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Genetic Organization of the Basic Replicon of Plasmid pMTH4 of a Facultatively Methylotrophic Bacterium *Paracoccus methylutens* DM12

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Abstract. Two functional regions within the basic replicon of plasmid pMTH4 of *Paracoccus methylutens* DM12 have been distinguished that are responsible for the replication of the plasmid (REP) and its stabilization (STA). In the REP region, a gene encoding the putative replication initiation protein RepA has been identified, with the highest similarity to the replication protein of plasmid pALC1 (*Paracoccus alcaliphilus*). The potential origin of replication (*oriV*), consisting of five long repeated sequences (iterons) as well as putative DnaA and IHF boxes, has been localized in the promoter region of the gene *repA.* The STA region was found to ensure stability for heterogeneous plasmid pABW3 that is unstable itself in paracocci. The mini-STA region (850 bp) contains two short open reading frames, one of which shows similarity to the RelB protein of *Escherichia coli.* Our investigations suggest that the stabilizing system of pMTH4 is based on the toxin and antidote principle.

Paracoccus methylutens DM12 was isolated on dichloromethane-containing agar from an enrichment culture inoculated with biological material from organic solvent-contaminated ground-water [9]. This bacterium utilizes a wide range of organic substrates (including polycarbon compounds), but also dichloroamine, methanol, methylamine, formate, CO_2 + H_2 as the carbon and energy sources. No detailed genetic characterization of this species has been undertaken so far. The only information in this respect is that *P. methylutens* DM12 carries three plasmids: pMTH1 (40 kb), pMTH2 (100 kb), and megaplasmid pMTH3 (approx. 450 kb) [1]. Detailed analysis allowed, however, identification in the strain of the additional plasmid pMTH4 (approx. 23 kb), an object of the present studies.

Out of several paracoccal species, the closest phylogenetic relatives of *P. methylutens,* as shown by bootstrap analysis, are *Paracoccus pantotrophus* and *Paracoccus versutus* [13]. Despite the close phylogenetical relation of the species, none of the *P. methylutens* DM12 plasmids contained the *repABC*-type replicon found in several plasmids of *P. pantotrophus* and *P. versutus* [2], which was previously shown by hybridization analysis [4].

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We describe here identification and genetic organization of the basic replicon of pMTH4, which allowed us to distinguish two functional regions responsible for plasmid replication and stable maintenance. These modules (functional in all tested paracocci) can be used for construction of specific vectors for this group of bacteria. Concerning the potential application of *P. methylutens* in the bioremediation process, such vectors may be of great importance for obtaining genetically modified derivatives, with the improved capacity for degradation of the carcinogenic and mutagenic substances.

Materials and Methods

Bacterial strains and growth conditions. *P. methylutens* DM12, *Escherichia coli* TG1, and all other paracoccal strains used in this study, as well as *Agrobacterium tumefaciens* LBA 1010, were grown in Luria Broth [16], while *Rhizobium leguminosarum* 1062 was grown in TY medium [6]. The concentration of rifampicin and kanamycin included in the media was 50 μg/mL.

DNA manipulations. Plasmid DNA was isolated according to Birnboim and Doly [7] and, when necessary, was purified by CsCl-ethidium bromide gradient centrifugation. Cloning experiments, digestion with restriction enzymes, ligation, and agarose gel electrophoresis were conducted in accordance with standard procedures as described by [16]. For Southern hybridization [16], DNA probes were labeled with digoxigenin (Roche). Hybridization and visualization of hybridization products were carried out as recommended by the supplier.

Introduction of plasmid DNA into bacterial cells. Electroporation of *P. pantotrophus* KL100 was performed as previously described [5]. Competent cells for chemical transformation of *E. coli* TG1 were prepared as described by Kushner [14]. Transformants were selected on solidified LB medium supplemented with antibiotic. Triparental mating procedure was used in host range assay experiments for transfer of tested plasmid from *E. coli* to paracoccal hosts as described in [5].

Plasmid stability. Stationary phase cultures of strain *P. versutus* UW225 carrying tested plasmid were diluted in fresh medium without antibiotic selection and were cultivated for approx. 10, 20, and 30 generations. Samples taken at these time intervals were diluted and plated onto solid medium free of selective drugs. One hundred colonies were tested by replica plating with the use of Km^r marker. The retention of plasmids after 30 generations was used as plasmid stability indicator (expressed as percentage of kanamycin-resistant colonies).

Host-range determination. The assay was done with the use of pMSZ1 mobilizable plasmid, composed of an *E. coli*-specific mobilizable, Km^r, pABW1 vector (non-replicating in *Paracoccus* spp. [5]) and the 4.7-kb *Hin*dIII fragment of the mini-replicon pMTH100. Plasmid pMSZ1 was transferred conjugationally into nine paracoccal species as well as into *A. tumefaciens* and *R. leguminosarum* and was tested exactly as described in [5].

DNA sequencing and analysis. The nucleotide sequence was determined by using a terminator sequencing kit and an automatic sequencer (ABI 377 Perkin Elmer). Sequence analysis was done with programs included in the UWGCG Package [8]. Comparison searches through the databases were performed with BLAST programs provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

Results and Discussion

Construction of a mini-replicon of pMTH4. The mini-replicon of pMTH4 was constructed by a classical method, i.e., digestion of the plasmid DNA with a restriction enzyme (in our case *Hin*dIII was used), ligation with an appropriate form of resistance marker (Km^r cassette derived from pKRP11 [15]), and electroporation of the ligation mixture to the paracoccal host. In order to avoid the predicted incompatibility between pMTH4 and its incoming mini-derivative, we performed the construction in a phylogenetically related strain *P. pantotrophus* KL100. Km^r electroporants carrying a plasmid pMTH100, composed of a 4.7-kb *Hin*dIII restriction fragment of pMTH4, and 1.4 kb Km^r cassette were obtained. The identity of the mini-replicon was proved by DNA hybridization of pMTH100 (as a probe) with the parental pMTH4 (data not shown).

Host range of pMTH100. For the studies of the host range of pMTH100 mobilizable hybrid plasmid pMSZ1 (constructed as described in Materials and Methods) was used. We found that pMSZ1 can be transferred by triparental mating and maintained in the nine paracoccal species, the same as tested in [5], as well as in the two representatives of the *Rhizobiaceae* family. This indicates that the host range of pMTH100 embraces besides *Paracoccus* spp. also representatives of two other genera of Alphaproteobacteria. It is worth to mention that the mini-replicon pMTH100 could not be electroporated into *E. coli* TG1, which proved that its replicator system is not functional in this host.

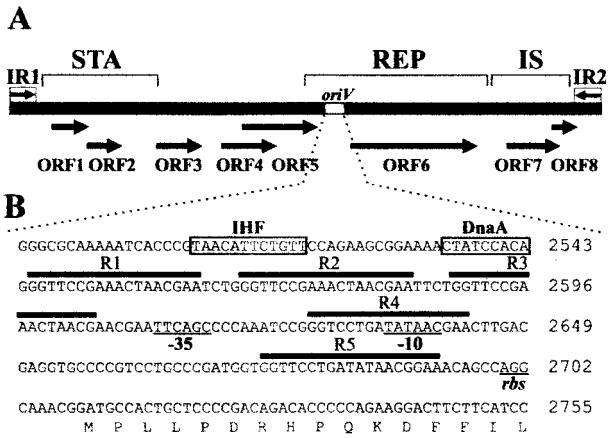

Fig. 1. (A) Genetic organization of a 4.7-kb *Hin*dIII fragment of pMTH4 included in the mini-replicon pMTH100. The open reading frames and their transcriptional orientation are indicated by arrows. The terminal inverted repeats (IR1 and IR2) are shown as boxed arrows. Two distinguished regions involved in plasmid replication [REP] and stabilization [STA] as well as the putative insertion sequence [IS] are marked and adequately indicated. Localization of the AT-rich region containing a putative origin of replication (*oriV*) is marked. (B) The nucleotide sequence of the predicted origin of replication. The amino acid sequence of the proximal part of RepA protein is given below the DNA sequence. Putative ribosome binding site (*rbs*), as well as potential −10 and −35 boxes of the predicted *repA* promoter are underlined and appropriately marked. Horizontal thick lines indicate the R1-R5 repeated sequences. Putative DnaA and IHF binding sequences are boxed. Numbers given on the right-hand side of the sequence refer to the nucleotide sequence of pMTH100 deposited within GenBank.

DNA sequence analysis of pMTH100. To examine the genetic organization of pMTH100, the complete nucleotide sequence of the mini-replicon was determined (GenBank accession no. AY337272). The sequence (4692 bp) has a calculated G + C content of 59%, which is lower than the G + C content of total DNA of *P. methylutens* (67%) [9], which may suggests its relatively recent acquisition by lateral transfer. The terminal parts of the sequenced region of pMTH4 were shown to contain approx. 200 bp-long identical inverted repeats, designated IR1 and IR2 (Fig. 1A). Restriction analysis of pMTH4 strongly suggests that IR1 and IR2 repeats are much longer and comprise a considerable part (at least 4 kb of each side) of the pMTH4 genome (data not shown). However, more detailed analysis is needed to define precisely the end points of these repeats. Computer analysis revealed that pMTH100 encodes eight open reading frames (Fig. 1A), which predicted protein products

showed similarities to other bacterial protein sequences. From the similarity searches, we distinguished two putative regions: REP—responsible for plasmid replication (containing ORF6), and STA—involved in plasmid stabilization (carrying ORF1 and ORF2) (Fig. 1A). More detailed analysis of the REP and STA regions is presented below. The remaining ORF7 showed similarities with a number of transposases encoded by several insertion sequences (IS), while ORF3, ORF4, ORF5, and ORF8 were similar to hypothetical bacterial proteins of unknown function (data not shown).

Characterization of replicator region of pMTH100. The sequence analysis of the REP region revealed the presence of one major open reading frame (ORF6) encoding protein (RepA) of a predicted molecular mass 39.2 kDa, with substantial similarity with four replication proteins encoded by plasmids: pALC1 of *Paracoccus alcaliphilus* (51% identity/61% similarity) [3], pRS241a of *Rhodobacter sphaeroides* (45%/56%) [accession no. ZP00007730], pSD20 of *Ruegeria sp.* (46%/54%) [17], and pSW500 of *Pantoea stewartii* (35%/48%) [10]. Within the promoter region of the *repA* gene we identified a region, with structural similarity to the iteron containing origin of replication found in many theta-replicating plasmids of Gram-negative bacteria. It contains five iteron-like repeated sequences (R1-R5), located within a short AT-rich region (Fig. 1B). R1–R3 sequences (18 bp long) are identical (5′-GGTTCCGAAACTAACGAA-3′), while some mismatches were observed within R4 and R5 (Fig. 1B). Moreover, the predicted *oriV* carries also the putative DnaA box (5′-CTATCCACA-3′; matching the consensus sequence 5′-TTATCCACA-3′) as well as a sequence (5′-TAACATTCTGTT-3′) similar to the IHF-binding site [12]. The replicator regions of pALC1, pRS241a, pSD20, and pSW500 also carry similarly situated sets of repeats (iterons) as well as the DnaA boxes (data not shown), which indicates the high conservation of the origin structure within this plasmid family. It seems thus probable that these replicons might originate from a common ancestor plasmid.

Identification of stabilizing, an addiction type system in pMTH100. The mini-replicon pMTH100 is very stably maintained in a bacterial population. We were not able to detect spontaneous loss of the plasmid after approx. 30 generations of growth in non-selective conditions, which may suggests that pMTH100 encodes a stabilizing system(s). Analysis of the amino acid sequences of the putative pMTH100 encoded proteins revealed that two of them (ORF1 and ORF2 products) show similarity to several putative proteins that share conserved domains of RelB and ParE proteins, respectively (data not shown). RelB and ParE homologous are encoded by several different plasmid addiction systems [11]. Such systems usually consist of two genes that form an operon. One of them codes for a protein that is toxic to the cell (toxin), and the other is responsible for the production of a factor that precludes the manifestation of the lethal effect of the toxic protein (antidote). In the cell cured of plasmid by aberrant segregation, neither the toxin protein nor antidote is synthesized, but since antidote is less stable than toxin, its preventing action will be short-lasting, and plasmid-less cells are killed [11]. To prove the presence of the stabilizing system within pMTH100, we cloned several selected restriction fragments of this replicon (Fig. 2) into an *E. coli-Paracoccus* spp. shuttle mobilizable vector pABW3 [2] and tested the stability of the resulting plasmids in *P. versutus* UW225. The pABW3 carries a replicator region that is unstable in paracocci (only about 4% plasmid retention after 30 generations of growth in non-selective conditions). We found that the presence of a 2.5-kb *Hin*dIII-*Eco*RI restriction fragment of pMTH100 (Fig. 2) ensured a high degree of stability of the resulting plasmid (approx. 99%). Analogous results were obtained for other restriction fragments tested: (a) 1 kb *Hin*dIII/*Nde*I, (b) 1.1 kb *Eco*RV/*Sma*I, and (c) 1.3 *Sma*I fragment (Fig. 2). This analysis allowed us to delineate the stabilizing region to 855 bp *Eco*RV-*Nde*I restriction fragment, carrying both ORF1 and ORF2. To prove the role of the individual ORFs in functioning of the analyzed system, we performed mutational analysis. We found that not in frame

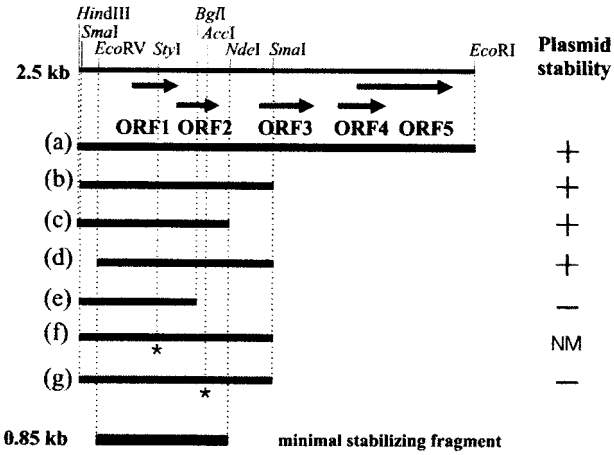

Fig. 2. Mutational analysis of the STA region of pMTH100. Only the restriction sites used to construct recombined plasmids are shown. The open reading frames are indicated by arrows. The thick boxes represent DNA restriction fragments of pMTH100 cloned into pABW3. The minimal stabilizing fragment and its size are matched. Mutations introduced into ORF1 and ORF2 are indicated as asterisks. (+) indicates full stability and (−) indicates instability of pABW3-derived recombinant plasmids in *P. versutus* UW225. NM, no mutants obtained.

mutation introduced into ORF2 (2 bp insertion generated by *Acc*I digestion followed by filling in the ends by Klenow fragment of DNA polymerase I and self-ligation; Fig. 2) completely abolished stabilization functions of the mutated plasmid (stability was at the level of pABW3 vector). On the other hand, all attempts to introduce an analogous mutation within the *Sty*I site of ORF1 (Fig. 2) met with failure.

In the light of these results, it seems highly probable that the investigated stabilizing system belongs to the group of plasmid addiction systems. It can be assumed that the role of the ORF1 (*staA*) product in the cell is that of an antidote for a potential toxin coded for by ORF2 (*staB*), whose inactivation drastically decreases the stability of pABW3 derivative. Inactivation of the protein being an antidote in the presence of the toxin results, thus, in death of the cell. More detailed characterization of the identified stabilizing system is in progress.

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