(De)regulation of key enzyme steps in the shikimate pathway and phenylalanine-specific pathway of the actinomycete Amycolatopsis methanolica


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Prephenate dehydratase (PDT), chorismate mutase (CM) and 3-deoxy-D-arabino-7-heptulosonate 7-phosphate (DAHP) synthase are key regulatory enzymes in aromatic amino acid biosynthesis in the actinomycete Amycolatopsis methanolica. Deregulated, feedback-control-resistant mutants were isolated by incubation of A. methanolica on glucose mineral agar containing the toxic analogue p-fluoro-DL-phenylalanine (pFPhe). Several of these mutants had completely lost PDT sensitivity to Phe inhibition and Tyr activation. Mutant characterization yielded new information about PDT amino acid residues involved in Phe and Tyr effector binding sites. A. methanolica wild-type cells grown on glucose mineral medium normally possess a bifunctional CM/DAHP synthase protein complex (with DS1, a plant-type DAHP synthase). The CM activity of this protein complex is feedback-inhibited by Tyr and Phe, while DS1 activity is mainly inhibited by Trp. Isolation of pFPhe-resistant mutants yielded two feedback-inhibition-resistant CM mutants. These were characterized as regulatory mutants, derepressed in (a) synthesis of CM, now occurring as an abundant, feedback-inhibition-resistant, separate protein, and (b) synthesis of an alternative DAHP synthase (DS2, an E. coli-type DAHP synthase), only inhibited by Tyr and Trp. DS1 and DS2 thus are well integrated in A. methanolica primary metabolism: DS1 and CM form a protein complex, which stimulates CM activity and renders it sensitive to feedback inhibition by Phe and Tyr. Synthesis of CM and DS2 proteins appears to be controlled co-ordinately, sensitive to Phe-mediated feedback repression.

INTRODUCTION

In micro-organisms and plants the biosynthesis of aromatic compounds proceeds via the common seven-step aromatic or shikimate pathway to the branch point intermediate chorismate. This intermediate is subsequently converted to the three aromatic amino acids via specific terminal pathways (Fig. 1). Many other (aromatic) compounds are derived either partially or entirely from chorismate or from other pathway intermediates or end products, e.g. pyrroloquinoline quinone, lignin, ubiquinone, plastiquinone, enterochelin, vitamin K and 3-amino-5-hydroxybenzoic acid, the precursor of the mC7N units found in mitomycin and ansamycin antibiotics (Bentley, 1990; Knaggs, 1999; Arakawa et al., 2002). Aromatic amino acid biosynthesis in bacteria is strictly regulated via feedback control mechanisms. Limited information is available about these enzymes or their regulation in actinomycetes, Gram-positive bacteria that are well-known producers of numerous antibiotics derived from aromatic amino acids or their pathway intermediates (Hodgson, 2000). Antibiotic biosynthesis may require specific metabolic adaptations, e.g. expression of isoenzymes that serve to avoid feedback regulation by aromatic amino acids.

We are interested in the enzymology and regulation of aromatic amino acid biosynthesis in the nocardioform actinomycete Amycolatopsis methanolica. This bacterium is closely related to Amycolatopsis mediterranei, producing the antibiotic rifamycin via 3-amino-5-hydroxybenzoic acid (Kim et al., 1996a, b), and to Amycolatopsis orientalis, producing the glycopeptide antibiotics chloroeremomycin and vancomycin, containing for instance Tyr (van Wageningen et al., 1998). Previous studies of aromatic amino acid biosynthesis in A. methanolica have resulted in the isolation of a large number of auxotrophic mutants and the biochemical and molecular characterization of several
The shikimate pathway and the Phe-specific pathway are controlled by three regulatory enzymes: 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase, chorismate mutase (CM) and prephenate dehydratase (PDT). DAHP synthase, the first enzyme of the shikimate pathway, is responsible for the condensation of the pentose phosphate pathway intermediate D-erythrose 4-phosphate and the glycolytic pathway intermediate phosphoenolpyruvate to DAHP. Carbon flow through the shikimate pathway, where tested in other microbes, is generally controlled by feedback inhibition of DAHP synthase activity and/or repression of DAHP synthase synthesis. DAHP synthase enzymes are present either as a monofunctional di- or tetrameric protein, e.g. in *Escherichia coli* (Ray & Bauerle, 1991; McCandliss *et al.*, 1978; Schoner & Herrmann, 1976), or as a bifunctional protein exhibiting both DAHP synthase and CM activities, e.g. in *Brevibacterium flavum* (Sugimoto & Shiio, 1980) and in *A. methanolica* (Euverink *et al.*, 1995a). Several organisms contain two or three isoenzymes of DAHP synthase, each displaying a specific feedback inhibition pattern.

Based on their highly divergent primary structures, two DAHP synthase families are distinguished. *E. coli*-type DAHP synthase enzymes have only been found in microorganisms. Plant-type DAHP synthase proteins are mainly found in plants, but an increasing number of these enzymes are found in bacteria, e.g. in the aurachin-producing Gram-negative bacterium *Stigmatella aurantiaca* (Silakowski *et al.*, 2000), and in the phenazine biosynthetic gene cluster of *Pseudomonas aureofaciens* (*phzF*). It has been speculated that the *phzF* gene product serves to bypass feedback-inhibited DAHP synthase protein(s) in *P. aureofaciens* to ensure sufficient intracellular levels of chorismate for phenazine production (Pierson *et al.*, 1995). Plant-type DAHP synthase enzymes are also found in actinomycetes; e.g. the *Streptomyces avermitilis* (BAC73797) (Ikeda *et al.*, 2003) and *Streptomyces coelicolor* (P80574, CAB38581) (Bentley *et al.*, 2002) genomes encode one and two (putative) plant-type DAHP synthase proteins, respectively. The *Corynebacterium glutamicum* genome encodes single copies of both DAHP synthase protein families (BAB99571, BAB988383), similar to the rifamycin producer *A. medierranei* (AAK28148, AAC01718). The ORF encoding the plant-type DAHP synthase, located in the rifamycin biosynthetic gene cluster, is involved in aminoDAHP synthesis (August *et al.*, 1998).

Biochemical studies already have shown that two DAHP synthase isoenzymes and single CM and PDT enzymes are present in *A. methanolica*. DAHP synthase 1 (DS1) is a 160 kDa enzyme associated non-covalently with a dimeric CM protein, thus forming a heteromeric two-enzyme complex. The two enzyme activities can be separated by Q-Sepharose anion-exchange chromatography, yielding a dimeric CM protein with a fivefold reduced activity that is no longer feedback-inhibited by Phe and Tyr, and a 160 kDa DAHP synthase that is still feedback inhibition sensitive to its effectors Phe, Tyr and (most strongly) Trp (Euverink *et al.*, 1995a).

Characterization of a leaky Phe auxotrophic mutant (GH141) of *A. methanolica* revealed that it had lost 90% of the Phe aminotransferase activity, resulting in Phe-limited growth in mineral medium. Mutant GH141 expressed an additional Tyr-sensitive DAHP synthase activity (DS2), accompanied by a strongly elevated CM activity. In mutant GH141 the CM protein was not associated with DS1 activity, but occurred as a separate dimeric protein (Euverink *et al.*, 1995a). Supplementing Phe to the growth medium of GH141 restored wild-type activity levels of both CM and DAHP synthase. An o-fluorodL-phenylalanine-resistant mutant (oFPhe83) was subsequently characterized that showed high levels of DS2 and dimeric CM activity, in both the presence and absence of Phe, suggesting a regulatory mutation that derepressed the synthesis of both proteins (Euverink *et al.*, 1995a). Thus, whereas DS1 activity is sensitive to Phe, Tyr and
(most strongly) Trp feedback inhibition, DS2 activity is sensitive to Tyr (but not Phe) feedback inhibition whereas its synthesis is sensitive to feedback repression mediated by Phe.

Prephenate dehydratase (PDT) enzymes occur as monofunctional proteins in Gram-positive bacteria or as bifunctional proteins (P-proteins) in Gram-negative bacteria (Bentley, 1990). PDT enzymes are generally sensitive to feedback regulation by Phe and/or Tyr (Bentley, 1990). PDT of A. methanolica is allosterically inhibited by Phe and activated by Tyr (de Boer et al., 1989; Euverink et al., 1995b). Previously, we reported characterization of the A. methanolica pdt gene (Vrijbloed et al., 1995).

Here we report the isolation and characterization of a number of spontaneous A. methanolica PDT mutants insensitive to Phe feedback inhibition and Tyr feedback activation, providing new information about amino acid residues involved in PDT allosteric control. Furthermore, we report the molecular and biochemical characterization of both DAHP synthases of A. methanolica, revealing that this organism contains a single representative of both currently recognized families of DAHP synthase enzymes. We believe this to be the first report of a plant-type DAHP synthase that forms a protein complex with a CM, thereby stimulating CM activity and making it sensitive to feedback inhibition.

**METHODS**

**Micro-organisms, cultivation and DNA manipulations.** Amycolatopsis methanolica wild-type (NCIB 11946) and derived phenylalanine-analogue-resistant mutants (this study) were cultivated as described by de Boer et al. (1988). E. coli strains DH5α and BL21(DE3) were grown in Luria–Bertani (LB) medium (ampicillin and chloramphenicol, 100 μg ml⁻¹; IPTG, 0-5 mM) at 37 °C and 30 °C, respectively. Methods for DNA handling, modification and cloning were as described by Sambrook et al. (1989). Plasmids and cloning vectors used are listed in Table 1.

**Isolation of phenylalanine-analogue-resistant mutants.** A. methanolica mutants resistant to the toxic analogue p-fluoro-DL-phenylalanine (pFPhe; 5 mg ml⁻¹, 32.8 mM) were isolated on 10 mM glucose mineral agar plates with filter-sterilized analogue. Plates were inoculated with approximately 5 x 10⁷ cells. After 5–7 days the spontaneously resistant colonies that had appeared were transferred to fresh agar plates with the same composition.

**Preparation of extracts and enzyme assays.** Cells were washed in buffer (50 mM Tris/HCl pH 7-5) and disrupted by three passages through a French pressure cell at 140 MPa. Unbroken cells and debris were removed by centrifugation at 40 000 g for 30 min at 4 °C and the supernatant was used for enzyme assays. Unless otherwise stated enzyme assays were performed at 37 °C.

PDT (EC 4.2.1.51) activity was assayed by measuring phenylpyruvate formation. The reaction mixture (0-5 ml) contained 1 mM potassium prephenate, 50 mM Tris/HCl pH 7-5 and protein. At appropriate time intervals 0-5 ml 2-0 M NaOH was added and the absorbance of phenylpyruvate was measured at 320 nm (ɛ₅₃₀[phenylpyruvate]= 17.5-×10⁶ M⁻¹ cm⁻¹) (Patel et al., 1977). CM (EC 5.4.99.5) activity was assayed by measuring the amount of prephenate formed after its conversion to phenylpyruvate (Dopheide et al., 1972). The reaction mixture (0-1 ml) contained 50 mM Tris/HCl, pH 7-5, 2-0 mM chorismate and protein. After 10 min, 10 μl 4-5 M HCl was added and the reaction mixture was incubated for 15 min at 37 °C. After addition of NaOH (890 μl of a 1:58 M solution) the phenylpyruvate formed was determined by measuring A₂₅₀. DAHP synthase (EC 2.5.1.54) activity was assayed as described by de Boer et al. (1989). Phe, Tyr and Trp inhibition/activation of PDT, CM and DAHP synthase activity was determined in the presence of 1 mM of each amino acid (Euverink et al., 1995a, b).

**DNA sequence analysis.** Nucleotide sequencing was done using dye-primers in the cycle sequencing method (Murray, 1989) with the thermosequenase kit RPN 2538 from Amersham Pharmacia Biotech. The samples were run on the ALF-Express sequencing robot. Analysis of nucleotide sequence was done using CloneManager version 4.01. Protein sequence comparisons were performed using the facilities of the BLAST server (Altschul et al., 1990) at NCBI (National Library of Medicine, Washington, DC, USA).

**PCR primers used to clone aroG.** Using alignments of plant-type (putative) DAHP synthase proteins (see Fig. 3), degenerate primers were designed on the basis of two conserved motifs: PLDS1 [5’-GG(G/C)(A/C)G(C/G)ATCGC(G/C)GG(G/C)CA(G/A)-3’] and PLDS2 [5’-(G/C)GTGTG(T/C)C(C/G)CCGTGCAT(G/C)GG-3’]. PCR experiments with these primers, using A. methanolica DNA as template, yielded a single DNA fragment of approximately 730 bp with strong sequence similarity to plant-type DAHP synthase genes. Specific primers PLDS3 and PLDS4 were designed (Fig. 3), and used to screen an A. methanolica genomic library.

**Table 1. Plasmids and vectors used in this study**

<table>
<thead>
<tr>
<th>Plasmid or vector</th>
<th>Characteristics</th>
<th>Reference/origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBlueScript II KS⁻</td>
<td>Cloning vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pET3b</td>
<td>Expression vector</td>
<td>Novagen</td>
</tr>
<tr>
<td>P2ErO-2.0</td>
<td>Cloning vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pHK200</td>
<td>pdt cloned in pBlueScript II KS⁻</td>
<td>This study</td>
</tr>
<tr>
<td>pHK255(1-10)</td>
<td>pdt wild-type and nine mutants in pET3b</td>
<td>This study</td>
</tr>
<tr>
<td>pHK276</td>
<td>aroF cloned in pET3b</td>
<td>This study</td>
</tr>
<tr>
<td>pHK276-0</td>
<td>aroF containing stop codon at bp position</td>
<td>This study</td>
</tr>
<tr>
<td>pHK287</td>
<td>aroG cloned in pET3b</td>
<td>This study</td>
</tr>
</tbody>
</table>

http://mic.sgmjournals.org
Construction and PCR screening of genomic DNA library. An A. methanolica genomic DNA library was constructed using partially Sau3A-digested chromosomal DNA, sized by sucrose gradient centrifugation. Fragments of 8–12 kb were cloned in the BamHI site of pZErO-2.0, allowing positive selection of recombinants. Transformation of E. coli DH5α with ligation mixture generated a DNA library of ~5000 independent transformants (mean insert size 7.8 kb; insert frequency 92%).

Gene library screening for the aroG gene was performed by PCR, using primers PLDS3 (5'-TGCCACTCTGCTGATG-3') and PLDS4 (5'-TGCCAGATGACCTTGTG-3'). Plates with ~100 gene library transformants were replica-plated. Subsequently, all cell material from the original plate was resuspended in 1 ml LB medium, from which plasmid DNA was isolated and subjected to PCR analysis. Colonies originating from plates with a positive outcome were subjected to individual PCR analysis. This resulted in identification of a positive clone containing an insert of approximately 8 kb.

Southern hybridization of aroF. Digested chromosomal DNA from A. methanolica was separated on a 0.8% (w/v) agarose gel and blotted onto a high-bond nylon membrane (Qiagen), via an alkaline transfer method (Sambrook et al., 1989). Southern hybridization was performed at 65 °C, using the entire aroF gene (for DS2) of A. methanolica as a probe. Radioactive probe labelling was performed with the high prime DNA labelling kit from Boehringer Mannheim. Following hybridization the membrane was washed at 65 °C with 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) containing 0.5% (w/v) SDS for 10 min, twice with 1× SSC containing 0.5% (w/v) SDS at room temperature and three times with 0.3× SSC containing 0.5% (w/v) SDS at room temperature.

Estimation of the molecular mass of native proteins. The molecular masses of AroF and AroG were estimated by loading E. coli cell-free extracts on a Superdex-200 column (OX 16/60) equilibrated with Tris/HCl buffer (pH 7.5). Bio-Rad gel filtration standard proteins (670, 158, 44 and 17 kDa proteins) were used as standards.

Analytical methods. Protein concentrations were determined with bovine serum albumin as standard (Bradford, 1976).

Table 2. Characteristic properties of deregulated PDT mutant proteins in spontaneous pFPhe and oFPhe analogue resistant mutants of A. methanolica

<table>
<thead>
<tr>
<th>Mutant strain</th>
<th>Phe inhibition† (%)</th>
<th>Tyr activation† (%)</th>
<th>Mutation (nucleotide posn)</th>
<th>Mutation (amino acid posn)</th>
</tr>
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<tbody>
<tr>
<td>PDT-Wt</td>
<td>30</td>
<td>225</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pFPhe32*</td>
<td>89</td>
<td>209</td>
<td>C625T</td>
<td>L209F</td>
</tr>
<tr>
<td>oFPhe84*</td>
<td>81</td>
<td>96</td>
<td>C374G</td>
<td>A125G</td>
</tr>
<tr>
<td>pFPhe17</td>
<td>86</td>
<td>202</td>
<td>C625T</td>
<td>L209F</td>
</tr>
<tr>
<td>pFPhe54</td>
<td>117</td>
<td>110</td>
<td>C593T</td>
<td>A198V</td>
</tr>
<tr>
<td>pFPhe55</td>
<td>108</td>
<td>101</td>
<td>G592C</td>
<td>A198P</td>
</tr>
<tr>
<td>pFPhe60</td>
<td>98</td>
<td>114</td>
<td>A680G</td>
<td>H227R</td>
</tr>
<tr>
<td>pFPhe82</td>
<td>97</td>
<td>210</td>
<td>C600G</td>
<td>N200K</td>
</tr>
<tr>
<td>pFPhe114</td>
<td>129</td>
<td>117</td>
<td>C593T</td>
<td>A198V</td>
</tr>
<tr>
<td>pFPhe118</td>
<td>105</td>
<td>113</td>
<td>A680G</td>
<td>H227R</td>
</tr>
</tbody>
</table>

*Isolated by Euverink et al. (1995b).
†Activity relative to incubation without effector.

RESULTS

Isolation of A. methanolica pFPhe-resistant mutants

The PDT and CM enzymes of A. methanolica are sensitive to inhibition by (pF)Phe. Growth of wild-type A. methanolica on glucose mineral agar with pFPhe at 32.5 mM is completely inhibited. Previously we have shown that this is due to inhibition of Phe synthesis and can be overcome by adding Phe to the growth medium (de Boer et al., 1990). This allowed straightforward selection of A. methanolica mutants with deregulated PDT/CM enzymes. Colonies of A. methanolica started to appear after 4 days of incubation on glucose mineral medium agar plates with pFPhe. The maximum number of pFPhe-resistant mutants was reached after 7 days (mutant frequency ~10^{-3}). Transfer to fresh agar plates with the same pFPhe-containing medium resulted in growth of almost 80% of the pFPhe-resistant colonies. Cell extracts of mutant strains harvested from glucose mineral medium cultures were assayed for PDT and CM activities and analysed for Phe and Tyr feedback inhibition.

A. methanolica wild-type PDT activity is inhibited up to 70% by 1 mM Phe, and stimulated by a factor of two by a similar amount of Tyr (Table 2) (Euverink et al., 1995b). Of 76 colonies tested, seven had (completely) lost Phe feedback inhibition sensitivity, and five of those seven mutants had also completely lost the stimulatory effect of

Accession numbers. The DAHP synthase encoding nucleotide sequences of A. methanolica presented in this paper were entered into GenBank under accession numbers AY382157 (aroF) and AY382158 (aroG). The accession number of the A. methanolica pdt gene sequence is Q44104.
Tyr. Similar results were obtained for PDT enzymes in two previously (Euverink et al., 1995b) isolated FPh-resistant *A. methanolica* mutants, strains pFPhe32 and oFPhe84. None of these nine strains carrying PDT mutants were affected in CM sensitivity to Phe inhibition. No PDT mutants were detected that had lost the stimulatory effect of Tyr only.

A further two of the 76 mutants tested were affected in CM feedback inhibition activity. Both mutants (strains pFPhe4 and pFPhe25) were completely insensitive to the inhibitory effect of Phe (50 % in wild-type situation), while Tyr (1 mM) inhibition was reduced to 25 % (50 % in wild-type situation). The presence of both Phe and Tyr (1 mM each) in the reaction mix yielded 35 % relative inhibition (85 % in wild-type situation).

CM only displays its feedback inhibition sensitivity when bound within the CM–DAHP synthase enzyme complex (with DS1; see below) (Euverink et al., 1995a). The inhibitor-binding domain involved in this phenomenon thus may be located on the DAHP synthase moiety. In this situation, two types of CM and DAHP synthase feedback inhibition resistant mutants may occur: (I) a DS1 mutation resulting in loss of feedback inhibition sensitivity of both CM activity and DS1 activity, and (II) a regulatory mutation leading to derepression of (Phe feedback inhibition insensitive) DS2 and CM synthesis, similar to mutant oFPhe83 (Euverink et al., 1995a). The two isolated mutant strains with deregulated CM enzymes were therefore tested for their DAHP synthase activities and feedback inhibition patterns. Mutants pFPhe4 and pFPhe25 both possessed 9–10-fold higher DAHP synthase activity levels. Similar to mutant oFPhe83, this DAHP synthase activity was most strongly feedback-inhibited by Tyr, indicating the presence of (derepressed) DS2 activity (Table 3). Screening of the remaining 74 pFPhe-resistant mutant strains did not yield a single mutant with deregulated DS1 activity.

**Sequence analysis and heterologous expression of deregulated PDT mutants**

Cloning and nucleotide sequencing of the nine mutant *pdt* genes revealed point mutations in six different nucleotides (mutants A125G, A198P, A198V, N200K, L209F and H227R (Table 2). Mutants N200K and L209F were still activated by Tyr (Table 2). The *A. methanolica* wild-type and mutant *pdt* genes were cloned into pET3b, resulting in plasmids pHK255 (1–10). PDT activity in *E. coli* strain BL21(DE3)/pHK255 expressing the *A. methanolica* wild-type *pdt* gene reached levels of 19 U mg\(^{-1}\), while PDT activity in the *E. coli* BL21(DE3)/pET3b control strain was 0–009 U mg\(^{-1}\). Effects of Phe and Tyr on mutant PDT proteins were similar in *E. coli* BL21(DE3)/pHK255 (1–10) extracts and in *A. methanolica* mutant strains.

**PDT sequence alignments**

Sequence alignments revealed that *A. methanolica* PDT shares considerable similarity with other (monofunctional) actinomycete PDT sequences (Fig. 2). Also the PDT-encoding domains of P-proteins aligned well with *A. methanolica* PDT, albeit with lower relative sequence similarity.

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**Table 3.** Specific DAHP synthase activities and feedback inhibition patterns in *A. methanolica* wild-type and derived mutant strains, and in *E. coli* BL21(DE3) strains expressing *A. methanolica* DAHP synthase proteins, grown in different media

Assays were performed with cell-free extracts of *A. methanolica* or *E. coli*. Sensitivity to feedback inhibition by the aromatic amino acids (1 mM final concentrations) is indicated by the relative residual activities.

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Medium</th>
<th>Sp. act. [mU (mg protein)](^{-1})*</th>
<th>Activity (%) with 1 mM:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phe</td>
</tr>
<tr>
<td><em>A. methanolica</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>Mineral medium</td>
<td>22</td>
<td>65</td>
</tr>
<tr>
<td>oFPhe83</td>
<td>Mineral medium</td>
<td>188</td>
<td>100</td>
</tr>
<tr>
<td>pFPhe4</td>
<td>Mineral medium</td>
<td>215</td>
<td>103</td>
</tr>
<tr>
<td>pFPhe25</td>
<td>Mineral medium</td>
<td>204</td>
<td>95</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pET3b (neg. control)</td>
<td>LB</td>
<td>4</td>
<td>ND†</td>
</tr>
<tr>
<td>pHK276</td>
<td>LB</td>
<td>363</td>
<td>ND</td>
</tr>
<tr>
<td>pHK276-0</td>
<td>LB + 0.5 M sorbitol</td>
<td>3267</td>
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</tr>
<tr>
<td>pHK287</td>
<td>LB</td>
<td>377</td>
<td>ND</td>
</tr>
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<td></td>
<td>LB + 0.5 M sorbitol</td>
<td>1880</td>
<td>81</td>
</tr>
</tbody>
</table>

*One U is defined as 1 μmol phosphoenolpyruvate consumed min\(^{-1}\). ND, Not determined.*
Analysis, expression and characterization of A. methanolica AroF

Previously (Alves et al., 1996), we reported the fortuitous cloning of an E. coli-type DAHP synthase gene from A. methanolica. Completion of the nucleotide sequence of this DAHP synthase gene revealed that it potentially encodes a protein with a subunit molecular mass of 37-8 kDa (Table 4). Alignments of this A. methanolica DAHP synthase with other E. coli-type DAHP synthase proteins revealed significant similarities. However, some residues within the AIGARTTESQ motif, highly conserved in E. coli-type proteins, are different in the A. methanolica and A. mediterranei DAHP synthase proteins (Table 4; URL: http://www.sanger.ac.uk/Software/Pfam).

Cloning of the A. methanolica DAHP synthase gene in the expression vector pET3b (pHK276) resulted in its successful expression in E. coli BL21(DE3). The DAHP synthase activity expressed was strongly feedback-inhibited by Tyr (similar to E. coli AroF: Shultz et al., 1984) and the protein was therefore referred to as AroF. It also displayed intermediate sensitivity toward Trp but was unaffected by the addition of Phe (Table 3). These characteristics resemble the feedback inhibition pattern of A. methanolica DS2 activity, as observed in mutant strain oPhe 83 (Table 3).

Expression of AroF was stimulated by one order of magnitude when 0.5 M sorbitol was added to the LB growth medium as a compatible solute (Table 3). The amount of AroF present in extracts of cells grown on LB with 0.5 M sorbitol was estimated to be about 20% of total protein as observed on SDS-PAGE gels (data not shown).

During PCR amplification of the aroF gene, a PCR fragment was isolated with an opal stop codon mutation at nucleotide position 907 of the ORF. Expression of this mutated aroF (pHK276-0), encoding a protein with a subunit molecular mass of 31.5 kDa instead of 37.8 kDa, yielded a protein insensitive to feedback inhibition. Rather, Phe and Trp now had a stimulatory effect on the activity of the truncated protein (Table 3).

Cloning and characterization of a second DAHP synthase gene

Attempts to clone the DS1 gene from chromosomal DNA of A. methanolica in Southern hybridization experiments using aroF as a probe failed (data not shown). Using PCR screening, we therefore searched for a plant-type DAHP synthase gene in A. methanolica.

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Fig. 2. Amino acid sequence alignment of PDT of A. methanolica with other monofunctional (putative) PDT proteins or bifunctional P-proteins. The CM domain of P-proteins has been deleted to acquire an unbiased alignment (apparent from residue numbering). '*' indicates positions with a single, fully conserved residue. ':' indicates that one of the following 'strong' groups is fully conserved: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY and FYW. '.' indicates that one of the following 'weaker' groups is fully conserved: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHRK, NEQHRK, FVLIM and HFY. Bold, overlined amino acid residues within the PDT sequence of A. methanolica indicate positions involved in Phe and Tyr binding. The conserved GALV and ESRP regions (Pohnert et al., 1999) are underlined. The percentage identity and similarity between A. methanolica PDT and each of the other (putative) PDT proteins is shown underneath the alignment.
Table 4. Partial alignment of amino acid sequences of (E. coli-type DAHP synthase) AroF of A. methanolica, DAHP synthase proteins closely related to AroF and the three E. coli DAHP synthase isozymes

The percentage identity and similarity between A. methanolica AroF and each of the other DAHP synthase proteins is shown. Aberrant residues within the AIGARTTESQ motif are shown in bold.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Protein</th>
<th>Accession no.</th>
<th>Total identity/similarity</th>
<th>AIGARTTESQ motif</th>
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<td>Amycolatopsis mediterranei</td>
<td>AroG</td>
<td>AAK28148</td>
<td>80/86</td>
<td>$IGARTAESQ$</td>
</tr>
<tr>
<td>Amycolatopsis orientalis</td>
<td>AroF</td>
<td>T17477</td>
<td>60/72</td>
<td>AIGARTVESQ</td>
</tr>
<tr>
<td>Ralstonia solanacearum</td>
<td>AroG</td>
<td>NP518864</td>
<td>58/72</td>
<td>AIGARTTESQ</td>
</tr>
<tr>
<td>Escherichia coli</td>
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<td>NP286475</td>
<td>53/67</td>
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<td>Escherichia coli</td>
<td>AroH</td>
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<td>49/64</td>
<td>AIGARTTESQ</td>
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<tr>
<td>Escherichia coli</td>
<td>AroF</td>
<td>NP289154</td>
<td>48/62</td>
<td>AIGARTTESQ</td>
</tr>
</tbody>
</table>

DNA sequence determination of 4.5 kb of the insert of the positive clone selected (see Methods), revealed an ORF of 1391 bp potentially encoding a 50.687 kDa protein, showing a high degree of sequence similarity toward other plant-type DAHP synthase proteins (Fig. 3). Heterologous expression of this ORF, designated aroG, was achieved in E. coli by cloning the gene into the expression vector pET3b (pHK287). DAHP synthase activity in cell extracts of E. coli strain BL21(DE3)/pHK287 was very sensitive to Trp inhibition, as observed for DS1 of A. methanolica (Table 3, wild-type situation). The amount of AroG present in extracts of cells of the heterologous host was estimated to be approximately 15%, as observed on SDS-PAGE gels (data not shown).

**Gel filtration analysis of A. methanolica AroF and AroG**

Extracts of mutant oFPhe83 expressed DS2 DAHP synthase protein in a monomeric form (Euverink et al., 1995a). The native molecular masses of both A. methanolica DAHP synthase proteins expressed in E. coli were determined by gel filtration chromatography on a Superdex-200 column. Elution times of the AroF and AroG activity peaks corresponded to molecular masses of 160 and 200 kDa, respectively (data not shown). In view of their subunit molecular masses (37.8 and 50.7 kDa, respectively), these results indicate that both proteins are expressed as tetramers in E. coli. No (significant) monomeric DAHP synthase activity could be determined in either of the extracts, corresponding to the DS2 activity found earlier in A. methanolica mutants GH141 and oFPhe83 (Euverink et al., 1995a).

**Effects of AroG on CM activity and feedback inhibition sensitivity**

Mutant strain GH141 displayed a strongly increased level of deregulated CM activity, not associated with a DAHP synthase enzyme. The dimeric CM activity of this mutant could be activated and its feedback control restored by addition of gel filtration fractions containing DAHP synthase activity (Euverink et al., 1995a).

Titration of Superdex-200 gel filtration fractions containing DS1 AroG (purity approx. 60%) to cell extracts of mutant GH141 stimulated CM activity by a factor of 5, also restoring its feedback inhibition sensitivity (Fig. 4). These effects were not observed in titration experiments with DS2 AroF to cell extracts of GH141.

**DISCUSSION**

This study shows that the phenylalanine analogue pFPhe can be used efficiently to screen for spontaneous A. methanolica mutants carrying mutant forms of PDT that have retained full activity but are devoid of Phe feedback control. Similar approaches have been applied to create Amycolatopsis strains overproducing primary and secondary metabolites originating from Phe or its precursors (data not shown). Furthermore, the results presented provide new information about amino acid residues directly or indirectly involved in Phe and Tyr binding by PDT. Four of the six mutations were found to affect both the stimulatory effects of Tyr and the inhibitory effects of Phe, indicating that both effector molecules bind at the same effector binding site of PDT. In the E. coli P-protein Phe binding was shown to occur in the C-terminal domain (Zhang et al., 1998) and, using isothermal titration calorimetry, interactions between Phe and a 12 kDa C-terminal P-protein fragment (residues 286 to 386) were established (Pohnert et al., 1999). Alignments of A. methanolica PDT and the E. coli P-protein indicate that all PDT feedback-inhibition-resistant mutants, except for the oFPhe84 PDT mutation A125G, are located within this (regulatory) domain (Fig. 2). The overall primary structures of A. methanolica PDT and the E. coli P-protein are similar, which may suggest a similar 3D structure of both proteins. Effector binding sites may thus be well conserved.
All other mutations found to affect A. methanolica PDT feedback inhibition control are located in the vicinity of – but not within – the highly conserved GALV and ESRP regions (E. coli P-protein residues 309–312 and 329–332, respectively) (Fig. 2). Mutations in the hydrophobic GALV region, decreasing its hydrophobic nature or introducing large side chains, impaired Phe binding in the E. coli P-protein (Pohnert et al., 1999). Conserved residues within the GALV and ESRP regions may also be important in Phe and Tyr binding in A. methanolica PDT, but mutations in these residues may well be detrimental for PDT activity due to incorrect protein folding. Obviously, such mutations will not be found when screening for fully active, deregulated PDT mutants.

The A. methanolica PDT mutant H227R is located near the ESRP region; mutants A198P, A198V, N200K and L209F are all located near the GALV region. A198, which is not a conserved residue, may be part of the hydrophobic binding pocket and the introduction of the more bulky Pro and Val side chains may prevent Phe and Tyr binding. The Lactococcus lactis and Aquifex aeolicus putative PDT proteins, however, do contain a Pro and a Val residue at their equivalent A198 positions, respectively. These proteins have not been characterized yet, however, and their sensitivity to feedback inhibition by Phe remains to be studied. It is unlikely that the charged N200, which is conserved in various PDT proteins, is (directly) involved in Phe (or Tyr) side chain binding. Also binding of the carboxyl group of the effector molecule is unlikely, since it is still activated by Tyr. The P-protein of E. coli contains a Gln at this position, which was shown to be essential for feedback inhibition.
regulation (Nelms et al., 1992). The well-conserved L209 may be part of a hydrophobic pocket, involved in binding of the Phe (and Tyr) side chain. Why Phe feedback inhibition-resistant mutant L209F is still capable of Tyr binding remains unclear.

Only two CM and DAHP synthase mutants were identified among the pFpHe-resistant strains, both with derepressed CM and DS2 activity levels. We failed to clone the A. methanolica gene encoding CM. Sequence analysis of the surrounding regions of the aroF and aroG genes revealed that the CM gene is not organized in an operon with one of these DAHP synthase genes. The derepression of both activities suggests that a common (unidentified) transcriptional regulator of both genes has been mutated. No AroG feedback-inhibition-resistant mutants were isolated, which also would have resulted in a deregulated CM activity. Mutant selection was directed toward active proteins, and deregulated AroG mutants may possess no or only low activities. Such mutants therefore may have escaped detection in the selection procedure used.

Several plant-type DAHP synthases have been identified in actinomycetes, e.g. in Streptomyces species (Walker et al., 1996). This type of DAHP synthase is often involved in the biosynthesis of secondary metabolites, such as the ansamycin antibiotic rifamycin in A. mediterranei (Yu et al., 2001), phenazine in P. aeruginosa (Pierson et al., 1995), auracin in Stigmatella aurantiaca (Silakowski et al., 2000), chloramphenicol in Streptomyces venezuelae (He et al., 2001) and ansatin and naphthomycin in Streptomyces coelicolor (Chen et al., 1999). In contrast, in Xanthomonas campestris a plant-type enzyme functions as the sole DAHP synthase supporting aromatic amino acid biosynthesis (Gosset et al., 2001).

Expression of both A. methanolica DAHP synthase proteins in E. coli was successful. Addition of 0-5 M sorbitol even further increased DAHP synthase activity by a factor of 8. The inhibition pattern of AroG was similar to that of DS1 activity, with Trp as its main effector, and that of AroF was similar to DS2 activity, with Tyr showing the strongest inhibition.

Gel filtration experiments show that AroF is expressed in E. coli as a tetramer. The protein responsible for DS2 activity found in mutant A. methanolica strain oFpHe83, however, was monomeric with a molecular mass of approximately 42 kDa. Possibly, AroF expressed in E. coli is assembled as a tetramer, while it remains a monomer in its original host. PCR fragment analysis did not indicate the presence of a second plant-type DAHP synthase gene, since several cloned PCR fragments were identical in DNA sequence. Furthermore, feedback inhibition patterns of DAHP synthase activities in wild-type and E. coli/pHK276 were similar (Table 3).

From the CM–DAHP synthase titration experiments we conclude that AroG is associated with CM to form a heterohexameric enzyme complex. AroG clearly activates CM activity up to a factor of 5 and restores its feedback sensitivity to Phe and Tyr, as observed in the A. methanolica wild-type strain. The close association of the AroG and CM proteins, and the feedback inhibition pattern of AroG, show that AroG activity supports aromatic amino acid biosynthesis in A. methanolica. However, AroF activity also potentially makes an important contribution, becoming derepressed under Phe limiting conditions (this study; Euverink et al., 1995a).

### REFERENCES


