Transformation of the Methylophilic Actinomycete *Amicycolatopsis methanolica* with Plasmid DNA: Stimulatory Effect of a pMEA300-Encoded Gene

J. W. VRIJBLOED, J. MADON*,¹ AND L. DIJKHUIZEN²

Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands; and *Mikrobiologisches Institute, ETH-Zentrum Universitätsstrasse 2, CH-8092 Zürich, Switzerland

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Amicycolatopsis methanolica contains a 13.29-kb plasmid (pMEA300) present both in the free state and integrated at a unique genomic location. A pMEA300-free derivative (strain WV1) was selected, allowing further analysis of pMEA300-encoded functions. Whole cells of strain WV1 could be transformed at high frequencies (approximately 10^6 transformants per microgram of plasmid DNA) with both circular and linear plasmid DNA, provided that the pMEA300-encoded *stf* (stimulation of transformation frequency) gene was present. *stf* would encode a putative protein of 373 amino acids with M_r 40,201, resembling putative regulatory proteins involved in sporulation of *Streptomyces griseus* and *Streptomyces coelicolor*. © 1995 Academic Press, Inc.

The nocardioform actinomycete *Amicycolatopsis methanolica* (De Boer *et al.*, 1990) contains a 13.29-kb plasmid (pMEA300; Fig. 1A) present both in the free state and integrated at a unique genomic location (Vrijbloed *et al.*, 1994). Integrated elements have been described for various actinomycetes (Cohen *et al.*, 1985; Hopwood *et al.*, 1984; Bibb *et al.*, 1977, 1981; Moretti *et al.*, 1985; Pernodet *et al.*, 1984; Brown *et al.*, 1988), but clear physiological roles for these elements remain to be established. Here we report the isolation of an *A. methanolica* derivative devoid of both integrated and free pMEA300 sequences and an efficient transformation protocol for whole cells.

A. methanolica strains (Table 1) were maintained and grown in complex medium (CM)³ (Vrijbloed *et al.*, 1994) unless stated otherwise. Other rich media used, TS broth, S27M,

and R2L, were as described (Madoñ and Hütter, 1991). A modification of the method described for *Amicycolatopsis mediterranei* (Madoñ and Hütter, 1991) was used for transformation of *A. methanolica*. Cells grown in TS broth were harvested in the stationary phase at an OD₄₃₃ of 5.0–8.0 and used immediately. Cells were centrifuged (10 min, 3600g, room temperature), washed once in 1 volume of TE, and resuspended in TE to OD₄₃₃ 160. The standard transformation mixture (400 μ l), containing 100 μ l cell suspension (final OD₄₃₃ = 40), 5 mM MgCl₂, 0.625 M CsCl, 7.5 μ g of sonicated calf thymus DNA, 0.1 μ g of plasmid DNA, and 32.5% (wt/vol) polyethylene glycol (PEG 1000; Koch Light), prepared as described (Madoñ and Hütter, 1991), was incubated for 40 min at 37°C. S27M medium (1 ml, minus agar) was added to the suspension, which was centrifuged after gentle mixing. The cell pellet was gently resuspended in S27M (1 ml), centrifuged, and resuspended in S27M medium (400 μ l). Plating of cells on S27M agar medium in the R2L low-melting-point agarose overlay and selection for pock formation or antibiotic (thiostrepton) resis-

¹ Current address: Department of Internal Medicine, Medical Clinic B, University Hospital, Rämistrasse 100, CH-8091 Zürich, Switzerland.

² Corresponding author. Fax: +31.50.632154. E-mail: L.Dijkhuizen@biol.rug.nl.

³ Abbreviations used: CM, complex medium.

FIG. 1. Restriction maps of pMEA300 (A), pWV129, and derivative deletion plasmids (B). Numbers between brackets indicate the restriction sites on pMEA300 and pWV129. Other numbers indicate distances (in kb) from the unique *Bam*HI site. Closed arrows indicate open reading frames on pMEA300. The bars in A represent the various functional regions identified (Vrijbloed *et al.*, 1994, 1995a,b,c). The open arrow (B) indicates the restriction site (*Hind*III[15]) disrupted with Klenow.

TABLE 1

Amycolatopsis methanolica STRAINS, VARIOUS ACTINOMYCETE PLASMIDS, AND pMEA300-DERIVED PLASMIDS USED IN THIS STUDY

Strain or plasmid	Description	Source
<i>A. methanolica</i>	NCIB 11946 wild-type strain	De Boer <i>et al.</i> (1990)
WV1	pMEA300 free derivative strain of <i>A. methanolica</i>	This work
WV2	Spectinomycin-resistant (150 µg/ml) derivative strain of strain WV1	This work
Plasmids	Deletion or modification	
pIJ702		Katz <i>et al.</i> (1983)
pIJ922		Lydiate <i>et al.</i> (1985)
pIJ41		Thompson <i>et al.</i> (1982)
pMEA100	Wild-type plasmid of <i>A. mediterranei</i>	Moretti <i>et al.</i> (1985)
pMEA300	Wild-type plasmid of <i>A. methanolica</i>	Vrijbloed <i>et al.</i> (1994)
pMEA301	pMEA300 containing the <i>tsr</i> gene as a 1.1-kb <i>Bcl</i> I fragment of pIJ702 in the unique <i>Bgl</i> II[9] site (Fig. 1A)	This work
pMEA302	A spontaneously occurring 20.8-kb hybrid plasmid of pMEA300 and pMEA301, except for a 6.4-kb deletion extending over the <i>attP</i> site, with one endpoint located between the unique <i>Pst</i> I [16] and <i>Pvu</i> II[17] sites and the other between the <i>Bcl</i> II[7] and the <i>Sal</i> II[8] sites of pMEA300 (Fig. 1A).	This work
pWV113	<i>Bam</i> HI[1]– <i>Hind</i> III[15] fragment of pMEA300 inserted in <i>Bam</i> HI[1]– <i>Hind</i> III[2] of pHSS6 (Seifert <i>et al.</i> , 1986)	Vrijbloed <i>et al.</i> (1995b)
pWV111	<i>Bam</i> HI[1]– <i>Pvu</i> II[17] fragment of pMEA300 inserted in <i>Bam</i> HI[1]– <i>Sam</i> I[4] of pHSS6	Vrijbloed <i>et al.</i> (1995b)
pWV129	<i>Bam</i> HI[1]– <i>Apa</i> I[21, blunt] fragment of pMEA300 inserted in <i>Bam</i> HI[1]– <i>Eco</i> RI[1, blunt] of pHSS6	Vrijbloed <i>et al.</i> (1995b)
pWV375	<i>Hind</i> III[15] site of pWV129 filled in with Klenow	Vrijbloed <i>et al.</i> (1995a)

tance were performed as described (Madoñ and Hütter, 1991). After 5 h incubation at 37°C a thiostrepton solution (final concentration 10 µg/ml) was added to the plates. The plates were allowed to dry for 1.5 h in a laminar flow cabinet and incubated at 37°C.

Escherichia coli strain JM110 (Norlander *et al.*, 1983) was grown on Luria broth (Sambrook *et al.*, 1989) and transformed via a PEG-Mg²⁺ mediated method (Chung *et al.*, 1989). Strain JM110 was used for the isolation of *E. coli*–*A. methanolica* shuttle plasmids (Table 1), using 50 µg/ml kanamycin to select for the plasmids.

Linear plasmid DNA was isolated as described (Bibb *et al.*, 1980). Other DNA isolation, sequencing, blotting, and hybridization methods were as described (Vrijbloed *et al.*, 1994). The nucleotide sequence data deter-

mined in this report will appear in the GenBank database under Accession No. L36679.

None of the existing actinomycete plasmid vectors tested (Table 1) were stably maintained in *A. methanolica*, differing from observations made with *Amycolatopsis orientalis* (Matsushima *et al.*, 1994) but similar to the situation reported for *A. mediterranei* (Madoñ and Hütter, 1991). The thiostrepton-resistant colonies obtained following transformation with pMEA301 (e.g., strain AM301) and pMEA302 (e.g., strain AM302), however, could be transferred readily to fresh selective media. In view of these promising results we decided to develop cloning vectors for *A. methanolica* based on the indigenous plasmid pMEA300. No naturally occurring pMEA300-free strains of *A. methanolica* are known at present, necessitating the isolation of a deriva-

tive of the NCIB 11946 strain that had lost both integrated and free pMEA300 sequences.

Protoplast formation and regeneration, as used for *A. mediterranei* (Moretti *et al.*, 1985), did not result in loss of pMEA300 from *A. methanolic* wild type. The following procedure, however, was successful. Strain AM302 was cultivated for 30 generations in CM medium with thiostrepton, protoplasted, and regenerated to obtain colonies arising from individual cells. Southern hybridization showed that pMEA302 had replaced both the integrated and the free forms of pMEA300 in all AM302-derived strains. Subsequent loss of plasmid could now be detected by loss of thiostrepton resistance. Strain AM302/1 was therefore cultivated for 30 generations in CM medium without thiostrepton, protoplasted, and regenerated. Approximately 1000 colonies were tested, yielding 20 thiostrepton-sensitive colonies. Of these only strain WV1 was completely devoid of both free and integrated forms of pMEA300. No differences were noticed between strain WV1 and wild type with respect to antibiotic sensitivity, ability to use methanol, or growth rates on various media.

Transformation of strain WV1 with pMEA300 and pMEA301 also resulted in pock formation, the appearance of zones of growth inhibition when plasmid-carrying donor cells transfer the plasmid by conjugation within a confluent lawn of plasmid-free recipient cells (Bibb *et al.*, 1977). The diameters of the pocks were about 4 mm for pMEA300 and 1–2 mm for pMEA301. Southern hybridization revealed that both pMEA300 and pMEA301 re-integrated into the original (single) genomic site; no DNA rearrangements were detected (results not shown). Further evidence for conjugational plasmid transfer was obtained using strain WV2 (spectinomycin resistant) as recipient and strain WV1/pMEA301 as donor in mixed cultures on agar. Spectinomycin- and thiostrepton-resistant transconjugants were routinely obtained in mating experiments involving S27M agar without these antibiotics, allowing growth of both donor and recipient strains.

FIG. 2. Effects of varying cell density (A, CsCl, PEG concentrations according to Madoñ and Hütter, 1991) and concentrations of CsCl (B, optimal cell concentrations as under A; PEG concentrations according to Madoñ and Hütter, 1991) and PEG 1000 (C, optimal cell and CsCl concentrations as under A and B) in the transformation frequency of strain WV1 with pMEA300.

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NarI [14] . . . . .
1  GGGCCCCAGCACCTTCGGCAGTAGCCCGCTCCACCAGTCGGGACCGGAATCATGTTGGC
61  CTTCTTGCCCATGGCTACCTCCCAGGTTCTGCGGCTCGGCGGTTTCCGTTGCCATGCCTC
121  AAGATTCTGCTCTGGTTCCGGCTTCTTGGGGAGATGAGTCCCTGGTGAGTCGTTGGTGATTC
181  GCTCGACTCACCAGGGGTGACCAGGCACAATGCCCGCAAGGGAAGTGACCGTGGGGAC
                                     fm G D
HindIII [15] . . . . .
241  AGCGGGGCAACCGAGAGCAAGCTTCGCCGTGCTCGCCTTGCCGCCGGGATGACGCAAGGC
    S G A T E S K L R R A R L A A G M T Q G
301  GAGGTCCGGGCCAAGCTCACGCAGGCTCGCCGTCGCCGGGGCAAGATCCCCCGAAGGAA
    E V R A K L T Q A R R R G K M P P K E
361  GCCAGCTTGAAGCGGATGTACACGTCGTGGGAGACCGGCGCGGTGATCCCGACGGACTGG
    A S L K R M Y T S W E T G A V I P T D W
421  CGGGACGAACTGTGCGAGGTATTTCGAGCTTCCACCGGCCGCGCTCGGGTTGGTCGAGACC
    R D E L C E V F E L P P A A L G L V E T
481  ACACCGCCACCTGCGCTCGACCTCCCGAGCACATTCGAGGTGGTCCGGCTCGATCCAGCG
    T P P P A L D L P S T F E V V R L D P A
PstI [16] . . . . .
541  GTGATTTCGCTGCTAGACCAGCAGACGAACTTCTACCGGCTGCAGGACCGGCTGCTGGGG
    V I S L L D Q Q T N F Y R L Q D R L L G
601  GCGGCGATCATTCGCGAGACCGAAGCCACGTCGCGCAACCTTGAGCAGATGCTGCGAAAT
    A A I I P Q T E A H V R N L E Q M L R N
661  GCGCTGCCGAGCGGCCACCTTCCGACAGCGCGGTGACCTCGCTGAGGCGGGTGCCTC
    A L P S G H L P T A A V T L A E A A A L
721  GCCGGTTGGCAAGCGCTCGATGCGGGTGATCTCCGGAAGCGTGGGACCTGCACGACATC
    A G W Q A L D A G D L R K A W D L H D I
781  GCGAAGTCCGAGCAGCGGAGGGGAGAACCAGCCGTGCTCGCGCACGTGACGGCGCAG
    A K S A A R Q G E N P A V L A H V T A Q
841  CAGGCTTACGTTCTGCTCGATGCCGGCCGGCCGCCGATGCGGTGGAGCTGGTCGAGTAT
    Q A Y V L L D A G R A A D A V E L V E Y
901  GCAAGCGAAGCCAGGCTGCTCGGACAGGTCCCGCACGCTTCGGTCGTGGTTGGCCGCG
    A S E P R L L G Q V P A R L R S W L A A
961  GCGCACGCCGAGTTCCTGGCCGCGGGGGGACCGATCCGGCGCGATGCGGCGGCTCGAT
    A H A E F L A A A G D R S G A M R R L D
PvuII [17] . . . . .
1021  CAAGCGGCCGAGTGTGCCAGCTGGCGACAACGACCCGTGAGTTGCCGTACCTGATGCTG
    Q A A D V L P A G D N D P E L P Y L M L

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FIG. 3. Nucleotide sequence of the 1.53-kb *NarI*[14]–*SphI*[18] fragment of pMEA300 encoding *stf*. The deduced amino acid sequence (single-letter code) is shown below the DNA sequence. Restriction sites are shown above the sequence, numbered as shown in Fig. 1A. The predicted ribosome-binding site is shown in boldface.

Transformation of *A. methanolica* was successful in selecting for pock formation using the cell density and PEG and CsCl concentrations previously described (Madoñ and Hütter, 1991). The number of transformants of *A. methanolica*, however, further increased with cell density up to an OD₄₃₃ of 40 (Fig. 2A) with stationary phase cells. The fact that more

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1081 AACGGCGCGCACCTCGCCCGGTGGCGGGGCAACTGCTTGGCGCGACTCGGCGAAGACCAG
    N G A H L A R W R G N C L A R L G E D Q
1141 GCGATCGAGGACCTGACAGCGGCGCTCGATGGGCTCACCACGCTCACCTCACGGCGAGCA
    A I E D L T A A L D G L T T L T S R R A
1201 GAGGCGGGGCTTCGTGTAGACCTCGCGCTTGCCCTGCGGAAGCGCGGCGACCTGGACGAG
    E A G L R V D L A L A L R K R G D L D E
1261 TCGCGCGTCGAGGCCCGACAAAGCCCGAGCTGGCCGGCACAACAGGCTCAGCCCGGCAG
    S R V Q A R Q A A E L A G T T G S A R Q
1321 CGAGCCCGGATCGCGGAGCTACTTGCCGCCCTAGCGCGAGAACGTGCAGCAGCCCGATCAG
    R A R I A E L L A A -
1381 AGCGCCTGAGTTCCCGACCTGGCCTGCTCGGATGAGGTCGGGAACGTGCGGAAGGGCAT
1441 CCACTGGAAGGTGCCTTCGTTCTGCTCGGTCGGGTCGGCGCACTTGCTCGAGCCCTCGGAC
    SphI [18]
1501 GACAAAGAGGTGGTTCGGGTTGCGCAGCATGC 1532

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FIG. 3—Continued

transformants were obtained when the transformation mixture was further diluted for plating led to the identification of CsCl as a toxic compound, causing a fivefold reduction in plating efficiency and number of transformants at a final concentration of 30 mM. This problem was overcome by introducing a wash step before plating cells in the R2L overlay. CsCl and PEG were essential for transformation (Figs. 2B and 2C). The optimal CsCl concentration was 0.625 M, as for *A. mediterranei* (Madoñ and Hütter, 1991), but approximately 60% lower than for *Nocardia lactamdurans* (Kumar *et al.*, 1994). Further optimization of the transformation protocol for strain WV1 with pMEA300 was obtained by changing the PEG concentration (Fig. 2C). PEG exhibited its stimulatory effect in a narrow concentration range of 30–37.5%, the optimal concentration being 32.5% (Fig. 2C). In addition to the normal nutrients, mannitol (0.4 M) in the R2L and S27M media appeared essential for efficient transformation. With this optimized transformation protocol (see above), pMEA300 routinely yielded 9×10^5 transformants per microgram of DNA in the plasmid-free strain WV1 and 10^4 in the wild-type strain.

Evidence for transformation of strain WV1 with linear plasmid DNA was obtained using

pMEA300 DNA digested with eight different restriction enzymes, all recognizing only a single site on pMEA300, and pMEA301 linearized with *EcoRV* at its single site in the thiostrepton-resistance gene. Strain WV1 was transformed with the digested DNA without dephosphorylation and ligation. Undigested, supercoiled pMEA300 and pMEA301 DNA were used as controls. DNA cut with *SphI*, *SacI*, and *KpnI*, creating 3' protruding ends, gave transformation frequencies comparable to those of the control. In contrast, the frequency was 10% of the control with *BamHI*, *BglII*, *XhoI*, *Asp718*, and *ClaI*, creating 5' protruding ends, while it was 1% of the control with *PvuII* and *EcoRV*, which yield blunt ends. Approximately 70% of the pocks originating from transformation with the *EcoRV* digest of pMEA301 contained cells that were unable to grow on thiostrepton-containing media, indicating that a disruption of the *EcoRV* site had occurred. Transformation with linearized plasmid DNA has been reported for *E. coli* (Chua *et al.*, 1992; Conley and Saunders, 1984) and *Streptomyces* (Bibb *et al.*, 1980); 10^2 – to 10^3 -fold and 10^1 – to 10^2 -fold reduction in transformation frequency occurred compared to CCC plasmid DNA in these two organisms. *A. methanolica* is the first example of an actinomycete that can be transformed

served may well be due to processing and subsequent establishment of such linear molecules. Ligase overproducing *E. coli* strains display a 3-fold higher transformation frequency with linear DNA (Conley and Saunders, 1984). The transformation frequency with linear plasmid DNA with cohesive ends in *A. methanolica* is so high that it clearly provides problems in cloning experiments. The data suggest that the organism possesses a ligase displaying higher activity with cohesive ends than with blunt ends. The difference between 5' and 3' ends may depend on the presence of a polymerase that is active on 5' ends, making these blunt and thus more difficult to ligate.

Regions on pMEA300 encoding functions for site-specific chromosomal integration (Vrijbloed *et al.*, 1994) (2.1 kb), autonomous replication (Vrijbloed *et al.*, 1995b) (maintenance) (2.4 and 0.8 kb), conjugational transfer (Vrijbloed *et al.*, 1995c) (6.2 kb), and high frequency of spontaneous mutagenesis (Vrijbloed *et al.*, 1995a) (0.6 kb) have been identified previously (Fig. 1A). Deletions in the remaining 1.53-kb *NarI*[14]–*SphI*[18] fragment (pWV111, pWV113, pWV375) (Vrijbloed *et al.*, 1995a,b) (Fig. 1B; Table 1) resulted in strongly reduced transformation frequencies. The number of transformants of strain WV1 obtained with pMEA300 and pWV129 was $5 \cdot 10^4$, but only 10^3 with pWV113, pWV111, and pWV375, using saturating concentrations ($>1.0 \mu\text{g}$) of plasmid DNA.

Sequence analysis of the 1532-bp *NarI*[14]–*SphI*[18] fragment deleted from pWV113, pWV111, and pWV375 revealed one complete ORF, designated *stf* (stimulation of transformation frequency). *stf* starts at GTG-232 and terminates at TAG-1351 and would encode a protein of 373 amino acids with M_r 40,201; it is preceded by a recognizable ribosome-binding site (GGAGG) (Fig. 3). Database searches revealed considerable similarities (42% overall similarity) between the putative Stf protein and putative regulatory proteins involved in sporulation of *Streptomyces griseus* (Babcock *et al.*,

1990) and *Streptomyces coelicolor* (McCue *et al.*, 1992) (Fig. 4).

Current studies aim to resolve the regulatory role of *stf* on pMEA300-encoded functions.

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