

## Research Article



## Cloning and expression of a D-alanine-D-alanine ligase gene from *Bacillus megaterium* in *Escherichia coli*

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Accepted on: 03-02-2014; Finalized on: 31-03-2014.

### ABSTRACT

PCR analysis of isolated DNA from *Bacillus megaterium* showed a 1kb amplification band upon using specific primers for D-alanine-D-alanine ligase enzyme gene. Consequently, the target fragment was sub-cloned in pET 29a (+) expression vector and under strong T7 bacteriophage inducible promoter. The recombinant plasmid pET 29a /ddl was introduced into BL21DE3pLysS *E. coli* strain. The engineered bacteria harboring the construct pET 29a (+) / ddl were cultivated. When the Expression of ddl gene was induced by 1 mM IPTG and analyzed on SDS-PAGE, a prominent band of approximately 40 kD was observed after induction which matched the predicted molecular weight of full-length product. However, no expression was observed in un-induced colonies. These results suggested that, the 40 KD most probably represents the expression of ddl gene. However, further investigations will be carried out in order to evaluate the interaction of the inhibitor (heptyl phosphinate) to the protein expressed to be a novel slow-binding inhibitor of the ligases from *Bacillus megaterium* to identify novel drug targets that are required to design new defenses against antibiotic resistant pathogens.

**Keywords:** Gen cloning, *B. megaterium*, ddl, *E. coli*.

### INTRODUCTION

D-Alanine:d-alanine ligase (ddl) is an essential enzyme that catalyses the ligation of d-Ala–d-Ala in the assembly of peptidoglycan precursors<sup>1</sup> and has been considered as an important antimicrobial drug target for years.<sup>2</sup> To date, nearly all the reported Ddl inhibitors are Ala analogues (e.g. d-cycloserine) or transition state analogues,<sup>3,4</sup> except one allosteric inhibitor binding to a hydrophobic pocket near the first d-Ala binding site of Ddl as revealed by its co-crystal structure with *St. aureus* Ddl.<sup>5</sup> and its homologue d-Ala-d-lactate ligase have been studied by X-ray crystallographic methods as these enzymes play key roles in the biosynthesis of the bacterial cell and are interesting targets for drug discovery. To date, the crystal structures of Ddls from *E. coli*,<sup>6</sup> *St. aureus*,<sup>5</sup> *T. caldophilus*<sup>7</sup> and *H. pylori*.<sup>1</sup> Those of d-Ala-d-lactate ligases from *L. mesenteroides*<sup>8</sup> and *Enterococci faecium*<sup>9</sup> have been reported in order to elucidate the structural and chemical bases of enzymatic catalysis and vancomycin resistance. These enzymes, which belong to the ATP-dependent carboxylate-amine/thiol ligase super family together with glutathione synthetase, biotin carboxylase and carbamoyl phosphate synthetase,<sup>10</sup> consist of N-terminal, central and C-terminal domains. ATP is bound to the interface between the central and C-terminal domains and the substrate d-Ala molecules are located at the intersection between the three domains. The central domain and the flexible loop have been suggested to move towards the C-terminal domain to close the active site based on structural comparison of Ddls.<sup>5,7</sup> Many efforts have been made to develop antibiotics which inhibit peptidoglycan

biosynthesis, as peptidoglycan polymers play a critical role in maintenance of the cell-wall structure.<sup>6</sup>  $\beta$ -Lactam antibiotics are covalently bound to the active site of the transpeptidase, resulting in inactivation of the enzyme, and have long been used for the treatment of infectious diseases.<sup>11</sup> However, methicillin-resistant *St. aureus*, which shows multi-drug resistance to  $\beta$ -lactam antibiotics, has emerged.<sup>12</sup> A glycopeptides antibiotic, vancomycin, was isolated and has been used as a drug of last resort. Vancomycin becomes hydrogen bonded to the terminal d-Ala-d-Ala of the growing peptidoglycan polymers, inhibiting the transpeptidation reaction and further cross-linking of the peptidoglycan polymers. However, vancomycin-resistant strains have also appeared. In these strains, d-Ala-d-lactate replaces the terminal d-Ala-d-Ala of the peptidoglycan polymers and prevents vancomycin from binding to the terminal peptide.<sup>13</sup> Understanding the molecular mechanisms of antimicrobial action is an important facet of developing new therapeutic strategies, particularly where drug resistance is a problem.<sup>14,15</sup> The present study, a gene encoding D-alanine-D-alanine ligase from *B. megaterium* was cloned, sequenced, and expressed in *E. coli*.

### MATERIALS AND METHODS

#### Strains, plasmids and media

*Bacillus megaterium* isolated from soil and identified was used as DNA donor and source of the gene encoding ddl enzyme strain.<sup>16</sup> *E. coli* DH5 $\alpha$  and BL21 (DE3) (Novagen, USA) were used as host cells for gene cloning and expression, respectively. The plasmid vectors used for



PCR product cloning and gene expression were pJET1.2/blunt Fermentas (Maryland, USA) pET-29a (+) (Novagen, Germany) (DNA sequencing (Macro Gen, Japan). *B. megaterium* was grown aerobically at 37°C in Lauria-Bertani medium (LB).<sup>17</sup> *E. coli* DH5 $\alpha$  and *E. coli* BL21 containing recombinant plasmids were cultured in LB medium supplemented with 50 mg/ml ampicillin.

### Chemicals and Primers design

All chemicals used were of reagent grade. Broad Range Protein Molecular Weight Markers and 1 kb DNA Ladder was purchased from Promega (Madison, USA). PCR Purification Kit, DNA Gel Extraction Kit, IPTG, Taq polymerase, T4 DNA ligase, pJET1.2/blunt cloning vector Kit and the restriction enzymes *Xho*I and *Xba*I were obtained from Fermentas (Maryland, USA) and were used according to the manufacturers' instructions. Based on the known gene sequence and homogenous analysis by BLASTN and the comparison of consensus sequence of *ddl* genes from 11 different bacteria two specific primers: *ddl*-F5'-GTGAAAATTAAGCTTGGCCT-3' and *ddl*-R5'-TTACATTGTGTGTTAATTTGCT-3' were designed and used for amplification of the genomic DNA encoding *ddl* gene.

### Cloning and expression of *ddl* gene

#### Genomic DNA isolation

Standard molecular cloning techniques were used throughout in this study.<sup>17,18</sup> The genomic DNA of *B. megaterium* was isolated by using Genomic DNA Purification Kit according to the manufacturer's instructions the quantity and quality of the nucleic acid was determined by measuring the absorbance at 260 nm and 280 nm. For DNA its  $A_{260} = 50 \mu\text{g/ml}$  the purity of the nucleic acid was determined by calculating the ratio of  $A_{260}/A_{280}$ .

#### D-alanine-D-alanine ligase (*ddl*) gene detection by PCR technique

Concentration of genomic DNA and plasmid DNA in PCRs was 100ng/ml. The DNA amplification was carried out in a thermal cycler. The PCR reaction (50  $\mu\text{l}$ ) contained 15  $\mu\text{l}$  of template DNA, 5  $\mu\text{l}$  of Taq polymerase reaction buffer reaction, 5  $\mu\text{l}$  of deoxynucleotide triphosphate (dNTP), 5  $\mu\text{l}$  of each primer, 5  $\mu\text{l}$  of 5U Taq DNA polymerase (Fermentus, Lithuania) and 10  $\mu\text{l}$  of sterile H<sub>2</sub>O. Template DNA was preheated at 94°C for 5 min. Then it was denatured at 94°C for 1 min, annealed to primers at 48°C for 1 min and extension of PCR products were achieved at 72°C for 3 min. The PCR was done for 35 cycles. Negative (without DNA template) and positive (with a standard template) controls were set for the primers used. An aliquot of the reaction mixture (5  $\mu\text{l}$ ) was analyzed by (1%) agarose gel electrophoresis, and the products were stained with ethidium bromide and visualized under UV light. DNA fragments were recovered from agarose gels using the DNA Gel Extraction Kit.

### DNA manipulations

DNA was extracted from *B. megaterium* as described by Sambrook *et al.*,<sup>17</sup>. Products of the PCR reaction were cloned into pJET 1.2/ blunt vector according to the manufacturer's instructions. The recovered PCR product with 3'-dA overhangs generated using Taq DNA polymerase was blunted in 5 min with a proprietary thermostable DNA blunting enzyme (included in the kit) prior to ligation. Self-ligation at room temperature (22°C) for 5 min using T4 DNA ligase was occurred.

### Preparation of competent cells and transformation

Competent *E. coli* DH5 $\alpha$  cells were made by the protocol of Hanahan<sup>19</sup> ligation products were transformed into *E. coli* DH5 $\alpha$ . The resulting transformants were plated on Lauria-Bertani (LB) agar plates including 50 mg/ml ampicillin. The ampicillin-resistant clones were selected by blue/white testing and isolated by using DNA Purification Kit according to the manufacturer's instructions.

### Sequence and phylogenetic analysis

Recombinant vectors were sequenced by (Macro Gen Japan). Alignments were performed by using the online Blastx search engine at the NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>).

### Construction of pET29a-*ddl* recombinant vector

pJET-*ddl* clone and pET-29a(+) vector, were subjected to restriction digestion with *Xho*I and *Xba*I restriction enzymes (Fermentase, USA) According to the manufacturer's instructions. Recovered insert DNA fragment was ligated into *Xho*I and *Xba*I cut pET-29a (+) to generate vector pET-29a (+)-*ddl*. Transformed *E. coli* BL21 (DE3) pLysS cells were cultured aerobically in LB plate's medium containing 50 $\mu\text{g/ml}$  kanamycin and incubated overnight at 37°C. Screening for positive colonies was confirmed using vector specific primers using the following PCR conditions: 2 min denaturation followed by 35 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 48°C, 2 min extension at 72°C, and a final extension at 72°C for 10 min. The nucleotide sequences of the used primers are as follow: pET14b5: 5'-GGAATCAAGCTTGAGC-3' and pET14b3: 5'-GGTATAGTTCCTCCTTCT-3'.

### Expression of *ddl* in *E. coli* BL21 (DE3) under T7- inducible promoter

Expression was performed as described previously with some modifications<sup>20</sup>. Briefly, the *E. coli* BL21 with DE3 containing the transformed pET-*ddl* vector was mass cultured in LB medium and was allowed to grow overnight at 37°C in a shaker incubator at 160 rpm. The following day, the cultured bacteria was inoculated into a 50 ml flask and incubated at 37°C in a shaker at 200 rpm. Cultures in logarithmic phase (O.D<sub>600</sub> of 0.6) were induced for 6 hr with 1mM (IPTG). After induction, cells were lysed in 5x sample buffer [100 mmol Tris HCl pH 8, 20% glycerol, 4% sodium dodecylsulfate (SDS), 2%  $\beta$ -mercapto-

ethanol and 0.2% bromophenol blue] and centrifuged for 15 min at room temperature. The supernatant was collected and analyzed with 12% SDS polyacrylamide gel electrophoresis.

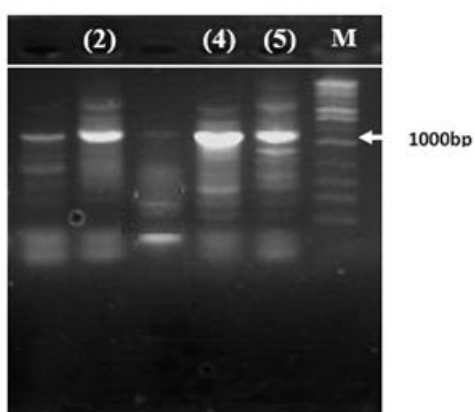
### SDS-polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed along with molecular size standard proteins (Broad Range Protein Molecular Weight Markers, Promega) in P8DS Electrophoresis Unit (Owl Scientific Inc., Woburn, USA). Gel having 12% acrylamide concentration was prepared according to Laemmli.<sup>21</sup> Coomassie brilliant blue R-250 was used for staining and the molecular weight of the polypeptide was determined using a calibration curve of the protein standards. After electrophoresis, the gel was incubated at 45 °C for 45 min in Tris–HCl buffer pH 7.0.

## RESULTS

### Cloning and sequence analysis of the *ddl* gene from *B. megaterium*

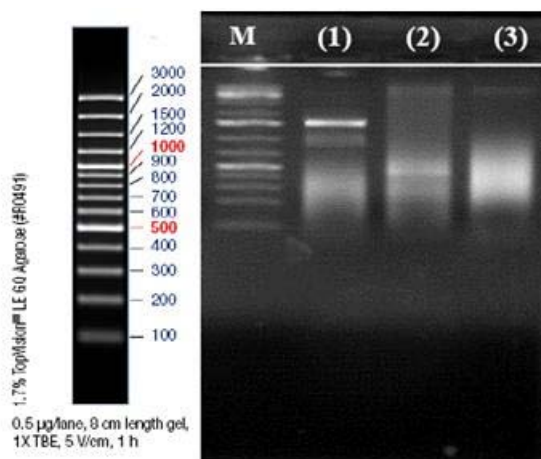
Genomic DNA was isolated from *B. megaterium*, a 1000 bp fragment was obtained as the major product of PCR amplification using primers *ddl*-F and *ddl*-R. Sequence alignment revealed that the amplified fragment shared 99 % identity with D-alanine-D-alanine ligase enzyme gene of different *B. megaterium* strains, DSM319, QM B1551 and WSH 002 and was therefore considered the actual *B. megaterium* *ddl* gene. The cloned gene was ligated at LacZ cassette/MCS of pJET1.2 vector (2.9 kb) which promote the blue/ white selection on Ampicillin plates. Confirmatory PCR was carried out by selecting the white or faint blue colonies for screening using plasmid specific pJET forward and reverse primers. Three colonies from the five screened colonies were found to be positive (harboring the construct pJET/*ddl*) and show amplification approximately around 1000 bp (Figure 1).



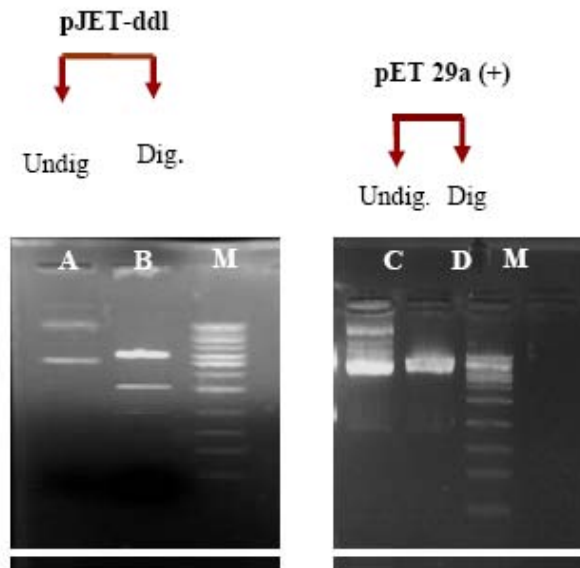
**Figure 1:** Cloning of PCR- Product fragment into pJET vector. The construct pJET/*ddl* was transformed into DH5 $\alpha$  competent cell. Confirmatory PCR with pJET1.2 specific forward and reverse primers was used to verify the cloning of *ddl* fragment. The colony screening was all positive except lane 3. Lane M 1 KB DNA ladder

The same positive three colonies were tested for insert orientation. When PCR was carried out using Forward

plasmid specific and *ddl*-F insert specific primers, It was revealed that one out of the three colonies contained insert in wrong direction (3' of fragment towards pJET forward primer) (Figure 2, lane 1), and the other two colonies in Right direction (5' of fragment towards pJET forward primer) (Figure 2, lane 2 & 3). According to pJET1.2/blunt and pET-29a (+) vectors cloning/ expression region the plasmids from wrong colonies were isolated with high purity. The undigested plasmid was run on 0.8% agarose gel in parallel to digested one (Figure 3). The digested fragments were cut from the gel and purified.



**Figure 2:** The positive colonies were tested for orientation using pJET1.2 forward plasmid specific primer and *ddl*-F specific primer. Lane 1 show gene insert in wrong orientation



**Figure 3:** gel showing restriction digests of pJET/*ddl* construct and pET 29a (+) plasmid by *Xho*I and *Xba*I. Lanes A and B represent pJET/*ddl* construct before and after restriction digestion. Lanes C and D pET29a before and after restriction digestion respectively

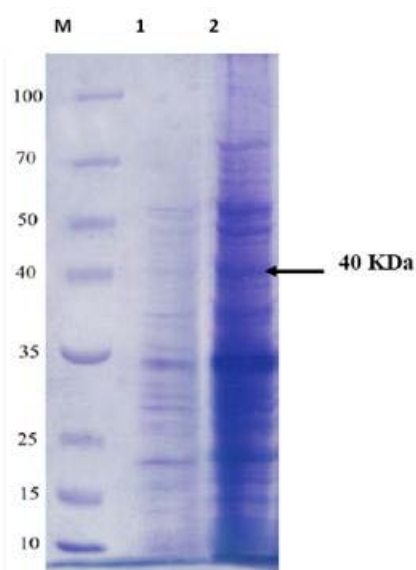
### Cloning of *ddl* fragment in pET 29a (+) His-tag expression system and transformation into *E. coli* BL21

The recovered *ddl* fragment was subcloned in pET29a (+) His-tag expression system at *Xho*I and *Xba*I sites and under strong control of T7 bacteriophage promoter in

which T4 ligase mediated ligation. The *E. coli* BL21 strain with DE3 (a  $\lambda$  prophage carrying the T7 RNA polymerase gene) was transformed with pET/ddl. Four out of seven colonies from blue/ white selection using X-gal-IPTG containing LB plates medium were found to contain ddl fragment when tested using pET14b5 and pET14b3 primers.

#### Testing the expression of ddl fragment in BL21 by SDS-PAGE

The recombinant protein was successfully produced in *E. coli* BL21 (DE3) pLysS at low temperature (37°C) by the induction with 1 mM IPTG under the control of T7 RNA polymerase promoter with 6 His-tagged. SDS-PAGE electrophoresis indicated the presence of only one major band with the molecular weight of 39 kDa when the gels were stained with Coomassie Blue (Figure 4).



**Figure 4:** SDS- PAGE of pET29-ddl colony. Lane (M): protein marker, lane (1); uninduced colony. Lane (2) induced colony with IPTG

#### DISCUSSION

According to the 2004 World Health Organization Report ([www.who.int/whr/2004/annex/topic/en/annex2en.pdf](http://www.who.int/whr/2004/annex/topic/en/annex2en.pdf)), 16.4 million people died worldwide in that year from bacterial infectious diseases. Although several antibiotics are currently available for each bacterial pathogen, the emerging drug-resistant strains of such pathogens make them difficult to control.<sup>22</sup> Drug target identification is the first step in the drug discovery process.<sup>23</sup> Because of the availability of both pathogen and host-genome sequences, it has become easier to identify drug targets at the genomic level for any given pathogen.<sup>24</sup> In recent years, the strategies are shifting progressively from a generic approach to genomic and metabolomic approaches.<sup>25</sup> To identify novel drug targets that are required to design new defenses against antibiotic resistant pathogens.<sup>26</sup> More importance has been given to targets related to pathways unique to bacteria.<sup>27</sup> Targets related to pathogens' unique pathways are 60–

70% in common, regardless of the genotype of the pathogen, and 30–40% of the targets genus or strain specific. D-alanine ligase is involved in the biosynthesis of the peptidoglycan component of the bacterial cell wall, and catalyses the following reaction:



The gene (ddl) for the *E. coli* enzyme has been cloned and sequenced by Neuhaus<sup>28</sup> and Robinson *et al.*<sup>29</sup> and not unexpectedly, this gene was shown to be essential for the growth of the organism. Subsequently a related gene from *S. typhimurium* was characterized but, since the gene is not essential in this organism, it was designated as ddIA.<sup>30</sup> The ligase encoded by the *S. typhimurium* ddIA gene has been expressed in a *S. typhimurium* bearing pDS4, and extensive studies on the purified enzyme have been performed.<sup>31</sup> The present study was done to evaluate the feasibility and availability of *B. megaterium* ddl gene expression in *E. coli* BL21. PCR analysis of isolated DNA from *B. megaterium* showed a 1000bp amplification band upon using specific primers for D-alanine-D-alanine gene. Consequently, the *Xho*I and *Xba*I fragment was sub-cloned in pET 29a (+) expression vector and under strong T7 bacteriophage inducible promoter. The recombinant plasmid pET 29a/ddl was introduced into BL21DE3pLysS *E. coli* strain (with a  $\lambda$  prophage carrying T7 polymerase). The engineered bacteria harboring the pET 29a (+)/ddl were cultivated. When the Expression of ddl gene was induced by 1mmol IPTG and analyzed on SDS-PAGE, A prominent band of approximately 40 kD was observed after induction which matched the predicted molecular weight of full-length product. However, no expression was observed in un-induced colonies. These results suggested that, the 40 KD most probably represents the expression of ddl gene which is in excellent agreement with the  $M_r$  of 32840 calculated from the deduced amino acid sequence.<sup>32</sup> Findings of Kitamura *et al.*<sup>33</sup> showed that the over expressed ddl from *T. thermophilus* HB8 (TtDdl) in *E. coli*, contains 319 residues and has a molecular mass of 34 670 Da. However, further investigations will be carried out in order to evaluate the interaction of the inhibitor (the heptyl phosphinate) to be a novel slow-binding inhibitor of the ligases from *B. megaterium* for more characterization of the expressed protein.

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Source of Support: Nil, Conflict of Interest: None.

