Biochemical and functional characterization of Nudix hydrolase enzymes with novel regulatory roles in Gram positive methylotrophic bacteria

Kloosterman, Harmen

Chapter 7

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

H. Kloosterman, G.I. Hessels, J.W. Vrijbloed, G.J. Euverink and L. Dijkhuizen

Microbiology (2003) 149(11): 3321-3330

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 *para*-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 bi-functional 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, de-repressed 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 microorganisms 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 mC_7N 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;Kim *et al.*, 1996b), and to *Amycolatopsis orientalis*, producing the glycopeptide antibiotics chloroeremomycin and vancomycin, containing for instance Tyr (van Wageningen AM *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 key regulatory enzymes (Euverink *et al.*, 1995a;Euverink *et al.*, 1995b;Vrijbloed *et al.*, 1995d;Euverink *et al.*, 1996).

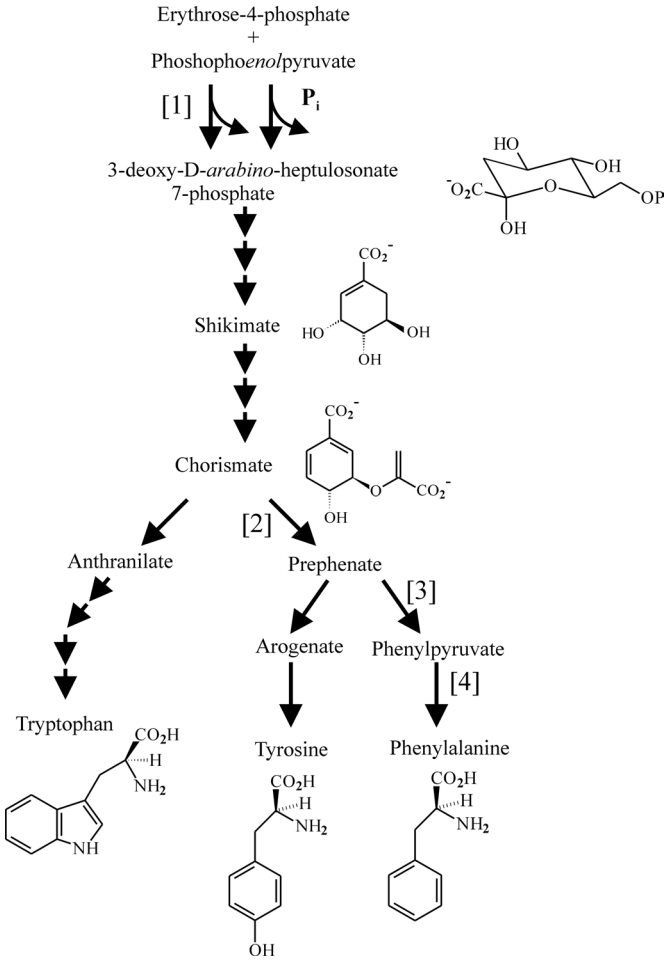

Figure 1. Biosynthesis of aromatic amino acids via the common shikimate pathway, and the specific amino acid terminal pathways. [1] DAHP-synthase, [2] chorismate mutase, [3] prephenate dehydratase and [4] phenylalanine amino-transferase.

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 (E4P) and the glycolytic pathway intermediate phospho*enol*pyruvate (PEP) 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 *E. coli* (Ray and Bauerle, 1991;McCandliss *et al.*, 1978;Schoner and Herrmann, 1976), or as a bifunctional protein exhibiting both DAHP-synthase and CM activities, e.g. in *Brevibacterium flavum* (Sugimoto and Shiio, 1980) and in *A. methanolica* (Euverink *et al.*, 1995a). Several organisms contain 2 or 3 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 *Ps. 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, BAB98383), similar to the rifamycin producer *A. mediterranei* (AAK28148, AAC01718). The plant type DAHP-synthase encoding ORF, 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. Both enzyme activities can be separated with Q-sepharose anion-exchange chromatography, yielding a dimeric CM protein with a 5-fold reduced activity that no longer is 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 *ortho*-fluoro-DL-phenylalanine resistant mutant (oFPhe83) subsequently was characterized that showed high levels of DS2 and dimeric CM activity, both in the presence and absence of Phe, suggesting a regulatory mutation that de-repressed 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 feedback inhibition (but not Phe) 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.*, 1995d).

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 a second DAHP-synthase in *A. methanolica* and molecular 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.

Materials and methods

Microorganisms, cultivation and DNA manipulations

Amycolatopsis methanolica wild-type (NCIB 11946) and derived phenylalanine analogue resistant mutants (this study) were cultivated as described (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; IPTG, 0.5 mM) at 37°C and 30°C, respectively. Methods for DNA handling, modification and cloning were performed as in (Sambrook *et al.*, 1989). Plasmids and cloning vectors used are listed in Table 1.

Table 1. Plasmids and vectors used in this study.

Plasmid or vector	Characteristics	Reference/origin
pBlueScript II KS-	Cloning vector	Stratagene, La Jolla, Ca
pET3b	Expression vector	Novagen, Madison, Wi
PZErO-2.0	Cloning vector	Invitrogen,Carlsbad, Ca
pHK200	*pdt* cloned in pBlueScript II KS-	This study
pHK255(1-10)	*pdt* wild type and 9 mutants in pET3b	This study
pHK276	*aroF* cloned in pET3b	This study
pHK276-0	*aroF* containing stop codon at bp position 907 cloned in pET3b	This study
pHK287	*aroG* cloned in pET3b	This study

Isolation of phenylalanine-analogue-resistant mutants

A. methanolica mutants resistant to toxic pFPhe (5 mg/ml, 32.8 mM) were isolated on 10 mM glucose mineral agar plates with filter-sterilized analogue. Plates were inoculated with approximately 5 x 10^7 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 of the lysate 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 of 2.0 M NaOH was added and the absorbance of phenylpyruvate was measured at 320 nm (ε_{320}[phenylpyruvate]= 17.5 *10^3 $M^{-1}.cm^{-1}$) (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 an1.58 M solution) the phenylpyruvate formed was determined at A_{320}. DAHP synthase (EC 2.5.1.54) activity was assayed as described (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;Euverink *et al.*, 1995b).

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 AB. The samples were run on the A.L.F-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 (Natl. Library of Medicine, Washington, D.C.).

PCR primers used to clone *aroG*

Using alignments of plant-type (putative) DAHP-synthase proteins (Fig. 4), degenerate primers were designed on the basis of two conserved motifs. PLDS1: 5'-GG(G/C)(A/C)G(G/C)ATCGC (G/C)GG(G/C)CA(G/A)-3' and PLDS2: 5'-(G/C)GTGTT(G/C)CCGTGCAT(G/C)GG-3'. PCR experiments with these primers, using *A. methanolica* DNA as template, yielded a single DNA-fragment of approx. 730 bp with strong sequence similarity to plant type DAHP-synthase genes. Specific primers PLDS3 and PLDS4 were designed (Fig. 4), and used to screen an *A.methanolica* genomic library.

Construction and PCR screening of genomic DNA library

An *A. methanolica* genomic DNA library was constructed using partially *Sau*3A digested chromosomal DNA, sized by sucrose gradient centrifugation. Fragments of 8-12 kb were cloned in the *Bam*HI 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 (average insert size 7.8 kb; insert frequency 92%).

Gene library screening for the *aroG* gene was performed by PCR, using primers PLDS3 (5'-TCGCACTTCCTGTGGAT-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 approx. 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 supplied by Qiagen (Basel, Switzerland), via an alkaline transfer method (Sambrook *et al.*, 1989). Southern hybridization was performed at 65^0C, using the entire *aroF* gene (for DS2) of *A. methanolica* as a probe. Radioactive probe labeling was performed with the high prime DNA labeling kit from Boehringer Mannheim. Following hybridization the membrane was washed at 65^0C with 2xSSC (1xSSC is 0.15M NaCl and 0.015M sodium citrate) containing 0.5% (w/v) sodium dodecyl sulphate (SDS) for 10 min, twice with 1xSSC containing 0.5% (w/v) SDS at room temperature and three times with 0.3xSSC containing 0.5% (w/v) SDS at room temperature.

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

Analytical methods
Protein concentrations were determined with the protein determination kit from Bio-Rad, using bovine serum albumin as standard (Richmond, CA, USA) (Bradford, 1976).

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 amino acid sequence is Q44104.

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 *A. methanolica* wild type 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 L. *et al.*, 1990b). 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 seven days (mutant frequency ~10^{-5}). 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 analyzed 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 two by a similar amount of Tyr (Table 2) (Euverink *et al.*, 1995b). Of 76 colonies tested, 7 had (completely) lost Phe feedback inhibition sensitivity, while 5 of those 7 mutants also had completely lost the stimulatory effect of Tyr. Similar results were obtained for PDT enzymes in 2 previously (Euverink *et al.*, 1995b) isolated FPhe resistant *A. methanolica* mutants, strains pFPhe32 and oFPhe84. None of these 9 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 de-repression 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 therefore were 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 (de-repressed) DS2 activity (Table 3). Screening of the remaining 74 pFPhe resistant mutant strains did not yield a single mutant with deregulated DS1 activity.

Table 2. Characteristic properties of deregulated prephenate dehydratase mutant proteins in spontaneous pFPhe and oFPhe analogue resistant mutants of *Amycolatopsis methanolica*. Feedback inhibition pattern of PDT enzymes in *A. methanolica* cell extracts determined in the presence of 1 mM Phe or Tyr.

Mutant strain	Phe inhibition[b] (%)	Tyr activation[b] (%)	Mutation (nucleotide pos.)	Mutation (amino acid pos.)
PDT-Wt	30	225	-	-
pFPhe32[a]	89	209	C625T	L209F
oFPhe84[a]	81	96	C374G	A125G
pFPhe17	86	202	C625T	L209F
pFPhe54	117	110	C593T	A198V
pFPhe55	108	101	G592C	A198P
pFPhe60	98	114	A680G	H227R
pFPhe82	97	210	C600G	N200K
pFPhe114	129	117	C593T	A198V
pFPhe118	105	113	A680G	H227R

[a] Isolated by Euverink et al. (Euverink *et al.*, 1995b); [b] Activity relative to incubation without effector

Sequence analysis and heterologous expression of deregulated PDT mutants

Cloning and nucleotide sequencing of the 9 mutant *pdt* genes revealed point mutations in 6 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.

```
Aqaeol   91 -----IKVAYLGPKATFTHQAALE-----FFGFSAH----YTPCSTIRDVFVEVETKRADYGVVPVENTIEGVVNYTLD
Psstu    95 -----LRVAYLGPEGTFSQAAALK-----HFGHSVI----SKPMAAIDEVFREVVAGAVNFGVVPVENSTEGAVNHTLD
Myclep    1 --MSVARIAYLGPEGTFTEAALLRMTAAGLVPDTGPDGLRRWPTESTPAALDAVRGGAADYACVPIENSIDGSVAPTLD
Myctub    1 ----MVRIAYLGPEGTFTEAALVRMVAAGLVPETGPDALQRMPVESAPAALAAVRDGGADYACVPIENSIDGSVLPTLD
Corglu    1 MSDAPTVVAYLGPAGTFTEEALYKFADAGVFGD-GE--IEQLPAKSPQEAVDAVRHGTAQFAVVAIENFVDGPVTPTFD
Scoel     1 ---MPASYAYLGPEGTFTEVAL-R-----TLPETAT--RELVPYVSVQSALDAVRTGEAEAAFVPIENSVEGGITTTLD
Ameth     1 ----MSRIAYFGPVGTFTEQAA-R-----TFMAAGD---ELVAAETIPKALDAVRRGEADAACVPVENSVEGAVPATLD
Ecoli   104 -----ARIAFLGPKGSYSHLAA-R-----QYAARHFEQFIESGCAKFADIFNQVETGQADYAVVPIENTSSGAINDVYD
Haein   104 -----LHIAFLGKRGSYSNLAA-R-----NYAARYQKQFVELGCQSFEQVFEKVQTGEADFGVLPLENTTSGAINEVYD
Bacsu     1 -----MKVGYLGPAATFTHLAVSS-----CFQNGAE----HVAYRTIPECIDAAVAGEVDFAFVPLENALEGSVNLTID
Lacla     1 -----MKIAYLGPRGSFCSVVAEA-----AFKS-EE----LYSYATILDVIEAYNEGECDFALVPIENSTEGTVNMSID
                     .::*  .::   .                              .        : . :.:**  .* :    *

Aqaeol      MF-LESDVKIAGEIVIPITLHLLS-AS--DSIENVEKVYSHKMALAQCRSWLEKNLPSVQVIEVESTAKACEIALEDER
Psstu       SF-LEHDIVICGEVELRIHHHLLVGET--TKTDRITRIYSHAQSLAQCRKWLDAHYPNVERVAVSSNADAAKRVKSEWN
Myclep      NLAIGSPLQVFAETTLDVEFNIVVKPG--ITAADIRTLAAFPVAAAQVRQWLAAHLAGAELRPAYSNADAARQVAYGQV
Myctub      SLAIGVRLQVFAETTLDVTFSIVVKPG--RNAADVRTLAAFPVAAAQVRQWLAAHLPAADLRPAYSNADAARQVADGLV
Corglu      ALDQGSNVQIIAEEELDIAFSIMVRPG--TSLADVKTLATHPVGYQQVKNWMATTIPDAMYLSASSNGAGAQMVAEGTA
Scoel       ELVAGQPLMIYREVLLSITFALLVRPG--TKLSDIKTVTAHPAAQPQVRNWIKAHLPDVAWESAASNADGARLVQEGRY
Ameth       SLAVGEPLIGVAEALLPVHFSVLTR----DDVGEIRTVASHPHALAQVRKWLEDNLPGARVV<b>A</b>AGSTAAAAVAVQAGEF
Ecoli       LL-QHTSLSIVGEMTLTIDHCLLVSGT--TDLSTINTVYSHPQPFQQCSKFLNR-YPHWKIEYTESTSAAMEKVAQAKS
Haein       LL-QHTDLSLVGELAYPIKHCVLVNDK--TDLNQIDTLYSHPQVIQQCSQFIHS-LDRVHIEYCESSSHAMQLVASLNK
Bacsu       YLIHEQPLPIVGEMTLPIHQHLLVHPSRENAWKELDKIYSHSHAIAQCHKFLHRHFPSVPYEYANSTGAAAKFVSDHPE
Lacla       KIFHDSNAKVVAEFVLPISQNLLAVS----KEQKIEHIYSHPQALAQTRVYLRKFYPQAQVEITESTSAAAEFVKNNPD
             :         *    :   ::          :  : :.     *   ::            *.. .   .

Aqaeol      A--GAVASEVAAYTYHLNILARNIQDSGDNFTRFLVIAKR------D-LKPTGSDKTSILFG--VKDEPGALYKALEVF
Psstu       S--AAIAGDMAAQLYGLSKLAEKIEDRPDNSTRFLIIGSQ------E-VPPTGDDKTSIIVS--MRNKPGALHELLMPF
Myclep      D--AAVTSPLAATRWGLIALAAGIVDEPNARTRFVLVGMP-----GPPPARTGTDRTSAVLR--IDNAPGMLVAALAEF
Myctub      D--AAVTSPLAAARWGLAALADGVVDESNARTRFVLVGRP-----GPPPARTGADRTSAVLR--IDNQPGALVAALAEF
Corglu      D--AAAAPSRAAELFGLERLVDDVADVRGARTRFVAVQAQ-----AAVSEPTGHDRTSVIFS--LPNVPGSLVRALNEF
Scoel       D--AAFAGEFAAERYGLEVLEADIHDAENAQTRFVLVGRP-----ARPAAPTGADKTSVVLWQ-RDDHPGGLRDLLGEF
Ameth       D--AAVTAPVAVEHYPLKVLATEVADVRDARTRFLLMRRPP----VVLPEPTGADRTSIVAA--<b>A</b>A<b>N</b>RTGTLAEL<b>L</b>TEL
Ecoli       PHVAALGSEAGGTLYGLQVLERIEANQRQNFTRFVVLARK-----AINVSDQVPAKTTLLMA--TGQQAGALVEALLVL
Haein       PNIAALGNEDGGKLYGLSVLKTNIANQENNITRFIVVAKE-----PREVSSQIPTKTLLLMT--TSQQAGALVDALLVF
Bacsu       LNIGVIANDMAASTYELKIVKRDIQDYRDNHTRFVILSPDENISFEVNSKLSSRPKTTLMVMLPQDDQSGALHRVLSAF
Lacla       LPAAAVANSFAAKMYDLEFIAENIQDLAGNSTRFWLLGKEK----QSFDLNQTKDKVTLALT-LPDNLPGALHKAISVF
               ..     .   : *  :     :     ***  :                  :.         : .* *   :  :

Aqaeol      YKHGINLTKIESRPSKKKAWDYVFFVDLEGHKEE-ERVEKALKELKEKTQFLKVLGSYPKALLQE--------------
Psstu       HSNGIDLTRIETRPSRSGKWTYVFFIDCMGHYQD-PLIKDVLEKIDHEAVALKVLGSYPKAVL----------------
Myclep      GIRGIDLTRIESRPTRTELGTYLFFVDCVGHIDD-GVVAEALKALHRRCADVCYLGSWPAGLATGPTVSPPPPDEASRW
Myctub      GIRGIDLTRIESRPTRTELGTYLFFVDCVGHIDD-EAVAEALKAVHRRCADVRYLGSWPTGPAAG--AQPPLVDEASRW
Corglu      AIRGVDLTRIESRPTRKVFGTYRFHLDISGHIRD-IPVAEALRALHLQAEELVFVGSWPSNRAED--S-TPQTDQ----
Scoel       ATRGINLMLLQSRPTGAGIGNYCFCIDAEGHISD-RRVADALMGLKRTCLQVRYLGSYPRAEVTP-----ADVDALRPG
Ameth       ATRGINLTRLDARP<b>H</b>KQNFGEYRFFIDFEGHVAE-PRIADALAALRRRCRDVRFLGSFARADGVA-----ATIEPAAR-
Ecoli       RNHNLIMTRLESRPIHGNPWEEMFYLDIQANLES-AEMQKALKELGEITRSMKVLGCYPSENVVP-------VDPT---
Haein       KKHQINMTKLESRPIYGKPWEEMFYLEIEANIHH-PDTKQALEELKNYSNYLKILGCYPSEIVKP-------VSV----
Bacsu       SWRNLNLSKIESRPTKTGLGHYFFIIDIEKAFDD-VLIPGAMQELEALGCKVRLLGAYQSYQL----------------
Lacla       AWRDIDMTKIESRPLRTRLGQYFFIIDLENNATNSLKIPYALEELAGLGVNVRLLGNYSVYSLGEV--------------
              . : :  :::**         * ::             .:  :      :  :* :

Aqaeol      ------------------------
Psstu       ------------------------
Myclep      LARLRAGKPDQASE-PG--GGKL-
Myctub      LARLRAGKPEQTLVRPDDQGAQA-
Corglu      LAKLH--KADEWVRAASE-GRKLN
Scoel       TSDEAFVAAADWVARCQD-GRF--
Ameth       --NEDFTDAADWVAAVQR-GEQA-
Ecoli       ------------------------
Haein       ------------------------
Bacsu       ------------------------
Lacla       ------------------------
```

(continued on page 107)

Organism	Abbrev.	Accession	Identity	Similarity
Amycolatopsis methanolica	Ameth	Q 44104	100	100
Streptomyces coelicolor	Scoel	NP 628146	46	59
Mycobacterium leprae	Myclep	NP 301183	44	58
Mycobacterium tuberculosis	Myctub	NP 338499	45	59
Corynebacterium glutamicum	Corglu	NP 602088	37	56
Aquifex aeolicus	Aqaeol	NP 213648	36	51
Pseudomonas stutzeri	Psstu	A 44764	36	49
Bacillus subtilis	Bacsu	NP 390668	33	51
Lactococcus lactis	Lacla	NP 267898	32	51
Escherichia coli	Ecoli	NP 311489	30	46
Haemophilus influenzae	Haein	NP 439303	26	46

Figure 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, NDEQHK, NEQHRK, FVLIM and HFY.
Bold face 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.

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 either of the aromatic amino acids (1 mM final concentrations) is indicated by the relative residual activities.

Strain/plasmid	Medium	Spec. Act. (mU.mg prot^{-1})*	Activity with 1 mM: Phe (%)	Tyr (%)	Trp (%)
A. methanolica:					
Wild type	Mineral medium	22	65	75	10
oFPhe83	Mineral medium	188	100	17	61
pFPhe4	Mineral medium	215	103	13	100
pFPhe25	Mineral medium	204	95	21	91
E. coli:					
pET3b (neg. control)	LB	4	n.d.$^{\#}$	n.d.	n.d.
pHK276	LB	363	102	16	34
	LB+0.5 M sorbitol	3267	n.d.	n.d.	n.d.
pHK276-0	LB	377	371	76	181
pHK287	LB	274	83	91	38
pHK287	LB+0.5 M sorbitol	1880	81	92	35

*One U is defined as 1 μmol PEP consumed .min^{-1},$^{\#}$ not determined.

Chapter 7

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 Mr 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* strain 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 oFPhe 83 (Table 3).

Expression of *aroF* was stimulated 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).

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.

Organism	Protein	Accession	Total Identity/similarity	AIGARTESQ motif
Amycolatopsis methanolica	AroF	AY382157	100/100	**S**IGART**AA**SQ
Amycolatopsis mediterranei		AAK28148	80/86	**S**IGART**AA**SQ
Amycolatopsis orientalis		T17477	60/72	AIGARTVESQ
Ralstonia solanacearum		NP518864	58/72	AIGARTTESQ
Escherichia coli	AroG	NP286475	53/67	AIGARTTESQ
Escherichia coli	AroH	NP288138	49/64	AIGARTTESQ
Escherichia coli	AroF	NP289154	48/62	AIGARTTESQ

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.*

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).

```
Ameth      MNWTVDVPVDTLPELPPLPPELRARLDDALSRPAAQQPEWPDADAVGRVRHLLEAVPPITVPAEIDRLQDRLAMVARGEA
Amedi      MNWTVDVPVDTLPELPPLPPELRTRLDKALALPAAQQPEWPDPEATRRVRGVLESVPPITVPAEIDRLKSRLAMVARGEA
Actpre     MNWTVDVPVDTLPEL-------RSRLDDALGRPAAQQPEWPDADLARRVRAVLESVPPITVPAEIDRLRENLAAVARGEA
Myctub     MNWTVDIPIDQLPSLPPLPTDLRTRLDAALAKPAAQQPTWP-ADQALAMRTVLESVPPVTVPSEIVRLQEQLAQVAKGEA
Corglu     MSWTVDIPKEVLPDLPPLPEGMQQQFEDTISRDAKQQPTWD-RAQAENVRKILESVPPIVVAPEVLELKQKLADVANGKA
Scoel      ----MTVNAKTSPSAG----------NTWRDLPAAQQPEYPDTEALRAVIADLESYPPLVFAGECDQLRARMAAVAKGEA
Hepylo     -------MSNTTWSP-----------TSWHSFKIEQHPTYKDKQELERVKKELHSYPPLVFAGEARNLQERLAQVIDNKA
Psfluor    -----------MEDL----------LKRVLKCEALQQPQWSEPSQLHDAQAYLRDSASLIRVEDILVLRATLARVAAGEA
                      .                     *:* :           *.  ..:    :   *:  :* *  .:*

Ameth      FLLQGGDCAETFESNTEPHIRANLRTLLQMAVVLTYGASLPVVKV<b>GRIAGQ</b>YAKPRS---NSTDALGLPVYRGDIVNSLV
Amedi      FLLQGGDCAETFESNTEPHIRANLRTLLQMAVVLTYGASLPVVKV<b>GRIAGQ</b>YAKPRS---AATDALGLPVYRGDIINSLV
Actpre     FMLQGGDCAETFADNTEPHIRANVRTLLQMAVVLTYGASLPVVKI<b>GRIAGQ</b>YAKPRS---SGTDALGLPSYRGDIVNSLV
Myctub     FLLQGGDCAETFMDNTEPHIRGNVRALLQMAVVLTYGASMPVVKVA<b>RIAGQ</b>YAKPRS---ADIDALGLRSYRGDMINGFA
Corglu     FLLQGGDCAETFESNTEPHIRANVKTLLQMAVVLTYGASTPVIKMA<b>RIAGQ</b>YAKPRS---SDLDGNGLPNYRGDIVNGVE
Scoel      FLLQGGDCAEAFDAVSADHIRNKLKTLLQMGAVLTYAASVPVVKV<b>GRIAGQ</b>YSKPRSKPTETRDGVTLPTYRGDSVNGFD
Hepylo     FLLQGGDCAESFSQFSANRIRDMFKVMMQMAIVLTFAGSIPIVKV<b>GRIAGQ</b>FAKPRSNATEMLDNEEVLSYRGDIINGIS
Psfluor    MIIQCGDCAEDMDESAADHVTRKAALLDMLAGTFRLVTQQPVVRV<b>GRIAGQ</b>FAKPRSNHSERIGDVELPVYRGDMVNGRD
           :::* ***** :   :  ::      :  :. .:    . *:::::.*****::****      .   :  **** :*.

Ameth      AKPELRVPDPGRMIRAYANSGAAMNLVRALTGAGMADLAQVHDWNKDFVRTSPAGERYEALAGEIDRGLRFMSACGVSDT
Amedi      AKPELRVPDPGRMIRAYANAGAAMNLVRALTGAGMADLHQVHDWNKDFVSASPAAQRFEALANEIDRGLRFMSACGVTDT
Actpre     PTPEARVADPGRMIRAYANAGAAMNLLRAMTTAGMADLHRLHDWNKDFVRTSPAGERYEALAAEIDRGLRFMSACGVNDS
Myctub     PDAAAREHDPSRLVRAYANASAAMNLVRALTSSGLASLHLVHDWNREFVRTSPAGARYEALATEIDRGLRFMSACGVADR
Corglu     ATPEARRHDPARMIRAYANASAAMNLVRALTSSGTADLYRLSEWNREFVANSPAGARYEALAREIDSGLRFMEACGVSDE
Scoel      FTEAARIPDPERLKRMYHASASTLNLVRAFTTGGYADLRQVHAWNQDFVKSSPSGQRYEQLAREIDNALNFMRACGTDPA
Hepylo     KK--EREPNPERMLKAYHQSVATLNLIRAFAQGGLADLEQVHRFNLDFVKNNDFGQKYQQIADRITQALGFMRACGVEIE
Psfluor    AVLGHRQHDAQRLVRGYR---AAQDIMQHL---G---------WK------EPSGQ--EQLT---------------GSP
                *  :. *: : *    :: :::: :   *         ::      .  .   : ::

                                                    PLDS3
                                                    ----->
Ameth      S---LHSTEIFASHEALLLDYERAMLRMDNANAGNPKLYNLSSHFLWIGERTRQLDGAHIALAELLANPIGVKIGPTTTP
Amedi      S---LQSTEIFASHEALLLDYERSMLRLDQADATNPKLYNLSSHFLWVGERTRQLDGAHIAFAELLANPIGLKIGPTTTP
Actpre     S---LHTTEIFASHEALLLDYERALLRLDTR-GDEPKLYDLSSHFLWIGERTKQLDGAHIAFAELLANPIGLKIGPSTTP
Myctub     N---LQTAEIYASHEALVLDYERAMLRLSDGDDGEPQLFDLSAHTVWIGERTRQIDGAHIAFAQVIANPVGVKLGPNMTP
Corglu     S---LRAADIYCSHEALLVDYERSMLRLATDEEGNEELYDLSAHQLWIGERTRGMDDFHVNFASMISNPIGIKIGPGITP
Scoel      E---FQTVEFFSSHEALLLDYESALTRVDSR---TGQLYDVSGHMVWIGERTRQLDHAHIEFASRIRNPIGIKLGPSTTA
Hepylo     RTPILREVEFYTSHEALLLHYEEPLVRKDSL---TNQFYDCSAHMLWIGERTRDPKGAHVEFLRGVCNPIGVKIGPNASV
Psfluor    A---------WTSHEMLVLDYELPQVRRDEQ----GRTFLGSTHWPWIGERTRQLTGAHVALLSEVLNPVACKVGPDITQ
                   : *** *::.** .  *         . :  * *  *:****:     *: :   : **:. *:**  :

                                                      PLDS4
                                                     <-----
Ameth      EQAVEYVERLDPRNQPGRVTLISRMGNGKVRDVLPAIVEKVEATGHKVIWQC<b>DPMHGNT</b>HESSSGYKTRHFDRIIDEVQG
Amedi      EQALEYVERLDPRSEPGRLTLIARMGNGKVREVLPAIVEKVEASGHKVIWQC<b>DPMHGNT</b>HESSTGYKTRHFDRIVDEVQG
Actpre     EMAVEYVERLDPHNQPGRLTLIIRMGNGKVRDVLPAIVEKVTASGHKVIWQC<b>DPMHGNT</b>HESSTGYKTRHFDRIVDEVQG
Myctub     ELAVEYVERLDPHNKPGRLTLVSRMGNHKVRDLLPPIVEKVQATGHQVIWQC<b>DPMHGNT</b>HESSTGFKTRHFDRIVDEVQG
Corglu     EEAVAYADKLDPNFEPGRLTIVARMGHDKVRSVLPGVIQAVEASGHKVIWQS<b>DPMHGNT</b>FTASNGYKTRHFDKVIDEVQG
Scoel      EEALQYIERLDPEREPGRLTFIVRMGADKIRDKLPELVEKVTASGATVAWIT<b>DPMHGNT</b>YEAASGHKTRRFDDVLDEVKG
Hepylo     SEVLELCDVLNPRNIKGRLNLIVRMGSKMIKERLPKLLQGVLEEKRHILWSI<b>DPMHGNT</b>VKTSLGVKTRAFDSVLDEVKS
Psfluor    DQLLSLCERLDPRREPGRLTLIARMGAHKVADRLPPLVEAVRRAGHKIIWLS<b>DPMHGNT</b>IVAPCGNKTRMVQTITDEITA
           .  :   : *:*.   **:.:: ***   : . ** ::: *      : *  *******  :. * *** .: : **: .

Ameth      FFEVHHKLGSYPGGIHIELTGEDVTECLGGAQEISDSDLAGRYETACDPRLNTQQSLELAFLVAEMLRG-----
Amedi      FFEVHNKLGTYPGGIHVELTGEDVTECLGGAQEISDVDLSGRYETACDPRLNTQQSLELAFLVAEMLRG-----
Actpre     FFEVHRRLGTHPGGIHIELTGEDVTECLGGAQEISDTDLAGRYETACDPRLNTQQSLELAFLVAEMLRS-----
Myctub     FFEVHRALGTHPGGIHVEITGENVTECLGGAQDISETDLAGRYETACDPRLNTQQSLELAFLVAEMLRD-----
Corglu     FFEVHRALGTHPGGIHIEFTGEDVTECLGGAEDITDVDLPGRYESACDPRLNTQQSLELAFLVAEMLRN-----
Scoel      FFEVHKSLGTHPGGIHVELTGDDVTECVGGGDEIFVDDLHQRYETACDPRLNRSQSLDLAFLVAEMYRDQ----
Hepylo     FFEIHRAEGSLASGVHLEMTGENVTECIGGSQAITEEGLSCHYYTQCDPRLNATQALELAFLIADMLKKQHA--
Psfluor    FKHAVVSAGGVAGGLHLETTPDDVSECASDAAGLG--QVGSHYKSLCDPRLNPWQAITAVMAWKACPPPSFVSL
           * .     *  ..*:*:* * ::*:** ...  :    :  :* : ******  *::  .:
```

(continued on page 110)

Organism	Abbreviation	Accession	Identity	Similarity
Amycolatopsis methanolica	Ameth	AY382158	100	100
Amycolatopsis mediterranei	Amedi	AAF 70331	89	95
Actinosynnema pretiosum	Actpre	AAC 13561	85	91
Mycobacterium tuberculosis	Myctub	NP 216694	74	86
Corynebacterium glutamicum	Corglu	BAB 99571	67	81
Streptomyces coelicolor	Scoel	NP 626372	60	73
Helicobacter pylori	Hepylo	NP 206934	47	65
Pseudomonas fluorescens	Psfluor	Q 51789	37	50

Figure 3. Amino acid sequence alignment of plant-type DAHP-synthase proteins. For the meaning of alignment indicators, see legend Fig. 2. The percentage similarity between AroG and each of the other DAHP-synthase proteins is shown underneath. Bold characters refer to conserved motifs used to design PCR primers PLDS1 and PLDS2. The positions of primers PLDS3 and PLDS4 are indicated by arrows.

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 feedback 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 approx. 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 weights 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 Mr's of 160 and 200 kDa, respectively (data not shown). In view of their subunit Mr (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 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.

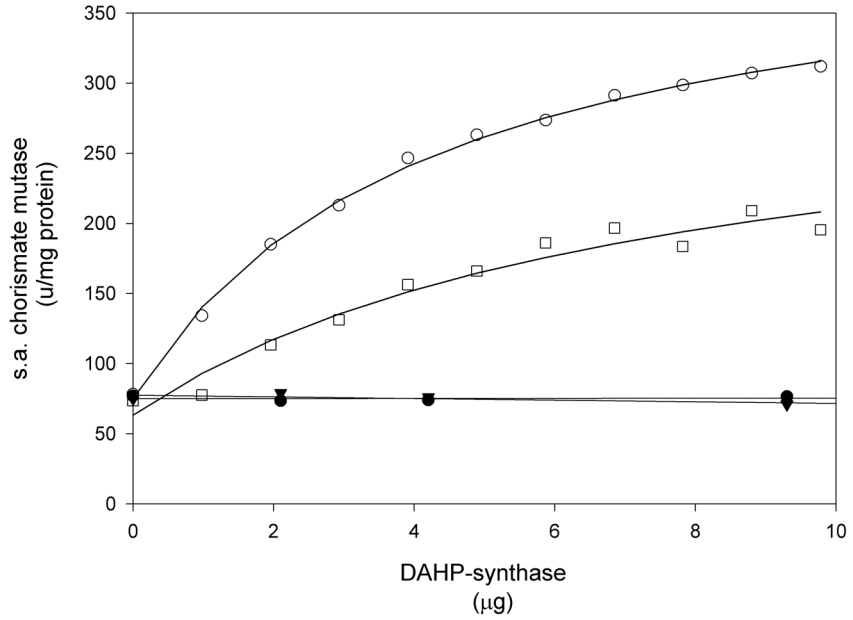

Figure 4. Titration experiment of Superdex-200 gel filtration fractions containing *A. methanolica* AroG and AroF proteins to cell extracts (67 μg protein) of mutant GH141. CM activities were determined with AroG in the presence (open squares) or absence (open circles) of 1 mM Phe and Tyr, and with AroF in the presence (closed triangles) or absence (closed circles) of 1 mM Phe and Tyr.

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 (precursors of) Phe (data not shown). Furthermore, the data presented provides 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 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 -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 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 de-repressed 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 de-repression 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 *Ps. aureofaciens* (Pierson *et al.*, 1995), aurachin in *S. aurantiaca* (Silakowski *et al.*, 2000), chloramphenicol in *Streptomyces venezuelae* (He *et al.*, 2001) and ansatrienin and naphthomycin in *Streptomyces collinus* (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 Mr of approx. 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 hetero hexameric enzyme complex. AroG clearly activates CM activity up to a factor 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*. Also AroF activity, however, potentially makes an important contribution, becoming de-repressed under Phe limiting conditions (this study) (Euverink *et al.*, 1995a).