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## GENOMICS. TRANSCRIPTOMICS. PROTEOMICS

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# The Replication System of Plasmids from *Bacillus subtilis* Environmental Isolates

**A. V. Lagodich[1], Ya. V. Shtaniuk[1], A. A. Prozorov[2], and M. A. Titok[1]**

[1] *Belarussian State University, Minsk, 220050 Belarus*
*e-mail: lagodichav@bsu.by*
[2] *Vavilov Institute of General Genetics, Russian Academy of Sciences, Moscow, 119991 Russia*

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**Abstract**—Restriction enzyme analysis, cloning, and sequencing showed that large (more than 90 kb) plasmids isolated from different *Bacillus subtilis* strains are identical in structure of the region ensuring stable inheritance of plasmid replicons and are widespread in Belarussian environmental strains of *B. subtilis*.

*Key words*: *Bacillus subtilis* plasmids, replicon, cloning

## INTRODUCTION

Bacterial plasmids similar in the replication system belong to one incompatibility group. Their distinguishing feature is the inability to persist simultaneously in one bacterial cell [1]. Notwithstanding the similarity of replication systems, plasmids of one group commonly vary in size and in the set of genetic determinants. In addition, the origin of vegetative replication and the gene for the replication initiation protein, which together constitute the basal replicon, are often polymorphic in nucleotide sequence [2].

Cases are known of absolute identity of plasmids isolated from taxonomically different bacterial strains. In particular, restriction mapping and heteroduplex analysis have demonstrated the identity of plasmids RP4, RP1, and R68 isolated from clinical strains of *Pseudomonas aeruginosa* and plasmid RK2 of *Klebsiella aerogenes* [3, 4]. These plasmids all belong to the IncP1α incompatibility group. The genome structure and the restriction map are identical in IncQ plasmids RSF1010, R1162, and R300B isolated from different bacteria of the family Enterobacteriaceae [5]. Distribution of similar extrachromosomal elements among environmental strains of bacteria are possibly due to the specific features of their replication systems and to the certain genetic determinants (*tra* and *mob* genes), which provide for horizontal and vertical transfer of plasmid replicons circulating in environmental strains.

The objective of this work was to compare the replication system for similarly sized (more than 90 kb) plasmids, which are often present in *Bacillus subtilis* strains isolated from various natural sources of Belarus.

## EXPERIMENTAL

**Strains and plasmids.** We used 55 *B. subtilis* strains isolated in Belarus; *B. subtilis* type strain 168 *trp*C2 (provided by A.A. Prozorov); and *Escherichia coli xl-1 Blu F' proAB lac lacZ ΔM15 Tn10 ($Tc^R$), recA1, endA1, gurA96 ($Nal^R$), thi1, hsd, R17 ($r^-m^+$), supE44, relA1lac* (provided by E.A. Nikolaichik). We used pMTL21C [6] for cloning minireplicons and fragments amplified from their replication regions (rep) and pUC19 [7] for cloning DNA fragments to be sequenced.

Bacteria were grown in liquid or on solid LB [8] or on a minimal medium [9]. As a source of carbon, glucose was used at 0.2 (*E. coli*) or 0.5% (*B. subtilis*). Commercial ampicillin (Ap) and chloramphenicol (Cm) were used at 50 and 5 μg/ml, respectively. IPTG and X-Gal were prepared as recommended by Fermentas (Lithuania) and used at 0.5 mM and 50 μg/ml, respectively.

**Manipulations with DNA.** Total DNA was isolated according to the standard procedure [8].

Plasmid DNA was isolated by alkaline lysis with modification [10]. Transformation of competent *E. coli* and *B. subtilis* cells was carried out as described in [8] and [11], respectively.

Digestion with restriction enzymes, treatment with phosphatase, and ligation were performed as recommended by Fermentas.

Electrophoresis followed the standard procedure [8]. The sizes of DNA fragments were estimated against markers λ DNA/*Hin*dIII Marker 2 or GeneRuler 1-kb DNA ladder (Fermentas). DNA fragments were isolated from gel as in [8].

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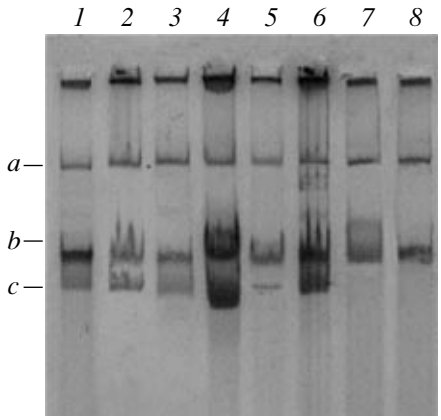


**Fig. 1.** Electrophoresis of plasmid DNAs found in environmental *B. subtilis* strains (*1*) BS57 isolated from the Bobrik River, Brest Region; (*2*) BSN1 isolated from the Naroch' Lake, Minsk Region; (*3*) BS2 isolated from the same lake; (*4*) BS4 isolated from the Rislovskoe Lake, Gomel' Region; (*5*) BS8 isolated from a meadow, Grodno Region; (*6*) BS15 isolated from the Rislovskoe Lake; (*7*) BS19 isolated from a forest, Grodno Region; and (*8*) BS72 isolated from a flowerbed, Minsk. Positions of (*a*, *c*) plasmid and (*b*) chromosomal DNAs are indicated.

The rep regions of plasmids of environmental *B. subtilis* strains were cloned and plasmids tested for stable inheritance according to published protocols [10].

The polymerase chain reaction (PCR) included 30 cycles of 10 s at 94°C, 15 s at 50°C, and 4 min at 65°C, with first denaturation for 5 min, and was carried out with a Takara kit (Japan). We used primers BS1-direct (5'-GCT AGC TTG ACT TTA GGG ACC CTG-3') and BS2-reverse (5'-GCT AGC AAA TTC TGG CAG CAT CC-3').

Sequencing with a CycleReader auto DNA sequencing kit (Fermentas) was performed in an ALFexpress II automated sequencer. The results were analyzed with the BLASTP 2.2.1 (available from NCBI site http://www.ncbi.nlm.nih.gov) and ALFwin Sequence Analyzer ver. 2.10 programs.

## RESULTS AND DISCUSSION

Four open reading frames (ORF) were found in a 3081-bp replicon, which has previously been cloned and sequenced from a large (more than 90 kb) plasmid pBS72 of an environmental *B. subtilis* strain. Replication of the minireplicon depends on the function of a gene starting with *orf1* and the origin of vegetative replication, which is in the intergenic spacer between *orf1* and *orf2*. Nucleotide sequence analysis revealed significant homology between the C-terminal region of the *orf1* protein product (271 residues) and the N-terminal region of bacterial DnaA, which participates in replication initiation on the bacterial chromosome [12, 13]. However, the minireplicon of pBS72 showed no homology to the rep regions of known plasmids of Gram-positive or Gram-negative bacteria [14–16]. Hence it is possible that pBS72 is unique and belongs to a new class of extrachromosomal bacterial elements. In addition, single-stranded intermediates were not observed during replication of pBS72, suggesting the θ replication mechanism. Since the cloned replicon, pMTLBS72, showed inheritance in *B. subtilis polA*$^{-}$ cells, its initial replication is independent of DNA polymerase I [10, 17].

Cells of 55 environmental *B. subtilis* strains were subjected to alkaline lysis and tested for extrachromosomal elements. Unexpectedly, plasmids similar in size to pBS72 were found in seven strains originating from different natural sources (Fig. 1). It was of interest to compare the replication system of these plasmids and pBS72. The plasmids were isolated and, along with pMTL21C, digested with a restriction enzyme (*Kpn*I, *Sma*I, *Bam*HI, *Sal*I, *Hind*III, *Bgl*II, *Xho*I, *Eco*RI, *Pst*I, *Sac*I, or *Sty*I), which had a site in the polylinker. After ligation, the mixture was used to transform *B. subtilis* cells. This method allows direct selection of *B. subtilis* cells that inherited certain recombinant plasmids. Hybrid plasmids were isolated from the selected clones and used to transform *E. coli xl-1 Blu*. Transformants were used to isolate the plasmids for further analysis.

In these experiments, the 2.9-kb rep region was cloned from pBS57 with *Eco*RI. The pBS57 minireplicon (hereafter referred to as pMTLBS57) showed stable inheritance in *B. subtilis* cells grown under nonselective conditions for 20 generations (inheritance stability 98%). To study the molecular genetic structure of the rep region of pBS57, the cloned replicon

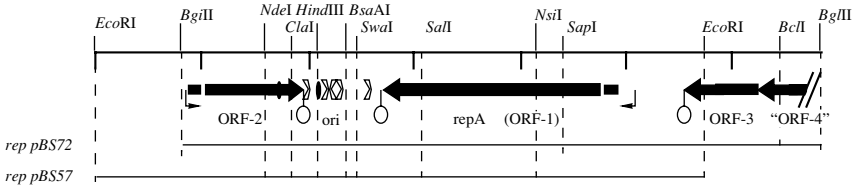


**Fig. 2.** Minireplicons of pBS72 (3081 bp) and pBS57 (2892 bp). The sequence established contains three full-length (OFR 1–3) and one truncated ("ORF 4") ORF. Designations: promoter; ■, Shine–Dalgarno sequence; , terminator; 》, right-oriented repeats; 《, left-oriented repeats; ▮, DnaA-binding site.

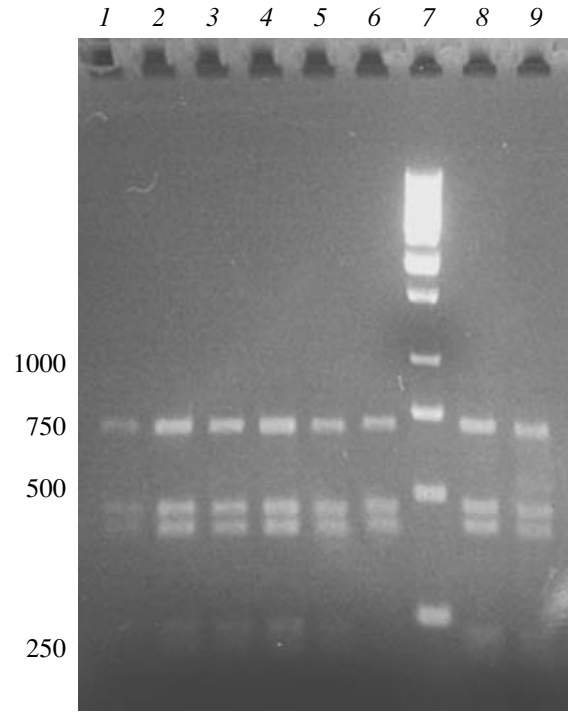


**Fig. 3.** Restriction enzyme analysis of the rep regions amplified from large plasmids of environmental *B. subtilis* strains (*1*) BS23, (*2*) BSN1, (*3*) BS2, (*4*) BS4, (*5*) BS15, (*6*) BS7A-1, (*8*) BS57, and (*9*) BS72. Total DNAs were used as PCR templates. Lane *7*, molecular weight markers (MBI, Fermentas).

was sequenced. The 1.3- and 1.6-kb fragments obtained by pMTLBS57 digestion with both *Sal*I and *Eco*RI were cloned in pUC19. The resulting constructs were used as sequencing templates. The pBS57 replicon and the pBS72 minireplicon showed 100% homology of regions containing *orf1* and *orf2*. The *orf2–orf1* (putative oriV) and *orf1–orf3* intergenic regions also proved to be identical. Compared with the minireplicon of pBS72, the cloned sequence lacked the 529-bp *Eco*RI-*Bgl*II fragment containing *orf3* and *orf4* and had an additional 340-bp *Eco*RI-*Bgl*II fragment immediately upstream of *orf2* (Fig. 2).

Nucleotide sequence analysis of the *Bgl*II-*Eco*RI fragment revealed neither ORF nor homology to database DNA sequences. Functional analysis showed that, unlike the minireplicon of pBS72, that of pBS57 was not replicated when having defective *orf1*, which codes for the replication initiation protein: deletion derivatives Δ*Sal*I-*Eco*RI and Δ*Eco*RI-*Sal*I could not be maintained in *B. subtilis* cells.

Structural identity of the rep region in pBS72 and pBS57 made it possible to assume that replicons of this type are widespread among environmental *B. subtilis* strains of Belarus. To check, we constructed primers allowing amplification of the rep region of pBS72 to yield the 2.1-kb amplification product. Total DNAs of 55 environmental *B. subtilis* isolates were used as templates in PCR. Specific amplification products were obtained with eight isolates, which were previously observed to possess plasmids, and with strains BS7A-1 and BS23. Restriction enzyme analysis with *Rsa*I demonstrated complete identity of the amplified fragments (Fig. 3). This testifies that large plasmids with similar rep regions are indeed widespread in Belarussian environmental strains of *B. subtilis*.

To verify whether the amplified DNA fragments contained the rep region ensuring plasmid replication, one of the fragments was cloned in pMTL21C, and the resulting construct tested for the ability to transform *B. subtilis* 168 cells. The fragment amplified from total DNA of strain BS4 was ligated with pMTL21C digested with *Sma*I, and the ligation mixture used to transform *E. coli xl-1 Blu* cells. Selection was performed in the presence of X-Gal, IPTG, and ampicillin to obtain constructs carrying the additional DNA fragment. Plasmid DNA isolated from transformed *E. coli xl-1 Blu* was subjected to restriction enzyme analysis. This showed that a 2.1-kb fragment was cloned in pMTL21C. The recombinant plasmid was used to transform *B. subtilis* 168 cells. The transformant clones showed stable inheritance of the plasmid, indicating that the cloned fragment contained a region sufficient for plasmid replication.

Thus, similarly sized (more than 90-kb) plasmids with identical replication systems were revealed in the *B. subtilis* strains isolated from various natural sources in Belarus. The wide distribution of these similar replicons may be explained by their capability of conjugal mobilization [18, 19] or the presence of genetic determinants conferring a selective advantage on the host bacteria.

## REFERENCES

1. Datta N. 1979. Plasmid classification: incompatibility grouping. In: *Plasmid of medical, environmental and commercial importance*. Eds. Timmis K.N., Puhler A. Amsterdam: Elseviers/Noeth Holland Publ. Co., 3–12.
2. Osborn M., Bron S., Fieth N., Holsappel S., Huddleston A., Kiewiet R., Meijer W., Seegers J., Skurray R., Terpstra P., Thomas C.M., Thorsted P., Tietze E., Turner S.L. 2000. The evolution of bacterial plasmids. In: *The Horizontal Gene Pool: Bacterial Plasmids and Gene Spread*. Ed. Thomas C.M. Harwood Acad. Publ., 301–363.
3. Burkardt H.J., Riess G., Puhler A. 1979. Relationship of group P1 plasmids revealed by heteroduplex experiments: RP1, RP4, R68 and RK2 are identical. *J. Gen. Microbiol.* **114**, 341–348
4. Currier T.C., Morgan M.K. 1981. Restriction endonuclease analysis of the incompatibility group P–1 plasmids RK2, RP1, RP4, R68, R68.45. *Curr. Microbiol.* **5**, 323–327.
5. Barth P.T., Grinter N.J. 1974. Comparison of the deoxyribonucleic acid molecular weights and homologies of

plasmids conferring linked resistance to streptomycin and sulphonamide. *J. Bacteriol*. **120**, 618–630.

6. Chambers S.P., Prior S.E., Barstow D.A., Minton N.P. 1988. The pMTL cloning vectors. I. Improved polylinker regions to facilitate the generation of sonicated DNA for nucleotide sequencing. *Gene*. **68**, 139–149.
7. Yanisch-Perron C., Vieira J., Messing J. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene*. **33**, 103–119.
8. Sambrook J., Fritsch E.F., Maniatis T. 1989. *Molecular Cloning: A Laboratory Manual*. N.Y.: Cold Spring Harbor Laboratory Press.
9. Anagnostopoulos C., Spizizen J. 1961. Requirements for transformation in *Bacillus subtilis. J. Bacteriol.* **81**, 741–746.
10. Titok M.A., Chapuis J., Selezneva Y.V., Lagodich A.V., Prokulevich V.A., Ehrlich S.D., Janniere L. 2003. *Bacillus subtilis* soil isolates: plasmid replicon analysis and construction of a new theta-replicating vector. *Plasmid*. **49**, 53–62.
11. Bron S. 1990. Plasmids. In: *Molecular Biological Methods for Bacillus*. Eds. Harwood C.R., Cutting S.M. Chichester: John Wiley and Sons Ltd., 75–174.
12. Sutton M.D., Carr K.M., Vicente M., Kaguni J.M. 1998. Escherichia coli DnaA protein. The N-terminal domain and loading of DnaB helicase at the *E. coli* chromosomal origin. *J. Biol. Chem.* **273**, 34255–34262.
13. Weigel C., Schmidt A., Seitz H, Tungler D., Welzeck M., Messer W. 1999. The N-terminus promotes oligomerization of the *Escherichia coli* initiator protein DnaA. *Mol. Microbiol.* **34**, 53–66.
14. Tanaka T., Ogura M. 1998. A novel Bacillus natto plasmid pLS32 capable of replication in *Bacillus subtilis. FEBS Lett.* **422**, 243–246.
15. Meijer W.J., de Boer A.J., van Tongeren S., Venema G., Bron S. 1995. Characterization of the replication region of the *Bacillus subtilis* plasmid pLS20: a novel type of replicon. *Nucleic Acids Res.* **23**, 3214–3223.
16. Espinosa M., Cohen S., Couturier M., del Solar G., Diaz-Orejas R., Giraldo R., Janniere L., Miller C., Osborn M., Thomas C.M. 2000. Plasmid replication and copy number control. In: *The Horizontal Gene Pool: Bacterial Plasmids and Gene Spread*. Ed. Thomas C.M. Harwood Acad. Publ., 1–47.
17. Titok M.A., Lagodich A.V. 2003. Molecular genetic analysis of the rep region of θ-type plasmid pBS72 of *Bacillus subtilis*. *Dokl. Nats. Akad. Nauk Belarusi*. **47**, 67–70.
18. Lotareva O.V., Nezametdinova V.Z., Fedorina E.A., Poluektova E.U., Titok M.A., Prozorov A.A. 2001. Conjugal mobilization occurring at a high rate in environmental *Bacillus subtilis* isolates carrying a large plasmid. *Genetika*. **37**, 1598–1603.
19. Lotareva O.V., Poluektova E.U., Titok M.A., Prozorov A.A. 2001. A large plasmid of a soil *Bacillus subtilis* strain allows a high rate of conjugal mobilization. *Dokl. Akad. Nauk*. **379**, 130–131.