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Isolation and analysis of mutants of the methylotrophic actinomycete *Amycolatopsis methanolica* blocked in aromatic amino acid biosynthesis

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Abstract

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 amino acid supplements. Mutant characterization provided unambiguous evidence that L-tyrosine is synthesized via arogenate and that L-phenylalanine is synthesized via phenylpyruvate. The efficiency of chromosomal DNA marker exchange was highest in matings with mutant strains that lacked the previously characterized 13.3-kb integrative plasmid pMEA300.

Keywords: Actinomycete; *Amycolatopsis methanolica*; Aromatic amino acid biosynthesis; Auxotrophic mutant

1. Introduction

Current knowledge of the biochemistry and regulation of primary metabolism in actinomycetes is limited but is required for the rational improvement of strains overproducing aromatic amino acids and derived secondary metabolites (e.g. antibiotics) [1]. We have initiated studies of glucose [2], methanol (H.J. Hektor and L. Dijkhuizen, unpublished) [3], and aromatic amino acid metabolism [4–7] in the actinomycete *Amycolatopsis methanolica* [8].

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 [9]. Selection of auxotrophic mutants of pseudomycelium-forming bacteria such as actinomycetes necessitates isolation of spores or protoplasts [10]. This was rather difficult for *A. methanolica* and during our studies of L-phenylalanine biosynthesis we therefore applied new and rapid procedures for the isolation of a large number of auxotrophic mutants, covering virtually every step in aromatic amino acid biosynthesis.

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2. Materials and methods

2.1. Microorganisms and cultivation

Amycolatopsis methanolica wild-type (NCIB 11946), the plasmid pMEA300 [11] deficient strain WV1 [12], the spectinomycin resistant derivative of WV1, strain WV2 [12], and auxotrophic mutants derived from wild-type, from strain WV1 or WV2 (this study) [5], were used. Procedures for batch cultivation have been described [5].

2.2. Mutant isolation

Late-exponential phase cells (5 ml, 10^9 colony forming units) grown in glucose mineral medium were sonicated for 15 s at an amplitude of 6 μm with a MSE sonicator, using an ethanol sterilized probe (10 mm diameter). Samples were spread on glucose mineral agar with the aromatic amino acids and quinate (final concentration 1 mg l^{-1} of each), than irradiated for 10–30 s 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 l^{-1} each) and characterized for growth requirements and enzyme lesions.

2.3. Mating of auxotrophic mutant strains

Mutants were grown to an OD_{430} of 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 of 50 mM K_2HPO_4 (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 K_2HPO_4 (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 ($2–16 \times 10^7$), after correction for revertants (less than 100 in each experiment).

2.4. Preparation of extracts and enzyme assays

Cell-free extract was prepared as described [5]. All enzyme assays were performed in triplicate; data presented are averages with a standard deviation of less then 10%. Specific enzyme activities were determined at 37°C according to published methods: transketolase (EC 2.2.1.1) [2]; 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) synthase (EC 4.1.2.15) and anthranilate synthase (EC 4.1.3.27) [13]; 3-dehydroquinate 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) [5]; chorismate mutase (EC 5.4.99.5) [7]; prephenate dehydratase (EC 4.2.1.51) [6]; arogenate dehydratase (EC 4.2.1.91) [14]; prephenate dehydrogenase (EC 1.3.1.12/13) [15]; prephenate aminotransferase (EC 2.6.1.-), aromatic amino acid aminotransferase (EC 2.6.1.57), arogenate dehydrogenase (EC 1.3.1.43) [4]. Shikimate kinase (EC 2.7.1.71) was assayed with 50 mM Tris · HCl (pH 7.5), 10 mM $MgCl_2$, 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.

2.5. 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) OsO_4 and 2% (w/v) K_2CrO_7 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).

2.6. Analytical methods

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

2.7. Biochemicals

DAHP was prepared as previously described [5]. 3-Dehydroquinate, and the *Escherichia coli* enzymes dehydroquinate synthase, dehydroquinate dehydratase and shikimate dehydrogenase, were a kind gift of J.R. Coggins, University of Glasgow, Scotland.

3. Results and discussion

3.1. Activities of L-phenylalanine, L-tyrosine and L-tryptophan 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 [5]. 5-*Enol*pyruvyl-shikimate-3-phosphate synthase and chorismate synthase 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).

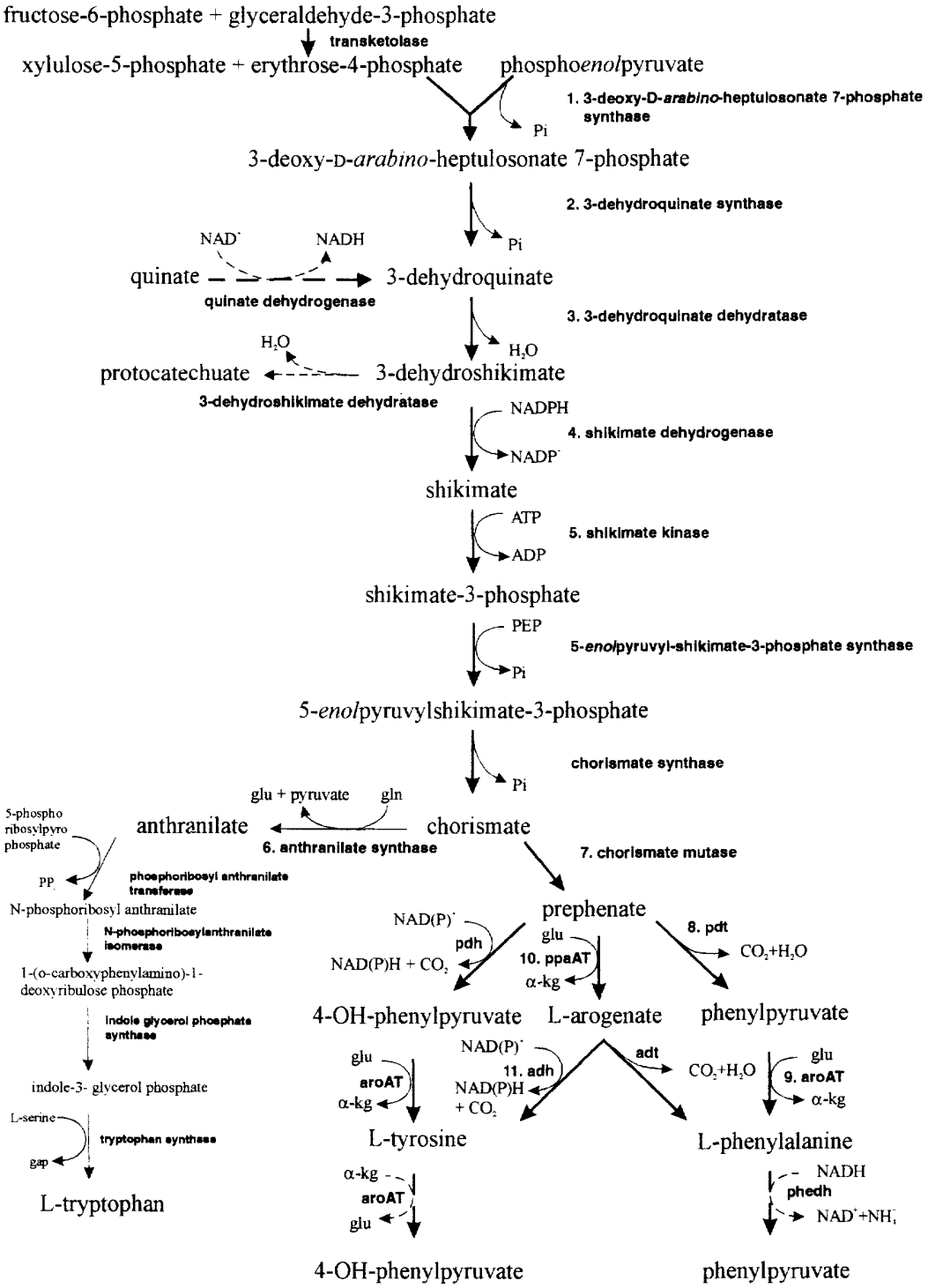
Table 1
Specific activities (mU (mg protein)$^{-1}$) of enzymes involved in L-Phe, L-Tyr and L-Trp biosynthesis in extracts of *A. methanolica* strain WV2 cells grown in glucose mineral medium, and the number of auxotrophic mutants isolated in the separate enzyme steps

Enzyme [a]		Specific activity	Number of mutants	Class
	Transketolase	550	22	I [b]
1.	DAHP synthase	36	0	
2.	3-Dehydroquinate synthase	8	15 [c]	I
3.	3-Dehydroquinate dehydratase	203	8 [d]	II [e]
4.	Shikimate dehydrogenase ($NADP^+$)	25	0	
5.	Shikimate kinase	7	0	
	5-*Enol*pyruvyl-shikimate-3-phosphate synthase	ND	8 [f]	
	Chorismate synthase	ND		
6.	Anthranilate synthase	1	18	III [g]
	L-Trp pathway (Ant → L-Trp)	–	23	III
7.	Chorismate mutase	7	2	IV [h]
8.	Prephenate dehydratase	19	2	V [i]
9.	Phenylpyruvate aminotransferase	51	2	V
	4-Hydroxyphenylpyruvate aminotransferase	44	2 [j]	
10.	Prephenate aminotransferase	1	0	
11.	Arogenate dehydrogenase (NAD^+)	2	10	VI [k]

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/dehydroquinate synthase mutants; unable to grow on quinate.
[e] II, Growth occurs with all three aromatic amino acids only.
[f] 5-*Enol*pyruvyl-shikimate-3-phosphate synthase and/or chorismate synthase deficient mutants.
[g] III, Growth occurs with L-Trp.
[h] IV, Growth occurs with L-Phe plus L-Tyr only.
[i] V, Growth occurs with L-Phe.
[j] 4-Hydroxyphenylpyruvate aminotransferase and phenylpyruvate aminotransferase are identical [4].
[k] VI, Growth occurs with L-Tyr or 4-hydroxyphenylpyruvate.

3.2. Isolation of single cells of A. methanolica

In liquid media, *A. methanolica* grows in chains of tightly linked cells but with clearly visible cell walls (Fig. 2). Instead of isolating spores or protoplasts, we tried to separate cells from each other by vortexing, pipetting at high shear, ultrasonic treatments in a sonication bath or with a sonication probe. 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 s 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 mutants of *A. methanolica* blocked in aromatic amino acid biosynthesis (this paper; [4,5,7]), methanol metabolism and the pentose phosphate pathway ([2]; H.J. Hektor and L. Dijkhuizen, unpublished) were isolated.


fructose-6-phosphate + glyceraldehyde-3-phosphate
transketolase
xylulose-5-phosphate + erythrose-4-phosphate
phosphoenolpyruvate
1. 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase
Pi
3-deoxy-D-arabino-heptulosonate 7-phosphate
2. 3-dehydroquinate synthase
Pi
NAD+
NADH
quinate
quinate dehydrogenase
3-dehydroquinate
3. 3-dehydroquinate dehydratase
H2O
H2O
protocatechuate
3-dehydroshikimate dehydratase
3-dehydroshikimate
NADPH
4. shikimate dehydrogenase
NADP+
shikimate
ATP
5. shikimate kinase
ADP
shikimate-3-phosphate
PEP
5-enolpyruvyl-shikimate-3-phosphate synthase
Pi
5-enolpyruvylshikimate-3-phosphate
chorismate synthase
Pi
glu + pyruvate
gln
5-phospho ribosylpyro phosphate
anthranilate
6. anthranilate synthase
chorismate
7. chorismate mutase
PP
phosphoribosyl anthranilate transferase
N-phosphoribosyl anthranilate
N-phosphoribosylanthranilate isomerase
1-(o-carboxyphenylamino)-1-deoxyribulose phosphate
indole glycerol phosphate synthase
indole-3- glycerol phosphate
L-serine
tryptophan synthase
gap
L-tryptophan
NAD(P)+
pdh
NAD(P)H + CO2
prephenate
8. pdt
CO2+H2O
glu
10. ppaAT
α-kg
4-OH-phenylpyruvate
L-arogenate
phenylpyruvate
glu
aroAT
α-kg
NAD(P)+
11. adh
NAD(P)H + CO2
adt
CO2+H2O
glu
9. aroAT
α-kg
L-tyrosine
L-phenylalanine
α-kg
aroAT
glu
NADH
phedh
NAD+ +NH4+
4-OH-phenylpyruvate
phenylpyruvate

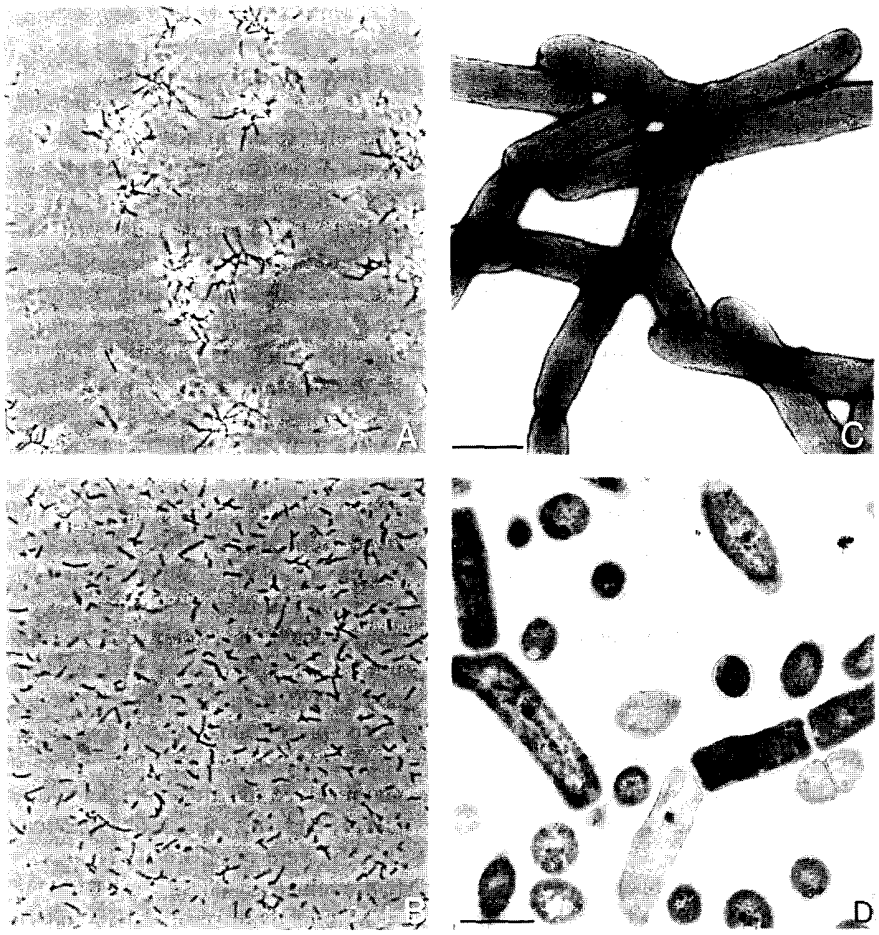


Fig. 2. Pseudo-mycelium formed by *A. methanolica* in the late-exponential growth phase in glucose mineral medium (phase contrast micrograph: ×1240). (**A**) Before sonication; (**B**) after 15 s sonication; (**C**) electron micrograph of pseudo-mycelium; (**D**) electron micrograph prepared from ultrathin sections of pseudo-mycelium. Bar represents 1.0 μm.

3.3. 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 mutants isolated (150 in total) displayed stable genotypes and represented six classes of growth requirements (Table 1; Fig. 1). Enzyme analysis showed that Class I mutants were blocked in transketolase or dehydro-

Fig. 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, arogenate dehydrogenase; adt, arogenate dehydratase; pdt, prephenate dehydratase; phedh, phenylalanine dehydrogenase; ppaAT, prephenate aminotransferase; aroAT, aromatic amino acid aminotransferase; gln, L-glutamine; glu, L-glutamate; α-kg, α-ketoglutarate.

quinate synthase. DAHP synthase mutants were not identified. Recent biochemical studies provided evidence that DAHP synthase isoenzymes are present in *A. methanolica* [7].

The eight Class II mutants unable to grow on quinate as carbon source (Fig. 1) lacked dehydroquinate dehydratase; this enzyme thus has not only a biosynthetic function but is also required in quinate catabolism. Each of these eight mutants had also lost dehydroquinate synthase, indicating that these two steps are genetically linked [5]. No shikimate dehydrogenase and shikimate kinase mutants were identified. As mentioned above, *A. methanolica* is able to synthesize NAD^+ and $NADP^-$ dependent shikimate dehydrogenase isoenzymes. Shikimate kinase isoenzymes have been reported for *E. coli* and *Salmonella typhimurium* [16], but no information is available for *A. methanolica*. The remaining eight Class II mutants probably lack 5-*enol*pyruvyl-shikimate-3-phosphate synthase or chorismate synthase.

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 (AroAT); only the prephenate dehydratase mutants also grew with phenylpyruvate as supplement. AroAT mutants also had lost the ability to grow on L-Tyr as carbon source. The in vivo role of this AroAT enzyme thus is in L-Phe biosynthesis as well as in L-Tyr catabolism [4]. All Class VI mutants lacked arogenate dehydrogenase and also grew with 4-hydroxyphenylpyruvate (transaminated into L-Tyr by AroAT) as supplement. Prephenate aminotransferase mutants were not identified. Biochemical studies showed that *A. methanolica* possesses several aminotransferase enzymes converting prephenate into arogenate [4]. The remaining 40 mutants 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.

3.4. 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 [9,10,17,18]. 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 dehydroquinate synthase, were derived from wild-type (containing pMEA300) and strain WV1 (pMEA300 cured), respectively, and are spectinomycin sensitive. Mutant GH70 lacks arogenate dehydrogenase and was derived from strain WV2 (pMEA300 cured, spectinomycin resistant). When characterizing the prototrophic recombinants selected, we assumed that the non-selected spectinomycin resistance marker had not been transferred as well. Mating of strains GH1 and GH70 yielded $13 \times 10^{-3}\%$ prototrophs. From these prototrophs 12.8% were resistant towards spectinomycin (12.8% thus were originally GH70 and received the arogenate dehydrogenase gene from GH1) and 87.2% were spectinomycin sensitive (87.2% were originally GH1 and received the 3-dehydroquinate synthase gene from GH70).

Matings with strains GH3 and GH70 yielded 1% prototrophs. Of these prototrophs 28.3% were resistant towards spectinomycin (28.3% thus were originally GH70 and received the arogenate dehydrogenase gene from GH3) and 71.7% were spectinomycin sensitive (71.7% thus were originally GH3 and received the 3-dehydroquinate synthase gene from GH70). Interestingly, matings with plasmid pMEA300 [11] cured strains showed a 77-fold ($1\%/13 \times 10^{-3}\%$) higher efficiency in exchanging chromosomal markers than pMEA300 containing cells.

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* [19]. Alternatively, mating mechanisms in nocardiaform bacteria may involve specific phages [20]. 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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