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

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Chapter 6

Identification and characterisation of the minimal replicon of the indigenous plasmid pMEA300 of the actinomycete *Amycolatopsis methanolica*

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actinomycete cccDNA plasmids belong either to the pC194 plasmid family (Seery *et al.*, 1993;Muth *et al.*, 1995;Hagege *et al.*, 1993a;Servin-Gonzalez, 1993;Billington *et al.*, 1998;Suzuki *et al.*, 1997), which is widely distributed in Gram-positive and Gram-negative bacteria, or to a recently recognized fifth family of RCR replicons (Venkova-Canova *et al.*, 2003), with only *Corynebacterium* plasmids, pGA1, pSR1 and pNG2 (Nesvera *et al.*, 1997;Archer and Sinskey, 1993;Zhang *et al.*, 1994).

The actinomycete *Amycolatopsis methanolica* (Vrijbloed *et al.*, 1995b) harbours a 13.3 kb plasmid, pMEA300. The complete sequence of pMEA300 has been determined previously (GenBank database accession number L36679), revealing the presence of 20 putative ORFs. Construction and characterisation of deletion derivatives allowed the identification of genes required for conjugation (Vrijbloed *et al.*, 1995c), regulation and integration (Vrijbloed *et al.*, 1994). The region of pMEA300 required for autonomous replication was previously minimised to two unlinked DNA fragments, encoding OrfA and OrfB, and KorA (Fig. 1) (Vrijbloed *et al.*, 1995a), a putative transcriptional regulator of the genes involved in conjugative transfer. Like other plasmid-encoded Kor proteins (Hagege *et al.*, 1993b), KorA is similar to GntR-type transcriptional regulators (Vrijbloed *et al.*, 1995c).

In this paper we report a further reduction of the minimal replicon size of pMEA300 to (part of) *orfA* and *orfB* (*rep*). Interestingly, the amino acid sequence of the identified replication protein (Rep) does not share any significant homology with Rep proteins of the pC194 plasmid family, nor with any (Rep) protein identified so far. Furthermore, Rep of pMEA300 does not contain any of the conserved motifs found in pC194 or in the 3 other families of RCR plasmids (del Solar *et al.*, 1998). The Rep protein binds specifically, however, to a pMEA300 fragment containing a DNA sequence motif with similarity to the pC194 nicking site region (del Solar *et al.*, 1998).

Material and Methods

Microorganisms and cultivation.

Amycolatopsis methanolica wild-type (NCIB 11946) (De Boer L. *et al.*, 1990a) and the plasmid pMEA300 deficient strain WV1 (Vrijbloed *et al.*, 1994;Vrijbloed *et al.*, 1995b) were used. The procedures followed for cultivation in batch cultures, harvesting of cells, and growth measurements have all been described previously (De Boer *et al.*, 1988).

E. coli strains DH5α (*recA*) and BL21(DE3) were grown in Luria-Bertani (LB) medium at 37^{o}C and 30^{o}C, respectively. When necessary, the following supplements were added: 100µg/ml ampicillin/chloramphenicol; 0.4 mM IPTG. Agar was added for solid media (1.2 % (wt/vol)).

Rhodococcus erythropolis SQ1 (Quan and Dabbs, 1993) was cultivated at 30^{o}C in liquid LBP-medium containing 1% (wt/vol) Bacto Peptone (Difco, Detroit, Mich.), 0.5% (wt/vol) yeast extract and 1% (wt/vol) NaCl. *Streptomyces lividans* 1326 was cultivated at 30^{o}C on R5-medium (Kieser *et al.*, 2000).

DNA manipulations.

Plasmid DNA from *E. coli* and *R. erythropolis* SQ1 was isolated using commercially available spin-prep kits (Sigma), which are based on the alkaline lysis method (Birnboim and Doly, 1979). Prior to plasmid DNA isolation from *R. erythropolis* SQ1, cultures were incubated with ampicillin (600 µg/ml) for 2 h, to improve cell lysis. Transformation of *R. erythropolis* SQ1 was performed as described previously (van der Geize *et al.*, 2000). *S. lividans* protoplast preparation and transformation were performed as described (Kieser *et al.*, 2000). DNA-modifying enzymes were obtained from commercial sources and used as recommended by the manufacturer. All other DNA manipulations were done according to standard protocols (Sambrook *et al.*, 1989).

Limited information is available on the mechanism of plasmid replication in actinomycetes, especially in the genus *Amycolatopsis*. Plasmid pMEA300 of *Amycolatopsis methanolica* site-specifically integrates into its genome but also can replicate autonomously. The complete nucleotide sequence of the plasmid has been determined previously. Functional characterisation of deletion derivatives of pMEA300 resulted in identification of its minimal replicon, constituted by *orfB* (*rep*) and (part of the) *orfA* genes. The pMEA300 Rep and OrfA proteins show no similarity with any other Rep protein in databases, but Rep shows significant similarity to a putative protein encoded by *orf1* of the *Saccharopolyspora erythraea* plasmid pSE211. Purified pMEA300 Rep protein binds specifically to the 3' end region of the *rep* gene, containing a stem loop structure with a DNA sequence motif (also present in *orf1* of pSE211 of *S. erythraea*) with similarity to the nicking site of the Double Strand Origin (DSO) of the pC194 family of rolling circle replication (RCR) plasmids. However, the functional organisation of pMEA300, with the putative DSO located within the *rep* gene, differs from that of the pC194 RCR plasmid family. The data suggest that pMEA300 *rep* (and *orf1* of pSE211 of *S. erythraea*) constitute the first examples of *rep* genes belonging to a new family of RCR plasmids.

The pMEA300 minimal replicon (devoid of regulatory features), with only the *rep* (plus DSO) and (part of the) *orfA* genes, but not pMEA300 itself, also replicated in *Rhodococcus erythropolis* SQ1, but not in *Streptomyces lividans*. This is the first report of an *Amycolatopsis* plasmid derivative that also can replicate in a *Rhodococcus* species.

Introduction

Actinomycetes are Gram-positive, mycelium-forming, soil bacteria that play an important role in mineralisation processes in nature and are abundant producers of antibiotics and other secondary metabolites. Genetic studies, which have focussed on *Streptomyces*, *Saccharopolyspora* and *Amycolatopsis* (*Nocardia*) species, provided evidence that self-transmissible plasmids are widespread in these organisms. These plasmids may be present as covalently closed circular (ccc), or as linear DNA molecules, and some of them are capable of site-specific integration into the genome (Brown *et al.*, 1988;Vrijbloed *et al.*, 1994;Vrijbloed *et al.*, 1994). So far, only a single phenotype was found to be associated with transfer of these plasmids: i.e. the development of inhibition zones (also referred to as pock formation) when plasmid-carrying donor cells grow in a confluent lawn of plasmid-lacking potential recipient cells (Bibb *et al.*, 1977). In contrast, plasmids from other bacteria may encode a large variety of other functions. They frequently have been shown to carry genes conferring antibiotic resistance, or proteins for catabolic pathways (Jacoby and Shapiro, 1977).

Where studied, actinomycete plasmids (pSAM2, pSG5, pAP1, pSN22, and pNG2) were found to replicate via the rolling circle replication (RCR) mechanism (Hagege *et al.*, 1993a) and, because of their sequence similarity, this is also assumed to be the case for pJV1, pIJ101, pSR1 and pGA1 (Servin-Gonzalez, 1993;Suzuki *et al.*, 1997;Archer and Sinskey, 1993;Nesvera *et al.*, 1997). RCR is initiated when the replication initiator (Rep) protein nicks the plus strand of the Double Strand Origin (DSO). The Rep protein is covalently attached to the 5' phosphate at the nick site, as leading strand replication is initiated from the 3' OH end. After the leading strand has been fully displaced, the Rep protein cleaves the displaced ssDNA at the regenerated nick site. After a series of cleavage/joining reactions one double stranded plasmid and one circular, single stranded form is generated. The latter one is converted into dsDNA using the single stranded origin (SSO) and the host replication machinery. Based on Rep sequence alignments and DSO nicking site similarities, RCR plasmids can be categorised into four main families: pT181, pC194, pMV158 and pSN2 (del Solar *et al.*, 1998). All characterised

DNA sequencing.
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 6.00.

Transformation of *A. methanolica* WV1.
Media used for growth have been described previously (Madon and Hutter, 1991). A simplified version of the method previously described (Vrijbloed *et al.*, 1995b) was used to transform *A. methanolica* WV1. This improved method, omitting the (soft agar) overlay steps, is much less laborious and yields similar transformation frequencies as reported previously (3-5.10^4 transformants, using saturating concentrations >1.0 µg of plasmid DNA) (Vrijbloed *et al.*, 1995b). Additionally, with the new procedure background growth on agar plates became significantly reduced.
Preparation of competent cells: Overnight cultures of *A. methanolica* WV1 on Tryptic Soy Broth (TSB) medium (25 ml, grown to an OD_{430} of 5.0) were centrifuged (5 min, at 3,600 g), washed in 25 ml $T_{10}E_1$ and resuspended in $T_{10}E_1$ to an OD_{430} of 160.
Transformation of *A. methanolica* WV1: to 100 µl cell suspension, 10 µl 0.2 M $MgCl_2$, 60 µl 4.17 M $CsCl_2$, target DNA and $T_{10}E_1$ to a total volume of 20 µl was added and mixed by pipetting. Then 200 µl 65% (w/v) PEG-1000 (Koch Light) was added and gently mixed by pipetting. The transformation mixture was incubated for 40 min at 37°C. Following incubation, 1 ml of T27M (3% (wt/vol) trypticase soy broth (BBL), 7.3% (wt/vol) mannitol) (37°C) was added, gently mixed and centrifuged (1 min, 10,000 x g). After a second wash step with 1 ml T27M, cells were resuspended in 500 µl T27M and incubated for 5-7 h at 37°C on a shaking incubator. Finally, an appropriate amount of cell suspension was transferred on T27M agar plates containing the antibiotics kanamycin (20 µg /ml). Transformants appeared after approx. 3 days of incubation at 37°C.

Analysis of autonomous replication.
Autonomous replication of the pMEA300 derivatives in *A. methanolica* WV1 was checked by Southern hybridisation. Total DNA was isolated from transformants grown overnight in 2 ml of TSB supplemented with 20 µg/ml kanamycin. After DNA digestion with appropriate restriction enzymes, Southern hybridisation was performed with a DIG DNA Labeling and Detection Kit (Roche) using the *orfB* (*rep*) gene of pMEA300 as probe. Replication of pMEA300 constructs also was checked by isolation of total DNA of *A. methanolica* WV1 followed by transformation to *E. coli* DH5α and subsequent restriction analysis of plasmid DNA of *E. coli* transformants.
Autonomous replication of pMEA300 derivatives in *R. erythropolis* SQ1 was checked by isolation of plasmid DNA from *R. erythropolis* SQ1 transformants, its transformation to *E. coli* DH5α, followed by restriction analysis of plasmid DNA of *E. coli* transformants obtained.

Construction of Rep expression plasmids.
The *rep* gene (*orfB*) and *orfA* were PCR-amplified from pMEA300 plasmid DNA using the following primer pairs: for *rep*: 5'-GCGCATATGACCGCCAACCCCGGAGC-3' and 5'-CGC GGATCCTCAGGCGTTGTTGCCGACGAC-3' and for *orfA*: 5'-GCGCATATGCCTACCGCC ACAGCG-3' and 5'-CGCGGATCCTCAGGCGGCCACCTCCAGGT-3'. In both cases the first primer introduces a *Nde*I-restriction site (underlined) at the start codon, and the second a *Bam*HI site (underlined) at the termination codon. Reactions were performed with Vent DNA

polymerase (Biolabs, New-England). Restriction-digested PCR fragments were cloned into the *Nde*I-*Bam*HI sites of pET15B (Novagen), introducing an N-terminal His_6-tag in both proteins, yielding the expression plasmids pHisRep and pHisOrfA. Sequencing of the pHisRep construct showed that no amplification errors had been introduced.

Rep and OrfA protein purification.
A. methanolica pMEA300 Rep and OrfA proteins were overproduced in *E. coli* BL21(DE3)-pHisRep or *E. coli* BL21(DE3)-pHisOrfA, respectively. Cells were grown at 30ºC in 50 ml LB medium containing 50 µg/ml ampicillin. Protein expression was induced by addition of 0.5 mM IPTG at an OD_{660} of 0.3 and subsequent incubation at room temperature for 24 h. Cells were harvested by centrifugation and resuspended in 1 ml of 25 mM Tris-HCl pH 8.5. Cell free extracts were obtained by passage over a French pressure cell operating at $1.4*10^5$ $kN.m^{-2}$ and subsequent centrifugation (1 min, 10,000 x g). His-tagged Rep and OrfA protein were purified with nickel nitrilotriacetic acid (Ni-NTA) resin (Qiagen), using the protocol of the manufacturer.

Gel retardation assays.
Purified His-tagged Rep or His-tagged OrfA protein (0.13 µg and 0.25 µg, respectively) were incubated with 0.5 µg restriction-digested pHK315 plasmid DNA (unless stated otherwise) for 30 min at 37ºC in 15 µl of reaction buffer (10 mM Tris-HCl pH 8; 5 mM $MgCl_2$; 100 mM NaCl, 1 mM 2-mercaptoethanol; 5 mM DTT; 50 µg/ml BSA). Binding of plasmid DNA by Rep or OrfA was studied by analysing DNA mobility in 1.2 % agarose gels. Following electrophoresis, gels were stained with ethidium bromide and photographed under UV illumination.

Database accession number.
The nucleotide sequence of pMEA300 presented in this paper has been deposited in the GenBank database under the accession number L36679.

Results and Discussion
We previously reduced the *A. methanolica* indigenous plasmid pMEA300 to two unlinked DNA fragments (fragments A and B in Fig. 1) that appeared essential for plasmid replication. Firstly, a *Cla*I -*Nar*I fragment of 774 bp, which contains the complete *korA* gene, and the *korA*-*traA* intergenic region (putatively with overlapping promoters), and secondly, a 2.6 kb *Nsp*HI-*Aat*II fragment, containing *orfA* and *orfB* encoding (putative) proteins of 170 and 416 amino acids, respectively (Fig. 1) (Vrijbloed *et al.*, 1995a).

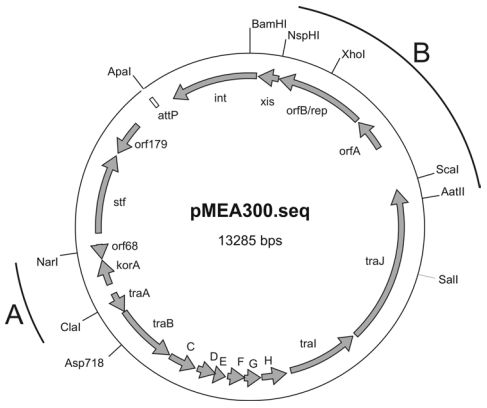

Figure 1. Restriction map of pMEA300. The plasmid regions indicated by A and B were previously identified as essential for replication (Vrijbloed *et al.*, 1995a).

The pMEA300 *Nsp*HI-*Aat*II fragment carries all genes essential for plasmid replication

To further identify the region involved in pMEA300 replication, deletion derivatives were constructed from the *E. coli*-*A. methanolica* shuttle vector pWV136 (Table 1, Fig. 2) (Vrijbloed *et al.*, 1995a). Firstly, the remaining parts of the transfer genes were removed via a *Cla*I-*Sca*I deletion in pWV136, yielding pHK315 (Table 1, Fig. 2). As anticipated, this construct was still capable of autonomous replication in *A. methanolica* WV1, which was demonstrated using the standard tests, i.e. by Southern hybridisation on total DNA of *A. methanolica* WV1-pHK315 and by transforming *E. coli* cells with total DNA extracted from *A. methanolica* WV1-pHK315 cells, followed by *E. coli* plasmid DNA analysis. Subsequent deletion of the *Sac*I-*Bst*EII(2) fragment of pWV136 (pHK314, Fig. 2, Table 1), completely abolished replication in *A. methanolica* WV1, since no transformants could be acquired when the strain was transformed with pHK314. This indicates that the *Sca*I-*Bst*EII(2) fragment is essential for replication. Disruption of the ORF of *orfA* (pHK313, Fig. 2, Table 1) did not result in loss of autonomous replication. Thus, (part of) the *Sca*I-*Bst*EII(2) fragment is essential for pMEA300 replication, but a functional OrfA protein is not required. Interestingly, Southern hybridisation on total DNA of *A. methanolica* WV1-pHK313 revealed a much stronger hybridisation signal compared to the hybridisation signal obtained with *A. methanolica* WV1-pHK315 total DNA, indicating that the copy number of pHK313 is much higher than that of pHK315. OrfA thus may down-regulate pMEA300 copy number, e.g. via inhibition of Rep protein activity.

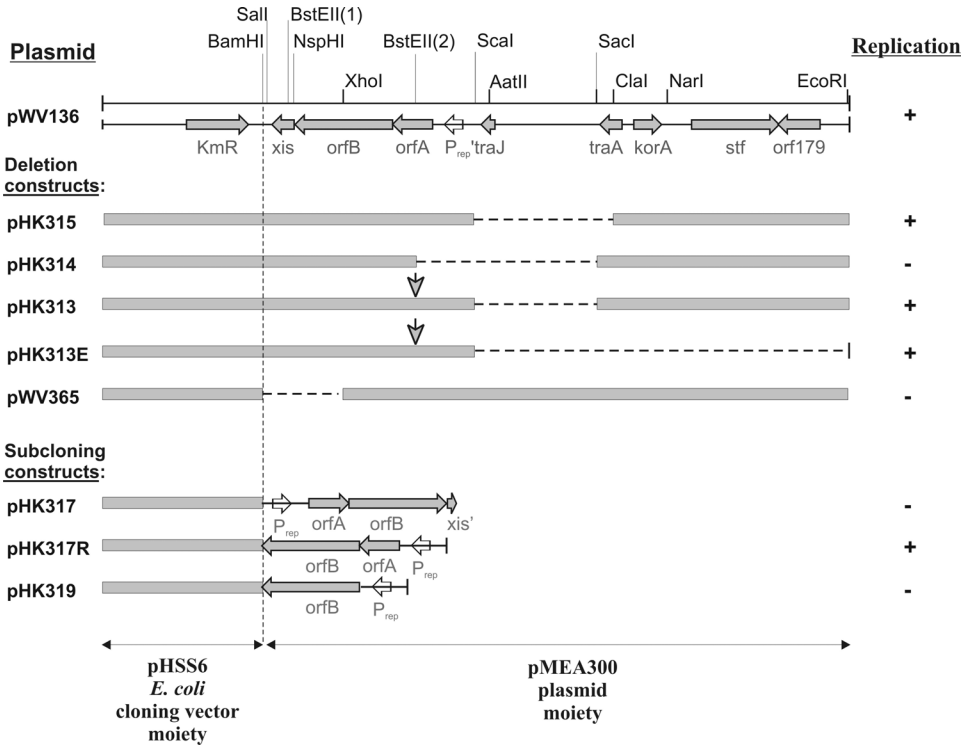

Figure 2. Linear representation of (deletion) derivatives of the pMEA300-derived shuttle vector pWV136. Dotted lines indicate deletions made in the various constructs. Vertical arrows indicate disruption of the ORF of *orfA* by digestion and subsequent Klenow DNA polymerase fill-in of the *Bst*EII(2) site in *orfA*.

Table 1. Deletion constructs and subclones of pWV136.

Plasmid	Description
pWV136	*E. coli*-*A. methanolica* shuttle vector (Vrijbloed *et al.*, 1995a), containing the 11.9 kb *Bam*HI-*Apa*I fragment, with a *Sal*I-*Asp*718 deletion of the region involved in conjugative transfer, of pMEA300 (Fig. 1), cloned into the *Bam*HI-*Eco*RI sites of the *E. coli* cloning vector pHSS6 (Seifert *et al.*, 1986).
pHK315	*Cla*I-*Sca*I deletion construct of pWV136 (Fig. 2).
pHK314	*Sac*I-*Bst*EII(2) deletion construct of pWV136 (Fig. 2).
pHK313	*Sac*I-*Sca*I deletion construct of pWV136, containing a disruption of the ORF of *orfA* by digestion and subsequent Klenow DNA polymerase fill-in of the *Bst*EII(2) site in *orfA*. The frameshift starts at amino acid residue 66. Total length of OrfA: 170 amino acid residues (Fig. 2).
pHK313E	Deletion construct of pHK313 in which the *Eco*R1-*Sca*I fragment has been deleted (Fig. 2).
pHK317	*Sca*I-*Bam*HI DNA fragment of pWV136 cloned into the *Bam*HI-*Sma*I site of pHSS6, followed by deletion of the *Sal*I-*Bst*EII(1) fragment (Fig. 2).
pHK317R	PCR fragment stretching from the *Sca*I site to the stop codon of *orfB* cloned into the *Sma*I site of pHSS6, with *orfA* and *orfB* positioned in the reverse orientation in pHSS6 as pHK317 (Fig. 2).
pHK319	Constructed by joining two PCR products, one flanked by the *Sca*I-site and the start codon of *orfA*, and the other starting 20 bp upstream of *orfB* (including the ribosome binding site of *orfB*) and ending at the stop codon of *orfB*, and subsequent cloning in the *Bam*HI and *Eco*RI sites of the pHSS6 cloning vector (Fig. 2).

No transformants were obtained when attempting to transform *A. methanolica* WV1 cells with pHK317, which only contains the (*orfA* and *orfB*) genes located on the *Nsp*HI-*Aat*II fragment of pMEA300 (Fig. 2, Table 1). However, plasmid pHK317R, in which *orfA* and *orfB* are positioned in the reverse orientation in pHSS6 (Table 1, Fig. 2), could be transformed to *A. methanolica* WV1 and replicated autonomously. This data thus showed that all pMEA300 genes necessary for replication, and its origin of replication, are located on the pMEA300 segment flanked by *Sca*I and the stop codon of *orfB*. *KorA* and the *korA*-*traA* intergenic region, putatively with overlapping promoters, are not essential for replication. The dispensability of the *traA* promoter also indicated that the promoter of the replication gene *orfB* (and of *orfA*) must be located on the region flanked by *Sca*I and the start codon of *orfA*.
The differences between pHK317 and pHK317R may be explained by a polar effect of the

kanamycin promoter. In case of pHK317 this would stimulate expression of the *orfA* and *orfB* genes. Because no replication is observed with pHK317, this may suggest that OrfA negatively regulates replication, e.g. via inhibition of Rep protein activity.

***orfA* sequences are essential for pMEA300 replication**

As shown for pHK313, disruption of the pMEA300 *orfA* gene did not affect replication. This was also shown with construct pHK313E (Fig. 2, Table 1), a deletion construct of pHK313 in which the *Eco*R1-*Sca*I fragment had been deleted. Like pHK317R, pHK313E could replicate in *A. methanolica* WV1. Complete deletion of *orfA* abolished replication of pMEA300 (derivatives), however. This was shown with pHK319, which completely lacks *orfA* sequences (Fig. 2, Table 1). With disruption of *orfA* (pHK313E) not affecting plasmid replication, we conclude that the OrfA protein is not required for replication. However, the inability of pHK319 to replicate suggests that *orfA* contains DNA sequences that are essential for replication, e.g. a Single Strand Origin (SSO). Protein database searches, using the Blast search tool (Altschul *et al.*, 1990), did not reveal any protein with significant homology to OrfA.

To confirm whether *orfA* indeed encodes a protein, this gene was cloned into the expression vector pET15b and transformed to *E. coli* BL21(DE3). Cell extracts from *E. coli* transformed with pHisOrfA showed an additional protein band of approx. 20 kDa on SDS-PAA gels compared to an extract of *E. coli* BL21(DE3) transformed with the pET15b expression vector, which is in agreement with the expected size of OrfA (18.4 kDa). OrfA protein was purified from *E. coli* cell extracts using Ni-NTA column chromatography. To determine whether OrfA functions as a transcriptional regulator, purified OrfA preparations were incubated with pHK315, either as cccDNA or fragmented with various restriction enzymes. No DNA binding activity of OrfA could be demonstrated in gel retardation experiments. A putative regulatory role of OrfA as a DNA binding protein cannot completely be ruled out yet, however, and possibly requires presence of specific (low molecular weight) effector molecules.

***orfB* encodes the pMEA300 replication protein**

Previously, we have shown that *orfB* is required for autonomous replication: construct pWV365, derived from pWV136 by deleting part of *orfB* (the *Xho*I-*Bam*HI fragment, Fig. 2), was incapable of autonomous replication (Vrijbloed *et al.*, 1995a). The total data set thus allows us to conclude that *orfB* encodes the replication protein of pMEA300. Its minimal replicon thus consists of only one complete gene, namely *orfB*, and (part of) the sequence of *orfA*. From here on, the OrfB protein will be referred to as Rep. Interestingly, Rep of pMEA300 shows no similarity to any other Rep protein found. Extensive sequence similarity (52%), however, exists between Rep and the putative protein encoded by the *orf1* gene (with unknown function) of *Saccharopolyspora erythraea* plasmid pSE211 (Fig. 4). This high similarity suggests that *orf1* may be the replication gene of pSE211.

Rep binds to the 3' end of its own coding sequence

Initiation of plasmid replication involves binding of a plasmid-encoded Rep initiator protein to a specific sequence (origin of replication; *ori*) of the plasmid. To determine whether Rep is able to bind pMEA300 DNA, gel retardation experiments were performed with purified Rep protein. To obtain Rep protein, the *rep* gene was cloned into the expression vector pET15b and transformed to *E. coli* BL21(DE3). Cell extracts from *E. coli* transformed with pHisRep showed an additional band of approx. 48 kDa on SDS-PAA gels compared to an extract of *E. coli* BL21(DE3) transformed with the pET15b expression vector, which is in agreement with the expected size of Rep (45.3 kDa). Rep was purified from *E. coli* cell extracts using Ni-NTA column chromatography. Plasmid pHK315 DNA was used in the gel retardation assays.

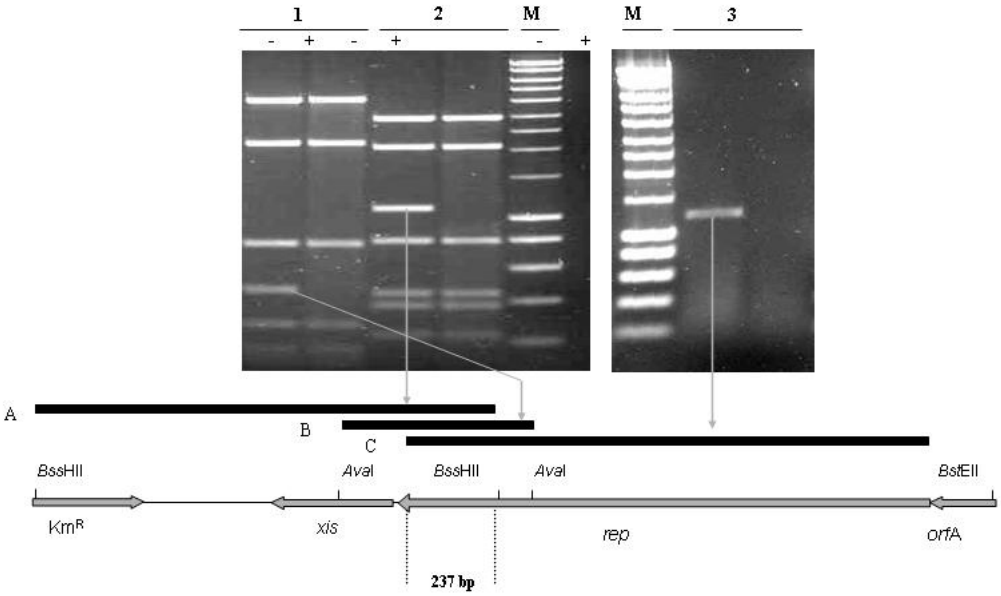

Figure 3. Gel retardation experiments carried out with (+) or without (-) 0.13 µg of purified His_6Rep protein and a *Ava*I (1) and a *Bss*HII (2) digest of pHK315 (0.5 µg), and with 0.02 µg of the PCR product of *rep* (3). The positions of the retarded DNA restriction fragments (A, B) or PCR product (C) on pHK315 are indicated by bars. M: SmartLadder (Eurogentec) DNA molecular weight marker (10,000, 8000, 6000, 5000, 4000, 3000, 2500, 2000, 1500, 1000, 800, 600, 400, 200 bp).

Specific binding of Rep was shown to the pMEA300 moiety of the shuttle plasmid pHK315, while no binding was observed with the pHSS6 part of pHK315. Addition of purified OrfA to the reaction mix did not affect the DNA binding (and DNA nicking) activity of Rep. To locate the region of pMEA300 to which Rep binds, gel shift assays were performed with purified Rep protein and restriction fragments of pHK315. Rep specifically binds a 374 bp *Ava*I-*Bss*HII fragment of pHK315, whereas the negative controls - *E. coli* BL21(DE3)-pET15b and a reaction mixture without Rep – did not show any binding (Fig. 3). Rep-bound DNA did not migrate into the agarose gel and remained in the slots of the gels, evident from DNA (ethidium bromide) staining and protein (Coomassie Brilliant Blue) staining (not visible in Fig. 3). Furthermore, Rep was shown to bind to a PCR product of *rep*. The binding region of Rep was thus narrowed down to 237 bp (Fig. 3). Gel shift assays were also performed with restriction fragments of other constructs covering the entire pMEA300-sequence, but Rep was only shown to bind to the 237 bp *rep* gene fragment flanked by the *BssH*II site and the *rep* stop codon (data not shown). The binding region in *rep* contains a strong stem loop structure (Fig. 4), which is often found in the *bind* region of the DSO (del Solar *et al.*, 1998). This stem loop structure (138 bp) produced by a 23 bp inverted repeat shows high similarity (56% identity) to a 174 bp stem loop structure within *orf1* of plasmid pSE211 of *S. erythraea* (Fig. 4). The loop of this putative stem loop structure carries two DNA sequence motifs with clear similarity to the nicking site (5'-CTTGATA-3') of the pC194 RCR family. One motif was found on the sense strand and the other on the anti-sense strand; the same observation was made for *orf1* of pSE211 (Fig. 4).

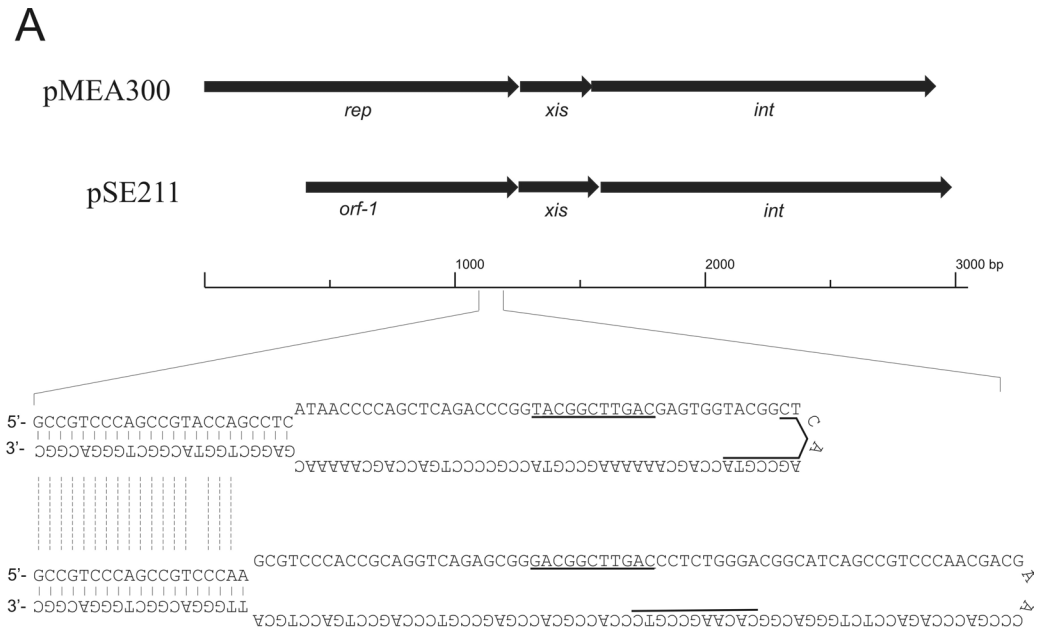

Figure 4. (A) Stem loop structure present in the DSO in the *rep* gene of pMEA300 and its similarity with a stem loop structure present at the same position in *orf1* of pSE211. The putative pC194 type nicking sites present on the sense (s) and antisense (as) strands are underlined. (B) Alignment of pC194 type nicking sites found in actinomycete plasmids. The nicking position is indicated by an arrow.

Further biochemical analysis is needed to determine whether one of these sites is indeed nicked by Rep. However, besides similarity to the pC194 family nicking site, the Rep amino acid sequence does not share any significant homology with the pC194 family of Rep proteins, nor with any Rep protein identified so far. We were also unable to detect any of the consensus sequences characteristic for RCR Rep proteins (Ilyina and Koonin, 1992;del Solar *et al.*, 1998). Furthermore, the DSO of pMEA300 appears to be located within the *rep* gene. This functional organisation differs from the one found on plasmids belonging to the pC194 family, in which the DSO is located upstream of the *rep* gene. This atypical location of the pMEA300 DSO at the distal end of the *rep* gene was also found on plasmid pGA1 of the actinomycete *Corynebacterium glutamicum*. This plasmid is suggested to belong to a new family of RCR plasmids (Abrhamova *et al.*, 2002). The Rep proteins of pGA1 and pMEA300 do not show any significant sequence similarity, however. These results suggest that the pMEA300 *rep* and pSE211 *orf1* replication genes are the first examples of a new family of RCR plasmids.

Replication of pMEA300 (derivatives) in *Rhodococcus erythropolis*

Attemps to transform other actinomycetes (*S. lividans* and *R. erythropolis* SQ1) with pMEA301, a pMEA300 derivative carrying the thiostrepton resistance gene (Vrijbloed *et al.*, 1995b), always failed. Apparently, the host range specificity of pMEA300 is high, limited to its original host. To determine whether a pMEA300 derivative plasmid only containing the minimal replicon (pHK313E) could replicate in *S. lividans* and *R. erythropolis* SQ1, this construct was transformed to both strains. We were unable to transform *S. lividans*, but transformation of *R. erythropolis* SQ1 was successful. To determine whether pHK313E replicated autonomously in its new host, plasmid DNA was extracted from *R. erythropolis*-pHK313E cells and used to transform *E. coli* cells. Restriction enzyme analysis of plasmid DNA isolated from *E. coli* transformants confirmed the presence of the pHK313E plasmid. Similarly, pHK317 was shown to replicate autonomously in *R. erythropolis* SQ1. The presence of a functional *orfA* gene did not significantly affect transformation frequencies. Surprisingly, we could not demonstrate the presence of pHK317R as an autonomously replicating plasmid in *R. erythropolis* SQ1. Plasmids pHK317(R) thus provide strongly different results in *A. methanolica* and *R. erythropolis* SQ1 as hosts (Fig. 2), which may be due to different levels of expression of OrfA and Rep, as a result of the positioning of the kanamycin promoter.

The regulatory mechanism of OrfA in autonomous replication of pMEA300 remains unclear at this point. The results obtained from our experiments with *A. methanolica* indicate that OrfA functions as a regulatory protein, negatively affecting the *orfB* encoded replication function. No evidence was obtained, however, that OrfA protein binds to the promoter region of *orfA*/*rep*. Furthermore, OrfA did not affect the DSO binding activity of Rep (data not shown). Possibly, OrfA interferes in cleavage or joining reactions of Rep. The precise role of the regulatory mechanism of OrfA will be a subject of future studies.

This is the first report of a plasmid replicating in both *Rhodococcus* and *Amycolatopsis* host strains. In future studies we aim to elucidate what pMEA300-based factor(s) prevents replication of pMEA300 itself in *R. erythropolis* SQ1, and thus determines host range specificity. Possibly, the presence of a *kil-kor* system on pMEA300 (Vrijbloed *et al.*, 1995a) is involved in host range specificity. An unbalanced expression of genes involved in this phenotype may be detrimental for its host.