{12}$, while the number of cells after 10 hours of growth is less, only $4x10^{11}$. The amount of cadaverine produced is therefore significantly larger after 10 hours of growth. The survival of the *E. coli* cells during the stationary phase of growth may therefore be due to production of cadaverine.

Figure 12 Viability of cells subjected to acid stress. Plates 1, a, b, c and d show the growth of *E. coli* BL21 after serial dilution after 15 min, 1 hour, 4 hours and 10 hours of growth respectively in minimal medium pH 7. Plates 2 a, b, c, and d show the growth of *E. coli* BL21 after serial dilution after 15 min, 1 hour, 4 hours and 10 hours of growth respectively in minimal medium pH 4.5




The growth studies suggest that both the forms of lysine decarboxylase are essential for the normal growth of *E. coli*. Deletion of even one of the enzymes retards the growth. It was also observed that the two forms of the enzyme might be expressed at different phases of growth in these cells. The significance of Ldc is evident from these experiments.

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