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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 44·1 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 θ-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 α-*Proteobacteria*. Paracocci are very interesting organisms because of their versatile metabolism. They are chemoorganotrophs or facultative chemolithoautotrophs, which oxidize reduced sulphur compounds as well as molecular hydrogen. They can also grow methylotrophically. 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-

Abbreviations: Km, kanamycin; *oriT*, origin of conjugative transfer; *oriV*, origin of replication; Rif, rifampicin.

The GenBank accession number for the sequence reported in this paper is AF482428.

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Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics*	Reference/source
Strain		
Paracoccus pantotrophus DSM 11072	Host strain of pWKS1 (2·7 kb) and pWKS3 (>400 kb)	Jordan *et al.* (1997)
Paracoccus pantotrophus DSM 11072R	Rif^r derivative of DSM 11072	This work
Paracoccus pantotrophus UWP1	Derivative of DSM 11072R deprived of its natural plasmid, pWKS1	This work
Paracoccus pantotrophus KL100	Rif^r derivative of DSM 11073 deprived of its natural plasmid, pKLW1; contains pKLW2 (>400 kb)	Bartosik *et al.* (2002)
Paracoccus pantotrophus DSM 65R	Rif^r derivative of DSM 65; contains pHG16-a (approx. 70 kb) and pHG16-b (>400 kb)	This work
Paracoccus pantotrophus LMD 82.5R	Rif^r derivative of LMD 82.5; contains pPAN1 (approx. 100 kb) and pPAN2 (>400 kb)	This work
Paracoccus alcaliphilus JCM 7364R	Rif^r derivative of JCM 7364; contains pALC1 (approx. 70 kb)	Bartosik *et al.* (2002)
Paracoccus alkenifer JCM 7364R	Rif^r derivative of JCM 7364; no plasmids detected	Bartosik *et al.* (2001b)
Paracoccus aminophilus JCM 7686R	Rif^r derivative of JCM 7686; contains pAMI3 (6 kb), pAMI2 (17 kb), pAMI1 (100 kb) and pAMI4 (>400 kb)	Bartosik *et al.* (2002)
Paracoccus aminovorans JCM 7685R	Rif^r derivative of JCM 7685; contains pAMV2 (4 kb), pAMV1 (>100 kb) and pAMV3 (>400 kb)	Bartosik *et al.* (2002)
Paracoccus denitrificans LMD 22.21R	Rif^r derivative of LMD 22.21; contains pDEN1 (approx. 40 kb)	This work
Paracoccus denitrificans DSM 413	Contains plasmid pHG18 (approx. 400 kb)	Rainey *et al.* (1999)
Paracoccus kocurii CCM 4331	Contains pKOC1 (approx. 40 kb)	Ohara *et al.* (1990)
Paracoccus methylutens DM 12R	Rif^r derivative of DM 12; contains pMTH1 (approx. 40 kb), pMTH2 (approx. 100 kb) and pMTH3 (>400 kb)	Bartosik *et al.* (2002)
Paracoccus solventivorans DSM 11592R	Rif^r derivative of DSM 11592; contains pSOV1 (5·6 kb)	This work
Paracoccus solventivorans DSM 6637R	Rif^r derivative of DSM 6637; contains pSOS1 (approx. 70 kb)	This work
Paracoccus thiocyanatus IAM 12816R	Rif^r derivative of IAM 12816; contains pTHI1 (<40 kb), pTHI2 (>100 kb) and pTHI3 (>400 kb)	Bartosik *et al.* (2002)
Paracoccus versutus UW1R	Rif^r derivative of wild-type UW1 strain (first described as *Thiobacillus* sp. strain A2); contains pTAV1 (107 kb), pTAV3 (>400 kb) and pTAV4 (>400 kb)	Bartosik *et al.* (2002)
Agrobacterium tumefaciens LBA 1010	Rif^r	Koekman *et al.* (1982)
Rhizobium leguminosarum 1062	Str^r	Hirsch *et al.* (1980)
Rhodobacter sphaeroides 2.4.1R	Rif^r derivative of strain 2.4.1	This work
Escherichia coli TG1	Host strain for recombinant plasmids	Sambrook *et al.* (1989)
Escherichia coli DH5α	Host strain for helper plasmid pRK2013	Hanahan (1983)
Escherichia coli DH5R	Rif^r derivative of DH5α	This work
Escherichia coli S17-1	RP4 transfer genes integrated into the chromosome	Simon *et al.* (1983)
Plasmid		
pWKS1	2·7 kb, cryptic, natural plasmid of *P. pantotrophus* DSM 11072	Baj *et al.* (2000)
pWKS10	Km^r, 6·4 kb, shuttle plasmid composed of *E. coli*-specific vector pBGS18 and *Pst*I linearized pWKS1	This work
pWKS12	Km^r, 6·4 kb, shuttle plasmid composed of *E. coli*-specific vector pBGS18 and *Eco*RI linearized pWKS1	This work
pWKS18	Km^r, 4 kb, pWKS1 derivative containing Km^r cassette (from pUC4K) cloned into unique *Pst*I site	This work
pWKS19	Km^r, 4 kb, pWKS1 derivative containing Km^r cassette (from pUC4K) cloned into unique *Eco*RI site	This work
pWKS20	Km^r, 7·3 kb, mobilizable shuttle plasmid composed of pABW1 and *Pst*I linearized plasmid pWKS1	This work

Table 1 continued.

Strain/plasmid	Relevant characteristics*	Reference/source
pWKS21	Km^r, 6·4 kb, derivative of pBGS18 containing *Sma*I linearized pWKS1	This work
pWKS24	Km^r, 4·9 kb, derivative of pABW1 containing 380 bp *Sau*3AI fragment (with *oriV*) of pWKS1	This work
pWKS25	Km^r, 5·5 kb, derivative of pBGS18 containing 1·8 kb *Eco*RI–*Pst*I restriction fragment of pWKS1	This work
pABW1	Km^r, mobilizable cloning vector based on pBGS18, *oriT* of RK2	Bartosik *et al.* (1997)
pUC4K	Source of Km-resistance cassette	Vieira & Messing (1982)
pBGS18	Km^r, 3·7 kb, cloning vector, ColE1 *oriV*	Spratt *et al.* (1986)
pBBR1MCS	Cm^r, 4·7 kb, cloning vector based on pBBR1	Kovach *et al.* (1994)
pRK2013	Km^r, helper plasmid carrying RK2 *tra* genes	Ditta *et al.* (1980)

* Str^r, streptomycin-resistant; Cm^r, chloramphenicol-resistant.

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 harboured 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 (Km), 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 *Pst*I or *Eco*RI 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 Ω (for *E. coli*) or 400 Ω (for *P. pantotrophus* KL100) in a gene pulser apparatus (Bio-Rad), according to the modified Bio-Rad procedure (Wlodarczyk *et al.*, 1994). Electrotransformants 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 triparental 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 was 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

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

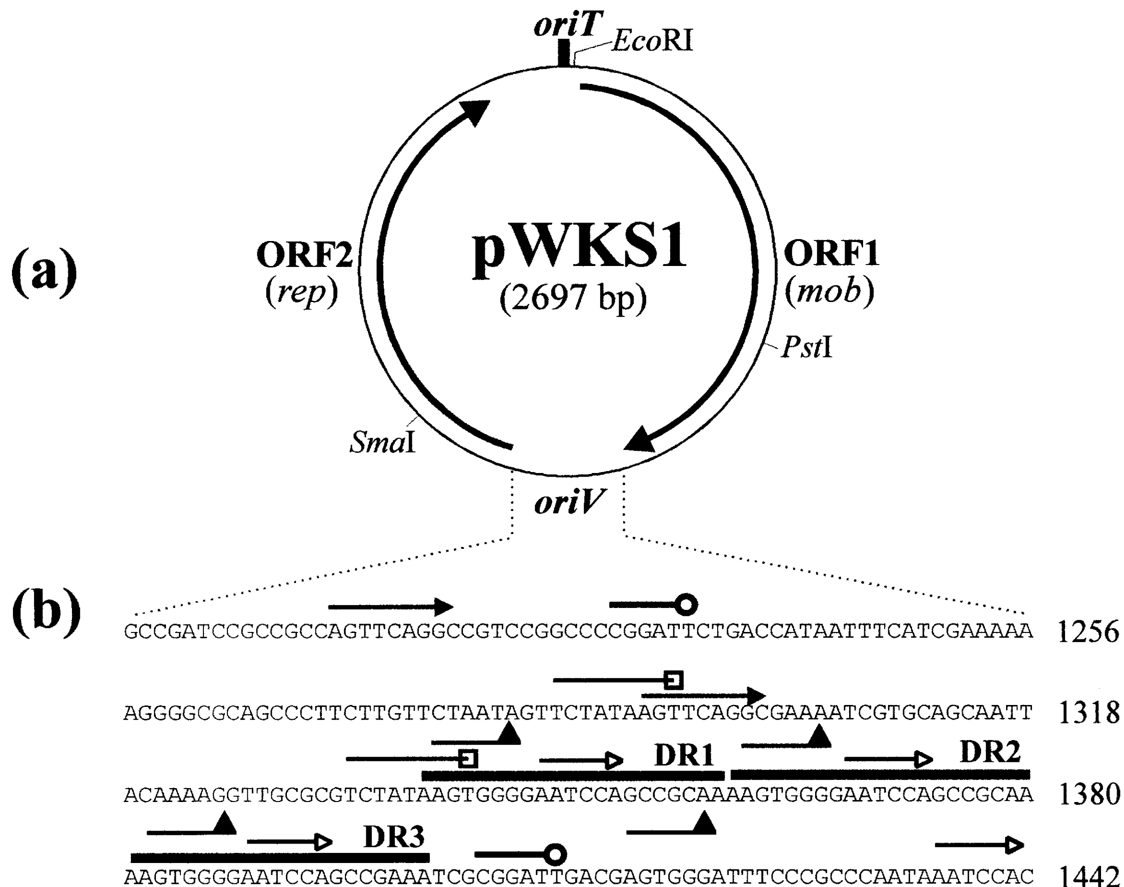

Fig. 1. Genetic organization of pWKS1. (a) Circular map of pWKS1. The unique *Eco*RI site was arbitrarily assigned as co-ordinate 1. Only the unique restriction sites used for the construction of plasmid derivatives are given on the pWKS1 restriction map. The arrows indicate two ORFs, designated ORF1 (*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.

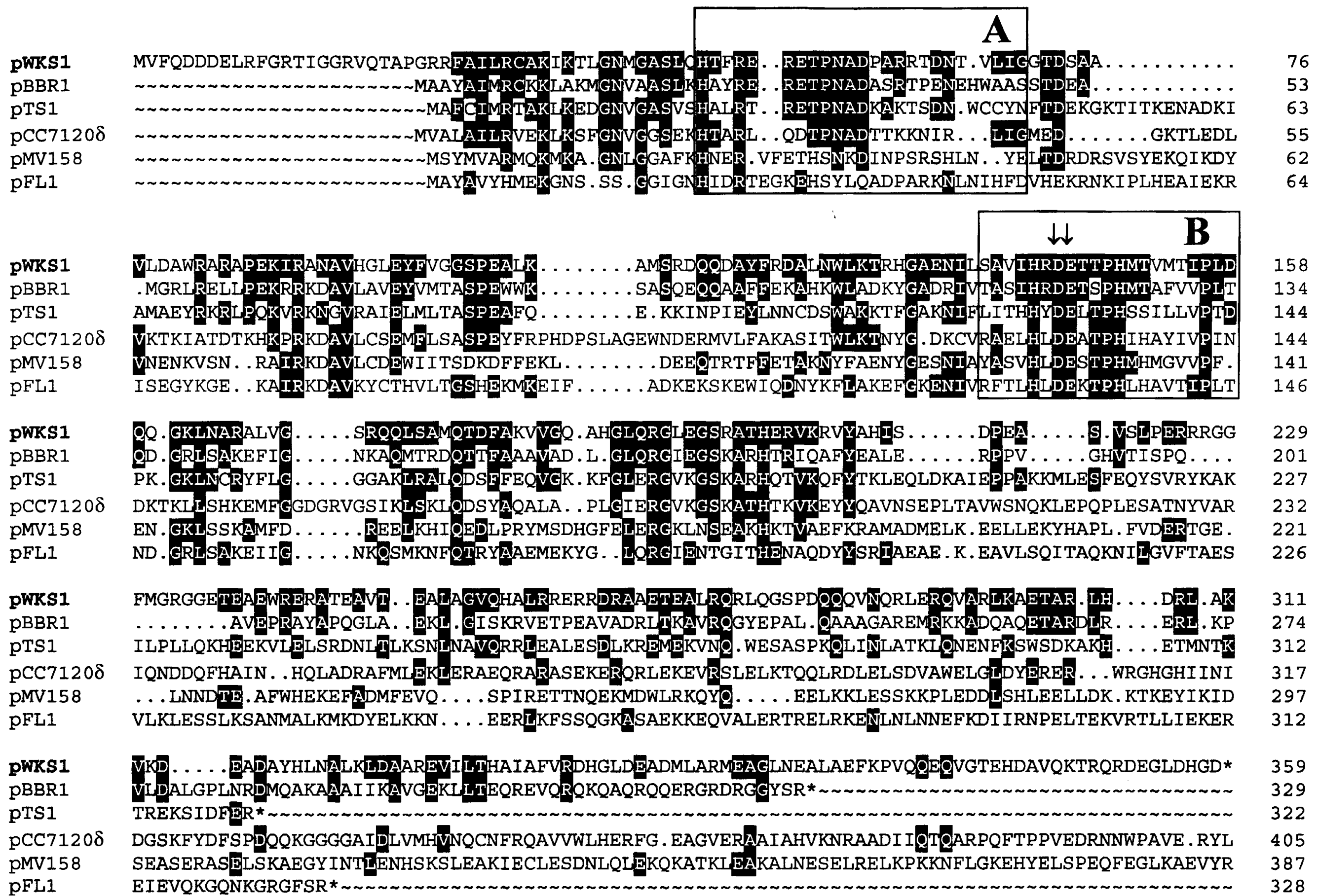

Fig. 2. Alignment of the deduced amino-acid sequences of the Mob proteins from several plasmids. pWKS1 is shown in bold. The other plasmids included in this alignment were: pBBR1 from *Bordetella bronchiseptica* (accession no. S25246); pTS1 from *T. denticola* (accession no. NP_073756); pCC7120δ from *Anabaena* sp. PCC 7120 (accession no. NP_489420); pMV158 from *Streptococcus agalactiae* (accession no. P13925); pFL1 from *Flavobacterium* sp. KP1 (accession no. NP_052877). Dots indicate gaps introduced to maximize the alignment. Amino acids identical to those in the Mob sequence of pWKS1 are shown against a dark background. An asterisk at the end of a sequence indicates a stop codon. Amino-acid numbering is shown at the right-hand side of the sequence lines. Two conserved regions (termed A and B), as described by Guzman & Espinosa (1997), are shown boxed. The conserved residues (aspartate and glutamate) shown to be crucial for the mobilization of pBBR1 (Szpirer *et al.*, 2001) are indicated by vertical arrows. The sequences of pCC7120δ and pMV158 have been truncated at their C termini.

several plasmids from Gram-negative bacteria, e.g. pTS1 from *Treponema 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 Tn*5520* 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 pWKS1. In their studies on the mobilization mechanism of pBBR1, Szpirer *et al.* (2001) identified two amino acids (aspartate and glutamate) in the Mob protein, located in the B region, which were

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 θ-replicating plasmids from *Lactococcus lactis*, e.g. pFV1201, pJW565 and pJW566 (accession nos CAA65650, 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 (*oriV*)] may be distantly related. However, in the sequence of the ORF2-encoded protein, we could find neither an obvious DNA-binding domain (helix–turn–helix motif) nor a leucin-zipper motif, which are typical for many Rep proteins of θ-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 ρ-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, such as those in *Lactobacillus* spp. (Bates & Gilbert, 1989), *Streptococcus* spp. (Priebe & Lacks, 1989), *Staphylococcus* spp. (Projan & Novick, 1988) and *Bacillus* spp. (Meijer *et al*., 1998). 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 *Pst*I (located within ORF1) and *Eco*RI (located 5 bp upstream of ORF1) sites of pWKS1, respectively. The *Eco*RI 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 (*Pst*I) and pWKS19 (*Eco*RI) (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 *Sma*I 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 *Sma*I-digested linear form of pWKS1 (plasmid pWKS21) and a 1·8 kb *Eco*RI–*Pst*I 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, pBGS18. 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′-AAGTGGGGAATCCAGCCGCAA-3′), are tandemly repeated (without any spacer sequences) three times. In many θ-replicating plasmids, direct repeats (iterons), located within *oriV*, 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 *oriV* of many plasmids) were identified within this region. To check whether the intergenic sequence mentioned above contains *oriV*, we cloned this region (on a 380 bp *Sau*3AI fragment of pWKS1) into the *Bam*HI 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 UWP1 (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

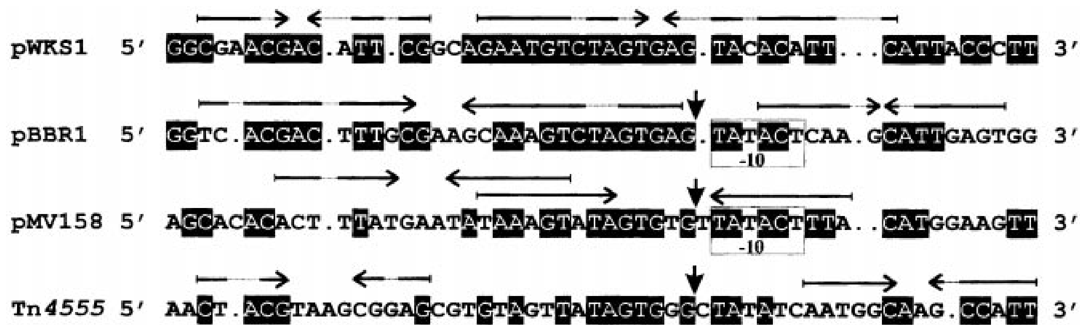

Fig. 3. DNA sequence alignment of the putative *oriT* sequence of pWKS1 with the *oriT* sequences of pBBR1 from *Bordetella bronchiseptica* (accession no. X66730), pMV158 from *Streptococcus agalactiae* (accession no. X15669) and mobilizable transposon Tn*4555* (accession no. U75371). Inverted-repeat sequences are indicated by horizontal arrows. The nick sites are shown by vertical arrows. Conserved nucleotides are shown against a dark background. The −10 boxes determined for the *mob* promoters of pBBR1 and pMV158 are boxed.

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 transformants 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 transformants 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 2×10^{-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 (Crellin & 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 (Farías *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* (Farías & Espinosa, 2000) and in *oriT* sequence of the mobilizable transposon Tn*4555* 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 *Leuconostoc 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 pKWS1, 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 alkenifer*, *Paracoccus aminophilus*, *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 *α-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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