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GENETICS OF MICROORGANISMS

Organization and Maintenance Features of IncP-7 Naphthalene Degradation Plasmid pFME5 Basic Replicon

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Abstract—A basic replicon of the naphthalene degradation plasmid pFME5 (80 kb, IncP-7) has been constructed and sequenced. The nucleotide sequence of pFME5mini is almost identical to replicons of the pND6-1 subgroup, which was separated based on the *repA–oriV* homology in our previous work. The basic replicon of pFME5 is capable of replication and stable maintenance exclusively in *Pseudomonas* species. An analysis of the deletion mutation indicated that, in contrast to the *parWAB* region, the *parC* gene is not essential for the stability of pFME5mini and this can be a common feature of IncP-7 replicons. We revealed that *par*-defective mutants of pFME5mini were slowly eliminated from the bacterial population in a nonselective medium compared to their pCAR1-based counterparts. Designed primers specific to the *repA* and *parC* genes can be used to detect IncP-7 plasmids, while primers specific to two variants of *parA* can be used for intragroup classification.

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INTRODUCTION

During recent decades, there has been persistent interest from the scientific community in extra-chromosomal genetic elements that spread the determinants of antibiotic and heavy metal resistance, as well as pollutant biodegradation genes. The variety of these plasmids also includes bacterial biodegradation plasmids (D-plasmids) of the *Pseudomonas* genus allowing their hosts to compete successfully in soils contaminated with petroleum derivatives and in the wastewater of the chemical industry [1]. Plasmids are traditionally classified according to their incompatibility. Incompatibility is determined according to the way their basic replicons operate, i.e., by the region responsible for plasmid replication and maintenance in growing bacterial population [2]. The classification system of *Pseudomonas* plasmids (IncP) numbers 14 incompatibility groups [1]. The P-1, P-7, and P-9 groups are of particular interest as they include the plasmids of biodegradation of xenobiotics and oil components (halogen compounds, caprolactam, aromatic hydrocarbons, etc). An incompatibility group determines a bacterial host range, the ability for the long-lasting coexistence with other plasmids in the same host, and hence the fate of biodegradation/resistance genes in certain microcosms. Unlike plasmids of the P-1 and P-9 groups [3, 4], the maintenance mechanisms and structural diversity of the plasmids that belong to the IncP-7 group have not yet been studied in detail. A distinctive feature of the IncP-7 plasmids is that they are frequently found in contaminated soil and water samples, whereas the representatives of the IncP-9 group are found anywhere, including pure samples [4, 5]. The association of this feature with the replication and maintenance mechanisms of P-7 replicons and, consequently, with their host range also cannot be excluded. The completed nucleotide sequences are known for four IncP-7 plasmids, carrying genes of carbazole/dioxin (pCAR1), toluene (pWW53, pDK1), and naphthalene (pND6-1) biodegradation. The sequence of another toluene biodegradation plasmid, pL6.5, is partial. We previously determined the sequence of the replication initiation region for streptomycin resistance plasmid Rms148 (archetype of group IncP-7) [6]. However, the involvement of the certain plasmid regions and genes in the maintaining of P-7 replicons was only studied for pCAR1. This information is not sufficient to determine the general mechanisms of replication and segregation, as well as to establish the intragroup classification of the P-7 plasmids. In this work, we constructed the basic replicon of the naphthalene biodegradation plasmid pFME5 (pFME5mini), estimated its ability to maintain in homo- and heterologous hosts, sequenced pFME5mini, and accomplished the deletion analysis in order to evaluate how various pFME5 regions contribute to the replicon stable maintenance.

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Description	Reference/source
Strains		
Comamonas acidovorans B-1251	Type strain	VKM
E. coli K-12 DH5α	F$^-$ Φ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*) *U169 endA1 recA1 hsdR17*(r_K^- m_K^+) *deoR thi-1 supE44* λ *gyrA96 relA1*	Takara Bio, Japan
Pseudomonas putida BS394	Cys$^-$, Sm^R	Laboratory collection
P. aureofaciens BS1393 (reclassified as *P. chlororaphis* subsp. *aureofaciens*)	Phenazine antibiotic producer	The same
P. chlororaphis PCL1391 (*P. chlororaphis* subsp. *chlororaphis*)	Phenazine-1-carboxamide producer	[7]
P. fluorescens 38a	Pioluteorine producer	Laboratory collection
P. aeruginosa VKM B588		The same
P. fluorescens FME5	Naphthalene and salicylate destructor harboring the pFME5 plasmid	[8]
Plasmids		
pFME5	Nah$^+$Sal$^+$2MeN$^+$ Tra$^+$IncP-7, 80 kb	[8]
pFME5mini	7 kb pFME5 *Hin*dIII fragment with *repA-oriV-parWABC* carrying the tetracycline resistance gene	This work
pFME5miniΔ*parC*	5.9 kb pFME5mini *Hin*dIII–*Pci*I fragment with intact *repA-oriV-parWAB*	The same
pFME5miniΔ*parBC*	5.2 kb pFME5mini *Eco*RI fragment with intact *repA-oriV-parWA*	"
pFME5miniΔ*parABC*	2.6 kb pFME5mini fragment with intact *repA-oriV-parW*	"
pFME5miniΔ*parWABC*	1.9 kb pFME5mini fragment with intact *repA-oriV*	"
pFME5miniΔ*parA*	pFME5mini with the defective *parA* gene	"
pFME5miniΔ*parW*	pFME5mini with the defective *parW* gene	"
pUC19	Ap^R *lacZ* replicon of pMB9	[9]

Note: Encoded degradation traits: Nah$^+$ is naphthalene, Sal$^+$ is salicylate, MeN$^+$ is methyl naphthalene. Encoded resistance traits: Sm^R is streptomycin, Tc^R is tetracycline, Ap^R is ampicillin. Tra$^+$ is the proven ability to conjugation transfer. Cys$^-$ is cysteine auxotrophy. VKM is All-Russian Collection of Microorganisms.

MATERIALS AND METHODS

Research objects. Bacterial strains and plasmids used in this work are presented in Table 1. Bacteria were grown in LB medium or in mineral Evans medium [9] at 28°C. In order to maintain pFME5 plasmid, 0.1 g/L of naphthalene was added to Evans medium. When cultivated on agar medium, naphthalene was placed on the inner surface of an inverted Petri dish. To select the pFME5 basic replicon in the *Pseudomonas* and *Comamonas* genera, tetracycline was added to LB to 30 μg/mL of final concentration and, for selection in *E. coli*, 10 μg/mL of tetracycline were used. Constructs derived from pUC19 and transformed into the *E. coli* DH5α strain were selected on agar plates with 50 μg/mL ampicillin.

DNA of large plasmids was isolated by the alkaline lysis method [9]. The pFME5mini plasmid DNA from *Pseudomonas* and other constructs made in this study were isolated by employing ZR Plasmid Miniprep™ Classic Kit (Zymo Research, United States).

Polymerase chain reaction (PCR) and DNA visualization. PCR machine of Mastercycler Gradient (Eppendorf, Germany) was used for PCR. Primers used in this study are presented in Table 2. Reaction was run under standard conditions with *Taq*-polymerase. DNA electrophoresis was run conventionally in 0.8% or 1.5% agarose gel with Tris-acetate buffer [9]. DNA visualization was done by staining in ethidium bromide solution. DNA was purified from gel by use of Zymoclean Gel DNA Recovery Kit (Zymo Research, United States) according to the Instruction Manual.

DNA was digested by endonucleases obtained from Fermentas (Lithuania) for 2–6 h at 37°C according to the manufacturer's instructions.

pFME5 basic replicon construction. In order to obtain a basic replicon, pFME5 plasmid was digested by *Hin*dIII. To select regions capable of independent replication, a mix of *Hin*dIII fragments was ligated to a Tc-resistant cassette originated from p34S-Tc vector.

Table 2. PCR primers

Gene/region	Primers	Nucleotide sequence (5' ⟶ 3')	PCR product size, bp	Reference
repA (IncP-7)	RepAP7F	GCCCATGCCGAAAAAGGTGTC	412	[6]
	RepAP7R	GAATCGTTGATAGGCATCCGAC		
parA pND6-1 type	ParANDF	TCACCTCAAGGGCTTATACG	355	This work
	ParANDR	ATCGGATCAAAGCATCTCACC		
parA pCAR1 type	ParACARF	ATTGTTTTGGATTGACTACCCG	518	The same
	ParACARR	ATCCTCGAGGTACGCATCAGC		
parC (IncP-7)	ParCP7F	TGGTGCAACCGCAGACACG	267	"
	ParCP7R	GTGTTTGGTTGAATTGCTCATAGA		
repA-oriV (IncP-7)	4292F	CGA***AAGCTT***CAGTATTCATTGGGTTC*	1900	"
	6216R	GAC***GGATCC***TAATCTGGTTGCTCTC*		
repA-oriV-parW (IncP-7)	3622F	GA***GGATCC***CTGCATTTGCTGAATAC*	2600	"
	6216R	GAC***GGATCC***TAATCTGGTTGCTCTC*		

* Introduced restriction enzyme recognition sites *Hin*dIII and *Bam*HI are marked in bold italics, substituted nucleotides are underlined.

Vector DNA was preliminary digested with *Hin*dIII. DNA ligase of phage T4 was used for ligation according to the manufacturer's protocol (Fermentas, Lithuania). After 10–12 h of incubation at 18°C, 5–8 μL of the mix was added to the fresh and precooled cell suspension of the *P. putida* BS394 strain for electroporation (1.7 kV, 0.1-cm electrode distance). The MicroPulser electroporator (Bio-Rad, United States) was used for this purpose. Next, cells were grown in L broth for 1–1.5 h at 28°C and placed on agar media with tetracycline. After 1–2 days, grown colonies were checked for the presence of plasmid DNA.

Strains of the *Pseudomonas* and *Comamonas* genera were transformed with the basic replicon construct under the same conditions. The *E. coli* K-12 DH5α strain was transformed conventionally [9].

Construction of deletion mutants of the par locus in pFME5mini is presented in Fig. 1. Two mutant variants of pFME5mini*ΔparC* and pFME5mini*ΔparBC* have been obtained by deletion of corresponding *par* genes (Fig. 1a). The pFME5mini*ΔparWABC* and pFME5mini*ΔparABC* mutants (Fig. 1b) were obtained by amplification using primers presented in Table 2, i.e., 4292F and 6216R for the first variant and 3622F and 6216R for the second one. All mutated replicons, except pFME5mini*ΔparABC*, were treated with Klenow fragment and ligated with the tetracycline resistance cassette cut from the p34S-Tc vector with *Sma*I. When making pFME5mini*ΔparABC*, primers with *Bam*HI sites were used.

The pFME5mini*ΔparW* and pFME5mini*ΔparA* variants were obtained using insertions that shifted the open reading frame (ORF) towards *Aat*II and *Nsi*I restriction sites, respectively (Fig. 1c). Insertion DNA has been obtained by PCR with primers containing introduced *Aat*II or *Nsi*I restriction sites, on matrices besides the pFME5 *par* locus.

The stability of the pFME5 basic replicon, as well as its mutant variants in the *P. aureofaciens* BS1393 strain, was verified by the consecutive transfer of the cell suspension to the fresh LB medium without tetracycline. Separate colonies were grown in 5 mL of medium until the late logarithmic growth phase. Then, 50 μL of suspension were transferred each day to fresh medium for 10 days in two sample repeats for intact pFME5mini or for 6 days in three sample repeats for the *par* defective pFME5mini variants. Sampling was conducted on 1, 3, 6, and 10 days in the first case and daily in the second case. After corresponding dilutions, the cell suspension was transferred to nonselective LB agar plates with the subsequent replication of 100 colonies on LB plates with tetracycline. The plasmid stability was determined as the percentage of the ratio of Tc-resistant clones to all examined clones.

Plasmid DNA mutagenesis using hydroxylamine was fulfilled according to [10].

For sequencing, 3.5 kb *Eco*RI–*Kpn*I, 1.7 kb *Kpn*I–*Eco*RI, and 1.6 kb *Eco*RI-*Hin*dIII DNA fragments of pFME5mini were cloned in the pUC19 vector by standard technique [9]. Furthermore, the basic replicon regions were amplified using various primer combinations while sequencing was in progress. DNA sequencing was accomplished using the ABI PRISM® BigDye Terminator v. 3.1 kit and the ABI PRISM 3730 Applied Biosystems automated DNA sequencer, which is located in the GENOME center (interinstitute center for common use facilities supported by the Russian Foundation for Basic Research; http://www.genome-center.narod.ru).

An analysis of nucleotide and amino acid sequences was carried out using the DNAStar software packet in combination with BLAST, Jpred3, PredictProtein,

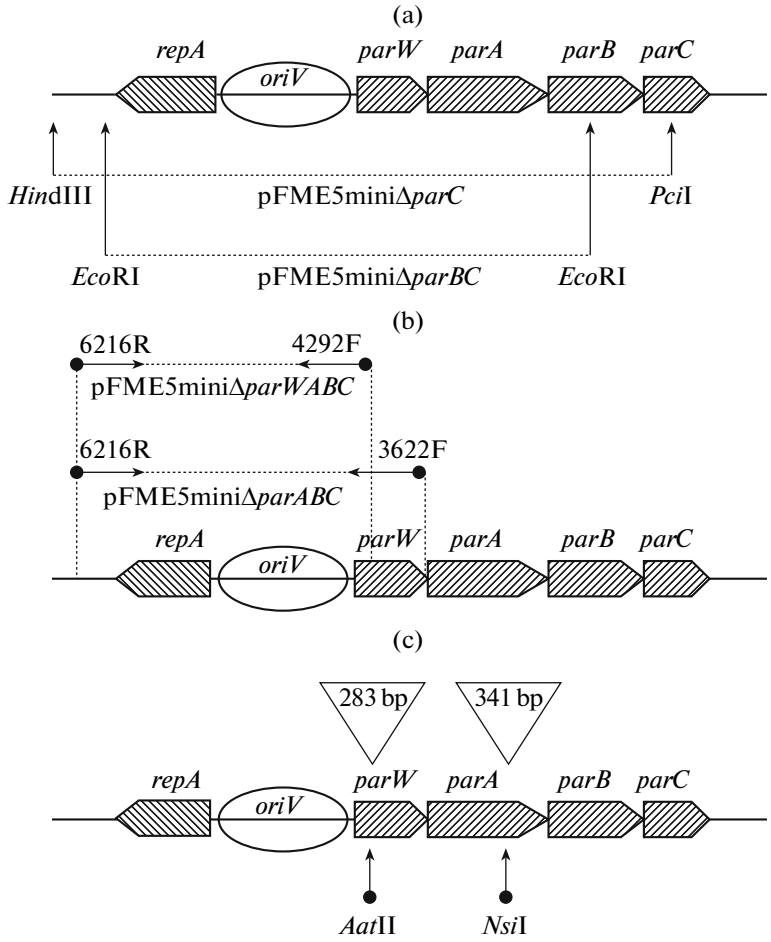

Fig. 1. Construction of *par* gene mutants of pFME5mini. (a) Deletion mutants obtained using restriction endonucleases. (b) Mutants obtained by PCR amplification of the basic replicon fragments. PCR primers are shown above arrows. (c) Insertional mutants obtained by ORF shift. DNA fragments were inserted in the restriction enzyme recognition sites located in the *parW* gene (*Aat*II in the case of pFME5miniΔ*parW*) or in the *parA* gene (*Nsi*I in the case of pFME5miniΔ*parA*). Insertion size is designated in triangles.

SOSUI, and BPROM (Softberry) software available online.

RESULTS AND DISCUSSION

pFME5 Basic Replicon Structure

The 80-kb pFME5 naphthalene and salicylate biodegradation plasmid was found in the *Pseudomonas fluorescens* FME5 strain isolated from a sludge storage pit of OAO Nizhnekamskneftechim. The plasmid was later found to belong to the P-7 incompatibility group [8]. It was chosen to be a model plasmid for studying the structural and functional properties of the IncP-7 basic replicon because of its stable maintenance in the innate host and easy purification by the alkaline lysis method. Up to date, the only naphthalene biodegradation plasmid that belongs to the IncP-7 group, pND6-1, has been sequenced. The plasmid host was isolated from industrial wastewater in China. Interestingly, the pND61, as well as the pWW53 TOL plasmid, have lost the region of conjugative transfer during their evolution [11]. Conversely, pFME5 can be transferred into the *P. aureofaciens* BS1393 strain with a high frequency and, therefore, it belongs to conjugative plasmids (data not shown). Large, low-copy plasmids, such as biodegradation plasmids, also incorporate a plethora of genetic material associated with the conjugative transfer and biodegradation. For this reason, constructing basic replicons derived from the large plasmids and carrying regions for both DNA replication and the stable maintenance of the plasmid (correct copy segregation) alone is of considerable interest. The term "basic replicon" is used repeatedly as a synonym for "minimal replicon." In this work, we consider the region required for the initiation of replication to be a minimal replicon, while a region that carries the minimal replicon together with the genes responsible for segregation is considered to be a basic replicon.

The sequence analysis of the pND6-1, pWW53, pL6.5, and pCAR1 plasmids (GenBank IDs AY208917, AB238971, AJ250853 and AB088420 respectively) revealed that the region in question might be reduced by *Hin*dIII digestion, though the region size varied among the representatives of the P-7 group. In fact, the ligation of the pFME5 *Hin*dIII fragment with the Tc-resistant cassette resulted in a short plasmid version, pFME5mini, capable of replication in the *P. putida* BS394 cells. Clones that harbor the mini plasmid were selected for tetracycline resistance. Plasmid DNA was isolated from the *P. putida* BS394 (pFME5mini) cells, the Tc cassette was deleted after digestion with *Hin*dIII and ramained replicon by itself was estimated as fragment of around 7 kb in size. It could not be excluded that a large plasmid might incorporate regions of replication initiation that come from several incompatibility groups and, thus, represents a mixed replicon. For this reason, it was necessary to exclude the presence of genetic determinants other than IncP-7. The *repA* gene, which encodes the replication initiator is conservative within the P-7 group. Using the *repA* specific primers (Table 2), we showed that PCR was positive; also, we needed confirmation that pFME5mini had a complete set of genes responsible for the stable replicon maintenance, i.e., the *par* (partitioning) locus. For this purpose, using known sequences of P-7 plasmids, we designed three primer pairs specific to *parC* (the last gene of the *par* locus), *parA* of the pND6-1 type, and *parA* of the pCAR1 type (Table 2). We did not design universal primers suitable for the whole group because of the low homology between the *parA* gene from pCAR1 plasmid and its homologs from the other P-7 plasmids. PCR analysis showed that pFME5mini carried genes of the *par* locus (at least *parA* and *parC*) in addition to the *repA* gene (Fig. 2). It should be noted that, similar to the *parA* genes from the pND6-1, pWW53, pL6.5, and pDK1 plasmids, pFME5mini had a *parA* gene of the pND6-1 type. Previously, comparing *repA–oriV* replication initiation regions, we defined three subgroups within the IncP-7 group, i.e., pND6-1 (includes pND6-1, pWW53, pL6.5, and pDK1), pCAR1, and Rms148 [6]. This division seems to be acceptable regardless of some differences in the *par* locus of different subgroups. Moreover, primers specific to the *parA* gene can be used for the intragroup classification of the IncP-7 plasmids.

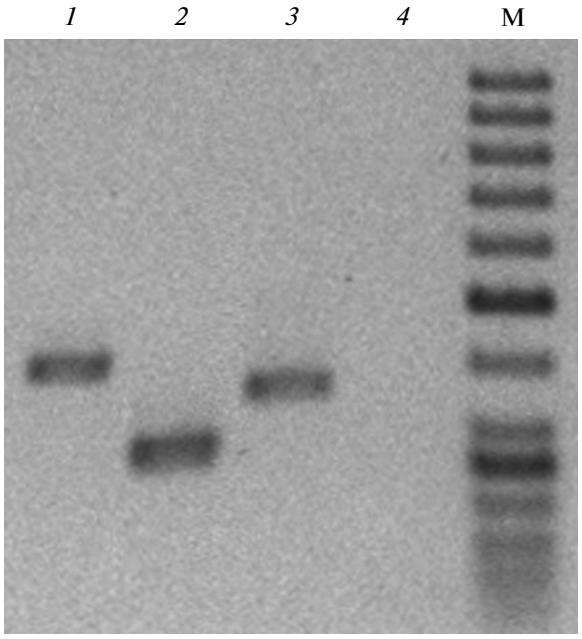

Fig. 2. Electropherogram of amplified DNA fragments of the genes localized in the region of replication initiation and segregation of pFME5mini. *1*, *repA* (412 bp); *2*, *parC* (267 bp); *3*, *parA* of pND6-1 type (355 bp); *4*, *parA* of pCAR1 type (518 bp, no amplification); M is molecular mass marker (50-bp DNA ladder).

Maintenance of pFME5mini in Homo- and Heterologous Hosts

In order to estimate the stability of pFME5mini in different hosts, various strains of the *Pseudomonas* genus (γ-proteobacteria, Pseudomonadaceae), as well as the *E. coli* DH5α strain (γ-proteobacteria, Enterobacteriaceae) and the *Comamonas acidovorans* B-1251 strain (β-proteobacteria), have been transformed by this plasmid. The results showed that the pFME5 basic replicon was capable of replication in all representatives of the *Pseudomonas* genus (Table 1, homologous hosts). We did not observe the transformation of the suggested heterologous hosts (*E. coli* DH5α and *C. acidovorans* B-1251), which is consistent with the results of other authors and defines the host range of IncP-7 plasmids, including only the *Pseudomonas* genus [11, 12]. The single known exclusion is the transformation of the pCAR1 plasmid into *Stenotrophomonas* representatives, which also belong to γ-proteobacteria [13]. Previously, we found some structural similarity between RepA proteins that originated from P-7 plasmids and the unclassified plasmid pPS10 [6]. It was shown that point mutations in the *repA* gene induced by hydroxylamine enlarged the pPS10 host range [14]. Unfortunately, the hydroxylamine treatment of pFME5mini did not result in mutants capable of replication in *E. coli* cells at either 37°C or 28°C.

The stability of pFME5mini in the *Pseudomonas* transformants was evaluated during 10 days of culture growth (approximately 200 generations) under nonselective conditions. The stability of the replicon differed slightly among *Pseudomonas* species (Fig. 3a), which seems to be due to the effect of the genetic background on the maintenance of extrachromosomal elements. The evaluation of the stability of the pCAR1 basic replicon during 5 days gave similar results where pCAR1mini was maintained in 96% of the *P. putida* DS1 population. However, the plasmid was quickly eliminated from the KT2440 strain, which belongs to

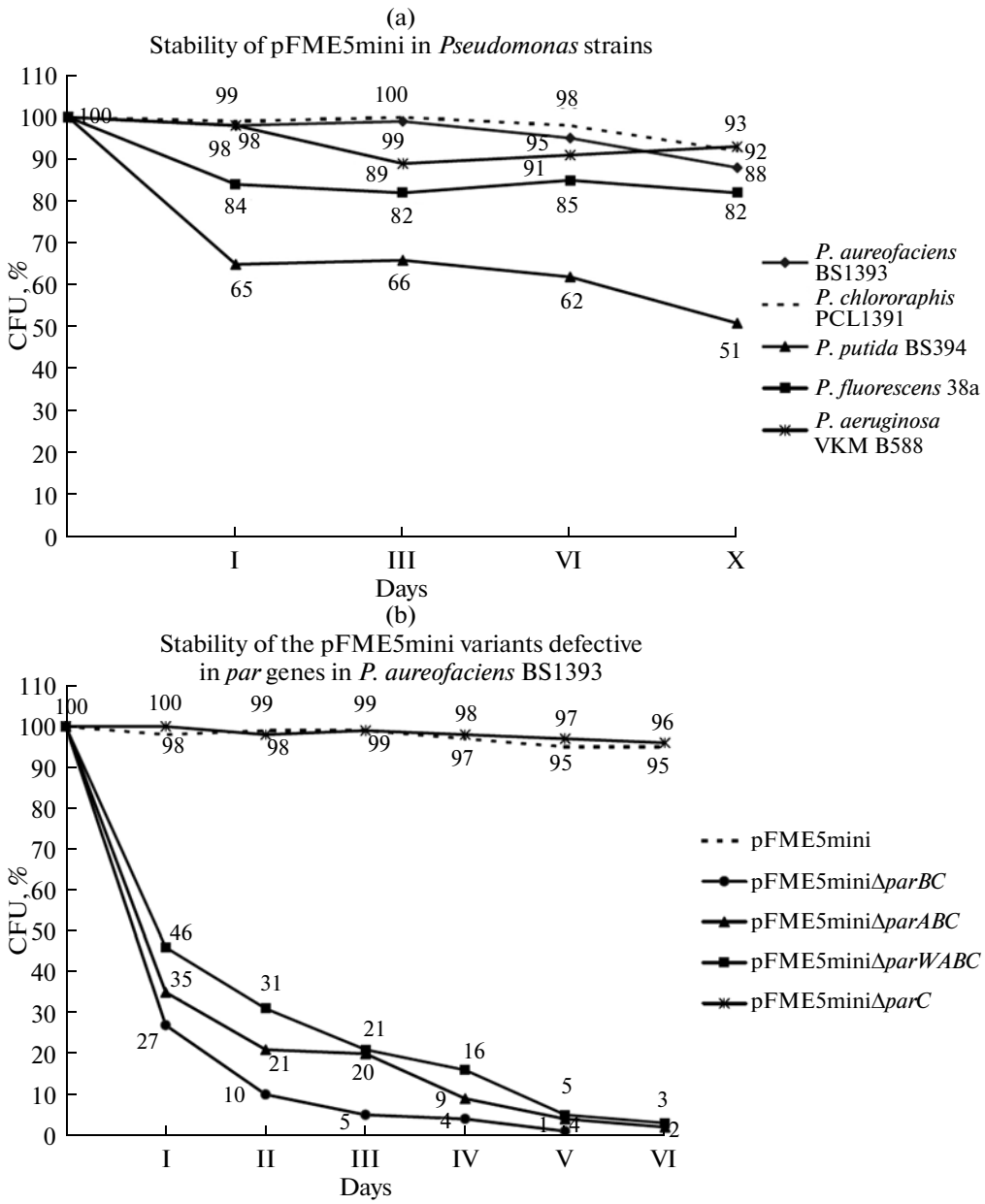

Fig. 3. Stability of pFME5mini in various *Pseudomonas* strains (a) and of pFME5mini *par* mutants in *P. aureofaciens* BS1393 (b). Stability was monitored under nonselective conditions for (a) 10 days or (b) 6 days. Diagrams for pFME5miniΔ*parW* and pFME5miniΔ*parA* are not shown, as they are similar to the diagrams of pFME5miniΔ*parWABC* and pFME5miniΔ*parABC*, respectively.

the same species [15]. Previously, we studied both physiological and biodegradation traits of several host–plasmid combinations using bacteria of the *Pseudomonas* genus [16, 17]. Interestingly, the pFME5 plasmid was stable in its native host and was conjugated into the *P. aureofaciens* BS1393 strain with a high frequency, but was eliminated after two days of growth (not shown). However, the pFME5 basic replicon was stably inherited in this host for 10 days. Thus, all other conditions being equal, the survival of plasmid depends not only on the basic properties of the replicon, but also on some other ("loading") genetic material.

Since the stable maintenance of the pFME5mini replicon was observed in four out of five nonrelated *Pseudomonas* strains, it can be concluded that there is the complete set of genes and noncoding regions that control plasmid autonomous replication and segregation within the *Pseudomonas* genus.

pFME5mini Sequence Analysis

For the preliminary intragroup classification of the IncP-7 plasmids, as well as for the mutation analysis strategy, the pFME5 basic replicon was completely

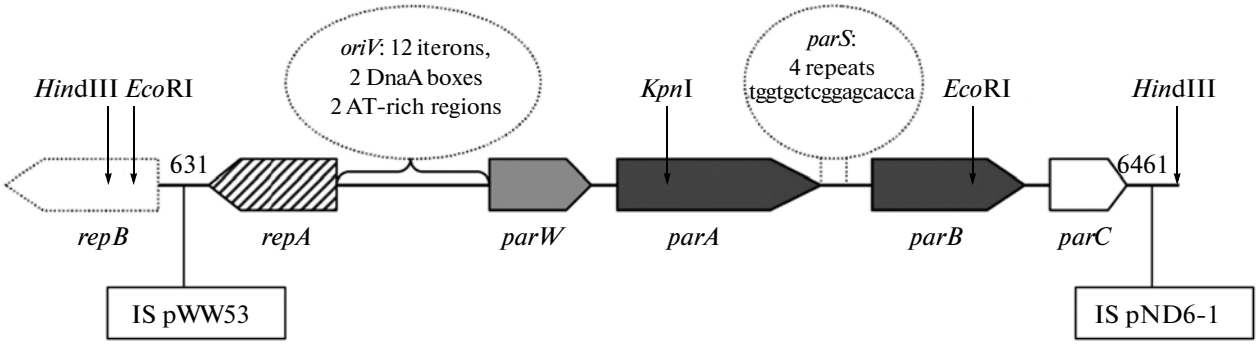

Fig. 4. Genetic map of pFME5mini. The entire *Hin*dIII fragment (6952 bp) was sequenced. Genes needed for replication and stable maintenance of the pFME5 basic replicon are highlighted. Insertions located in corresponding sites of basic replicons of other IncP-7 plasmids are shown in rectangles: IS*Ppu20* is located in pWW53 and IS*222* is located in pND6-1. Numbers above the insertion sites designate nucleotide positions of pFME5mini where IS elements were inserted in the homologous plasmids.

sequenced. Furthermore, we accomplished the restriction analysis of pFME5mini and compared its restriction profiles with the sequences of the plasmids that belong to the pND6-1 subgroup. This allowed us to find convenient restriction sites for the subcloning of replicon fragments in the pUC19 vector for further sequencing.

The entire *Hin*dIII fragment (6952 bp), including the basic replicon, has been sequenced (GenBank submission ID JQ929663). Although the hosts of pFME5 and other IncP-7 plasmids inhabit geographically distant areas, the basic replicons of these extrachromosomal elements turned out to be highly conservative. The pFME5mini sequence has 99–100% homology on an average compared to the plasmids of the pND6-1 subgroup and 84% homology compared to pCAR1. The plasmid carries the intact *repA*, *parW*, *parA*, *parB*, and *parC* genes, as well as the 5' end of *repB* (Fig. 4). The last gene encoding DNA helicase is present in all known P-7 plasmids; however, as was found, it is not the obligate component for plasmid maintenance, at least in *Pseudomonas* cells. Moreover, the *repB* gene from pDK1 was inactivated by the IS*1162* insertion. Interestingly, in terms of phylogeny (taking into account the number of base changes), pFME5mini is closer to the basic replicons of the toluene biodegradation plasmids pWW53 and pL6.5 than that of the naphthalene biodegradation plasmid pND6-1. Unlike pWW53 and pND6-1, the pFME5mini region in question is intact, i.e., it does not carry any insertions of transposons or other insertional elements (Fig. 4). The pND6-1 plasmid has IS*222* insertion of 1235 bp downstream of the *parC* gene with the duplicated GGT triplet while pWW53 has IS*Ppu20* insertion of 1598 bp between the *repB* and *repA* genes with the duplicated ATTAA sequence.

The structure of the regions of replication initiation of the P-7 plasmids Rms148 and pCAR1 was described in detail earlier [6, 12]. The pFME5mini *oriV* region has 12 iterons (binding sites of the replication initiator protein RepA) with the consensus sequence 5'-CGACTACA(G/A)ATT(A/C)CGGCT-3'. In addition, there is a sequence homologous to two reduced inverted iterons that overlap with the suggested −35 and −10 boxes of the *repA* gene promoter. Moreover, *oriV* of pFME5mini contains two AT-rich regions with 12-bp direct repeats of the TTTTNTGTTTTT consensus separated by DnaA boxes. The first DnaA box corresponds to the consensus sequence of TT(A/T)TNCACA in all representatives of the group, whereas the second DnaA box sequence is variable with the minimal divergence from the consensus in pFME5 and in other representatives of the pND6-1 subgroup. It is unknown whether both boxes are necessary for replication; however, in the case of the P1 plasmid DNA, melting in *oriV* was shown to be more effective in the presence of several DnaA boxes [18].

The *repA* gene of pFME5mini (867 bp) is 100% identical to all *repA* genes of the pND6-1 subgroup members, 96% to pCAR1, and 93% to that of Rms148. No considerable homology has been found with replication-initiator genes outside of the P-7 group. We also found a similar replication initiator in *Pseudomonas psychrotolerans* L19 using the deduced RepA amino acid sequence from pFME5mini (69% identity with 81% overlapping). Homologous replication initiators from pECB2 and pPS10 were described previously [6]. Due to the low homology of almost all ORFs of the pFME5mini with genes of plasmids other than IncP-7, below, we will only compare their amino acid sequences.

The *par* locus of pFME5mini includes the *parA* and *par*B genes, which encode a classic pair of the *trans*-acting segregation proteins, which enables the stable maintenance of many plasmids, as well as, under special conditions, the maintenance of bacterial chromosomes [19]. For P-7 plasmids, the unique 17-bp palindrome 5'-TGGTGCTCgGAGCACCA-3' was suggested as the most probable candidate for the *cis*-acting element *parS* (centromere-like sequence binding segregation proteins) [11]. This palindrome capa-

ble of binding the ParB protein was found between the *parA* and *parB* genes in all P-7 plasmids. The pFME5mini plasmid, as well as pWW53, pL6.5, and pND6-1, contain four palindrome repeats, while pCAR1 and pDK1 contain less of those (three and two, respectively). The location of the *parS* site in P-7 replicons is unique because, in other plasmids, it is located upstream or downstream of the *par* operon.

The calculated amino acid sequence of ParA (377/394 aa), which is encoded by the corresponding pFME5mini gene, is homologous to its counterparts originated from the pND6-1 subgroup (100%) and pCAR1 (83%). In addition, it has 63–65% homology to ParA proteins of various pathovars of *P. syringae*, *Pseudomonas* sp. TJI-51 (EGB 99664), and *P. putida* DOT-T1E (pGRT1) (AEK25429). It is noteworthy that the available computer programs find various *parA* translational start signals for members of the pND6-1 subgroup and the pCAR1 plasmid. This means that N-termini of ParA proteins of all plasmids with the exception of pCAR1 may be 5% longer. That is, unlike ParA of pCAR1 with 377 aa, the entire gene product can comprise 394 aa, which is consistent with GenBank annotations for plasmids of the pND6-1 subgroup. The longer variant does not suggest transcription from the promoter found by the BPROM computer program close to the start codon of the shorter ORF, though this promoter is responsible for the synthesis of the transcript containing *parA, parB*, and *parC* genes of pCAR1 [15]. However, the same authors showed that the longer transcript with the same genes can also be synthesized with slightly less efficacy, but together with the *parW* gene. The promoter responsible for the longer transcript synthesis is localized just before the *parW* gene. Therefore, the translation of the longer variants of ParA from the transcripts started before *parW* in representatives of the pND6-1 subgroup cannot be excluded. Recently, we have sequenced the *parW–parA* region of Rms148 (results not published) and found that, as in the case of pCAR1, a point mutation before the *parA* gene forbids longer polypeptide synthesis. Nevertheless, the phylogenetic distance of plasmids that belong to different subgroups raises the possibility of modifications concerning the function of their basic replicons.

Considering the ParA amino acid sequence of pFME5mini, there is a deviant Walker motif (P-loop representing ADP/ATP binding site) located between the protein N-terminus and its central part. Furthermore, the protein has two binding sites for magnesium cations. Since the ParA proteins of all plasmids that compose the group belong to the Walker ATPases and have sizes close to 400 aa, the IncP-7 segregation system can be classified the type Ia according to the conventional classification. The type Ia also includes the ParA proteins of the P1 plasmid, as well as the SopA protein of the F factor [19].

The size of the ParB protein of pFME5mini is 338 aa, that accords with the type-Ia segregation system as well. This protein identity with the pND6-1 subgroup plasmids, pCAR1 (342 aa), *Pseudomonas* sp TJI-51, together with several pathovars of *P. syringae*, is 99–100, 71, and 42% respectively. ParB usually interacts with plasmid DNA in the *parS* centromere region, which seems to be represented by 17-bp palindromes in P-7 plasmids, as was described previously in the text. The ParB-*parS* complex is recognized by the ParA ATPase with consequent polymerization and the formation of filamentary structures. The latter move the plasmid copies apart to different quarters of the cell, which enable the active segregation of plasmid clusters with the same *origin* of replication. It is generally assumed that the ParA ATPase is able to measure the cell space, which means that, for the correct positioning of plasmid copies before cell division, there is no need for specific membrane receptors encoded by the host genome [19]. Interestingly, an operon that encodes the ParAB homologs and belongs to the IncP-9 plasmids includes a gene for a TolA membrane protein with an unknown function [20]. The *parW* gene, which encodes a membrane protein with no homology to the TolA, was found in all IncP-7 plasmids.

The ParW protein of pFME5mini (159 aa) is 99–100% identical to its homologs from the pND6-1 subgroup. This protein has lower identity compared to homologs of pCAR1 (95%) and Rms148 (85%). The ParW is also 22% identical (85% overlap) to a CNPT3 (EAS38234) hypothetical protein of *Psuchromonas* sp., which is encoded by a gene located between two transposase genes and next to *parA*. Moreover, the central part of ParW from pFME5mini has some homology (32% identity) with an ascomycetes *Ajellomyces capsulatus* H88 protein, which contains trichoplein-like domain. Trichoplein/mitostatin bound to the cytoskeleton ensures the correct localization of centrosome proteins in the eukaryotic cell, as well as regulation of the cell cycle in vertebrates [21]. Using the PredictProtein and SOSUI computer programs, we identified a transmembrane domain in the ParW N-terminus of pFME5mini that consists of 12–29 amino acids, while the sequence of 1–20 aa was identified as an α-helix signal peptide. Similar structures have been predicted for all ParW representatives of the IncP-7 group. It is likely that membrane proteins encoded by the IncP-7 replicons interact with the ParA–ParB–*parS* complex, which makes it possible to precisely address the delivery of segregating plasmids to zones located under the cell membrane and/or they participate in the compaction of the segregated copies.

The *parC* gene product is a hypothetical protein that takes into account the absence of experimentally studied homologs. The ParC amino acid sequence (106 aa) of pFME5mini is conservative within the P-7 group. Compared to the corresponding proteins of the pND6-1 subgroup, pCAR1, and hypothetical protein of *P. psychrotolerans* L19, it has identity of 100, 94, and 36% (51% overlapping), respectively.

Mutation Analysis of pFME5mini

The stable maintenance of the pFME5 basic replicon in most *Pseudomonas* species (Fig. 3a) made it possible to search for pFME5mini regions responsible for replication or segregation by mutation analysis. In order to compare the stability of mutant plasmids, we employed the *P. aureofaciens* BS1393 strain as a host because of its brilliant orange colonies, which are easily distinguished from contamination when growing on nonselective media.

Figure 3b presents the results of evaluating the maintenance stability of pFME5mini *par*-defective variants. As was suggested, the *repA–oriV* region was sufficient for autonomous replication; however, this construct was lost for 24 h in more than 50% of clones. Interestingly, the minimal pCAR1 replicon, unlike pFME5, was completely eliminated from the bacterial population of *P. putida* DS1 for 2 days [12]. Slower elimination over 5–6 days was observed in the case of all defective pFME5mini variants. This can follow from the divergence of the nucleotide sequence of replicons that belong to the two subgroups, the choice of host or selective marker. In this study, we used a tetracycline-resistance marker, whereas other authors used a marker of kanamycin resistance [12]. It was shown that the elimination rate of a low-copy plasmids with the same *oriV* but different antibiotic resistance cassettes varied considerably [22]. Nevertheless, plasmids that carry Tc resistance genes were more stable than plasmids that express Km resistance.

The *parC* gene did not take part in pFME5mini stabilization as was observed for pCAR1too. This can be concluded from the same stability values of the replicon with both the intact and deleted gene. The pFME5miniΔ*parC* plasmid was successively isolated from the growing cells after 6 days of cultivation, which excludes its integration in the host chromosome.

Notably, the faster loss of pFME5miniΔ*parBC* was observed compared to pFME5miniΔ*parWABC* with the completely deleted *par* locus and to the rest of the mutant variants. It could be explained by the absence of the ParB protein, which suggests the failure of the ParB–*parS* complex to assemble. The unoccupied *parS* sequence has four large palindromes probably capable of further destabilizing the defective construct.

Thus, using the deletion-mutation analysis, we have shown that the presence of all genes that constitute the *par* locus is needed for the stable plasmid inheritance with the exception of *parC*, despite that it was found in all IncP-7 plasmids. Currently, it is unclear whether this feature is common for all group members due to the lack of data on both the structural diversity and segregation mechanisms of the IncP-7 plasmids of other subgroups (Rms148 and possibly still unknown plasmids). To better understand the functioning of the P-7 replicon, the identification of transcription factors and their binding sites within operator regions of *par* operons is needed. The investigation of the pCAR1mini defective variants using RT-PCR showed that, if deletions in the *parW*, *parA*, and *parB* genes were introduced independently, the *parW* transcript was absent, while the *parA* and *parB* transcripts were always present [12]. As was noted previously, a later study [15] revealed two large transcripts of different sizes in the *par* locus of pCAR1. Since alternative transcripts can be used for the translation of *parAB*, mutations inconsistent with *parWABC* transcription possibly does not influence the expression of the *parABC* operon. However, it is unclear if and how Par proteins of IncP-7 plasmids participate in transcriptional regulation/autoregulation.

In summary, the construction of the basic replicon of the pFME5 (IncP-7) naphthalene biodegradation plasmid with the consequent sequence analysis revealed its identity with the replicons, which we previously assigned to the pND6-1 subgroup. Nevertheless, the replicon does not contain insertion elements in the regions adjacent to *repA* and *parWABC*. Also, it was shown that pFME5mini is capable of both replication and the stable maintenance exclusively in the cells of the homologous hosts within the *Pseudomonas* genus. Our results, as well as the results of other authors [11, 12], indicate that the narrow host range of the IncP-7 plasmids is defined by both the peculiarity of their conjugative transfer and inability to replicate in cells of heterologous hosts unrelated to *Pseudomonas*. The deletion-mutation analysis of pFME5mini also revealed the need of expression of the *parWAB* genes for stable plasmid maintenance, as was observed in the case of the pCAR1 carbazole biodegradation plasmid as well. These genes encode the ParW membrane protein with unknown function, the Walker ATPase ParA (engine of the segregation machine), and the ParB protein that binds to the *parS* centromere. However, the pFME5mini variants that are defective in the *par* locus were eliminated from the bacterial population a few days later than the corresponding pCAR1mini mutants. Designed primers specific to the *repA* and *parC* genes can be used to detect the IncP-7 plasmids. Also, primers specific to the variants of the *parA* gene can be used for intragroup plasmid classification.

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