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Suppression of Initiation Defects of Chromosome Replication in *Bacillus subtilis dnaA* and *oriC*-Deleted Mutants by Integration of a Plasmid Replicon into the Chromosomes

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We constructed *Bacillus subtilis* strains in which chromosome replication initiates from the minimal replicon of a plasmid isolated from *Bacillus natto*, independently of *oriC*. Integration of the replicon in either orientation at the *proA* locus (115° on the genetic map) suppressed the temperature-sensitive phenotype caused by a mutation in *dnaA*, a gene required for initiation of replication from *oriC*. In addition, in a strain with the plasmid replicon integrated into the chromosome, we were able to delete sequences required for *oriC* function. These strains were viable but had a slower growth rate than the *oriC*$^+$ strains. Marker frequency analysis revealed that both *pyrD* and *metD*, genes close to *proA*, showed the highest values among the markers (genes) measured, and those of other markers decreased symmetrically with distance from the site of the integration (*proA*). These results indicated that the integrated plasmid replicon operated as a new and sole origin of chromosome replication in these strains and that the mode of replication was bidirectional. Interestingly, these mutants produced anucleate cells at a high frequency (about 40% in exponential culture), and the distribution of chromosomes in the cells was irregular. A change in the site and mechanism (from *oriC* to a plasmid system) of initiation appears to have resulted in a drastic alteration in coordination between chromosome replication and chromosome partition or cell division.

Major events in the bacterial cell cycle include chromosome replication, chromosome partition, and cell division. These processes are coordinated to ensure that the proper number of chromosomes is maintained in each progeny cell over a variety of growth rates. In order to understand the regulation of the bacterial cell cycle, it is important to know the mechanisms coupling the above three processes. However, at present, little is known about these coupling mechanisms (6).

Initiation of chromosome replication takes place when the cell mass per replication origin (*oriC*) reaches a critical value, called the initiation mass (6). The DnaA protein and multiple repeats of the DnaA protein binding sequence (DnaA box) in the *oriC* region are well conserved in eubacteria (46), indicating that DnaA is executing a key role in initiation of bacterial chromosome replication. In *Escherichia coli*, overproduction of DnaA decreased the initiation mass (21). Therefore, it is postulated that either synthesis of a critical amount of DnaA per origin or the DnaA concentration in the cell determines the initiation mass (11). However, it was also demonstrated that phospholipids reactivated an inactive form of DnaA (ADP-DnaA) in vitro by exchanging ADP for ATP (37). Also, initiation from *oriC* seemed to be inhibited in vivo in the *pgsA* mutant in which synthesis of phospholipids was limited (43), suggesting that the activity of DnaA might also affect the time of initiation of replication. Although the role of DnaA as a key factor for regulation of initiation of replication is clear, the interpretation of how cells recognize their initiation mass is not clear.

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The molecular mechanism of chromosome partition in bacteria is less known than that of chromosome replication. The partition mutants isolated so far are roughly divided into two categories (13, 40). The mutants belonging to one category are defective in decatenation of interlinked chromosomes or resolution of dimer chromosomes formed during chromosome replication. These processes are necessary for preparing separable chromosomes for two progeny cells. The mutants of the other category are defective in processes concerned more directly in chromosome partition (e.g., chromosome movement) and produce anucleate cells at an enhanced level. Several *trans*-acting factors engaged in these processes (chromosome positioning) have recently been identified by Hiraga (13) and by Yamanaka et al. (45). However, *cis*-acting centromere-like sequences found in plasmids have not yet been identified in bacteria.

The mechanisms coordinating chromosome replication and cell division in the normal cell cycle of bacteria are poorly understood to date, although a special coordination mechanism induced by DNA damage is well documented in *E. coli* (22). In 1991, Bernander and coworkers noted that chromosome replication and cell division were not sequential steps in the bacterial cell cycle but were independent and parallel pathways with coordination between them (3, 32). They used an *E. coli* strain in which *oriC* was disrupted by insertion of the minimal replicon of plasmid R1, resulting in initiation of replication being dependent on the control system of the plasmid replication. Since the amount of the RepA protein was regulated by a temperature-sensitive λ*c*I repressor in this system, the initiation mass could be varied with temperature. They reported that cell size was not affected despite increasing DNA content with increasing temperature, and they concluded that chromosome replication did not trigger cell division (2). How-

TABLE 1. *Bacillus subtilis* strains used in this study

Strain	Genotype	Source or reference
CRK6000	*purA16 metB5 hisA3 guaB*	25
CRK3000	*leuB8 metB5 purA16 hisA3*	44
QB922	*trpC2 gltA292*	5
QB926	*pyrD1 hisA1 ilvA1 trpC2*	5
GSY1035	*ura1 metC hisA1*	C. Anagnostopoulos
QB858	*glyB133 metD1*	*Bacillus* Genetic Stock Center
1A12	*leuB8 metB5 purF6*	*Bacillus* Genetic Stock Center
NIS11	*ura1 hisA1 purF6*	This study
NIS21	*metD1 hisA3*	This study
CRK6010	*purA16 hisA3 guaB dnaA1*	25
BD54*dnaB19*	*metB5 ilvA dnaB19*	18, 34
CRK3199	CRK3000 *dnaC199*	9, 36
BD54*dnaD23*	*metB5 ilvA dnaD23*	4, 18
BD54*dnaN5*	*metB5 ilvA dnaN5*	18, 33[a]
NIS6060	*purA16 metB5 guaB dnaN5*	This study
NIS6061	NIS6060 *proA*::pBPA23	This study
NIS6062	NIS6060 *proA*::pBPA21	This study
NIS6011	CRK6010 *proA*::pBPA23	This study
NIS6012	CRK6010 *proA*::pBPA21	This study
NIS6100	CRK6000 *proA*::pBPA23	This study
NIS6200	CRK6000 *proA*::pBPA21	This study
NIS6101	*purA16 metB5 guaB proA*::pBPA23 Δ*oriC*	This study
NIS6201	*purA16 metB5 guaB proA*::pBPA21 Δ*oriC*	This study
NIS6102	NIS6101 *rtp*::*erm*	This study
NIS6202	NIS6201 *rtp*::*erm*	This study
NIS6111	*purA16 metB5 guaB proA*::pBPA23 *dnaA*(*ochre*)	This study
NIS6211	*purA16 metB5 guaB proA*::pBPA21 *dnaA*(*ochre*)	This study

[a] Formerly *dnaG5*.

ever, it is still generally accepted that normal cell division requires completion of replication and nucleoid segregation (6, 29). Recently, Wu et al. found that septum formation and separation of chromosomes to both sides of the septum took place without completion of replication in *Bacillus subtilis* (although movement of the chromosomes to correct positions was inhibited) (42). These results suggested that signals for septation and separation of daughter chromosomes were generated within a period during which half the chromosome was replicated.

To analyze mechanisms coupling chromosome replication and cell division in *B. subtilis*, especially between the mode and site of initiation of replication and cell division, we attempted to construct a strain in which replication would be independent of *oriC* (by integrating a plasmid replicon into the chromosome, at a locus apart from *oriC*). We used a plasmid isolated from *Bacillus natto* (39) for this purpose, because it is a low-copy-number plasmid (1 to 2 copies/chromosome) and seemed to be replicated by a theta mechanism (39a). The *oriC* sequence could be deleted after integration of the miniplasmid into the chromosome at 115° on the *B. subtilis* genetic map. Interestingly, the mutant produced anucleate cells at a high frequency (about 40% in an exponentially growing cell population), suggesting impairment in chromosome partition.

MATERIALS AND METHODS

Bacterial strains. *E. coli* JM103, DH5α, and XL-1 blue were used for molecular cloning. *B. subtilis* strains used in this study are listed in Table 1. NIS11 (*ura1 hisA1 purF6*) and NIS21 (*metD1 hisA3*) were constructed by transformation (congression) of GSY1035 (*ura1 metC hisA1*) and CRK6000 (*purA16 metB5 hisA3 guaB*) with chromosomal DNA of 1A12 (*leuB8 metB5 purF6*) and QB858 (*glyB133 metD1*), respectively. NIS6060 (*purA16 metB5 guaB dnaN5*) was constructed by transformation of CRK6000 with chromosomal DNA from BD54*dnaN5*, selection for *his*$^+$, and testing for temperature sensitivity due to *dnaN5*. NIS6011 and NIS6012 were obtained as chloramphenicol-resistant strains by integration of mini-pLS32 plasmids pBPA23 and pBPA21, respectively, into the *proA* locus of CRK6010 (*purA16 hisA3 guaB dnaA1*). These integrated forms of the plasmids were transferred by transformation into the NIS6060 and CRK6000 chromosomes, resulting in NIS6061 (NIS6060 *proA*::pBPA23), NIS6062 (NIS6060 *proA*::pBPA21), NIS6100 (CRK6000 *proA*::pBPA23) and NIS6200 (CRK6000 *proA*::pBPA21) strains.

Plasmids. Two plasmids, pBPA21 and pBPA23, were constructed to integrate a minimal replication region of pLS32 isolated from *B. natto* (39) into the *proA* locus of the *B. subtilis* chromosome. Both are pUC18 derivatives and contain a 1.07-kb fragment (*Bcl*I-*Eco*RI) of the coding region of *proA* (35) in the *Sma*I site, a 2.5-kb fragment including the minimal replication region (*oripLS32*) (1.5 kb; *Eco*RI-*Hin*dIII), and the chloramphenicol acetyltransferase (CAT) gene of pC194 (1.0 kb; *Msp*I-*Sau*3AI) (15) in the *Eco*RI site of pUC18 (see Fig. 1). Both *Xba*I and *Eco*RI sites at both sides of the CAT fragment were generated during construction of the plasmids by use of multicloning sites.

pNO41Δ*oriC* was constructed to delete 103 bp within *oriC* on the *B. subtilis* chromosome. This plasmid was obtained by insertion of an *Eco*RI fragment containing the C-terminal 784 bp of the *dnaA* gene [nucleotide numbers 1175 (*Eco*RI site) to 1959 in reference 23] and a multicloning sequence of pSPT18 (Pharmacia, Uppsala, Sweden) into the *Eco*RI site of pSM4060dL5 (24) which carried the N-terminal region of the *dnaN* gene. As a result, a 103-bp (nucleotides 1960 to 2063) (23) fragment containing three DnaA boxes within the downstream untranslated region of *dnaA* gene was replaced by the multicloning sequence in the plasmid (see Fig. 2).

A plasmid, pAH1, was constructed for disruption of the *rtp* gene in *B. subtilis*. A fragment of 795 bp from the upstream region of the *rtp* gene, including the first 6 bp of its N-terminal coding region, was amplified by PCR with primers *terC*1 (nucleotides 1162 to 1181) (1) and *terC*2 (nucleotides 1937 to 1956, designed to contain an artificial *Bam*HI site). Another fragment of 836 bp from the downstream region, including the last 78 bp of the C-terminal coding region of the *rtp* gene, was amplified by PCR with primers *terC*3 (nucleotides 2227 to 2258, also designed to contain a *Bam*HI site) and *terC*4 (nucleotides 3043 to 3062) (1). These two fragments were digested with *Bgl*II (nucleotides 1184 to 1189) and *Bam*HI, and with *Bam*HI and *Eco*RI (nucleotides 3011 to 3016) respectively, ligated to each other at the central *Bam*HI site, and then inserted between the *Bam*HI and *Eco*RI sites of a plasmid, pBEST703 (17), a derivative of pBR322. Finally, the erythromycin-resistant gene from the *Bsp*MII-to-*Cla*I region of pE194 (14) was inserted into the *Bam*HI site of the plasmid described above after the ends of the fragments were repaired by Klenow fragment.

Integration of mini-pLS32 plasmids into the *B. subtilis* chromosome. Plasmid pBPA21 or pBPA23 was introduced into CRK6010 (*purA16 hisA3 guaB dnaA1*) cells by transformation. As these plasmids could replicate in *B. subtilis*, chloramphenicol-resistant colonies obtained by transformation were grown overnight twice on agar plates of antibiotic medium 3 (Penassay broth) (Difco, Detroit, Mich.) without chloramphenicol to allow loss of free plasmids. The colonies were then again spread on plates containing chloramphenicol (10 μg/ml) to select clones in which the plasmids were integrated into the chromosome. Chromosomal DNA was extracted from several clones to confirm this integration by Southern hybridization. After chromosomal DNA was digested with appropriate restriction enzymes, the resultant DNA fragments were separated in a 1% agarose gel and transferred to a nylon membrane. Labeling of probes, hybridization, and detection of the signals were carried out with the ECL direct nucleic acid labeling and detection system (Amersham, Buckinghamshire, United Kingdom) according to the supplier's instructions.

Marker frequency analysis. The frequency of various genetic markers on the *B. subtilis* chromosome was estimated by transformation as described previously (38). Transformation of *B. subtilis* cells was carried out by a conventional method as described previously (8) or the modified method of Kunst et al. (20). The *purA16* mutation in *oriC*-deleted strains (NIS6101 and NIS6201) was replaced by the wild-type sequence by transformation before this analysis.

Chromosomal DNA was extracted from exponentially growing cultures and used as donor DNA for this analysis. The following strains were used as recipients to obtain the following transformants for the marker frequency analysis: CRK6000 (*purA16 metB5 hisA3 guaB*) for *purA*$^+$ and *hisA*$^+$ transformants; CRK3000 (*leuB8 metB5 purA16 hisA3*) for *leuB*$^+$ and *hisA*$^+$ transformants; QB926 (*pyrD1 hisA1 ilvA1 trpC2*) for *pyrD*$^+$, *ilvA*$^+$, and *hisA*$^+$ transformants; NIS11 (*ura1 hisA1 purF6*) for *purF*$^+$ and *hisA*$^+$ transformants and NIS21 (*metD1 hisA3*) for *metD*$^+$ and *hisA*$^+$ transformants. Ratios of various markers to *hisA* were calculated as the number of transformants for a given marker divided by the number of transformants for *hisA*, and these were normalized to the ratio obtained using DNA made from nonreplicating cells [strain QB922 (*trpC2 gltA292*)] that were in stationary phase.

Microscopic observation. *B. subtilis* cells grown in Penassay broth were harvested at an optical density at 600 nm of 0.2 by centrifugation. After washing with 10 mM Tris buffer (pH 7.4), cells were suspended in 1 ml of 70% ethanol for fixation. The fixed cells were collected by centrifugation, washed with the same buffer, and spread on a slide glass pretreated with polylysine. Cells were stained

with a DAPI (4′,6-diamidino-2-phenylindole) solution (1 μg/ml in 50% glycerol) (10), and chromosome distribution in cells was observed by a method described by Hiraga et al. (12) with fluorescence microscopy (Olympus BX50 with a UPlanApo-IrisPH3 100× oil immersion objective and a U-MWU mirror cube unit). The image was captured by a color-chilled 3CCD camera (C5810; Hamamatsu, Japan).

Sporulation efficiency. *B. subtilis* cells were grown in 2×SG medium (30) supplemented with adenine, guanosine (requirements for growth; 20 μg/ml), and chloramphenicol (5 μg/ml) at 37°C (with shaking). At 12 h after the end of exponential growth (T_{12}), the total number of viable CFU (viable cells) and the number of heat-resistant (80°C for 10 min) CFU (spores) were determined. Sporulation efficiency was calculated as the percentage of spores per viable cell.

Immunoblotting. *B. subtilis* cells growing exponentially were collected by centrifugation, and cell lysates were prepared as described previously (25). Cell lysates containing approximately equal amounts of total protein (20 μg) were applied to each lane of a sodium dodecyl sulfate-polyacrylamide gradient (10 to 20%) gel, and resolved proteins were transferred to a nitrocellulose membrane. DnaA protein was detected by using the ECL Western blotting detection reagents (Amersham) after the nitrocellulose membrane was treated with anti-DnaA rabbit antiserum (25) followed by treatment with a second antibody (goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate) (Bio-Rad Laboratories, Richmond, Calif.). To know the limit of detection for the DnaA protein in this immunoblot assay, cell lysate from a *dnaA*-null mutant was mixed with a cell lysate from the wild-type strain in various proportions, and an equal amount of total protein (40 μg) was resolved and analyzed as described above.

RESULTS

Suppression of the temperature-sensitive growth of a *B. subtilis dnaA* mutant by integration of a plasmid containing a minimal replication region of pLS32 into the chromosome. Plasmid pLS32 isolated from *B. natto* IAM1163 is a low-copy-number plasmid (1 to 2 copies/chromosome) of about 70 kb. A 1.5-kb fragment of the plasmid was identified as the minimal region required for replication. An open reading frame (ORF), presumably encoding a Rep protein, and 6 repeats of a 10-bp sequence within the ORF were found in this 1.5-kb fragment (39a). The ORF showed no significant homology with Rep proteins of plasmids known to replicate by a rolling-circle mechanism (39a), suggesting that pLS32 might be replicated by a theta mechanism. DnaA box sequences were also not found in the minimal replication region. This observation suggests strongly that DnaA protein is not required for replication of pLS32. We therefore postulated that this plasmid could suppress the temperature sensitivity of a *B. subtilis dnaA* mutant (25) when integrated into the chromosome. As shown in Fig. 1, two plasmids (pBPA21 and pBPA23) were constructed for integration of the pLS32 replicon into the chromosome. Both contain the minimal region for replication of pLS32 (1.5 kb), a chloramphenicol resistance gene as a selection marker in *B. subtilis*, and a 1.0-kb fragment of the *proA* gene (35) to allow integration of the plasmids into the *B. subtilis* chromosome.

The *dnaA* mutant cells were transformed with these plasmids, and the resultant chloramphenicol-resistant transformants were grown in medium lacking chloramphenicol to segregate replicating plasmids from cells. Chloramphenicol-resistant colonies were then selected again, and integration of the plasmid into the chromosome was analyzed by Southern hybridization. When chromosomal DNA was digested with *Nco*I (unique in the plasmid) and a DNA fragment (*Bcl*I-*Eco*RI) in the *proA* gene was used as a probe (Fig. 1A), two bands were detected in the transformants (lanes 1 to 4), in contrast to one band in the parent (lane 5). Moreover, the sizes of the DNA fragments varied depending on the plasmid used for integration (Fig. 1A; compare lanes 1 and 2 with lanes 3 and 4). These results clearly indicated that the plasmid had been integrated into the *proA* locus of the chromosome. DNA bands corresponding to the size of the free plasmids (Fig. 1A, lane 6) were not detected in any transformants, indicating that no free plasmids were present and that a single copy of the plasmid was integrated at the *proA* locus. When the *Eco*RI fragment containing *oripLS32* and CAT was used as a probe (Fig. 1B), no additional bands were detected (Fig. 1B, lanes 1 to 4, compared with Fig. 1A, lanes 1 to 4), indicating that the plasmid was integrated only at the *proA* locus.

In order to confirm that the whole plasmid was integrated, chromosomal DNA was digested with *Xba*I, and an *oripLS32* fragment (*Eco*RI-*Xba*I) (shown in Fig. 1C) or pUC18 DNA (shown in Fig. 1D) was used as a probe. Bands of the expected sizes were detected, indicating integration of the whole plasmid into the *proA* locus. Integration of a single copy of the whole plasmid into the *proA* locus was also confirmed by PCR with primers located outside the *proA* fragment in pBPA21 and pBPA23 (data not shown).

The resultant strains (NIS6011 and -6012) could grow at the nonpermissive temperature of the *dnaA* mutant, irrespective of the orientation of the minimal replication region of the mini-pLS32 plasmids. Furthermore, when this integrated mini-pLS32 was again transferred into the *dnaA* mutant by transformation, almost all (24 of 25 examined) chloramphenicol-resistant transformants could grow at the nonpermissive temperature (Table 2). To confirm that the DnaA protein is not required for replication of mini-pLS32, we constructed *dnaA*-null mutants of *B. subtilis* strains containing mini-pLS32 at *proA*. A plasmid, pSM5050, which contained a *dnaA* gene with two ochre mutations in the 5th and 10th codons and also a wild-type *dnaN* gene (26) was used to transform *B. subtilis* NIS6061 (*dnaN5 proA*::pBPA23) and NIS6062 (*dnaN5 proA*::pBPA21) cells which were temperature sensitive (Table 1). As the *dnaA* gene is located adjacent to *dnaN*, it was expected that these ochre mutations could be introduced efficiently into the chromosome together with the wild-type *dnaN* sequence during transformation. About 30% of the temperature-resistant transformants selected at 48°C showed a slow-growth phenotype, as found in strains NIS6011 and 6012. Therefore, these were expected to be likely *dnaA*-null mutants, and 10 of them from NIS6061 were examined by immunoblotting. It was found that all of them expressed amounts of the DnaA protein less than 1% of that in the wild-type strain (Fig. 2). These results strongly suggested that the integrated mini-pLS32 served as a new origin of chromosome replication in the absence of the DnaA protein. However, when this integrated mini-pLS32 was transferred into various other *dna* mutants, *dnaB19* (18, 34), *dnaC199* (9, 36), *dnaD23* (4, 18) and *dnaN5* (18, 33), their temperature sensitivity was not suppressed (Table 2). These results indicate that DnaB, DnaC, DnaD, and DnaN host proteins are necessary for replication from the plasmid origin integrated in the chromosome.

Deletion of the *oriC* sequence from the *B. subtilis* chromosome in which the plasmid replicon was integrated. To confirm that mini-pLS32 integrated in the chromosome could act as an origin for chromosome replication, we deleted part of the *oriC* sequence from the *B. subtilis* chromosome in which mini-pLS32 was integrated at the *proA* locus. Two untranslated regions upstream and downstream of the *dnaA* gene contain multiple repeats of the DnaA box and are required for initiation of chromosome replication (26). Replication initiates within the downstream region, both in vivo (28) and in vitro (27). We replaced 103 bp of the downstream region, including all DnaA boxes, in a plasmid, pNO41Δ*oriC*, with a 20-bp fragment that contains an *Eco*RI site (Fig. 3). As this region is located upstream of the *dnaN* gene, the *oriC* mutation could be easily recombined from the plasmid to the chromosome by using the same strategy adopted in constructing the *dnaA*-null mutants, described above. As shown schematically in Fig. 3, pNO41Δ*oriC* was used to transform the *dnaN5* mutant derivatives (NIS6061 and NIS6062) in which mini-pLS32 was inte-

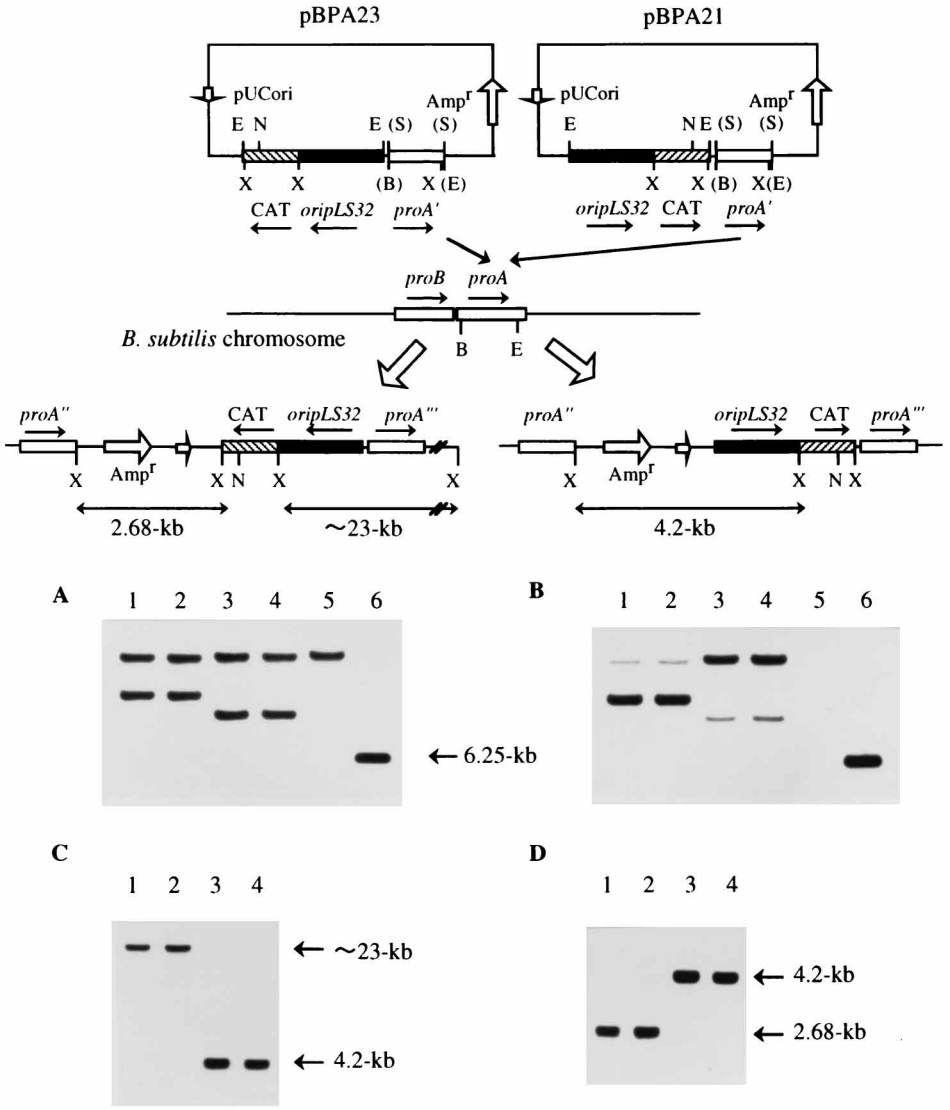

FIG. 1. Integration of mini-pLS32 plasmids into the *proA* locus of the chromosome of a *B. subtilis dnaA* mutant. Two mini-pLS32 plasmids, pBPA21 and pBPA23, which are pUC18 derivatives and differ in orientation of the minimal replication region of pLS32, were used to transform the *dnaA* mutant (CRK6010). Transformants were obtained on Penassay plates containing chloramphenicol (10 μg/ml), and candidates that had the integrated form of the plasmid on the chromosome were selected as described in Materials and Methods. In the upper part of this figure, changes at the *proA* locus caused by integration of plasmids are schematically shown and the lengths of *Xba*I fragments detected by Southern hybridization are given. Arrows below genes indicate the direction of transcription. B, *Bcl*I; E, *Eco*RI; N, *Nco*I; S, *Sma*I; X, *Xba*I. Chromosomal DNA was extracted from two candidates in each orientation and digested with *Nco*I (A and B) or *Xba*I (C and D). Integration of the plasmids was examined by Southern hybridization. (A) The *proA* fragment from plasmid pBPA23 was used as a probe. Lanes 1 and 2, two clones of orientation A (pBPA23); lanes 3 and 4, two clones of orientation B (pBPA21); lane 5, parent chromosome (without integration); lane 6, pBPA23 DNA digested with *Nco*I. (B) An *Eco*RI fragment (2.5 kb) containing the replication origin region (*oripLS32*) and the CAT gene was used as a probe. Lanes are the same as in panel A. (C and D) *oripLS32* and pUC18 DNA were used as probes, respectively. Lanes 1 and 2, two clones of orientation A; lanes 3 and 4, two clones of orientation B.

grated. Temperature-resistant transformants were selected at 48°C, and deletion of the *oriC* sequence on the chromosome was examined by Southern hybridization. Two *Eco*RI fragments (890 bp and 770 bp) instead of a 1.7-kb fragment were expected to be detected when the 103 bp in the *oriC* sequence was replaced by pNO41Δ*oriC* DNA (Fig. 3). About 30% of temperature-resistant transformants showed the expected pattern, indicating deletion of *oriC* in the transformants (Fig. 3). These *oriC*-deleted mutants showed a significantly slower growth rate compared to that of the wild-type strain (CRK6000); the doubling time of the mutants in nutrient broth at 37°C was 110 min, compared with 35 min for CRK6000. In contrast, integration of mini-pLS32 into the chromosome alone did not cause any change in growth rate. To confirm that the 103-bp fragment within *oriC* is essential for initiation of chromosome replication in the wild-type cells, chromosomal DNA of the *oriC*-deleted mutant was used as a donor to transform NIS6060 (*dnaN5*) and NIS6062 (*dnaN5 proA*::pBPA21) cells. Among 100 temperature-resistant transformants from NIS6060, none showed slow growth caused by *oriC* deletion. On the other hand, 32 of 100 temperature-resistant transformants from NIS6062 grew slowly. Thirty transformants from NIS6060 and 10 slow growers from NIS6062 were examined to detect the deletion within *oriC* by Southern hybridization as described above. None of the 30 from NIS6060 contained the deletion within *oriC* but all the slow growers from NIS6062 did (data

TABLE 2. Temperature sensitivity of various *dna*-ts mutants in which mini-pLS32 was integrated[a]

dna-ts mutant	No. of temperature-resistant clones[b] in orientation[c]:	
	A	B
dnaA1	25	24
dnaB19	0	1
dnaC199	1	2
dnaD23	1	0
dnaN5	0	1

[a] Chromosomal DNA of NIS6011 and NIS6012 in which mini-pLS32 plasmids pBPA23 and pBPA21, respectively, were integrated was used for transformation of the *dna*-ts (temperature-sensitive) mutants listed in this table. Twenty-five transformants (chloramphenicol resistant) were streaked onto a Penassay plate containing chloramphenicol (10 μg/ml) and incubated at the nonpermissive temperature (49°C for *dnaA1* and 48°C for the others) to examine suppression of the temperature sensitivity by integration of the plasmid.

[b] Of 25 transformants examined.

[c] Orientation of the mini-pLS32 replication region integrated into the chromosome.

not shown). These results demonstrated that the minimal replication region of pLS32 integrated into the *B. subtilis* chromosome was able to act as a new and sole replication origin for chromosome replication.

Chromosome replication initiates near the integration site of the plasmid replicon and proceeds bidirectionally. To confirm that the replication origin of mini-pLS32 that had integrated into the chromosome was actually working as a new initiation site for chromosome replication (in the absence of *oriC*), we measured the gene dosage of various genetic markers on the chromosome by transformation. The *hisA* marker was chosen as a tentative terminus marker because it is located opposite to *proA*. In Fig. 4, values of gene dosage of various markers relative to that of *hisA* are presented. Two markers, *pyrD* and *metD*, which are close to the integration site (the *proA* locus at 115° on the genetic map) had the highest values among markers examined. However, a dip between *pyrD* and *ilvA* was observed both in NIS6101 and in NIS6201. The similarity in the pattern of marker frequency irrespective of the orientation of the plasmid origin integrated at *proA* suggested that bidirectional replication was occurring and that the clockwise fork was stalled at *terC*. To confirm this, *rtp* disruption strains were derived from NIS6101 and 6201 by transformation with plasmid pAH1 as the donor. As described in Materials and Methods, this plasmid contains an erythromycin-resistant gene flanked by both upstream and downstream regions of the *rtp* gene. As the Rtp protein binds to the recognition sequences within *terC* and arrests the movement of replication forks (40), the gene dosage of the *ilvA* marker should increase in the *rtp* disruption derivatives (erythromycin-resistant transformants). This expected result was obtained and, furthermore, the gene dosages of other markers decreased almost symmetrically with their distance from the site of integration (Fig. 4). These results indicated that in the *oriC*-deleted cells, chromosome replication began at the integration site of the plasmid and proceeded bidirectionally.

Production of anucleate cells with a high frequency. The *oriC*-deleted cells grew much slower as described above. This

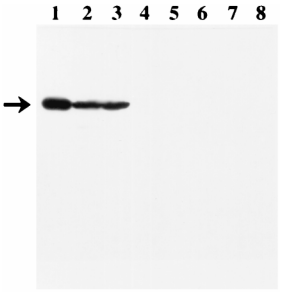

FIG. 2. DnaA protein level in *dnaA*-null mutants detected by immunoblotting. Ten independent clones of the mutants derived from NIS6061 were analyzed to detect DnaA protein as described in the text. Five of them are shown in this figure. Cell lysates containing approximately equal amounts of protein were loaded in lanes. Lane 1, DnaA protein (50 ng) purified as described previously (7); lanes 2 and 3, cell lysates from two independent temperature-resistant transformants that grew normally; lanes 4 to 8, cell lysates from five independent transformants (candidates for *dnaA*-null mutants) that grew slowly.

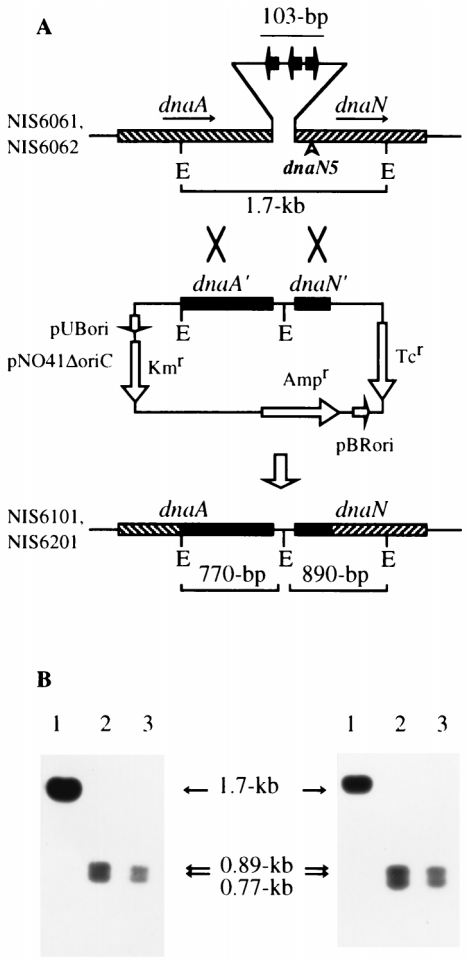

FIG. 3. Deletion of the *oriC* sequence from the *B. subtilis* chromosome in which mini-pLS32 was integrated. (A) Schematic presentation of the strategy for deletion of the *oriC* sequence. In pNO41Δ*oriC*, a 103-bp fragment within *oriC* (noncoding region downstream of the *dnaA* gene) was replaced by a multicloning sequence including an *Eco*RI site. Solid arrows indicate DnaA boxes which are required for initiation of chromosome replication in *B. subtilis* (24, 26). Linearized plasmid DNA was used to transform NIS6061 (*dnaN5 proA*::pBPA23) and NIS6062 (*dnaN5 proA*::pBPA21) cells. The location of the *dnaN5* mutation is shown by the arrowhead. (B) Southern hybridization of *oriC*-deleted transformants. Chromosomal DNA was digested with *Eco*RI, and the resultant DNA fragments were separated in a 1% agarose gel by electrophoresis. Southern hybridization was carried out as described in Materials and Methods. The 1.7-kbp *Eco*RI fragment shown in the figure was used as a probe. Lanes: 1, parent; 2 and 3, two *oriC*-deleted clones in which mini-pLS32 plasmid was integrated at the *proA* locus in orientation A (left panel) or orientation B (right panel) (see Fig. 1).

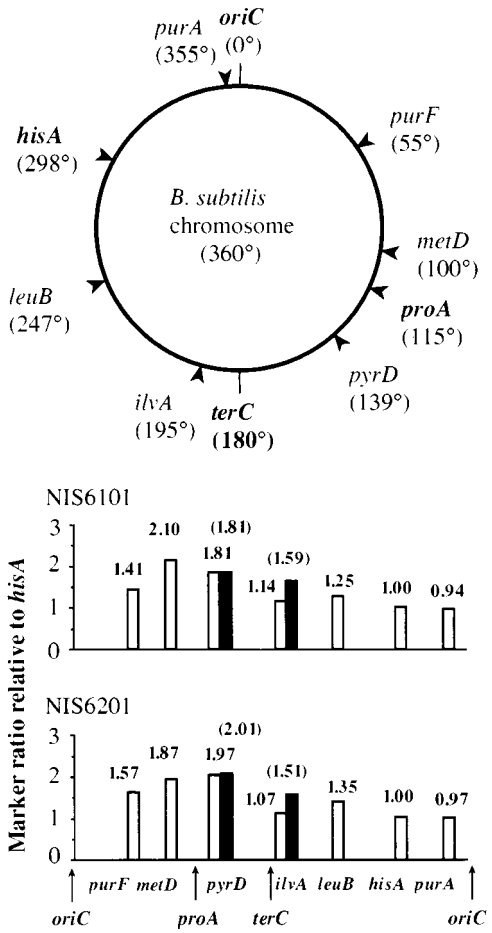

FIG. 4. Marker frequency analysis of *oriC*-deleted mutants (NIS6101 and NIS6201). Ratios of various markers on the chromosome relative to *hisA* were measured by transformation as described in Materials and Methods. The values are averages of two to five separate experiments. Solid bars indicate the results obtained from the *rtp*-disruption derivatives of NIS6101 and 6201, and the ratios are shown in parentheses. The position (degree on a 360-degree map) of each genetic marker used in this study is schematically indicated on a circular map of the *B. subtilis* chromosome. The mini-pLS32 plasmid was integrated at the *proA* locus.

might suggest impairment of chromosome segregation in the *oriC*-deleted cells. We, therefore, examined chromosome distribution in cells under fluorescence microscopy after staining chromosomes with DAPI by using a method described by Hiraga et al. (12). Many anucleate cells were found in exponentially growing culture of the *oriC*-deleted strain (NIS6101) (Fig. 5C and D). Moreover, the chromosome distribution in the cells appeared to be irregular compared to the distribution in wild-type cells (CRK6000) (Fig. 5A) and in the derivative containing the mini-pLS32 integrated into the chromosome without deletion of *oriC* (NIS6100) (Fig. 5B). About 40% of the cells were anucleate in the *oriC*-deleted strains (NIS6101 and NIS6201), irrespective of the orientation of the plasmid replication region (Table 3). In contrast, anucleate cells were hardly detected in the wild-type strain (CRK6000) and its derivatives containing the integrated mini-pLS32 (NIS6100 and NIS6200). These results indicate that anucleate cells were produced by disruption of the *oriC* sequence and were not due solely to integration of mini-pLS32 into the chromosome. Furthermore, *rtp* disruption did not cause any change in anucleate cell production (data not shown), indicating that arrest of the clockwise replication fork at *terC* was not the cause for production of anucleate cells. These observations suggest that the *oriC* deletion disrupted a site required for the machinery of chromosome partition, or that changes in the initiation mechanism of chromosome replication (from *oriC* to plasmid origin) and/or in the site of initiation on the chromosome (0 to 115° on the genetic map) disrupted regulatory mechanisms of chromosome partition, leading to the frequent production of anucleate cells.

Sporulation efficiency of the cells lacking *oriC*. Wu and Errington suggested that one of two chromosomes in a mother cell moved into the prespore compartment beginning with the *oriC* region of the chromosome during the early stage of sporulation in *B. subtilis* (41). We, therefore, were interested in the sporulation efficiency of the *oriC*-deleted cells obtained in this study. As shown in Table 4, only about 1 to 3% of viable cells formed spores, irrespective of the orientation of the plasmid replication region integrated into the chromosome, in *oriC*-deleted strains (NIS6101 and NIS6201), compared with 40 to 50% sporulation efficiency in the isogenic wild-type strain (CRK6000) and the derivatives in which mini-pLS32 was integrated at the *proA* locus with either orientation (NIS6100 and NIS6200). These results clearly indicate that initiation of chromosome replication from *oriC* is essential for normal sporulation.

DISCUSSION

We have shown that a phenomenon called integrative suppression can occur in *B. subtilis*. In *E. coli*, the conditional lethality of temperature-sensitive *dna* mutants defective in initiation of chromosome replication can be suppressed by integration of other replication origins (P2, R1, and F) into the chromosome (19). Such cells can survive by initiating chromosome replication from the plasmid origin instead of the native origin (*oriC*). In this study, we used a minimal replicon of plasmid pLS32 isolated from *B. natto* for integrative suppression of the defect in initiation of chromosome replication in a *B. subtilis dnaA* mutant. The *dnaA* derivatives in which mini-pLS32 was integrated into the chromosome (at *proA*) could grow at the nonpermissive temperature, and *dnaA*-null mutants could be isolated in the presence of the integrated plasmid origin. These results indicate that replication from the integrated mini-pLS32 plasmid initiates independently of the DnaA protein. We also could delete the *oriC* sequence from the chromosome in which mini-pLS32 was integrated. It was demonstrated by marker frequency analysis that chromosome replication was initiated near the site of integration and proceeded bidirectionally. The deletion within *oriC* seemed not to have activated other latent origins on the chromosome, since this deletion caused death in an otherwise wild-type strain. These results indicate clearly that the replication region of mini-pLS32 integrated into the chromosome acts as a new and sole origin for *B. subtilis* chromosome replication. Although the replication mode of plasmid pLS32 has not been reported, our result strongly suggests that this plasmid is replicated bidirectionally. Our study also showed that DnaB, DnaC helicase, and DnaD (proteins engaged in initiation of *B. subtilis* chromosome replication) were required as host factors for replication of the plasmid.

Interestingly, cells lacking the *oriC* sequence produced anucleate cells with a high frequency. This might suggest that *oriC* plays an important role in chromosome partition. In fact, not only production of anucleate cells but also an irregular distribution of chromosomes were observed in the *oriC*-deleted cells. The observation that chromosome DNA moved from the mother cell into the prespore compartment headed by the *oriC* region during sporulation (41) suggested that this region might

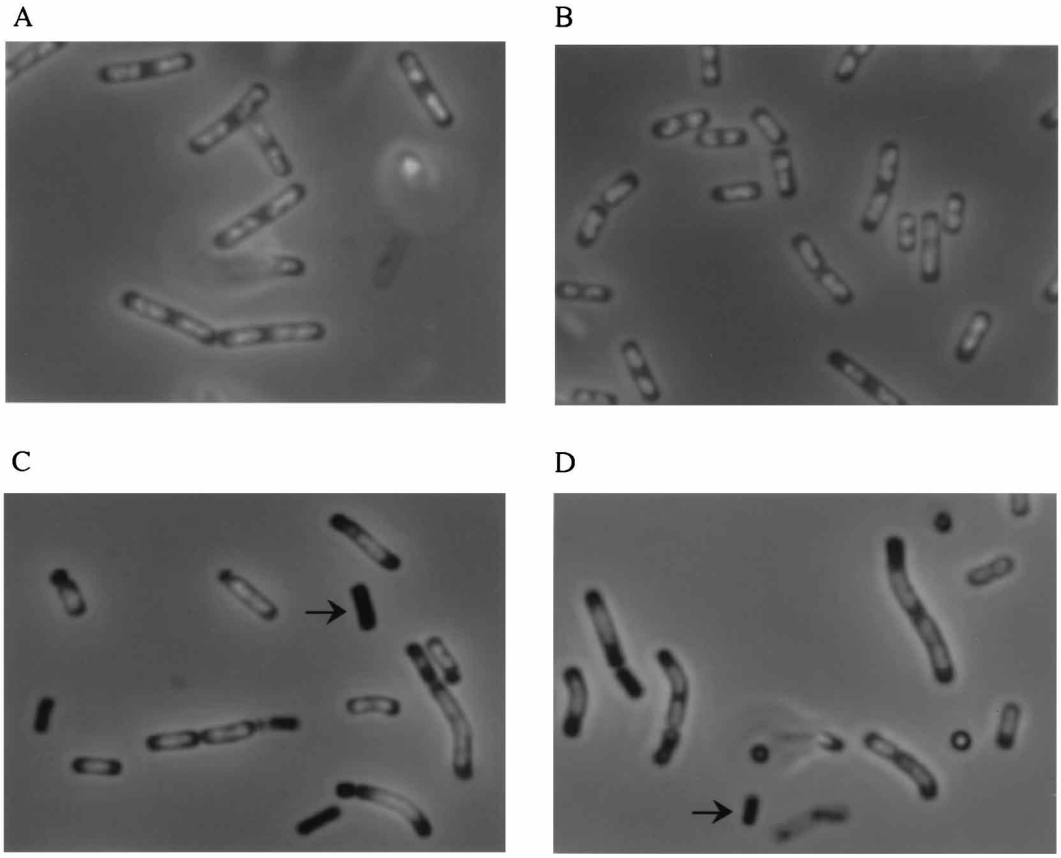

FIG. 5. Photographs of cells observed by fluorescence microscopy after staining with DAPI. Cells were grown in Penassay broth at 37°C, harvested at mid-log phase, and fixed in ethanol. (A) Parent strain (CRK6000). (B) A derivative (NIS6100) in which the mini-pLS32 plasmid pBPA23 is integrated at the *proA* locus. (C and D) Two foci of an *oriC*-deleted mutant (NIS6101). Arrows indicate anucleate cells.

behave as a "bacterial centromere." Consistent with this observation, it was found in our study that the *oriC*-deleted strains had a very low frequency of sporulation. This might suggest that *oriC* is equally important for chromosome segregation in both sporulation and vegetative cell growth. Furthermore, Wu et al. have recently reported that two daughter chromosomes have already been separated on both sides of a new septum without completion of chromosome replication, although correct positioning of chromosomes is inhibited (42). This result may further support the idea that *oriC* plays an important role in chromosome segregation in *B. subtilis*. As the *dnaA*-null mutants (NIS6111 and -6211 in Table 1) maintain *oriC*, analysis of these strains was expected to answer the important question whether *oriC* was required for chromosome partition. However, these mutants showed an unstable phenotype. In the primary transformants, some showed mainly elongated cells with only a few anucleate cells and the others showed almost the same phenotype (frequent production of anucleate cells) as the *oriC*-deleted mutants. Moreover, the former cells changed to shorter ones during prolonged incubation, although no DnaA protein was found. This instability seemed not to be due to insufficient replication in the absence of DnaA protein because the *pyrD*/*hisA* ratios in these mutants were similar (1.92 and 1.90 in NIS6111 and -6211, respectively) to those in *oriC*-deleted mutants (1.81 and 1.97) (Fig. 4). Similar instability in cell morphology was also observed in NIS6011 (*dnaA1 proA*::pBPA23) cells when they were incubated at the nonpermissive temperature of the *dnaA* mutant. Thus, at

TABLE 3. Frequency of anucleate cell production in various *B. subtilis* strains

Strain	No. of cells examined	No. of anucleate cells (% of total)
CRK6000 (wild type)	2,679	1 (0.04)
NIS6100 (*proA*::pBPA23)	1,384	5 (0.36)
NIS6200 (*proA*::pBPA21)	1,163	4 (0.34)
NIS6101 (*proA*::pBPA23 Δ*oriC*)	2,289	1,018 (44)
NIS6201 (*proA*::pBPA21 Δ*oriC*)	2,130	893 (42)

TABLE 4. Effect of deletion of *oriC* sequence in sporulation[a]

Strain	Heat-resistant spores/ml	Viable cells/ml	Sporulation efficiency (%)
CRK6000 (wild type)	4.24×10^8	9.86×10^8	43
NIS6100 (*proA*::pBPA23)	4.84×10^8	1.00×10^9	48
NIS6200 (*proA*::pBPA21)	4.30×10^8	1.03×10^9	42
NIS6101 (*proA*::pBPA23 Δ*oriC*)	1.80×10^6	3.00×10^8	0.6
NIS6201 (*proA*::pBPA21 Δ*oriC*)	1.80×10^7	6.04×10^8	2.9

[a] Numbers of viable cells and heat-resistant spores were measured at T_{12} as described in Materials and Methods. Sporulation efficiency was calculated as the percentage of spores per viable cell. Cells were cultivated in the presence of chloramphenicol to maintain the integration of the plasmid replicon. However, the same sporulation efficiency was obtained in all strains when they were cultivated without chloramphenicol (data not shown).

present, it is difficult to explain the segregation phenotype of the *dnaA*-null mutants. Moreover, even if the *dnaA*-null mutants produced anucleate cells as well as *oriC*-deleted mutants, the following reason suggests that it is difficult to conclude that *oriC* is not required for chromosome partition. If *oriC* is required for chromosome partition, it is not so strange to assume that DnaA protein is also engaged in the partition as one of the *trans*-acting factors because of the presence of multiple repeats of the binding sequence in *oriC* (26). In this case, *dnaA*-null mutants would show the same phenotype defective in chromosome partition as *oriC*-deleted mutants.

Alternatively, the frequent production of anucleate cells observed in the *oriC*-deleted mutants might be due to the change in initiation site of chromosome replication (from 0 to 115° on the *B. subtilis* genetic map) and/or the change in the initiation mechanism (from *oriC* to the plasmid replication system). Also, as marker frequency analysis showed that chromosome replication seemed to terminate around the *hisA-purA* region and away from *terC*, this change in the position of replication termination caused by the change in the initiation site of replication might be related to anucleate cell production. Approximate origin/terminus ratios (*pyrD*/*hisA* and *metD*/*hisA*) in log-phase cells of the *oriC*-deleted mutants were a little lower (1.93 on average) than the origin/terminus ratio (2.4) obtained in isogenic wild-type cells growing in the same conditions. If the change in initiation mechanism of chromosome replication (from *oriC* to the plasmid system) destroys coupling of DNA replication and cell division, this slight decrease in initiation frequency of replication might lead to production of anucleate cells in the *oriC*-deleted mutants. This insufficient replication could give a smaller number of chromosomes at the time of cell division, and cells which have lost the mechanism coupling cell division to chromosome number could neither recognize this deficiency nor delay cell division. In view of the facts that other mutants defective in chromosome partition, *mukB106* in *E. coli* (31) and *spoOJ*-null in *B. subtilis* (16), produced anucleate cells at relatively low frequencies, ~5 and 1.4%, respectively, most of the anucleate cells found in *oriC*-deleted mutants might be produced by the mechanism suggested above. Experiments are in progress to determine the effects of change in initiation site (including change in the position of replication termination) and mechanism on anucleate cell production by putting the plasmid origin at different locations around the chromosome or transferring *oriC* into the *proA* locus (115°).

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