Biosynthesis of phenylalanine and tyrosine in the methylotrophic actinomycete amycolatopsis methanolica
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1995

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 2

ISOLATION AND ANALYSIS OF MUTANTS OF THE METHYLOTROPIC ACTINOMYCETE AMYCOLATOPSIS METHANOLICA BLOCKED IN AROMATIC AMINO ACID BIOSYNTHESIS


This chapter has been submitted to FEMS Microbiology Letters
Mutants of the actinomycete Amycolatopsis methanolica blocked in aromatic amino acid biosynthesis were isolated using brief ultrasonic treatments to obtain single cells. After UV irradiation auxotrophic mutants were selected as pinpoint colonies on mineral agar with only 1 mg L\(^{-1}\) of the supplements. Mutant characterization provided unambiguous evidence that L-Tyr is synthesized via arogenate only and L-Phe via phenylpyruvate only. The efficiency of chromosomal DNA marker exchange was highest in matings with A. methanolica mutants that lacked the plasmid pMEA300.

**INTRODUCTION**

Current knowledge of the biochemistry and regulation of primary metabolism in actinomycetes is limited but required for the rational improvement of strains overproducing aromatic amino acids and derived secondary metabolites (e.g. antibiotics) (Bushell, 1989; Kell et al., 1989; Rowlands, 1984). We have initiated studies of glucose (Alves et al., 1994), methanol (H.J. Hektor and L. Dijkhuizen, unpublished; Van Ophem et al., 1991), and aromatic amino acid metabolism (Abou-Zeid et al., 1995; Euverink et al., 1992, 1995a, 1995c) in the methylotrophic actinomycete Amycolatopsis methanolica (De Boer et al., 1990a).

The isolation of mutants in biosynthetic pathways allows identification of the separate enzyme steps and their overall organization. Attempts to isolate auxotrophic mutants of Amycolatopsis mediterranei, however, have met with limited success only (Ghisalba et al., 1984). Selection of auxotrophic mutants of pseudomycelium-forming bacteria such as actinomycetes necessitates isolation of spores or protoplasts (Hopwood et al., 1985). This turned out to be rather difficult for A. methanolica (De Boer et al., 1990a) and during our studies of L-Phe biosynthesis we therefore applied new and rapid procedures for mutant isolation, combined with a simple protocol for the identification of metabolic lesions. This has allowed the isolation and characterization of a large number of auxotrophic mutants, covering virtually every step in aromatic amino acid biosynthesis.

**MATERIALS AND METHODS**

**Microorganisms and cultivation**

Amycolatopsis methanolica wild-type (NCIB 11946) (De Boer et al., 1990a), the plasmid pMEA300 (Vrijbloed et al., 1994) deficient strain WV1 (Vrijbloed et al., 1995), the spectinomycin resistant derivative of WV1, strain WV2 (Vrijbloed et al., 1995), and auxotrophic mutants derived from wild-type, strain WV1 or WV2 (this study; Euverink et al., 1992), were used. The procedures
for batch cultivation have been described previously (De Boer et al., 1988; Euverink et al., 1992).

**Mutant isolation**
Late-exponential phase cells (5 ml, 10⁹ colony forming units) grown in glucose mineral medium were sonicated for 15 sec at an amplitude of 6 microns with a MSE sonicator, using an ethanol sterilized probe (10 mm). Samples were spread on glucose mineral agar with the aromatic amino acids and quinate (final concentration 1 mg.1⁻¹ of each), than irradiated for 10-30 sec with an UV lamp (Philips TAW 15 W) at a distance of 20 cm. After 7 days incubation, pinpoint colonies were purified on glucose agar with excess of the above supplements (25 mg.1⁻¹ each) and characterized for growth requirements and enzyme lesions.

**Mating of auxotrophic mutant strains**
Mutants were grown to OD₄₃₀ 3-4 in glucose mineral medium with the appropriate amino acids (50 mg.l⁻¹). Cells (2.0 ml) of two strains were mixed, pelleted, resuspended in 0.1 ml 50 mM K₂HPO₄ pH 7.2, and transferred to sterile 0.2 µm filter paper. After drying, the filter was placed on glucose agar with supplements that allowed growth of both mutants. After 24 h incubation at 37°C, cells were resuspended in 50 mM K₂HPO₄, pH 7.2 and spread on glucose mineral agar. Prototrophic recombinant strains were selected and subsequently tested for spectinomycin (125 µg.ml⁻¹) resistance. Mating efficiencies were calculated as the number of recombinant colonies divided by the total number of colonies, corrected for revertants.

**Preparation of extracts and enzyme assays**
Cell-free extract was prepared as described (Euverink et al., 1992). Specific enzyme activities were determined at 37°C according to published methods. All enzyme assays were performed in triplicate; data presented are averages with a standard deviation of less than 10%. Transketolase (EC 2.2.1.1) (Alves et al., 1994); 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase (EC 4.1.2.15) and anthranilate synthase (EC 4.1.3.27) (De Boer et al., 1989); 3-dehydroquininate synthase (EC 4.6.1.3), shikimate:NADP⁺ 3-oxidoreductase (NADP⁺-dependent shikimate dehydrogenase) (EC 1.1.1.25), 3-dehydroquinate dehydratase (EC 4.2.1.10) (Euverink et al., 1992); chorismate mutase (EC 5.4.99.5) (Euverink et al., 1995a); prephenate dehydratase (EC 4.2.1.51) (Euverink et al., 1995c); arogenate dehydratase (EC 4.2.1.91) (Fischer and Jensen, 1987c); prephenate dehydrogenase (EC 1.3.1.12/13) (Fischer and Jensen, 1987b); prephenate aminotransferase (EC 2.6.1.1-), aromatic amino acid aminotransferase (EC 2.6.1.57), arogenate dehydrogenase (EC 1.3.1.43) (Abou-Zeid et al., 1995). Shikimate kinase (EC 2.7.1.71) was assayed with 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 5 mM ATP, 5 mM shikimate, and limiting amounts of extract. At appropriate time intervals the reaction was stopped by placing the mixtures on ice and shikimate was assayed with NADPH (0.15 mM) and shikimate dehydrogenase (1 U) at 340 nm at 37°C.

**Electron microscopy**
Cells were air-dried and negatively stained with 1% uranylacetate (negative staining). Cells were fixed overnight with 0.5% glutaraldehyde at 0°C followed by post-fixation in a mixture of 1% (w/v) OsO₄ and 2% (w/v) K₂CrO₇ in a sodium-cacodylate buffer for 1 h at 0°C and stained with 1% uranylacetate. After dehydration in a graded ethanol series, the samples were embedded in Epon; ultrathin sections were cut with a diamond knife and examined in a Philips CM10 transmission electron microscope (ultrathin sections).
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).

Biochemicals
DAHP was prepared as previously described (Euverink et al., 1992). 3-Dehydroquinate and the *Escherichia coli* enzymes 3-dehydroquinate synthase, 3-dehydroquinate dehydratase, and shikimate dehydrogenase, were a kind gift of J.R. Coggins, University of Glasgow, Scotland.

RESULTS AND DISCUSSION

Activities of L-Phe, L-Tyr, and L-Trp biosynthetic enzymes

The specific activities of enzymes involved in the biosynthesis of aromatic amino acids could be measured reproducibly in *A. methanolica* (Table 1; Fig. 1). Glucose-grown cells possessed an NADP⁺-dependent shikimate dehydrogenase. Growth on quinate resulted in additional synthesis of an NAD⁺-dependent quinate/shikimate dehydrogenase (Euverink et al., 1992). 5-Enolpyruvyl-shikimate-3-phosphate synthase and chorismate mutase were not measured, due to lack of a (commercial) source for their substrates. The presence of prephenate dehydratase and arogenate dehydrogenase, and the absence of prephenate dehydrogenase and arogenate dehydratase, provided evidence that L-Phe and L-Tyr are exclusively synthesized via phenylpyruvate and L-arogenate, respectively (Fig. 1).

Isolation of single cells of *A. methanolica*

In liquid media *A. methanolica* grows in long chains of individual cells containing clearly visible cell walls (Fig. 2). Instead of isolating spores or generating protoplasts, we tried to separate cells from each other using vortexing, pipetting at high shear, ultrasonic treatments in a sonication bath or with a sonication probe. Individual cells appeared to be tightly linked together. Only sonication with a probe directly inserted into the cell suspension, a rather harsh and potentially lethal treatment, was successful in generating single cells of *A. methanolica* (Fig. 2). No cell lysis was observed under optimal conditions (15 sec sonication), with the number of colony forming units even increasing 10-fold. In the late-exponential growth phase one colony forming unit of *A. methanolica* thus is the result of 10 cells on average. Using this approach a large number of recessive mutants of *A. methanolica* were isolated blocked in aromatic amino acid biosynthesis (this paper; Abou-Zeid *et al.*, 1995; Euverink *et al.*, 1992, 1995a), methanol metabolism,
### Table 1.
Specific activities (mU mg protein⁻¹) of enzymes involved in L-Phe, L-Tyr and L-Trp biosynthesis in extracts of *A. methanolica* strain WV2 cells grown in glucose mineral medium and the number of auxotrophic mutants isolated in the separate enzyme steps.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity</th>
<th>Mutants</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transketolase</td>
<td>550</td>
<td>22</td>
<td>Iᵇ</td>
</tr>
<tr>
<td>1 DAHP synthase</td>
<td>36</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2 3-Dehydroquinate synthase</td>
<td>8</td>
<td>15c</td>
<td>I</td>
</tr>
<tr>
<td>3 3-Dehydroquinate dehydratase</td>
<td>203</td>
<td>8d</td>
<td>IIᵉ</td>
</tr>
<tr>
<td>4 Shikimate dehydrogenase (NADP⁺)</td>
<td>25</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5 Shikimate kinase</td>
<td>7</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5-Enolpyruvyl-shikimate-3-phosphate synthase</td>
<td>ND</td>
<td>8f</td>
<td></td>
</tr>
<tr>
<td>Chorismate synthase</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 Anthranilate synthase</td>
<td>1</td>
<td>18</td>
<td>IIIᵍ</td>
</tr>
<tr>
<td>L-Trp pathway (Anthranilate → L-Trp)</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 Chorismate mutase</td>
<td>7</td>
<td>2</td>
<td>IVᵇ</td>
</tr>
<tr>
<td>8 Prephenate dehydratase</td>
<td>19</td>
<td>2</td>
<td>V</td>
</tr>
<tr>
<td>9 Phenylpyruvate aminotransferase</td>
<td>51</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>10 Prephenate aminotransferase</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>11 Arogenate dehydrogenase (NAD⁺)</td>
<td>2</td>
<td>10</td>
<td>VIᵏ</td>
</tr>
</tbody>
</table>

ND, not determined.

ᵃ Enzyme numbers refer to reactions shown in Fig. 1.
ᵇ I, Growth occurs with quinate or all three aromatic amino acids.
ᶜ Intracellular accumulation of 3-deoxy-D-arabino-heptulosonate 7-phosphate.
ᵈ 3-Dehydroquinate dehydratase/3-dehydroquinate synthase mutants; unable to grow on quinate.
ᵉ II, Growth occurs with all three aromatic amino acids only.
ᶠ 5-Enolpyruvyl-shikimate-3-phosphate synthase and/or chorismate synthase deficient mutants.
ᵍ III, Growth occurs with L-Trp.
ᵇ IV, Growth occurs with L-Phe plus L-Tyr only.
¹ V, Growth occurs with L-Phe.
+j 4-Hydroxyphenylpyruvate aminotransferase and phenylpyruvate aminotransferase are identical (Abou-Zeid et al., 1995).
ᵏ VI, Growth occurs with L-Tyr or 4-hydroxyphenylpyruvate.
fructose-6-phosphate + glyceraldehyde-3-phosphate
\[ \text{transketolase} \]
\[ \text{xylulose-5-phosphate + erythrose-4-phosphate} \]
\[ \text{phosphoenolpyruvate} \]
\[ \text{3-deoxy-D-arabino-heptulosonate 7-phosphate} \]
\[ \text{NAD}^+ \]
\[ \text{NADH} \]
\[ \text{Pi} \]
\[ \text{quinatde} \]
\[ \text{dehydroquinatde} \]
\[ \text{proto} \]
\[ \text{catechuate} \]
\[ \text{3-dehydroshikimate dehydratase} \]
\[ \text{shikimate-3-phosphate} \]
\[ \text{5-enolpyruvylshikimate-3-phosphate synthase} \]
\[ \text{5-enolpyruvylshikimate-3-phosphate} \]
\[ \text{anthranilate} \]
\[ \text{chorismatde} \]
\[ \text{5-phosphoanthranilate synthetase} \]
\[ \text{N-phosphoribosylanthranilate} \]
\[ \text{phenylalanine dehydrogenase} \]
\[ \text{phenylalanine dehydratase} \]
\[ \text{phenylalanine aminotransferase} \]
\[ \text{phenylalanine dehydratase} \]
\[ \text{phenylalanine aminotransferase} \]
\[ \text{L-tryptophan} \]
\[ \text{phenylalanine dehydrogenase} \]
\[ \text{phenylalanine dehydratase} \]
\[ \text{phenylalanine aminotransferase} \]
\[ \text{phenylalanine dehydratase} \]
\[ \text{NAD}^+ \]
\[ \text{NADH} \]
\[ \text{CO}_2 \]
\[ \text{4-OH-phenylpyruvate} \]
\[ \text{L-phenylalanine} \]
\[ \text{L-tyrosine} \]
\[ \text{4-OH-phenylpyruvate} \]
\[ \text{phenylpyruvate} \]
\[ \text{Figure 1. Biosynthesis of aromatic amino acids (solid arrows) and catabolism of quinate, L-Phe and L-Tyr (dashed arrows). Numbers indicate enzyme steps involved in L-Phe, L-Tyr, and L-Trp biosynthesis in A. methanolica identified via enzyme and mutant studies (Table 1). pdh, prephenate dehydrogenase; adh, aragenate dehydrogenase; adt, aragenate dehydratase; pdd, prephenate dehydratase; phedh, phenylalanine dehydrogenase; ppaAT, prephenate aminotransferase; aroAT, aromatic amino acid aminotransferase; gln, L-glutamine; glu, L-glutamate; α-kg, α-ketoglutarate.} \]
Characterization of auxotrophic mutants

The UV irradiation procedure resulted in 70-90% killing. A typical experiment yielded about 10% pinpoint colonies among the survivors, using 1 mg.l⁻¹ of the supplements. Further growth tests showed that 0.1-0.5% of these strictly depended on the presence of aromatic amino acids. The isolated mutants (150 in total) displayed stable genotypes and represented 6 classes of growth requirements (Table 1; Fig. 1). Enzyme analysis showed that Class I mutants were blocked in transketolase or 3-dehydroquinate synthase. DAHP synthase mutants were not identified. Recent biochemical studies provided evidence that DAHP synthase isoenzymes are present in A. methanolica (Euverink et al., 1995a).

The 8 Class II mutants unable to grow on quinate as carbon source (Fig. 1) lacked 3-dehydroquinate dehydratase; this enzyme thus has not only a biosynthetic function but is also required in quinate catabolism. Each of these 8 mutants had also lost

Figure 2. Pseudo-mycelium formed by A. methanolica in the late-exponential growth phase in glucose mineral medium (Phase contrast micrograph; × 200). A, Before sonication; B, after 15 sec sonication; C, Electron micrograph of pseudo-mycelium; D, Electron micrograph prepared from ultrathin sections of pseudo-mycelium; Bar = 1.0 µm.
3-dehydroquinate synthase, indicating that these two steps are genetically linked (Euverink et al., 1992). No shikimate dehydrogenase and shikimate kinase mutants were identified. As mentioned above, *A. methanolica* is able to synthesize both NAD⁺- and NADP⁺-dependent shikimate dehydrogenase isoenzymes. Shikimate kinase isoenzymes have been reported for *E. coli* and *Salmonella typhimurium* (Berlyn and Giles, 1969), but no information is available for *A. methanolica*. The remaining eight Class II mutants probably lack 5-enolpyruvyl-shikimate-3-phosphate synthase or chorismate synthase activity.

A large number of Class III mutants were obtained; several of these also grew with anthranilate as supplement and lacked anthranilate synthase activity. The other Class III mutants have not been studied in more detail yet. Class IV mutants were blocked in chorismate mutase. Class V mutants lacked prephenate dehydratase or phenylpyruvate aminotransferase (aro AT); only the prephenate dehydratase mutants also grew with phenylpyruvate as supplement. Aro AT mutants also had lost the ability to grow on L-Tyr as a carbon source. The *in vivo* role of this aro AT enzyme thus is in L-Phe biosynthesis as well as in L-Tyr catabolism (Abou-Zeid et al., 1995). All Class VI mutants lacked arogenate dehydrogenase and also grew with 4-hydroxyphenylpyruvate (transaminated into L-Tyr by aro AT) as supplement. Prephenate aminotransferase mutants were not identified. Biochemical studies showed that *A. methanolica* possesses several aminotransferase enzymes converting prephenate into arogenate (Abou-Zeid et al., 1995).

The remaining 40 mutants grew in the presence of any two out of three aromatic amino acids and did not show significant changes in enzyme activities. These strains may possess (leaky) mutations in general metabolism; the supply of aromatic amino acids, the synthesis of which is expensive, may stimulate their growth.

The available set of well-characterized aromatic amino acid auxotrophic mutants of *A. methanolica* will enable cloning of the corresponding genes via complementation.

**Mating of auxotrophic mutants**

Improvement of industrial strains generally involves repeated mutagenic treatments. However, this may also result in accumulation of unwanted mutations. Actinomycetes are able to exchange chromosomal DNA via intra- and interspecific matings (Ghisalba et al., 1984; Hopwood et al., 1985; Mirdamadi-Tehrani et al., 1992; Stoycheva et al., 1994). Separate mutations, therefore, also may be introduced into the desired background via mating. To analyse the mating potential of *A. methanolica*, two experiments were performed with auxotrophic mutants derived from different parent strains. Mutants GH1 and GH3 lack 3-dehydroquinate synthase and were derived from wild-type and strain WV1, respectively. Mutant GH70 lacks arogenate dehydrogenase and was derived from
strain WV2. When characterizing the prototrophic recombinants selected we assumed that the non-selected spectinomycin resistancy marker had not been transferred as well. Mating of strains GH1 and GH70 yielded \(13 \times 10^{-3}\%\) prototrophs. From these prototrophs 12.8\% was resistant towards spectinomycin (12.8\% thus was originally GH70 and received the arogenate dehydrogenase gene from GH1) and 87.2\% was spectinomycin sensitive (87.2 \% was originally GH1 and received the 3-dehydroquinate synthase gene from GH70) (Table 2).

Matings with strains GH3 and GH70 yielded 1\% prototrophs. From these prototrophs 28.3\% was resistant towards spectinomycin (28.3\% thus was originally GH70 and received the arogenate dehydrogenase gene from GH3) and 71.7\% was spectinomycin sensitive (71.7\% thus was originally GH3 and received the 3-dehydroquinate synthase gene from GH70) (Table 2).

Interestingly, matings with plasmid pMEA300 (Vrijbloed et al., 1994) cured strains showed a 77-fold (\(1/13 \times 10^{-3}\)) higher efficiency in exchanging chromosomal markers than pMEA300 containing cells (Table 2).

The mechanism for chromosomal recombination in actinomycetes is currently not known. It has been proposed that interactions between the chromosome and plasmid SCP1 are involved in the recombination process in *Streptomyces coelicolor* (Hopwood and Wright, 1973a, 1973b; Vivian, 1971). Alternatively, mating mechanisms in nocardiaform bacteria may involve specific phages (Brownell and Adams, 1968). Also cell-fusion represents an alternative mechanism for the exchange of chromosomal DNA. Mobilization of *A. methanolica* chromosomal DNA obviously does not involve the plasmid pMEA300. Other plasmids or bacteriophages have not been identified thus far. Further studies aim to elucidate the mechanism by which pMEA300 exerts a negative effect on the mating process.

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**Table 2.** Characterization of prototrophic recombinants obtained in matings of strain GH70 (pMEA300 cured, spec\(^S\)) with strain GH1 (pMEA300 containing, spec\(^R\)) or strain GH3 (pMEA300 cured, spec\(^S\)).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cfu</th>
<th>Revertants</th>
<th>Prototrophs</th>
<th>Spec(^R)</th>
<th>Spec(^S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH1</td>
<td>(21 \times 10^6)</td>
<td>0</td>
<td>(106 \times 10^2) ((0.013%))</td>
<td>12.8%</td>
<td>87.2%</td>
</tr>
<tr>
<td>GH70</td>
<td>(60 \times 10^6)</td>
<td>88</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH3</td>
<td>(56 \times 10^6)</td>
<td>1</td>
<td>(215 \times 10^4) ((1.01%))</td>
<td>28.3%</td>
<td>71.7%</td>
</tr>
<tr>
<td>GH70</td>
<td>(157 \times 10^6)</td>
<td>39</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Chapter 2

REFERENCES

References are listed on pages 147-160.