Fate of unstable *Bacillus subtilis* subgenome: re-integration and amplification in the main genome

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Abstract The plastic *Bacillus subtilis* genome was dissected into two physically separate genomes, the 3.9 Mb main genome and the 0.3 Mb subgenome. DNA replication of the main genome was initiated from the normal replication origin (*oriC*) and that of the subgenome was from a 7.2 kb *oriN*-containing fragment artificially inserted. When the 7.2 kb fragment was shortened to a 1.5 kb fragment that contains *oriN* but lacks the segregational function, the subgenome became unstable and was rapidly lost from the cell, producing inviable cells due to the loss of essential genes carried by the subgenome. Stable survivors were isolated in which the subgenome had re-integrated and multiplied in the main genome. These results suggest that a reduced genetic stability of the subgenome induces size variation of the *B. subtilis* genome.

Key words: Subgenome; *repN*; *oriN*; Homologous recombination; Essential gene

1. Introduction

Bacillus subtilis 168 is an endospore-forming Gram-positive soil bacterium that normally has a single circular genome comprized of 4215 kb [1]. The genome was artificially dissected into the 3.9 Mb main genome and the stable 0.3 Mb subgenome by homologous recombination between the two identical 590 bp sequences located 300 kb apart and a 7.2 kb extrachromosomal origin of DNA replication (*oriN*) [2]. This simple process shown in Fig. 1 not only disclosed a plasticity of the genome of long laboratory cultivated strain *B. subtilis* 168 but also may mimic the multiple genomic states found in several bacteria [3–5]. It was suggested that the genetic stability of the subgenome was supported by a number of discriminatory factors [2] such as, (i) the replication function of the *oriN* segment by which subgenome DNA replicates, (ii) genes that allow the subgenome to be maintained as low as the copy number of the main genome, (iii) essential cognate genes carried by the subgenome, the loss of which renders the cell inviable. We thought that a study on the genetic stability of the subgenome expedites to understand the underlying principles with respect to DNA re-arrangement, horizontal transfer and genome evolution that may be mediated by the subgenome formation [2].

The 7.2 kb *oriN* fragment was originally isolated from a low copy plasmid pLS32 of *Bacillus natto* IFO1163 and cloned in the pBET131 plasmid [6]. Tanaka and Ogura sequenced the entire insert and located a minimal functional *oriN* sequence in as small as an 861 bp fragment by measuring the ability of various deletion derivatives of pBET131 to replicate as a plasmid in vivo [6]. The minimal replication origin is within an orf designated as *repN* whose expression was required for the initiation of DNA replication [6]. However, the stability of the minimal *oriN* plasmid was significantly reduced compared with the parental *oriN* plasmid pBET131 and it was suggested that there is a DNA region responsible for the plasmid segregation (T.T. and M. Ogura, unpublished). We are interested in the genetic stability of the subgenome when the stability region is removed, because the subgenome carries genomic genes indispensable for the viability of *B. subtilis*. In this report, we constructed strains having subgenomes with the minimal *oriN* sequence and examined their stability.

2. Materials and methods

2.1. Bacterial strains and plasmids

Bacterial strains and plasmid constructed in this study are described in the text. LB broth [7] was used for growth of a cloning host *Escherichia coli* (JA221; F$^-$ *hsdR hsdM*$^+$ *trp leu lacY recA1* [2]) and *B. subtilis*. Preparation and transformation of the competent *B. subtilis* were done as described previously [8].

2.2. Construction of mini-oriN plasmids and integration into the B. subtilis genome

A 1.5 kb *Eco*RI-*Hin*dIII fragment isolated from the 7.2 kb insert of the pBET131 was carried by pYBF2 that comprises of a 1.0 kb chloramphenicol-resistance gene from pBEST402 [9] and a 2.6 kb *E. coli* vector (pUC-KIXX, Pharmacia Biotech, USA). The 1.5 kb fragment has a minimal functional *oriN* sequence, hereafter designated as mini-*oriN* in contrast to the 7.2 kb sequence in pBET131 designated as *oriN*. The mini-*oriN* (pYBF2) was needed to be modified for integration of the genome of BEST5018 [2], a parental strain of BEST4173 shown in Fig. 1. The 5.1 kb pYBF2 was digested by *Sma*I and ligated to the *Sfi*I site of an *Sfi*I-linking clone (pSOFT4 [10]) that had been blunt-ended by Klenow DNA polymerase, resulting in pSO5. pSO5 was used to integrate the mini-*oriN* in the BEST5018 genome by transformation. Chloramphenicol-resistant BEST4141 was obtained from BEST5018 and the structure of mini-*oriN* in the *Sfi*I site close to the *proB* locus [2,10] was confirmed by Southern analysis (data not shown).

2.3. Physical map of the B. subtilis 168 genome

The *Not*I-*Sfi*I-I-*Ceu*I physical map of the *B. subtilis* 168 genome [10,11] was confirmed by a determined nucleotide sequence [1].

2.4. In vitro DNA manipulations

Type II restriction enzymes, alkaline phosphatases, T4 DNA polymerase and T4 DNA ligase were obtained from Toyobo (Tokyo, Japan), except for *Sfi*I (New England Biolab, Beverly, MA, USA) and *Not*I (Takara Shuzo, Kyoto, Japan). Homing endonucleases, I-*Sce*I and I-*Ceu*I were purchased from Boehringer-Mannheim (USA) and NEB, respectively. DNA manipulations in vitro were done according to the method described in [12] or the manufacturers' instructions unless otherwise specified. The Southern hybridization procedure has been described previously [10].

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2.5. Preparation of the B. subtilis genome DNA

Preparation and digestion of genome DNA for contour-clamped homogeneous electric field (CHEF) gel electrophoresis were carried out as described previously [10]. DNAs were run in TBE solution (45 mM Tris-borate, pH 8.0, 1.0 mM EDTA) under the constant voltage of 3 V/cm at 14°C. The pulse and running time are specified in each figure. Genomic DNA in a liquid form prepared by the method of Saito and Miura [13] was used for Southern hybridization experiments after cleavage by 6 base-cutting restriction enzymes. A densitometer (CS-9300PC, Shimadzu, Kyoto, Japan) was used to estimate the amounts of ethidium bromide-stained bands.

3. Results

To obtain strains carrying the mini-*oriN* subgenome, BEST4141 was grown in LB medium in the absence of antibiotics at 37°C. 100 μl of the stational culture was spreaded on LB-CBSN plates (LB plate containing chloramphenicol (5 μg/ml), blasticidin S (400 μg/ml), spectinomycin (25 μg/ml) and neomycin (5 μg/ml)). After incubation at 30°C for 24 h, 132 neomycin-resistant colonies were obtained. Four clones were transferred to fresh LB-CBSN medium and incubated for 24 h at 37°C from which genomic DNA was prepared and analyzed by site-specific endonucleases. Because an I-*Sce*I site is allocated to the subgenome as illustrated in Fig. 1, the subgenome can be linearized by I-*Sce*I cleavage and resolved as a 300 kb fragment by CHEF gel electrophoresis [2]. An example, BEST4150, is shown in Fig. 2a where a fragment with approximately 300 kb was observed and designated as mini-*oriN* subgenome. However, the amount of the fragment was significantly reduced compared with that of the *oriN* subgenome in BEST4175 [2]. The apparent reduction of the mini-*oriN* subgenome of BEST4150 was confirmed in a *Not*I digestion analysis (Fig. 2b). *Not*I fragments 11N (120 kb), 20N (85 kb) and 21N (84 kb) that are carried in the mini-*oriN* subgenome were significantly reduced in BEST4150, being approximately 4.7% of those of the parental strain BEST4141. Physical separation of the mini-*oriN* subgenome from the main genome was verified by the shortened fragment between *rrnD* and *rrnB* (Fig. 1), the DB fragment produced on I-*Ceu*I digestion (Fig. 2c) and an alteration of the AS fragment (*Sfi*I, [10]) associated with the subgenome formation (data not shown). Many long filamentous cells in the BEST4150 culture were observed under microscopic examination compared with the parental strain BEST4141 (data not shown). The genome structures of the other three isolates were similar to those of BEST4150 (data not shown).

These observations indicated that only 5% of the cells in the BEST4150 culture had the expected genomic state and the remaining 95% cells had lost the mini-*oriN* subgenome. This is consistent with an observation that the genetic stability of the pSO5 plasmid (mini-*oriN*) in *B. subtilis* is reduced to approximately 10% of that of the pBET131 plasmid (*oriN*) (T.T. and M. Ogura, unpublished).

To examine the genetic stability of the mini-*oriN* subgenome, the BEST4150 were inoculated into fresh 25 ml LB+CBSN medium at a dilution of 10^{-3}, 10^{-6}, 10^{-8} and 10^{-9} and incubated at 37°C. The 10^{-9} dilution that initially contained at least five cells as estimated under a microscopic measurement, did not become turbid after 88 h. As for the other three, the stational phase was reached after 18 h for 10^{-3} (BEST4150-3), 25 h for 10^{-6} (BEST4150-6) and 44 h for 10^{-8} (BEST4150-8). The genome structure of BEST4150-8 was similar to that of BEST4150 (Fig. 2a and b). This was consistent with the estimation that 95% of the BEST4150 culture had lost the mini-*oriN* subgenome and those cells were unable to start growing due to the lack of indispensable genes carried by the subgenome.

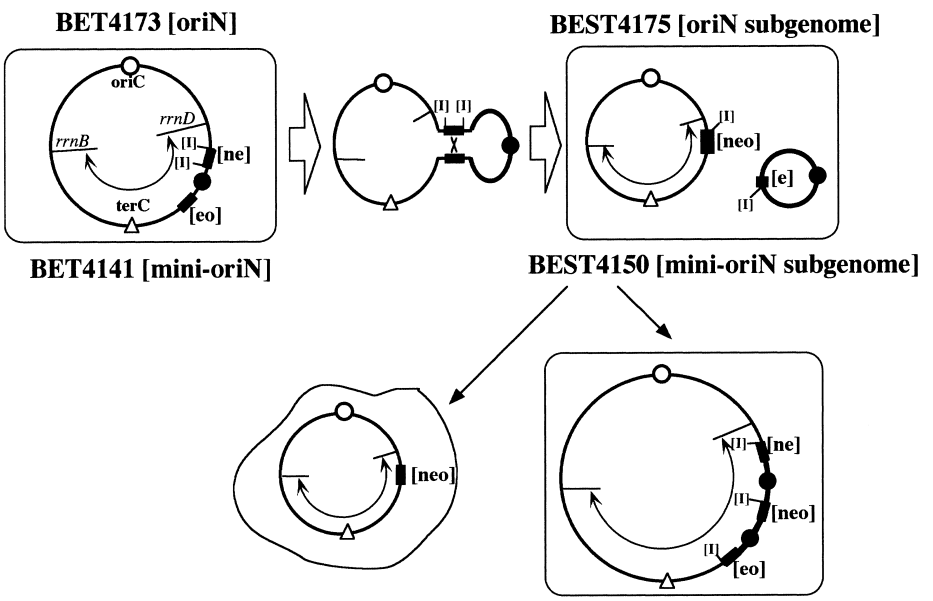

Fig. 1. A scheme to form subgenomes. *B. subtilis* strains BEST4173 [2] and BEST4141 have similar structures except for the *oriN* sequence shown as a closed circle. A single circular genome with the locations of *oriC*, *terC*, *rrnD*, *rrnB*, [ne] and [eo] is shown. A cross-over between the two homologous stretches [ne] or [eo] creates the subgenome, the DNA replication of which initiates from the *oriN* in BEST4175 [2] and from mini-*oriN* in BEST4150. BEST4175 and BEST4150 have recombinant [neo] rendering the cell neomycin-resistant [2]. One of the two I-*Sce*I sites [1] at the end of the [ne] is allocated to the subgenome. An I-*Ceu*I site resides in the *rrnD* and *rrnB* [11]. The DB fragments shown in Fig. 2c cover the genomic regions by double-headed curved arrows. BEST4150 contains cells that have lost the subgenome and are unable to start cell division (bottom left) and those in which subgenomes integrate and multiplied in the main genome and restore normal growth (bottom right).

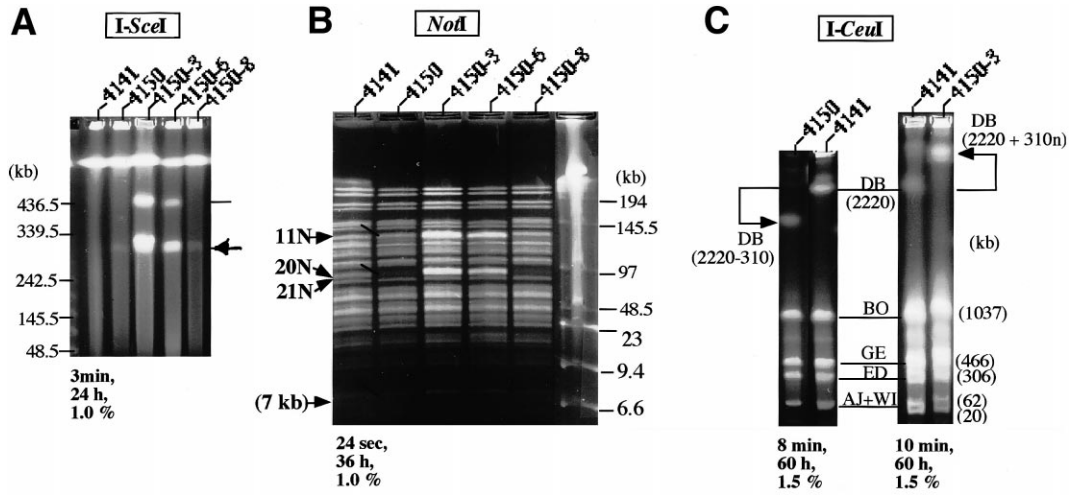

Fig. 2. Isolation and characterization of the state of the mini-*oriN* subgenome and the main genome. DNA isolated from the indicated strains is separated by CHEF gel electrophoresis after digestion by (a) I-*Sce*I, (b) *Not*I and (c) I-*Ceu*I. Concatemeric lambda DNA (48.5×n) and *Hin*dIII-digested lambda DNA are size markers. The pulse time, running time and gel concentration (%) are shown below each figure. (a) The position of the subgenome fragment is indicated by an arrow in the right. The slow migrating fragment observed in BEST4150-3 and BEST4150-6 (indicated by a bar) is described in the text. (b) The 310 kb DNA includes five *Not*I fragments, 120 kb (11N), 85 kb (20N), 84 kb (21N) and 7 kb [2] are indicated by arrows in the left. (c) The DB fragment (2220 kb indicated in Fig. 1) of BEST4141 was shortened in BEST4150 by excision of the 310 kb segment and elongated in BEST4150-3 by 310×*n*. Multiplicity (*n*) was estimated three.

To our surprise, the amounts of the 300 kb I-*Sce*I fragment of BEST4150-3 and 4150-6 were significantly increased (Fig. 2a). The corresponding *Not*I fragments, 11N, 20N, 21N and 7 kb, were also increased at a multiplicity of 3.7 for 11N and 4.4 for 20N and 21N compared with those of BEST4141 (Fig. 2b). However, the DB fragment (I-*Ceu*I) of BEST4150-3 was not shortened but elongated as seen in Fig. 2c. Thus, it is likely that these amplification was caused not by an increase of the copy number of the mini-*oriN* subgenome, but by re-integration of a multimer form of the subgenome as illustrated in Fig. 1. Simple re-integration of the single copy subgenome should restore BEST4141 and it becomes neomycin sensitive and cannot be selected for neomycin-resistance (Fig. 1). Since the neomycin was always present in the medium, only the cells having at least a duplicated form can carry an intact neomycin-resistance gene. The estimated multiplicity of four in the BEST4150-3 genome seems consistent with the need.

To investigate viable clones in the BEST4150 culture, we isolated two kinds of colonies, normal and small in size, formed on the LB-CBSN plate. Analyses of the genome structure of the two small colonies gave similar results to those of BEST4150 (data not shown). Two normal size colonies had similar genome structures to those of BEST4150-3 (data not shown). These observations indicated that the BEST4150 culture consists of heterologous cells with respect to the state of the mini-*oriN* subgenome. It seems that the re-integrated cells resumed a normal growth and overrode the slow growers that carry the mini-*oriN* subgenome, although accurate populations of the normal growers in the BEST4150 culture remained to be estimated.

There were slow migrating I-*Sce*I fragments (approximately 440 kb) observed in BEST4150-3 and BEST4150-6 (Fig. 2a). A preliminary investigation indicated that an I-*Sce*I site was created in association with a deletion of approximately a 30 kb region between the 5N and 16N fragment (*Not*I) in the genome [10]. Characterization of this DNA re-arrangement is under study.

4. Discussion

The mini-*oriN* subgenome was shown to be unstable in *B. subtilis* and rapidly lost during growth. The sharp contrast to the stable *oriN* subgenome [2] is rationalized due to the absence of segregational machinery in the mini-*oriN*[6]. This appears to indicate that the mini-*oriN*-dependent replication produces anucleate cells with respect to subgenome at a significantly high rate even under antibiotic selection conditions.

Moriya et al. applied our mini-*oriN* to the *B. subtilis* genome [14,15] and demonstrated that it allows replication of the entire *B. subtilis* genome when the *dnaA* gene is inactivated or the *oriC* region is deleted, both of which are absolutely required for the normal initiation of chromosome replication. The mini-*oriN*-dependent *B. subtilis* strains grow very slow and produce anucleate cells at extremely high rates, 2–6% of the cells under microscopic observation [14,15]. They discussed that both phenotypes may be caused by being uncoupled from regulation factors for the normal chromosomal segregation [16] and/or positional effects of the mini-*oriN* in the genome [15]. We think it is more feasible to attribute the anucleate cell production simply to the lack of a segregational machinery included in the *oriN*, although our speculations remain to be demonstrated.

There are numerous examples in bacteria that DNA segments exist as autonomously replicating and integrated forms in the host genome such as *E. coli* phage lambda, switching the lysogenic state and the lytic cycle [17] or the F-factor, shuttling between the Hfr and plasmid forms [18]. These conversions of states proceed by a stable origin of DNA replication and well-regulated gene functions. Our present study implied that a segment larger than 300 kb can shuttle between subgenomic and integrated states whose process does not seem to require other specific genes than normal homologous recombinational pathways. Our observations suggest that the combination of a functionally unstable origin of DNA replication and homologous sequences may accelerate the size var-

iation of the bacterial genomes or even eukaryotic genomes in which many replication origin sequences and repetitive sequences are present.

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