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Bacillus subtilis soil isolates: plasmid replicon analysis and construction of a new theta-replicating vector

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Abstract

We have searched for plasmids in a collection of 55 *Bacillus subtilis* strains isolated from various natural sources of the territory of Belarus. Twenty percent of the strains contained one or two plasmids of either 6–8 or ~90 kb. Small plasmids were shown to carry a rolling circle replicon of the pC194 type. Four out of the eight large plasmids contained a related theta replicon that has no homolog in databases as shown by sequence determination. A *B. subtilis*/*Escherichia coli* shuttle vector based on this replicon was constructed. It has a low copy number (6 units per chromosome) and is stably inherited in *B. subtilis*. It might thus be a useful tool for DNA cloning. These data extend previous observations, indicating that most of the small plasmids of *B. subtilis* replicate as rolling circles and belong to the pC194 family. On the contrary, large plasmids appear to form a large pool of theta-replicating determinants, since three different replicons have already been isolated from them.
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Keywords: Plasmid; Replication region; Cloning vectors; *Bacillus subtilis*; Soil isolates; Rolling circle; Theta; Segregational stability; DnaA protein

1. Introduction

Natural and industrial isolates of *Bacillus subtilis* species frequently harbor small (<10 kb) and large (>50 kb) plasmids (Nezametdinova et al., 1992; Poluektova et al., 1996; Tanaka and Koshikawa, 1977; Uozumi et al., 1980). Small plasmids, grouped into seven classes as a function of their size and restriction pattern (Meijer et al., 1998; Uozumi et al., 1980), replicate as rolling circles (RC plasmids) and carry a replication determinant related to that of the *Staphylococcus aureus* plasmid pC194 (Meijer et al., 1998; Nezametdinova et al., 1992). They also encode

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determinants involved in spreading and host metabolism. Similar to spreading functions, metabolism functions may contribute to plasmid dissemination among natural strains, as they might provide adaptive advantage to the host in its environment (Meijer et al., 1998; Thorsted et al., 1999). Large plasmids, found alone or co-resident with a small plasmid, are poorly characterized in *B. subtilis*, with the exception of pLS32 and pLS20. The pLS32 extrachromosomal element belongs to a family of replicons hosted by Gram-positive bacteria that depends on a plasmid encoded protein (Rep) for replication (the pAD1 family). Plasmid pLS20 has no equivalent in databases and is Rep-independent. Evidence that they both replicate via a theta mode was obtained (Meijer et al., 1995; Tanaka and Ogura, 1998). Here, we report on a systematic analysis of plasmid DNA content in a collection of 55 *B. subtilis* strains isolated from the territory of Belarus. The results extend the notion that small (<10 kb) plasmids from *B. subtilis*-type strains belong to the pC194 family of RC replicons. A replicon isolated from a large (~90 kb) plasmid appears to be widely represented in the Belarus collection. Sequence determination showed that it forms a new class of replicons that is likely Rep-dependent and replicates as theta circles. Based on this replicon, a segregationally stable, low copy number, *B. subtilis*/*Escherichia coli* shuttle vector was constructed. Altogether, these results show that replication functions from large plasmids are more diverse than those from small plasmids, forming a large pool of new replicons.

2. Materials and methods

2.1. Bacterial strains and manipulation

The 287 natural isolates of Gram-positive bacteria investigated in this study originate from various soil samples from Belarus. Laboratory strains were *E. coli* JM105 (Δ(*lac-proAB*) *supE thi rspL* (Str^r) *endA sbcB15 hsdR4* (r_K-m_K+)/ F'(*traD36* $proAB^+$ $lacI^q$ *lacZ*Δ*M15*)) (Sambrook et al., 1989) and *B. subtilis* 168 (*trpC2*) (Anagnostopoulos and Spizizen, 1961), 1A224 (*hisH2 pheA1 trpC2*), 1A226 (*hisH2 pheA1 trpC2 polA5*) (Bruand et al., 1993) and PPBJ65 (168 *priA*::pAPJ12::erm, PriA+) (Polard et al., 2002). Bacteria were grown at 30 or 37 °C in Luria–Bertani broth (LB) or in Spizizen minimal medium supplemented with 0.05% (w/v) casein hydrolysate and 0.2% (w/v) glucose (controlled rich medium) (Anagnostopoulos and Spizizen, 1961; Sambrook et al., 1989). Competent *B. subtilis* and *E. coli* cells were prepared and transformed as described (Bron, 1990; Dagert and Ehrlich, 1979). Chloramphenicol (Cm), ampicillin (Ap), tetracycline (Tc), streptomycin (Sm), kanamycin (Km), and erythromycin (Ery) were used at 3–5, 50, 5, 10, 20, and 1 μg/ml, respectively, unless specified otherwise in the text. Strain identification was carried out by assaying cell sensitivity to *B. subtilis* specific phages (AR1, AR3, AR9, Θ105, and SP01), as previously described (Kozlowski and Prozorov, 1981). To cure cells from plasmids, tripophlavine (1, 5 or 10 μg/ml) was added to cultures (Miller, 1972). Plasmid segregational stability was determined by spreading cells grown about 20 generations without selective pressure on non-selective LB plates at 37 °C and tooth-picking ⩾ 100 colonies on the same medium supplemented or not with Cm.

2.2. DNA manipulation and plasmid description

The *E. coli* vector pMTL21C (Chambers et al., 1988) was used for cloning purposes. Reference plasmids were pHV1436 [pTB19 replicon (Jannière et al., 1990)], pMTL500E [pAMβ1 replicon (Oultram et al., 1988)], and pLS20 (Koehler and Thorne, 1987) for theta-replicating plasmids and pE194, pT181, pC194 (Gruss and Ehrlich, 1989), and p1414 (Kanapina et al., 1995) for RC-type replicons. Total DNA preparations, Southern blot analysis, and detection of single-stranded DNA (ssDNA) were standard protocols (te Riele et al., 1986a; Sambrook et al., 1989; Bron, 1990). Plasmids from *B. subtilis* soil isolates were prepared by an alkaline-lysis method. The method derived from that published previously (Bron, 1990) is as follows: (i) plasmid preparation was carried out from cells harvested from a 30 ml culture grown at 30 °C, (ii) cell lysis and protein denaturation/precipitation were performed in large volumes (15-fold larger than that from the

standard method) and successively added solutions were mixed gently, (iii) cells in lysis solution were kept at room temperature for 15 min, (iv) nucleic acids were precipitated with propanol-2 and dissolved in 700 μl Tris pH 7.5 10 mM, EDTA pH 8 1 mM before phenol extraction, and (v) the isolated DNA was purified further with a QIAquick PCR Purification Kit (Qiagen GmbH, Germany). Restriction enzymes, DNA ligase, and radiolabeling kits were used according to supplier's instructions (Boehringer–Mannheim, France SA and Biolabs, Ozyme, France). DNA sequencing of PCR products was carried out with the PRISM Sequencing Kit (Applied Biosystems, Warrington) and analyzed by an automated sequencing apparatus (Applied Biosystem 377A). Sequence analysis was carried out with the BLASTP 2.2.1 software and the BLOSUM62 matrix at the NCBI site (http://www.ncbi.nlm.nih.gov). HTH motifs were searched for using the HTH motif prediction software at the NPS@ site (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server. html).

2.3. Copy number measurement

To measure the plasmid copy number per chromosome, pMTLBS72 was introduced into a 168 strain (PPBJ65) that contains, at position 140° in the chromosome, a 1.9 kb sequence identical to a sub-region of pMTLBS72 (the origin and ampicillin resistance marker of pBR322). Total DNA was extracted from cells growing exponentially in LB + Cm and restricted with *Eco*RV and *Cla*I. This generated plasmid and chromosomal fragments of 6.6 and 4.2 kb, respectively, that encompass the pBR322 sequences. The cleaved DNA was then run in an agarose gel and analyzed by Southern blot analysis using as probe the 1.9 kb DNA fragment. After exposure of the radioactive membrane to a standard phosphorus screen (Molecular Dynamics), signals were revealed with a STORM 860 apparatus (Molecular Dynamics) and quantified with the ImageQuant software (Molecular Dynamics). The copy number of pMTLBS72, measured as the ratio plasmid/chromosomal signals from seven independent cultures, was 5.9 ± 1.1. Control experiments (based on Southern blot analysis of appropriately restricted total DNAs) showed that more than 95% of the plasmid DNA was free in the cells and that less than 10% of the chromosome contained an inserted copy of the plasmid at the pBR322 sequences. Finally, streaking and tooth-picking confirmed the absence of plasmid-free cells at the time of DNA extraction.

3. Results

3.1. Plasmid isolation from B. subtilis soil isolates

We have isolated 287 strains of Gram-positive bacteria from various soil samples in Belarus. Among these, 55 were identified as *B. subtilis* on the basis of their sensitivity to specific bacteriophages [AR1, AR3, AR9, Θ105, and SP01 (Kozlowski and Prozorov, 1981)]. With an alkaline-lysis method, 11 out of the 55 strains (20%) were shown to harbor plasmid DNA (Table 1). Five had a single extrachromosomal element of either 6.3 (strains 4K31 and 21Z), 8 (strain 1) or ~90 kb (strains 19 and 72). The remaining six contained two plasmids of 8 and ~90 kb (strains N1, 2, 4, 8, 15, and 57). The size of the large plasmid hosted by strain 19 was determined by Prozorov and coworkers (Lotareva et al., 2001). Gel electrophoresis showed that other large plasmids are of similar size (see below).

For verification of taxonomic position of plasmid-bearing strains, a transformation assay was carried out. For this, the total DNA of these strains was extracted and used to transform competent cells of the 168 *B. subtilis* strain. Trp^+ transformants were selected on a controlled rich medium lacking tryptophan. In all cases, numerous Trp^+ transformants were obtained (Table 1), confirming the fact that the isolated microorganisms are of *B. subtilis*-type (Seki et al., 1975). All the plasmid-harboring strains are sensitive to Cm, Ap, Tc, Sm, Km, and Ery (final concentration of 5–20 μg/ml) except strains 15, 8, and 72 which are Sm^R (10 μg/ml). Attempts to eliminate this phenotype by an acrydine dye (tripophlavine) known to cure cells of plasmids (Miller, 1972), were unsuccessful, suggesting that the determinant is chromosome-borne.

Table 1
Characteristics of *B. subtilis* plasmid-bearing strains isolated from natural sources

Strain number	Source	Sensivity to specific *B. subtilis* phages[a]					Number of Trp^+ transformants per μg of DNA[b]	Plasmid size (kb)
		AR1	AR3	AR9	Θ105	SP01		
4K31	Rainwater (Minsk Street)	+	−	+	−	+	0.8×10^6	6.3
21Z	Field (Minsk region)	+	−	−	−	+	1.3×10^6	6.3
1	Lac Sviatskoe (Gomel region)	+	−	−	−	+	0.9×10^6	8
8	Meadow (Grodno region)	+	±	±	±	+	1.5×10^6	8 >90
57	Bobrik river (Brest region)	+	−	−	±	+	1.9×10^6	8 >90
2	Lac Naroch (Minsk region)	+	±	±	−	+	1.1×10^6	8 >90
4	Lac Rislovskoe (Gomel region)	±	−	−	−	±	1.3×10^6	8 >90
15	Lac Rislovskoe (Gomel region)	±	±	±	±	+	0.2×10^6	8 >90
N1	Lac Naroch (Minsk region)	±	−	±	±	+	0.3×10^6	8 >90
19	Forest (Grodno region)	±	−	−	−	+	1.1×10^6	>90
72	Flower bed (Minsk region)	+	+	+	+	+	1.3×10^6	>90

[a] (+) Clear plaques; (±) turbid plaques; (−) no plaque.
[b] Values are determined with 1 ng DNA. A *B. subtilis* Trp^+ DNA gave 0.8×10^6 transformants/μg.

3.2. Cloning and primary characterization of plasmid replicons

To characterize further the detected plasmids, their replication region was cloned. For this purpose, the purified plasmid DNA and the *E. coli* vector pMTL21C (Chambers et al., 1988) were cleaved by a restriction enzyme (*Kpn*I, *Sma*I, *Bam*HI, *Sal*I, *Hin*dIII, *Bgl*II, *Xho*I, *Eco*RI, *Pst*I, *Sac*I or *Sty*I), mixed, and ligated. Upon transformation of the *B. subtilis* 168 strain, Cm^R transformants were selected (the *cat* gene is carried by the vector). Hybrid plasmids were then extracted and amplified in *E. coli* cells. Plasmids isolated from *E. coli* carried insert 1–6.3 kb long that originated from nine small and two large plasmids, as shown by Southern blot analysis (Table 2). Small plasmids from strains 8 and 57 might be identical, since the native element and the cloned *Eco*RI replicon are of similar size. The lack of detailed restriction analysis prevents to draw any conclusion on this matter for other extrachromosomal elements. Three observations suggested that the cloned fragments encompass most of the plasmid replication and maintenance functions. First, the *E. coli*-purified hybrids efficiently transformed *B. subtilis*. Second, 9 out of the 11 constructs displayed a rather good segregational stability as less than 10% of the cells lost the plasmid (i.e., were Cm^S) after 20 generations of growth without selective pressure (Table 2). Third, the copy number of the constructs generally mirrored that of the original replicon. In case of the hybrid carrying the replicon of the large plasmid of strain 72, the copy number was measured and shown to be 6 units per chromosome (see Section 2).

To gain insight into the mode of replication, the relatedness of the cloned replication regions to each other and to typical Gram-positive replicons was investigated by hybridization. Regular stringent conditions were set up in this experiment (Sambrook et al., 1989). Results obtained with the replicon of the 8 kb N1 plasmid as a probe (pMTLN1, Fig. 1) showed that small plasmids of the collection hybridize with the probe as do reference RC plasmids pC194 and p1414. On the contrary, no hybridization was detected with

Table 2
Properties of cloned replicons[a]

Strain number	Plasmid size (kb)	Enzyme used for cloning	Cloned replicon (size, kb)	Construct designation	ssDNA production	Segregational stability (%)	DNA polymerase I dependence
4K31	6.3	*Hin*dIII	6.3	pMTL4K31	+	96	–
21Z	6.3	*Hin*dIII–*Eco*RI	3.9	pMTL 21Z	–	98	–
1	8	*Hin*dIII	2.5	pMTL1	+	90	–
8	8	*Eco*RI	2.8	pMTL8	–	72	–
57	8	*Eco*RI	2.9	pMTL57	–	82	–
2	8	*Hin*dIII	1	pMTL2	+	98	–
4	8	*Pst*I	3.9	pMTL4	+	93	–
15	8	*Eco*RI	1.6	pMTL15	+	98	–
N1	8	*Eco*RI	1	pMTLN1	+	96	–
19	>90	*Eco*RI	5.5	pMTL19	–	98	–
72	>90	*Bgl*II	3.1	pMTLBS72	–	100	–

[a] See Section 2 for more details.

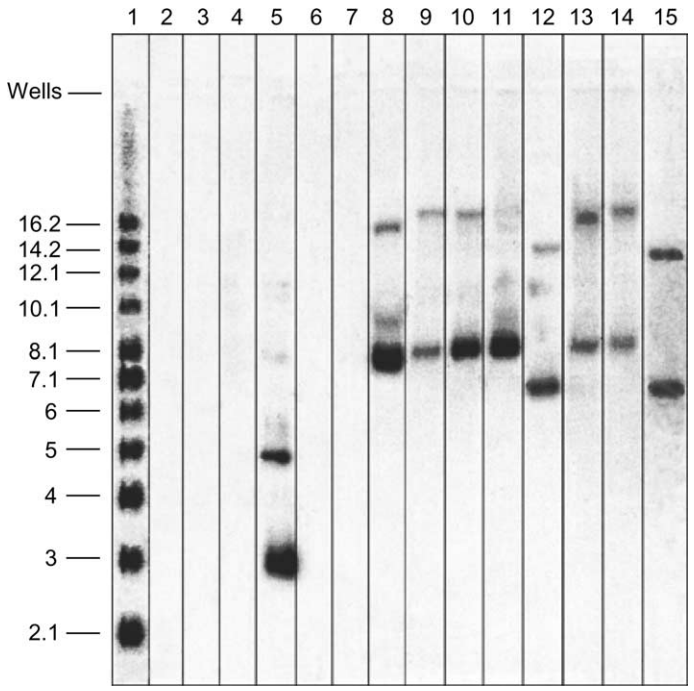

Fig. 1. Replication determinants of small natural plasmids belong to the pC194 family of RC replicons. Plasmid DNA of reference plasmids and natural strains were extracted, electrophoresed, transferred to a membrane, and hybridized against labeled pMTLN1 DNA. 1, Supercoiled DNA ladder; 2–8, reference plasmid DNAs (2, pAMβa1 replicon; 3, pTB19 replicon; 4, pT181; 5, pC194; 6, pE194; 7, pLS20; 8, p1414); 9–15, natural strains (9, strain 15; 10, strain 4; 11, strain 2; 12, strain 21Z; 13, strain 1; 14, strain 57; 15, strain 4K31). Numbers on the left refer to DNA size (kb).

either large natural plasmids or with reference theta replicons (pAMβ1, pTB19, and pLS20) and RC plasmids (pT181 and pC194). This shows that small plasmids of the Belarus collection are of RC type and belong to the pC194 family, as expected from previous studies [see as review (Meijer et al.,

1998)]. That these plasmids do replicate as rolling circles was confirmed by direct detection of ssDNA, a hallmark of RC replication (te Riele et al., 1986b), with six out of the nine constructs (Fig. 2). Interestingly, the difference in ssDNA production from natural plasmids (Fig. 1) and constructs (Fig. 2) indicates that the conversion of ssDNA to double-stranded DNA is less efficient in the second situation than in the former. This may result from the lack of the single-stranded origin in the cloned fragment or from a reduced activity of the single-stranded origin in the *B. subtilis* 168 context.

The replicon cloned from the large plasmid detected in strain 72 (pBS72) hybridized to large plasmids from strains N1, 8, and 19 (Fig. 3) and to the autonomously replicating fragment cloned from the pBS19 element (not shown). It, however, did not hybridize to reference RC- or theta-replicating replicons (pC194, pT181, pE194, pTB19, pAMβ1, and pLS20, Fig. 3). That the pBS72-type replicon differs from that of pAMβ1 is enforced by the fact that the cloned pBS72 and pBS19 fragments direct autonomous replication in a *B. subtilis* DNA polymerase I mutant (Table 2) that does not allow pAMβ1 replication (Bruand et al., 1993). Overall, this shows that the pBS72 replicon is widely represented among natural *B. subtilis* strains of the Belarus territory and suggests that it forms a new family of theta-replicating plasmids.

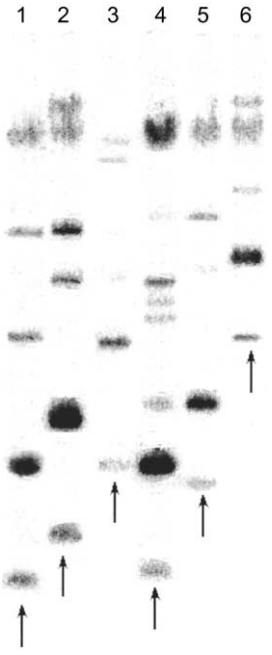

Fig. 2. Natural small plasmids replicate as rolling circles. Total DNA from *B. subtilis* strains harboring constructs containing replicons from small plasmids was electrophoresed and hybridized against a homologous (pMTL21C) radiolabelled probe. 1, pMTL2; 2, pMTL15; 3, pMTL4; 4, pMTLN1; 5, pMTL1; 6, pMTL4K31. (↑) Position of ssDNA.

3.3. Sequence analysis of the pBS72 replicon

To characterize further the pBS72 replicon, its nucleotide sequence was determined. Results show that the cloned *Bgl*II fragment is 3081 bp long and contains three complete and one truncated ORFs (ORFs 1–3 and 4, respectively, Fig. 4, top; Accession No. AY102630). ORF-1 and -2 are expected to be expressed, as they are preceded by conventional expression signals (Vellanoweth, 1993; Jarmer et al., 2001). The lack of promoter upstream of the two remaining ORFs, which are predicted to form an operon in the native plasmid, makes their expression in the construct unlikely. A rho-independent transcription terminator lies downstream of each complete ORF. The C-terminal part of ORF-1 is homologous to the N-terminal part of bacterial replication initiators DnaA (Fig. 4, bottom). To our knowledge, such a homology has no precedent in the literature. The region ensures oligomerization of DnaA at *oriC* and stable association of the replicative helicase to the prepriming complex in the *E. coli* system (Sutton et al., 1998; Weigel et al., 1999). No related protein for the remaining 271 residues of ORF-1 and for the other two complete ORFs (2 and 3) was detected in databases. The truncated protein (ORF-4) displays a strong homology with the C-terminus of bacterial proteins of the ParA/Soj family, suggesting its involvement in the partitioning of the native plasmid (Gerdes et al., 2000). However, since the ORF is truncated in the construct and since the construct lacks the *parB*-type gene required, in addition to *parA*, for partitioning (Gerdes et al., 2000), it is most likely that the putative partitioning system of pBS72 is not functional in the vector. Search for peptide motifs showed that ORF-1 product likely contains a

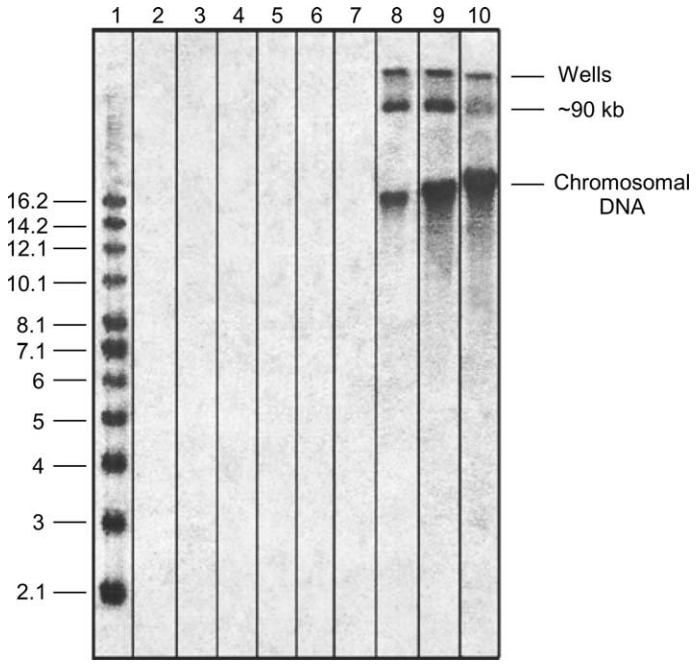

Fig. 3. Large plasmids of the Belarus collection contain a new type of replicon. Plasmid DNAs were analyzed as indicated in Fig. 1 using as a probe the pMTLBS72 DNA. Fragmentation of large plasmids during DNA preparation results in hybridization at the level of chromosomal DNA. 1, Supercoiled DNA ladder; 2–7, reference plasmid DNA (2, pAMβ1 replicon; 3, pTB19 replicon; 4, pT181; 5, pE194; 6, pC194; 7, pLS20); 8–10, natural strains (8, strain N1; 9, strain 8; 10, strain 19).

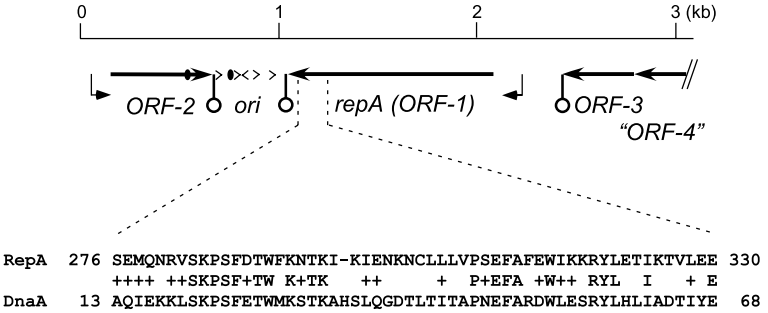

Fig. 4. Sequence analysis of the pBS72 replicon. Top: Genetic organization. The 3081 bp *Bgl*II fragment containing the pBS72 replicon encompasses three complete ORFs (1–3) and one truncated (4, horizontal arrows), conventional expression signals (promoters, bent arrows; transcription terminators, lollipops), iterons oriented rightwards (>) or leftwards (<) and *dnaA* boxes (black ovals). ORF-1, -2, -3, and -4 are 344, 168, 113, and 87 amino acid long, respectively. Bottom: Alignment of the C-terminal region of RepA with the N-terminal domain of the DnaA protein of *B. subtilis*. Identities (letters) and similarities (+) are indicated between the two protein sequences. The level of identity, similarity, and the *E*-value are 35%, 63%, and 1×10^{-5}, respectively. The best match was obtained with *B. halodurans* DnaA protein (identity, 42%; similarity, 64%; *E*-value, 9×10^{-9}). It corresponds to the ProDom domain PD002363 (Altschul et al., 1997).

HTH motif (probability of 50%) between coordinates 111 and 132. The homology to DnaA chromosomal initiators and the presence of a DNA binding motif strongly suggest that ORF-1 encodes the plasmid replication initiator that was termed RepA.

The ~370 bp intergenic region between ORF-1 and ORF-2 carries a typical DnaA binding site, TTATCCACA (Schaper and Messer, 1995), and five copies of a core consensus sequence ATTAAAT(T/A)TT(A/G)A(T/C). It might thus correspond to the origin of replication and repeated sequences might be the binding site for the plasmid initiator (iterons). An extra DnaA box is located at the 3′ end of ORF-2 (Fig. 4). Other noticeable DNA motifs [extra iterons, other repeats, replication terminus, and *dif*-like sequences (Smith et al., 1996; Sciochetti et al., 2001)] were not detected in the insert. The lack of homology with previously described RC and theta-replication functions established the fact that the cloned replicon is of a new type. The detection of a region carrying hallmarks of theta-replicating plasmids (*dnaA* boxes and iterons) indicates that it replicates by a theta mechanism.

4. Discussion

Our analysis of plasmid distribution in *B. subtilis* strains isolated from different environments of the Belarus territory shows that the strain collection frequently contains plasmids of small (<10 kb) and/or large (~90 kb) sizes. All small plasmids belong to the pC194 family and replicate as rolling circles. This is consistent with previous similar studies in *B. subtilis* [see as review (Meijer et al., 1998)]. The wide distribution of this class of replicons in this group of bacteria might result from a combination of (i) horizontal transfer and (ii) selective advantage of the plasmid harboring strains. It is known that small *B. subtilis* plasmids encode a *mob*/*pre* determinant (Gruss and Ehrlich, 1989; Meijer et al., 1998; Thorsted et al., 1999) that allows plasmid mobilization during conjugation processes (Oskam et al., 1991). The host advantage might result from various plasmid encoded products that participate in cellular metabolism and optimize host establishment in natural environments (Meijer et al., 1998; Thorsted et al., 1999). What still lacks a reasonable explanation is the absence of other types of RC replicons in *B. subtilis* species. So far, in addition to the pC194 family, at least four classes of RC replication functions have been identified (Gruss and Ehrlich, 1989; Jannière et al., 1993; Khan, 1997). Most of them have a broad host range and were shown to be active in *B. subtilis* laboratory strains. So, their absence in natural *B. subtilis* species cannot result from a major defect in plasmid replication in this organism. This is enforced by in silico analysis of completely sequenced genomes that does not pinpoint major differences between replication functions of *B. subtilis* and other Gram-positive bacteria (Lemon et al., 2002). The surprising bias in RC replicon distribution in *B. subtilis* may thus be caused by subtle interplays between pC194 RC determinants and host functions. The key elements involved in this fine-tuning are not known.

Large plasmids are also likely to contain a related replicon, as cloned pBS72 (~90 kb) replication functions are homologous to those of the large plasmid of strain 19 and display similarity to two other extrachromosomal elements of the same size from strains N1 and 8. As for small plasmids, the wide distribution of this replicon might be caused by its linkage to conjugative determinants (Koehler and Thorne, 1987; Lotareva et al., 2001; Poluectova et al., 2000) and to genes providing a selective advantage to microorganisms containing them. The size of the natural plasmids, the lack of ssDNA production with small pBS72 derivatives (data not shown), and the sequence analysis of the replicon argue for a theta mode of replication. In addition, these plasmids do not depend on DNA polymerase I, showing that their replication mechanism is different from that of pAMβ1-, ColE1-, and ColE2-types plasmids from Gram-positive and Gram-negative bacteria in which replication is initiated by DNA polymerase I (Espinosa et al., 2000).

The lack of homology with sequences contained in databases shows that the isolated replicon is of a new type. Sequence analysis indicates that the product of ORF-1 and the intergenic region between ORF-1 and 2 correspond to the plasmid initiator and the origin of replication, respectively. The ORF-1 product (RepA) displays homology to DnaA chromosomal initiators and carries a DNA binding (HTH) motif and the intergenic region encompasses repeated sequences (iterons) and a *dnaA* box. It is predicted that binding of RepA to the iterons is a prerequisite for initiating plasmid

replication (Espinosa et al., 2000; Helinski et al., 1996; Jannière et al., 1993).

Interestingly, the pBS72 derived plasmid has a low copy number (6 units per chromosome) and is stably inherited in *B. subtilis* cells. According to theoretical calculations (Summers, 1991), these results strongly suggest that it encodes stabilization functions. However, our sequence analysis did not reveal any functional stabilization determinant like partition, resolution or killing functions (Gerdes et al., 2000; Helinski et al., 1996; Kobayashi, 2001; Lemon et al., 2002). Additional experiments are required to identify plasmid elements involved in this process.

Despite relatively few investigations on large plasmids, three different types of theta replicons (pBS72, pLS20, and pLS32) have already been described in *B. subtilis* species [(Meijer et al., 1995; Tanaka and Ogura, 1998) this work]. Among these, pLS20 and pBS72 have no equivalent in databases. This high diversity of theta replicons is in sharp contrast with the universality of the pC194-type RC replicon of small plasmids. As *B. subtilis* is a safe and important industrial microorganism and as theta-replicating-based vectors are in general much more useful for DNA cloning purposes than RC-based vectors (Bron, 1990; Jannière et al., 1990; Jannière et al., 1993), further analyses of these replicons and development of plasmid tools appear interesting and required goals for the near future. The segregationally stable, low copy number, *B. subtilis*/*E. coli* shuttle vector (pMTLBS72) described here, might be one of these tools. It can be ordered from the *Bacillus* Genetic Stock Center (http://bacillus.biosci.ohio-state.edu) along with its nucleotide sequence and genetic map.

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