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EXPERIMENTAL ARTICLES

The Structure of the Transposable Genetic Element IS*Bsu2* from the Cryptic Plasmid p1516 of a Soil *Bacillus subtilis* Strain and the Presence of Homologues of This Element in the Chromosomes of Various *Bacillus subtilis* Strains

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Abstract—A cryptic plasmid from a soil strain of *Bacillus subtilis* was found to contain a sequence having features of an IS element. Homologous sequences were also found in the chromosome of this strain and in the chromosomes of some other *B. subtilis* strains.

Key words: *Bacillus subtilis*, plasmids, IS elements, additional taxonomic criterion.

Transposable genetic elements (IS elements, transposons) are widespread in nature and occur in the chromosomes and plasmids of various bacteria. Until recently, however, no IS elements have been reported for *Bacillus subtilis* strain 168 (Marburg), whose complete genome sequence has been determined [1]. The first *B. subtilis* IS element, IS*4Bsu*1, was found in the chromosome of *B. subtilis (natto)*, a starter strain used in Japan for the manufacture of natto (fermented soybeans) [2]. This strain was earlier considered as an independent bacillar species. The transfer of transposable elements is relatively frequent and involves no fewer than 1% of cells in a population. In such cells, the synthesis of the capsular polymer poly-γ-glutamic acid is impaired. The mutants that lack poly-γ-glutamic acid have an altered colonial morphology [3]. It is the investigation of the causes of morphological variability that has led to the discovery of the transposable element IS*4Bsu*1 [2].

Earlier, we studied small cryptic plasmids from *B. subtilis* 168 and related soil bacillar strains. To assess the degree of relation of these strains to *B. subtilis* 168, we compared their susceptibility to phages, analyzed their isoenzymes, and evaluated the ability of DNA from these strains to transform the recipient *B. subtilis* 168 cells [4–6].

The aim of this work was to study the structure of some of these plasmids and to analyze them for the presence of transposable genetic elements. One of the strains studied, *B. subtilis* 1516, was found to contain two plasmids. Restriction analysis showed that the larger plasmid differed from the smaller one in the presence of an additional fragment about 1.4 kbp in size. The determination of the nucleotide sequence of this fragment showed that it has features of an IS element. Homologues of this sequence were revealed by blot hybridization in the chromosomes of *B. subtilis* 1516 and some other *B. subtilis* strains of different origin.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids studied in this work are summarized in Table 1.

Cultivation conditions. The strains were cultivated in liquid LB broth or on LB agar (Fluka).

Isolation and analysis of DNA. Plasmid DNA was isolated as described in the handbook by Maniatis *et al.* [8]. Chromosomal DNA was isolated by the method used in our laboratory [9]. DNA was cleaved by restriction endonucleases (Promega) according to the manufacturer's instructions. DNA was subjected to electrophoresis in 1% agarose gel and Tris–borate buffer.

Cloning procedure. The *Eco*RI-*Hin*dIII fragments of plasmids p1516S and p1516L were cloned in *Escherichia coli* DH5α cells using the commercial vector pBluescriptII.

PCR amplification. The transposable element IS*Bsu2* present in plasmid p1516L was amplified by PCR using the forward primer CGTACGGCTGGAAT-

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GCCTAG (SH1516fw) and the reverse primer GGAAGGGCAATTGATTGCCCG (SH1516rev), which are homologous to plasmid DNA near the ends of IS, and an Expand high-fidelity PCR system kit from Roche. PCR products were analyzed by electrophoresis in 1% agarose gel and purified using a High-pure PCR product purification kit purchased from Boehringer Mannheim.

DNA–DNA hybridization. DNA was labeled with [^{32}P]dATP (obtained from the Energy Physics Institute, Obninsk, Russia) using Prime-a-gene statistical primers (Promega). DNA was transferred to Hybond^{TM}-N filters (Amersham) by Southern blotting [8]. DNA–DNA hybridization was carried out at 65°C on the filters, followed by their washing with 0.1 SSPE at the same temperature.

Nucleotide sequence determination. DNA was sequenced using an automated ALFexpress II DNA analysis system (Amersham Pharmacia Biotech), a Labstation thermo sequenase labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham), and the Cy5-forward and Cy5-reverse universal primers (Pharmacia Biotech).

Nucleotide sequence analysis. The nucleotide sequence of IS*Bsu*2 was compared with sequences available in GenBank (http://www.ncbi.nlm.nih.gov) with the aid of the Pairwise BLAST program (options blastn and blastx) [10].

Deposition of the nucleotide sequence. The nucleotide sequence of IS*Bsu*2 has been deposited in GenBank under the accession number AY099458.

Table 1. Bacterial strains and plasmids used in the work

Bacterial strains and plasmids	Source
B. subtilis 604	Isolated from Moscow soil in our laboratory by Kozlovskii [4]
1315(p1315)	The same
1385(p1385)	»
1356	»
1414(p1414)	»
1431	»
1513	»
1516(p1516S; p1516L)	»
IFO3022(pTA1060)	Described by Uozumi *et al.* [7]
19(p19; pV)	Isolated from soil by M.A. Titok, Belarussian State University
168	Laboratory collection
W-23	The same
B. aterrimus VKM B-992	»
B. licheniformis B-933	»
B. pumilus VKM B-508	»
E. coli DH5α	Product of Life Technologies
pBluescriptII	Product of Stratagene

RESULTS

The Plasmid Profile of B. subtilis 1516

Small cryptic plasmids are widespread in *B. subtilis* and related bacilli [11], each organism usually containing one such plasmid [12]. However, the electropherogram of *B. subtilis* 1516 lysate exhibited two bands of plasmid DNA, which often merged. Presumably, these two bands corresponded to two plasmids close in size. To determine whether they occur in one cell or in different cells of *B. subtilis* 1516, we obtained 28 colonies of this strain grown from single cells and analyzed them for the presence of plasmids. Among these colonies, 12 contained only a larger plasmid (it was called p1516L), 15 colonies only a smaller plasmid (it was called p1516S), and one colony had both plasmids. Three colonies (one of each kind) were plated out to give individual colonies, which were again analyzed for the presence of the plasmids. All three tested colonies of those grown after plating out the colony with plasmid p1516L turned out to contain only this plasmid. Likewise, all three tested colonies of those grown after plating out the colony with plasmid p1516S contained only this plasmid. At the same time, the colony that contained two plasmids gave rise to colonies containing either plasmid p1516L or plasmid p1516S. Colonies with both of these plasmids were not detected. Thus, it is most likely that cells of *B. subtilis* 1516 contain only one plasmid at a time (either small or large). However, the possibility of spontaneous conversion of these two kinds of plasmids into each other in cells cannot be completely excluded.

We constructed the restriction maps of plasmids p1516S and p1516L. Judging from the sum of restriction fragments, plasmids p1516S and p1516L are 9.9 and 11.3 kbp in size. The plasmids were almost identical in the position of the restriction sites and differed only in that plasmid p1516L had an additional fragment about 1.4 kbp in size (Fig. 1).

Additional Nucleotide Sequence in Plasmid p1516L

The large and small plasmids of *B. subtilis* 1516 differed only in the size of *Eco*RI–*Hin*dIII fragment (Fig. 1), which was 2.1 kbp in plasmid p1516L and 0.7 kbp in plasmid p1516S. We determined the nucleotide sequence of the *Eco*RI–*Hin*dIII fragments of both plasmids and found that the *Eco*RI–*Hin*dIII fragment of plasmid p1516L differed from the respective fragment of plasmid p1516S in that it had an insertion of 1383 bp. The structure of the insertion and its flanking regions is schematically shown in Fig. 2. The insertion was flanked by 7-bp repeats (one on the insertion and the other outside of it) and had two pairs of perfect inverted

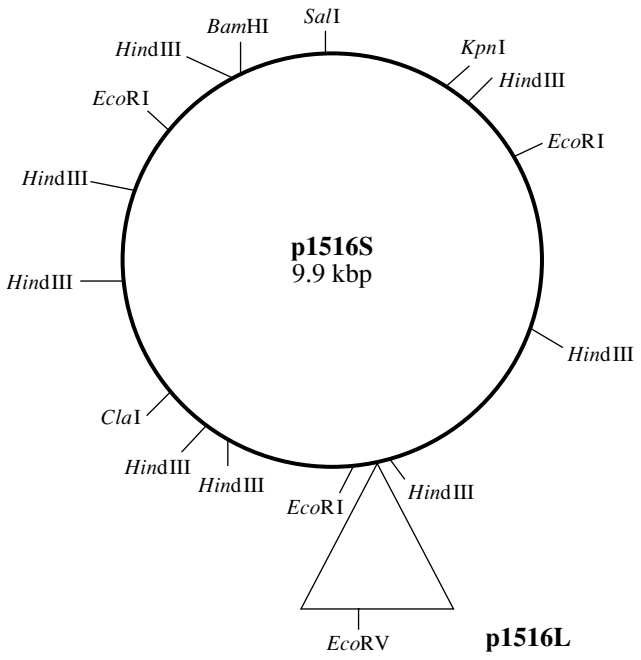

Fig. 1. The restriction map of plasmids p1516S and p1516L.

10-bp repeats at the ends. The G+C content of the insertion was found to be 40%. The insertion was 70% homologous to fragments of the *Enterococcus faecalis* and *Enterococcus faecium* genomes (the GenBank sequences TIGR_1351/gef_11370 and DOE_1352/Contig719, respectively). The amino acid sequence corresponding to the nucleotide sequence of the insertion was found to be similar to many microbial proteins (transposases, putative transposases, and unidentified proteins). However, the insertion contained no full-length ORF and exhibited multiple translational frameshifts. This insertion was not homologous to IS*4Bsu*1. The results of a comparison of the putative protein that corresponds to the insertion with some transposases are summarized in Table 2.

Thus, the insertion in plasmid p1516L has the specific properties of an IS element: the presence of direct and inverted repeats at the ends and homology of the putative amino acid sequence of its central part to microbial transposases. The insertion was called IS*Bsu*2. The gene of IS*Bsu*2 transposase is likely defective (i.e., is a pseudogene). The transposase IS*Bsu*2 is homologous to the transposases IS*285*, IS*1356*, and IS*RM3* from the IS*256* family [13]. Like some transposases of this family, IS*Bsu2* has two pairs of inverted repeats, which is not typical of most transposases. Presumably, IS*Bsu2* belongs to the IS*256* family.

Homologues of ISBsu2 in the Chromosomal DNA of B. subtilis Strains

The question arose of whether or not IS*Bsu*2 is present in the chromosome of strain 1516 harboring plasmid p1516L and in the chromosomes of other *B. subtilis* strains and other bacillar species. To answer this question, we tested the chromosomal DNA of various bacilli for the presence of IS*Bsu*2 by blot hybridization. For this purpose, IS*Bsu*2 was amplified in plasmid p1516L by PCR with specific primers and then labeled with [^{32}P]dATP. The labeled product was used as a probe in the Southern blot hybridization with bacillar chromosomal DNA. Chromosomal DNA for Southern blot hybridization was preliminarily cleaved with *Eco*RV restriction endonuclease. IS*Bsu*2 has one recognition site for *Eco*RV, located close to the center of this element, whereas the other part of plasmid p1516L does not contain such sites (Fig. 1).

Southern blot hybridization with the chromosomal DNA of the *B. subtilis* strain harboring plasmid p1516S gave rise to 18–20 hybridization bands of different

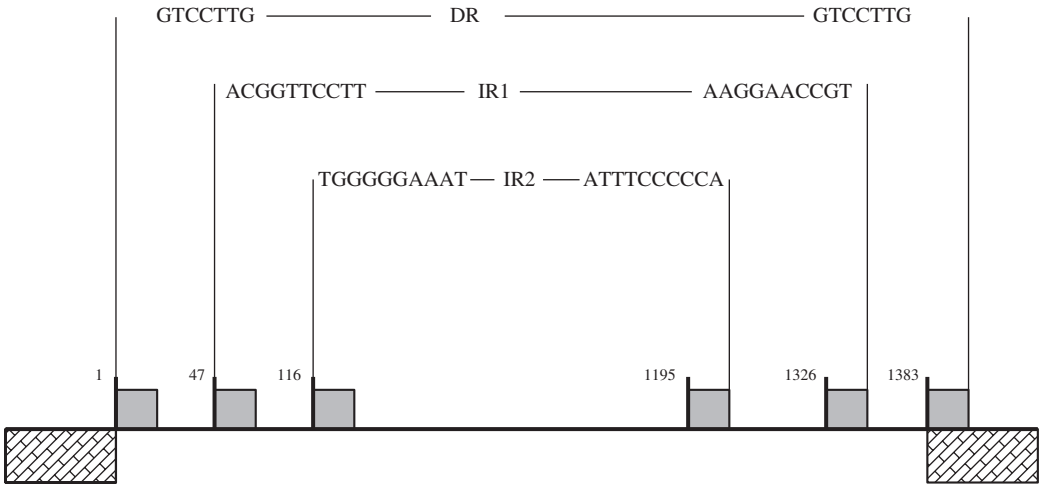

Fig. 2. Schematic representation of the transposable element IS*Bsu2*. DR and IR stand for "direct repeat" and "inverted repeat," respectively. The cross-hatched areas below the solid line represent the regions of plasmid p1516L lying outside IS*Bsu2*.

Table 2. Various microbial transposases homologous to the putative transposase encoded by IS *Bsu*2

GenBank accession number	Protein	Organism	Identity level, %	Similarity level, %	Significance level	Homologous region size, bp
AF336309	Putative transposase ISYen1	*Yersinia enterocolitica* serotype 08, plasmid pYVe8081	36–40	55–62	6e–57	247
AF447814	Putative transposase	*Escherichia coli* CFT073 pathogenicity island	40–42	60–64	2e–42	229
AF339846	Putative transposase MS103	*Microscilla* sp. PRE1 plasmid pSD15	27–34	51–54	4e–40	374
AE006980	Putative transposase IS1554	*Mycobacterium tuberculosis*	32–33	53	3e–38	306
AAC82722*	Transposase IS285	*Yersinia pestis*	25–32	45–52	1e–21	383
AP001508	Putative transposase (04)	*Bacillus halodurans*	23–29	44–48	3e–16	370

Note: Identity and similarity levels are given as intervals and the total sizes of homologous region sizes are shown because the regions compared are divided into segments by frameshift sites.

*The functional activity of this transposase was shown experimentally.

intensity (Fig. 3). The possible presence of plasmid DNA in the preparation of chromosomal DNA could not affect the results of this hybridization analysis, because plasmid p1516S does not contain IS*Bsu2*. Since the restriction enzyme *Eco*RV cleaves IS*Bsu2* into two parts, this endonuclease must also cleave into two parts any nucleotide sequence in the chromosomal DNA homologous to IS*Bsu*2 giving rise to two hybridization bands. If so, the number of sequences in chromosomal DNA homologous to IS*Bsu*2 and revealed with the aid of *Eco*RV must be two times smaller (in the given case, nine or ten) than the number of hybridization bands. The occurrence of hybridization bands in different regions of electrophoretic lanes suggests that IS*Bsu2* homologues occurred in different DNA fragments and, hence, are presumably located in different regions of the chromosome. The hybridization analysis of the chromosomal DNA of the *B. subtilis* strains harboring plasmids p1414, p1315, p1385, and pTA1060 (the complete sequence of these plasmids showed that none of them contains IS elements [11, 14]) also revealed hybridization bands. The number, intensity, and position of these bands, which are produced by the *Eco*RV fragments of chromosomal DNA hybridized with IS*Bsu2*, were unique for each particular strain subjected to hybridization analysis. The different intensities of hybridization bands may reflect the different degrees of homology between IS*Bsu2* and the respective regions of the chromosome. Strain 1414 exhibited the maximum number of hybridization bands, whereas two strains, 1385 and IFO3022, showed the minimum number. It should be noted that the *B. subtilis* strain harboring plasmid p1516L also gave rise to many hybridization bands, one of which could be due to the contamination of chromosomal DNA by plasmid DNA. Of the other chromosomal DNA tested (DNA from *B. subtilis* strain 19, harboring the large conjugative plasmid p19 and the small cryptic plasmid pV; from the type laboratory plasmidless strain *B. subtilis* 168; from the plasmidless soil strains *B. subtilis* 604, 1356, 1431, and 1513; from other bacilli related to *B. subtilis* 168 (*B. subtilis* W23, *B. aterrimus* VKM B-992, *B. licheniformis* B-993, and *B. pumilus* VKM B-508); and from *E. coli* DH5α), only the DNA isolated from *B. subtilis* 1513 exhibited hybridization bands (Fig. 3). It should be noted that Figure 3 presents data only for one of the bacterial strains whose DNA did not produce hybridization bands, namely, *B. subtilis* 168.

Thus, the chromosomal DNA of some soil *B. subtilis* strains contain homologues of IS*Bsu2*. Most of these strains were isolated from the soils of Moscow and Moscow oblast [4], with the exception that the strain *B. subtilis* (*natto*) IFO3022 with plasmid pTA1060 was isolated in Japan.

DISCUSSION

This paper describes the second IS element found in the genome of *B. subtilis*, IS*Bsu2*. For the first time, this transposable element has been found not only in the chromosomal but also in the plasmid DNA of *B. subtilis*. IS*Bsu2* has all of the major characteristics of IS elements, including a putative amino acid sequence homologous to that of microbial transposases and the presence of direct and inverted repeats in the flanking regions. At the same time, the amino acid sequence of IS*Bsu2* that is homologous to sequences of some transposases has several frameshift sites, indicating that the transposase gene is presently nonfunctional, i.e., represents a pseudogene. In the past, however, IS*Bsu2* itself or its chromosomal analogues might have been functional, as is evident from the presence of IS*Bsu2* or its homologues not only in one of the two plasmids of *B. subtilis* 1516 but also in the chromosome of this strain (in several copies) and in the chromosomes of some other *B. subtilis* strains. It should be noted that the

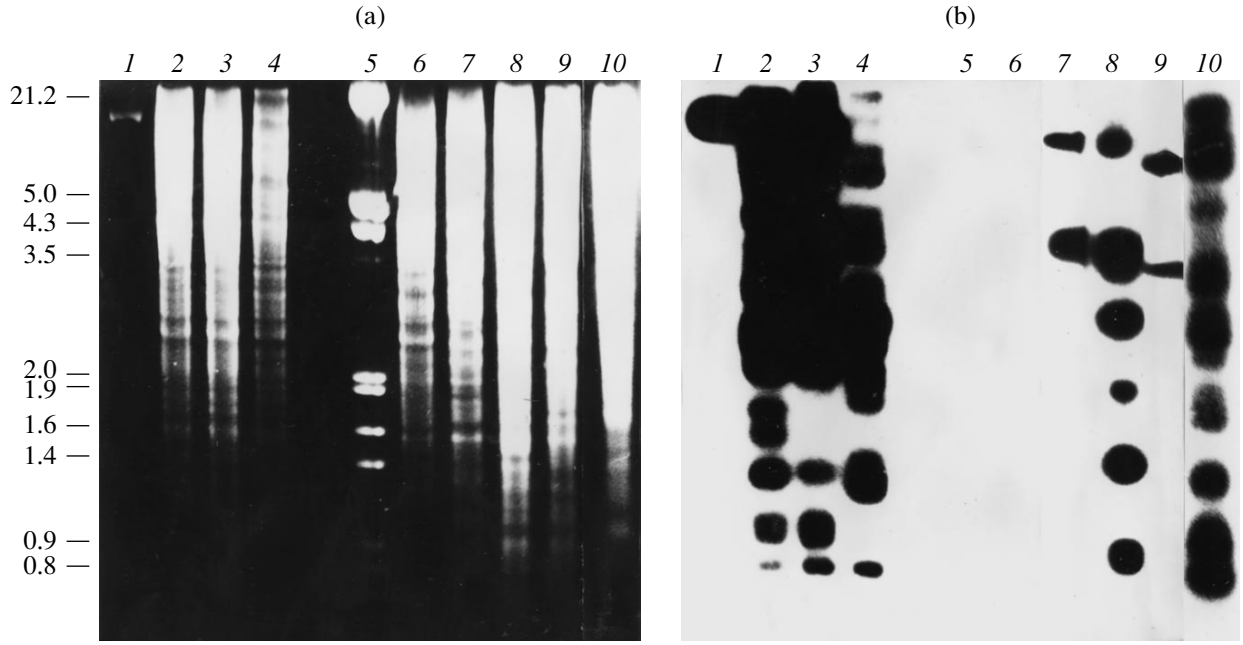

Fig. 3. The blot hybridization of chromosomal DNA from various *B. subtilis* strains with the [^{32}P]dATP-labeled DNA of IS*Bsu2*: (a) the *Eco*RV fragments of chromosomal and plasmid DNA and the *Eco*RI–*Hin*dIII fragments of the phage λ DNA; (b) the blot hybridization of these fragments with the radioactive probe. Lanes: (*1*) plasmid p1516L DNA; (*5*) phage λ DNA; (*2–4, 6–10*) chromosomal DNA from *B. subtilis* strains (*2*) 1516(p1516S), (*3*) 1516(p1516L), (*4*) 1414(p1414), (*6*) 168, (*7*) IFO3022(pTA1060), (*8*) 1315(p1315), (*9*) 1385(p1385), and (*10*) 1513. Figures at the left of panel a indicate the molecular mass (in kbp) of the *Eco*RI–*Hin*dIII fragments of the phage λ DNA.

enterococcal nucleotide sequences that are highly homologous to IS*Bsu2* contain full-length ORF and presumably are not pseudogenes. The hybridization bands of different strains have different intensity, likely due to a divergence of the nucleotide composition of IS*Bsu2* copies in the chromosomes of these strains. Under the conditions we used (hybridization temperature 65°C), Southern blot hybridization is able to detect about 80% homology of nucleotide sequences.

Of interest is the fact that IS*Bsu2* was found in the chromosomes of six *B. subtilis* strains, five of which harbor small cryptic plasmids. At the same time, only one of the nine plasmidless *B. subtilis* strains tested was found to contain chromosomal copies of IS*Bsu2*. If this is not a coincidence, these data suggest that IS*Bsu2* chiefly occurs in bacillar strains prone to the transfer of small cryptic plasmids. Such strains may be good recipients upon the conjugational and transformational transfer of small plasmids. The occurrence of IS*Bsu2* in the plasmid and chromosomal DNA of *B. subtilis* strains isolated from the soils of Moscow and its environs may be explained by the local horizontal transfer of plasmids due to transformation or conjugation under natural conditions. However, this sequence (or its analogues) was also found in the chromosome of the *B. subtilis* (*natto*) strain, isolated far from Moscow (in Japan), and in the chromosomes of two different enterococci, indicating that IS*Bsu2* may propagate worldwide through horizontal gene transfer, as was shown for mercury resistance genes [15].

The presence of IS*Bsu2* in the chromosomes of *B. subtilis* strains may serve as an additional taxonomic criterion for the classification of different strains belonging to this species.

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