Full Length Research Paper

Isolation, recombinant expression and characterization of the dprA 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 dprA gene was cloned and heterologously expressed in *Escherichia coli* BL21 (DE3) and the gene product was purified and analysed spectrophotometrically. A dprA 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 dprA mutant regained its capability of mycelial growth, however no significant effect on spore formation was observed confirming that dprA 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, dprA 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 β-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 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*...
coelicolor (accession codes: NP 626178.1), Streptomyces avermitilis (accession 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 dprA 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 dprA genes in related bac-terial species. Also, the dprA gene coded for DHDP2 in S. rimosus was amplified via PCR, cloned, sequenced and heterologously expressed in E. coli BL21 (DE3). The biochemical activity of the expressed dprA gene product was determined, characterized and the phenotypic changes that occurred in the dprA- 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.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant markers/Product</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DH5α</td>
<td>F-, Φ80. lacZ ΔM15, endA1, recA1, hsdR17 (r-, m-), gyrA96, thi, relA1 supE44, deoR</td>
<td>Hanahan, 1983</td>
</tr>
<tr>
<td>E. coli ET12567</td>
<td>F-, dam13, dcm6, hsdM, hsdR, recF143, galT22, ara14, lacY1, hisG4</td>
<td>MacNeil et al., 1992</td>
</tr>
<tr>
<td>E. coli BL21 (DE3)</td>
<td>F-, ompT, λ-prophage-T7-polymerase gal, dcm</td>
<td>Studier et al., 1990</td>
</tr>
<tr>
<td>S. rimosus subsp. paromomycinus</td>
<td>paromomycin producer</td>
<td>NRRL 2455</td>
</tr>
<tr>
<td>S. ribosidificus</td>
<td>ribostamycin producer</td>
<td>NRRL B-11466</td>
</tr>
<tr>
<td>S. coelicolor M145</td>
<td>actinorhodin producer</td>
<td>Wuppertal, Germany</td>
</tr>
</tbody>
</table>

Cloning and DNA sequencing of dprA 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 MgCl₂, 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'-CCATGATACGCGCCGTAC-3' and DHP-R: 5'-CGCCTTGGCGGTGACCGC-3' as well as the two homologous primers, DHP2-F (Ndel): 5'-TCCGACTATGCAGCTGATC-3' and DHP2-R (BanHI): 5'-CGCAAGATCCGAGGAG-3' were designed for amplification of both dprA probe (745 bp) and dprA 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 dprA probe (or 52°C, 45 sec for dprA 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 dprA probe was treated with T4-polynucleotide kinase followed by Klenow DNA polymerase (Sigma-Aldrich Co.) and cloned into pUCPU21 (Smal), while dprA gene was digested with Ndel/BanHI and inserted into pUCPU21 (Ndel/BanHI) 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 dprA probe and dprA gene were sequenced according to Sanger et al. (1970) using the A.L.F. DNA extraction kit (Amersham-Bioscience Europe, Freiburg, Germany) before being transformed.

Construction of cosmid banks

The chromosomal DNA of S. rimosus was partially digested with Sau3A1, and ligated into the BanHI sites of dephosphorylated pOJ436 vector (Beye et al., 1998; Burgdorf et al., 1998; Bierman et al., 1992) After desalting and packaging (Strategene), the DNA was transfected into E. coli DH5α (Invitrogen). The transfer of colonies into microtiter plates and onto nylon membranes (Amersham Bioscience Europe, Freiburg, Germany; Combiblend
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 dprA 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 mg ml⁻¹) 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 μg ml⁻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 were allowed to grow at 30°C on shaker incubator 100 rpm to an OD₆₀₀ of 0.5 - 0.6. Induction of T7-RNA polymerase was achieved by the addition of 0.5 mM IPTG.

Cells before, and 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 MgCl₂; 3.0 mM β-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 1.5 gl⁻¹, methanol 450.0 ml⁻¹; acetic acid 100.0 ml⁻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 ml⁻¹; acetic acid 100.0 ml⁻¹; distilled water 650 ml⁻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 recomended 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 Ni²⁺-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 sonication 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⁻¹; 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 (control 1); and control 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 dprA knock-out mutant

The pUDP4 plasmid was restricted with Smal and ligated into the apramycin resistances cassette (aacC4; Rouault et al., 1997) restricted with Smal at both ends to form the construct pUDC4P1. The pUDC4P1 was prepared from methyldeficient 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 μg ml⁻¹ and reinoculated 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 Map and Online analysis tools (http://www.restrictionmapper.org/). PCR amplification temperatures were computed using pDRAWS2 (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 dprA probe (0.745 kb) was detected. Therefore, results obtained from PCR products had confirmed primer selectivity. The dprA probe (0.75 kbp) 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 dprA probe, have been fully sequenced via creation of
three subclones (Figure 3) and the entire DNA sequences have been assembled using the Staden package programme. The final DNA sequence have been annotated, submitted to EMBL GenBank under the accession code EU617017. Analysis of the submitted DNA fragment revealed that, the dprA 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_05014619, 62% identity) and *S. coelicolor* A3(2) (613 aa, NP_626641, 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 dprA 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 *Staphylococcus aureus* (295 aa, YP_186282 , 55 identity).

For DHDPS overexpression, the dprA 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 database (accession code: EU617017). SP = putative large secreted protein, IP = integral membrane protein, *dprA* = gene coded dihydrodipicolinate 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 corresponding to the molecular mass of the expressed DHDPS protein.

dprA gene into the promoter/ribosome-binding site cassettes of pET16b expression vector. On the other hand, the reverse primer was designed for the introduction of a BamHI 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 decrease in absorbance in the test experiment as a result of utilization of pyruvate by DHDPS as compared to the control experiments (Figure 5). In order to study the role of this enzyme, the *dprA* 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 *dprA* knock-out mutant was verified by its growth on TSB agar in the presence or apramycin (50 µg/ml) 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, dprA mutant. M: 1.0 kb DNA ladder (Invitrogen, Germany).

The dprA 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 dprA mutant. This would mean that the dprA gene within the dprA mutant strain was disrupted as a result of homologous recombination of the pUDC4P1 plasmid. Moreover, spore count of both wild and mutant *S. rimosus* (dprA-) 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 dprA 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 dprA gene product in the biosynthesis of dihydrodipicolinate, the main intermediate of peptidoglycan biosynthesis. Since the dprA 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 dprA gene in *S. rimosus* as in case of *S. griseus* (Ohnishi et al., 2008). In order to confirm this hypothesis, both wild and dprA- mutant strains were allowed to grow on minimal medium in the presence and absence of commercially available meso-DAP (50 μg/ml; Sigma; D1377). Results showed that the dprA 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 dprA mutant to consume meso-DAP and regained its normal growth as the wild strain. However, biosynthesis of dihydrodipicolinic acid intermediate was stopped. Therefore, formation of dihydrodipicolinic acid was an essential requirement for sporulation by *S. rimosus*. Accordingly, the obtained results confirmed the role of dprA gene product in the biosynthesis of dihydrodipicolinate in *S. rimosus* NRRL 2455. Moreover, the future perspective of this research is to study the influence of dprA 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 dpA mutant in the presence and absence of meso-DAP.

<table>
<thead>
<tr>
<th>Dry weight (g/50 ml suspension)</th>
<th>Spore count (CFU/ml) X 10^4</th>
</tr>
</thead>
<tbody>
<tr>
<td>In absence of meso-DAP</td>
<td>In presence of meso-DAP</td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>S. rimosus NRRL 2455 (wild strain)</td>
<td>2.3</td>
</tr>
<tr>
<td>S. rimosus dpA mutant</td>
<td>1.3</td>
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<tr>
<td>% Reduction</td>
<td>43</td>
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</table>

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REFERENCES


