ISSN 0026-8933, Molecular Biology, 2013, Vol. 47, No. 2, pp. 205–214. © Pleiades Publishing, Inc., 2013.
Original Russian Text © O. V. Volkova, A.V. Panov, I.A. Kosheleva, A.M. Boronin, 2013, published in Molekulyarnaya Biologiya, 2013, Vol. 47, No. 2, pp. 232–242.

GENOMICS. TRANSCRIPTOMICS

UDC 576.851.132

Classification of IncP-7 Plasmids Based on Structural Diversity of Their Basic Replicons

O. V. Volkova[a], A. V. Panov[a], I. A. Kosheleva[a,b], and A. M. Boronin[a,b]

[a] *Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow Region, 142290 Russia*

[b] *Pushchino State Institute of Natural Sciences, Pushchino, Moscow Region, 142290 Russia; e-mail: volkova_o_v@inbox.ru*

Received June 30, 2012; in final form, September 27, 2012

Abstract—The structural diversity of basic replicons and *repB* gene was analyzed for the first time in a large collection of IncP-7 plasmids by PCR, restriction endonuclease analysis, and partial sequencing. It was found that the DNA fragment that contains the gene for UvrD-like helicase RepB is a part of all known P-7 replicons, but often acts as a hot insertion spot for different IS-elements. Based on the detected divergence of the *repA-oriV-parWABC* nucleotide sequence, the first system of P-7 plasmid classification has been proposed. Most degradation plasmids were classified in the β subgroup; the streptomycin resistance plasmid Rms148 (IncP-7 archetype) was placed into the α subgroup. The γ subgroup included the carbazole degradation plasmid pCAR1 and NAH/SAL-plasmids from the pY line (Yamal oil deposits), and the CAP plasmid pBS270 with a presumably reduced P-7 replicon was classified into a tentative δ subgroup. It was shown that, in most cases, the character of molecular organization of IncP-7 basic replicons did not correlate with particular phenotypic traits; that is, a given P-7 subgroup can include plasmids that encode different phenotypic markers.

DOI: 10.1134/S0026893313020167

Keywords: *Pseudomonas*, IncP-7 group plasmids, basic replicon, restriction, nucleotide sequence, *repA-oriV-parWABC* polymorphism, classification

INTRODUCTION

Ubiquitous bacteria of the genus *Pseudomonas* are known for their ability to degrade various organic compounds, including toxic and carcinogenic pollutants. On the other hand, multidrug resistance acquired by some opportunistic species of the genus can constitute a serious health problem [1]. These phenotypic traits are commonly determined by extrachromosomal genetic elements as follows: degradation plasmids and resistance plasmids (D- and R-plasmids), respectively [2, 3]. Plasmids can be classified according to phenotypic traits acquired by their host, their size, ability to conjugation transfer, etc. However, from a molecular-genetic point of view, the most informative plasmid classification system is the one by incompatibility groups, since it takes into account the differences in the structure and function of their basic replicons (regions responsible for plasmid replication and stable maintenance in bacterial generations) and reflects the evolution of these genetic elements [4]. Pseudomonad plasmids are classified into 14 incompatibility groups (IncP) [3].

Most of the known plasmids of xenobiotic and aromatic carbohydrate degradation belong to the groups P-1, P-7, and P-9. The structure of P-1 replicons and the mechanisms of their maintenance in different hosts are relatively well studied, mostly because this group contains a large number of R-plasmids and because their small size facilitates sequencing [5]. Based on the phylogenetic analysis of the plasmid backbone sequences (conserved regions free from inserted sequences and controlling plasmid replication, stable maintenance, and mobilization/conjugation transfer), six subgroups were identified within the P-1 plasmid group (α-ζ) [6]. Within the IncP-9 group, a correlation was established between the structure of basic replicons and the general structure of these plasmids, which led to the identification of nine IncP-9 subgroups (α-ι) [7, 8]. The structural diversity of IncP-7 plasmids has not yet been evaluated, and no subgroup classification has been proposed. An RFLP analysis of several degradation plasmids of the group P-7 did not produce results that allow one to identify individual subgroups [9], possibly due to the large number and diversity of mobile genetic elements introduced into the backbone or to polymorphism of the backbone itself. Complete nucleotide sequences are only known for four members of this group

Abbreviations: NAH, plasmid of naphthalene degradation; SAL, plasmid of salicylate degradation; TOL, plasmid of toluene degradation; CAP, plasmid of ε-caprolactam degradation; ORF, open reading frame.

Table 1. Bacterial strains and plasmids used in the study

Strain	Plasmid (IncP-7)	Plasmid characteristics	Source
P. fluorescens OS18	pOS18, 135 kb	Nah Sal Tra^+	Sludge collector, Nizhnekamskneftekhim, Nizhnekamsk
P. fluorescens FME4	pFME4, 77 kb	Nah Sal	
P. fluorescens FME5	pFME5, 80 kb	Nah Sal Tra^+	
P. putida AK5	pAK5, 115 kb	Nah Sal Gen	
	pEx4	Sal Cap Tra^+	Exogenous isolate, Puschino overpass
	pY1-3	Nah Sal Gen Tra^+	Exogenous isolates, oil field soils
	pY1-7	Sal Gen Tra^+	
	pY5-6	Sal Gen Tra^+	Yamal peninsula
	pBS270, 105 kb	Sal Cap Tra^+	*Pseudomonas* strains chemical plant soil Kemerovo [11]
P. fluorescens S6f	pS6f	Sal Cap Tra^+	Chemical plant soil Shchekino, Tula region
P. aureofaciens BS1393*			LPB collection
	Rms148, 180 kb	Sm^R Tra^+	Courtesy of Dr. S. Mitsuhashi (Japan)
P. aureofaciens BS1393	Rms148 mini-replicon, 2053 bp	*repA-oriV* Rms148, fused with Tc^R	[10]
P. aureofaciens BS1393	pFME5 mini-replicon, 1918 bp	*repA-oriV* pFME5, fused with Tc^R	O.V. Volkova (unpublished)
P. aureofaciens BS1393	pY1-3, ~1980 bp	*repA-oriV* pY1-3, fused with Tc^R	This study

* Species has been reclassified as *P. chlororaphis* subsp. *aureofaciens*.

Note: Encoded degradation traits: Nah is naphthalene, Sal is salicylate, Cap is caprolactam, and Gen is gentisate. Encoded resistance traits: Sm^R is streptomycin and Tc^R is tetracycline. Tra^+ is the proven ability to conjugation transfer. LPB is the Laboratory of Plasmid Biology, Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences.

(pCAR1, pND6-1, pWW53, and pDK1), and partial sequences are available for another three plasmids (pL6.5, pFME5, Rms148). The Laboratory of Plasmid Biology of the Skryabin Institute possesses a large collection of degradation and resistance plasmids of several IncP groups. From this collection, we selected 11 plasmids classified into the IncP-7 group by microbiological incompatibility tests and/or PCR analysis. The structural diversity of their basic replicons was investigated using PCR with designed specific primers, restriction endonuclease analysis, and partial sequencing; these data, along with the P-7 sequences available from the GenBank database and the results of our previous work [10], were used to propose the first subgroup structure of the IncP-7 group.

EXPERIMENTAL

Bacterial strains and plasmids used in the study are characterized in Table 1. Some plasmids were obtained previously by exogenous isolation using indigenous soil bacteria as plasmid donors and the laboratory *P. putida* strain KT2442 (courtesy of K. Smalla) as the recipient. Bacteria were grown in LB medium and in mineral saline medium [12] at 28°C. Naphthalene, salicylate, and caprolactam were supplemented to the concentration of 1 g/L. For Rms148 support, LB was supplemented with streptomycin to a final concentration of 200 μg/mL and, to select the basic plasmid replicons in *Pseudomonas* spp., it was supplemented with tetracycline to 30 μg/mL.

Plasmid DNA. Large plasmids were isolated from pseudomonads by alkaline lysis [12]; basic replicons were isolated using a ZR Plasmid Miniprep™ Classic kit (Zymo Research, United States).

PCR was performed in a Mastercycler Gradient thermal cycler (Eppendorf). Oligonucleotide primers used in the study are listed in Table 2. The reaction was performed under standard conditions using *Taq* DNA polymerase. PCR products were separated by electrophoresis in a 0.8% or 1.5% agarose gel in Tris–acetate buffer according to the conventional protocol [12]. GeneRuler 1-kb DNA Ladder (SM0311) and GeneRuler 50 bp DNA Ladder (SM0371) (Fermentas,

Table 2. PCR primers used in the study

Gene/region	Primer	Nucleotide sequence, 5' → 3'	PCR product, bp	Reference
repA	RepAP7F	GCCCATGCCGAAAAAGGTGTC	412	[10]
(IncP-7)	RepAP7R	GAATCGTTGATAGGCATCCGAC		
parA	ParANDF	TCACCTCAAGGGCTTATACG	355	O.V. Volkova
type pND6-1	ParANDR	ATCGGATCAAAGCATCTCACC		
parA	ParACARF	ATTGTTTTGGATTGACTACCCG	518	O.V. Volkova
pCAR1 type	ParACARR	ATCCTCGAGGTACGCATCAGC		
parC	ParCP7F	TGGTGCAACCGCAGACACG	267	O.V. Volkova
(IncP-7)	ParCP7R	GTGTTTGGTTGAATTGCTCATAGA		
repA-oriV	4210F	ATCGTCGTAACGCCTACAG	~1900	[10]
	6216R	GAC***GGATCC***TAATCTGGTTGCTCTC*		
repB	RepBF	TT***CTGCAG***CAAATCGCTTGAG*	2344	This study
(IncP-7)	RepBR	CT***GGATCC***ATGTGTTCAGTTTCG*		
parW	ParWF	ATCATCGACAAGATTGGTAC	299	This study
(IncP-7)	ParWR	TTGACTGTGAATTGGGTGTC		
parB	ParBNDF	AATCTCAGGGTTTCCGTTG	495	"
type pND6-1	ParBNDR	CACGAATCGAACTTTCTTCC		
parB	ParBCARF	ACCCAATTTAAGAGTTTCGG	437	"
pCAR1 type	ParBCARR	CTTCTTGGCCTTCGTTGTC		
oriV (IncP-7)	4292F	CGA***AAGCTT***CAGTATTCATTGGGTTC*	~1000	"
	5292R	CTGTAGTCGATACCCAGGAG		
oriV	4210F	ATCGTCGTAACGCCTACAG	~700	"
fragment (IncP-7)	ori630R	GAAAGACTATTTATTGGCG		

* Recognition sites of BamHI, PstI, and HindIII designed for cloning that were not used in this study are shown in bold (substituted nucleotides are underlined).

Lithuania) were used as molecular weight markers. DNA was visualized by ethidium bromide staining; PCR products were isolated from the gel using a Zymoclean Gel DNA Recovery Kit as recommended by Zymo Research.

Endonuclease treatment. DNA was cleaved with restriction endonucleases (Fermentas) for 2–6 h at 37°C (at 30°C for SmaI) as recommended by the manufacturer.

Minimal replicon of pY1-3 was constructed as described in [10]. The *repA-oriV* region was amplified with the primers 4210F and 6216R (Table 2); the tetracycline resistance gene was used as a selective marker for *P. aureofaciens* BS1393 clones that contain autonomously replicating constructs. The minimal replicon of pFME5 had been obtained previously in the same manner using the primers 4292F and 6216R (Volkova et al., in press).

Maintenance stability of Rms148 [10], pFME5, and pY1-3 minimal replicons in *P. aureofaciens* BS1393 was determined by sequential passages in liquid LB in the absence of tetracycline. Individual colonies were grown in 5 mL of broth to the late log phase; for 6 days, 50 μL of cell suspension were daily transferred into fresh broth in three replicates. Samples were taken daily; after a series of dilutions, cells were planted on LB agar plates, and 100 colonies were transferred with a replicator onto tetracycline-containing LB agar. Plasmid stability was determined as the portion of clones that retained tetracycline resistance among all clones tested (%).

DNA sequencing was performed on an automated ABI Prism 373 3130XL Genetic Analyzer (Perkin-Elmer) by the Synthol Company (Moscow, Russia). Purified PCR products obtained for different regions of minimal IncP-7 replicons with primers listed in Table 2 were used as sequencing templates.

Nucleotide sequences and derived amino acid sequences were analyzed using the DNAStar software package and pDRAW32 (ACACLONE software), as well as the BLASTN and BLASTP programs available on the NCBI site.

RESULTS AND DISCUSSION

repAB-oriV Polymorphism

The affiliation of a plasmid with a particular incompatibility group is usually determined by a standard microbiological incompatibility test; however, it is labor consuming and often inapplicable because of the peculiarities of plasmid transfer and the presence or absence of selective markers in replicons analyzed. Based on the rapidly growing nucleotide sequence databases, an increasingly popular approach is to design specific primers to most conserved plasmid backbone regions within the incompatibility group (usually minimal replicon structures). In this work, we use the term "minimal replicon" to define a plasmid region that contains the gene of a replication initiator protein (*rep*) and the site of replication origin (*oriV*), i.e., the minimal fragment required for plasmid reproduction. A minimal replicon together with partition genes (*par*) that stabilize it in the bacterial population will be considered as basic replicon. For IncP-9 plasmids, it was possible to design common primers to amplify *rep* and *oriV* regions by PCR [13]; however, for the IncP-1 group, it turned out to be impossible because of the high divergence among nucleotide sequences of minimal replicons [6].

An analysis of complete or partial nucleotide sequences of IncP-7 plasmids pND6-1, pWW53, pL6.5, pDK1, and pCAR1 (GenBank acc. nos. AY208917, AB238971, AJ250853, AB434906, and AB088420, respectively) showed that their putative basic replicon was well conserved, which enabled us to design primers to detect the *repA* gene and construct minimal replicons for this group of plasmids [10]. A PCR–RFLP analysis of *repA* fragments that represent different plasmids from our collection showed that the restriction patterns of the *repA* internal region (412 bp) were identical for all plasmids studied, except Rms148. The nucleotide sequence of the Rms148 minimal replicon was determined and compared to known *repA-oriV* sequences of IncP-7 plasmids, which enabled the construction of a phylogenetic tree with separate branches (preliminary subgroups) formed by pCAR1, Rms148, and a cluster of pND6-1-like plasmids (pND6-1, pWW53, pL6.5, and pDK1) [10]. Altogether, we analyzed *repA* amplicons from 11 plasmids in our collection (Table 1); they were isolated from the bacteria of geographically distant origins at different points in time and determine different phenotypes. It was found that, for all plasmids except Rms148, the PCR–RFLP patterns of *repA* fragments were identical and coincided with the theoretical restriction patterns of *repA* amplicons of pCAR1 and pND6-1 (electrophoregram is not shown because it is identical to that published in [10]). Thus, in contrast to sequencing, PCR–RFLP analysis of *repA* fragments is incapable of discriminating between pCAR1- and pND6-1-like replicons.

Downstream from *repA*, the four completely sequenced P-7 plasmids contain *repB*, a gene that putatively encodes a DNA helicase of the DNA and RNA helicase superfamily I. The calculated RepB amino acid sequences of these four plasmids (693 amino acids) show 99–100% identity; their C-terminal region (44% of total RepB size in IncP-7) is absolutely identical to the helicase encoded by pGRT1 (GenBank acc. no. AEK25429) and has 54–56% identity (for 69% of total IncP-7 RepB length) to helicases from different *P. aeruginosa* and *P. syringae* strains. However, no significant homology was found with functionally characterized proteins. The secondary structure of RepB contains classical ATP-binding and C-terminal domains of 3'–5'-UvrD helicases. In contrast to the Rep helicase, the functional activity of UvrD helicases is associated with reparation (excision reparation together with UvrABC and methyl-directed reparation of replication errors together with MutSLH) rather than with normal replication [14, 15]. UvrD helicases can also restart replication in the vicinity of blocked replication forks by removing RecA molecules bound to ssDNA or Tus protein bound to sites *ter* of replication termination [16, 17]. It should be noted that *tus* is not present in some IncP-7 plasmids, and conserved sequences of known *ter* sites [17] were not detected. In all P-7 plasmids, regions of the origin of replication contain several potential methylation sites GATC; in case of erroneous nucleotide incorporation in the course of replication, they can be nicked by MutH-like endonucleases of MutSLH complexes, and become starting points for DNA unwinding by an UvrD-type helicase (the mechanism of methyl-directed reparation was described for *E. coli* in [15]). It is unknown which helicases perform this function in IncP-7 plasmids (if it is significant for these replicons at all), i.e., plasmid-specific (e.g., RepB) or homologues encoded by the host chromosome. It was shown for the basic replicon of pCAR1 that the region essential for its stable maintenance in *P. putida* DS1 cells is *repA-oriV-parWAB* [18]. However, it cannot be ruled out that the *repB* product functions in other bacterial strains, under some specific conditions, or at particular stages of reproduction of these 50–200-kb plasmids.

Although *repB* is generally well conserved and was present in all four sequenced P-7 plasmids, in pDK1, *repB* ORF was interrupted by a large inserted sequence, and, in pWW53, an IS element was incorporated between the putative *repB* promoter and the translation start codon (Figs. 1a, 1c). To determine whether this gene is a part of the conserved P-7 plasmid backbone and how often it presents intact, we designed a pair of primers, RepBF and RepBR (Table 2) complementary to sequences lying 100–160 bp upstream and downstream from *repB* ORF. Many PCR products obtained with DNA of 11 IncP-7 plasmids as templates had the expected size of 2344 bp (Fig. 1b). Moreover, HaeIII and RsaI restriction pat-

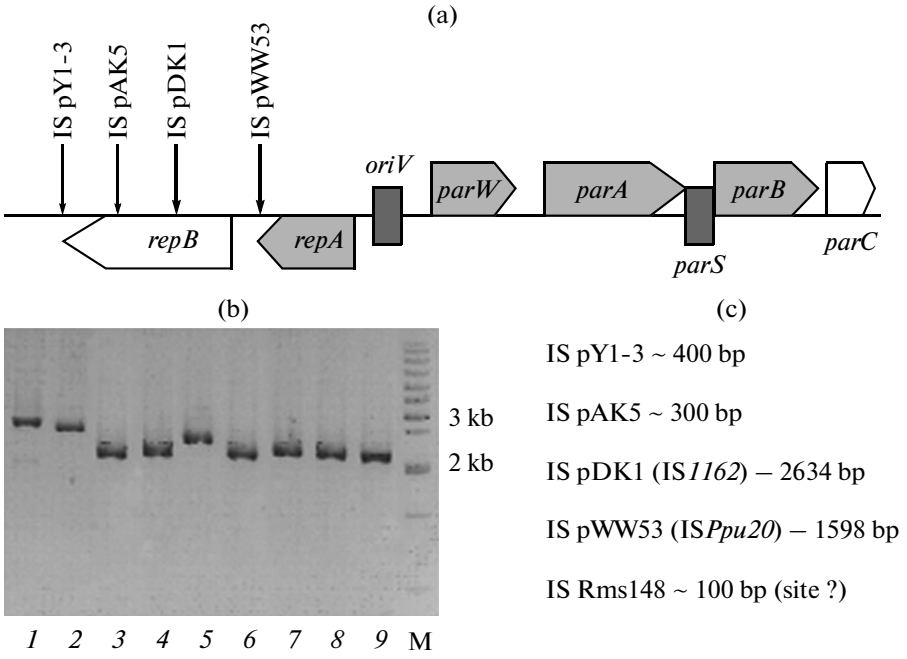

Fig. 1. Polymorphisms of *repB* and adjacent regions in IncP-7 plasmids. (a) Basic P-7 replicon and insertion loci in the *repB* region found in different plasmids of this group. Genes and regions essential for replication and stable maintenance are shaded. (b) PCR products of *repB* fragments obtained with P-7 plasmids from laboratory collection (amplified with primers RepBF–RepBR; fragment size without insertions 2344 bp): *1*, pY1-3*; *2*, pAK5; *3*, pFME4; *4*, pFME5; *5*, Rms148; *6*, pOS18; *7*, pS6f; *8*, pEx4; *9*, pBS270; *M*, molecular weight marker (1 kb DNA ladder). (c) Insertion sequences of the *repB* region of IncP-7 plasmids. *PCR products representing *repB* of pY1-7 and pY5-6 are identical to *repB* of pY1-3.

terns of all products of this size did not differ from theoretical restriction maps of the homologous region of pCAR1 and pND6-1 (data not shown). PCR products obtained with Rms148, pAK5, pY1-3, pY1-7, and pY5-6 were 100–400 bp longer than expected but, judging by their size, the incorporation of full-size IS elements or transposons in respective *repB* or adjacent sequences could be ruled out. Using crude sequencing, it was determined that the larger size of pAK5 and pY1-3 amplicons was not associated with a high divergence of *repB* sequences. pY1-3 was found to contain a unique inserted sequence of approximately 400 bp located 14 bp downstream from the *repB* stop codon without template nucleotide duplication. In pAK5, an inserted sequence of ~300 bp was incorporated in *repB* ORF 180 bp upstream from the stop codon (also without nucleotide duplication). A small part of the insert was homologous to some IncQ/IncP-4 plasmid regions adjacent to class 3 integrons or internal integron/IS element structures, and its 3'-terminal sequence had 87% identity to the 5'-inverted repeat of Tn*4654*. Apparently, both inserts are the remains of one or several IS elements/transposons that had previously been incorrectly excised from the point of insertion. The presence of some deficient integrons in the region is also possible. Thus, most IncP-7 plasmids carry intact conserved *repB* genes; furthermore, in two plasmids (pAK5 and pDK1), ORFs are disrupted by insertions and, in pWW53 and pY1-3 (and probably in its homologues, pY1-7 and pY5-6), inserts were found in direct vicinity of *repB*, i.e., this region is a hot spot for foreign DNA insertion. It is possible that, in the course of their adaptation to a narrow host range (genus *Pseudomonas*), IncP-7 plasmids ceased to require RepB helicase for vital plasmid functions. When necessary, the functions of this helicase could be performed by cellular homologues. The presence and nature of inserts do not correlate with the plasmid's assignment to any particular preliminary subgroup, but rather reflect the individual history of the replicon.

As was noted previously [10], the region of replication origin (*oriV*) slightly varies in size among plasmids of the three subgroups and is less conserved than the *repA* sequence, which suggests that plasmid subgroups could be identified by the PCR–RFLP analysis of this region. Using the pDRAW32 program, we considered theoretical restriction patterns that could be obtained by frequently cutting endonucleases for 4292F–5292R fragments of P-7 plasmids with known *oriV* sequences. The HaeIII digestion of the 4292F–5292R amplicon would produce the following fragments:

for Rms148 (1055 bp), 302 + 261 + 240 + 95 + 79 + 52 + 14 + 12 bp;

for pND6-1-type plasmids (999 bp), 220 + 205 + 117 + 116 + 114 + 112 + 52 + 38 + 14 + 11 bp;

for pCAR1 (980 bp), 539 + 246 + 117 + 52 + 14 + 12 bp.

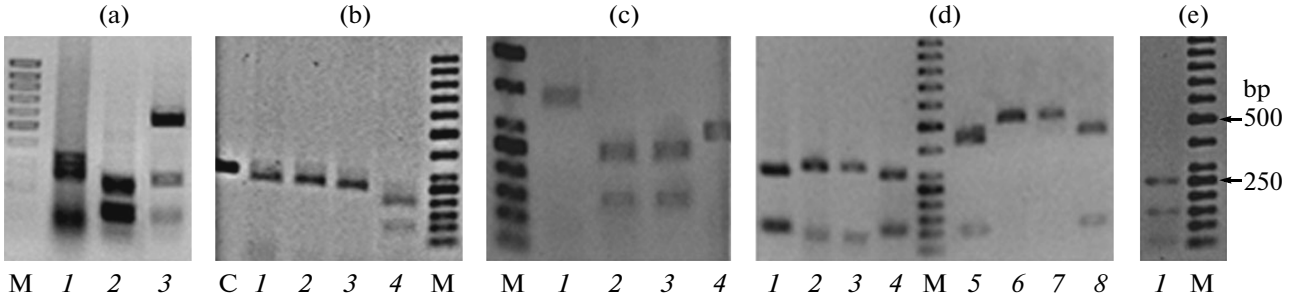

Fig. 2. PCR–RFLP analysis of *oriV* fragments (a) and genes of the *par* locus (b–e) of IncP-7 plasmids (most demonstrative examples). (a) HaeIII fragments of *oriV* PCR products (4292F–5292R) of three types of P-7 replicons: *1*, Rms148 (type I); *2*, pFME5 (type II); *3*, pY1-3 (type III). (b) RsaI fragments of *parW* PCR products (C, intact fragment of pEx4): *1*, pEx4; *2*, pS6f; *3*, Rms148; *4*, pY1-3. (c) RsaI fragments of pND6-1 type *parA* PCR products (*1–3*), and HpaII fragments of a pCAR1 type *parA* PCR product (*4*): *1*, Rms148; *2*, pEx4; *3*, pS6f; *4*, pY1-3. (d) HaeIII (*1–4*) and HpaII (*5–8*) fragments of PCR products of pND6-1 type *parA*: *1*, *5*. pFME5; *2, 6*. Rms148; *3, 7*. pS6f; *4, 8*. pAK5. (e) HpaII fragments of the PCR product of pCAR1 type *parA*: *1*, pY1-3. Here and in Fig. 3, M is molecular weight marker (50 bp DNA ladder).

Using PCR with primers 4292F and 5292R, we obtained *oriV* fragments (~1000 bp) for all plasmids of the laboratory collection except the CAP plasmid pBS270. The HaeIII treatment of these amplicons produced restriction patterns of all three types (Fig. 2a). The only amplicon that produces a type-I (Rms148) pattern was Rms148 itself, while pFME4, pFME5, pAK5, pOS18, pEx4, and pS6f amplicons produced type-II (pND6-1) patterns, and pY1-3, pY1-7, and pY5-6 (pY line) produced type-III (pCAR1) patterns.

An analysis of the known P-7 plasmid sequences revealed a variable *oriV* region adjacent to the *par* locus. The amplification and sequencing of this region could help to determine the level of homology among *oriV* of pY1-3, pY1-7, pY5-6, and pCAR1, since pCAR1 was previously considered to be a unique P-7 plasmid that is phylogenetically remote from other members of the group. The sequencing of 700-bp amplicons obtained with primers 4210F and ori630R on pY plasmid templates (Yamal peninsula) did not detect any differences from the homologous region of pCAR1 (Japan). In pBS270, the 4292F–5292R fragment could not be amplified, probably because of the lacking 4292F binding site distal from *repA* or because of the low homology between the primer and pBS270 sequences, which is less probable. The 1400-bp fragment, the only one that could be amplified on the pBS270 template, included *repA* and the adjacent *oriV* fragment (approximately up to the putative *par* promoter). Its sequence differed from the homologous region of pND6-1 type replicons by several substituted nucleotides. The 1400 bp fragment does not include all elements necessary for replication initiation [18], but it contains seven interons, which are plasmid incompatibility determinants. Thus, PCR–RFLP analysis of the *oriV* region makes it possible to classify IncP-7 plasmids in three types, which coincide with the subgroups suggested in our previous work [10]. The CAP plasmid pBS270 is an exception that is probably related to the potential reduction of the basic P-7 replicon and tentatively classified into a separate subgroup.

Polymorphism of *par* Locus

Since *par* locus is a part of the conserved plasmid backbone and its certain regions (in particular, *parS*, i.e., the centromere) are involved in the incompatibility phenomenon [4], and *parWABC* genes of the partition apparatus were found in all known plasmids of the P-7 group, we thought it promising to investigate whether intragroup classification could be improved based on the polymorphism of these genes. Specific primers were designed to internal sequences of *parW* and *parB*. Since *parB* genes of pCAR1 and plasmids of the pND6-1 subgroup have only 73% similarity, primers were designed so as to differentiate between these two gene variants (Table 2). Two primer pairs for amplifying different *parA* variants (pCAR1 and pND6-1 types showed 83% identity) and a pair of primers specific to *parC* common for the whole group were previously designed. The results of PCR with these primers (except for *parC* amplicons) are shown in Fig. 3. PCR products of *parW* and *parC* were obtained with all plasmid templates except pBS270. It should be noted that *parW* amplification from the Rms148 template could not always be reproduced, probably because ParWF and/or ParWR are not sufficiently specific to the gene sequence in Rms148. In plasmids of the line Y, both *parA* and *parB* fragments could only be amplified with primers specific to pCAR1 genes. Interestingly, primers specific to pND6-1 type variants of *parA* and *parB* were efficient in the amplification of gene fragments not only from the pFME4, pFME5, pAK5, pOS18, pEx4, and pS6f templates, but also from Rms148. No elements of the *par* locus were found in pBS270.

Similar to the *oriV* analysis, we used pDRAW32 to develop the best differentiating PCR–RFLP protocol

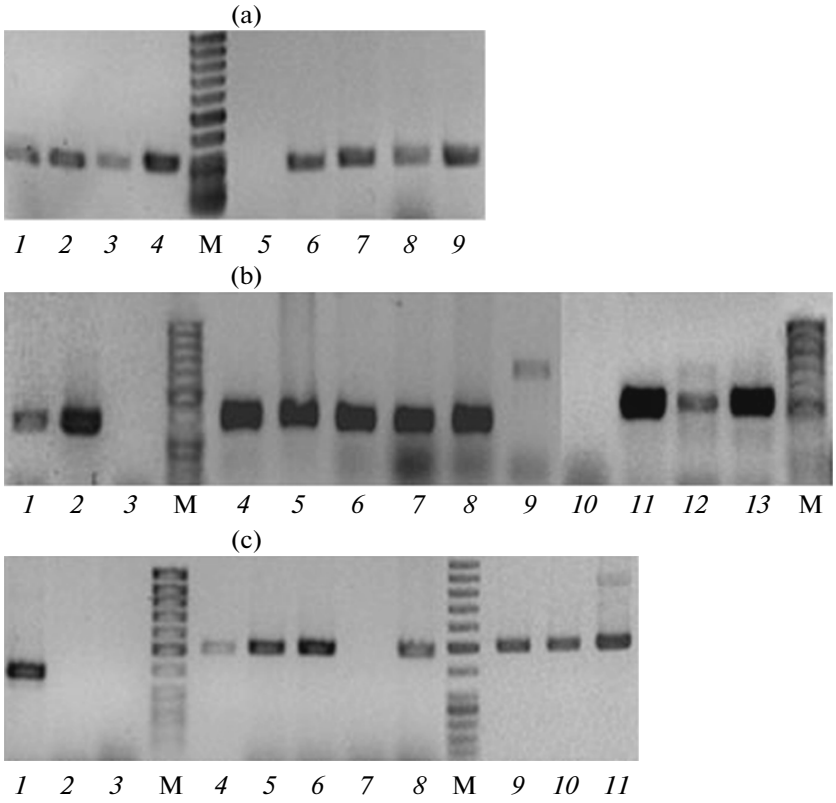

Fig. 3. Amplified gene fragments of the *par* locus of IncP-7 plasmids. (a) PCR products representing *parW* (299 bp): *1*. pFME4; *2*. pY1-3; *3*. Rms148; *4*. pFME5; *5*. pBS270; *6*. pOS18; *7*. pAK5; *8*. pS6f; *9*. pEx4. (b) PCR products representing *parA* type pND6-1 (355 bp, *1–9*) and type pCAR1 (518 bp, *10–13*): *1*. pFME4; *2*. Rms148; *3*. pBS270; *4*. pFME5; *5*. pOS18; *6*. pAK5; *7*. pS6f; *8*. pEx4; *9*. pY1-3; *10*. pBS270; *11*. pY1-3; *12*. pY1-7; *13*. pY5-6. (c) PCR products representing *parB* of pCAR1 type (437 bp, *1–3*) and pND6-1 type (495 bp, *4–11*): *1*. pY1-3; *2*. pBS270; *3*. Rms148; *4*. pFME4; *5*. Rms148; *6*. pFME5; *7*. pBS270; *8*. pOS18; *9*. pAK5; *10*. pS6f; *11*. pEx4.

for *parWAB* genes. The *parW* amplicon (299 bp) is hydrolyzed by RsaI into fragments of 258, 23, and 18 bp for pND6-1- type plasmids and in 183, 75, 23, and 18 bp for pCAR1 type plasmids. Figure 2b shows examples of PCR–RFLP patterns for *parW* amplicons of several plasmids. Restriction patterns of pFME4, pFME5, pAK5, pOS18, pEx4, pS6f, and Rms148 belonged to the pND6-1 type, and those of pY1-3, pY1-7, and pY5-6 belonged to the pCAR1 type. However, we determined the nucleotide sequence of Rms148 *parW* and found that it was different from other *parW* sequences of the pND6-1 type; it lacked one RsaI recognition site (258 + 41 bp), which could not be detected by electrophoresis. *parW* nucleotide sequence identity was 87% for Rms148 and pND6-1 type plasmids, 82% for Rms148 and pCAR1, and 91% for pCAR1 and pND6-1 type plasmids. Thus, the restriction analysis of the ParWF–ParWR fragment did not enable discrimination between Rms148 and pND6-1-like replicons, although the corresponding *parW* sequences differ considerably.

parA amplicons of the pND6-1 type (355 bp) were cleaved by RsaI in two fragments (233 + 122 bp), while the *parA* amplicon of Rms148 remained intact (Fig. 2c). It was found that the first third of the *parA* nucleotide sequence of Rms148 had 90–91% identity to the corresponding region of pND6-1 type plasmids and 71% identity to that of pCAR1. *parA* amplicons of pY plasmids (518 bp) were cleaved by HpaII in two fragments that were identical in size to those theoretically expected for the corresponding pCAR1 sequence, i.e., 255 + 263 bp.

Amplified *parB* fragments of the pND6-1 type (495 bp) were treated independently with two endonucleases, HaeIII and HpaII (Fig. 2d). RFLP patterns obtained with HpaII were more informative concerning *parB* polymorphism, even within the pND6-1 cluster. For all plasmids of this group, except pS6f, HpaII hydrolysis of *parB* amplicons produced fragments 419 and 76 bp long. The *parB* fragment of pS6f was not cleaved by this enzyme, and neither was the one of Rms148. Similar differences were observed when *parB* fragments were treated with HaeIII. *parB* amplicons of pY plasmids (437 bp) were cleaved by HpaII in three fragments (Fig. 2e) that corresponded to the calculated fragments of the pCAR1 amplicon, i.e., 238, 114, and 85 bp.

Interestingly, the phylogenetic similarity between the *par* loci of Rms148 and pND6-1 type replicons is closer than between those of Rms148 and pCAR1, or between pND6-1 and pCAR1. At the same time, the identity level between *parW* genes of pCAR1 and pND6-1 type plasmids (91%) is higher than between those of Rms148 and pND6-1 (87%). On one hand, this fact may suggest that minimal P-7 replicons have independently acquired *parW* and *parAB* modules that had already slightly diverged from their common ancestor variant; on the other hand, these modules might have undergone different nucleotide adaptation (amelioration) to the genetic environment of their bacterial host. Indeed, the main function of *parAB* products is specific interaction with particular regions of plasmid DNA ensuring their efficient partition, whereas, judging by its predicted secondary structure, the *parW* product is a membrane-bound partition protein and is probably involved in closer interactions with bacterial cell structures than the ParAB complex. Initial hosts of the streptomycin resistance plasmid Rms148 are clinical isolates of *P. aeruginosa* [19], and P-7 degradation plasmids originate from soil and aquatic pseudomonad strains phylogenetically remote from *P. aeruginosa*. Thus, the nucleotide divergence of *parW* sequence could correlate with the difference in the historic host range of IncP-7 plasmids, especially considering that the variation of the *parW* region that encodes the transmembrane domain (N-terminal amino acids 12–29) is probably the highest. Remarkably, the three *parW* types also use different translation stop codons (UAA in the pND6-1 subgroup, UGA, in pCAR1, and UAG in Rms148), whereas all other *par* genes in all types of plasmids use the UGA stop codon.

In general, the results of PCR–RFLP analysis of the *par* locus (accompanied with sequencing for dubious cases) confirm the classification of IncP-7 plasmids into four subgroups based on the minimal replicon polymorphism (pBS270 was isolated into an individual subgroup because of the negative results of PCR with primers to *par* genes and the adjacent *oriV* region).

Minimal Replicons of the pCAR1 Type Are Eliminated from the Pseudomonad Population Faster than Other Types of Minimal Replicons

An analysis of the pCAR1 and Rms148 minimal replicons (*repA-oriV* fused to a selective marker) [10, 18] showed that their replication in pseudomonads did not require further plasmid genes and regions, but their stable maintenance depended on the expression of *par* genes [18]. We have previously obtained the minimal replicon of the naphthalene degradation plasmid pFME5 and shown that it was completely eliminated from the *P. aureofaciens* BS1393 population several days later than the minimal pCAR1 replicon was eliminated from *P. putida* DS1 (Volkova et al., in press). However, pFME5 and pCAR1 were studied in different hosts and with different selective markers, so the phenomenon could not be definitely linked to the structure and properties of the replicons as such. After pCAR1-like plasmids (pY line) were discovered, the experiment could be repeated in standardized conditions. The stability of *repA-oriV* maintenance in *P. aureofaciens* BS1393 in nonselective conditions was evaluated for 6 days for replicons derived from Rms148 and pY1-3 and compared to a similar chart obtained for the minimal replicon of pFME5 (Fig. 4). As was expected, all replicons were gradually eliminated from the bacterial population due to the lack of the *par* locus; however, the *repA-oriV* of pY1-3 was lost more rapidly. No significant differences were found between the rates of Rms148 and pFME5 minimal replicon elimination. Most likely, the structure of the region of replication origin that is characteristic of pCAR1 type plasmids does not support efficient interactions with some host factors that could partially balance the lack of an own *par* locus. Interestingly, all pY plasmids, as well as pCAR1, contained a 18-bp deletion in the *oriV* region adjacent to *parW* and including the first AT-rich fragment (compared to other types of replicons). Possible differences in the number of copies of these minimal replicons could also play a role.

IncP-7 Replicons Are Classified in Four Subgroups that Do Not Follow the Rule of One Subgroup to One Encoded Phenotype

Using PCR–RFLP analysis and, in some cases, the sequencing of basic replicon fragments, we classified plasmids of the incompatibility group P-7 in four clusters (subgroups) as follows: Rms148, pND6-1 type replicons, pCAR1 type replicons, and pBS270. The IncP classification system implies the subgroup notation using Greek letters; therefore, the above clusters should be referred to as subgroups α, β, γ, and δ, respectively. Subgroup α is represented by the only plasmid of antibiotic resistance, Rms148 (P-7 archetype). Subgroup β includes most P-7 degradation plasmids, i.e., pND6-1, pWW53, pL6.5, pDK1, pOS18, pAK5, pFME4, pFME5, pEx4, and pS6f. This is the largest subgroup, but basic replicon sequences of these plasmids are highly conserved, except for the difference in the *parS* palindrome repeats number [20] and for *parB* polymorphism (the *parB* amplicon of pS6f showed a restriction pattern similar to that of Rms148). Subgroup γ includes pCAR1, pY1-3, pY1-7, and pY5-6. For some reasons, in nonselective conditions, *repA-oriV* minimal replicons of this type are eliminated from a pseudomonad population faster than α or β type replicons. The caprolactam/salicylate degradation plasmid pBS270 was classified into a tentative subgroup δ. Apparently, it contains a reduced P-7 replicon, which probably cannot support autonomous plasmid maintenance, but shows incompatibility with other IncP-7 plasmids [11]. It is also possible that, due to a recombination event, the minimal P-7

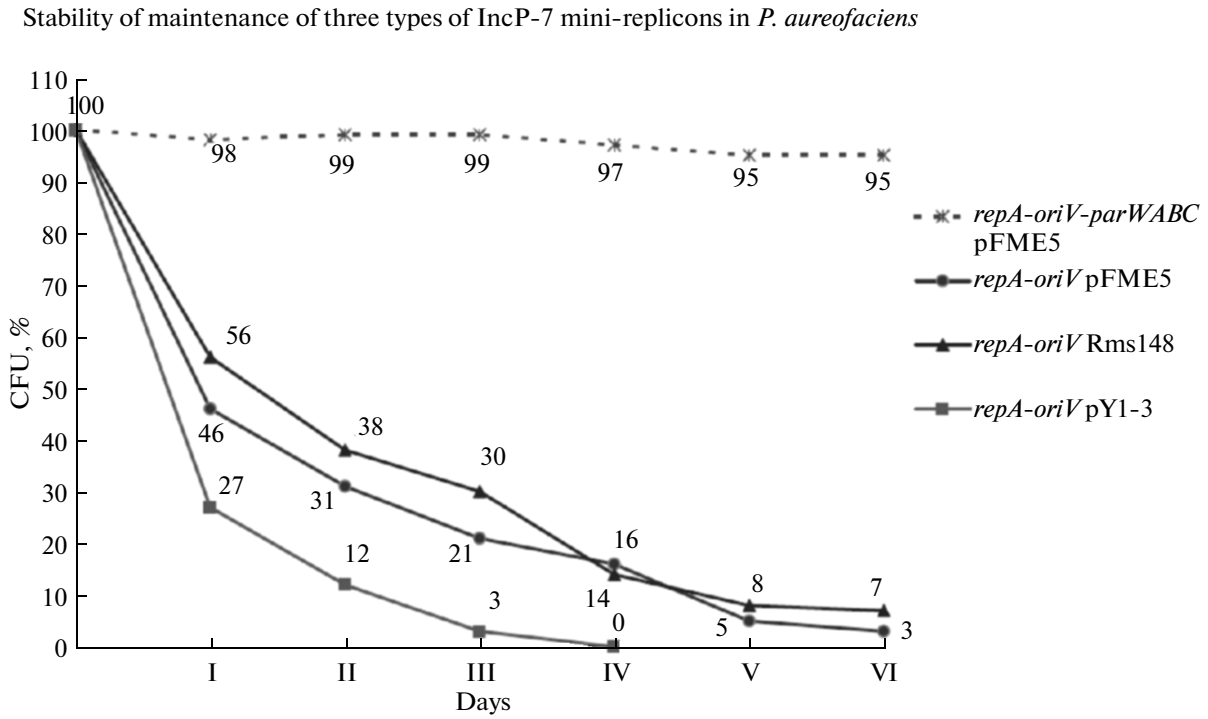

Fig. 4. Maintenance stability of pFME5, Rms148, and pY1-3 minimal replicons (*repA-oriV*) representing different IncP-7 phylogenetic clusters. The *repA-oriV-parWABC* variant of pFME5mini is given for comparison, since it contains the stabilizing *par* locus.

replicon of pBS270 includes a partition system of a different type (including the promoter-operator region). In any case, pBS270 is stably maintained in pseudomonads. Recently, we found this plasmid to comprise a replication initiation region that was not homologous to IncP-7 minimal replicons (data not published). It is still unknown whether this region is associated with any partition system. Moreover, the observed weak incompatibility between pBS270 and IncP-9 plasmids [11] may suggest that pBS270 comprises (possibly partially) a third replicon, i.e., a P-9 homologue (it could not be detected by PCR with standard primers to *rep-ori* of IncP-9 type).

In general, basic replicons of known P-7 plasmids are well conserved, despite their wide range of geographic origin. The level of nucleotide sequence divergence among subgroups is only 7–8% for *repA* (compared to 8–26% among P-9 plasmids), and 24–27% for *oriV* (7–36% in IncP-9). This is not surprising taking into account that the host range of IncP-7 plasmids is narrow; actually restricted to the genus *Pseudomonas*; and, at the extreme, closely related bacteria. However, the general polymorphism of these plasmids is very high [9], which is probably related to a large number of inserted sequences and their remnants present throughout the plasmid backbone (in particular in the *repB* region) and to the high frequency of recombination events between P-7 replicons and replicons of other types (e.g., pBS270).

A characteristic feature of the IncP-7 group is the lack of correlation between the basic replicon structure and the phenotypic traits encoded by plasmids of the same subgroup. For instance, one of IncP-9 subgroups includes the caprolactam degradation plasmids, while another includes naphthalene degradation plasmids; antibiotic resistance plasmids fall into a third subgroup (the only exception is subgroup β that includes both TOL and NAH plasmids) [8]. No such correlation was found for most P-7 plasmids, i.e., naphthalene and salicylate degradation are encoded by plasmids of both β (pND6-1, pOS18, pAK5, pFME4, pFME5) and γ subgroups (pY1-3, pY1-7, pY5-6); the β subgroup includes also TOL (pWW53, pL6.5, pDK1) and CAP/SAL plasmids (pEx4, pS6f), and the γ subgroup includes a carbazole/dioxin degradation plasmid pCAR1. It is difficult to say whether the α subgroup is an exception in this respect because no homologues of the Rms148 basic replicon were found and other IncP-7 R-plasmids are unavailable for study.

Thus, we have for the first time investigated the structural diversity of basic replicons and *repB* sequences in a large sample of IncP-7 plasmids by PCR–RFLP and selective sequencing. The evaluation of basic replicon polymorphism among plasmids of our laboratory collection and replicons available in the GenBank database enabled us to propose the first system of the intragroup classification of P-7 plasmids;

the four subgroups of this system were identified taking into account the divergence of both *repA-oriV* and *par* nucleotide sequences. In contrast to the incompatibility group P-9, in most cases, the organization of IncP-7 basic replicons was not correlated with particular phenotypic traits encoded by these plasmids. Primer sets designed in this study can be used to detect and classify new IncP-7 plasmids.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (project no. 12-04-90041-Bel_a).

REFERENCES

1. Strateva T., Yordanov D. 2009. *Pseudomonas aeruginosa*: A phenomenon of bacterial resistance. *J. Med. Microbiol.* **58**, 1133–1148.
2. Top E.M., Moenne-Loccoz Y., Pembroke T., Thomas C.M. 2000. Phenotypic traits conferred by plasmids. In: *The Horizontal Gene Pool: Bacterial Plasmids and Gene Spread*. Ed. Thomas C.M. Amsterdam: Harwood, pp. 246–285.
3. Boronin A.M. 1992. Diversity of *Pseudomonas* plasmids: To what extent? *FEMS Microbiol. Lett.* **79**, 461–467.
4. Novick R.P. 1987. Plasmid incompatibility. *Microbiol. Rev.* **51**, 381–395.
5. Adamczyk M., Jagura-Burdzy G. 2003. Spread and survival of promiscuous IncP-1 plasmids. *Acta Biochimica Polonica*. **50**, 425–453.
6. Norberg P., Bergstrom M., JethavaV., Dubhashi D., Hermansson M. 2011. The IncP-1 plasmid backbone adapts to different host bacterial species and evolves through homologous recombination. *Nature Commun*. **2**, 268. Epub. www.nature.com/ncomms/journal/v2/n4/full/ncomms1267.html
7. Izmalkova T.Yu., Mavrodi D.V., Sokolov S.L., Kosheleva I.A., Smalla K., Thomas C.M., Boronin A.M. 2006. Molecular classification of IncP-9 naphthalene degradation plasmids. *Plasmid*. **56**, 1–10.
8. Sevastsyanovich Y.R., Krasowiak R., Bingle L.E.H., Haines A.S., Sokolov S.L., Kosheleva I.A., Leuchuk A.A., Titok M.A., Smalla K., Thomas C.M. 2008. Diversity of IncP-9 plasmids of *Pseudomonas*. *Microbiology* (UK). **154**, 2929–2941.
9. Izmalkova T.Yu., Sazonova O.I., Sokolov S.L., Kosheleva I.A., Boronin A.M. 2005. The P-7 incompatibility group plasmids responsible for biodegradation of naphthalene and salicylate in fluorescent pseudomonads. *Microbiology* (Moscow). **74** (3), 290–295.
10. Volkova O.V., Kosheleva I.A., Boronin A.M. 2012. Structure of replication initiation region in *Pseudomonas* IncP-7 streptomycin resistance plasmid Rms148. *Mol. Biol*. (Moscow). **46**, 542–547.
11. Esikova T.Z., Grishchenkov V.G., Boronin A.M. 1990. Plasmids responsible for ε-caprolactam biodegradation. *Mikrobiologiya*. **4**, 547–552.
12. Sambrook J., Russel D.W. 2001. *Molecular Cloning: A Laboratory Manual*, 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Lab. Press.
13. Greated A., Thomas C.M. 1999. A pair of PCR primers for IncP-9 plasmids. *Microbiology* (UK). **145**, 3003–3004.
14. Kumari A., Minko I.G., Smith R.L., Lloyd R.S., McCullough A.K. 2010. Modulation of UvrD helicase activity by covalent DNA-protein cross-links. *J. Biol. Chem*. **285**, 21313–21322.
15. Yamaguchi M., Dao V., Modrich P. 1998. MutS and MutL activate DNA helicase II in a mismatch-dependent manner. *J. Biol. Chem*. **273**, 9197–9201.
16. Veaute X., Delmas S., Selva M., Jeusset J.,Le Cam E., Matic I., Fabre F., Petit M.-A. 2005. UvrD helicase, unlike Rep helicase, dismantles RecA nucleoprotein filaments in *Escherichia coli*. *EMBO J*. **24**, 180–189.
17. Neylon C., Kralicek A.V., Hill T.M., Dixon N.E. 2005. Replication termination in *Escherichia coli*: Structure and antihelicase activity of the Tus–*Ter* complex. *Microbiol. Mol. Biol. Rev.* **69**, 501–526.
18. Shintani M., Yano H., Habe H., Omori T., Yamane H., Tsuda M., Nojiri H. 2006. Characterization of the replication, maintenance, and transfer features of the IncP-7 plasmid pCAR1, which carries genes involved in carbazole and dioxin degradation. *Appl. Environ. Microbiol*. **72**, 3206–3216.
19. Sagai H., Hasuda K., Iyobe S., Bryan L.E., Holloway B.W., Mitsuhashi S. 1976. Classification of R plasmids by incompatibility in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **10**, 573–578.
20. Yano H., Miyakoshi M., Ohshima K., Tabata M., Nagata Y., Hattori M., Tsuda M. 2010. Complete nucleotide sequence of TOL plasmid pDK1 provides evidence for evolutionary history of IncP-7 catabolic plasmids. *J. Bacteriol*. **192**, 4337–4347.