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SHORT COMMUNICATIONS

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A New Gene of a TrfA Type Replication Initiator Found in a Caprolactam/Salicylate Degradation Plasmid pBS270

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Abstract—A mini-replicon was obtained for the caprolactam/salicylate degradation plasmid pBS270 (105 kb) of incompatibility group P-7 from *Pseudomonas* bacteria, and its nucleotide sequence was determined. A new gene that encodes a TrfA-like replication initiator was found on this replicon. The level of homology between this replication initiator and known proteins of the TrfA family suggests that the obtained replicon can be classified as IncP-1-like. The pBS270mini was shown to be chimeric.

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INTRODUCTION

The ability of *Pseudomonas* bacteria to utilize different organic substrates, including oil-derived carbohydrates and xenobiotics is often due to D-plasmids (degradation plasmids). In contrast to several naphthalene and toluene degradation plasmids, the structure of extrachromosomal elements encoding caprolactam catabolism enzymes has not been characterized. Regions responsible for the replication (*rep-ori*) and stable maintenance (*par*) of the plasmid and, thus, for the incompatibility features of most known CAP plasmids have not been studied either. Using microbiological incompatibility tests, the team of the Laboratory of Plasmid Biology of the Skryabin Institute showed that CAP plasmids isolated from pseudomonads (75–450 kb, conjugative) belong to the groups IncP-2, IncP-7, and IncP-9 [1].

Using molecular genetic techniques, it was shown that three CAP plasmids belong to the P-9 group and form a separate subgroup γ [2].

pBS270 is a CAP plasmid whose host is *Pseudomonas* sp. strain BS838 isolated from soil polluted with sewage of a caprolactam-producing plant, Kemerovo; it shows weak incompatibility with IncP-9 and IncP-7 replicons [1] and, as was recently found, carries genes of both caprolactam and salicylate degradation. P-9 incompatibility determinants were not found in pBS270 by PCR. The plasmid was found to contain an IncP-7 minimal replicon, including a *repA* replication initiator gene and, partially, an *oriV* region of replication origin. However, the *oriV* fragment, which is adjacent to the *par* locus and, as suggested in [3], is necessary for the initiation of replication, as well as elements of the IncP-7 *par* locus, could not be amplified (Volkova et. al., accepted for publication), although pBS270 is stably maintained in pseudomonads. For this reason, the functional activity of the P-7 replicon remained an open question. The purpose of this study was to identify the functional mini-replicon of pBS270 and to analyze its structure.

EXPERIMENTAL

Bacteria. *Pseudomonas putida* strain KT2442 (pBS270) was used to isolate pBS270; *P. putida* BS394, *P. aureofaciens* BS1393, *P. fluorescens* 38a, *P. aeruginosa* VKM B588, *Comamonas acidovorans* B-1251, and *Escherichia coli* K-12 DH5α were plasmidless recipient strains used to evaluate the host range and the stability of pBS270mini maintenance. Bacteria were grown in LB medium [4] at 28°C (*E. coli* cells were grown at 37°C). To select the mini-replicons, growth medium was supplemented with tetracycline (Tc) to the final concentration of 30 μg/mL (for *Pseudomonas* and *Comamonas* strains) or 10 μg/mL (for *E. coli*).

Plasmid pBS270 was isolated by alkaline lysis [4], and its mini-replicon was isolated using a ZR Plasmid Miniprep™ Classic kit (Zymo Research, United States).

PCR, DNA visualization, endonuclease treatment, ligation, and mini-replicon cloning into a pUC19 vector for sequencing were performed according to standard protocols [4] with enzymes purchased from Fer-

Abbreviations: CAP/SAL plasmid, caprolactam and salicylate biodegradation plasmid; ORF, open reading frame; GI, genomic island.

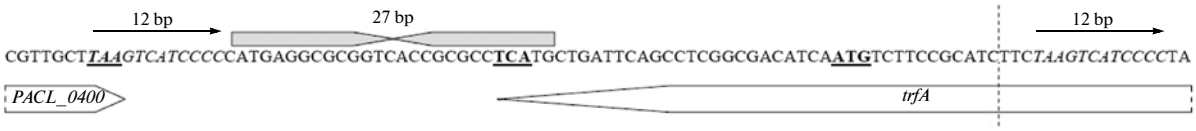

Fig. 1. Chimeric region in pBS270mini. Sequence to the left of the dashed line is homologous to genomic islands of *P. aeruginosa*; sequence to the right is unique to pBS270. Features shown in bold and underlined are: TAA, stop codon of ORF1 homologous to *PACL_0400* of genomic islands; ATG, initiating codon of *PACL_0399* (*terD*) of genomic islands (in pBS270mini this ORF is incomplete due to the chimeric nature of the plasmid); TCA, triplet complementary to *trfA* stop codon (ORF2). Direct repeats with 100% identity between two different loci are shown in italics and designated with a top arrow; a perfect palindrome differing from genomic island palindromes by one or two nucleotides is shown with opposed shaded arrows.

mentas (Lithuania). DNA fragments were isolated from a gel using a Zymoclean Gel DNA Recovery Kit (Zymo Research).

DNA sequences were obtained on an automated ABI Prism 373 3130XL Genetic Analyzer sequencer (Perkin-Elmer) by the Synthol Company (Moscow). Nucleotide and derived amino acid sequences were analyzed using DNAStar, pDRAW32 (ACACLONE software), as well as BLASTN and BLASTP programs available at the NCBI site. Phylogenetic trees were constructed using CLUSTAL X and TREECON [5].

pBS270 mini-replicon. To identify the plasmid region capable of independent replication, it was cleaved with restriction endonucleases BamHI, HindIII, SalI, and PstI. The fragment mixture was ligated to a Tc resistance cassette (from p34S-Tc) used as a selective marker. *Comamonas* cells were transformed by the same method as *Pseudomonas* [6]; *E. coli* cells were transformed using the conventional technique [4].

Stability of pBS270mini maintenance was determined by sequential passages in liquid LB without Tc for 6 days; samples were taken daily. Following a series of dilutions, cells were planted on LB agar plates and 100 colonies were transferred onto Tc-containing LB agar. The plasmid stability was defined as the percentage of clones that retained tetracycline resistance in the total number of clones tested.

RESULTS AND DISCUSSION

In our experiments aimed at limitation of the pBS270 mini-replicon using restriction endonucleases, in *P. putida* BS394, we obtained different constructs that lack IncP-7 replicon markers (*repA* and *oriV* regions). For a detailed analysis, we chose a 4.3-kb PstI fragment, which is the smallest fragment capable of independent replication in all *Pseudomonas* strains studied. The transformation of *E. coli* and *C. acidovorans* with this mini-replicon termed pBS270mini did not result in colony formation, which suggests that this replicon has a narrow host range or lacks regions required for replication in taxons other than *Pseudomonas*. It should be noted that pBS270mini maintenance in pseudomonads was unstable, and the replicon was fully eliminated from bacterial populations in 4–6 days (depending on the strain), which suggests that the pBS270mini lacks plasmid stabilization systems or that they do not function properly.

The complete nucleotide sequence of pBS270mini (4290 bp) was deposited in GenBank, acc. no. JX840344. A sequence analysis using pDRAW32 detected three open reading frames (ORF), two of them incomplete. The amino acid sequence derived from ORF1 (138 C-terminal residues) had 93% identity to the *PACL_0400* product from the genomic island (GI) of *P. aeruginosa* PACS171b (GenBank acc. no ACD39188), as well as to products of *PACL_0400* homologues from GIs of other pathogenic *P. aeruginosa* strains/isolates (CIG1, C79, 37308). The similarity between the ORF1 product and the hypothetical protein encoded by the chromosome of *P. putida* ND6 (host of pND6-1, an IncP-7 naphthalene degradation plasmid), was significantly lower (51%). In all GIs, downstream of *PACL_0400* there lies a gene (*PACL_0399* in PACS171b) encoding a TerD-like protein (stress response/tellurium resistance). pBS270mini contains but a short 5'-terminal fragment of this gene, and the subsequent sequence lacks homology to GIs (Fig. 1). The perfect palindrome and direct repeats found in immediate vicinity of this region can be associated with some recombination events or act as regulatory elements.

ORF2 seems particularly interesting because, on one hand, it is apparently chimeric (its 3' end lies within the pBS270mini region, which is homologous to the *P. aeruginosa* GI, Fig. 1) and, on the other hand, it encodes a TrfA-type protein of 363 amino acids related to replication initiators of IncP-1 plasmids. The involvement of this particular gene in the initiation of pBS270mini replication was confirmed by MfeI-mediated deletion of a 161-bp fragment in the central part of ORF2 (*trfA*). Despite the 28–80% overlap in amino acid sequences, the TrfA protein encoded by pBS270mini has only 24–33% identity to known proteins of the TrfA family. In the phylogenetic tree, the nearest neighbors of TrfA of pBS270mini are proteins encoded by plasmids (and genes anchored in some proteobacterial chromosomes) that belong to the extended γ subgroup of IncP-1 (Fig. 2).

However, the significant divergence between plasmids of this subgroup and classical IncP-1 plasmids is apparent, not only from the comparison of their repli-

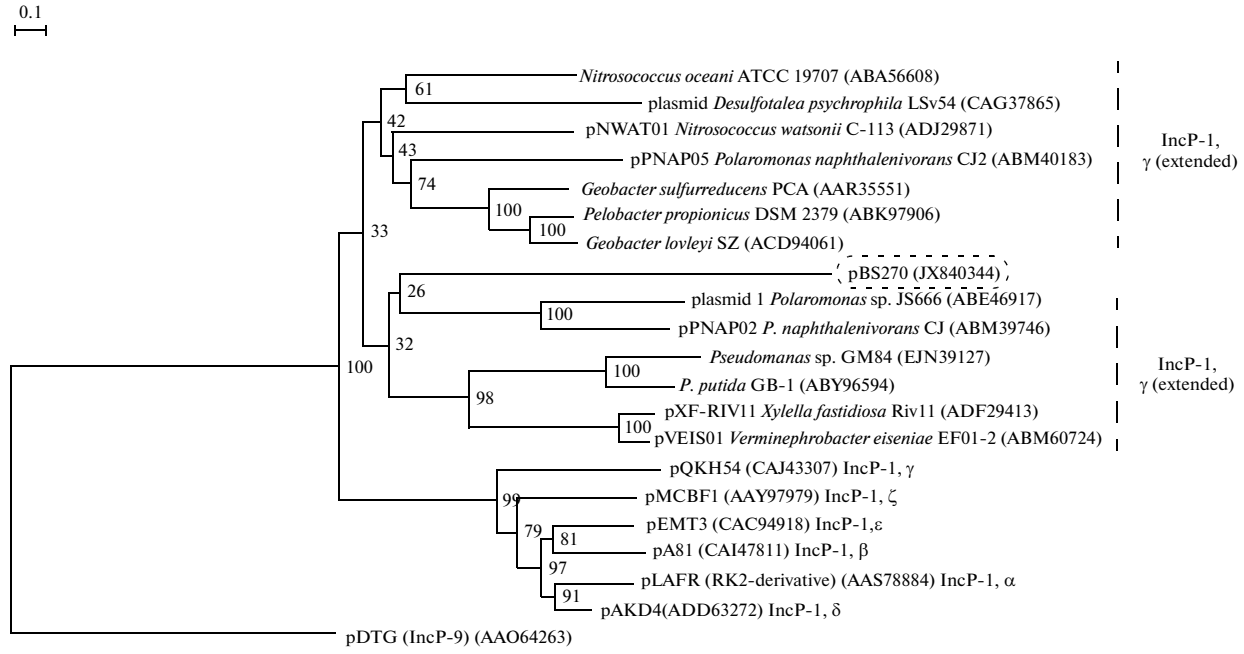

Fig. 2. Dendrogram of evolutionary relationships among the amino acid sequences of TrfA proteins encoded by classic IncP-1 plasmids (subgroups α, β, γ, δ, ε, ζ), representatives of the extended γ subgroup, and the CAP/SAL plasmid pBS270. If a TrfA protein is encoded by the chromosome, or the plasmid location of the gene is unknown, only the strain name is provided. The tree is rooted by the Rep protein of pDTG1 (IncP-9). GenBank accession numbers are given in parentheses. The tree was constructed using the TREECON program by neighbour-joining and bootstrap analysis.

cation initiators represented on the dendrogram, but also from the analysis of the structure and replication features of pXF-RIV11 and pVEIS01, plasmids of the extended γ subgroup [8, 9]. First of all, these plasmids lack fully functional active partition systems characteristic of P-1 plasmids, and their inheritance is only maintained by *pemI/pemK* modules (toxin/antitoxin system). In addition, these plasmids (especially pVEIS01) have a narrower host range than most members of classical P-1 subgroups. Moreover, the initiation of mini-pXF-RIV11 replication in some hosts depended on the presence of a complete ORF located upstream from *trfA* and encoding a resolvase (serine recombinase). Interestingly, the incomplete ORF3 of pBS270mini also encodes a recombinase, although a tyrosine one. The derived amino acid sequence of ORF3 (209 C-terminal residues) showed 58–62% identity to XerD-type site-specific recombinases (phage integrase family) from several pseudomonad strains, including *P. putida* ND6 (GenBank acc. no. AFK72703), as well as to an integrase encoded by the pQBR103 mega-plasmid (IncP-3) from *P. fluorescens* SBW25 (CAM96137). It is possible that the presence of the complete ORF3 within pBS270mini could affect the host range or stability of the replicon.

The exact location of the *ori* site in pBS270mini has not been identified so far, but, most likely, it is located between *trfA* and ORF3 (upstream from *trfA*, which is untypical for IncP-1 plasmids), since this region contains potential regulatory elements: four pairs of inverted repeats (22, 20, 18, and 17 bp) and a small perfect palindrome (14 bp). Inverted repeats and palindromes are also present in *oriV* of pXF-RIV11, but they are not homologous to the elements found in pBS270mini. Direct repeats (TrfA binding sites) homologous to IncP-1 iterons were not found in pBS270mini, except for the nonrepetitive sequence TTACCGTGGCAATAGGTT, where underlined nucleotides correspond to the iteron consensus sequence of pXF-RIV11.

Considering that members of the extended γ subgroup are phylogenetically remote from classical IncP-1 plasmids and that the level of homology between TrfA of pBS270mini and known replication initiators is low, even for this polymorphic subgroup (the evolutionary distance is also reflected in the phylogenetic tree), as well as taking into account that pBS270 and RP4 (IncP-1, α) [1] are absolutely compatible and lack *oriV* homology, it would be more correct to classify the *trfA*-containing replicon within pBS270 as IncP-1-like. The system that ensures the stable maintenance of pBS270 has not yet been identified, but it is certainly not the ParWABC system of the IncP-7 group. The identification of this system could help to specify the position of pBS270 in the IncP classification. Nevertheless, an analysis of the region of the initiation of replication of the CAP/SAL plasmid pBS270 enabled us to detect a new replication initiator gene of the *trfA* family, as well as to reveal the complicated evolutionary history of this plasmid, rich in

events of horizontal transfer between different genetic elements, including at least an IncP-7 plasmid, a carrier of an IncP-1-like replicon (plasmid or chromosome), and a GI of *P. aeruginosa*.

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