Polish Journal of Microbiology
2006, Vol. 55, No 4, 261–270

Replication System of Plasmid pMTH4 of *Paracoccus methylutens* DM12 Contains an Enhancer

MICHAŁ SZYMANIK[1], RENATA WELC-FALĘCIAK[2], DARIUSZ BARTOSIK and MIROSŁAWA WŁODARCZYK*

Department of Bacterial Genetics, Institute of Microbiology, Warsaw University, Warsaw, Poland

Received 19 September 2006, revised 17 October 2006, accepted 23 October 2006

Abstract

The replication system of plasmid pMTH4 (22 kb) of dichloromethane-degrading *Paracoccus methylutens* DM12 (Alphaproteobacteria) has been cloned within a mini-replicon pMTH100 (4.7 kb) and preliminarily characterized. Functional analysis, performed with a series of mutated plasmid mini-derivatives, showed that the replicator region consists of three elements: (i) gene *repA* coding for a replication initiation protein RepA, (ii) origin of replication (*oriV*), placed in the promoter region of *repA* and containing a set of imperfect directly repeated sequences (iterons) together with putative DnaA and IHF-binding DNA sequences as well as (iii) an enhancer (0.65 kb) upstream of *oriV*. We showed that the enhancer was not crucial for plasmid replication, however, it was necessary to assure the proper plasmid copy number. Additionally its presence has increased the strength of a determinant of incompatibility (located within the *oriV* region) as well as the level of transcription carried from the *repA* promoter. The enhancer region was shown not to encode any proteins or promoter sequences. We speculate that this region might constitute a site of binding of plasmid or host-encoded proteins that are able to interact with the origin, which positively regulates the initiation of replication.

Key words: *Paracoccus methylutens*, enhancer, iteron, plasmid replication

Introduction

The majority of plasmids of Gram-negative bacteria replicate according to the so called theta model. Among them five classes have been distinguished (Bruand *et al.*, 1993; Meijer *et al*., 1995), including the iteron-containing plasmids (class A). Replication initiation of class A plasmids depends on a plasmid-encoded initiation replication protein (Rep). The DNA region comprising the origin of replication of these plasmids contains several direct nucleotide repeats (iterons) recognized by Rep protein as well as host-encoded DnaA and IHF (and occasionally Fis, Ici, SeqA) binding sites (reviewed in Kruger *et al*., 2004). The well known representatives of the class A plasmids are: F, P1, R6K, pSC101, RK2 or pPS10 (Chattoraj, 2000).

Saturation of the iteron sequences with monomeric forms of Rep proteins as well as binding of the host proteins results in a local destabilization of DNA sequence at the AT rich region of replication origin, which results in open complex formation (Messer *et al.*, 2001). The frequency of formation of the complex is an important factor that regulates plasmid copy number. In some cases (R6K, pSC101 and pCD3.4) it has been shown that the class A replicator regions encode additional regulatory elements involved in regulation of initiation of replication (van Belkum and Stiles, 2006; Kelley *et al*., 1992; Miller and Cohen, 1993; Sugiura *et al*., 1993). In all cases the enhancers do not encode for additional proteins engaged directly in the regulatory process but encode sequences recognized by plasmid- or host-encoded proteins that interact with the *oriV*.

Paracoccus methylutens DM12, the natural host of plasmid pMTH4, is a Gram-negative, oxidative, facultatively methylotrophic bacterium, able to utilize many compounds commonly considered as a highly

* Corresponding author: M. Włodarczyk, Department of Bacterial Genetics, Institute of Microbiology, Warsaw University, Miecznikowa 1, 02-096 Warsaw, Poland; e-mail: miraw@biol.uw.edu.pl

[1] Current address: Adamed Sp z o.o., Pieńków 149, 05-152 Czosnów, Poland

[2] Current address: Department of Parasitology, Institute of Zoology, Warsaw University, Miecznikowa 1, 02-096 Warsaw, Poland

toxic (*eg*. dichloromethane, dichloroamine, methanol, methylamine, and formate (Doronina *et al*., 1998). We have found that the strain harbors 4 native plasmids, and one of them – pMTH4 (not detected in our preliminary search; Baj *et al*., 2000) has become the object of our interest. Plasmid pMTH4 is ~22 kb in size. A mini-replicon of this plasmid has been constructed (pMTH100 – carries a 4.7 kb HindIII restriction fragment of pMTH4 and a kanamycin resistance cassette) and shown to be stably maintained in different representatives of the Alphaproteobacteria. *In silico* analysis of the nucleotide sequence of pMTH100 allowed us to distinguish two potential structural modules: (1) REP – coding for replication system, (2) STA – carrying stabilizing module as well as a truncated insertion sequence (IS) (Szymanik *et al*., 2004).

The REP module carries (i) a *repA* gene whose putative product shows similarity to the replication initiation proteins of several iteron plasmids and (ii) the putative origin of replication (*oriV*) located upstream of *repA* gene, containing a set of directly repeated sequences (iterons) as well as presumptive DnaA and IHF binding sites (Fig. 1). The iterons R1-R3 (18 bp) are identical, while the other two R4 (17 bp in length) and R5 (19 bp) contain some differences at the nucleotide sequence. The distances between R1 and R2 as well as between R2 and R3 are identical (4 bp), while the distance between R4 and R5 is as long as 31 bp (Szymanik *et al.*, 2004). The STA module of pMTH100 (~1000 bp) is located upstream of REP and carries two putative genes *staA* and *staB* (Fig. 1A) which represent a proteic toxin-antitoxin system, with *staA* gene encoding antitoxin and *staB* toxin products, respectively (Szymanik, 2006).

The present paper deals with the functional characteristics of pMTH4 replication system, which led to the identification of an enhancer region that seems to positively influence the process of initiation of plasmid replication.

Experimental

Materials and Methods

Bacterial strains, plasmids, media and growth conditions. Bacterial strains and plasmids used (but not constructed) in this work are listed in Table I. Plasmids constructed in this work are described under Results and in the appropriate figures. Bacteria were grown in Luria-Bertani (LB) medium (Sambrook and Russell, 2001) at 30°C (*P. pantotrophus* KL100) or 37°C (*E. coli* strains). When necessary, the medium was supplemented with antibiotics: kanamycin (Km) – 50 μg/ml, rifampicin (Rif) – 50 μg/ml, tetracycline (Tc) – 0.1 μg/ml for *P. pantotrophus* KL100 with derivatives of pABW2 (minimal selective concentration), 0.5 μg/ml for *P. pantotrophus* KL100 with derivatives of pRK415, and 20 μg/ml for *E. coli*.

Table I
Bacterial strains and plasmids used (but not constructed) in this study

	Relevant characteristics	Source or reference
Bacterial strains		
E. coli DH5α	F^- Φ80d *lacZ* ΔM15 (*lacZYA-orgF*)U169 *deoR recA*1 *endA*1 *hsdR*17 *phoA supE*44 λ^- *thi*-1 *gyrA*96 *relA*1 (host strain for helper plasmid pRK2013)	Hanahan, 1983
E. coli TG1	*supE*44 *hsd*Δ5 *thi* Δ(*lac-proAB*) F' [*traD*36 $proAB^+$ $lacI^q$ *lacZ*ΔM15] (host strain for recombinant plasmids)	Sambrook and Russell, 2001
P. pantotrophus KL100	Rif^r derivative of *P. pantotrophus* DSM 11073 deprived of its natural plasmid pKLW1; contains pKLW2 (>400 kb)	Bartosik *et al.*, 2002
Plasmids		
pABW2	Tc^r; mobilizable cloning vector based on pBGS18, *oriT* of RK2	Bartosik *et al.*, 1997
pBGS18	Km^r; cloning vector, ColE1 origin of replication	Spratt *et al.*, 1986
pCM132	Km^r; mobilizable promoter probe vector (promoter-less *lacZ* gene) carrying ColE1 and RK2 replicator regions, *oriT* of RK2	Marx and Lindstrom, 2001
pKRP11	Source of Km^r cassette	Reece and Phillips, 1995
pMTH100	Km^r; mini-replicon of pMTH4, composed of 4692-bp HindIII fragment of pMTH4 (coordinates 1 – 4692*) and Km^r cassette	Szymanik *et al.*, 2004
pRK415	Tc^r; mobilizable broad-host-range cloning vector, *oriT* and origin of replication of RK2	Keen *et al.*, 1988
pRK2013	Km^r; helper plasmid carrying RK2 *tra* genes	Ditta *et al.*, 1980

* – Acc. no. AY337272

DNA manipulation. DNA techniques including plasmid purification, digestion with restriction enzymes, ligation and agarose gel electrophoresis were conducted according to standard procedures (Sambrook and Russel, 2001). All enzymes were purchased from Fermentas and used according to the manufacturer's instructions.

PCR amplification. For amplification of the origin fragment of pMTH100, containing all iterons, DNA box, and incomplete IHF binding site (ORILP fragment), the following pair of forward and reverse oligonucleotide primers was used: ORIL (5'-GCGATATCACATTCTGTTCCAGAAGCGG-3') and ORIP (5'-ATGGATCCCATCCGTTTGCCTGGCTGTT-3'). The introduced EcoRV (ORIL) and BamHI (ORIP) restriction sites are underlined. Amplification was performed in a Mastercycler Personal (Eppendorf) using the above primers, OptiTaq polymerase from EUR_X (with supplied buffer) and template DNA (pMTH100). The reaction mixture (25 µl) contained 10 ng template DNA, 2 mM $MgCl_2$, 200 µM dNTPs, 50 pmol of each primer and 0.5 u OptiTaq polymerase. The amplification cycle was: 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 60°C for 0.5 min and 72°C for 1 min; the last cycle was followed by a final extension step of 10 min.

Electroporation and transformation. Electroporation was carried out at 2500 V, 25 F and 400 Ω for *P. pantotrophus* KL100 in a gene pulser apparatus (Bio-Rad), based on modified Bio-Rad procedure (Wlodarczyk *et al*., 1994). Electrotransformants were selected on solidified LB medium supplemented with the appropriate antibiotic. Competent cells for transformation of *E. coli* TG1 were prepared and transformed as described by Kushner (1978).

Triparental mating. For triparental mating three overnight cultures (cells harvested by centrifugation and washed to remove antibiotics) of the donor strain *E. coli* TG1 (carrying a mobilizable plasmid), the plasmid-less *P. pantotrophus* KL100 strain or *P. pantotrophus* KL100 strain carrying Km^r minireplicon of pMTH4 as the recipient, and *E. coli* DH5α strain carrying the helper plasmid pRK2013, were mixed at a ratio of 1:2:1. An aliquot of 100 µl of this mixture was spread on a plate of LB medium. After overnight incubation at 30°C, the bacteria were washed off the plate and suitable dilutions were plated on selective LB medium containing rifampicin (selective marker of the recipient strain) and kanamycin or rifampicin, kanamycin and tetracycline to select for transconjugants.

Transcription and translation *in vitro* assay. The *in vitro* transcription and translation reaction was done with the *E. coli* Extraction System for Circular DNA kit (Promega) according to the manufacturer's procedure. The obtained proteins were separated as described by Schagger and von Jagow (1987) and visualized according to the kit manufacturer's procedure.

β-galactosidase assay. β-galactosidase activity in *P. pantotrophus* KL100 cell extracts was measured by the conversion of o-nitrophenyl-β-D-galactopyranoside (ONPG) to nitrophenol as described by Miller (1972), with slightly modified bacterial cells lysis procedure – the addition to 100 µl of bacterial culture of 50 µl TE containing lysozyme in concentration 6 mg/ml and 2.5 µl of 0.5 M EDTA (pH 8.0). The samples were then incubated 5 min at room temperature. β-galactosidase activity assays were carried out in triplicate.

Incompatibility testing. The incompatibility characteristics of two plasmids were examined by conjugational transfer (triparental mating) of tested recombinant Tc^r plasmids based on vector pRK415 (containing PCR-amplified or restriction fragments of pMTH100) into the recipient strain (*P. pantotrophus* KL100) carrying pMTH105 (Km^r) mini-replicon. Transconjugants were selected for the incoming and resident plasmids. The plasmid patterns of the transconjugants were verified by screening 10 colonies using a rapid alkaline extraction procedure and agarose gel electrophoresis. The incompatibility behaviour of pMTH105 (which coexisted *in trans* with pRK415-based plasmids) was tested during growth for approximately 30 bacterial generations (72 h). Every 24 h cultures were diluted in fresh medium with tetracycline and without kanamycin. At this time intervals, samples were diluted and plated onto solid medium with tetracycline and without kanamycin. Two hundred colonies were tested for the presence of the Km^r marker by replica plating. The retention of pMTH105 was defined by determining the percentage of kanamycin-resistant colonies among 200 Tc^r clones (containing a pRK415-based plasmid).

Plasmid stability. The stability of plasmids (Km^r) during growth in non-selective conditions was tested as described previously (Bartosik *et al*., 2002). Briefly, every 24 h stationary phase cultures were diluted in fresh medium without antibiotic selection and cultivated for approximately 30 generations (72 h). At time intervals, samples were diluted and plated onto solid medium in the absence of selective antibiotic. Two hundred colonies were tested for the presence of the Km^r marker by replica plating. The retention of plasmids, after approximately 30 generations, was determined from the percentage of kanamycin-resistant colonies among 200 clones.

Results

Identification of the minimal replicon of pMTH4. *In silico* analysis of the nucleotide sequence of the mini-replicon pMTH100 allowed to distinguish a putative replication system (REP) composed of *repA* gene and origin of replication, placed upstream of *repA*, containing iteron-like repeated sequences.

To prove the importance of the *repA* product in pMTH100 replication, mutational analysis was performed. To this end an *E. coli-Paracoccus* spp. shuttle vector pMTH102 was constructed, composed of (i) an *E. coli*-specific pBGS18 vector (Km^r; 3.7 kb; unable to replicate in paracocci) and (ii) the replicator region of pMTH4 (non-functional in *E. coli*) encoded by the 4.7 kb HindIII fragment of the mini-replicon pMTH100 (Fig. 1B). This plasmid (constructed in *E. coli*) could be easily introduced by electroporation into *P. pantotrophus* KL100 (a strain routinely used in our laboratory as a host for paracoccal plasmids). It appeared that a short not-in-frame insertion introduced within the XhoI restriction site of *repA* (4 bp) completely abolished the ability of the this mutated plasmid (pMTH102; Fig. 1B) to replicate in *P. pantotrophus*, as concluded from the lack of Km^r electroporants of the KL100 strain. This points to the crucial role of RepA protein in plasmid replication.

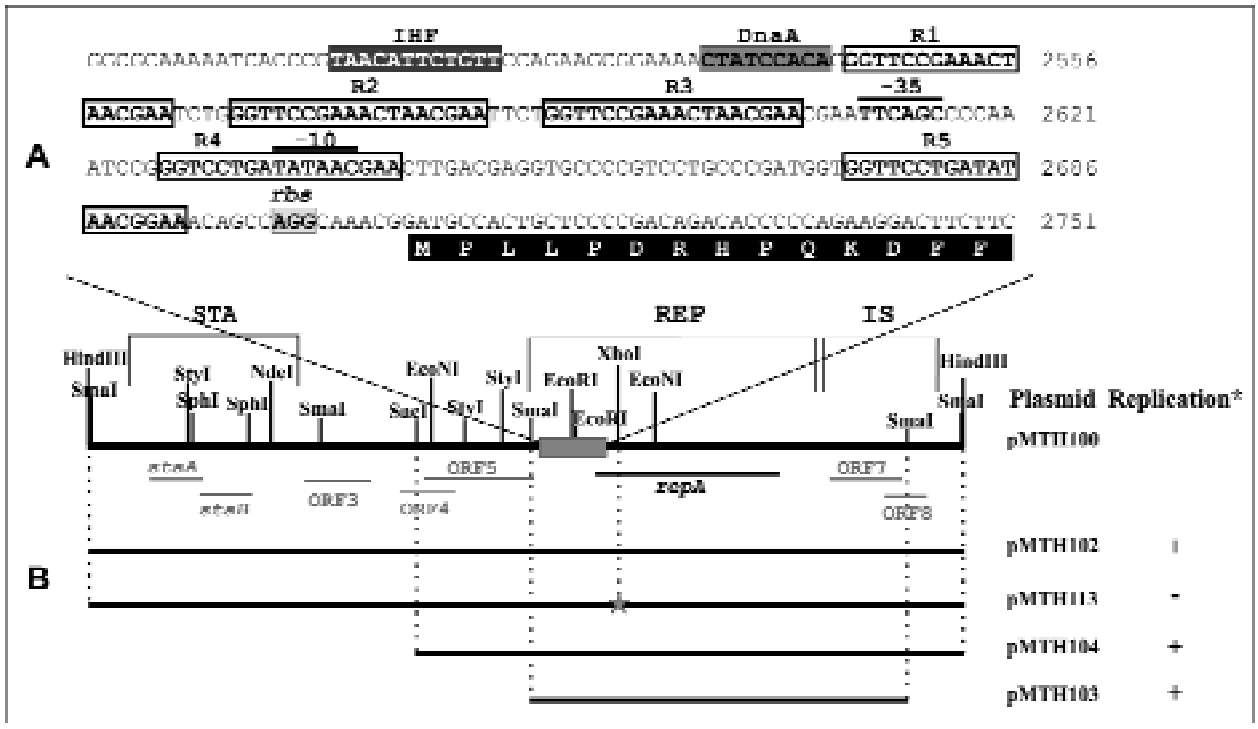

Fig. 1. Genetic organization of mini-replicon pMTH100. The distinguished replication module (REP), stabilization module (STA), and truncated IS are appropriately marked.

A. Nucleotide sequence of the iteron-containing region. The putative ribosome binding sequence (*rbs*), integration host factor (IHF) and DnaA (DnaA) binding boxes, –35 and –10 hexamers of the *repA* promoter and iterons (R1-R5) are marked. The deduced amino acid sequence of the N-terminal part of RepA is given below the sequence in a single-letter amino acid code in the first position of each codon. The numbers on the right refer to the deposited nucleotide sequence (Acc. no. AY337272).

B. Localization of the minimal replicon of plasmid pMTH100. The restriction fragments shown in this figure were cloned into pBGS18 vector or ligated with the Km^r cassette derived from pKRP11. Not-in frame mutation introduced into *repA* is marked by an asterisk.

* – plasmid replicating in *P. pantotrophus* KL100 (+) or unable to replicate (–).

In order to define the minimal replicon of pMTH100 we constructed diminished forms of the plasmid. To do this, two selected restriction fragments of pMTH100 (i) a 3 kb SacI-HindIII fragment and (ii) SmaI fragment (2 kb) (Fig. 1B) were ligated with a kanamycin resistance cassette derived from plasmid pKRP11, and the ligation mixtures were introduced by electroporation into *P. pantotrophus* strain KL100. In both cases electroporants were obtained, containing mini-derivatives pMTH104 and pMTH103, respectively. As shown in Fig 1B, the smallest mini-derivative (pMTH103) carried the *repA* gene, and the AT rich, iteron-containing region (289 bp) as well as terminal part of a putative insertion sequence located downstream of *repA* (most probably non-essential for replication). Plasmid pMTH103 might be thus considered a minimal replicon of pMTH4. This confirmed the initial *in silico* predictions.

Localization of the replication origin of pMTH4 and identification of an enhancer. To confirm experimentally that origin of replication of pMTH100 (and entire pMTH4) is located upstream of the *repA* several selected restriction fragments of pMTH100 (Fig. 2) containing the presumptive *oriV* were cloned into pABW2 vector (mobilizable Tc^r derivative of pBGS18) and introduced by triparental mating into *P. pantotrophus* KL100 containing the pMTH100, with the hope that mini-replicon present in recipient would provide *in trans* RepA protein enabling the replication of the pABW2-derivatives.

In control experiments we showed that plasmid pMTH232 (pABW2 carrying mutated version the 4.7 kb HindIII fragment of pMTH100, recovered from plasmid pMTH102; *repA* mutant) was capable of autonomous replication; the analyzed *oriV* can be thus *trans* activated by pMTH100. In contrast, empty vector pABW2 (carrying *E. coli*-specific replicator region) could not be introduced into the paracoccal hosts, which proved its inability to replicate in them. The results of the performed analysis are summarized in Fig. 2. Shortly, (i) no Tc^r transconjugants of KL100 (pMTH100) were selected with pMTH239 (pABW2 containing amplified by PCR 202 bp region of pMTH100 carrying all the iterons, DnaA box, and incomplete IHF box) (Fig. 2), (ii) Tc^r transconjugants were obtained upon conjugation of pMTH233, pMTH237 and pMTH235 (the plasmids contain the complete predicted origin, together with the adjacent sequences of pMTH100 of different length; Fig. 2). However, an autonomous form of the plasmid was visible after agar-

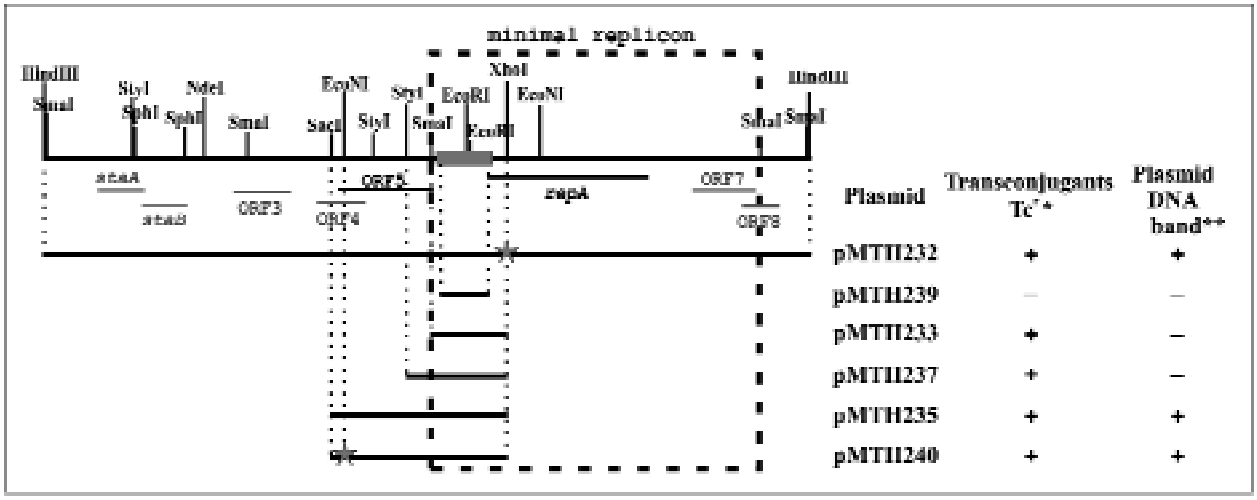

Fig. 2. Defining of the origin of replication of pMTH100 by construction of two plasmid system [pABW2 (Tc^r) + pMTH100 (Km^r)]. The restriction fragments of pMTH100, shown in this figure, were cloned into pABW2 vector and the resulting plasmids were introduced into *P. pantotrophus* KL100 (pMTH100). Insertion mutations introduced into *repA* and ORF5 are indicated with asterisks. Grey box indicates location of the iterons-containing region.

* (+) – obtained Rif^r Km^r Tc^r transconjugants of *P. pantotrophus* KL100 (pMTH100) carrying the introduced plasmid; (–) – lack of transconjugants;
** (+) – the introduced plasmid visible after agarose gel DNA electrophoresis; (–) – plasmid DNA not visible after ethidium bromide staining.

ose gel DNA electrophoresis exclusively in case of pMTH235. We speculated that this might result from deviations in copy number of the three tested plasmids. Out of the tested plasmids pMTH235 contains the largest DNA fragment (0.65 kb) adjacent to the 5'end of the predicted iteron-containing origin (Fig. 2). The obtained results suggested that this region (called the enhancer) might be responsible for determination of the accurate pMTH100 copy number.

Analysis of ORF5 carried by the enhancer. The enhancer region carries complete ORF5, which is one of the longest ORFs (642 bp) distinguished in pMTH100 sequence by *in silico* analysis (Szymanik *et al*., 2004). To find out whether it encodes a protein product that is expressed, the coupled *in vitro E. coli*-derived transcription-translation system was used. For this purpose a restriction fragment SacI-XhoI of pMTH100 (Fig. 2) containing the iteron region together with the adjacent enhancer was cloned, in opposite orientations, into pABW2 vector, to yield pMTH235 and pMTH236. Both plasmids together with pABW2 (as a control) were used for the analyses. Visualization of the obtained proteins showed that the enhancer region does not encode any protein products that are expressed in *E. coli* (data not shown).

Since the transcription-translation system used by us is based on *E. coli* cellular components it was still probable that ORF5 might be preferentially expressed in the native paracoccal host. This might be, for instance, due to the presence of specific promoters that are functional in paracocci but not in *E. coli*. To test the importance of the putative ORF5 product in the activity of the enhancer, plasmid pMTH240 was constructed. This plasmid was nearly identical to the described above pMTH235 – it carried however a 2 bp insertion generated within the terminal part of ORF5 (Fig. 2). The mutated plasmid was introduced into *P. pantotrophus* KL100 containing pMTH100. In this case we observed *trans* activation of the cloned origin of pMTH240, as observed previously for pMTH235. Since no differences in copy number were detected (as judged from the level of DNA recovery of pMTH240 and pMTH235 from obtained KL100 transconjugants) it seems probable that ORF5 does not encode a protein that is crucial for enhancer activity.

Influence of the enhancer on expression carried from *repA* promoter. Analysis of the nucleotide sequence of the REP region of pMTH100 allowed to distinguish a probable promoter of the *repA* gene (P_{repA}), located 70 bp upstream of the *repA* coding sequence (Fig. 1A). The predicted promoter was amplified by PCR within a 105 bp DNA fragment and inserted upstream of the promoter-less reporter *lacZ* gene in a broad host range promoter probe vector pCM132, to generate a transcriptional fusion. The resulting plasmid pMTH246 (Fig. 3A) was introduced into *P. pantotrophus* KL100 and β-galactosidase activity assay was used to examine promoter strength. The obtained results confirmed the presence of a weak promoter within the cloned fragment, as judged from the increased level of β-galactosidase activity, compared with the enzyme activity observed for the negative control – the empty vector pCM132 (Fig. 3B).

To test whether the enhancer influence expression driven from the *repA* promoter, several selected DNA fragments of pMTH100 (all containing P_{repA} with upstream sequences of different length – 0.1 kb, 0.4 kb,

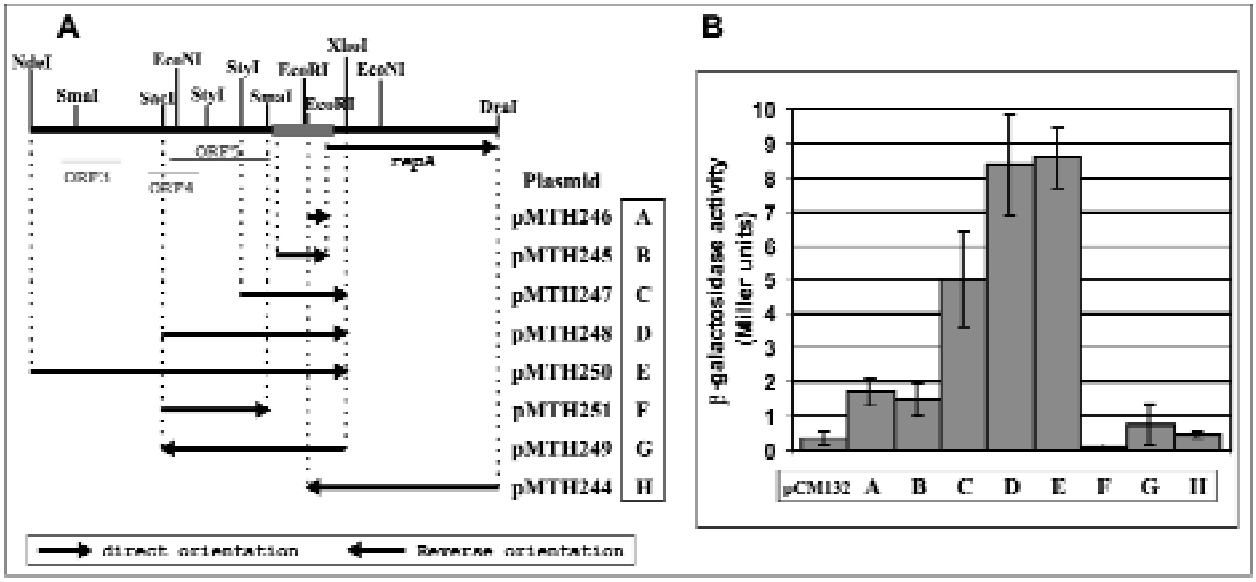

Fig. 3. Search for the promoters encoded within the REP module of pMTH100.
A. Several selected restriction fragments were cloned into promoter probe vector pCM132. The orientation of the cloned fragments refer to the *lacZ* gene of pCM132 is shown by arrows.
B. β-galactosidase activity in *P. pantotrophus* KL100 cells containing pCM132-derived plasmids listed in panel A.

0.85 kb and 1.6 kb) were cloned in a proper orientation into pCM132 vector, to yield pMTH245, pMTH247, pMTH248 and pMTH250, respectively (Fig. 3A). Analysis of the β-galactosidase activities determined in strain KL100 (Fig. 3B) allowed to conclude that the longer the enhancer region in the tested plasmids, the higher the enzyme activity (see pMTH245, pMTH247, pMTH248 in Fig. 3B). It also appeared that the presence of DNA sequences adjacent to the 5' end of the enhancer region did not additionally increase β-galactosidase expression driven by P_{repA} in comparison to that specified by the enhancer itself (compare pMTH250 and pMTH248 in Fig. 3B). By construction of an additional plasmid pMTH251 (pCM132 carrying 1.15 kb SmaI fragment of pMTH100, lacking P_{repA} but containing the overall enhancer) we showed that enhancer itself does not encode internal promoters, as judged from the lack of the β-galactosidase activity (Fig. 3B).

Additional analysis showed that the analyzed replicator region (including enhancer) does not carry promoter sequences placed in the opposite orientation towards P_{repA}. The presence of such promoters might suggest formation of antisense RNA or small regulatory RNAs involved in regulation of the initiation of pMTH100 replication (see plasmids pMTH244 and pMTH249 in Fig. 3A). Within the analyzed region we did not identify any putative terminators of transcription, whose presence might block β-galactosidase expression carried from the searched promoters.

Enhancer modulates the strength of a determinant of incompatibility (*inc*). In general two incompatible plasmids (*i.e.* carrying related replication or partitioning systems) cannot be stably maintained in a bacterial cell in the absence of selective pressure. To identify the incompatibility determinant within pMTH100, several selected DNA fragments of the mini-replicon (covering the whole mini-replicon; see Fig. 4) were cloned into the broad-host-range vector pRK415 (Tc^r; compatible with pMTH100) in *E. coli* TG1 and the resulting constructs were transferred by triparental mating into *P. pantotrophus* KL100 containing pMTH4 mini-derivative. It was expected that the presence of the *inc* region on the incoming plasmid would cause the exclusion of the residing parental replicon.

In the above experiments mini-replicon pMTH100 could not be used as the residing plasmid, since it encodes a stabilizing system that act on the toxin-antitoxin (TA) principium (Szymanik *et al*., 2004; Szymanik, 2006), which is responsible for post-segregational elimination of plasmid-less cells from bacterial population. The presence of such a system would exert a killing effect against the cells from which the plasmid was removed by reason of incompatibility. To avoid this, the mini-replicon pMTH105 was used as the recipient plasmid, which is a pMTH100 deletion derivative deprived of the functional TA system. Despite the inactivation of the TA locus, pMTH105 was very stably maintained in the bacterial population (after 72 h of growth in non-selective conditions 97% of the cells still carried the mini-replicon).

The performed analysis revealed that pMTH100 carries at least one determinant of incompatibility (see pMTH201 in Fig. 4). The minimal region exerting the Inc phenotype was shown to be present within a 0.4 kb

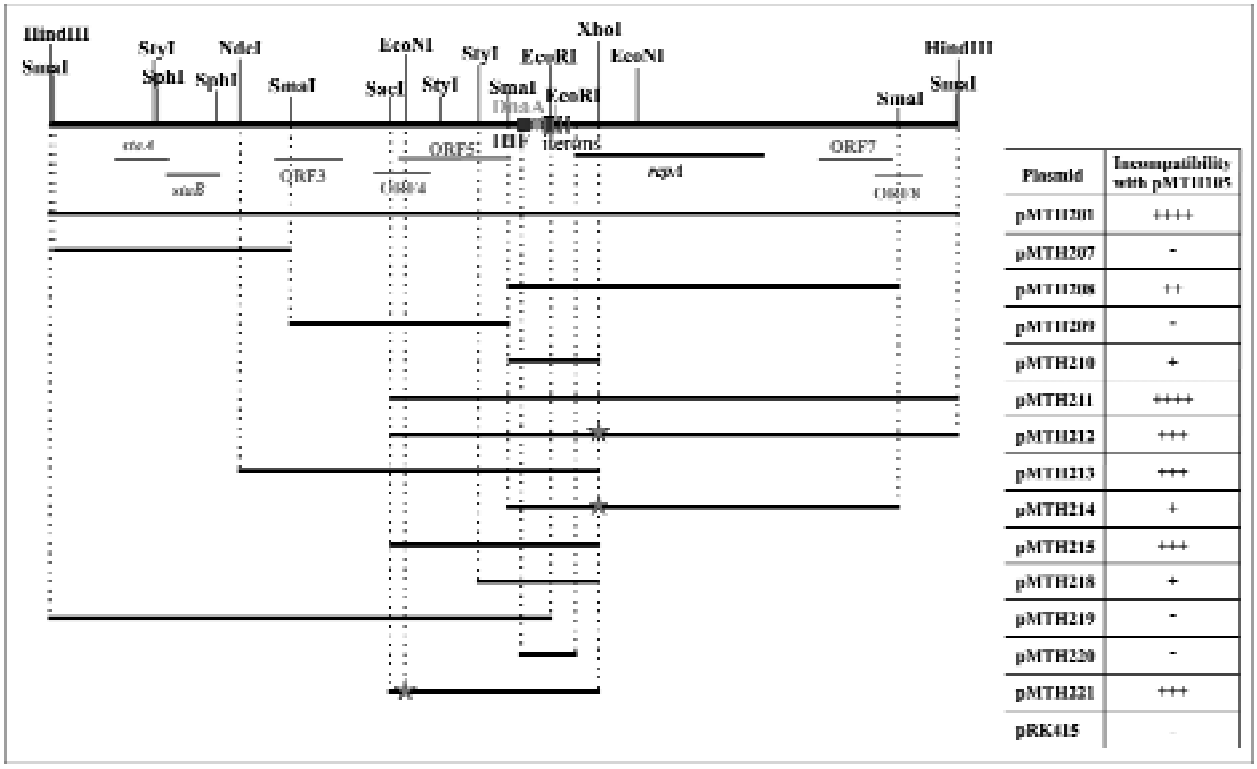

Fig. 4. Identification and characterization of incompatibility determinants of pMTH100.

Selected DNA fragments of the mini-replicon were cloned in pRK415 vector and introduced into *P. pantotrophus* KL100 (pMTH105). The incompatibility of the introduced pRK415-derivatives toward pMTH105 are shown on the right: (–) compatible plasmids; (+) incompatible plasmids. The number of "+" indicates the strength of the observed incompatibility [*e.g.* (+) – the weakest incompatibility; (++++) lack of the residing plasmid (pMTH105) tested after 72 h of growth in non-selective conditions]. Insertion mutations introduced into *repA* or ORF5 are marked by asterisks. The putative IHF binding site, DnaA box and iterons have been appropriately indicated.

SmaI-XhoI restriction fragment (pMTH210; Fig. 4). Interestingly, a part of this restriction fragment (202 bp), containing all the iterons, DnaA box and incomplete IHF box (pMTH220), did not express incompatibility towards pMTH105. The presence of enhancer at the 5' end of the minimal *inc* region strengthens the Inc phenotype (see plasmids pMTH218 and pMTH215 in Fig. 4). However, the enhancer itself does not express incompatibility (see pMTH209 in Fig. 4). It was also shown that mutation within ORF5 of enhancer did not influence the strength of the observed incompatibility (see pMTH215 and pMTH221 in Fig. 4).

Interestingly, the obtained results demonstrated that the presence within the analyzed DNA fragments of the functional *repA* increases incompatibility, which points to the regulatory role of RepA protein in the regulation of plasmid replication (compare pMTH211 and pMTH212 as well as pMTH208 and pMTH214 in Fig. 4).

Discussion

The replication system of pMTH4 has a typical structure for the plasmid iteron-containing replicator regions (*e.g*. Chattoraj, 2000; Kruger *et al*., 2004). It contains a gene coding for replication initiation protein RepA and origin of replication placed upstream of the *repA* gene. The origin of pMTH4 encodes five putative iterons R1-R5, as well as two sequences similar to the IHF binding sequence and the DnaA box of *E. coli*. These sequences are conserved within a group of related replication systems carried by plasmids: (i) pALC1 of *Paracoccus alcaliphilus* (Bartosik *et al*., 2001), (ii) pRS241a of *Rhodobacter sphaeroides* (Acc. no. EAP62516) and (iii) pSD20 from *Ruegeria* sp. (Zhong *et al*., 2003), which suggests that they are functional and may play an important role in plasmid replication. In each of the listed above plasmids the iterons are placed upstream of the *rep* gene, however their number and arrangement within the origins varies (data not shown).

Among the iterons of pMTH4 two groups could be distinguished: (i) identical repeats R1-R3 and (ii) R4 and R5 repeats placed within the *repA* promoter region. The R1, R2 and R3 repeats are separated by 22 bp

– a distance which seems to enable the location of these sequences on the same side of the DNA helix, which consequently might enable cooperative binding of the Rep protein molecules (del Solar *et al*., 1998). It is thus probable that R1-R3 repeats are functional iterons involved in initiation of plasmid replication. The R4 and R5 repeats are not identical. The R4 carries a putative –10 hexamer of the *repA* promoter, while R5 is placed close to *rbs*, which suggests their putative role in regulation of expression of the *repA* gene. In this case the binding of RepA proteins to R4 and R5 might block (or decrease) transcription driven from P_{repA} promoter. Analogous negative regulation caused by replication proteins and iterons is observed in other iteron-containing replication systems, *e.g.* of prophage P1 (Sozhamannan and Chattoraj, 1993). The true role of the particular R1-R5 repeats in pMTH4 replication and replication regulation needs, however, to be experimentally verified.

In this study we confirmed experimentally that the origin of replication of pMTH4 is located within the iteron-containing region. We also showed the presence of an additional element – termed an enhancer (present at the 5' end of the origin within a 0.65 bp DNA region), which seems to regulate the process of initiation of plasmid replication. This region does not encode any proteins, therefore we speculate that it might contain a specific sequence or sequences which are recognized by a not yet identified plasmid- or host-encoded factors. As shown, the enhancer increases (i) the plasmid copy number, (ii) the strength of determinant of incompatibility (placed within the origin) and (iii) activity of the *repA* promoter.

Enhancers are not commonly found in iteron-containing plasmids. So far such elements have been shown to be present merely in three plasmids: (i) pSC101 *Salmonella panama* (Miller and Cohen, 1993; Sugiura *et al*., 1993), (ii) R6K *E. coli* (Kelley *et al*., 1992) and (iii) pCD3.4 *Carnobacterium divergens* (van Belkum and Stiles, 2006). In all cases, the frequency of replication initiation is increased in the presence of the enhancer, this being manifested by higher plasmid copy number. The molecular basis of this phenomenon is not common for the studied plasmids. In the case of R6K, the enhancer (106 bp) is placed upstream of the origin of replication and contains a DnaA box. It is assumed that after the binding of protein IHF within the origin, subsequent bending of DNA enables direct interactions between the DnaA molecules bound to the enhancer and the proteins (DnaA and Rep) bound to the origin of replication (Lu *et al*., 1998). The enhancer of pCD3.4 carries a single iteron, which is not involved in initiation of replication but its presence increases plasmid copy number. In this case it is speculated that a replication protein binds to the enhancer region and then may probably interact with protein Rep molecules bound to the origin region (van Belkum and Stiles, 2006).

Interestingly, the replicator region of plasmid pSC101 encodes two enhancers: one called *par*, placed upstream of the origin of replication (approx. 200 bp), and the second one placed between the iterons and a *rep* gene and composed of inverted repeats IR1 and IR2, which are the binding site for the Rep protein. Gyrase can bind to the *par* region – the protein can then change the negative superhelicity of DNA, resulting in destabilization of the DNA helix within the origin, which may stimulate the initiation of replication (Ohkubo and Yamaguchi, 1995).

The common feature of the enhancers is thus the presence of sequences recognized by proteins that either interact with analogous proteins bound to the origin, or influence the structure of the origin. Enhancers are not homologous elements, for this reason they cannot be distinguished as a result of nucleotide sequence analysis. Furthermore, their mode of action has been not unequivocally determined. It is probable that DnaA or Rep mediated interactions between enhancer and origin might potentially hinder the access of Rep protein dimers to the origin (dimers negatively regulate the process of initiation of replication), which may lead to the prolonged time of the life of the open complex, and in effect bring about the initiation of a larger number of replication rounds. In this case negative regulation would be blocked by handcuffing, in which a key role would seem to be played by Rep protein dimers. These speculations might be confirmed by the results obtained for R6K. It was shown that R6K enhancer counteracts inhibition of initiation of replication caused by excess of replication protein π (such conditions favour the occurrence of dimers that inhibit the replication process) (Wu *et al*., 1994).

In this study we have shown that the enhancer of pMTH4 increases the activity of the *repA* promoter, however it itself does not encode any promoter sequences. It is probable that increased activity of P_{repA} (and consequently increased level of Rep protein) might potentially enhance the level of initiation of replication, however, such a directly proportional dependence might be observed exclusively in the absence of handcuffing. It would be interesting to know whether the activity of P_{repA} would be increased in the presence *in trans* of a RepA protein, which might potentially negatively regulate expression of *repA* gene at the level of transcription. It is also probable that the increased activity of P_{repA}, may be a secondary effect, caused by the potential binding of cellular proteins to the enhancer with subsequent destabilization of the helix with the origin region, thus facilitating the access of RNA polymerase.

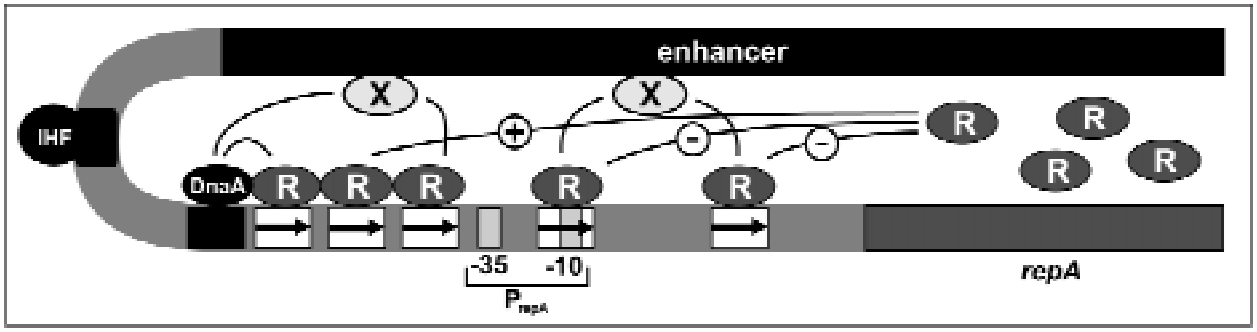

Fig. 5. Hypothetical model for functioning of the replication system of pMTH4.

The RepA molecules are marked with the letter "R". The RepA protein binds to the iterons (boxes with arrows), which activates initiation of replication (+) or negatively regulates expression of the *repA* gene (–). The DnaA and IHF molecules are appropriately indicated. A putative, not yet identified protein binding to the enhancer and able to interact with the origin, is marked with an "X". The –35 and –10 hexamers of the *repA* promoter (P_{repA}) are indicated. See text for details.

Based on the obtained results and data concerning other enhancer-containing replicons we constructed a hypothetical model of functioning of the replication system of pMTH4 (Fig. 5). It assumes that (i) RepA proteins binds the R1-R3 iterons, which activates the origin, (ii) RepA also binds the R4 and R5 repeats, which negatively regulate expression of the *repA* gene, (iii) DnaA protein binds within the origin and directs chromosomally encoded replication proteins to the *oriV*, (iv) IHF binds within the origin and bends DNA, which consequently enable interactions between not yet identified putative proteins bound in enhancer with proteins bound within the origin. This might result in destabilization of the DNA helix within the origin, which facilitates open complex formation (or prolongs the time of its life). This directly translates to increased frequency of initiation of replication, and thus an increase in plasmid copy number.

The iteron-containing replicons are usually low copy number plasmids. Their stable maintenance is determined by the presence of the partitioning systems (*par*), which are responsible for proper distribution of plasmid copies to daughter cells at cell division. All the plasmids that are closely phylogenetically related with pMTH4 (pALC1, pRS241a, pSD20) carry the partitioning modules placed in the close proximity to their replication systems. Genomic analysis of plasmid pMTH4 revealed that this replicon does not encode the *par* region (data not shown), therefore the only stabilizing system of this plasmid (placed within the *sta* region) is a toxin-antitoxin cassette, which is responsible for the post-segregational elimination of plasmid-less cells.

It is thus probable that the pMTH4 copies are randomly segregated at cell division. Thus if pMTH4 was a low copy number plasmid, then each round of replication would be followed by the formation of a large number of plasmid-less cells being eliminated from the population by the action of the toxin-antitoxin system. This would ultimately lead to elimination of the whole bacterial population. The presence of enhancer that increases plasmid copy number seems to compensate for the lack of an active partitioning system in the plasmid.

Literature

Baj J., E. Piechucka, D. Bartosik and M. Wlodarczyk. 2000. Plasmid occurrence and diversity in the genus *Paracoccus*. *Acta Microbiol. Pol.* **49**: 265–270.

Bartosik D., A. Bialkowska, J. Baj and M. Wlodarczyk. 1997. Construction of mobilizable cloning vectors derived from pBGS18 and their application for analysis of replicator region of a pTAV202 mini-derivative of *Paracoccus versutus* pTAV1 plasmid. *Acta Microbiol. Pol.* **46**: 387–392.

Bartosik D., M. Witkowska, J. Baj and M. Wlodarczyk. 2001. Characterization and sequence analysis of the replicator region of the novel plasmid pALC1 from *Paracoccus alcaliphilus*. *Plasmid* **45**: 222–226.

Bartosik D., J. Baj, A.A. Bartosik and M. Wlodarczyk. 2002. Characterisation of the replicator region of megaplasmid pTAV3 of *Paracoccus versutus* and search for plasmid-encoded traits. *Microbiology* **148**: 871–881.

Bruand C., E. Le Chatelier, S.D. Ehrlich and L. Janniere. 1993. A fourth class of theta-replicating plasmids: the pAM1 family from Gram-positive bacteria. *Proc. Natl. Acad. Sci. USA* **90**: 11668–11672.

Chattoraj D.K. 2000. Control of plasmid DNA replication by iterons: no longer paradoxical. *Mol. Microbiol.* **37**: 467–476.

del Solar G., R. Giraldo, M.J. Ruiz-Echevarria, M. Espinosa and R. Diaz-Orejas. 1998. Replication and control of circular bacterial plasmids. *Microbiol. Mol. Biol. Rev.* **62**: 434–464.

Ditta G., S. Stanfield, D. Corbin and D.R. Helinski. 1980. Broad host-range DNA cloning system for Gram-negative bacteria: construction of a bank of *Rhizobium meliloti*. *Proc. Natl. Acad. Sci. USA* **77**: 7347–7351.

Doronina N.V., Y.A. Trotsenko, V.I. Krausova and N.E. Suzina. 1998. *Paracoccus methylutens* sp. nov. – a new aerobic facultatively methylotrophic bacterium utilizing dichloromethane. *Syst. Appl. Microbiol.* **21**: 230–236.

Hanahan D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**: 557–580.

Keen N.T., S. Tamaki, D. Kobayashi and D. Trollinger. 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene* **70**:191–197.

Kelley W.L., I. Patel and D. Bastia. 1992. Structural and functional analysis of a replication enhancer: separation of the enhancer activity from origin function by mutational dissection of the replication origin γ of plasmid R6K. *Proc. Natl. Acad. Sci. USA* **89**: 5078–5082.

Kruger R., S.A. Rakowski and M. Filutowicz. 2004. Participating elements in the replication of iteron-containing plasmids, pp. 25–45. In: B.E. Funnell and G.J. Phillips (eds), Plasmid Biology, ASM Press, Washington, D.C.

Kushner S.R. 1978. An improved method for transformation of *E. coli* with ColE1-derived plasmids. pp. 17–23. In: H.B. Boyer and S. Nicosia (eds), Genetic Engineering, Elsevier/North-Holland, Amsterdam.

Lu Y.B., H.J. Datta and D. Bastia. 1998. Mechanistic studies of initiator-initiator interaction and replication initiation. *EMBO J.* **17**: 5192–5200.

Marx C.J. and M.E. Lidstrom. 2001. Development of improved versatile broad-host-range vectors for use in methylotrophs and other Gram-negative bacteria. *Microbiology* **147**: 2065–2075.

Meijer W.J.J., A.J. de Boer, S. van Tongeren, G. Venema and S. Bron. 1995. Characterization of the replication region of the *Bacillus subtilis* plasmid pLS20: a novel type of replicon. *Nucleic Acids Res.* **23**: 3214–3223.

Messer W., F. Blaesing, D. Jakimowicz, M. Krause, J. Majka, J. Nardmann, S. Schaper, H. Seitz, C. Speck, C. Weigel, G. Wegrzyn, M. Welzeck and J. Zakrzewska-Czerwinska. 2001. Bacterial replication initiator DnaA. Rules for DnaA binding and roles of DnaA in origin unwinding and helicase loading. *Biochimie* **83**: 5–12.

Miller J.H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor, New York.

Miller C.A. and S.N. Cohen. 1993. The partition (*par*) locus of pSC101 is an enhancer of plasmid incompatibility. *Mol. Microbiol.* **9**: 695–702.

Ohkubo S. and K. Yamaguchi. 1995. Two enhancer elements for DNA replication of pSC101, *par* and a palindromic binding sequence of the Rep protein. *J. Bacteriol.* **177**: 558–565.

Reece K.S. and G.J. Phillips. 1995. New plasmids carrying antibiotic-resistance cassettes. *Gene* **165**: 141–142.

Sambrook J. and D.W. Russell. 2001. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor, New York.

Schagger H. and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**: 368–379.

Sozhamannan S. and D.K. Chattoraj. 1993. Heat shock proteins DnaJ, DnaK, and GrpE stimulate P1 plasmid replication by promoting initiator binding to the origin. *J. Bacteriol.* **175**: 3546–3555.

Spratt B.G., P.J. Hedge, S. te Heesen, A. Edelman and J.K. Broome-Smith. 1986. Kanamycin-resistant vectors that are analogues of plasmids pUC8, pUC9, pEMBL8 and pEMBL9. *Gene* **41**:337–342.

Sugiura S., S. Ohkubo and K. Yamaguchi. 1993. Minimal essential origin of plasmid pSC101 replication: requirement of a region downstream of iterons. *J. Bacteriol.* **175**: 5993–6001.

Szymanik M., D. Bartosik and M. Wlodarczyk. 2004. Genetic organization of the basic replicon of plasmid pMTH4 of a facultatively methylotrophic bacterium *Paracoccus methylutens* DM12. *Curr. Microbiol.* **48**: 291–294.

Szymanik M. 2006. Ph.D. Thesis. Warsaw University, Warsaw.

van Belkum M.J. and M.E. Stiles. 2006. Characterization of the theta-type plasmid pCD3.4 from *Carnobacterium divergens*, and modulation of its host range by RepA mutation. *Microbiology* **152**: 171–178.

Wlodarczyk M., E.K. Jagusztyn-Krynicka, D. Bartosik and I. Kalinowska. 1994. Electroporation of *Thiobacillus versutus* with plasmid DNA. *Acta Microbiol. Pol.* **4**3: 223–227.

Wu F., I. Levchenko and M. Filutowicz. 1994. Binding of DnaA protein to a replication enhancer counteracts the inhibition of plasmid R6K γ origin replication mediated by elevated levels of R6K π protein. *J. Bacteriol.* **176**: 6795–6801.

Zhong Z., R. Caspi, D. Helinski, V. Knauf, S. Sykes, C. O'Byrne, T.P. Shea, J.E. Wilkinson, C. DeLoughery and A. Toukdarian. 2003. Nucleotide sequence based characterizations of two cryptic plasmids from the marine bacterium *Ruegeria* isolate PR1b. *Plasmid* **49**: 233–252.