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Identification and Functional Analysis of the Transfer Region of Plasmid pMEA300 of the Methylotrophic Actinomycete Amycolatopsis methanolica

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Amycolatopsis methanolica contains a 13.3-kb plasmid (pMEA300) that is present either as an integrated element or as an autonomously replicating plasmid. Conjugational transfer of pMEA300 results in pock formation, zones of growth inhibition that become apparent when plasmid-carrying donor cells develop in a confluent lawn of plasmid-lacking recipient cells. A 6.2-kb pMEA300 DNA region specifying the functions of conjugal pock formation was sequenced, revealing 10 open reading frames. This is the first sequence of the transfer region of a plasmid from a nonstreptomycete actinomycete. No clear similarities were found between the deduced sequences of the 10 putative Tra proteins of pMEA300 and those of Streptomyces plasmids. All Tra proteins of pMEA300 thus may represent unfamiliar types. A detailed mutational analysis showed that at least four individual proteins, TraG (9,488 Da), TraH (12,586 Da), TraI (40,468 Da), and TraJ (81,109 Da), are required for efficient transfer of pMEA300. Their disruption resulted in a clear reduction in the conjugal transfer frequencies, ranging from (5.2 × 10^6)-fold (TraG) to (2.3 × 10^3)-fold (TraJ), and in reduced pock sizes. At least two putative proteins, TraA (10,698 Da) and TraB (31,442 Da), were shown to be responsible for pock formation specifically. Specific binding of the pMEA300-encoded KorA protein to the traA-korA intragenic region was observed.

Genetic studies carried out with various actinomycetes (mainly Streptomyces, Saccharopolyspora, and Nocardia species) have provided evidence that self-transmissible plasmids are common in these gram-positive bacteria. These plasmids may be present as covalently closed circular or linear DNAs, autonomously replicating or chromosomally integrated (6, 16, 23, 34). A common phenotype associated with these actinomycete plasmids is that of pock formation, zones of growth inhibition that become apparent when plasmid-carrying donor cells develop in a confluent lawn of plasmid-lacking recipient cells (lethal zygosis) (2, 3, 7, 9, 15, 22, 23). The donor cells develop in a confluent lawn of plasmid-lacking recipient mycelium (14). In general, very few genes appear to be involved in pock formation in Streptomyces species. The mechanisms of plasmid transfer in representatives of this genus, and in other actinomycetes, remain to be elucidated (16).

We are interested in the physiological role of pMEA300, a 13.3-kb conjugal plasmid from the methylotrophic actinomycete Amycolatopsis methanolica (10, 28–31). A pMEA300-deficient derivative strain of A. methanolica has been isolated (31), allowing an analysis of pMEA300-encoded functions. This has resulted in the identification of a pMEA300-encoded mutator system (mut [28]) and of a region stimulating transformation frequency (sif [31]). Depending on growth conditions, pMEA300 is maintained either as an integrated element in the genome of A. methanolica or as an autonomously replicating plasmid, coexisting with the integrated form (29). All the plasmid-encoded information required for site-specific integration of pMEA300 was found on a DNA fragment carrying the attP and the genes encoding the integrase (Int) and excisionase (Xis) proteins (30). Two separate DNA fragments are required for maintenance of pMEA300: these contain two open reading frames (ORFs) with unknown functions (orfA and orfB) and a putative transcriptional repressor protein (KorA [29]). The requirement of the korA gene product for maintenance of free pMEA300 indicates that a kil-kor system is associated with autonomous replication (29).

Here we report the identification, nucleotide sequence, and functional analysis of the pMEA300 region involved in conjugal transfer and demonstrate that the putative proteins involved in pock formation and transfer of pMEA300 are encoded by different genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions, and transformation. The Amycolatopsis methanolica strains and plasmids used are listed in Table 1. Maintenance, cultivation, and transformation of A. methanolica NCIB 11946 wild-type (10) and pMEA300-free derivative strains have been described previously (30, 31). A. methanolica contains a very efficient methyl-dependent restriction barrier; plasmid DNA to be used for transformation of A. methanolica therefore was isolated from Escherichia coli JM110 (dam dam supE44 leu2 thi leu rpsL lacY galT glnA ara tonA thr tis A(lac-pro)B F′ (traD36 proAB lacZΔM15) (31). Transformation of strain JM110 was accomplished as previously described (8). The kanamycin concentration was 10 μg/ml for A. methanolica and 30 μg/ml for strain JM110.

Mating experiments. Strain Wv2 was used as the recipient, and strain Wv1 containing pWV129, a nonintegrative derivative of pMEA300 (29), or carrying further derivatives of pWV129 (Table 1) was used as the donor strain. The abilities of pWV129 derivatives to yield pocks were determined after transformation and plating on nonselective media. The transfer efficiencies of different
RESULTS AND DISCUSSION

Identification of the DNA region involved in pMEA300 pock formation. Previously we characterized DNA regions on pMEA300 specifying the functions of site-specific integration, located on a 2.1-kb fragment (deleted from pWV129 [30]), and plasmid maintenance (29), encoded by two unlinked DNA fragments of 2.4 kb (NspIII[2]-ScaI[3]) and 0.8 kb (ClaI[3]-NarI[18]) (Fig. 1; see the legend for an explanation of the numbers in brackets). Insertion of the tsr gene at the BglII[10] site (pMEA301) resulted in relatively small pocks (31). Deletion of the SalI[6]-Asp718[12] fragment (pWV136) and the ScaI[3]-ClaI[13] fragment (pWV368) of pWV129 (29) yielded plasmids deficient in pock formation. Deletions in the HindIII [19]-ApaI[22] region did not affect pock formation. A genetic and functional analysis of the pMEA300 transfer region (the ScaI[3]-ClaI[13] fragment) subsequently was carried out.

Sequence analysis of the ScaI[3]-ClaI[13] fragment of pMEA300. The nucleotide sequence of the 6.2-kb ScaI[3]-ClaI[13] region was determined (Fig. 2). For convenience, the 5′ end of the previously identified orf′ (29), now renamed traA, is shown. The previously identified orf2 (29) is now renamed traJ (see below). Sequence analysis with the computer program GCWIND (26) revealed 10 ORFs, designated traA to traJ, all transcribed in the same direction (Fig. 1). Four of these ORFs are separated (if at all) by very short untranslatable sequences, and only a single inverted repeat that might serve as a transcription stop site (ΔG = −22 kcal [ca. −4.2 × 10^3 J]) was detected, downstream of the traJ gene. Some characteristics of each ORF are summarized in Table 2. Analysis of putative Shine-Dalgarno sequences of Streptomyces genes revealed the conserved sequence (A/G)-G-G-A-G-G (27). Similar sequences were found near the predicted initiation codon of each ORF. Either ATG or GTG start codons were found at the predicted initiation codon (Table 2). 30

pWV129 derivatives were determined as follows. Overnight cultures of the donor strain (1 × 10^9 to 2 × 10^9 cells) and recipient strains (1 × 10^9 to 2 × 10^9 cells) grown in complete medium (CM [30]) were mixed. After centrifugation in an Eppendorf centrifuge, the pellet was resuspended in 0.1 ml of CM. This solution was applied in the center of a 0.2-μm-pore-size membrane filter with a diameter of 25 mm (Schleicher and Schuell, Dassel, Germany). The filter was placed on a sterilized tissue paper to remove excess liquid and incubated on CM agar at 37°C for 20 h. The cells were subsequently removed from the filter and resuspended in 5 ml of liquid CM. Various dilutions were plated on CM agar containing 10 and 125 μg of kanamycin and spectinomycin per ml, respectively. The numbers of colonies resistant to spectinomycin and to both antibiotics were determined, and the transfer frequencies relative to that of pWV129 were calculated.

DNA isolations, manipulations, and sequencing. Preparation of plasmid DNA of E. coli was done by the alkaline lysis (24). DNA sequencing was done as previously described (30). The nucleotide sequence data were analyzed with the programs supplied in the PC/GENE software package (IntelliGenetics, Mountain View, Calif.). ORFs coding for putative proteins were identified with the computer program GCWIND (26). This program is based on the biased codon usage that occurs in organisms with a high G+C content. The deduced amino acid sequences of the identified ORFs were compared with sequences in protein databases by using the BLAST program (1).

Nucleotide sequence accession number. The nucleotide sequence data determined in this report have been deposited in the GenBank database under the accession number L36679.

TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. methanolica strains</td>
<td>Wild-type strain, contains pMEA300 in a free and integrated form</td>
<td>30</td>
</tr>
<tr>
<td>NCIB 11946</td>
<td>pMEA300-free derivative strain of A. methanolica</td>
<td>31</td>
</tr>
<tr>
<td>WV1</td>
<td>Spectinomycin-resistant derivative of WV1</td>
<td>31</td>
</tr>
</tbody>
</table>

Plasmids

pBluescript (II) KS * | ColE1-derived plasmid carrying an IPTG-inducible lacZ promoter (Stratagene, Westburg, Leusden, The Netherlands) | 1 |
| pHSS6 | ColE1-derived plasmid carrying the kanamycin resistance gene of Tn5 | 25 |
| pKT245-Ap | Contains ampicillin resistance gene flanked by omega elements | 11 |
| pMEA300 | Wild-type plasmid | 30 |
| pMEA301 | Insertion of tsr gene (BclI fragment of pL702) in the BglII[10] site of pMEA300 | 31 |
| pWV306 | Asp718[12]-PvuII[20] fragment (2.3 kb) of pMEA300 inserted in the EcoRV-Asp718 fragment of pBluescript (II) KS | This paper |
| pJB1 | Insertion of korA of pMEA300 (as a 390-bp Eco47-3[15]-HindII[17] fragment; Fig. 1) in the correct orientation in front of the IPTG-inducible lacZ promoter of pBluescript (II) KS* | This paper |
| pWV129 | BamHI[1]-ApaI[22] (blunt) fragment of pMEA300 inserted in BamHI-EcoRI (blunt) fragment of pHSS6; contains the kanamycin resistance gene | 29 |

pWV129-derived plasmids

| pWV136 | del SalI[6]-Asp718[12] | 29 |
| pWV368 | del ScaI[3]-ClaI[13] | 29 |
| pMAT103 | del ScaI[3]-ClaI[13] | 29 |
| pMAT104 | del ScaI[3]-ClaI[13] | 29 |

* Numbers between brackets refer to pWV129 restriction enzyme sites shown in Fig. 1.
FIG. 1. Restriction map of pWV129 (29) and functional analysis of pWV129-derived plasmids. Only restriction sites (numbers between brackets) relevant to plasmid construction are shown. Other numbers indicate distances (in kilobases) of restriction sites from the unique BamHI site. Arrows indicate ORFs on pWV129 (open arrows, previously described [28–31]; solid arrows, described in this work; dotted arrow and box, vector-related features). Arrowheads indicate restriction sites disrupted with Klenow. Bars indicate the various deletions in pWV129. Ω indicates a restriction site disrupted with the omega interposon of pKT254[11]. The results of transformation experiments with strain WV1, selecting for pock formation (31), and conjugation experiments with donor strain WV1 and recipient strain WV2, with pWV129 or further derivative plasmids, are shown. Transfer frequencies were determined by counting the number of colonies resistant to both spectinomycin and kanamycin. Data are expressed relative to the observations made with pWV129.
Neither the nucleotide sequences nor the predicted amino acid sequences of these 10 ORFs showed similarity to any sequence in the databases searched. Similarity was not even observed with the most closely related system studied thus far, the integrative plasmid pSAM2 of S. ambofaciens (14). The average G+C content (71.2%) of the \( Sca^1 - Cla^1 \) region is similar to that of the replication region (72.7%) but relatively high compared with that of the region involved in plasmid integration (65.2%) (29, 30). Also, for plasmid pSAM2 of S. ambofaciens the G+C content of the transfer (71.6%) and replication regions (71.7%) were similar but significantly higher than that of the integration region (68.6%) (4, 13, 14). If the G+C content of a gene reflects the efficiency of its translation (33), then it may be that the \( tra \) genes of these plasmids translated more efficiently than their integration genes.

Functional analysis of the \( Sca^1 - Cla^1 \) fragment. A series of pWV129-derived plasmids was constructed for a further functional analysis of the pMEA300 DNA region involved in pock formation. The restriction sites \( Cla^1 \), \( Asp^718 \), \( Bsm^I \), \( Asc^I \), \( Bsi^W \), and \( Bsp^HI \) were disrupted by filling in the 5'-overhanging ends with Klenow fragment (Table 1, Fig. 1). This caused a disruption of the \( traA \) gene, whereas the \( traB \), \( traG \), \( traH \), \( traI \), and \( traJ \) genes remained intact. The putative transcription stop located downstream of \( traI \) is indicated by arrows. Boldfaced and underlined amino acids indicate putative membrane-spanning regions (21).

Table 2. Characteristics of ORFs involved in pMEA300 transfer and pock formation

<table>
<thead>
<tr>
<th>ORF</th>
<th>No. of amino acids</th>
<th>Position of putative SD sequences*</th>
<th>Predicted ( M_r ) of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>( traA )</td>
<td>97</td>
<td>AGGAGA--3-ATG</td>
<td>10,698</td>
</tr>
<tr>
<td>( traB )</td>
<td>292</td>
<td>AGGGCG--10-ATG</td>
<td>31,442</td>
</tr>
<tr>
<td>( traC )</td>
<td>143</td>
<td>AGGAGG--5-GTG</td>
<td>15,436</td>
</tr>
<tr>
<td>( traD )</td>
<td>86</td>
<td>AGGAGG--4-GTG</td>
<td>9,457</td>
</tr>
<tr>
<td>( traEb )</td>
<td>58</td>
<td>CGGAGG--7-ATG</td>
<td>6,334</td>
</tr>
<tr>
<td>( traF )</td>
<td>79</td>
<td>AGGGCG--6-GTG</td>
<td>8,660</td>
</tr>
<tr>
<td>( traGb )</td>
<td>84</td>
<td>GTAGAC--4-GTG</td>
<td>9,488</td>
</tr>
<tr>
<td>( traHb )</td>
<td>115</td>
<td>AGGAGG--6-GTG</td>
<td>12,586</td>
</tr>
<tr>
<td>( traIb )</td>
<td>374</td>
<td>GGGAGG--7-GTG</td>
<td>40,468</td>
</tr>
<tr>
<td>( traJb )</td>
<td>737</td>
<td>GGGAGG--6-GTG</td>
<td>81,109</td>
</tr>
</tbody>
</table>

* The putative Shine-Dalgarno (SD) sequences and their distances to the predicted start codon of each ORF are indicated.

Analysis of the deduced amino acid sequence (Fig. 2) revealed the presence of membrane-spanning regions (21).
KorA binds to the traA-korA intergenic region. Gel retardation assays were performed to investigate possible binding of KorA to the putative promoter of the tra genes (29). For this purpose, the KorA protein was expressed in *E. coli* DH5α (24) cells harboring pJB1 (Table 1). A 13-kDa protein was found in extracts of these cells, grown overnight in Luria-Bertani medium with IPTG (isopropyl-β-D-thiogalactopyranoside) (0.3 mM); this corresponded well with the expected KorA size of 12.9 kDa (29). Gel retardation experiments demonstrated that KorA binds specifically to the *Sal*I[-14] - *Sal*I[-16] intergenic region between *korA* and *traA* (Fig. 1 and 4). The appearance of more slowly migrating bands with higher concentrations of KorA also indicated that KorA protein binds most likely at multiple sites in this region. KorA of pMEA300 (29) thus may play a key role in transcriptional control of the pMEA300 tra genes. Transfer of some *Streptomyces* plasmids has been shown to be associated with a *kil-kor* system, e.g., *tra-korA* in pJJ101 (19), *traB-traR* in pSN22 (18), and *traSA-korSA* in pSAM2 (14). In each case, the plasmid transfer region thus carries the *kil* locus. Conceivably, *traA/traB-korA* constitutes a *kil* system associated with pMEA300 transfer. This, the mechanism for pock formation, and transcriptional regulation of the tra genes are currently the subjects of further analysis.

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We are indebted to P. Terpstra for assistance with the sequence analysis and to R. van den Bergh for valuable discussions.

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