Video Article

An Assay for Measuring the Activity of *Escherichia coli* Inducible Lysine Decarboxyase

Usheer Kanjee, Walid A. Houry
Department of Biochemistry, University of Toronto

Correspondence to: Walid A. Houry at walid.houry@utoronto.ca

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Abstract

Escherichia coli is an enteric bacterium that is capable of growing over a wide range of pH values (pH 5 - 9)[1] and, incredibly, is able to survive extreme acid stresses including passage through the mammalian stomach where the pH can fall to as low as pH 1 - 2[2]. To enable such a broad range of acidic pH survival, *E. coli* possesses four different inducible amino acid decarboxylases that decarboxylate their substrate amino acids in a proton-dependent manner thus raising the internal pH. The decarboxylases include the glutamic acid decarboxylases GadA and GadB[3], the arginine decarboxylase AdiA[4], the lysine decarboxylase LdcI[5, 6] and the ornithine decarboxylase SpeF[7]. All of these enzymes utilize pyridoxal-5'-phospate as a co-factor[8] and function together with inner-membrane substrate-product antiporters that remove decarboxylation products to the external medium in exchange for fresh substrate[2]. In the case of LdcI, the lysine-cadaverine antiporter is called CadB. Recently, we determined the X-ray crystal structure of LdcI to 2.0 Å, and we discovered a novel small-molecule bound to LdcI the stringent response regulator guanosine 5'-diphosphate,3'-diphosphate (ppGpp) [14]. The stringent response occurs when exponentially growing cells experience nutrient deprivation or one of a number of other stresses[9]. As a result, cells produce ppGpp which leads to a signaling cascade culminating in the shift from exponential growth to stationary phase growth[10]. We have demonstrated that ppGpp is a specific inhibitor of LdcI [14]. Here we describe the lysine decarboxylase assay, modified from the assay developed by Phan et al.[11], that we have used to determine the activity of LdcI and the effect of pppGpp/ppGpp on that activity. The LdcI decarboxylation reaction removes the α-carboxy group of L-lysine and produces carbon dioxide and the polyamine cadaverine (1,5-diaminopentane)[5]. L-lysine and cadaverine can be reacted with 2,4,6-trinitrobenzensulfonic acid (TNBS) at high pH to generate N,N'-bistrinitrophenylcadaverine (TNP-cadaverine) and N,N′-bistrinitrophenyllysine (TNP-lysine), respectively[11]. The TNP-cadaverine can be separated from the TNP-lysine as the former is soluble in organic solvents such as toluene while the latter is not (See Figure 1). The linear range of the assay was determined empirically using purified cadaverine.

Protocol

1) Reagents and Equipment

1. First, prepare the following three solutions: 1 mL of Solution A which is made up of 8 mM L-lysine, 100 mM sodium 2-(N-Morpholino)ethanesulphonic acid (MES) pH 6.5, 0.2 mM nucleotide, where the nucleotide is either: guanosine diphosphate (GDP), guanosine triphosphate (GTP), guanosine 5'-diphosphate,3'-diphosphate (ppGpp), or guanosine 5'-triphosphate,3'-diphosphate (pppGpp), 0.1 mM pyridoxal 5'-phosphate (PLP), and 1 mM β-mercaptoethanol/ (β-ME). 1 mL of Solution B which consists of 100 mM sodium MES pH 6.5, 0.1 mM PLP, 1 mM β-ME, and 50 nM LdcI. LdcI was purified as described in Snider *et al*.[6] and Kanjee *et al*. [14].
   1 mL of Solution C which is identical to Solution B but does not contain LdcI.
2. Prepare 100 mL of the stop solution consisting of 1 M sodium carbonate (10.6 g/100 mL) and aliquot 50 µL into each well of a 96-well polystyrene plate using a multi-channel pipette. Add 30 µL of water to the plate and then cover. Note that the sum of the volume of water added and the volume of sample extracted during the enzyme reaction must equal 50 µL. In this case, 20 µL of enzyme reaction sample will be removed.
3. Prepare 5 mL of TNBS solution at 10 mM by diluting 294 µL of 5% (w/v) TNBS stock with 4706 µL water, wrap in aluminium foil and keep on ice.
4. Equilibrate the Eppendorf ThermoStat Plus at 37°C. The ThermoStat Plus has 24 wells in 6 columns. See Figure 2 for schematic. Label 1.5 mL Eppendorf tubes indicating the identity of the nucleotide that will be tested (one per column): GTP (column A), GDP (column B), ppGpp (column C), pppGpp (column D), no nucleotide (column E), and protein samples (column F).
5. Rack several boxes of 200 µL tips such that each alternate row is omitted giving a total of four rows of tips. This is necessary for the use of the multi-channel pipette with the ThermoStat Plus heatblock during the enzyme assay.
6. Equilibrate Digital Heatblock (VWR) at 42°C.
7. Place a 96-well 2.0 mL polypropylene plate on ice to cool.

2) LdcI Assay

1. Aliquot 50 µL of Solution A with the appropriate nucleotide to 1.5 mL Eppendorf tubes in each of the five columns of the Eppendorf ThermoStat.
2. Add 330 µL of Solution B to three tubes in column F and add 330 µL of Solution C (the no-protein control) to the final tube in column F.
3. Equilibrate the solutions at 37°C for five minutes. Tip: after 5 minutes, cut off the caps of the tubes in the center of the heating-block to prevent them from interfering with the multichannel pipette to be used next.
4. Using a 5-50 µL VWR multi-channel pipette, transfer 50 µL from the tubes in column F into each of the tubes in columns A-E. Start the timer as soon as the first tube is mixed.
5. At 2, 4, and 6 minutes remove 20 µL of sample using the multi-channel pipette and add to the stop solution.

6. Note: samples in stop solution can be covered in plastic wrap and frozen at -20°C for subsequent processing. The plates should be thawed at room temperature before reaction with TNBS.

3) TNBS Reaction and Color Development

1. Add 50 µL of 10 mM TNBS solution to the stop solution using the multi-channel pipette and then incubate at 42°C for 6 minutes. The solution will turn a dark yellow/orange color as the TNBS reacts with the lysine and the cadaverine.
2. After 6 minutes, cool the plate on ice to slow down the reaction.
3. Remove 100 µL of sample using a VWR 20-200 µL multi-channel pipette and place in the 2 mL deep well plate that was cooled on ice. Transfer ice-bucket to the fumehood.
4. Add 500 µL toluene to each well using a HandyStep (Brand) repeat pipettor and 12.5 mL pipette tip (Plastibrand).
5. Wipe away any excess toluene using a kimwipe and cover the plate with strips of packaging tape. Make sure to press down firmly to obtain a good seal. Cover the 96-well plate with a flat lid and then shake vigorously for 1 minute and 30 seconds.
6. Leave the solutions to settle for 5 minutes. The TNP-cadaverine is now in the upper toluene phase, while the TNP-lysine remains water soluble (see Figure 1).
7. Remove 200 µL of toluene using the 20-200 µL multi-channel pipette into a 96-well plate for reading. Note: make sure to check that each sample being removed is clear and does not have any of the bottom aqueous phase. Note: use barrier tips to prevent damage to multi-channel pipette by the toluene.
8. Read absorbance at 340 nm in a SpectraMax 340 plate reader.
9. To clean the quartz plate, rinse out the toluene with water and place the quartz plate in a large glass tray in the fume hood. Pour over 100 mL of a 7:3 mixture of 70% (v/v) nitric acid:95% (v/v) ethanol and cover the quartz plate with a second glass tray. Warning: the reaction is highly exothermic and sometimes explosive wear appropriate protection and ensure that the fume hood sash is set to the lower 6" level. After cooling, wash out nitric acid with water and then 95% ethanol.

4) Representative Results

1)Cadaverine Standard Curve (Figure 3)
The assay was performed as described but without any L-lysine or LdcI and instead various concentrations of cadaverine were used to empirically determine the linear range of the assay. The assay was linear to an OD_{340} of 0.25, corresponding to ~ 22 nmoles of cadaverine (Figure 3).

2)Activity of LdcI (Figure 4)
The activity of LdcI alone was determined to be 153.5 (± 18.1) nmoles cadaverine min-1 µg LdcI-1 at pH 6.5. The activity of LdcI is unaffected in the presence of 100 µM GTP or GDP but is strongly inhibited (>10-fold) in the presence of pppGpp and ppGpp (Figure 4).

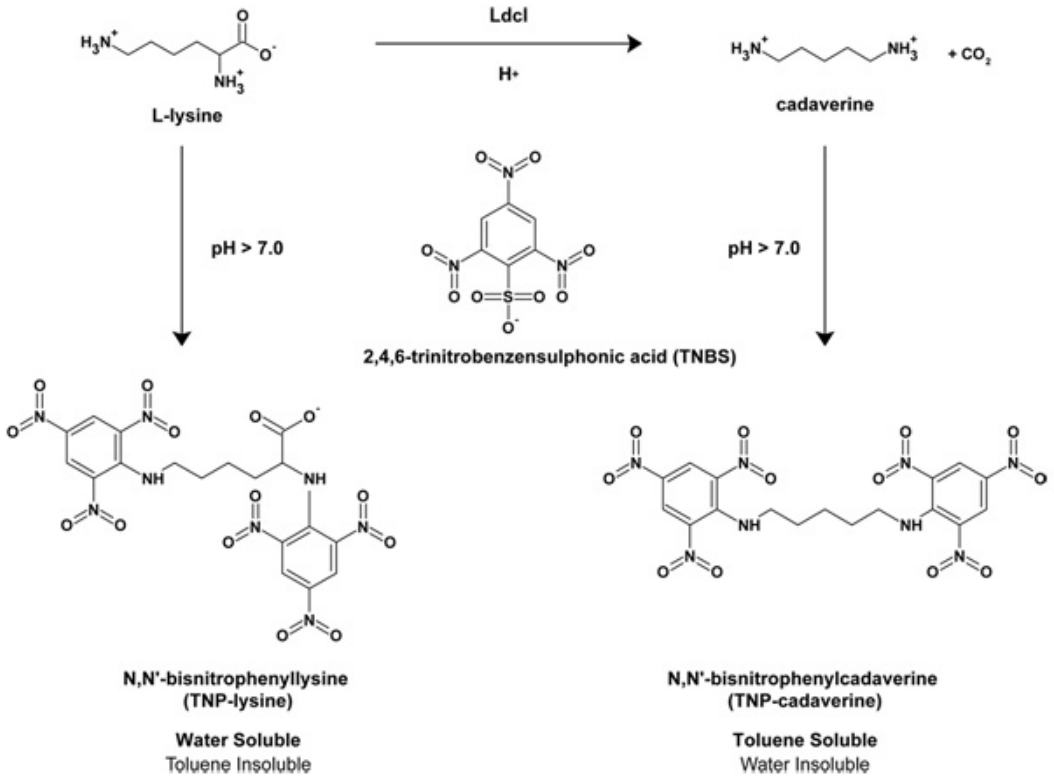

Figure 1. Schematic diagram of the LdcI reaction. The decarboxylation reaction of L-lysine to generate CO_2 and cadaverine is shown as well as the subsequent reaction with TNBS at high pH to generate N,N'-bistrinitrophenylcadaverine (TNP-cadaverine) and N,N'-bistrinitrophenyllysine (TNP-lysine). Based on Phan et al.11

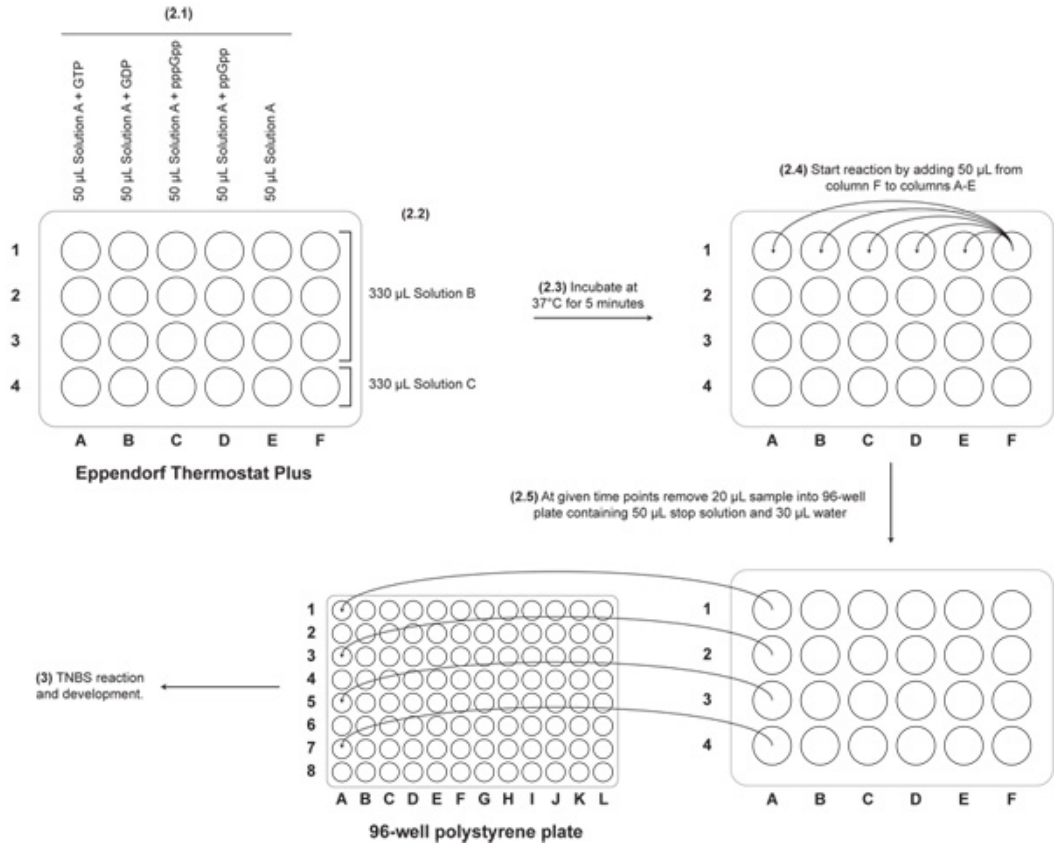

Figure 2. Setup of Eppendorf Thermostat. The layout of the samples in the 24-well Eppendorf ThermoStat is shown along with description of the steps of the assay (indicated in bold). Arrows indicate the transfer of reaction solutions according to the protocol. Removal of the reaction solution to the stop solution in the 96-well plate is also indicated.

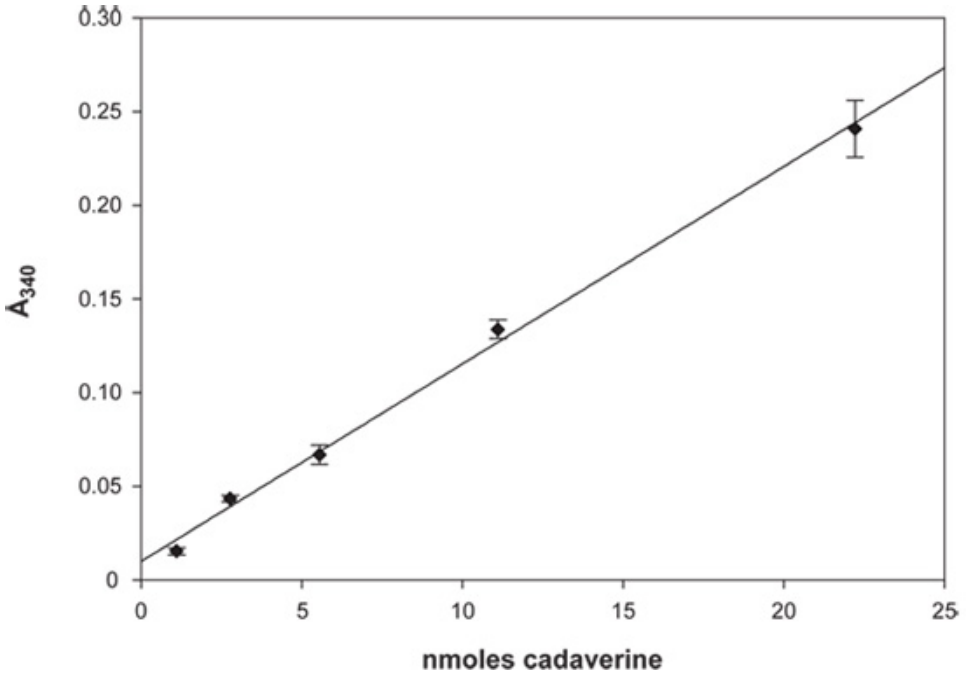

Figure 3. Cadaverine Standard Curve. A plot of absorbance at 340 nm versus nmoles of cadaverine is shown. Error bars represent the standard deviation of at least three independent measurements. The line of best fit is also shown and has an R2 of 0.996.

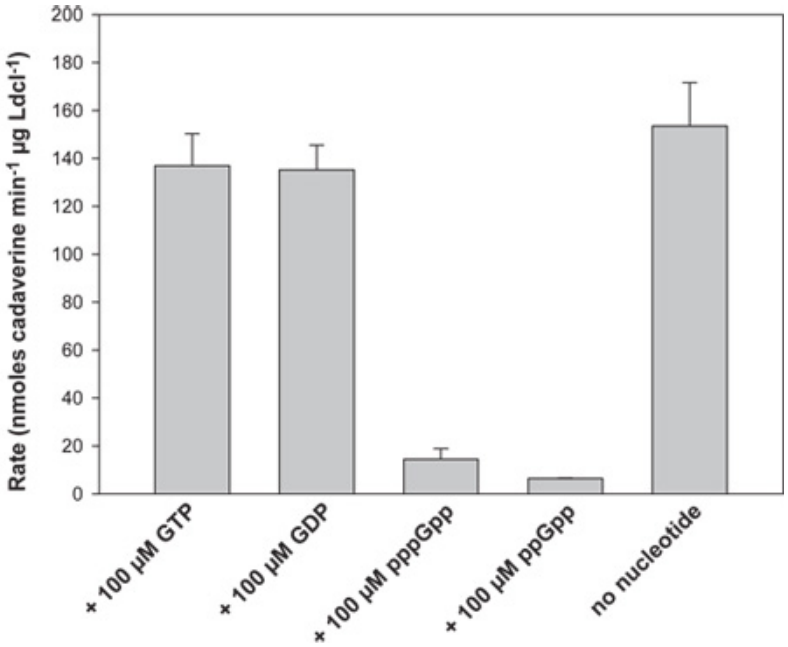

Figure 4. LdcI assay results. A plot of the rate of LdcI activity (in nmoles cadaverine produced min-1 µg-1 LdcI) is shown in the presence of 100 µM GTP, GDP, pppGpp, ppGpp and in the absence of nucleotide. The stringent response nucleotides (p)ppGpp are capable of significantly inhibiting LdcI activity. The error bars represent the standard deviation of at least six independent measurements.

Disclosures

No conflicts of interest declared.

Discussion

In the lysine decarboxylase assay, TNBS is reacted with the primary amines of L-lysine and cadaverine to form TNP-lysine and TNP-cadaverine adducts (Figure 1). Due to the presence of the carboxylic acid group on TNP-lysine, this adduct remains soluble in water while the TNP-cadaverine, lacking the carboxylic acid group, is capable of partitioning into toluene[11]. This type of assay can be utilized more broadly on other types of amino acids where the loss of a carboxylic acid group occurs during the reaction. This occurs during the decarboxylation of L-ornithine by the inducible ornithine decarboxylase SpeF to form the polyamine putrescine[7] and the decarboxylation of L-arginine by the inducible arginine decarboxylase AdiA to form the polyamine agmatine[4].

The LdcI assay described here provides a relatively fast method for the determination of the activity of the purified protein in vitro. The major advantages of this assay are:

i)Use of multiple replicates per experiment improves the precision of each measurement;

ii)The assay may be conducted over a wide range of buffer conditions (different pH, salt, reducing agent etc.) without modification of the protocol;

iii)The assay may be modified for measuring the *in vivo* activity of LdcI by determining the amount of cadaverine excreted during cell growth.

The major limitations of this assay are:

i)The sensitivity of the experiments are limited by the linear range of absorbance of the TNP-adducts;

ii)The multiple processing steps increase the magnitude of experimental errors;

iii)Not all amino acid decarboxylases are amenable to this type of protocol. For example, the decarboxylation of L-glutamic acid by the inducible glutamic acid decarboxylases GadA/GadB generates γ-amino-butyric acid[2], the TNBS-adduct of which will be soluble in water due to the presence of the side-chain carboxylic acid group.

The biochemical investigation of the acid stress response of *E. coli* is an expanding area of research and will allow us to better understand the molecular basis of stress response in *E. coli* and related γ-proteobacteria that have similar acid stress response systems such as *Salmonella enterica* serovar Typhimurium[12] and *Vibrio cholera*[13]. The discovery that LdcI activity is inhibited by the stringent response regulator ppGpp has provided us with a previously unknown insight into the regulation of this protein.

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