Molecular characterization of functional modules of plasmid pWKS1 of *Paracoccus pantotrophus* DSM 11072

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The complete nucleotide sequence of the small, cryptic plasmid pWKS1 (2697 bp) of *Paracoccus pantotrophus* DSM 11072 was determined. The G+C content of the sequence of this plasmid was 62 mol%. Analysis revealed that over 80% of the plasmid genome was covered by two ORFs, ORF1 and ORF2, which were capable of encoding putative peptides of 44 kDa and 37-8 kDa, respectively. Mutational analysis showed that ORF2 was crucial for plasmid replication. The translational product of ORF2 shared local homologies with replication proteins of several \( \theta \)-replicating lactococcal plasmids, as well as with the Rep proteins of plasmids residing in Gram-negative hosts. An A+T-rich region, located upstream of the rep gene and containing three tandemly repeated 21 bp long iteron-like sequences, served as the origin of replication (oriV). ORF1 encoded a putative mobilization protein with similarities to mobilization proteins (Mob) from the broad-host-range plasmid pBBR1 and plasmids of Gram-positive bacteria. A plasmid bearing the MOB module of pWKS1 (the mob gene and the oriT sequence) could be mobilized for transfer (by IncP RP4 transfer apparatus) at low frequency between different strains of *Escherichia coli*. MOB modules of pWKS1 and pBBR1 were functionally complementary to each other. Hybridization analysis revealed that only plasmid pSOV1 (6-5 kb), among all of the paracoccal plasmids identified so far, carries sequences related to pWKS1. Plasmid pWKS1 could replicate in 10 species of *Paracoccus* and in *Agrobacterium tumefaciens*, *Rhizobium leguminosarum* and *Rhodobacter sphaeroides*, but it could not replicate in *E. coli*.

**Keywords**: plasmid replication, mobilization

**INTRODUCTION**

The genus *Paracoccus*, currently embracing 14 described species (Baj, 2000; Kelly *et al*., 2000), belongs to the \( \alpha \)-Proteobacteria. Paracocci are very interesting organisms because of their versatile metabolism. They are chemooorganotrophs or facultative chemolithoautotrophs, which oxidize reduced sulphur compounds as well as molecular hydrogen. They can also grow methylothrophically. Moreover, most species of *Paracoccus* can use nitrate as an alternative electron acceptor. Some species belonging to this genus have been isolated from polluted soil, water or sewage purification units. All of these features indicate the potential application of paracocci to bioremediation. So far, very little is known about the genomic localization (chromosomes, megaplasmids or plasmids) and genetic structure of the loci responsible for the majority of the versatile metabolic traits of paracocci.

The increasing interest in bacteria belonging to the genus *Paracoccus* is paralleled by an increasing demand for appropriate tools (e.g. suitable advanced cloning vectors) that would facilitate the genetic analysis of these bacteria. With the construction of an optimal vector for *Paracoccus* spp. in mind, an analysis of the plasmid content of paracoccal strains has been conducted by Baj *et al*. (2000). A study of strains repre-
Table 1. Bacterial strains and plasmids used in this study

<table>
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<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics*</th>
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<tr>
<td><strong>Strain</strong></td>
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<tr>
<td><em>Paracoccus pantotrophus</em> DSM 11072</td>
<td>Host strain of pWKS1 (2-7 kb) and pWKS3 (&gt;400 kb)</td>
<td>Jordan et al. (1997)</td>
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<tr>
<td><em>Paracoccus pantotrophus</em> DSM 11072R</td>
<td>Riff derivative of DSM 11072</td>
<td>This work</td>
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<tr>
<td><em>Paracoccus pantotrophus</em> UWPI</td>
<td>Derivative of DSM 11072R deprived of its natural plasmid, pWKS1</td>
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<tr>
<td><em>Paracoccus pantotrophus</em> KL100</td>
<td>Riff derivative of DSM 11073 deprived of its natural plasmid, pKLW1, contains pKLW2 (&gt;400 kb)</td>
<td>Bartosik et al. (2002)</td>
</tr>
<tr>
<td><em>Paracoccus pantotrophus</em> DSM 65R</td>
<td>Riff derivative of DSM 65; contains pHG16-a (approx. 70 kb) and pHG16-b (&gt;400 kb)</td>
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<td><em>Paracoccus pantotrophus</em> LMD 82.5R</td>
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<tr>
<td><em>Paracoccus alcaliphilus</em> JCM 7364R</td>
<td>Riff derivative of JCM 7364; contains pALC1 (approx. 70 kb)</td>
<td>Bartosik et al. (2002)</td>
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<td><em>Paracoccus alkenifer</em> JCM 7364R</td>
<td>Riff derivative of JCM 7364; no plasmids detected</td>
<td>Bartosik et al. (2001b)</td>
</tr>
<tr>
<td><em>Paracoccus aminophilus</em> JCM 7686R</td>
<td>Riff derivative of JCM 7686; contains pAM1 (6 kb), pAM12 (17 kb), pAM11 (100 kb) and pAM14 (&gt;400 kb)</td>
<td>Bartosik et al. (2002)</td>
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<td><em>Paracoccus aminovorans</em> JCM 7685R</td>
<td>Riff derivative of JCM 7685; contains pAMV2 (4 kb), pAMV1 (&gt;100 kb) and pAMV3 (&gt;400 kb)</td>
<td>Bartosik et al. (2002)</td>
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<tr>
<td><em>Paracoccus denitrificans</em> LMD 22.21R</td>
<td>Riff derivative of LMD 22.21; contains pDEN1 (approx. 40 kb)</td>
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<td><em>Paracoccus denitrificans</em> DSM 413</td>
<td>Contains plasmid pHG18 (approx. 400 kb)</td>
<td>Rainey et al. (1999)</td>
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<td><em>Paracoccus kocurii</em> CCM 4331</td>
<td>Contains pKOC1 (approx. 40 kb)</td>
<td>Ohara et al. (1990)</td>
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<tr>
<td><em>Paracoccus methylutens</em> DM 12R</td>
<td>Riff derivative of DM 12; contains pMTH1 (approx. 40 kb), pMTH2 (approx. 100 kb) and pMTH3 (&gt;400 kb)</td>
<td>Bartosik et al. (2002)</td>
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<td><em>Paracoccus solventivorans</em> DSM 11592R</td>
<td>Riff derivative of DSM 11592; contains pSOV1 (5-6 kb)</td>
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<td><em>Paracoccus solventivorans</em> DSM 6637R</td>
<td>Riff derivative of DSM 6637; contains pSOS1 (approx. 70 kb)</td>
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<td><em>Paracoccus thiocyanatus</em> IAM 12816R</td>
<td>Riff derivative of IAM 12816; contains pTH1 (≥40 kb), pTH2 (&gt;100 kb) and pTH3 (&gt;400 kb)</td>
<td>Bartosik et al. (2002)</td>
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<td><em>Paracoccus versutus</em> UW1R</td>
<td>Riff derivative of wild-type UW1 strain (first described as <em>Thiobacillus</em> sp. strain A2); contains pTAV1 (107 kb), pTAV3 (&gt;400 kb) and pTAV4 (&gt;400 kb)</td>
<td>Bartosik et al. (2002)</td>
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<td><em>Agrobacterium tumefaciens</em> LBA 1010</td>
<td>Riff*</td>
<td>Koekman et al. (1982)</td>
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<td><em>Rhizobium leguminosarum</em> 1062</td>
<td>Str*</td>
<td>Hirsch et al. (1980)</td>
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<tr>
<td><em>Rhodobacter sphaeroides</em> 2.4.1R</td>
<td>Riff derivative of strain 2.4.1</td>
<td>This work</td>
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<td><em>Escherichia coli</em> TG1</td>
<td>Host strain for recombinant plasmids</td>
<td>Sambrook et al. (1989)</td>
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<td><em>Escherichia coli</em> DH5α</td>
<td>Host strain for helper plasmid pKK2013</td>
<td>Hanahan (1983)</td>
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<tr>
<td><em>Escherichia coli</em> DH5R</td>
<td>Riff derivative of DH5α</td>
<td>This work</td>
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<tr>
<td><em>Escherichia coli</em> S17-1</td>
<td>RP4 transfer genes integrated into the chromosome</td>
<td>Simon et al. (1983)</td>
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<td><strong>Plasmid</strong></td>
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<td>pWKS1</td>
<td>2.7 kb, cryptic, natural plasmid of <em>P. pantotrophus</em> DSM 11072</td>
<td>Baj et al. (2000)</td>
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<td>pWKS10</td>
<td>Km', 6-4 kb, shuttle plasmid composed of <em>E. coli</em>-specific vector pBG518 and PstI linearized pWKS1</td>
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<td>pWKS12</td>
<td>Km', 6-4 kb, shuttle plasmid composed of <em>E. coli</em>-specific vector pBG518 and EcoRI linearized pWKS1</td>
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<td>pWKS18</td>
<td>Km', 4 kb, pWKS1 derivative containing Km' cassette (from pUC4K) cloned into unique PstI site</td>
<td>This work</td>
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<td>pWKS19</td>
<td>Km', 4 kb, pWKS1 derivative containing Km' cassette (from pUC4K) cloned into unique EcoRI site</td>
<td>This work</td>
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<tr>
<td>pWKS20</td>
<td>Km', 7-3 kb, mobilizable shuttle plasmid composed of pABW1 and PstI linearized plasmid pWKS1</td>
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senting 11 paracoccal species revealed the presence of over 30 plasmids, including megaplasmids (Baj et al., 2000). So far, only three Paracoccus-carried plasmids have been studied in great detail – pTAV1 (107 kb; Bartosik et al., 1998, 2001a) and pTAV3 (approx. 400 kb; Bartosik et al., 2002) of Paracoccus versutus, and pALC1 of Paracoccus alcaliphilus (70 kb; Bartosik et al., 2001b). However, the mini-replicons of these three plasmids are not suitable for vector construction.

Our attention was drawn to the small multicopy plasmid pWKS1 harbouré by Paracoccus pantotrophus DSM 11072 that is capable of utilizing carbon disulphide (Jordan et al., 1997). In this study, we present an analysis of the genetic structure of pWKS1. Two functional modules of the plasmid were distinguished that are responsible for its replication and mobilization. These modules show significant similarity to analogous cassettes found in different replicon combinations in plasmids residing in Gram-positive and Gram-negative bacteria.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** All of the bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria–Bertani (LB) medium (Sambrook et al., 1989) at 30 °C (all paracoccal strains, Agrobacterium tumefaciens and Rhodobacter sphaeroides) or 37 °C (Escherichia coli) and in TY medium (Beringer, 1974) at 30 °C (Rhizobium leguminosarum). Antibiotics included in media were used at the following final concentrations (µg ml⁻¹): kanamycin (Kn), 50; rifampicin (Rif), 50; streptomycin, 400 for Rhizobium leguminosarum; tetracycline, 3 for P. pantotrophus and P. versutus, and 20 for E. coli. All Rif-resistant derivatives of the strains used in this work were obtained as spontaneous mutants selected on LB agar supplemented with Rif.

**DNA sequencing and sequence analysis.** To determine the complete nucleotide sequence of pWKS1, the plasmid was digested with *EcoRI* or *EcoRI* and then cloned into the pBGS18 vector; the derived plasmids were designated pWKS10 and pWKS12, respectively. The derived plasmids were then sequenced on both strands using an automated DNA sequencer (model 377; Perkin Elmer). Sequence analysis was done by using programs included within the GCG Package (Wisconsin Genetics Computer Group Sequence Analysis Software Package, version 8.1). Comparison searches through the databases were performed by using the BLAST program (Altschul et al., 1990) provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST).

**DNA manipulations.** Plasmid DNA was isolated according to Birnboim & Doly (1979), and if required purified by CsCl/ethidium bromide gradient centrifugation. Other molecular biological procedures were done as described by Sambrook et al. (1989). All enzymes were purchased from either Promega or Roche. DNA restriction fragments were recovered from agarose gels by using the DNA Gel-Out Kit (DNA Gdansk). For Southern hybridization (Sambrook et al., 1989), DNA probes were labelled with digoxigenin (Roche). Hybridization and visualization of the hybridization products was done as recommended by the supplier.

**Electroporation and transformation.** Electroporation was carried out at 2500 V, 25 µf and 200 ° (for E. coli) or 400 ° (for P. pantotrophus KL100) in a gene pulser apparatus (Bio-Rad), according to the modified Bio-Rad procedure (Wlodarczyk et al., 1994). Electrotrotransforms were selected on solidified LB medium supplemented with the appropriate antibiotic. Competent cells of E. coli TG1 were prepared and transformed as described by Kushner (1978).

**Mating.** The overnight cultures were spun down and washed twice to remove antibiotics. For biparental mating, the donor strain E. coli TG1 (carrying a mobilizable vector), a suitable recipient strain and E. coli DH5α(pRK2013) were mixed at a ratio of 1:2:1. An aliquot (100 µl) of this mixture was spread onto TY or LB agar, depending on the recipient strain. After overnight incubation of the plates at 30 °C, the bacteria were washed off of the plates and suitable dilutions of the cultures were plated onto selective media containing Rif or streptomycin. Km had also been added to the media. Rif and streptomycin were selective markers for the recipient strains; Km had to be added to the media to select for transconjugants. Diparental matings were made with E. coli S17-1 carrying a mobilizable plasmid (as a donor) and a suitable recipient strain of E. coli or Paracoccus sp. The strains were mixed at a ratio of 1:2 and plated onto LB agar. Transconjugants were selected as described above. The plasmid pattern

<table>
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<th>Strain/plasmid</th>
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<tr>
<td>pWKS21</td>
<td>Km', 6-4 kb, derivative of pBGS18 containing SmaI linearized pWKS1</td>
<td>This work</td>
</tr>
<tr>
<td>pWKS24</td>
<td>Km', 4-9 kb, derivative of pABW1 containing 380 bp SmaAI fragment (with oriV) of pWKS1</td>
<td>This work</td>
</tr>
<tr>
<td>pWKS25</td>
<td>Km', 5-5 kb, derivative of pBGS18 containing 1 kb EcoRI–PstI restriction fragment of pWKS1</td>
<td>This work</td>
</tr>
<tr>
<td>pABW1</td>
<td>Km', mobilizable cloning vector based on pBGS18, oriT of RK2</td>
<td>Bartosik et al. (1997)</td>
</tr>
<tr>
<td>pBGS18</td>
<td>Km', 3-7 kb, cloning vector, ColEl oriV</td>
<td>Spratt et al. (1986)</td>
</tr>
<tr>
<td>pBRR1MCS</td>
<td>Cm', 47 kb, cloning vector based on pBRR1</td>
<td>Kovach et al. (1994)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Km', helper plasmid carrying RK2 tra genes</td>
<td>Ditta et al. (1980)</td>
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* Str', streptomycin-resistant; Cm', chloramphenicol-resistant.
of the transconjugants was verified by screening several colonies by using a rapid alkaline extraction procedure and agarose-gel electrophoresis. Spontaneous resistance of the recipient strains to the selective markers was undetectable under these experimental conditions.

**Plasmid stability.** The stability of plasmids during growth under non-selective conditions was tested as previously described (Bartosik *et al*., 1998). Briefly, stationary-phase cultures were diluted in fresh medium without antibiotic selection and cultivated for approximately 10, 20 and 30 generations. Samples taken at these times were diluted and plated onto solid medium in the absence of selective drugs. Two-hundred colonies were tested with the use of a Km-resistance marker by replica plating. The retention of plasmids after approximately 30 generations was defined as the percentage of Km-resistant colonies.

**RESULTS AND DISCUSSION**

**Nucleotide sequence of pWKS1**

The complete nucleotide sequence of pWKS1 was determined (accession no. AF482428). The G+C content of this plasmid was found to be 62 mol%, which is slightly lower than the G+C content of the total DNA of *P. pantotrophus* (64–68 mol%) (Rainey *et al*., 1999). No significant DNA sequence similarity was found between pWKS1 and any of the nucleotide sequences held within the databases. Computer analysis of the sequence of pWKS1 (2697 nt) revealed the presence of two major ORFs (ORF1 and ORF2), which were in the same transcriptional orientation (Fig. 1a) and together covered 82% of the plasmid genome.

ORF1 (mapped at positions 7–1194; Fig. 1a) encodes a 395 aa polypeptide with a predicted molecular mass of 44-1 kDa and a pi value of 7.98. The transcriptional start codon of ORF1 (ATG) is preceded (7 bp) by a potential ribosome-binding site (RBS) AGG (Shine & Dalgarno, 1975). A BLAST comparison revealed that the ORF1 translational product showed the highest sequence similarity to a Mob protein involved in mobilization of the broad-host-range plasmid pBBR1 (46% similarity and 37% identity) from *Bordetella bronchiseptica* (Antoine & Locht, 1992). Similarities were also observed between the ORF1 product and the Mob proteins of

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**Fig. 1.** Genetic organization of pWKS1. (a) Circular map of pWKS1. The unique EcoRI site was arbitrarily assigned as coordinate 1. Only the unique restriction sites used for the construction of plasmid derivatives are given on the pWKS1 restriction map. The arrows indicate two ORFs, designated ORF1 (mob) and ORF2 (rep), and their transcriptional orientation. The positions of oriV and oriT are also indicated. (b) Nucleotide sequence of the A+T-rich intergenic region containing oriV of pWKS1. Several short repeated sequences identified within this region are indicated by different lines. Three long iteron-like repeated sequences are marked with thick lines and are designated DR1–DR3. Numbers on the right-hand side of the sequence refer to the nucleotide sequence of pWKS1 deposited within GenBank.
Fig. 2. Alignment of the deduced amino-acid sequences of the Mob proteins from several plasmids. pWK1 is shown in bold. The other plasmids included in this alignment were: pBBR1 from *Bordetella bronchiseptica* (accession no. S25246); pTS1 from *T. denticola* (accession no. NP_073756); pCC7120δ from *Anabaena* sp. PCC 7120 (Kaneko et al., 2001) and pFL1 from *Flavobacterium* sp. KP1 (Ashiuchi et al., 1999), and between the ORF1 product and the *Mob*} Pre protein family (recombinases involved in plasmid mobilization} recombination processes) encoded by many plasmids found in Gram-positive hosts (Fig. 2). The highest similarities between the translational product of ORF1 and other proteins were observed for the *Mob* proteins of plasmids p1414 (37% similarity and 25% identity) (Thorsted et al., 1999), pTA1015 and pTA1040 from *Bacillus subtilis* (Meijer et al., 1998), the *Mob* protein of pMV158 from *Streptococcus agalactiae* (Priebe & Lacks, 1989) and the Pre protein of pT181 from *Staphylococcus aureus* (Gennaro et al., 1987). The ORF1 product was also similar to the mobilization protein *BmpH* of the small mobilizable transposon Tn5520 from *Bacteroides fragilis* (Vedantam et al., 1999). A sequence comparison of various *Mob* proteins of plasmids residing in Gram-positive bacteria showed that the highest similarities in these proteins were observed within two regions (termed A and B) located in their N-terminal parts (Guzman & Espinosa, 1997). These conserved sequences correspond to the catalytic domains previously described by Koonin & Ilyina (1993) and Pansegrau et al. (1994). As shown in Fig. 2, regions analogous to those described by Guzman & Espinosa (1997) can also be distinguished in the N-terminal part of the *Mob* protein of pBBR1 and the ORF1 product of pWK1. In their studies on the mobilization mechanism of pBBR1, Szipier et al. (2001) identified two amino acids (aspartate and glutamate) shown to be crucial for the mobilization of pBBR1 (Szipier et al., 2001) are indicated by vertical arrows. The sequences of pCC7120δ and pMV158 have been truncated at their C termini.
necessary for the activity of this protein. These residues are also conserved in the ORF1 product of pWKS1 and in sequences derived from other plasmids (Fig. 2).

The second ORF (ORF2) of pWKS1, located between nucleotides 1444 and 2463 (250 bp downstream of ORF1), encodes a 339 aa polypeptide with a predicted molecular mass of 37.8 kDa and a pI value of 9.51. The start codon of ORF2 (ATG) is not preceded by a typical sequence resembling a consensus RBS. Searches done using the BLASTP program demonstrated that the putative product of ORF2 showed local similarity with replication proteins of many \( \theta \)-replicating plasmids from Lactococcus lactis, e.g. pFV1201, pJW565 and pJW566 (accession nos CAA65630, CAA65652 and CAA65651, respectively; Gravesen et al., 1997) or pUCL22 (accession no. S40058; Frere et al., 1993), with the Rep proteins of plasmid pOM1 from Francisella tularensis (accession no. NP_052243; Pomerantsev et al., 2001) and with plasmid pFA3 of Neisseria gonorrhoeae (accession no. A35257; Gilbride & Brunton, 1990). The similarities between the ORF2 product and these proteins were located mainly in the central part of the proteins (data not shown). Analogous similarities between different Rep proteins of various plasmids from Gram-positive and Gram-negative hosts have been observed by Gravesen et al. (1997), which suggests that the replication systems of these plasmids [in spite of certain differences in the organization of their origins of replication (ori\( V \))] may be distantly related. However, in the sequence of the ORF2-encoded protein, we could neither find an obvious DNA-binding domain (helix–turn–helix motif) nor a leucin-zipper motif, which are typical for many Rep proteins of \( \theta \)-replicating plasmids (del Solar et al., 1998).

We did not identify regions matching the consensus promoter sequence of E. coli (Greener et al., 1992) upstream of ORF1 and ORF2 of pWKS1. Also, we did not find (downstream of the two ORFs) any sequences resembling \( \rho \)-independent transcriptional terminators. However, we did localize two 17 bp long inverted-repeat sequences (mapped at positions 2548–2591, and separated by 10 bp) 85 bp downstream of ORF2 that were able to form a potential stem–loop structure (data not shown).

In summary, the data obtained from sequence analysis suggest the presence of two modules responsible for (i) replication (REP) and (ii) mobilization for conjugative transfer (MOB) in pWKS1. Such modules are commonly found, in different combinations, within many small multicopy plasmids residing in Gram-positive bacteria, as those in Lactobacillus spp. (Bates & Gilbert, 1989), Streptococcus spp. (Priebe & Lacks, 1989), Staphylococcus spp. (Projan & Novick, 1988) and Bacillus spp. (Meier et al., 1992). These modules also occur in some plasmids from Gram-negative hosts, e.g. those from Bordetella bronchiseptica (Antoine & Locht, 1992) and Ruminobacter amylophilus (Ogata et al., 1999). To confirm the presence of such modules in pWKS1, a detailed functional analysis of the plasmid was carried out in a later part of this study.

### Replication of pWKS1

To investigate the role of ORF1 and ORF2 in the replication of pWKS1, mutational analysis was performed. First, ORF1 was mutated by the insertion of a Km-resistance cassette into the unique \( \Psi \) site located within ORF1 and EcoRI (located 5 bp upstream of ORF1) sites of pWKS1, respectively. The EcoRI site is situated between a putative RBS and the start codon of ORF1, therefore a mutation within this site was supposed to inhibit the expression of the ORF1-encoded product. As expected for both cases, the replication and stability of the resulting plasmids pWKS18 (\( \Psi \)1) and pWKS19 (EcoRI) (Table 1), tested in P. pantotrophus KL100, was not affected. Conversely, we were unable to obtain a replicative form of pWKS1 that contained a Km-resistance cassette integrated into the unique Smal site located within ORF2, which might suggest that ORF2 encodes a protein which is crucial for pWKS1 replication. To confirm this hypothesis, we cloned a Smal-digested linear form of pWKS1 (plasmid pWKS21) and a 1.8 kb EcoRI–\( \Psi \) site restriction fragment of pWKS1 (Fig. 1) with complete ORF2 (plasmid pWKS25) into the multiple-cloning site of an E. coli-specific (unable to replicate in P. pantotrophus) vector, pBG518. After the electroporation of pWKS21 and pWKS25 (constructed in E. coli) into P. pantotrophus KL100, we obtained Km-resistant transformants containing only pWKS25. Based on the results of our mutational analysis and sequence similarity data, we conclude that ORF2 encodes the replication initiator protein (Rep) of pWKS1.

Analysis of the structural features of the pWKS1 sequence revealed the presence of an A+T-rich region located within the intergenic region upstream of rep. As shown in Fig. 1(b), this region is highly saturated with directly repeated sequences. The longest repeats (DR1–DR3; Fig. 1b), each of which contains a 21 bp sequence (‘\( 5'\)-AAGTTGGGGAATCCAGCGCAA\( -3'\)”), are tandemly repeated (without any spacer sequences) three times. In many \( \theta \)-replicating plasmids, direct repeats (iterons), located within ori\( V \), are required for the binding of the Rep protein and for the initiation of replication (Chattoraj, 2000). It is conceivable that the DR1–DR3 repeats might play a similar role in pWKS1 replication. However, no sequence corresponding to DnaA or IHF binding sites (also present within the ori\( V \) of many plasmids) were identified within this region.

To check whether the intergenic sequence mentioned above contains ori\( V \), we cloned this region (on a 380 bp Sau3AI fragment of pWKS1) into the BamHI site of an E. coli-specific (non-replicating in P. pantotrophus), mobilizable vector, pABW1. The resulting plasmid (pWKS24) was introduced via triparental mating (in the presence of the helper plasmid pRK2013) into two host strains – P. pantotrophus DSM 11072R(pWKS1) and UW1 (pWKS1-less). Transconjugants were obtained only with strain DSM 11072R, which indicates that the rep gene of pWKS1, when supplied in trans, can support the replication of pWKS24 that lacks a functional copy of its own rep gene. To verify that the Km-resistant transconjugants were not the result of
recombination between pWKS1 and pWKS24, plasmid DNA from several transconjugants was isolated and used to transform E. coli. Ten of the transformants were analysed for their plasmid content and plasmid-restriction pattern. All of these transconjugants contained intact pWKS24. This experiment provides evidence for the localization of oriV within the pWKS1 genome. The stable maintenance of co-residing autonomous forms of pWKS24 and pWKS1 in P. pantotrophus cells (under selective conditions for pWKS24) indicates that the cloned fragment carrying oriV (together with the potential promoter region of rep) does not carry incompatibility determinants that are frequently involved in the regulation of initiation of plasmid replication (del Solar et al., 1998).

Mobilization of pWKS1

The observed similarity of the ORF1 product to Mob proteins suggests the potential role of this protein in the conjugative transfer of pWKS1. The compact structure of pWKS1 (and its lack of convenient restriction sites) precludes the cloning of a selective marker into this plasmid without disruption of the replication or putative mobilization regions. This prevents studies on the functionality of the ORF1 protein in the mobilization of pWKS1 between different strains of Paracoccus spp. Consequently, this analysis was performed with strains of E. coli and plasmids pWKS10 and pWKS12 (both carrying an inactivated mob gene), and pWKS21 (with a disrupted rep gene). These plasmids were introduced, respectively, into E. coli S17-1 by transformation (E. coli S17-1 carried transfer genes of the IncP-plasmid RP4 which had been integrated into its chromosome). The Km-resistant transconjugants obtained were used as donors in diparental matings with the recipient strain E. coli DH5R. We observed that only pWKS21 could be mobilized for transfer. The frequency of transfer was, however, relatively low and averaged $2 \times 10^{-5}$ transconjugants (donor cell)$^{-1}$. The presence of pWKS21 in the transconjugants was confirmed as described in Methods. The lack of transconjugants carrying pWKS10 and pWKS12 points to the role of ORF1 (mob) in plasmid mobilization. We can state that the MOB module of pWKS1 enables the transfer of a hybrid plasmid (by donation) between various E. coli strains (in the presence, in trans, of transfer genes of the IncP-plasmid RP4) although the replication system of pWKS1 is not functional in this host (as demonstrated in a later part of this study).

Mobilizable plasmids possess a specific sequence (origin of conjugative transfer, oriT) from which the initiation of conjugative transfer occurs. A key role in this process is played by Mob proteins (relaxases) which mediate cleavage of the phosphodiester bond within the oriT sequence. Analysis of the nucleotide sequence of pWKS1 revealed the presence of a sequence, located upstream of mob, with significant similarity to oriT of pBBR1 and to the recombination site A sequence involved in the mobilization and recombination processes (mediated by Mob/Pre proteins) of several plasmids from Gram-positive organisms, e.g. pMV158, pE194, pT181 and pUB110 (van der Lelie et al., 1989; Gennaro et al., 1987; McKenzie et al., 1986). Sequences analogous to the oriT sequence of pWKS1 were also found in conjugative transposons (Crepin & Rood, 1998). The putative oriT sequence of pWKS1, like other sequences of this type, comprises inverted repeats (Fig. 3). As has already been shown for pBBR1 (Szpirer et al., 2000) and pMV158 (Farias et al., 1999), these inverted repeats are placed in close proximity to the −10 boxes of mob promoters. The nick site determined for the oriT sequence of pBBR1 is also strongly conserved in the potential oriT sequence of pWKS1 (Fig. 3). This analogous localization of nick sites has also been demonstrated in the oriT sequence of pMV158 from Streptococcus agalactiae (Farias & Espinosa, 2000) and in oriT sequence of the mobilizable transposon Tn4555 of Bacteroides sp. (Smith & Parker, 1998).

The visible similarity between the nucleotide sequences of the oriT genes of pWKS1 and pBBR1, as well as the
similarity of the amino-acid sequence motifs of Mob, suggests that the transfer systems of both of these plasmids are very closely related phylogenetically. The conjugative transfer of pBBR1 has been studied intensively (Szpirer et al., 2000, 2001); it has been demonstrated that pBBR1 is mobilized most efficiently by IncP plasmids (e.g. RP4). We decided to check whether the conjugative-transfer systems of pBBR1 and pWKS1 were complementary – i.e. whether Mob of the broad-host-range plasmid pBBR1 was able to interact with oriT of pWKS1. To this end we used pWKS25, a mob-deficient plasmid carrying oriT and the complete replicator region. pWKS25 was introduced into E. coli S17-1(pBBR1MCS), whose pBBR1MCS plasmid carried a compatible chloramphenicol-resistant Mob-positive derivative of pBBR1. As expected, pBBR1MCS was transferred efficiently between E. coli strains, as well as between E. coli and P. pantotrophus KL100 or E. coli and P. versutus UW1R. Interestingly, we also obtained Km-resistant transconjugants that contained pWKS25; this indicates that the plasmid was mobilizable in the constructed system. To exclude the possibility that the transfer of pWKS25 was due to conduction (recombinational fusion and transfer of plasmid co-integrates), 100 Km-resistant transconjugants were plated onto medium containing chloramphenicol. As many as 87% of the examined transconjugant colonies contained only pWKS25 (with this being confirmed by electrophoretic analysis of the plasmid DNA of the transconjugants). The possibility of the mobilization of vector pBGS18 by pBBR1MCS was excluded in a control experiment, as was the transfer of pWKS25 alone (without co-residing pBBR1MCS) between strains of E. coli.

The results obtained indicate that the RP4 transfer system in co-operation with the Mob protein of pBBR1 provides functions needed for conjugative mobilization of the plasmid containing oriT of pWKS1. The visible similarity of the oriT sequences observed between a number of mobilizable plasmids (and transposable elements) and the analogous localization of the strand-specific nick site (Fig. 3) may suggest that trans-mobilization by heterologous Mob proteins, derived from co-residing plasmids, might be common in natural genetic systems. It is worth mentioning that some plasmids do not carry a complete MOB module and only code a sequence similar to oriT/recombination site A, e.g. pA1 from Lactobacillus plantarum A112 (Vujcic & Topisirovic, 1993) or pCI411 from Leunconostoc lactis 533 (Coffey et al., 1994). Thus, the transfer of these plasmids depends fully on the presence in trans of a plasmid(s) that provides both mobilization and conjugation functions.

**Similarity of pWKS1 to other paracoccal plasmids and its host range**

To determine whether pWKS1 carries sequences related to other plasmids from Paracoccus spp., pWKS1 was digoxigenin-labelled and probed against the plasmid profiles of 15 strains of Paracoccus that represented 11 paracoccal species (Table 1). These strains had been shown previously to carry plasmids ranging from 5-6 to over 100 kb in size (Baj et al., 2000). Under the condition of high stringency, hybridization was observed only for pSOV1 (5-6 kb) of Paracoccus solventivorans (data not shown), which suggests that pWKS1 and pSOV1 carry related Mob and/or REP module(s). Also, pWKS1 did not hybridize with the total DNA from the strains tested, suggesting the absence of related sequences within the megaplasmids or chromosomes of these strains.

We did not succeed in introducing the Km-resistant derivatives of pWKS1 (pWKS18 and pWKS19) into E. coli TG1 by electroporation. To allow more detailed studies on the host range of pWKS1, and to overcome the restriction barrier of this plasmid, we constructed a convenient mobilizable hybrid plasmid that was composed of an E. coli-specific, mobilizable pABW1 vector and the replicator region of pWKS1. The resulting plasmid, pWKS20, was introduced by triparental mating into Rif-resistant derivatives of 10 different species belonging to the genus Paracoccus (P. alcaliphilus, Paracoccus alkemifer, Paracoccus amniphilus, Paracoccus aminovorans, Paracoccus denitrificans, Paracoccus methylutens, P. pantotrophus, P. solventivorans, Paracoccus thiocyanatus and P. versutus) as well as into Rhizobium leguminosarum, Agrobacterium tumefaciens and Rhodobacter sphaeroides (all of which belong to the alpha-Proteobacteria). The transfer of pWKS1 into the paracoccal hosts did not result in the loss of their natural plasmids, which suggests that the incoming plasmid was compatible with them.

The small size of pWKS1 and its compatibility with all of the previously studied plasmids of Paracoccus spp. indicates that this plasmid can serve as a base for the construction of vectors specific for this group of bacteria.

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**REFERENCES**


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