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Structure of Replication Initiation Region in *Pseudomonas* IncP-7 Streptomycin Resistance Plasmid Rms148

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Abstract—*Pseudomonas*' IncP-7 plasmids play significant role in the environmental biodegradative potential and sometimes carry antibiotic-resistance genes. Rms148 plasmid was used as archetypal P-7 plasmid in microbiological incompatibility studies for more then 30 years. However, the structure of its basic replicon was not described until now; furthermore, the phylogenetic relationships between all known plasmids within the IncP-7 group have not yet been studied. In the course of our study, we have constructed two pairs of primers to amplify the main components of the region of the initiation of P-7 replication, and the subsequent screening of *repA* intragenic polymorphism was performed using the laboratory collection of IncP-7 plasmids. The minimal replicon of Rms148 was determined and its nucleotide sequence was found to be 81–83% identical to *repA-oriV* of known P-7 plasmids and is considered to fall into a separate clade of the corresponding phylogenetic tree. Additionally, *repA* group members seem to be more conservative than the putative *oriV* region. The estimated amino acid sequence and predicted secondary and tertiary structures of Rms148 RepA protein allowed us to make the assumption that the initiation of replication in plasmids of the P-7 incompatibility group is described by the same model as for the unclassified cryptic plasmid pPS10.

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Nowadays, plasmids are usually classified using incompatibility features. The incompatibility phenomenon is associated with the special structure and functioning properties of the initial replicon, i.e., the region responsible for the replication and maintenance of the stable plasmid [1]. In addition to the whole block of unclassified extrachromosomal elements, there are 14 incompatibility groups in the classification of *Pseudomonas* (IncP) plasmids [2]. Several groups, including P-1, P-7, and P-9, are of major interest for biotechnological purposes, due to their pollutants, e.g., naphthalene, toluol, dioxin, as well as their biodegradation properties. The structure of replicons of the P-1 and P-9 incompatibility groups, as well as their replication, maintenance control, and host range have been studied quite well [3, 4]. As has been found, there is a correlation between the basic structure of replicons of the P-9 incompatibility group and the structure of plasmids as a whole [5], which formed the basis for classifications within subgroups of these plasmids [4]. An analysis of the restriction of P-7 group biodegradation plasmids has allowed us to identify separate subgroups inside of group [6], which could be associated with the high polymorphism of mobile genetic elements and the sites of their integration into the plasmid core, or the core itself, which includes the regions responsible for the replication, stable maintenance, and mobilization/conjugation transfer of the plasmids. Despite that the plasmids of this group are usually isolated from the oil-product polluted water and soil samples and play an important role in the biodegradation potential of the environment, there is a lack of information on their diversity, their range of natural and potential hosts, structural polymorphism, and the functioning principles of their basic replicons. Some antibiotic resistance plasmids (R-plasmids), which belong to the IncP-7 subgroup, have also been described [7, 8]; these plasmids are of major interest to the clinical microbiology.

Streptomycin phosphotransferase, which encodes the Rms148 plasmid (182 kbp) was isolated from clinical *Pseudomonas aeruginosa* isolates in one of the Frankfurt clinics and serves as an archetype of the P-7 incompatibility group in the clinical trial on the incompatibility of the plasmid for more then 30 years in the laboratories of different countries [8]. Nevertheless, the information on its basic replicon structure is still inaccessible. In addition, a comparison of the region of replication initiation of Rms148 and sub-

group representatives with known nucleotide structures is necessary to classify a P-7 group plasmids.

EXPERIMENTAL

Target subject. The presence of IncP-7 group plasmids was identified in the strains of different *Pseudomonas* species acquired from the collection of the Laboratory of Plasmid Biology, Institute of Biochemistry and Physiology of Microorganisms Russian Academy of Sciences. The *P. aureofaciens* BS1393 strain was used as a host for the Rms148 minireplicon. The bacteria were grown at 28°C in LB [9] medium. Tetracycline (for the Rms148 minireplicon selection) and streptomycin (for the selection of the complete Rms148) were added to the LB medium to a final concentration of 30 and 200 μg/mL, respectively.

Plasmid DNA was isolated using the alkaline lysis method according to [9] with some modifications. ZR Plasmid Miniprep™-Classic kit (Zymo Research, United States) was used to isolate the minireplicon from *P. aureofaciens* BS1393.

Polymerase chain reaction (PCR) and DNA visualization. PCR was carried out in a Mastercycler Gradient thermal cycler (Eppendorf, Germany). Two pairs of olygonucleotyde primers were used, i.e., RepAP7F1 (5'-GCCCATGCCGAAAAAGGTGTC) and RepAP7R1 (5'-GAATCGTTGATAGGCATCCGAC) for *repA* gene detection and 4210F (5'-ATCGTCGTAACGCCTACAG) and integrated site of BamHI restriction endonuclease recognition containing 6216R (5'-GACGgAtcCTAATCTGGTTGCTCTC); this site was used in the current work to acquire Rms148 minireplicons. The reaction was carried out under standard conditions with the use of Taq-DNA-polymerase with a primer annealing temperature of 53°C. Electrophoresis was carried out n the 0.8 and 1.5% agarose gels in Tris-acetate buffer according to standard protocol [9]. DNA was visualized with Ethidium Bromide gel staining.

DNA was isolated from the gel using Zymoclean Gel DNA Recovery Kit (Zymo Research, United States) according to the manufacturer's protocol.

Restriction analysis of the amplified *repA* gene fragments was performed using HaeIII enzyme at 37°C for 2 h in accordance with the manufacturer's recommendations (Fermentas, Lithuania).

Construction of minimal Rms148 replicon. A Rms148 DNA fragment 2 kbp long containing a region for controlling the replication of the IncP-7 group amplified by 4210F and 6216R was isolated from the agarose gel and treated with a Klenow fragment (Sibenzim, Russia) in order to acquire blunt ends. The fragment was then ligated to a tetracycline resistant cassette and excised from the p34S-Tc vector with SmaI restriction endonuclease (Fermentas, Lithuania). Ligation was carried using T4 phage DNA-ligase according to the manufacturer's protocol (Fermentas). After 10–12 h of incubation at 18°C; 5–8 μL of mixture were added to fresh, precooled *P. aureofaciens* BS1393 strain cells; and electroporation was performed (voltage was 1.7 kV and electrode distance of 0.1 cm) in a MicroPulser (BioRad) electroporator. The cells were incubated for 1–1.5 h at 28°C in LB medium, then seeded onto tetracycline containing agar medium; the colonies that formed after 1–2 days of incubation were tested to see whether they contained plasmid DNA.

DNA sequencing was performed on an automated DNA sequencer ABI Prism 373 3130XL Genetic Analyzer (Perkin-Elmer) at Synthol scientific-production company (Moscow, Russia).

Nucleotide and amino acid sequences were analyzed using DnaStar, BLAST, Jred3, PredictProtein, and BPROM software (Softberry). CLUSTAL and TREECON programs were used to determine the phylogenetic tree [10].

RESULTS AND DISCUSSION

repA Gene Polymorphism in P-7 Incompatibility Group Plasmids

The replication-initiation protein encoded by the plasmid (RepA is used for protein indication in the case of IncP-7 incompatibility) is needed for replication and the manifestation incompatibility in the *Pseudomonas* genus, since their plasmids usually demonstrate theta-type replication [11]. The nucleotide sequence of the Rep-protein gene is usually sufficient to reach a conclusion about whether the plasmid belongs to certain incompatibility group and, in some cases, the polymorphism of this gene allows one to establish an intragroup classification system. We used primers to inner region of the *repA* gene designed by us using the known nucleotide sequences of P-7 plasmids; a PCR was performed using Rms148 and biodegradation plasmids (D-plasmids) incompatible with Rms148 in standard microbiological test as a matrix. PCR fragments with estimated sizes of 412 bp were acquired in the majority of cases. *repA* gene polymorphism was evaluated by the restriction analysis of the corresponding amplified fragments of pFME4, pFME5, pBS270, pAK5, pS6f, and Rms148 plasmids.

It was discovered that only Rms148 (the only R-plasmid in the experiment) had a unique restriction map, i.e., different from other maps, of the inner region of the *repA* gene (Fig. 1). Notably, the restriction profiles of all plasmids, except for Rms148, were consistent with the virtual restriction maps of *repA* gene of partially or completely sequenced P-7 plasmids, including pND6-1, pWW53, pL6.5, pDK1, and pCAR1 (nucleotide sequences are available from GenBank under AY208917, AB238971, AJ250853, AB434906, and AB088420 accession numbers, respectively). The discovered differences in the protein

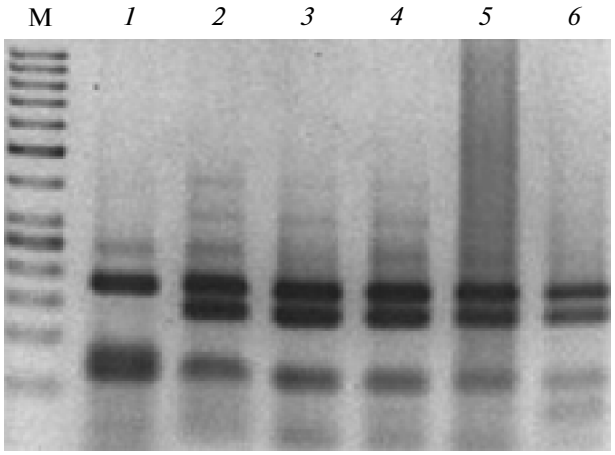

Fig. 1. Electrophoregram of IncP-7 group plasmids *repA* gene PCR fragments (412 bp) HaeIII restriction analysis. 500 bp DNA ladder was used as a molecular weight marker (M); the results for (*1*) Rms148 streptomycin resistance (Sm^R), (*2*) pFME4 naphthalene biodegradation (NAH), (*3*) pAK5 (NAH), (*4*) pFME5 (NAH), (*5*) pBS270 caprolactam biodegradation (CAP) and salycylate biodegradation (SAL), and (*6*) pS6f (CAP, SAL) are shown.

structure of the Rms148 initiator, as well as the fact that Rms148 is the only IncP-7 group antibiotic resistance plasmid available so far, were the reasons for the determination of the complete nucleotide sequence of the *repA* gene and the replication origin (*oriV*) of this plasmid.

Rms148 Minimal Replicon Structure

It is necessary to identify the minimal replicons of plasmids for the isolation and further analysis of the regions responsible for replication. If one has a minimal replicon, it is possible to claim that the specified plasmid region is sufficient for the plasmid amplification inside the host. There are several strategies for constructing minireplicons. The most widely used approach is the partial hydrolysis of plasmid DNA with frequently cutting of Sau3AI endonuclease and the subsequent ligation of a mixture of fragments with an antibiotic resistance cassette required for the further selection of plasmid containing bacteria clones. Plasmid fragments able to self-replicate are selected as a result of the transformation of bacteria cells with the acquired constructions. The specific feature of this method is the relative unpredictability of the size and, thus, the composition of selected fragments, which can cause problems in a study of the target region. Therefore, the most radical way to limit the replicon was chosen; i.e., the *repA-oriV* site was amplified without the adjacent *par* genes, which are required for correct segregation in order to maintain a stable replicon under nonselective conditions. In this case, the genome area between the *repA* gene and the preceding *orf* was considered to be *oriV*. The obtained Rms148 fragment of approximately 2 kbp in size (not counting tetracycline cassette) had the ability to self-replicate in the cells of the *P. aureofaciens* BS1393 strain, even after multiple passages under selective conditions.

The determination of the nucleotide sequence of the Rms148 minireplicon (deposited in GenBank under JN698877 accession number) revealed the following key components of replication initiation (Fig. 2):

1) thirteen 18-membered tandem repeats, potential iterons, which interact with the RepA protein in order to start the replication, with a unique CGACTACA(G/A)ATT(C/A)CGGCT consensus and additional reduced direct and inverted repeats (or one inverted repeat, partially homological to iterons), which overlaps with putative –35 and –10 *repA* gene promoter boxes;

2) two putative regions of interactions with the host DnaA protein, i.e., TTATCCACA, which is identical to DnaA boxes of *E. coli oriC* and *oriV* of the sequenced IncP-7 plasmids, and TGGTTCGCT, which is variable even inside the IncP-7 group; however, the maximal consensus deviation of four nucleotides is observed in the case of Rms148, which raises the question of the functionality of this region;

3) two AT-rich regions that are separated by DnaA boxes and contain 12-nucleotide direct repeats with the TTTTNTGTTTTT consensus, three of which are located in the first region distal to *repA* gene, while five are in the second, proximal region;

4) *repA* gene (867 bp), which encodes proteins 288 amino acid residues long, which falls into the Rep-3 superfamily of α-proteins.

It is possible that not all of the *oriV* components described are necessary to initiate replication; thus, this region should be considered an expanded *oriV*. There is only one known study, where the functioning of the P-7 plasmid basic replicon was studied, which, more specifically, is the only pCAR1 plasmid [12]. It was shown, that under the influence of in *trans* RepA protein, minimal *oriV* forms a 345-bp fragment, including the first AT-rich region, iterons 1–6, both DnaA-boxes, and two 12-member repeats of the second AT-rich region in *P. putida* DS1 cells. Unfortunately, this statement could not be extended to the other P-7 plasmids, since there is a certain phylogenetic distance between pCAR1 and the other P-7 plasmids, and, more importantly, changes in the requirements to *oriV* components for replication in different hosts, even inside one genus, could not be excluded [3].

An iteron (CGACTACAAATTCCGGCT, iteron 9, Fig. 2) considered to be excessive compared to other known P-7 plasmids was discovered during the Rms148 *oriV* nucleotide sequence analysis. Thus, Rms148 is the only P-7 group plasmid so far that contains a nontypical number of iterons. The role of this

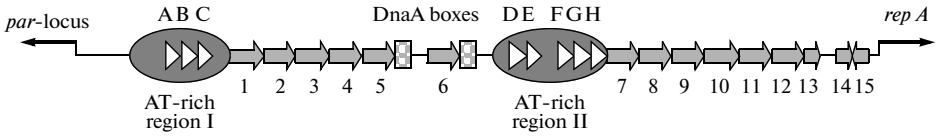

Fig. 2. Structure of region of initiation of Rms148 replication (*oriV*). Direct 12-member repeats (A–H) in left (I) and right (II) AT-rich regions, iterons, and iteron-like sequences (1–15) are also shown.

iteron in the cooperative interaction with replication intiator, as well as its influence on the manifestation of incompatibility and the number of plasmid copies is a subject for further investigation. As was shown for R1162 (IncP-4) plasmid, which contains the iteron, but has a high number of copies, Rep-protein titration took place with an increase in the number of iterons (in this case, the quantity of Rep-protein was the limiting factor), as a result of which fewer rounds of replication were initiated, the number of copies decrease, and the expression of incompatibility increased [13].

Phylogenetic Analysis of Rms148 *repA-oriV* Region

A comparison of Rms148 *repA-oriV* nucleotide sequences with other P-7 plasmids revealed the rather conservative nature of this region inside the group, unlike, e.g., P-9 [5]. Nevertheless, Rms148 and pCAR1 form independent clades of a phylogenetic tree built with a neighbor-joining method (Fig. 3). Since the differences among them are minimal, the pND6-1, pWW53, pL6.5, and pDK1 plasmids can be precombined into a pND6-1 subgroup. The nucleotide sequence of the analyzed Rms148 region is 83% identical to the *repA-oriV* of the pND6-1 subgroup plasmids and only 81% identical to pCAR1 plasmids.

It is worth mentioning that, unlike P-9, in the P-7 group, the *repA* gene seems to be more conservative than *oriV*. Rms148 *repA* gene identity to pND6-1 subgroup replication initiator genes is 93% and pCAR1 is 92%, and the *oriV* has only 75–76% and 73% identity, respectively. Even the sizes of extended *oriV* of plasmids that belong to different clades of phylogenetic tree differ; Rms148 has a length of 1080 bp, pND6-1 subgroup have 1024–1025 bp, and pCAR1 has 1005 bp.

It is interesting that Rms148 *oriV* fragment of 95 bp in length, which includes iterons 2–5 and the first DnaA-box, is 75% identical to the iteron-containing *oriV* part of pECB2, i.e., unclasified cryptic plasmid from *P. alcaligenes*. The GAGGTACAGATTC-CGG(C/G)T consensus sequence is repeated four times, and only the 5' part of this consensus is different from group IncP-7 iterons. This fact, as well as the homology of Rms148 and pECB2 RepA protein described below, may be proof of the possible relations of these plasmids. It cannot be excluded that plasmids homologous to cryptic and small (4480 bp) pECB2 were distant ancestors of the IncP-7 group plasmids.

Prediction of Protein Structure of Rms148 RepA Replication Initiator

The estimated amino acid sequence of Rms148 RepA protein is 99% identical to the RepA of pND6-1 subgroup and 98% identical to the pCAR1 one. In addition, this protein is homologous to the replication initiators of many iteron-containing plasmids, including *E. coli* F-factor, pSC101, and R6K, but the maximum similarity outside of the IncP-7 group is observed in the case of the pECB2 (46% identity) and pPS10 (41% identity) replication initiators with 77% overlap of the amino acid sequence.

Computer-predicted Rms148 RepA secondary and tertiary structures are characteristic of this group of the organization of the protein domain, including one winged helix (WH1-2 domains) at the N- and C-terminals (amino acid residues 4–142 and 145–231), the first of which is usually responsible for the protein–protein interactions, and the second contains the motif of the helix-turn-helix (HTH) type, which interacts with specific DNA sites (operator iterons) [14]. As is shown for the pPS10 cryptic plasmid, a hydrophobic N-terminal motif, the so-called leucine zipper (LZ, 26 amino acid residues), plays the main role in the RepA dimerization and in its interaction with the host factors. The estimated Rms148 RepA LZ motif is 58% identical to the corresponding pPS10 and pECB2 motifs, while the Rms148 HTH-core domain

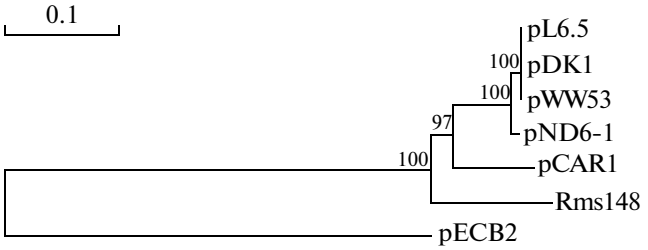

Fig. 3. Dendrogram of evolutionary relationships between nucleotide sequences of *repA-oriV* regions in IncP-7 plasmids. pECB2 sequence was used to root the tree. Tree was constructed by neighbor-joining method via bootstrap analysis using TREECON software.

(in WH2) is 52% identical to pECB2 and only 39% to pPS10, which is not surprising if the difference in *oriV* nucleotide sequences of these plasmids is taken into account.

IncP-7 RepA amino acid sequence has a 20–40-residue, elongated C-terminal region compared to the well functionally studied pPS10 and F-factor Rep-proteins. An analysis of the Rms148 RepA secondary structure suggests that this region contains an additional α-helical structure. In general, the structural similarity of Rep-proteins encoded by the P-7 plasmids with pPS10 RepA suggests a similar mechanism of their functioning, at least according to the model; i.e., a monomer binds to iterons that initiate the replication and dimer interacts with the promoter–operator region of its own gene (in the inverted repeat region), which performs autorepression.

An analysis of the *repA* gene structure and the consequences of its mutation may shed light on the reasons for the limited host range of the P-7 incompatibility group plasmids. So far, in the laboratory nobody has been able to transfer these plasmids into the bacteria of another group that does not belong to the *Pseudomonas* genus; however, the percentage of G+C in P-7 replicons is too low (53–54%) for evolution only within the limits of this genus (more then 60% G+C), although there could be other explanations for this phenomenon [15]. Indeed, the potential *repA* promoter is far from canonical, e.g., enterobacterial, which can cause a lack of replication initiator protein in these hosts. The increased number of Rep proteins expressed from the additional vector and compensated for the ineffectiveness of *rep*-promoter in case of pMT2 (IncP-9); as a result, the possibility of pMT2 replication in *E. coli* appeared [16]. Unlike P-9 plasmid with an excess of contributed in *trans* RepA, mini-pCAR1 did not replicate in the *E. coli* cells [12]. It can be assumed that the factors that limit the host range of IncP-7 group plasmids are not only the promoter features, but also inefficient interactions with the host's RepA initiation factors (DnaA, chaperones). It should be noted that there is some homology between Rms148 and pPS10 Rep-protein LZ-motifs, but only one T→C transition, which leads to the A31V replacement in the pPS10 RepA and allows the efficient replication of the plasmid in *E. coli* [14]. The experimental discovery of factors that limit the maintenance of P-7 replicons in certain genetic conditions or environments enables the more accurate assessment of the host range of these plasmids, the features of their evolution, and the potential distribution of antibiotic resistance and biodegradation determinants encoded by these plasmids.

In conclusion, it should be noted that not all of IncP-7 plasmids are conjugative; moreover, even conjugative copies are sometimes impossible to transfer within the *Pseudomonas* genus [17]. Since not all biodegradation plasmids could be maintained in the strains of *P. aeruginosa*, the recipient of R-plasmid on the stage of the IncP classification system development, we have developed a separate system of classifying D-plasmids in *P. putida* in our laboratory [18]. However, Rms148 shows a high frequency of conjugation transfer (10^{-1}) between strains of *P. aeruginosa* [19], as well as among the strains of other *Pseudomonas* species (in our laboratory), including the rhizospheric species. Therefore, the determination of potential hosts and retaining mechanisms for R-plasmids with the P-7 replicon is certainly of interest from a clinical point of view.

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