A novel rolling-circle-replicating plasmid from Pseudomonas putida P8: molecular characterization and use as vector

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In Pseudomonas putida P8, three cryptic circular plasmids were detected, i.e. pPP8-1 (2.5 kbp), pPP8-2 (42 kbp) and pPP8-3 (~100 kbp). Cloning and complete sequencing of pPP8-1 revealed a 2534 bp element harbouring four open reading frames (ORFs A, B, C and D). No function could be attributed to the latter three ORFs, whereas the predicted ORF A gene product is homologous to replication proteins known from small multicopy plasmids of Gram-positive bacteria and single-stranded (ss) phages, genetic elements replicating via a rolling circle (RC) mechanism involving characteristic ssDNA intermediates. Consistently, a double-strand origin of replication, highly conserved in rolling-circle-replicating (RCR) elements, was identified in pPP8-1, along with a putative single-strand origin. Beyond this, ss replication intermediates were confirmed by Southern analysis and mungbean-nuclease digestion. This being the first element of this type known in pseudomonads, a kanamycin-resistance gene was ligated into pPP8-1 and the resulting vector was successfully used for the transformation of both Escherichia coli and P. putida.

Keywords: extrachromosomal element, cryptic plasmid, cloning, pP8-1, repA

INTRODUCTION

Numerous relatively small plasmids have been identified in a wide range of Gram-negative bacteria. Most of these use the so-called ‘theta-replication’ system, in which – with some exceptions – a specific plasmid-encoded protein (Rep) is necessary for replication (del Solar et al., 1998). In contrast, most known small plasmids from Gram-positive bacteria replicate via the rolling-circle (RC) mechanism characterized by the synthesis of single-stranded (ss) DNA intermediates (Novick, 1989; Meijer et al., 1998). Indeed, RC replication was originally thought to be limited to small multicopy plasmids of Gram-positive bacteria and ss-phages (Baas & Jansz, 1988). However, some years ago, a wider distribution of RC replication was recognized (del Solar et al., 1993), but rolling-circle-replicating (RCR) plasmids were described only sporadically for Gram-negative bacteria; a compilation is given by Khan (1997). For an up-to-date and comprehensive list of RCR plasmids, the recently established Database of Plasmid Replicons established by M. Osborn (http://www.essex.ac.uk/bs/staff/osborn/DPR_home.htm) should be referred to. To our knowledge, no RC element replicating via ssDNA intermediates has been described for a member of the pseudomonads, though the number of plasmids known (Boronin, 1992) has increased remarkably over the past two decades.

The phenol-degrading Gram-negative bacterium Pseudomonas putida P8 has attracted considerable interest as a system for studying the degradation of harmful compounds such as phenol (Bettmann & Rehm, 1984; Zache & Rehm, 1989), particularly for elucidating the molecular mechanism by which bacteria adapt their membranes to such substances prior to degradation. Recently, we isolated and functionally characterized the chromosomal cti gene (Holtwick et al., 1997) encoding a cis/trans isomerase for unsaturated fatty acids – a cytochrome-c-type polypeptide (Holtwick et al., 1999) – enabling P. putida P8 to change membrane fluidity.

Abbreviations: ds, double-stranded; DSO, double-strand origin; IR, imperfect repeat; KmR, kanamycin resistance; RC, rolling circle; RCR, rolling circle replicating; S/D, Shine-Dalgarno; SSB, single-strand DNA-binding protein; ss, single-stranded; SSO, single-strand origin.

The EMBL and GenBank accession number for the nucleotide sequence of pPP8–1 is AJ289784.
Here, we report on the occurrence of three differently sized plasmids in *P. putida* P8, the cloning, complete sequencing and functional characterization of the smallest of these extrachromosomal elements, and its use as a cloning vector. On the basis of sequence comparisons with known elements and demonstration of the characteristic ssDNA intermediate, this extrachromosomal element has emerged as the first RC-type plasmid found in a representative of this physiologically extremely diverse bacterial group.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The wild-type *P. putida* P8 used throughout this study was originally isolated as a phenol-degrading bacterium (Bettmann & Rehm, 1984). *Escherichia coli* JM107 (Yanisch-Perron et al., 1985) served as the cloning host. *P. putida* and *E. coli* strains were routinely grown in complete medium (St I; Merck) in a rotary shaker (model G76; New Brunswick) at 200 r.p.m., at 30 and 37 °C, respectively. Solid (1.5% agar) and liquid media were supplemented, when required, with ampicillin (50 µg ml−1) and kanamycin (25–50 µg ml−1).

The commercially available pUCBM20 cloning plasmid (Boehringer Mannheim) was used for cloning pPP8-1 and for the construction of subclones.

**Recombinant DNA techniques.** *P. putida* DNA was prepared according to the protocol of Rodriguez & Tait (1983). Plasmid DNA was isolated from *E. coli* as described by Birnboim & Doly (1979). pUT-mini-Tn5-Km (de Lorenzo et al., 1990) served as the source of the kanamycin-resistance marker.

Restriction endonuclease digestions, ligation, agarose electrophoresis and additional recombinant DNA techniques were carried out as described by Sambrook et al. (1989) unless otherwise specified.

Southern blot hybridization for the verification of ssDNA replication intermediates was performed essentially as described by te Riele et al. (1986), using plasmid pPP8-1 as the probe. Label was added by random priming using a non-radioactive labelling kit (Boehringer Mannheim) according to the instructions of the supplier.

**Transformation procedures.** Plasmids were introduced into *E. coli* by the transformation of CaCl2-treated cells with purified plasmid DNA (Sambrook et al., 1989).

*P. putida* was transformed by electroporation, as described by Taghavi et al. (1994) but with the following modifications. *P. putida* strains were grown at 30 °C in complete medium to an OD600 of 0.8. Cells were collected by centrifugation (10 min, 7000 g) and washed twice with 1 vol. ice-cold buffer [10% (v/v) glycerol, 90% (v/v) Ho]. Subsequently, the cells were concentrated 100-fold. To 40 µl of the suspension, 4 µl plasmid DNA (total 1 µg) was added; the preparation was then transferred into a 2 mm electroporation cuvette (Elektroporator 2510; Eppendorf). The best results were obtained with the following settings: voltage, 2400 V; capacitance, 10 µF; resistance, 600 Ω. After electroporation, cells were incubated in 1 ml complete medium for 1 h at 30 °C before being plated onto selective medium.

**Sequencing.** Small fragments (0.5–1 kb) of pPP8-1 were subcloned in pUCBM20 and sequenced with IRD41-labelled universal and reverse primers by using a Thermo Sequenase fluorescent primer cycle sequencing kit with 7-deaza-dGTP (Amersham Buchler) and an automatic LI-COR sequencer.

Database searches were performed with the **BLAST** program provided by EMBL/Heidelberg, using the default parameter settings (Altschul et al., 1997). Multiple sequence alignments were carried out using the **Clustal-W** programs (Thompson et al., 1994). Further analysis of the sequence was performed by using the HUSAR Genius net service package of EMBL/Heidelberg.

**RESULTS**

**Detection of plasmid DNA in *P. putida* P8**

When bulk DNA was isolated from *P. putida* P8 and subjected to agarose gel electrophoresis, several bands were obtained in addition to the high-molecular-mass chromosomal DNA, indicating the presence of extrachromosomal genetic elements. Gel elution and restriction enzyme analysis (data not shown) revealed the existence of three circular plasmids, i.e. pPP8-1 (2.5 kbp), pPP8-2 (42 kbp) and pPP8-3 (approx. 100 kbp).

Since pPP8-1 was found to harbour a single HindIII site (see Fig. 1), the entire plasmid was inserted into the corresponding restriction enzyme site of the *E. coli* cloning vector pUCBM20, resulting in plasmid pUCPP81, which was subsequently used to construct subclones useful for sequencing.

![Fig. 1. Physical map of pPP8-1 and pPP8-1Km<sup>R</sup>. repA encodes the replication protein. ORFs B, C and D encode putative DNA-binding proteins.](Image)
Nucleotide sequence of pPP8-1

The plasmid was sequenced completely (GenBank accession number AJ289784), revealing a circular genome of 2534 bp, which accords well with the above-mentioned electrophoretic mobility of restriction fragments obtained in pPP8-1 digests. A physical map of the element harbouring four putative ORFs is presented in Fig. 1, whilst the corresponding sequence, emphasizing features of interest, is given in Fig. 2. Analysis of the sequence by using the HUSAR Genius net service package provided by EMBL/Heidelberg revealed the
presence of four putative ORFs, as outlined in Fig. 1. Putative coding regions were identified on the basis that an ORF should comprise at least 150 nt preceded by a ribosome-binding site (the Shine–Dalgarno sequence: $\text{S}_{D}$ in Fig. 2) at an appropriate distance from a common translational start codon. Further upstream of ORFs A, B, D, putative promoters were identified by comparing the corresponding sequences with $\text{RpoD}$-dependent promoters of $\text{Pseudomonas}$ (Ronald et al., 1992) and the $\sigma^{70}$ consensus sequence of $\text{E. coli}$ promoters. Putative promoter sequences are underlined and marked as $\text{fi}_{10}$ and $\text{fi}_{35}$ in Fig. 2. Upstream of ORF C, no such promoter sequence could be identified; possibly ORF C is cotranscribed from the promoter of the preceding ORF B. Downstream of ORFs A, C and D, there are inversely orientated sequences, which can form stable hairpin structures in their transcripts with calculated free energies ($\Delta G$) of $-47.6$ kJ mol$^{-1}$, $-19.6$ kJ mol$^{-1}$ and $-285.9$ kJ mol$^{-1}$, respectively (Tinoco et al., 1973). Since these inversely repeated sequences are followed by poly-U domains, they presumably constitute factor-independent transcriptional terminators. The fact that no potential terminator sequence was identified downstream of ORF B is further indicative of cotranscription of an operon comprising ORFs B and C.

**Analysis of predicted proteins encoded by the ORFs**

When the predicted polypeptides of the four ORFs identified in pPP8-1 were compared with protein databases by applying BLAST searches (Altschul et al., 1997), only the predicted polypeptide of ORF A showed significant similarities to known proteins. This putative ORF A gene product is homologous to replication proteins associated with small multicopy plasmids of Gram-positive bacteria. The highest homologies were found with replication proteins of the pC194 plasmid family (Horinouchi & Weisblum, 1982); accordingly, ORF A was designated as $\text{repA}$. Fig. 3 shows a sequence-alignment analysis of replication proteins belonging to that family. Motif IV is considered to be the enzymic active site; the tyrosine residue in this motif is invariant and plays a key role in the nicking process. Other conserved motifs (indicated by asterisks in Fig. 3) are also present (Khan, 1997).

**Identification of the double-strand origin of replication**

Having identified the gene encoding a potential Rep protein of the pC194 type, we searched for the corresponding origin of replication. In pC194-type plasmids, the double-strand origin (DSO or $\triangleright$ strand ori) of replication is highly conserved and located immediately upstream of the $\text{rep}$ genes (Novick, 1989). Such a sequence (35 bp) is located upstream of $\text{repA}$ at position 1234–1270 in pPP8-1 (Fig. 2). As depicted in Fig. 4, the DSO of replication of pPP8-1 contains the highly conserved sequence $\text{CTTGATA}$ and is thus identical to the origins of RC plasmids from Gram-positive bacteria and ssDNA $\text{E. coli}$ phages.

**Identification of a putative single-strand origin of replication**

Leading-strand synthesis mediated by the Rep protein of RCR plasmids results in the formation of ssDNA intermediates that are converted to double-stranded (ds) DNA by host proteins. Replication of the lagging strand of RC plasmids initiates from single-strand origins (SSOs) of replication. Those origins usually have extensive secondary structure, but, unlike the DSOs, their sequences are not homologous, even among representatives belonging to the same family (Khan, 1997; Kramer et al., 1999). At position 2363–156 of pPP8-1, five potential imperfect repeats (IRs) are located, possibly representing the SSO. The calculated free energies...
**RCR plasmid in Pseudomonas putida**

**Fig. 4.** Sequence alignments of DSOs of replication from several pC194-type elements. Boxed sequences are highly conserved; they harbour the site for introducing the nick as a prerequisite for the initiation of replication.

**Fig. 5.** Demonstration of ssDNA replication intermediates of pPP8-1. Gel electrophoresis (a) and corresponding Southern blotting using pPP8-1 as the probe (b). Lanes 1 and 4 contain a 1 kbp DNA ladder. Lanes 2 and 3 contain pPP8-1 preparations of 0.4 and 0.2 µg, respectively. Lanes 5 and 6 contain the same amount of plasmid DNA digested with the ss-specific mungbean nuclease. Bands visible in the gel correspond to dsDNA, i.e. covalently closed circular (ccc) and open circular (oc) DNA. The faint band at the top corresponds to contaminating high-molecular-mass DNA. ssDNA hybridizes just below the ccc DNA and disappeared almost completely after digestion (0.4 µg mungbean nuclease per µg DNA).

Demonstration of single-strand replication intermediates

When DNA is isolated from strains carrying RCR plasmids and subjected to gel electrophoresis, ssDNA can be detected by immediate blotting and UV cross-linking to nylon membranes without denaturation, since a plasmid-specific probe hybridizes to ssDNA rather than dsDNA. Digestion of ss intermediates by single-strand-specific nucleases, such as S1 nuclease or mungbean nuclease, prior to gel electrophoresis results in the disappearance of hybridization signals. The results of such experiments are shown in Fig. 3, clearly proving the existence of pPP8-1 ssDNA intermediates in *P. putida* P8.

Regulation of replication

In many RCR plasmids, replication, and hence copy number, is controlled by small divergently synthesized repA antisense transcripts (Wagner & Simons, 1994). Identification of a locus encoding antisense RNA on the basis of sequence data should make use of the fact that no ORF is present at the respective position but only a promoter and a corresponding terminator.

The putative promoter of repA is obviously located at position 1374–1402 and the repA terminator is located immediately 3′ downstream of the coding region at position 2272–2288, with a calculated free energy (∆G) of −47.6 kJ mol⁻¹. Apparently, a transcriptional terminator sequence with a calculated ∆G of −124.6 kJ mol⁻¹ is also located on the opposite strand at position 1276–1329. No ORF preceding this terminator could be identified; however, a potential promoter is situated at position 1542–1523. Accordingly, the repA mRNA and its antisense transcript overlap one another by 104 nt, comprising 28 nt 5′ upstream of the repA translational start and 76 nt of the repA coding sequence.

Vector construction

In our initial attempts to obtain a hybrid vector on the basis of pPP8-1, we tried to integrate the kanamycin-resistance (Km⁺) gene of Tn5 into the single HindIII site of pPP8-1. After Ca²⁺-mediated transformation, direct selection did not give rise to antibiotic-resistant *E. coli* clones (data not shown). Since the HindIII site is located within ORF B, disruption of the gene by integration of the antibiotic-resistance marker might have interfered with plasmid replication and maintenance, eventually resulting in the failure to obtain transformants. However, when the Km⁺ gene was ligated into the SmaI site located in the intergenic region between ORF D and
ORF B, E. coli transformants carrying the desired hybrid plasmid (pPP8-1Km$^R$ in Fig. 1) were easily obtained. As for the wild-type pPP8-1 in P. putida P8 (see Fig. 5), single-strand intermediates of the hybrid vector were detected in E. coli transformants. Plasmid DNA isolated from those transformants was characterized by restriction-enzyme analysis (see Fig. 1, pPP8-1Km$^R$) and used in electroporation transformation experiments with P. putida P8; the number of transformants obtained was up to $3 \times 10^4$ (µg DNA)$^{-1}$.

**DISCUSSION**

All plasmids hitherto described for pseudomonads are considered to replicate bidirectionally (theta replication). Thus, plasmid (pPP8-1) isolated from P. putida P8 and characterized in this study represents a novel type of extrachromosomal element for *Pseudomonas* because several lines of evidence support RC replication, as follows: (1) a highly conserved sequence representing the DSO of replication is located at the appropriate position; (2) a replication protein (RepA) is encoded, it being responsible for nicking the DNA at the DSO and instrumental in the formation of the single-strand replication intermediate; (3) a putative SSO was identified; (4) single-strand replication intermediates of the plasmid were found to occur in P. putida P8.

Plasmid pKB740 from *Pseudomonas* sp. (Altenschmidt *et al.*, 1992) may also replicate by an RC replication mechanism, on the basis of homology between the putative protein encoded by ORF 5 of this plasmid and that of the gene II protein, involved in RC replication of the filamentous bacteriophage I2-2 (Stassen *et al.*, 1991). By analogy with pC194 and ΦX174, the strictly conserved sequence CTT$^\uparrow$GATA carrying the indicated nick site (†) within the putative DSO was identified in pPP8-1, along with highly conserved amino acid residues (Tyr and Glu) in the catalytic domain of the predicted RepA polypeptide (Fig. 3, ‘IV’). Both amino acid residues were previously shown to be instrumental in the nicking process and in circularization of single-strand intermediates (Noriot-Gros *et al.*, 1994). Though the predicted RepA protein of pPP8-1 is only 25–30% similar to the corresponding proteins of the pC194-plasmid family, it harbourse the five functional domains identified in this type (Fig. 3). It is presumed that the strikingly conserved cysteines, as well as aromatic and charged amino acids (see Fig. 3), are instrumental in the recognition of, and binding to, the target sequence (Noriot-Gros *et al.*, 1994).

For RCR-type plasmids in general, the identified putative SSO shows no obvious sequence similarities to other RC elements. Also, blocks of nucleotides conserved in hitherto known SSOS, as shown by Kramer *et al.* (1999), could not be identified in pPP8-1. Since an SSO – generally characterized by extensive secondary structures (five inverted repeats in pPP8-1) – must be recognized by host-specific proteins for initiation and completion of synthesis of the double strand, it is probably adjusted to the respective host bacterium, ensuring proper replication (Boe *et al.*, 1987; Gruss *et al.*, 1987; del Solar *et al.*, 1987b).

Besides the repA locus, pPP8-1 harbours three additional ORFs, the predicted polypeptides of which share no significant similarities with known proteins. From BLAST searches, it became evident, however, that ORF C shares similarities with a possible ORF (position 75–333) of a recently described RCR-type plasmid of *Nitrosomonas* sp. (Yamagata *et al.*, 1999).

Since it was not possible to obtain hybrid plasmids in which the antibiotic-resistance marker was integrated into the single HindIII site, an intact ORF B seems to be essential. ORFs B and C apparently constitute a transcripational unit, encoding polypeptides of 105 and 101 aa, respectively. Only short regions of the predicted proteins slightly matched DNA-binding proteins such as DNA polymerases and regulator proteins (data not shown). Because of their small size, the gene products of both ORF B and ORF C cannot be polymerases; they presumably represent DNA-binding proteins of unknown function.

ORF D, though overlapping the putative SSO, was identified as a potential coding region because of a Shine–Dalgarno (S/D) sequence at an appropriate distance from the start codon, a possible promoter and a corresponding distinct transcriptional terminator. In computer-aided searches, at the DNA level we found a 97 nt region (244–367, Fig. 2) with 80% similarity to the functionally uncharacterized ORF 110 of single-strand phage Pf1 (Hill *et al.*, 1991). At the amino acid level, slight similarities to DNA-binding proteins such as polymerases and recombinases (data not shown) were obtained. In view of the small size of the predicted ORF D polypeptide (73 aa), it might represent a single-strand DNA-binding protein (SSB), as its size corresponds to known SSBS such as that of phage Pf3 (78 aa; Luiten *et al.*, 1985), phage IF1 (96 aa; Carne *et al.*, 1991) and *Bacillus thuringiensis* plasmid pTX14-3 (68 aa; Madsen *et al.*, 1993). In general, SSBS do not share noticeable structural similarities.

It remains to be determined whether plasmid-encoded proteins are instrumental in copy-number control in pPP8-1. However, regardless of such a possible involvement, copy-number control appears to be influenced by an antisense RNA overlapping the translational start (S/D and ATG) of the repA transcript. The size of the overlap corresponds well with other RCR plasmids, ranging from 40 nt in pJDB21 from *Selenomonas ruminantium* (Zhang & Broker, 1993), up to 148 nt in pLAB1000 of *Lactobacillus hilgardii* (Alonso & Tailor, 1987; Josson *et al.*, 1990).

A number of RCR plasmids from Gram-positive bacteria, such as pC194, pMV158 and pSA5700, but also pKYM of the Gram-negative *Shigella*, were found to replicate autonomously in *E. coli* (Goze & Ehrlich, 1980; Barany *et al.*, 1982; del Solar *et al.*, 1987a; Yasukawa *et al.*, 1991). The same holds true for the
hybrid plasmid pPP8-1KmR, which can be maintained in E. coli, albeit only under selective pressure. The cultivation of plasmid-containing E. coli cells overnight without kanamycin resulted in segregated loss of the plasmid (data not shown). Nevertheless, the plasmid fulfills several criteria deemed useful for a potential Pseudomonas cloning vector. It is relatively small (2534 bp), it contains restriction-enzyme sites (Smal) into which foreign DNA can be integrated without disrupting an essential locus, and standard cloning procedures can be performed in E. coli, from which the plasmid can be isolated and transferred to P. putida and possibly other hosts as well.

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