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Comparative characterization of *repABC*-type replicons of *Paracoccus pantotrophus* composite plasmids☆

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Abstract

The *repABC* replicons have an unusual structure, since they carry genes coding for partitioning (*repA*, *repB*) and replication (*repC*) proteins, which are organized in an operon. So far, the presence of these compact bi-functional modules has been reported only in the megaplasmids of the Rhizobiaceae and within the plasmid pTAV1 (107 kb) of *Paracoccus versutus*. We studied the distribution of *repABC*-type replicons within bacteria belonging to the genus *Paracoccus*. We found that *repABC* replicons occur only in the group of pTAV1-like plasmids: pKLW1, pHG16-a, pWKS2, and pPAN1, harbored by different strains of *Paracoccus pantotrophus*. A partial sequencing approach followed by phylogenetic analysis revealed that these replicons constitute a distinct evolutionary branch of *repABC* replicons. Incompatibility studies showed that they represent two incompatibility groups designated IncABC1 (pTAV1, pKLW1, and pHG16-a) and IncABC2 (pPAN1). Sequence comparison using available databases allowed the identification, within plasmid pRS241d of *Rhodobacter sphaeroides* 2.4.1, of an additional sequence highly homologous to the paracoccal *repABC* replicons, which has been included in comparative analyses.

Keywords: repABC replicon; *Paracoccus pantotrophus*; Incompatibility; Composite plasmid

1. Introduction

The *repABC* replicons have unique, identical genetic organization. They carry three genes (*repA*, *repB*, and *repC*) clustered in an operon and a short conserved intergenic sequence (*igs*) located between the *repB* and *repC* genes (Fig. 1; e.g., Bartosik et al., 2001a; Nishiguchi et al., 1987). The *repC* gene encodes for the main replication initiation protein (Bartosik et al., 1998; Tabata et al., 1989). The origin of replication (*oriV*) has not been experimentally localized but it is suggested that it might be present within the coding sequence of the *repC* gene (Bartosik et al., 1998; Palmer et al., 2000). The *repA* and *repB* protein products show sequence similarities to proteins involved in the partition of bacterial plasmids and

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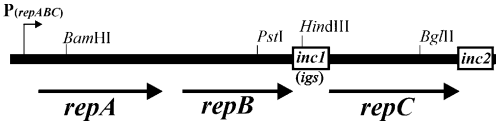

Fig. 1. Genetic organization of pTAV320 and other *repABC* replicons. The translational orientation of the three open reading frames is indicated by arrows. The highly conserved intergenic sequence between *repB* and *repC* genes is indicated as *igs*. P indicates promoter of the *repABC* operon. Two determinants of incompatibility are boxed and indicated *inc1* and *inc2* (see text for details). The restriction sites common for the *repABC* replicons of plasmids pTAV1 (*P. versutus*), pKLW1 and pHG16-a (*P. pantotrophus*) are indicated.

their chromosomal homologs (Gerdes et al., 2000). Recent studies have demonstrated that *repA*, *repB*, and the *cis* required partitioning site (located downstream of the *repC* gene) constitute a functional system for the active partitioning of a *repABC*-type replicon—pTAV320 (Bartosik et al., 2001a). Moreover, the results of studies by Ramírez-Romero et al. (2001) showed that the protein product of the *repA* gene of another *repABC* replicon (p42d) was recognized as a negative regulator of the operon. Therefore, like the homologous A-type proteins of other plasmid partitioning systems, RepA (which is also a *trans*-acting incompatibility factor) plays important regulatory functions. A search for other regulatory elements has led to the identification of two incompatibility determinants (*inc*). One of them is located within the intergenic sequence between *repB* and *repC* (probably involved in the regulation of expression of *repC* gene) and the second one (the above-mentioned partitioning site) is situated downstream of the *repC* gene (Bartosik et al., 2001a; Ramírez-Romero et al., 2000).

The *repABC* replicons are widely distributed in megaplasmids, harbored by the bacteria belonging to the Rhizobiaceae (e.g., Li and Farrand, 2000; Ramírez-Romero et al., 1997; Tabata et al., 1989; Turner and Young, 1995). Such a replicon has also been found in the plasmid pTAV1 of *Paracoccus versutus* (Bartosik et al., 1998) and within one of the chromosomes of the *Agrobacterium tumefaciens* strain C58 (Wood et al., 2001). Plasmid pTAV1 is a composite replicon carrying two distinct functional replication systems. These were cloned in the form of mini-replicons—pTAV203 (Bartosik et al., 1997b) and pTAV320 (Bartosik et al., 1998). Mini-replicon pTAV320 was shown to be the most divergent member of the *repABC* family (Li and Farrand, 2000; Palmer et al., 2000), while pTAV203 is not included in this class of plasmids. It encodes for a replication initiation protein (RepC′) with statistically significant homology to RepCs of the *repABC*-type replicons but no other structural similarities between these replicons have been found.

The distribution of *repABC* replicons has been intensively studied in the Rhizobiaceae. The first screening for *repABC* plasmids among *Rhizobium leguminosarum* field isolates was done with the use of conserved PCR primers, designed on the basis of the conserved nucleotide sequences of *repC* genes of plasmids pRL8Ji, pRiA4b, and pTiB6S. Phylogenetic analysis based on the amplified sequences allowed us to distinguish four distinct sequence groups, which (as suggested by Turner et al., 1996) correspond to the incompatibility groups of rhizobial plasmids. Modified *repC* primers allowed us to identify *repC* sequences in *Sinorhizobium* and *Mesorhizobium* as well as to distinguish additional incompatibility groups (Palmer et al., 2000; Rigottier-Gois et al., 1998).

So far, no studies have been undertaken to determine the dissemination and diversity of *repABC*-type replicons occurring in bacteria belonging to the genus *Paracoccus*, which currently embraces 14 species. Our preliminary analysis of plasmid patterns in several strains, representing 11 paracoccal species, enabled the identification of over 30 plasmids, including megaplasmids with sizes roughly determined at 400–500 kb (Baj et al., 2000). These plasmids have not yet been subjected to detailed analysis, except for the above-mentioned plasmid pTAV1 and megaplasmid pTAV3 (Bartosik et al., 2002) of *P. versutus*, as well as plasmid pALC1 of *P. alcaliphilus* (Bartosik et al., 2001b). The main objective of the research reported herein was to identify *repABC* replicons in plasmids of bacteria belonging to the genus *Paracoccus* and to carry out comparative studies embracing them.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

The following bacterial strains were used in this study: *P. alcaliphilus* JCM 7364 (Urakami et al., 1989); *P. alkenifer* JCM 7354 (Lipski et al., 1998); *P. aminophilus* JCM 7686 (Urakami et al., 1990); *P. aminovorans* JCM 7685 (Urakami

et al., 1990); *P. denitrificans* LMD 22.21 (Rainey et al., 1999); DSM 413 (Gerstenberg et al., 1982); *P. kocurii* CCM 4331 and CCM 4332 (Ohara et al., 1990); *P. methylutens* DM 12 (Doronina et al., 1998); *P. solventivorans* DSM 11592 and DSM 6637 (Siller et al., 1996); *P. thiocyanatus* IAM 12816 (Katayama et al., 1995); *P. versutus* UW1 (Taylor and Hoare, 1969), and eight strains of *P. pantotrophus*: DSM 11104, DSM 11072, DSM 11073 (Jordan et al., 1997); KL100 (Bartosik et al., 2002); DSM 65 (Gerstenberg et al., 1982); and LMD 82.5 (Baj et al., 2000). All Rif^r derivatives of the strains used in this work were obtained as spontaneous mutants selected on solidified LB medium supplemented with rifampicin. Cultures were grown in Luria Broth (LB) (Sambrook et al., 1989) at a temperature of 30 °C (*Paracoccus* spp.) or 37 °C (*Escherichia coli* TG1). The concentrations of antibiotics included in the media were as follows: kanamycin, 50 μg/ml; rifampicin, 50 μg/ml; tetracycline, 3 μg/ml for *P. versutus*, 0.3 μg/ml for *P. pantotrophus*, and 20 μg/ml for *E. coli*.

Bacterial plasmids used in this study are listed in Table 1.

2.2. DNA manipulation

Plasmid DNA was isolated according to Birnboim and Doly (1979) and when necessary purified by CsCl–ethidium bromide gradient centrifugation. Total DNA of *Paracoccus* spp. was isolated, as described by Williams et al. (1998). Cloning experiments, digestion with restriction enzymes, ligation, and agarose gel electrophoresis were conducted in accordance with standard procedures, as described by Sambrook et al. (1989). All enzymes were purchased from either Promega or Roche. DNA restriction fragments were purified from agarose with DNA-Gel-Out Kit (DNA Gdansk). For Southern hybridization (Sambrook et al., 1989), DNA probes were labeled with digoxigenin (Roche). Hybridization and visualization of hybridization products were carried out as recommended by the supplier.

Table 1
Plasmids used in this study

Plasmid	Relevant characteristics	References or source
pTAV1	107 kb	Bednarska et al. (1983)
pTAV203	Km^r; 5.3 kb mini-replicon of pTAV1, composed of 4.0 kb *Pst*I fragment of pTAV1 plus Km^r cassette	Bartosik et al. (1997b)
pTAV320	Km^r; 5.6 kb mini-replicon of pTAV1, composed of two *Pst*I fragments (2.1 and 2.2 kb) of pTAV1 plus Km^r cassette	Bartosik et al. (1998)
pHG16-a	≈70 kb	Gerstenberg et al. (1982)
pHGW100	Km^r; 8.0 kb mini-replicon of pHG16-a, composed of 6.7 kb *Pst*I fragment of pHG16-a plus Km^r cassette	This study
pHGW200	Km^r; 5.4 kb mini-replicon of pHG16-a, composed of two *Pst*I fragments (2.2 and 1.9 kb) of pHG16-a plus Km^r cassette	This study
pKLW1	≈100 kb	Baj et al. (2000)
pKLW100	Km^r; 6.8 kb mini-replicon of pKLW1, composed of 5.5 kb *Pst*I fragment of pKLW1 plus Km^r cassette	This study
pKLW200	Km^r; 6.1 kb mini-replicon of pKLW1, composed of two *Pst*I fragments (2.2 and 2.6 kb) of pKLW1 plus Km^r cassette	This study
pPAN1	≈100 kb	Baj et al. (2000)
pPAN200	Km^r; 11 kb mini-replicon of pPAN1, composed of three *Pst*I fragments (3.4, 1.3, and 5 kb) of pPAN1 plus Km^r cassette	This study
pUC4-K	source of Km^r cassette	Vieira and Messing (1982)
pBGS18	Km^r; cloning vector, ColE1 origin of replication	Spratt et al. (1986)
pABW1	Km^r; mobilizable cloning vector based on pBGS18, *oriT* of RK2	Bartosik et al. (1997a)
pRK415	Tc^r; mobilizable broad host range cloning vector, RK2 *oriT* and origin of replication	Keen et al. (1988)
pRK415/M1	Tc^r; pRK415 derivative carrying *inc1* (*igs*) of pTAV320	Bartosik et al. (2001a)
pRK415/M2	Tc^r; pRK415 derivative carrying *inc2* (partitioning site) of pTAV320	Bartosik et al. (2001a)
PRK415/M3	Tc^r; pRK415 derivative carrying the proximal part of pTAV320 with complete *repA* gene	Bartosik et al. (2001a)
pRK2013	Km^r; helper plasmid carrying RK2 *tra* genes	Ditta et al. (1980)

2.3. DNA sequencing and analysis

The nucleotide sequence was determined in the DNA Sequencing and Oligonucleotides Synthesis Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Science using a terminator sequencing kit and an automatic sequencer (ABI 377 Perkin–Elmer). For the purpose of sequencing, the chosen restriction fragments of the mini-replicons were cloned into *E. coli* pBGS18 vector and the inserts were sequenced starting with universal forward and reverse primers and then with primers complementary to the previously determined sequence. Sequence analysis was done with programs that were included in the UWGCG package (Devereux et al., 1984). Comparison searches through the databases were performed with BLAST program, provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). The alignment of sequences was performed with PILEUP program (GCG software). Phylogenetic tree of RepC was constructed using the Fitsch–Margoliash method with Dayhoff PAM distance matrix computed with the PROTDIST program of PHYLIP (Felsenstein, 1989). Bootstrap analysis (1000 replicates) was used to obtain confidence estimates for phylogenetic tree topologies. Significant bootstrap probability values are indicated (in percentage) at the branching points.

2.4. Electroporation and transformation

Electroporation was carried out at 2500 V, 25 μF, and 200 Ω (for *E. coli*) or 400 Ω (for *P. versutus* and *P. pantotrophus*) in a gene pulser apparatus (Bio-Rad Laboratories) according to a modified Bio-Rad procedure (Wlodarczyk et al., 1994). Electrotransformants were selected on solidified LB medium supplemented with appropriate antibiotics. Competent cells of *E. coli* TG1 were prepared and transformed as described by Kushner (1978).

2.5. Host range studies

To determine the host range of analyzed mini-replicons, we constructed hybrid plasmids composed of a mobilizable, *E. coli* specific (unable to replicate in the tested species of Alphaproteobacteria) vector pABW1 (Bartosik et al., 1997a) carrying the tested *repABC* module (cloned into appropriate sites of MCS of the vector). The thus constructed plasmids were introduced by means of triparental mating from *E. coli* TG1 into chosen bacterial strains, as described below. The presence of plasmid DNA in selected transconjugants was verified by the screening of 10 colonies using rapid alkaline lysis and agarose gel electrophoresis.

2.6. Triparental mating

The overnight cultures of the donor strain *E. coli* TG1 carrying the mobilizable recombinant vector based on pABW1 or pRK415 were grown, spun down, and washed twice with LB medium to remove antibiotics. These were mixed at a ratio of 1:2:1 with a suitable recipient strain and *E. coli* TG1 carrying the helper plasmid pRK2013. One hundred μl of such a mixture was spread on a plate with solidified LB medium. After overnight incubation at 30 °C, the bacteria were washed off the plate and suitable dilutions were plated on selective media containing rifampicin—selective marker of the recipient strain and an other appropriate antibiotic (Tc or Km) to select transconjugants. Spontaneous resistance of the recipient strains to Km and Tc was undetectable under these experimental conditions.

2.7. Plasmid stability

P. versutus or *P. pantotrophus* strains containing the studied plasmids were grown at 30 °C in LB medium supplemented with appropriate antibiotic. Stationary phase cultures were diluted in fresh medium without antibiotic selection and cultivated for approximately 10, 20, and 30 generations. Samples taken after these times were diluted and plated onto solid medium in the absence of selective drugs. From these plates, 200 colonies were tested by replica plating with the use of Km^r or Tc^r marker. The retention of plasmids, after approximately 30 generations, was determined as the percentage of kanamycin or tetracycline resistant colonies.

2.8. Incompatibility testing

The incompatibility characteristics of two plasmids were examined by conjugative transfer (triparental mating) of pRK415-derived plasmids (carrying incompatibility determinants of pTAV320) into recipient *P. pantotrophus* KL100 (harboring the studied *repABC*-type replicon). Transconjugants were selected for the incoming

plasmids (Tc^r) and for both incoming and residing plasmids (Tc^r and Km^r). The plasmid pattern of the transconjugants was verified by the screening of 10 colonies by rapid alkaline lysis and agarose gel electrophoresis. In control experiments, we showed that pRK415 itself is compatible with all tested *repABC* replicons.

3. Results

3.1. Identification of repABC replicons in Paracoccus spp. plasmids

To identify *repABC*-type replicons in the plasmids of *Paracoccus* spp. (ranging in size from 2.7 to ≈100 kb), hybridization analysis was performed employing DIG-labeled mini-replicon pTAV320 as a probe (Fig. 2). A positive result of hybridization was obtained for four plasmids: pHG16-a (≈70 kb), pKLW1 (≈100 kb), pWKS2 (≈100 kb), and pPAN1 (≈100 kb), occurring in strains DSM 65, DSM 11073, DSM 11104, and LMD 82.5, respectively (Fig. 2B, lanes 4–7), that have recently been classified to the species *P. pantotrophus* (the closest relative of *P. versutus*; Rainey et al., 1999).

Analogous hybridization analysis (with pTAV320 as a probe) was performed, involving the total DNA (digested with *Pst*I) of all paracoccal strains. The obtained results excluded the presence of other sequences homologous to pTAV320 in megaplasmids (which are very common among paracocci) and chromosomes (data not shown).

3.2. Construction and characterization of mini-replicons of plasmids pKLW1, pHG16-a, and pPAN1

As can be seen in Fig. 2A (lanes 3, 5, 6, and 7) plasmids pTAV1, pKLW1, pWKS2, and pPAN1 are of similar sizes (≈100 kb). Restriction analysis (performed with *Pst*I, *Eco*RI, *Bam*HI, *Sal*I, and *Bcl*I) has shown that pKLW1 and pWKS2 should be considered different isolates of the same plasmid because they gave the same restriction pattern after digestion with all the above-mentioned restriction enzymes (data not shown). In further studies, only pKLW1 was used.

To carry out a detailed analysis of the *repABC* replicons of *P. pantotrophus,* in the first stage mini-replicons of the analyzed plasmids were constructed according to a classical procedure. In short, the DNA of the parental plasmids was digested with *Pst*I, ligated with an appropriate form of the kanamycin resistance cassette (derived from pUC4-K), and then introduced by electroporation into plasmid-free *P. pantotrophus* strain KL100. In all cases, Km^r transformants carrying mini-derivatives of the parental plasmids were obtained. These were then taken for further analysis. Interestingly, in the case of plasmid pKLW1 as well as pHG16-a, two classes of replicons

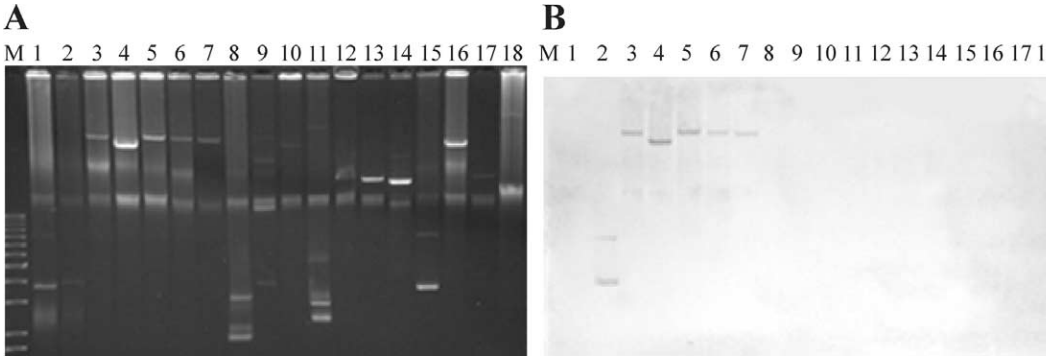

Fig. 2. (A) Agarose gel electrophoresis of plasmids isolated from *Paracoccus* spp. (B) Southern blot analysis of the same gel hybridized to the pTAV320 (*repABC* replicon of pTAV1) probe. M—DNA marker size (1 kb ladder). Lanes (1) pTAV203—negative control and (2) pTAV320—positive control. Lanes 3–18 natural plasmids isolated from paracocci by standard alkaline lysis procedure. Lane (3) *P. versutus* UW1 (pTAV1); (4) *P. pantotrophus* DSM 65 (pHG16-a); (5) *P. pantotrophus* DSM 11073 (pKLW1); (6) *P. pantotrophus* DSM 11104 (pWKS2); (7) *P. pantotrophus* LMD 82.5 (pPAN1); (8) *P. pantotrophus* DSM 11072; (9) *P. aminophilus* JCM 7686; (10) *P. alcaliphilus* JCM 7364; (11) *P. aminovorans* JCM 7685; (12) *P. kocurii* CCM 4332; (13) *P. kocurii* CCM 4331; (14) *P. methylutens* DM12; (15) *P. solventivorans* DSM 11592; (16) *P. solventivorans* DSM 6637; (17) *P. denitrificans* LMD 22.21; and (18) *P. thiocyanatus* IAM 12816.

containing *Pst*I restriction fragments of various lengths derived from the parental plasmids were identified (Table 1). This suggested that like pTAV1, the plasmids are composite ones, each of which carries two functional replication systems.

To identify the *repABC* replicons among the constructed mini-derivatives, hybridization analysis using pTAV320 as a probe was performed. A positive result of hybridization was obtained only with the DNA of mini-replicons pKLW200, pHGW200, and pPAN200. A similar hybridization analysis was repeated using mini-replicon pTAV203 carrying the second replication system (*repC*′-type) of plasmid pTAV1. In this case, hybridization was observed only with mini-replicons pKLW100 and pHGW100, as well as with the DNA of plasmid pPAN1 (Table 2). The obtained results indicate that all the *P. pantotrophus* plasmids (including pPAN1, for which attempts to construct a functional *repC*′-type replicon, using the same enzymes, proved futile) are composite plasmids, since each of them contains two kinds of replication systems related to those of pTAV1.

The identified *repABC* replicons included in pKLW200, pHGW200, and pPAN200 were, like pTAV320, very stably maintained in culture set up in the absence of selective pressure, whereas pKLW100 and pHGW100 were unstable, as was pTAV203. The characteristics of the mini-replicons are presented in Table 2. We also found that all of them have the same host range (tested as described in Section 2), encompassing all the studied *Paracoccus* species (*P. alkenifer*, *P. aminophilus*, *P. aminovorans*, *P. denitrificans*, *P. methylutens*, *P. solventivorans*, *P. thiocyanatus*, and *P. versutus*), as well as *A. tumefaciens*, *R. leguminosarum*—all belonging to the Alphaproteobacteria. Similar observations were made earlier in the case of the mini-replicons of pTAV1 (Bartosik et al., 1998; and unpublished results).

3.3. Analysis of sequence diversity of the paracoccal repABC replicons

In the next stage of the study, pTAV320 (probe) was hybridized with DNA of the tested mini-replicons digested with *Pst*I. The result of pTAV320 hybridization was positive for all restriction fragments of pKLW200 (2.2 and 2.6 kb) and pHGW200 (2.2 and 1.9 kb). However, in the case of pPAN1 a strong hybridization was observed exclusively with the 1.3 kb *Pst*I restriction fragment, which points to the high degree of sequence homology only in a part of this replicon (data not shown). Therefore, the 1.3 kb *Pst*I fragment of pPAN200 carries a sequence that seems to be conserved in *repABC* replicons of *Paracoccus* spp.

To identify the region of homology, the 1.3 kb *Pst*I restriction fragment of pPAN200 was cloned into vector pBGS18 and sequenced. An analysis of the obtained sequence (1364 bp) demonstrated that it embraces a significant part of gene *repC* (1237 bp), deprived of its proximal part. To specify the conserved region, a further part of replicon pPAN200 adjacent to the above-mentioned *Pst*I fragment was sequenced. Consequently, a sequence of the replicon embracing (i) terminal part of gene *repB*, (ii) *igs*, (iii) gene *repC*, and (iv) region downstream of *repC* (2005 bp; GenBank Accession No. AY033083) was obtained. A comparative analysis of the corresponding sequences of pPAN200 and pTAV320 revealed that in both replicons the *repC* gene sequence is conserved to be the strongest (92%

Table 2
Characteristics of mini-replicons derived from composite plasmids of *Paracoccus* spp

Parental plasmid	Mini-replicon	Stability (%)[a]	Hybridization with a probe derived from	
			pTAV320	pTAV203
pTAV1	pTAV320	99	+	–
	pTAV203	4	–	+
pKLW1	pKLW200	100	+	–
	pKLW100	7	–	+
pHG16-a	pHGW200	98	+	–
	pHGW100	5	–	+
pPAN1	pPAN200	100	+	–
	The second replicator region has not been cloned in the form of a mini-replicon[b]			

[a] After 30 generations of growth in non-selective conditions.
[b] pPAN1 hybridized with pTAV203; see text for details.

identity; 96.7% of similarity, and 95.5% of identity on amino acid sequence level). The *igs* (56% identity) as well as *repB* (47% of identity on a stretch of 279 bp; 40% of similarity and 31% of identity on protein sequence level) sequences were much less conserved. However, BLAST comparison showed that they are more similar to corresponding regions of pTAV320 than to rhizobial *repABC* replicons.

To analyze the sequence diversity of the *repABC* paracoccal replicons, we also sequenced analogous parts of pKLW200 and pHG200 (in both cases 2112 bp). We found that the sequenced regions of both plasmids (GenBank Accession Nos. AF397209 and AF397210, respectively) are identical but slightly depart from the sequence of pTAV320 (≈95% of identity within 2112 bp). The greatest similarity is within *repB* (100% identity in a stretch of 558 bp) and *igs* (99.5% identity) whereas *repC* was less conserved (91% identity; 96.7% similarity and 94% identity on protein sequence level). The results of comparative analyses (Table 3) therefore indicate that the sequence of gene *repC* is conserved to be the strongest in all the studied *repABC* replicons (over 90% identity). This sequence can thus be used as a specific probe enabling the identification of *Paracoccus*-type *repABC* replicons.

To detect other replicons of this type, we performed sequence searches in unfinished microbial genomes. Both BLASTN using the *repC* sequence of pTAV320 and TBLASTN using the predicted amino acid sequence of its translational product were carried out. This analysis allowed us to identify a homologous sequence in plasmid pRS241d (R.C. Mackenzie, personal communication) of *Rhodobacter sphaeroides* 2.4.1 (Accession No. NC002718; contig 195; www. rhodobacter.org), which showed a strongly significant similarity to *repC* of pTAV320 (85% of identity; 90% of similarity and 86% of identity on amino acid sequence level) (Table 3). The presence of conserved *igs* and two ORFs with similarity to *repA* and *repB* adjacent to *repC* indisputably indicates that this is a member of the *repABC* replicon family.

Based on amino acid sequences of RepC replication proteins, a phylogenetic analysis of all known *repABC* replicons was performed, including the above-mentioned replicon of *R. sphaeroides* 2.4.1. As seen in Fig. 3, the replicons of *Paracoccus* spp. (together with the replicon of *R. sphaeroides*) constitute an evolutionary branch of *repABC* replicons that is distinct from rhizobial plasmids.

3.4. Analysis of incompatibility of pTAV320 with repABC replicons of P. pantotrophus

In general, plasmids are considered incompatible if they cannot be co-maintained by a host cell under non-selective conditions. *repABC* replicons code several incompatibility determinants involved in the replication or plasmid partitioning (Bartosik et al., 2001a; Ramírez-Romero et al., 2000). As mentioned in Section 1, a detailed analysis of mini-replicon pTAV320 revealed the presence of two incompatibility determinants: *inc1* localized within *igs* and *inc2* (carrying *cis* required partitioning site) present in a short non-coding region downstream of the *repC* gene. It was also observed that RepA regulatory protein (when overexpressed *in trans*) was a *trans*-acting incompatibility factor (Bartosik et al., 2001a).

We decided to check whether *inc* regions of pTAV320 are able to express incompatibility towards other *repABC* replicons of *Paracoccus* spp. To this end, we used the pRK415-derived plasmids (Tc^r) carrying *inc* regions of pTAV320, constructed earlier (Bartosik et al., 2001a). These were pRK415/M1 (carrying *inc1* that is *igs*; Fig. 1), pRK415/M2 (carrying *inc2*; Fig. 1), and pRK415/M3 (overexpressing RepA protein; Table 1). The plasmids were transferred by triparental mating from *E. coli* TG1 into *P. pantotrophus*

Table 3
Analysis of sequence divergence of paracoccal-type *repABC* replicons

Analyzed element of the *repABC* replicons	Nucleotide sequence homology of the *repABC* replicon with corresponding parts of pTAV320 (%)		
	pPAN200	pKLW200/pHGW200	*R. sphaeroides* 2.4.1
repB	47[a]	100[b]	49
igs	56	99.5	53
repC	92	91	85

[a] Within the terminal part of *repB* (279 bp).
[b] Within the terminal part of *repB* (558 bp).

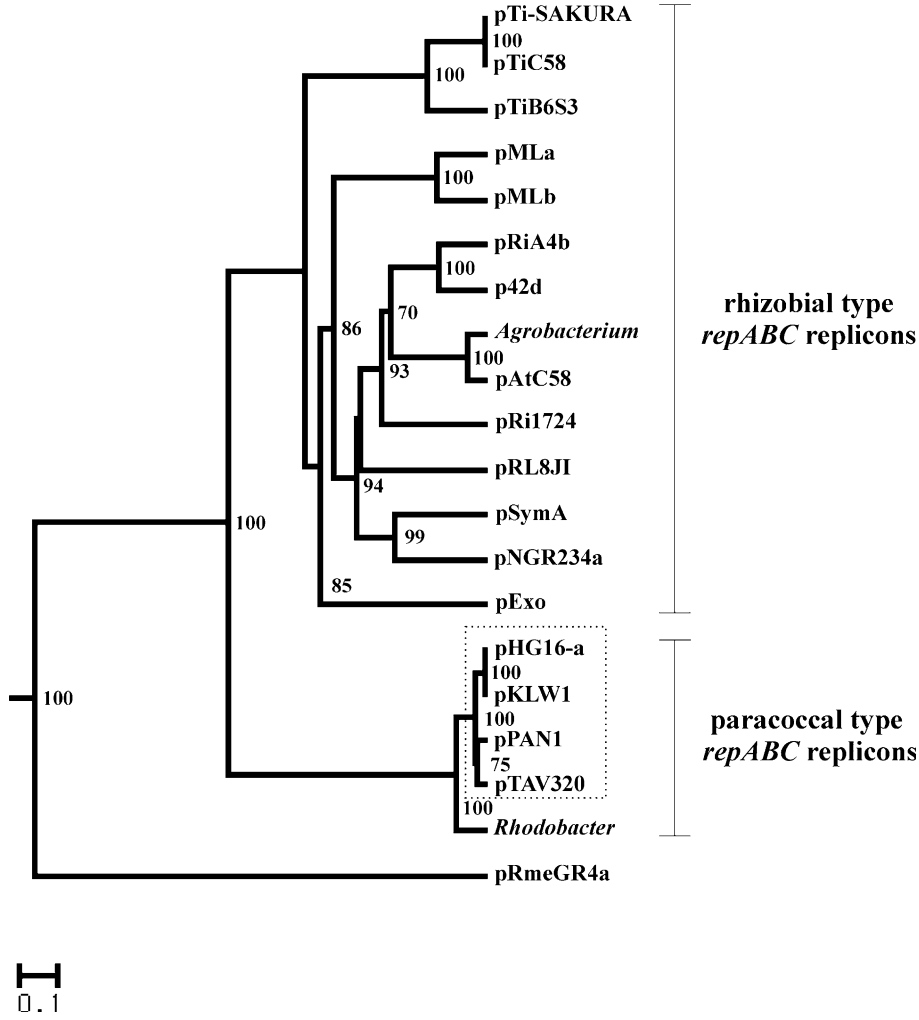

Fig. 3. Phylogenetic tree comparing the relatedness of RepC sequences. The data for each bootstrap value were resampled 1000 times, with the evolutionary distance scale (below) representing the number of fixed substitutions per site, as a measure of the horizontal distance. Support values for internal branches are shown as percentages. The tree was rooted using RepC of pRmeGR4a as the outgroup (pRmeGR4a codes for RepC homolog but does not belong to the *repABC* family). *repABC* replicons identified in *Paracoccus* spp. are boxed. The following sequences were included in the phylogenetic analysis (GenBank accession numbers are given in parentheses): pTi-SAKURA (NC 002147), pTiC58 (AF060155), pTiB6S3 (M24529), pMLb (NC 002682), pMLa (NC 002679), pRiA4b (P05684), p42d (U80928), *Agrobacterium* (linear chromosome of *A. tumefaciens* C58; NC 003064), pAtC58 (AF283811), pRi1724 (NC 002575), pRL8JI (X89447), pSymA (NC 003037), pNGR234a (NC 000914), pExo (NC 003078), pHG16-a (AF397210), pKLW1 (AF397209), pPAN1 (AY033083), pTAV320 (mini-replicon of pTAV1; U60522), *Rhodobacter* (pRS241d of *R. sphaeroides* 2.4.1; NC 002718), and pRmeGR4a (Y08790).

KL100 carrying tested mini-replicons (Km^r). In all cases, Km^r Tc^r transconjugants containing both incoming and residing plasmids were obtained. In the case of incompatibility, the tested mini-replicon was gradually lost by the culture of transconjugant that was grown in the absence of selective pressure (see Section 2 for details). We found that all three pTAV320 *inc* determinants express incompatibility towards pKLW200 and pHGW200. The strongest incompatibility was observed with pRK415/M2 (*inc2*), when already after 20 generations of growth no Km^r clones could be recovered. In the case of pRK415/M1 and pRK415/M3, 10–16% of the tested cells

carried both plasmids (after 30 generations). On the other hand, no *inc* phenotype was observed with pPAN200.

The nucleotide sequences of the incompatibility determinants of the studied replicons were compared (Fig. 4). It is evident that the *inc* sequences of pPAN200, in spite of the presence in them of several conserved motifs, significantly differ from analogous sequences in the remaining paracoccal plasmids. Interestingly, in all cases within *inc2* (partitioning site) occur conserved repeated sequences (R1 and R2; Fig 4B), which constitute sites that interact with partitioning protein RepB, as shown for pTAV320 (Bartosik et al., 2001a). Such repeats are also present in the novel *repABC* replicon of *R. sphaeroides* (Fig. 4B) but are not found within rhizobial *repABC* replicons (Bartosik et al., 2001a). The sequence comparison revealed that the *inc1* regions of pPAN200 and the *repABC* replicon of *R. sphaeroides* show a high level of identity (≈100%). The *inc2* regions of these replicons, although less conserved (58%), contain homologous R1 and R2 repeats (Fig. 4B). This suggests that these two plasmids belong to the same incompatibility group. Thus, *repABC* replicons of *Paracoccus* represent two incompatibility groups that we have designated IncABC1 (embracing pTAV320, pKLW200, and pHGW 200) and IncABC2 (pPAN200 and probably the *repABC* replicon of *R. sphaeroides*). However, the including the *R. sphaeroides* replicon in this group should be verified experimentally with genetic tests on the incompatibility.

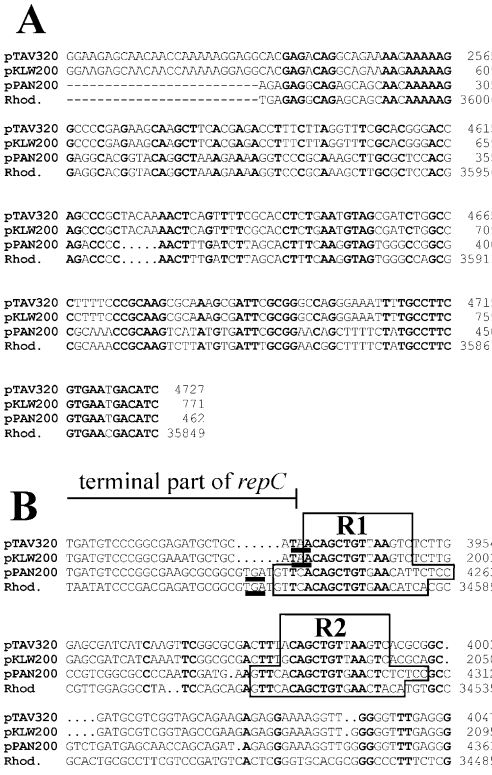

Fig. 4. Alignment of (A) *inc1* (*igs*) and (B) *inc2* regions from paracoccal-type *repABC* replicons (including *repABC* replicon of *R. sphaeroides*). The identical residues of the sequences are bolded. Terminal part of the *repC* genes is indicated. The stop codons of *repC*s are underlined. The R1 and R2 repeated sequences of *inc2* (the binding site for RepB partitioning protein) are boxed and adequately indicated. The numbers on the right refer to the deposited nucleotide sequences of pTAV320 (AF390867), pKLW200 (AF397209), pPAN200 (AY033083), and Rhod. (contig 195 of the genome of *R. sphaeroides* 2.4.1; NC 002718). The corresponding nucleotide sequences of pHG200 (AF397210) are identical with the sequences of pKLW1.

4. Discussion

repABC replicons have been previously recognized in megaplasmids of the Rhizobiaceae and plasmid pTAV1 of *P. versutus*. Although megaplasmids (over 400 kb) are also frequent among bacteria included in the genus *Paracoccus* (Baj et al., 2000), they seem not to contain *repABC* replicons of the rhizobial or paracoccal type (as we have shown by hybridization). This is in agreement with the results of recent studies, which showed that several megaplasmids of *Paracoccus* spp. carry replication systems of different types (Bartosik et al., 2002).

Our studies have allowed us to identify new members of the *repABC* family in four large plasmids (pKLW1, pWKS2, pHG16-a, and pPAN1), residing in different strains of *P. pantotrophus*. A comparative analysis of these replicons showed that they comprise (together with analogous replicon pTAV320 of pTAV1) a distinct evolutionary group of *repABC* replicons. This is the first such group to be identified outside the Rhizobiaceae.

As shown by the performed analysis, all the incompatibility determinants of pTAV320 express incompatibility towards *repABC* replicons of pKLW1 and pHG16-a (included in mini-replicons pKLW200 and pHGW200, respectively). The nucleotide sequences of the *inc* regions of these plasmids demonstrate a high degree of identity.

Interestingly, the sequence of the *repC* gene of pHG16-a and pKLW1 is conserved to a lesser degree than in the case of *repABC* replicon of pPAN1, which is compatible with pTAV320. Turner et al. (1996) proposed that the distinct *repC* groups of *repABC* replicons might correspond to different plasmid incompatibility classes. Based on this assumption, *repABC* plasmids of *Paracoccus* spp. should be classified to the same incompatibility group. However, our results indicate that incompatibility among *repABC* replicons is a far more complex phenomenon and that all suppositions regarding this subject should be preceded by detailed sequence analysis of *inc* determinants.

Although the role of the conserved *igs* in *repABC* replicons is still puzzling, the *inc2* was shown to carry a *cis* required partitioning site, which together with *repA* and *repB* constitutes a functional stabilizing system. We have previously shown the ability of the partitioning protein RepB of pTAV320 to bind to two short repeated sequences (R1 and R2), adjacent to the terminal part of the *repC* gene (*inc2*; Fig. 4B). The R1 and R2 sequences show similarities to the partitioning sites of several bacterial chromosomes, e.g., *Bacillus subtilis* (Bartosik et al., 2001a). Such repeats were not found within rhizobial *repABC*-type sequences but are highly conserved in the paracoccal composite plasmids and in a *repABC* replicon that is present in plasmid pRS241d of *R. sphaeroides*. The presence of these sequences may constitute an important element, allowing the preliminary distinguishing of non-rhizobial *repABC*-type replicons. The results obtained in this study indicate, however, that the sequence of the gene *repC*, which is the strongest conserved area in all of the studied replicons (including pRS241d), can be used as a specific probe allowing the identification of such replicons.

All of the studied plasmids of *P. pantotrophus* proved to be composite ones coding two different replication systems (types *repABC* and *repC'*) homologous to the replication systems of *P. versutus* plasmid pTAV1 that were characterized earlier (Bartosik et al., 1997b, 1998). Our more detailed analysis revealed that plasmid pRS241d also contains a sequence homologous to the replicator region of pTAV203 (*repC'*-type replicon of pTAV1). This finding strongly suggests that pRS241d is also a composite plasmid of the pTAV1-type. It thus seems probable that these plasmids have derived from a common ancestor. To confirm this assumption, we plan to perform a hybridization analysis involving pRS241d (probe) and pTAV1-like plasmids of *Paracoccus* spp., followed by the sequencing of selected fragments of the studied plasmids. We believe that the complete sequence of pRS241d will be the starting point for more convenient comparative analyses which, besides providing knowledge on the structure of a group of related plasmids, will allow us to draw conclusions on the evolution and variability of plasmid genomes.

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References

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

Bartosik, D., Bialkowska, A., Baj, J., Wlodarczyk, M., 1997a. Construction of mobilizable cloning vectors derived from pBGS18 and their application for analysis of replicator region of a pTAV203 mini-derivative of *Paracoccus versutus* pTAV1 plasmid. Acta Microbiol. Pol. 46, 379–383.

Bartosik, D., Wlodarczyk, M., Thomas, C.M., 1997b. Complete nucleotide sequence of the replicator region of *Paracoccus* (*Thiobacillus*) *versutus* pTAV1 plasmid and its correlation to several plasmids of *Agrobacterium* and *Rhizobium* species. Plasmid 38, 53–59.

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

Bartosik, D., Baj, J., Wlodarczyk, M., 1998. Molecular and functional analysis of pTAV320—*repABC* type replicon of a composite pTAV1 plasmid of *Paracoccus versutus*. Microbiology 144, 3149–3157.

Bartosik, D., Szymanik, M., Wysocka, E., 2001a. Identification of the partitioning site within the *repABC*-type replicon of the composite *Paracoccus*

versutus plasmid pTAV1. J. Bacteriol. 183, 6234–6243.

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

Bednarska, M., Jagusztyn-Krynicka, E.K., Popowski, J., Wlodarczyk, M., 1983. Extrachromosomal DNA in *Thiobacillus versutus*. FEMS Microbiol. Lett. 16, 183–185.

Birnboim, H.C., Doly, J., 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7, 1513–1519.

Devereux, J., Haeberli, P., Smithies, O., 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 11, 387–395.

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

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

Felsenstein, J., 1989. PHYLIP, phylogenetic inference package (version 3.2). Cladistics 5, 164–166.

Gerdes, K., Moller-Jensen, J., Jensen, R.B., 2000. Plasmid and chromosome partitioning: surprises from phylogeny. Mol. Microbiol. 37, 455–466.

Gerstenberg, C., Friedrich, B., Schlegel, H.G., 1982. Physical evidence for plasmids in autotrophic, especially hydrogen-oxidizing bacteria. Arch. Microbiol. 133, 90–96.

Jordan, S.L., McDonald, I.R., Kraczkiewicz-Dowjat, A.J., Kelly, D.P., Rainey, F.A., Murrell, J.C., Wood, A.P., 1997. Autotrophic growth on carbon disulfide is a property of novel strains of *Paracoccus denitrificans*. Arch. Microbiol. 168, 225–236.

Katayama, Y., Hirashi, A., Kuraishi, H., 1995. *Paracoccus thiocyanatus* sp. nov., a new species of thiocyanate-utilizing facultative chemolithotroph, and transfer of *Thiobacillus versutus* to the genus *Paracoccus* as *Paracoccus versutus* comb. nov. with emendation of the genus. Microbiology 141, 1469–1477.

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

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

Li, P.-L., Farrand, S.K., 2000. The replicator region of the nopaline-type Ti plasmid pTiC58 is a member of the *repABC* family and is influenced by the TraR-dependent quorum-sensing regulatory system. J. Bacteriol. 182, 179–188.

Lipski, A., Reichert, K., Reuter, B., Spröer, C., Altendorf, K., 1998. Identification of bacterial isolates from biofilters as *Paracoccus alkenifer* sp. nov. and *Paracoccus solventivorans* with emended description of *Paracoccus solventivorans*. Int. J. Syst. Bacteriol. 48, 529–536.

Nishiguchi, R., Takanami, M., Oka, A., 1987. Characterization and sequence determination of the replicator region in the hairy-root-inducing plasmid pRiA4b. Mol. Gen. Genet. 206, 1–8.

Ohara, M., Katayama, Y., Tsuzaki, M., Nakamoto, S., Kuraishi, H., 1990. *Paracoccus kocurii* sp. nov., a tetramethylammonium-assimilating bacterium. Int. J. Syst. Bacteriol. 40, 292–296.

Palmer, K.M., Turner, S.L., Young, J.P.W., 2000. Sequence diversity of the plasmid replication gene *repC* in the *Rhizobiaceae*. Plasmid 44, 209–219.

Rainey, F.A., Kelly, D.P., Stackebrandt, E., Burghardt, J., Hiraishi, A., Katayama, Y., Wood, A.P., 1999. A re-evaluation of the taxonomy of *Paracoccus denitrificans* and a proposal for the combination *Paracoccus pantotrophus* comb. nov. Int. J. Syst. Bacteriol. 49, 645–651.

Ramírez-Romero, M.A., Tellez-Sosa, J., Barrios, H., Perez-Oseguera, A., Rosas, V., Cevallos, M.A., 2001. RepA negatively autoregulates the transcription of the *repABC* operon of the *Rhizobium etli* symbiotic plasmid basic replicon. Mol. Microbiol. 42, 195–204.

Ramírez-Romero, M.A., Soberón, N., Pérez-Oseguera, A., Téllez-Sosa, J., Cevallos, M.A., 2000. Structural elements required for replication and incompatibility of the *Rhizobium etli* symbiotic plasmid. J. Bacteriol. 182, 3117–3124.

Ramírez-Romero, M.A., Bustos, P., Girard, L., Rodríguez, O., Cevallos, M.A., Dávila, G., 1997. Sequence, localization and characteristics of the replicator region of the symbiotic plasmid of *Rhizobium etli*. Microbiology 143, 2825–2831.

Rigottier-Gois, L., Turner, S., Young, J.P.W., Amarger, N., 1998. Distribution of *repC* plasmid-replication sequences among plasmids and isolates of *Rhizobium leguminosarum* bv. *viciae* from field populations. Microbiology 144, 771–780.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Siller, H., Rainey, F.A., Stackebrandt, E., Winter, J., 1996. Isolation and characterization of a new gram-negative, acetone-degrading, nitrate-reducing bacterium from soil, *Paracoccus solventivorans* sp. nov. Int. J. Syst. Bacteriol. 46, 1125–1130.

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

Tabata, S., Hooykaas, P.J.J., Oka, A., 1989. Sequence determinationand characterization of the replicator region in the tumor inducing plasmid pTiB6S3. J. Bacteriol. 171, 1665–1672.

Taylor, B.F., Hoare, D.S., 1969. New facultative *Thiobacillus* and re-evaluation of the heterotrophic potential of *Thiobacillus novellus*. J. Bacteriol. 100, 487–497.

Turner, S., Young, J.P.W., 1995. The replicator region of the *Rhizobium leguminosarum* cryptic plasmid pRL8JI. FEMS Microbiol. Lett. 133, 53–58.

Turner, S.L., Rigottier-Gois, L., Power, R.S., Amarger, N., Young, J.P.W., 1996. Diversity of *repC* plasmid-replication sequences in *Rhizobium leguminosarum*. Microbiology 142, 1705–1713.

Urakami, T., Araki, H., Oyanagi, H., Suzuki, K., Komagata, K., 1990. *Paracoccus aminophilus* sp. nov. and *Paracoccus aminovorans* sp. nov., which utilize *N*,*N*-dimethylformamide. Int. J. Syst. Bacteriol. 40, 287–291.

Urakami, T., Tamaoka, J., Suzuki, K., Komagata, K., 1989. *Paracoccus alcaliphilus* sp. nov., an alkaliphilic and facultatively methylotrophic bacterium. Int. J. Syst. Bacteriol. 39, 116–121.

Vieira, J., Messing, J., 1982. The pUC plasmids, and M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19, 259–268.

Williams, R., Macartney, D.P., Thomas, C.M., 1998. The partitioning activity of the RK2 central control region requires only *incC*, *korB* and KorB-binding site O_B3 but other KorB-binding sites form destabilizing complexes in the absence of O_B3. Microbiology 144, 3369–3378.

Wlodarczyk, M., Jagusztyn-Krynicka, E.K., Bartosik, D., Kalinowska, I., 1994. Electroporation of *Thiobacillus versutus* with plasmid DNA. Acta Microbiol. Pol. 43, 223–227.

Wood, D.W., Setubal, J.C., Kaul, R., Monks, D.E., Kitajima, J.P., Okura, V.K., Zhou, Y., Chen, L., Wood, G.E., Almeida Jr., N.F., Woo, L., Chen, Y., Paulsen, I.T., Eisen, J.A., Karp, P.D., Bovee, D. Sr, Chapman, P., Clendenning, J., Deatherage, G., Gillet, W., Grant, C., Kutyavin, T., Levy, R., Li, M.J., McClelland, E., Palmieri, A., Raymond, C., Rouse, G., Saenphimmachak, C., Wu, Z., Romero, P., Gordon, D., Zhang, S., Yoo, H., Tao, Y., Biddle, P., Jung, M., Krespan, W., Perry, M., Gordon-Kamm, B., Liao, L., Kim, S., Hendrick, C., Zhao, Z.Y., Dolan, M., Chumley, F., Tingey, S.V., Tomb, J.F., Gordon, M.P., Olson, M.V., Nester, E.W., 2001. The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. Science 294, 2317–2323.

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