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Full Length Research Paper

Isolation, recombinant expression and characterization of *the dpr*A gene product in *Streptomyces rimosus* NRRL 2455

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Dihydrodipicolinate synthase (DHDPS) catalyses the first enzymatic step in the lysine-biosynthetic pathway in bacteria, plants and some fungi. In this study, cloning and submission of 3639 bp from *Streptomyces rimosus* NRRL 2455 to the EMBL database under the accession code EU617017 were carried out. The entire *dpr*A gene was cloned and heterologously expressed in *Escherichia coli* BL21 (DE3) and the gene product was purified and analysed spectrophotometrically. A *dpr*A$^-$ knock-out mutant was created and showed a reduction of mycelia growth and spore formation by about 43 and 37%, respectively, as compared to the wild strain. Fed with *meso*-diaminopimelic acid, the *dpr*A$^-$ mutant regained its capability of mycelial growth, however no significant effect on spore formation was observed confirming that *dpr*A gene product was involved in the biosynthesis of dihydrodipicolinate in *S. rimosus* NRRL 2455. This is the first report about identification of dihydrodipicolinate synthase of the order streptomycetes.

Key words: Dihydrodipicolinate synthase, lysine biosynthesis, *Streptomyces rimosus* NRRL 2455, *dpr*A knock-out mutants

INTRODUCTION

The biosynthesis of the amino acid lysine from L-aspartate and pyruvate occurs in most plants, bacteria and in some fungi (Voss et al., 2009) . The lysine bio-synthetic pathway was first investigated by Gilvarg (1960) where several biosynthetic steps are involved. Dihydrodipicolinate synthase (DHDPS) catalyzes the formation of dihydrodipicolinate from pyruvate and L-aspartate - semialdehyde (ASA), the first enzymatic step unique to lysine biosynthesis via the diaminopimelate pathway (Bukhari and Taylor, 1970; Girish et al., 2008). DHDPS (EC 4.2.1.52) has been isolated and its activity was determined in various plant tissues and as well as in several bacteria species (Wallsgrove and Mazelis, 1980; Kumpaisal et al., 1987; Ghislain et al., 1990: Frisch et al., 1991; Dereppe et al., 1992). The activity of DHDP was found to be feedback inhibited by micromolar concentration of L-lysine (Frisch et al., 1991; Ghislain et al., 1990; Kumpaisal et al., 1987; Wallsgrove and Mazelis, 1980). Furthermore, the diaminopimelate pathway in bacteria acts as a branch-point for both lysine and cell wall components biosynthesis. This pathway involves also further enzymatic reactions with the generations of various intermediates such as *meso*-2,6-diamino- pimelate, a key component in bacterial peptidoglycan. More over, dipicolinic acid is an essential component of bacterial endspores which is mainly responsible for the spore resistance toward head and oxidizing agents (Prescott, 1993).

Therefore, this enzyme would play a crucial role in keeping the integrity of bacterial cells as well as their ability to form spores. Several investigators had determined the crystal structure of DHDPS from different sources including *Nicotina sylvestris*, *Escherichia coli*, *Thermotoga maritima*, *Bacillus anthracis*, *Mycobacterium tuberculosis* and *Staphylococcus aureus* (Mirwaldt et al., 1995; Blickling et al., 1997; Pearce et al., 2006; Girish et al., 2008; Kefala et al., 2008) exploring the different catalytic sites where both pyruvate and ASA bind. The amino acid sequences of some putative DHDPS homologous proteins are now available in the proteins bank database from different bacterial species. These include enzymes from related *Streptomyces* species such as *Streptomyces*

Table 1. List of bacterial strains used in this study.

Strain	Relevant markers/Product	Source/Reference
E. coli DH5	F^-, 80 *lac*Z M15, *endA1*, *recA1, hsdR17* (r^-, m^+), *gyrA96*, *thi*, *relA1 supE44*, *deoR*	Hanahan, 1983
E. coli ET12567	F^-, *dam13*, *dcm*6, *hsdM*, *hsdR*, *recF143*, *galT22*, *ara14*, *lacY1*,	MacNeil et al., 1992
E. coli BL21 (DE3)	*hisG4* F^-, *ompT,* -prophage-T7-polymerase *gal, dcm*	Studier et al., 1990
S. rimosus subsp. *paromomycinus*	paromomycin producer	NRRL 2455
S. ribosidificus	ribostamycin producer	NRRL B-11466
S. coelicolor M145	actinorhodin producer	Wuppertal, Germany

coelicolor (accesion codes: NP 626178.1), *Streptomyces avermitilis* (accesion code: BAC74054.1), *Streptomyces sviceus* (accession code: YP 00220875), *Streptomyces griseus* (accession code: 001827119) and *Streptomyces pristinaespiralis* (accession code: YP 002197764.1). However, the biochemical activities of the respective putative proteins have not been analysed.

In the present study, the biochemical activity of the *dpr*A gene product, putatively involved in the biosynthesis of dihydrodipicolinate in *Streptomyces rimosus* NRRL 2455 was biochemically analyzed. *S. rimosus* NRRL 2455 is a paromomycin producer, a 2 deoxystreptamine (2DOS)-containing aminocyclitol aminoglycoside antibiotic with broad spectrum activity against most of gram positive and gram negative bacteria.

For accomplishing this work, two universal heterologous primers were designed and were able to amplify various homologous *dpr*A genes in related bac-terial species. Also, the *dpr* A gene coded for DHDPS in *S. rimosus* was amplified via PCR, cloned, sequenced and heterologously expressed in *E. coli* BL21 (DE3). The biochemical activity of the expressed *dpr*A gene product was determined, characterized and the phenotypic changes that occurred in the *dpr*A^- knock out mutant were also studied.

MATERIALS AND METHODS

Bacterial strains, culture media and vectors

S. rimosus subsp. *paromomycinus* NRRL 2455 (paromomycin producer) was cultured in YEME liquid media or tryptic soy broth (TSB; Hopwood and Wright 1978; Kieser et al., 2000), or on soy bean mannit agar (SMA; Distler et al., 1987) at 28°C. *E. coli* DH5α (Hanahan, 1983) and *E. coli* BL21 DE3 (Studier et al., 1990) were used as a host for cloning and heterologous protein expression experiments, respectively. Bacterial strains used in this study are listed in Table 1. *E. coli* strains were grown on Luria Bertani (LB; Miller, 1972) liquid or agar medium at 37°C and selected with 100 μg ml^{-1} ampicillin, whenever necessary. The plasmids pUCPU21 (2.725 kb; U. Wehmeier, Wuppertal), and pET16b (5.711 kb; Novagen, Germany) were used for gene cloning and expression in *E. coli*, respectively.

Cloning and DNA sequencing of *dpr*A gene

DNA cloning and further manipulations were carried out according to methods described by Hopwood (2003), Kieser et al. (2000) and Sambrook and Russell (2001). Preparation, transformation and regeneration of competent *E. coli* cells were performed by standard protocols of Hanahan (1983). Isolation of chromosomal DNA of *S. rimosus* NRRL 2455 was achieved according to the method of Pospiech and Neumann (1995). PCR was performed in a Biometra Personal Cycler (Göttingen, Germany). Each assay (50 μl) consisted of 200 ng chromosomal DNA, 100 pmole of each appropriate primer, 0.2 mM dNTPs (Invitrogen, Karlsruhe, Germany), 3 mM $MgCl_2$, 10% DMSO to improve the denaturation of the template DNA and 2 U *Taq* DNA polymerase (Invitrogen, Karlsruhe, Germany). The two heterologous primers, DHP-F: 5'-CCATGATCACGCCGTTCACC- 3' and DHP-R: 5'-CGCCTTGGCGGTGACCGC-3' as well as the two homologous primers, DHP2-F (*Nde*I): 5'-TCCGCCATATGACCTCGATCTCG-3' and DHP2-R (*Bam*HI): 5' -CGCAGGATCCGGAGGAG-3' were designed for amplification of both *dpr*A probe (745 bp) and *dpr*A gene (959 bp), respectively. PCR was performed under the following conditions: (98°C, 5 min, then 30 cycles [95°C, 45 sec, 51°C, 45 sec for *dpr*A probe (or 52°C, 45 sec for *dpr*A *gene*), 72°C for 1 min] and 72°C for 5 min. The PCR products were subjected to agarose gel electrophoresis (0.8%) and the expected DNA fragments were extracted and purified using QIA quick Gel extraction kit (Qiagen, Hilden, Germany). The *dpr*A probe was treated with T4-polynucleotide kinase followed by Klenow DNA polymerase (Sigma-Aldrich Co.) and cloned into pUCPU21 (*Sma*I) while *dpr*A gene was digested with *Nde*I/*Bam*HI and inserted into pUCPU21 *(Nde*I/*Bam*HI) and pET16b to form pUDP4/pETDP4 recombinant plasmids, respectively. Plasmid DNA extraction from *E. coli* strains was carried out according to the method described by Birnboim and Doly (1979). The *dpr*A probe and *dpr*A gene were sequenced according to Sanger et al. (1970) using the A.L.F. DNA sequencer (Amersham-Pharmacia Biotech, Freiburg, Germany) and the Thermo-Sequenase sequencing kit (Amersham-Pharmacia Biotech, Freiburg, Germany) before being transformed.

Construction of cosmid banks

The chromosomal DNA of *S. rimosus* was partially digested with *Sau3*A1, and ligated into the *Bam*HI sites of dephosphorylated pOJ436 vector (Beye et al., 1998; Burgtorf et al., 1998; Bierman et al., 1992). After desalting and packaging (Strategene), the DNA was transfected into *E. coli* DH5 (Invitrogen). The transfer of colonies into microtiter plates and onto nylon membranes (Amersham Bioscience Europe, Freiburg, Germany; Combinature

Biopharm, Berlin, Germany) was robotically achieved. The bacterial colonies on the nylon membrane were allowed for growth and then were treated according to Nizetic et al. (1991) and non-radioactively hybridized using *dpr*A labelled probe using a standard protocol (Roche Diagnostics, Penzberg, Germany). The positively screened clones were confirmed by PCR and selected for DNA sequencing. The sequenced clones were assembled using the Staden package program (Staden, 1996). Transformants were grown onto LB agar plates containing the appropriate antibiotic and/or X-Gal (0.2 $mgml^{-}$) as a selective medium.

Nucleotide sequence accession numbers

The nucleotide sequence reported in this study is available in the GenBank database under the accession code: EU617017.

Heterologous expression of DHDPS in *E coli* BL21 (DE3)

Expression of DHDPS (pETDP4) in *E. coli* was carried out as described by Studier et al. (1990). Expression was performed under the control of the *T7* promoter using *E. coli* BL21 (DE3) strains. Single colony harbouring the plasmid pETDP4 (DHDPS), or pET16 (empty vector as a control) were grown overnight in 3 ml LB medium containing 100 μ gml^{-1} ampicillin at 37°C on a shaker incubator 300 rpm. About, 200 µl of these precultures were used to inoculate 20 ml fresh LB medium in 250 ml normal flasks and where allowed to grow at 30°C on shaker incubator 100 rpm to an OD_{600} of 0.5 - 0.6. Induction of *T7*-RNA polymerase was achieved by the addition of 0.5 mM IPTG.

Cells before, 1 and 2 h after IPTG induction were harvested by centrifugation (13,000 rpm/3 min), washed twice with ice cold 25 mM Tris-HCl, pH 7.5. Cell pellets were then suspended in the appropriate amounts of cell lyses buffer (50 mM Tris-HCl, pH 7.5; 1.0 mM dithiothreitol; 10.0 mM $MgCl_2$; 3.0 mM -mercapto-ethanol) and lysed by sonification. Protein concentrations were quantified according to process described by Bradford (1976). Electrophoresis of proteins was performed using SDS-PAGE according to Laemmli (1970) where the final concentration of acrylamide in the focus and separating gels was 5.5 and 12%, respectively.

Gels were stained with 10 - 15 ml gel staining solution (Coomassie Brilliant Blue R250 1.5gl^{-1}; methanol 450.0 mll^{-1}; acetic acid 100.0 mll^{-1}) for 2 - 3 h or overnight with gentle shaking at 55°C. The staining solution was discarded and the gels were then destained using gel destaining solution (methanol 250.0 mll^{-1}; acetic acid 100.0 mll^{-1} ; distilled water 650 $mll^{-}1$) for 1 - 2 h at 55°C. Western blotting was applied to detect His-tagged DHDPS and the method of immuno-detection of proteins was adapted as recommended by the provider of BM Chromagenic Western Blotting Kit (Roche-Mannheim) using an anti-His-tag antibody.

The supernatant after sonification was purified using protein purification Kit (Qiagen, Hilden, Germany) by passing through Ni^{2+}-NTA affinity columns, according to manufacture recommendation. The DHDPS was eluted with 200 mM imidazole in 50 mM Tris and 250 mM NaCl, pH 7.5 and retested onto SDS-PAGE.

Assay of DHDPS activity

The activity of DHDPS was determined using the purified enzyme prepared from *E. coli* BL21 (DE3)/pETDP4 after sonification and centrifugation (13,500 rpm, 30 min and 4°C) . The assay reaction (300 µl) consisted of 50 µg of DHDPS and 20 nm NADH di-sodium salt, 50 mM pyruvate, 20 mM ASA in 150 mM Tris- HC buffer pH 7.5. After 10 min, 10 µl L- lactate dehydrogenase (550 Umg^{-}; Roche-Mannheim) was added to the reaction. ASA was prepared according to the method described by Black and Wright (1954).

The non utilized pyruvate by DHDPS would be reduced to L-lactate in the presence of NADH and L-lactate dehydrogenase. The catalytic activity of DHDPS was determined as compared to two controls: control 1 exactly like the test experiment but in absence of pyruvate (contol 1); and contol 2, a negative control experiment in absence of DHDPS (*E. coli* BL21 (DE3) harbouring pET16b empty vector) . The activity of DHDPS results in the removal of pyruvate, thus a lower concentration of NADH was formed leading to a decrease in absorbance at wavelength 340 nm using the Perkin-ELMER UV/VIS lambda spectrophotometer.

Growth measurement through calculation of bacterial dry weight as well as spore preparation and counting was carried out according to method described by Kieser et al., 2000.

Construction of *dpr*A knock-out mutant

The pUDP4 plasmid was restricted with *Sma*I and ligated into the apramycin resistances cassette (*aacC4*; Rouault et al., 1997) restricted with *Sma*I at both ends to form the construct pUDC4P1. The pUDC4P1 was prepared from methylase deficient *E. coli* ET12456 (MacNeil et al., 1992) and was used for transformation of *S. rimosus* protoplasts according to protocol described by Babcock and Kendrick (1988) . The plates were incubated overnight at 28°C and thereafter were overlaid with apramycin 50 μgml^{-1} and reincubated for 3 - 6 days. The apramycin resistant transformants were selected and tested.

Computer-assisted analysis of DNA and protein sequence

The programs used for computer-assisted analysis of nucleotide and protein sequences were Staden package (Staden, 1996), FramePlot (Ishikawa and Hotta 1999), Online analysis tools (http://molbiol-tools.ca/) and Clustal W (Thompson et al., 1994). Restriction analysis was carried out using: Restriction Enzyme Site Mapper version 3 (http://www.restrictionmapper.org/). PCR amplification temperatures were computed using pDRAW32 (http://www.acaclone.com) or Primer X (http://bioinformatics. org/primer X).

RESULTS AND DISCUSSION

Two heterologous primers (DHP-F and DHP-R) have been created via multiple alignments of the known amino acid sequences of respective homologous proteins (Figure 1). Two areas of maximum conservation of the amino acid sequences have been selected and therefore back translated into their corresponding nucleotide sequences taking into consideration the codon usage for Actinomycetes (Bibb et al., 1984). The primer selectivity was also confirmed by PCR using three different chromosomal DNA of *Streptomyces* strains and used as templates (Figure 2). In each of the three PCR reactions, one DNA band of about 0.745 kb correspond in size to the expected *dpr* A probe (0.745 kb) was detected. Therefore, results obtained from PCR products had confirmed primer selectivity. The *dpr*A probe (0.75 kb) was verified by DNA sequencing and non-radioactively labelled to hybridize a constructed cosmid bank using a standard protocol (Roche Diagnostics, Penzberg, Germany). A DNA fragment of 3639 bp, hybridized with *dpr*A probe, have been fully sequenced via creation of

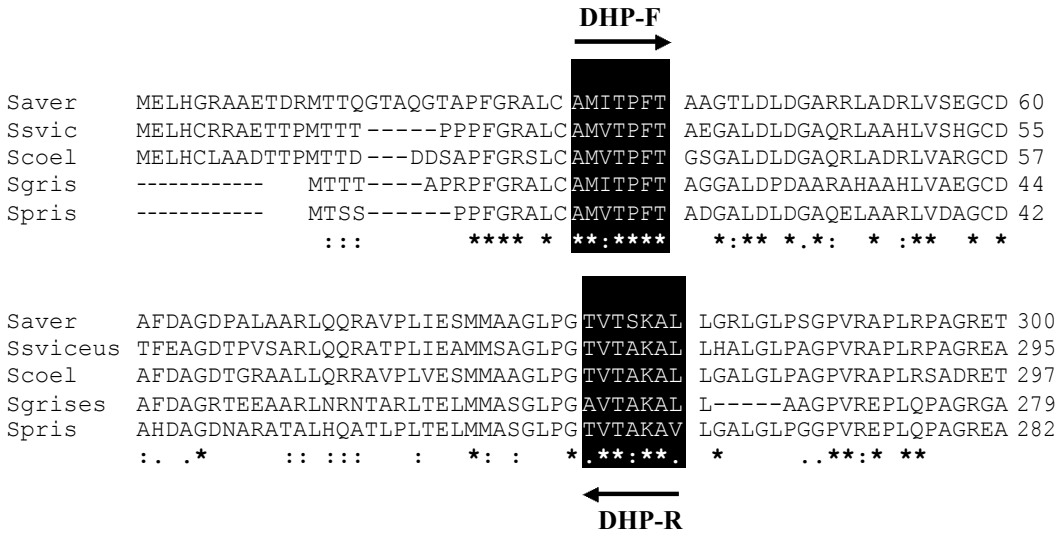

Figure 1. Partial alignment of dihydrodipicolinate synthase proteins for design of heterologous primers (Clustal W) . Boxes mark the areas which were used to design the heterologous primers. The numbers indicate the position within the corresponding proteins. Saver = *S. avermitilis*, Ssviceus = *S. sviceus*, Scoel = *S. coelicolor*, Sgrises = *S. griseus*, Spris = *S. pristinaespiralis*.

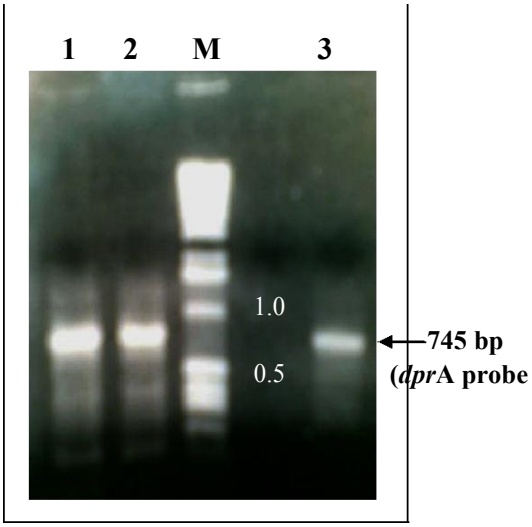

Figure 2. PCR amplification profile using DHP-F and DHP-R primers; lanes 1: *S. rimosus* NRRL 2455; 2: *S. ribosidificus* NRRL B-11466; 3: *S. coelicolor* M145. M: 1.0 kb DNA ladder (Invitrogen, Germany).

three subclones (Figure 3) and the entire DNA sequences have been assembled using the Staden package programme. The final DNA sequence have been analysed, annotated and submitted to EMBL GenBank under the accession code EU617017. Analysis of the submitted DNA fragment revealed that, the *dpr*A gene was flanked by two additional ORFs that are putatively coded with a large secreted protein (SP) and an integral membrane protein (IP) (Figure 3). The SP protein consisted of 581 amino acids (aa) and exhibited a good identity to the related ones in *Streptomyces hygroscopicus* ATCC 53653 (551 aa, ZP_05518737, 75% identity), *Saccharopolyspora erythraea* NRRL 2338 (602 aa, YP_001102513, 61% identity), *S. avermitilis* MA-4680 (608 aa, NP_827523, 61% identity), *S. pristinaespiralis* ATCC 25486 (615 aa, ZP_05012867, 62% identity) and *S. coelicolor* A3(2) (613 aa, NP_626174, 60% identity). With regard to the IP protein (271 aa), a good amino acid similarities were observed to homologous proteins of *S. avermitilis* MA-4680 (238 aa, NP_826957, 69% identity), *S. griseus* subsp. *griseus* NBRC 13350 (271 aa, YP_001826612, 64% identity), *S. pristinaespiralis* ATCC 25486 (273 aa, ZP_05014619, 61% identity) and *S. coelicolor* A3 (2) (274 aa, NP_626641, 63% identity). Whether the SP or IP plays a role in the biosynthesis of lysine or in its export still has to be verified. The three ORFs were found to be highly conserved (60% identity) in the completely analysed streptomycete genomes (Bentley et al., 2002; Ikeda et al., 2003).

The *dpr*A gene product consisted of 319 amino acids (aa) and exhibited a good identity to the related ones in *S. coelicolor* M145 (317 aa, CAB46413.1, 66% identity), *S. avermitilis* MA-4680 (315 aa, CAB46413.1, 67% identity), *S. sviceus* ATCC 29083 (309 aa, EDY55266.1, 64% identity), *S. pristinaespiralis* ATCC 25486 (306 aa, EDY65433.1, 64% identity), *S. griseus* subsp. *griseus* NBRC 13350 (294 aa, BAG22436.1, 59% identity) and *Staphylococcu aureus* (295 aa, YP_186282 , 55 identity).

For DHDPS overexpression, the *dpr*A gene was firstly amplified by PCR using DHP2-F and DHP2-R primers. The forward primer was designed for the introduction of an *Nde*I site, changing the sequence at the natural start codon for the ability to create start codon fusion of the

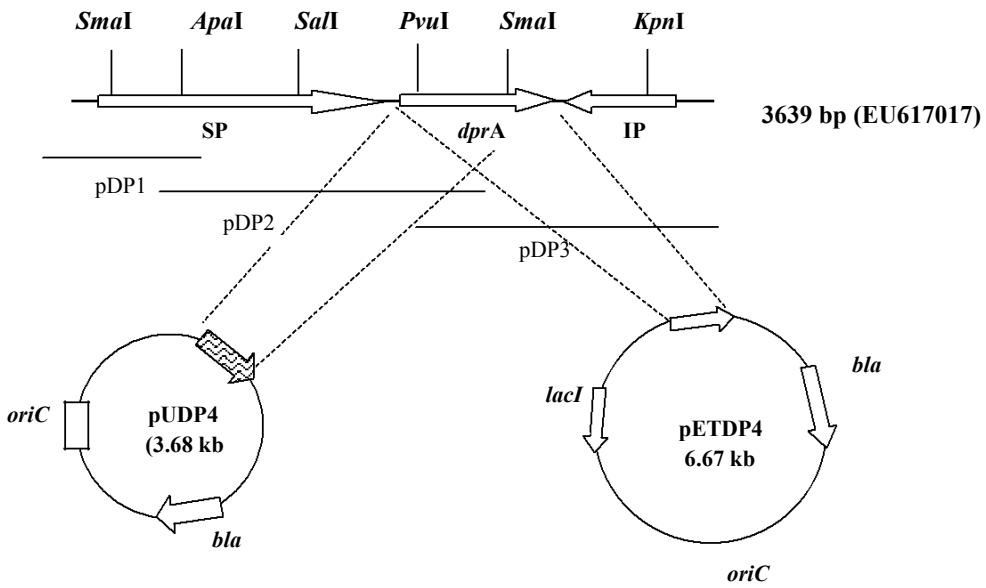

Figure 3. Restriction analysis profile of the DNA segment submitted to EMBL date base (accession code: EU617017). SP = putative large secreted protein, IP = integral membrane protein, *dprA* = gene coded dihdrodipicolinate synthase. Arrows indicate direction of the ORFs.

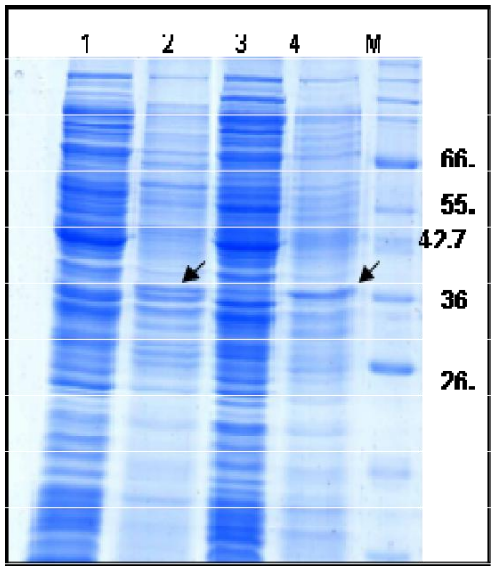

Figure 4. Laemmli SDS-PAGE gels (10%) of the cell -free extracts of *E. coli* BL21 (DE3) harbouring the following plasmids; Lane 1: pET16b (control; 1 h after IPTG induction); lane 2: pETB1 (pETDP4; 1 h); Lane 3: pET16b (2 h); lane 4: pETDP4 (2 h). M indicates protein marker (kDa; New England Biolabs). Arrows mark protein bands correspond-ing to the molecular mass of the expressed DHDPS protein.

*dpr*A gene into the promotor/ribosome-binding site cassettes of pET16b expression vector. On the other hand, the reverse primer was designed for the introduction of a *Bam*HI site located immediately downstream of the natural stop codon in order to allow orientated cloning into the pET16b expression vector.

The amplified DNA fragments were cloned into pET16b (N -terminal His-tagged fusion protein) under the control of the *T7* promoter.

Accordingly, DHDPS of *S. rimosus* was overproduced as a soluble N-terminal His-tagged protein in *E. coli* BL21 (DE3) as determined by SDS-PAGE (Figure 4). Additional band of about 35.7 kDa present in the soluble fraction of the cell-free extracts corresponded in size to the expected molecular masses of the respective His-tagged protein detected. Maximal overexpression of the DHDPS was 2 h after IPTG induction. Purification of the expressed protein had been performed and the biochemical activity of the purified expressed DHDPS was investigated spectrophotometrically at 340 nm. The catalytic activity of DHDPS was determined as compared to two controls: control 1 exactly like the test experiment but in absence of pyruvate (contol 1); and contol 2, a negative control experiment (*E. coli* BL21 (DE3) harbouring pET16b empty vector (Figure 5). Results revealed that DHDPS activity was observed via the gradual decease in absorbance in the test experiment as a result of utilization of pryuvate by DHDPS as compared to the control experiments (Figure 5). In order to study the role of this enzyme, the *dpr*A gene in *S. rimosus* was knocked-out via in-frame insertion of *aacC4* cassette coded for apramycin resistance (Rouault et al., 1997). The formation of *dpr*A knock- out mutant was verified by its growth on TSB agar in the presence or apramycin (50 μml^{-}) as well as by PCR using DHP2-F and DHP2-R

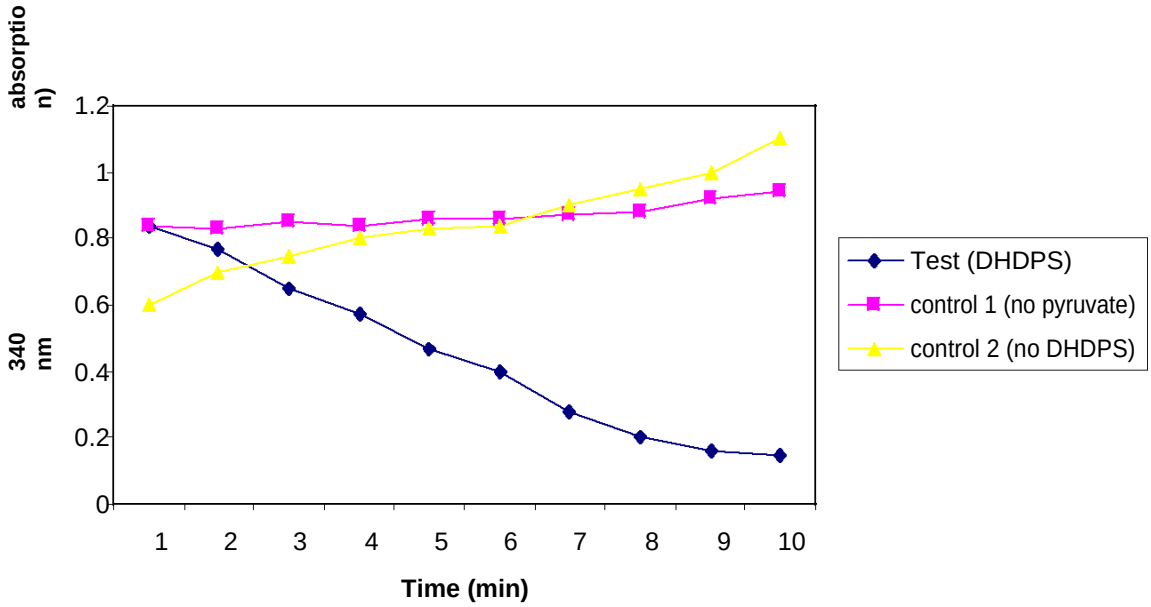

Figure 5. Spectrophotometric assay for measurement of the DHDPS activity. The enzyme assay was performed using the purified DprA recombinant protein of *E. coli* BL21 (DE3)/pETDP4 (Test). Control 1 was performed like in the test but in absence of pyruvate. Control 2 was performed using *E. coli* BL21 (DE3)/pET16b (negative control; no DHDPS).

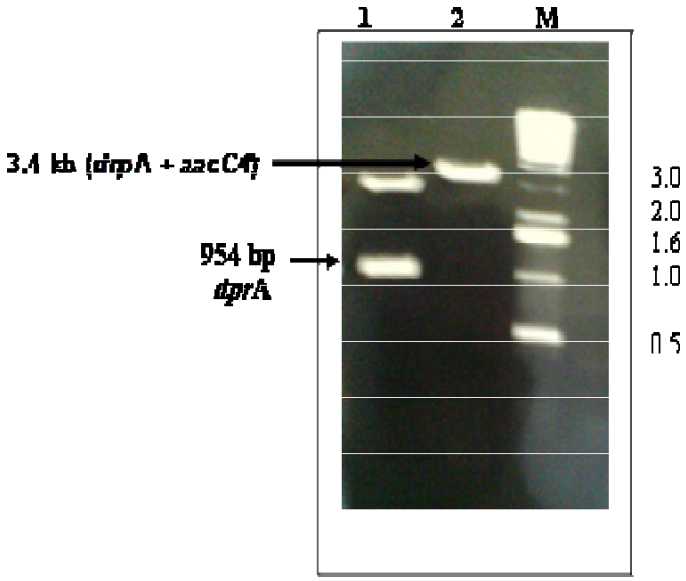

Figure 6. PCR amplification profile using DHP2-F and DHP2-R primers: lane 1, *S. rimosus* NRRL 2455; lane 2, *dpr*A$^-$ mutant. M: 1.0 kb DNA ladder (Invitrogen, Germany).

primers (Figure 6). The *dpr*A$^-$ mutant was able to grow in presence of apramycin due to the presence of apramycin gene cassette integrated in its chromosomal DNA. In addition, a PCR product of 0.95 kb was detected only upon using the chromosomal DNA of the wild strain as a template and was not found upon using the chromosomal DNA of the *dpr*A$^-$ mutant. This would mean that the *dpr*A gene within the *dpr*A$^-$ mutant strain was disrupted as a result of homologous recombination of the pUDC4P1 plasmid. Moreover, spore count of both wild and mutant *S. rimosus* (*dpr*A$^-$) showed that the mutant strain lost about 37% of its capability to form spores as determined by spore count of both the wild and mutant strains (Table 2). The growth rate of the *dpr*A$^-$ mutant was found to be significantly much slower in comparison to the wild strain (about 43% decrease as determined by dry weight of both mycelia) (Table 2).

These observations confirmed the role of *dpr*A gene product in the biosynthesis of dihydrodipicolinate, the main intermediate of peptidoglycan biosynthesis. Since the *dpr*A$^-$ mutant was still able to grow and form spores but at low rate, this could be interpreted by the presence of more than one chromosomal copy of the *dpr*A gene in *S. rimosus* as in case of *S. griseus* (Ohnishi et al., 2008). In order to confirm this hypothesis, both wild and *dpr*A$^-$ mutant strains were allowed to grow on minimal medium in the presence and absence of commercially available *meso*-DAP (50 μgml$^-$; Sigma; D1377). Results showed that the *dpr*A$^-$ regained its capability of mycelial growth as compared to the wild type; however no significant effect on the amount of spores formed was observed. These observations could be interpreted by the ability of the *dpr*A$^-$ mutant to consume *meso*-DAP and regained its normal growth as the wild strain. However, biosynthesis of dihydrodipicolinc acid intermediate was stopped. Therefore, formation of dihydrodipicolinc acid was an essential requirement for sporulation by *S. rimosus*. Accordingly, the obtained results confirmed the role of *dpr*A gene product in the biosynthesis of dihydro-dipicolinate in *S. rimosus* NRRL 2455. Moreover, the future perspective of this research is to study the influence of *dpr*A gene product on the level of paromomycin production by *S. rimosus*.

Table 2. Dry weight and spore count measurement of *S. rimosus* NRRL 2455 and of *S. rimosus dpr*A$^-$ mutant in the presence and absence of *meso*-DAP.

	Dry weight (g/50 ml suspension)		Spore count (CFU/ml) X 10^6	
	In absence of *meso*-DAP	In presence of *meso*-DAP	In absence of *meso*-DAP	In presence of *meso*-DAP
S. rimosus NRRL 2455 (wild strain)	2.3	2.45	243	255
*S. rimosus dpr*A$^-$ mutant	1.3	2.23	153	164
% Reduction	43	8.9	37	35.6

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REFERENCES

Babcock MJ, Kendrick KE (1988). Cloning of DNA involved in sporulation of *Streptomyces griseus*. J. Bacteriol. 170: 2802-2808.

Bentley SD, Chater KF, Cerdeno-Tarraga AM, Challis GL, Thomson NR, James KD, Harris DE, Quail MA, Kieser H, Harper D, Bateman A, Brown S, Chandra G, Chen CW, Collins M, Cronin A, Fraser A, Goble A, Hidalgo J, Hornsby T, Howarth S, Huang CH, Kieser T, Larke L, Murphy L, Oliver K, O'Neil S, Rabbinowitsch E, Rajandream MA, Rutherford K, Rutter S, Seeger K, Saunders D, Sharp S, Squares R, Squares S, Taylor K, Warren T, Wietzorrek A, Woodward J, Barrell BG, Parkhill J, Hopwood DA (2002). Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature, 417: 141-147.

Beye M, Poch A, Burgtorf C, Moritz RFA, Lehrach H (1998). A gridded genomic library of the honeybee (*Apis mellifera*): A reference library system for basic and comparative genetic studies of a hymenopteran genome Genomics, 49: 317-320.

Bibb MJ, Findlay PR, Johnson MW (1984). The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein-coding sequences. Gene, 30: 157-166.

Bierman M, Logan R, O'Brien K, Seno ET, Rao RN, Schoner BE (1992). Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* sp. Gene, 116: 3-49.

Birnboim HC, Doly J (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res., 7: 1513-1523.

Black S, Wright NG (1954). Aspartic -semialdehyde dehydrogenase and aspartic -semialdehyde. J. Biol. Chem., 213: 39-50.

Blickling S, Beisel HG, Bozic D, Knablein J, Laber B, Huber R (1997). Structure of dihydrodipicolinate synthase of *Nicotina sylvestris* reveals novel quaternary structure. J. Mol. Biol., 274: 608- 621.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Bukhari AI, Taylor AL (1970). Genetic analysis of diaminopimelic acid and lysine-requiring mutants of *E. coli*. J. Bacteriol., 105: 844-854.

Burgtorf C, Welzel K, Hasenbank R, Zehetner G, Weis S, Lehrach H (1998). Gridded genomic libraries of different chordate species: a reference library system for basic and comparative genetic studies of chordate genomes. Genomics, 52: 230-232.

Dereppe C, Bold G, Chisalba O, Ebert E, Schär (1992). Purification and characterization of dihydrodipicolinate synthase from pea. Plant physiol., 98: 813-821.

Distler J, Ebert A, Mansouri K, Pissowotzki K, Stockmann M, Piepersberg W (1987). Gene cluster for streptomycin biosynthesis in *Streptomyces griseus*: nucleotide sequence of three genes and analysis of transcriptional activity. Nucleic Acids Res., 15: 8041-8056.

Frisch DA, Gengenbach BG, Tommey AM, Sellner JM, Somers DA, Myers DE (1991). Isolation and characterization of DHDPS from Maize. Plant physiol., 96: 444-452.

Ghislain M, Frankard V, Jacobs M (1990). Dihydrodipicolinate synthase of nicontina sylverstris a chloroplast localized enzyme of the lysine pathway. Planta, 180: 480-486.

Gilvarg C (1960). Biosynthesis of diaminopimelic acid. Fin. Lakaresallsk. Handk. 19: 948-952

Girish TS, Sharma E, Gopal B (2008). Structural and functional characterization of *Staphylococcus aureus* dihydrodipicolinate synthase. FEBS Lett., 582 (19): 2923-2930

Hanahan D (1983). Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol., 166: 557-580.

Hopwood DA (2003). The *Streptomyces* genome-be prepared. Nat. Biotechnol., 21:505-506

Hopwood DA, Wright HM (1978). Bacterial protoplast fusion: recombination in fused protoplasts of *Streptomyces coelicolor*. Mol. Gen. Genet., 162: 307-317.

Ikeda H, Ishikawa J, Hanamoto A, Shinose M, Kikuchi H, Shiba T, Sakaki Y, Hattori M, Omura S (2003). Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. Nature Biotechnol., 21: 526-531.

Ishikawa J, Hotta K (1999). Frameplot: a new implementation of the Frame analysis for predicting protein-coding regions in bacterial DNA with a high G+C content. FEMS Microbiol. Lett., 174: 251-253.

Kefala G, Evans GL, Griffin MD, Devenish SR, Pearce FG, Perugini MA, Gerrard JA (2008). Crystal structure and kinetic study of dihydrodipicolinate synthase from *Mycobacterium tuberculosis*. Biochem. J., 411(2): 351-360.

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000). Practical Streptomyces genetics. The John Innes Foundation, John Innes centre, Norwich, UK.

Kumpaisal R, Hashimoto T, Yamada Y (1987). Purification and characterization of dihydrodipicolinate synthase from wheat suspension cultures. Plant Physiol., 85:145-151.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

MacNeil DJ, Gewain KM, Ruby CL, Dezeny G, Gibbons PH, Mac N (1992). Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. Gene, 111: 61-68.

Miller JH (1972). *Experiments in molecular genetics* Cold Spring Harbor Laboratory, Cold spring Harbor, p. 433.

Mirwaldt C, Korndorfer I, Huuber R (1995). The crystal structure of dihydrodipicolinate synthase from *Escherichia coli* at 2.5 Å resolution. J. Mol. Biol., 24: 227-239.

Nizetic D, Zehetner G, Monaco AP, Gellen L, Young BD, Lehrach H (1991). Construction, arraying, and high-density screening of large insert libraries of human chromosomes X and 21: their potential use as reference libraries. Proc. Natl. Acad. Sci., 88: 3233-3237.

Ohnishi Y, Ishikawa J, Hara H, Suzuki H, Ikenoya M, Ikeda H, Yamashita A, Hattori M, Horinouchi S (2008). Genome sequence of

the streptomycin-producing microorganism *Streptomyces griseus* IFO 13350. J. Bacteriol., 190(11): 4050-4060.
Pearce FG, Perugini A, McKerchar HJ, Gerrard JA (2006). Dihydrodipicolinate synthase from *Thermotoga maritima*. Biochem. J., 400(2): 359-366.
Pospiech A, Neumann B (1995) A versatile quick-prep of genomic DNA from Gram-positive bacteria. Trends Genet, 11: 217-218.
Prescott L (1993) Microbiology, Wm C Brown Publishers, ISBN 0-697-01372-3.
Rouault MHB, Weiser J, Lebrihi A, Branny P, Pernodet JL (1997). Antibiotic resistance gene cassettes derived from the interposon for use in *E coli* and *Streptomyces.* Gene, 190: 315-317.
Sambrook J, Russell DW (2001). Molecular cloning: a laboratory manual, 3rd Eds Cold Spring harbor laboratory Press, Cold Spring Harbor, New York.
Sanger F, Nicklen S, Coulsen AR (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci., 74: 5463-5467.
Staden R (1996). The Staden sequence analysis package. Mol. Biotechnol., 5:233-241.
Studier W, Rosenberg A, Dunn J, Dubendroff J (1990). Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol., 185: 61-89.
Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res., 22: 4673-4680.
Voss JE, Scally SW, Taylor NL, Dogovski C, Alderton MR, Hutton CA, Gerrard JK, Poarker MW, Dobson RC, Perugini MA (2009). Expression, purification, crystallization and preliminary X-ray diffraction analysis of dihydrodipiocolinate synthase from *Bacillus anthracis* in the presence of pyruvate. Acta. Crystallogr. Sect. F. Struct. Biol. Crys. Commun., 65(2): 188-191.
Wallsgrove RM, Mazelis M (1980). The enzymology of lysine biosynthesis in higher plants. FEBS Lett., 116: 189-192.