

Stability and Asymmetric Replication of the *Bacillus subtilis* 168 Chromosome Structure

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Chromosomal DNAs from a number of strains derived from *Bacillus subtilis* 168 were digested with restriction endonucleases *NotI* or *SfiI*, and the locations of chromosomal alterations were compared with the recently constructed standard *NotI-SfiI* restriction map (M. Itaya and T. Tanaka, *J. Mol. Biol.* 220:631-648, 1991). In general, the chromosome structure of *B. subtilis* 168 was found to be stable, as expected from the genetic stability of this species. DNA alterations, typically deletions, are formed in three limited loci on the chromosome. One of these alterations was characterized as a spontaneous deletion formed between *rrn* operons, and another occurred as a result of prophage SP β excision. I found that *oriC* and *terC* are not located on precisely opposite sides of the chromosome. Replication in the counter clockwise direction was 196 kb longer than replication in the clockwise direction. The characteristic of length difference is not changed by deletion formation.

Bacillus subtilis, a gram-positive bacterium, has been well studied genetically. Derivatives of *B. subtilis* 168 have been constructed in many laboratories. The pedigrees of various *B. subtilis* 168 strains can be determined by referring to the literature, although documentation for these organisms is not as complete as documentation for *Escherichia coli* K-12 (8). It seems likely that genetic variation, including alterations in chromosome structure, is present in isolates of *B. subtilis* 168. Very recently, a coworker and I published a complete detailed physical map of the chromosome of *B. subtilis* 168 (a *NotI-SfiI* restriction map of the entire genome) (22). On this map, all 26 *SfiI* fragments and as many as 72 *NotI* fragments were aligned unequivocally on the circular genome. The loci of 11 known genes were determined precisely, providing a good linear relationship between the physical and genetic linkage maps.

Altered genome structures which give rise to changes in the lengths of *SfiI*- and *NotI*-generated segments can now be identified as deviations from the standard *B. subtilis* chromosome, as suggested previously (22). Below, I present evidence that alterations of the *B. subtilis* chromosome have occurred in both expected and unexpected ways in derivatives of *B. subtilis* 168. These alterations include excision of lysogenized phage SP β , spontaneous deletions, and insertions (ranging in size from 4 to 130 kb). In the most extreme case found so far, 170 kb of DNA (about 4% of the entire genome) was lost. In addition, the distances between *oriC* and *terC* were found to be different for the clockwise and counterclockwise portions of the *B. subtilis* chromosome.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *B. subtilis* strains whose chromosomal DNAs were subjected to *NotI* or *SfiI* digestion analysis are listed in Table 1. Luria-Bertani (LB) broth (26) was used for the growth of *B. subtilis*. *E. coli* plasmids carrying the cloned *B. subtilis* DNA segments were used as probes for Southern hybridization analyses. *NotI* linking clones, (pNEXT49, pNEXT5, pNEXT59), an *SfiI* linking clone (pSOFT12), pWS10 (*terC*), and pBD56 (*cotA*) have been previously described (22). pBC275HE (*hut*) (29)

was obtained from Y. Fujita, and pMS102B7 (*rrnO*) (28) was obtained from N. Sueoka.

Isolation and digestion of chromosomal DNA from *B. subtilis*. Unsheared *B. subtilis* chromosomal DNA for *NotI* or *SfiI* digestion was prepared in a molten agarose gel as described previously, with the minor modifications described below (22). The cells were grown prior to harvest in LB medium (5 ml) instead of antibiotic medium no. 3 broth (Difco) with vigorous shaking at 37°C for at least 6 h after 5% inoculation with a preculture grown overnight in LB medium at 37°C. This protocol resulted in higher cell densities (higher DNA concentrations) and minimized DNA degradation generated from a fraction of sporulating or lysing cells. *SfiI* was purchased from New England Biolabs, Beverly, Mass., and *I-SceI* was purchased from Boehringer Mannheim, Indianapolis, Ind. Other type II restriction endonucleases, including *NotI*, were obtained from Toyobo, Tokyo, Japan. About 5 μ g of DNA in a 20- μ l agarose block was digested with *NotI* (10 U) at 37°C or with *SfiI* (15 U) at 50°C in a 50- μ l reaction mixture for 15 to 17 h. After the agarose block was melted at 65°C in the presence of spermine (0.3 mM) and spermidine (0.75 mM), the sample was gently loaded into a well in a 1% agarose gel made in TBE solution (45 mM Tris-borate [pH 8.0], 45 mM boric acid, 1 mM EDTA). Digestion with *I-SceI* was performed as suggested by the supplier, using 40 U of *I-SceI* per 50 μ l at 37°C for 1 h, and the gel block was placed directly into a well in a 1.5% agarose gel; this was followed by sealing with a 1.5% low-melting-point agarose gel solution kept at 43°C. Concatenated lambda DNA, which provided size markers (lambda cI857 Sam7), was purchased from New England Biolabs. The *Saccharomyces cerevisiae* chromosomal DNA used as a size marker was obtained from CLONTECH Laboratories, Palo Alto, Calif. The contour-clamped homogeneous electric field gel apparatus used was the same apparatus that was used previously (22). The running conditions used are described in the legends to the figures.

Southern analysis. DNAs from gels that were stained with ethidium bromide (1 μ g/ml) and photographed were transferred onto nylon membranes (type Nytran 13N; Schleicher & Shuell, Dassel, Germany) by capillary blotting as de-

TABLE 1. *B. subtilis* 168 strains analyzed in this study

Strain	Relevant genotype	Sizes of deletions (kb)			Source
		I	II	III	
168 <i>trpC2</i> (= 1A1)	<i>trpC2</i>				BGSC ^a
1A18 ^b	<i>dnaA13 ilvA1 metB5</i>			130	BGSC
1A19 ^b	<i>dnaB19 ilvA1 metB5</i>				BGSC
1A20 ^b	<i>dnaC30 ilvA1 metB5 Δ(rmH-rmG)</i>	5	23	130	BGSC
1A21 ^b	<i>dnaD23 ilvA1 metB5</i>		23	130	BGSC
1A22 ^b	<i>dnaE20 ilvA1 metB5 Δ(rmH-rmG)</i>	5	23	130	BGSC
1A23 ^b	<i>dnaF133 (= polC133) ilvA1 metB5</i>			130	BGSC
1A24 ^b	<i>dnaG34 ilvA1 metB5</i>				BGSC
1A25 ^b	<i>dnaH151 ilvA1 metB5</i>		23		BGSC
1A46	<i>trpC2 thr-5 recE4</i>				BGSC
1A54	<i>mtlB1 narB1 sacA321</i>		23		M. Nakano
JH642 (=1A96)	<i>trpC2 pheA1</i>	+4 ^c	20		Y. Kobayashi
60015	<i>trpC2 metC7</i>				Y. Fujita
QB944	<i>trpC2 cysA14 purA16</i>				Y. Fujita
GSY110	<i>tyrA1</i>				C. Anagnostopoulos
GSY2258	<i>hisH2 metB5 addA5</i>		23		C. Anagnostopoulos
GSY2266	<i>hisH2 metB5 addB72</i>				C. Anagnostopoulos
GSY2270	<i>hisH2 metB5 addB71</i>				C. Anagnostopoulos
BG126	<i>trpC2 metB5 amyE xin-1 attSPβ addB72</i>		23	130	J. Alonso
CU741	<i>trpC2 leuC7</i>		23		S. Zahler
OA101	Prototroph derived from CU741		23		T. Tanaka
YS11 ^b	<i>leuB8 arg15 purB6</i>		40	130	T. Uozumi
RM125 ^b	<i>leuB8 arg15 hsmM hrsM</i>		40	130	T. Uozumi
MI112	RM125 <i>thr-5 recE4 Δ(rmH-rmG)</i>	5	23	130	T. Tanaka
T24	<i>trpE</i>				T. Tanaka
BD170	<i>trpC2 thr-5 Δ(rmH-rmG)</i>	5			R. Rudner
BEST140	<i>gltA trpC2</i>				N. Ogasawara
BEST2069	1A1 <i>terC::neo(I-SceI)</i>				This study ^d
BEST2071	RM125 <i>terC::neo(I-SceI)</i>		40	130	This study ^e
BEST2076	1A1 <i>terC::neo(I-SceI) recF::cat(I-SceI)</i>				This study ^d
BEST2078	RM125 <i>terC::neo(I-SceI) recF::cat(I-SceI)</i>		40	130	This study ^e

^a BGSC, *Bacillus* Genetic Stock Center.

^b Strains having an additional *NotI* site at 543 kb.

^c The plus sign indicates a size increase.

^d Derived from strain 1A1 (= 168 *trpC2*).

^e Derived from strain RM125.

scribed previously (34). A nonradioactive DNA labeling and detection kit (Boehringer Mannheim), in which digoxigenin-11-dUTP was used as a labeling nucleotide, was used for DNA probe preparation, prehybridization, hybridization, and color development. The procedures used were the procedures described in the supplier's manual. The plasmid DNAs used for labeling were purified by CsCl-ethidium bromide ultracentrifugation.

RESULTS

Altered-size fragments detected after *SfiI* or *NotI* digestion. Since all of the *SfiI* sites and most of the *NotI* sites were determined on the entire chromosome of a *B. subtilis* 168 strain (22), it was possible to detect altered chromosome configurations in different *B. subtilis* 168 strains. The limit of fragment size resolution has been estimated to be about 1%; that is, fragments that are 100 and 101 kb long can be separated (unpublished data). The numbers and sizes of most *SfiI*-generated fragments in laboratory strains were found to be the same within the limits of fragment size resolution under various running conditions (Table 1 and Fig. 1). A comparison of fragment patterns after *NotI* digestion of the same DNAs verified the changes (Table 1 and Fig. 2). The alterations were located in three distinct regions of the chromosome (Fig. 1 and 2, regions I, II, and

III, and Fig. 3). In some strains multiple changes were found (Table 1).

Deletion site (deletion I) identical to *rmn* operons. A deletion that spanned about 5 kb in strains MI112, 1A20, 1A22, and BD170 was observed (data not shown) (Table 1). This deletion fell within the 24N fragment (*NotI*; 65 kb), and accordingly the FS fragment (*SfiI*; 238 kb) was also altered (Fig. 4C). The same region was 4 kb larger in strain JH642 (Table 1 and Fig. 1 and 2). The two flanking *NotI* fragments, fragments 9N (127 kb) and an unnumbered 5-kb fragment, did not appear to be altered when pNEXT49 and pNEXT5 were used as probes (data not shown), indicating that the changes were internal to the 24N fragment. These DNA alterations occurring on the 24N fragment were designated deletion I (Fig. 3). Several *rmn* operons reside in this region of the genetic map (30). Widom et al. described naturally occurring deletions at the *rmn* loci of *B. subtilis* (40). These authors estimated that there were 5.8- and 4.8-kb deletions between closely spaced operons, such as operons *rmnJ* and *rmnW* and operons *rmnH* and *rmnG*, respectively (40). A 4.8-kb deletion between *rmnH* and *rmnG* has been described in strain BD170 (40). Genomic Southern hybridization experiments were performed by using a DNA fragment carrying the *rmnO* gene as a probe (pMS102B7) to determine whether the deletions found in this study are identical. This probe hybridizes to all *B. subtilis* *rmn* operons. The loss of specific 4.8-kb *BclI* or

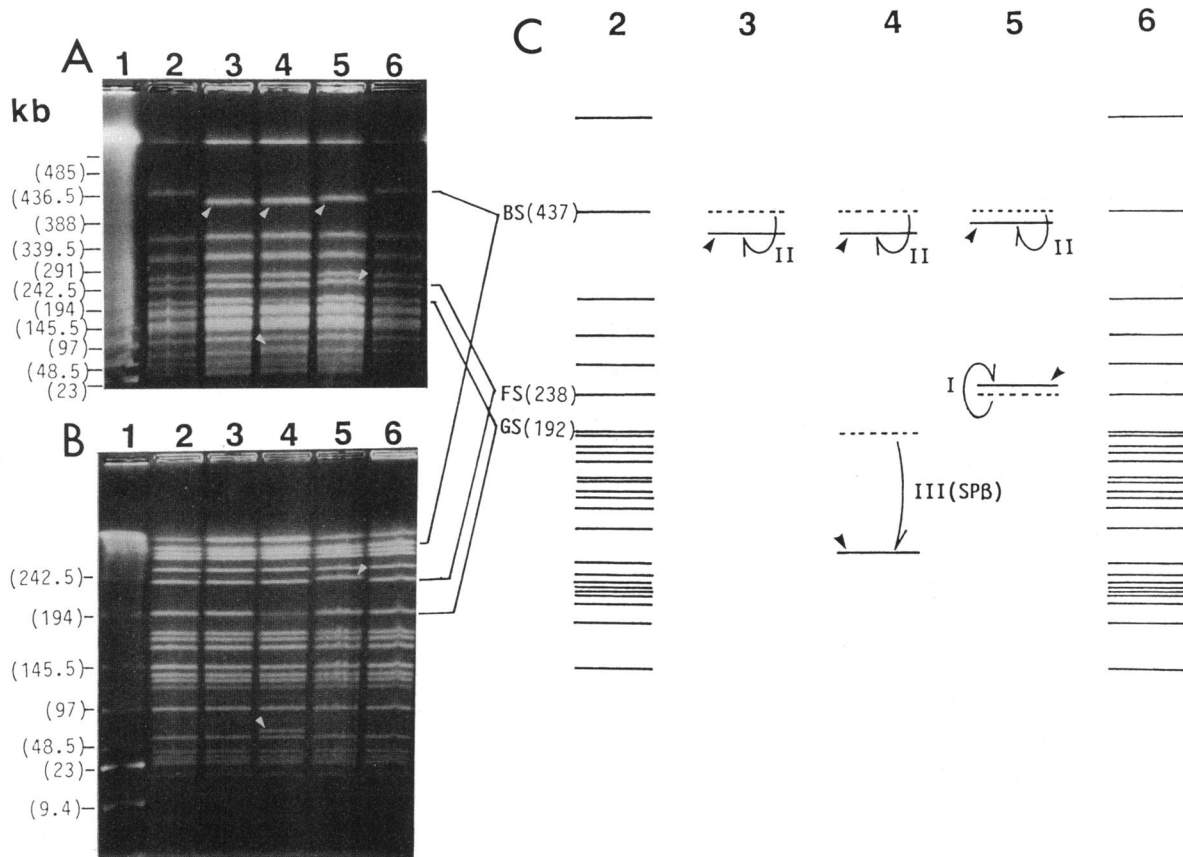


FIG. 1. Altered *SfiI* fragments in strains CU741, BD126, and JH642. For all panels, lane 1 contained concatemeric lambda DNA segments plus lambda DNA digested with *HindIII*; sizes (in kilobase pairs [kb]) are indicated in parentheses. Approximately 2 to 5 μ g of chromosomal DNA from strain 168 *trpC2* (lanes 2 and 6), CU741 (lane 3), BG126 (lane 4), or JH642 (lane 5) was loaded onto a gel after digestion with *SfiI*. The arrowheads indicate the positions of altered *SfiI* fragments. (A) Ethidium bromide-stained gel. The running conditions were as follows: 1% agarose, TBE solution (14°C), 90-s pulse time, 3 V cm^{-1} , 40-h running time. (B) Ethidium bromide-stained gel. The running conditions were the same as those described above for panel A except that a pulse time of 24 s was used to obtain greater resolution at sizes less than 240 kb. (C) Schematic representation of all of the *SfiI* fragments of 168 *trpC2* (lanes 2 and 6) and altered *SfiI* fragments (lanes 3 through 5) (solid bars with bent arrows). The dotted lines indicate the original positions of fragments BS, FS, and GS. I, II, and III (SPB) indicate the positions of regions I, II, and III (excision of SPB phage), respectively (see Fig. 3).

2.9-kb *EcoRI* fragments that was reported for strain BD170 (40) was also observed in strains MI112, 1A20, and 1A22 (data not shown). Thus, in these three strains deletion of approximately 5 kb occurred between *rmH* and *rmG*; this is similar to what happened in strain BD170. In contrast, the 4-kb increase in strain JH642 size (the only example of an increase in size in this study) was not related to *rm* operons (data not shown).

Deletion in strains CU741 and OA101 (deletion II). Strain CU741 (*leuC7 trpC2*) and its prototrophic derivative, strain OA101, were used as standard strains to construct the *B. subtilis* 168 physical map, and the estimated genome size for these two strains was 4,165 kb (22). In this study, however, it was found that both of these strains have a small deletion which was not noticed previously. This deletion (a 23-kb deletion) lies within fragment 18N (*NotI*; 97 kb) and fragment BS (*SfiI*; 414 kb) (Fig. 1 and 2), and its presence was verified by using pNEXT59 as a probe (Fig. 4). A similar 23-kb deletion in the 18N fragment was also found in other strains, but a deletion of a different size in the 18N fragment was observed in strains YS11 (40 kb), RM125 (40 kb), and JH642 (20 kb) (Fig. 1, 2, and 4 and Table 1). The location of the 18N fragment on the genetic map was estimated to be

between 32.6 degrees (483 kb) and 42.1 degrees (580 kb) by using the relationship of 11.57 kb per degree (22) from the *amyR* (25 degrees; 359 kb) and *cotA* (52 degrees; 695 kb) sites, as indicated in Fig. 4. No DNA rearrangement in this part of the genetic map has been clearly identified previously (30). There has been no evidence that these deletions could be formed by a single deletion at a fixed locus on the 18N fragment. Putative designation for deletion II as a single deletion that occurs within the 18N fragment is shown in Fig. 3 and 4. A typical 23-kb deletion may imply that an example of the deletion II could be formed via homologous recombination and/or possibly via an excision of an as-yet-unidentified prophage. Further characterization of the deletion will be performed in the future.

Deletion at the SPB locus (deletion III). Apparent loss of the normal GS fragment (*SfiI*; 192 kb) and the 1N fragment (*NotI*; 265 kb) was observed in strain BG126 (Fig. 1 and 2). These changes were confirmed by using two specific probes, pNEXT36 and pSOFT12 (Fig. 5). When pNEXT36 (a *NotI*-linking clone which hybridizes to two adjacent *NotI* fragments, fragments 1N [265 kb] and 15N [109 kb]), and to *SfiI* fragment KS [160 kb]) was used as the probe for strain BG126, a change in fragment 1N (from 265 to 135 kb) was

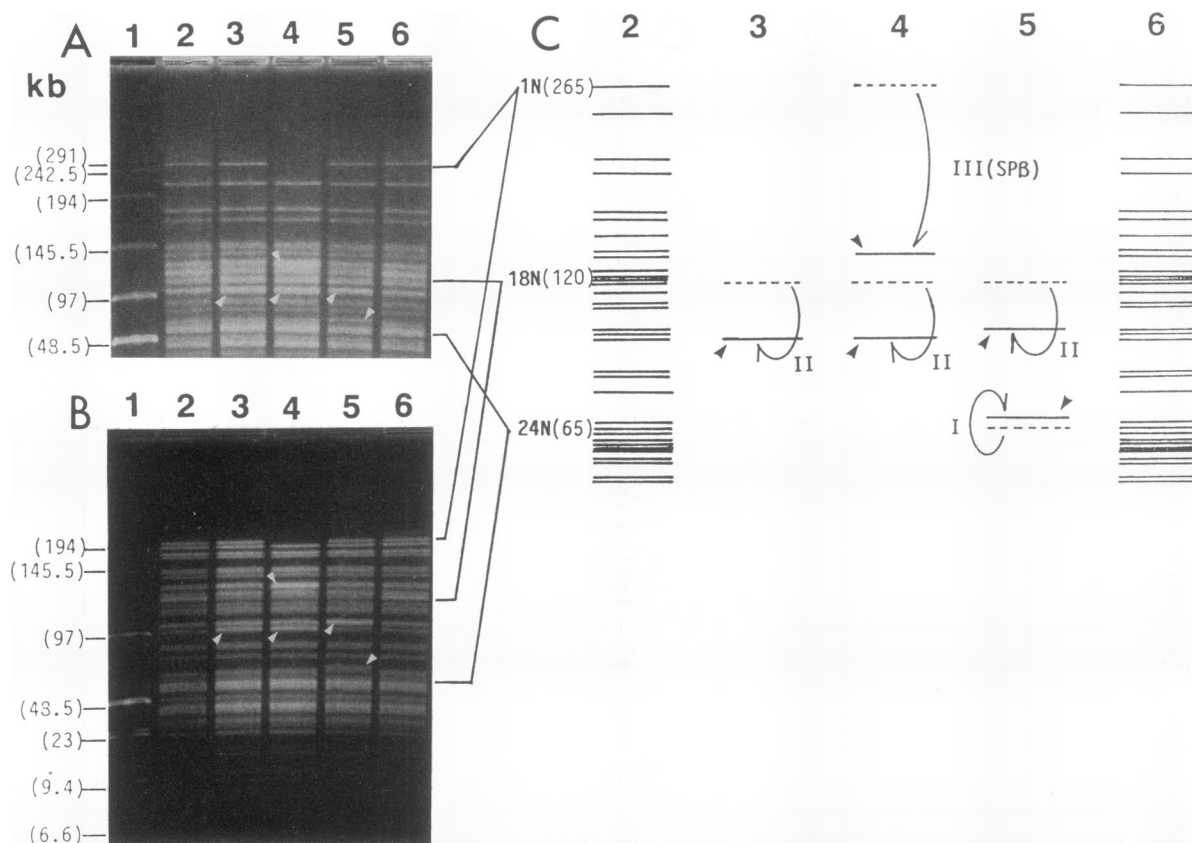


FIG. 2. Altered *NotI* fragments from strains CU741, BG126, and JH642. The samples used for the lanes and the labels are the same as those described in the legend to Fig. 1. Fractionation of most of the *NotI*-generated fragments in lanes 2 and 6 has been described previously (22); the only exception was fragment 18N. For details see the text. The running conditions were the same as those described in the legend to Fig. 1, except that the pulse time was 24 s for panel A and 12 s for panel B.

observed, whereas fragment KS (160 kb) was not changed (Fig. 5A). Similarly, when pSOFT12 was used as the probe (pSOFT12 is an *SfiI*-linking clone which hybridizes to two adjacent *SfiI* fragments, fragments KS [160 kb] and GS [192 kb], and to *NotI* fragment 1N [265 kb]), the fragment 1N (265-kb) and GS (192-kb) segments in strain BG126 were shortened to 135 and 60 kb, respectively, while fragment KS (160 kb) was unchanged (Fig. 5C). Fragments 1N and GS are smaller by 130 and 132 kb, respectively, in strain BG126 than in the standard strain (strain 1A1). The similar size changes found in these fragments suggest that the deletion is completely within these fragments. No size change in adjacent fragment AS or 10N was observed by using specific probe pWS10 carrying the *terC* region (data not shown) (Fig. 5E). Cloned DNA carrying the *cotD* gene (pBD56) hybridized to shortened fragments 1N (135 kb) and GS (60 kb) (data not shown) (Fig. 5E). Thus, I concluded that the 130-kb deletion is between positions 2129 and 2314 (Fig. 5). The genetic location of the deletion was estimated to be between 184 and 200 degrees by using a calculated value 11.57 kb per degree from *terC* (180 degrees; 2,012 kb) to *cotD* (200 degrees; 2,314 kb) (22). This locus on the genetic map clearly corresponds to lysogenized phage SP β (30). Strain BG126 was determined to be SP β nonlysogenic (*attSP β*) and sensitive to SP β phage (data not shown). I concluded that deletion III was formed by excision of lysogenized SP β phage. The loss of the original size of fragment 1N in strains YS11, RM125, and MI112 is shown in Fig. 4A, lanes 4 through 6; a similar loss

also occurred in several *dna* mutants listed in Table 1. Although *attSP β* was not described in these strains, the presence of the same 130-kb deletion was also confirmed (data not shown).

Size of the *B. subtilis* 168 chromosome. *B. subtilis* 168 derivatives CU741 and OA101, which were chosen as standard strains for the construction of the physical map, suffered only a 23-kb loss (deletion II) compared with *B. subtilis* 168 *trpC2* (= 1A1). DNAs from various other strains generated *NotI* or *SfiI* digestion patterns identical to the digestion pattern of *B. subtilis* 168 *trpC2*. Considering that *B. subtilis* 168 was isolated from a single ancestral cell, a *B. subtilis* Marburg cell (10), the original genome size of *B. subtilis* Marburg is most likely represented by the size of *B. subtilis* 168 *trpC2* (4,188 kb). Among the strains analyzed in this study the highest level of DNA alterations was found in strain RM125, in which about 4% of the total genome was lost; the mutations included deletions II (40 kb) and III (SP β ; 130 kb).

Origin and termination sites on the *B. subtilis* chromosome are located asymmetrically. The start of the physical map (0 kb) was defined as an *SfiI* site between fragments FS and NS (22), corresponding to the *SfiI* site on *SfiI*-linking clone pCIS7 described by Amjad et al. (4). The termination site for DNA replication, *terC*, has been determined on the physical map (*terC*; 2,012 kb, AS, 10N [122]) (22). Very recently, the site for *oriC* was precisely determined to be at 39 kb on the physical map (*oriC*; 39 kb, FS, 9N [127]) (21). Determination

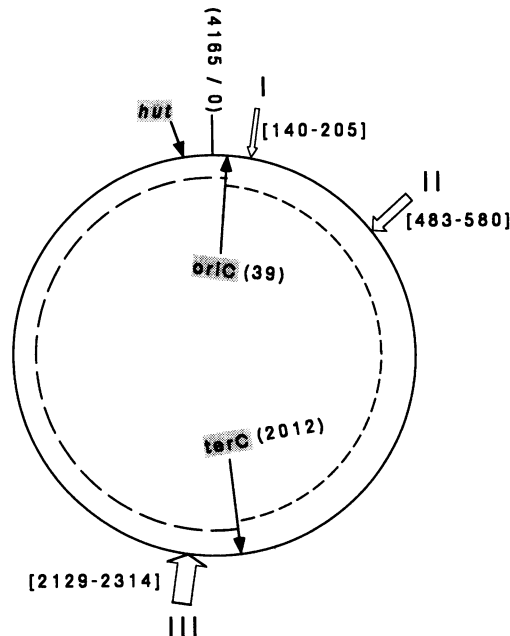


FIG. 3. Locations of DNA rearrangements on the *B. subtilis* 168 chromosome. Three DNA regions on the chromosome where size alterations occurred (regions I, II, and III) are indicated by the open arrows. The numbers in brackets and parentheses are the positions on the strain CU741 physical map (4,165 kb) (22). The widths of the open arrows indicate relative deletion sizes, as follows: deletion I, 5 kb; deletion II, 20 to 40 kb; and deletion III, 130 kb. The positions of *oriC* and *terC* have been determined previously (21, 22). The genome size of *B. subtilis* 168 *trpC2* was estimated to be 4,188 kb, 23 kb larger than the genome size of standard strain CU741 or OA101 (4,165 kb) (see text). The chromosome length on the right side between *oriC* and *terC* for strain 168 *trpC2* is 1,996 kb. The remaining part on the left side is 2,192 kb long. The zero point of the physical map was the *SfiI* site between fragments FS and NS (22) (see Fig. 4C). The location of the *hut* gene is indicated (see Fig. 6). Separation of the two segments is shown in Fig. 6.

of these two loci allowed me to estimate the lengths for clockwise DNA replication fork movement (1,996 kb) and counterclockwise DNA replication fork movement (2,192 kb) (Fig. 3). Two coworkers and I have described a new method to directly measure the physical length between two fixed loci on the *B. subtilis* chromosome (21). Briefly, creation of an 18-bp sequence recognized by meganuclease *I-SceI* at both the type II DNA-membrane binding site and the *recF* gene generated a fragment about 49 kb long after *I-SceI* digestion, indicating that the size of the new segment is equivalent to the physical distance between these two loci on the chromosome (21). Thus, the *B. subtilis* chromosome can be separated into two parts by creating the *I-SceI* recognition sequence at the *oriC* and *terC* loci.

To introduce the *I-SceI* site at the *terC* region, a neomycin resistance gene cassette (20) carrying the sequence *neo(I-SceI)*, (19) was inserted at the unique *BglII* site within the *terC* region cloned in pWS10 (*terC*) (18), resulting in pBMAP106SCB [*terC::neo(I-SceI)*]. pBMAP106SCB was used to transform *B. subtilis* 168 *trpC2* and RM125, resulting in neomycin-resistant strains BEST2069 and BEST2071, respectively (Table 1). An *I-SceI* site was created at the *terC* site in these strains (data not shown). Successive introduction of the second *I-SceI* site at the *recF* gene was accom-

plished by using plasmid pRecFC (21). Briefly, in pRecFC [*recF::cat(I-SceI)*] a chloramphenicol resistance cassette (23) carrying an *I-SceI* site [*cat(I-SceI)*] (21) was inserted at the unique *XhoI* site in the *recF* gene. The *recF* gene is about 2 kb from *oriC* (*recF*; 41 kb, FS, 9N [127]) (21, 27, 28). Subsequent transformation of strains BEST2069 and BEST2071 by using linearized pRecFC resulted in chloramphenicol-resistant strains BEST2076 and BEST2078, respectively (Table 1). The structures of genomic *terC::neo(I-SceI)* and *recF::cat(I-SceI)* were verified by a Southern analysis in which pWS10 and pRecFC were used as probes (data not shown). Two fragments were separated when chromosomal DNAs isolated from strains BEST2076 and BEST2078 were subjected to contour-clamped homogeneous electric field gel electrophoresis after digestion with *I-SceI* (Fig. 6). The Southern hybridization results obtained when pBC275HE (*hut* gene) (*hut*; 4029 to 4032 kb, XS, 30N [53]) was used as a probe showed that the larger fragment came from the left part of the *B. subtilis* chromosome and the shorter fragment came from the rest of the chromosome (Fig. 3 and 6). Estimates of the sizes of these fragments were based on the *SfiI-NotI* physical map (22). The different lengths of the two parts of the *B. subtilis* chromosome are manifest (Fig. 6), and more important, the size order was not affected despite deletion formation in a case as extreme as that of strain RM125 (Fig. 6, lane 2).

DISCUSSION

Stability of the *B. subtilis* genome. Essentially two kinds of physical maps have been constructed for bacterial chromosomes (22), maps that are collections of overlapping contiguous clones (contigs) and long-range restriction maps constructed by using rarely cutting restriction endonucleases. The former type of map (24) provides little information concerning the structural diversity among different isolates unless contigs from different strains of interest are prepared for comparison. Only a partial comparison between genomes of two closely related *E. coli* strains has been described previously (12). In contrast, a rarely cutting restriction endonuclease map should immediately be useful for analyses of entire genomes (for example, the genomes of *E. coli* strains) (13). A complete and detailed physical map of the *B. subtilis* 168 chromosome should allow workers to study the differences in chromosome structure compared with a standard strain. It is important to discriminate between genetic stability of the chromosome structure in a lineage of species and diversity of altered structures in different isolates classified as the same species. Examples of the latter have been reported frequently (7, 34) and are discussed below.

It has been suggested that the chromosome of *E. coli* K-12 is genetically stable (31). In contrast, the instability of the chromosome structure of *Streptomyces* spp. has been demonstrated (9). Is the *B. subtilis* 168 chromosome also stable? No alteration in the chromosome was observed in many cases. However, changes in DNA take place in three limited regions (deletions I through III) (Table 1 and Fig. 3), and these changes have been characterized. Formation of deletions I and III includes spontaneously occurring deletions in the *rnn* operons and SP β prophage induction, respectively. Deletion II is described in this paper for the first time, but its significance remains unknown. Such DNA rearrangements with concomitant losses of some genetic information should be neutral or at least not deleterious for the survival of cells, since no apparent growth defect has been observed. Inves-

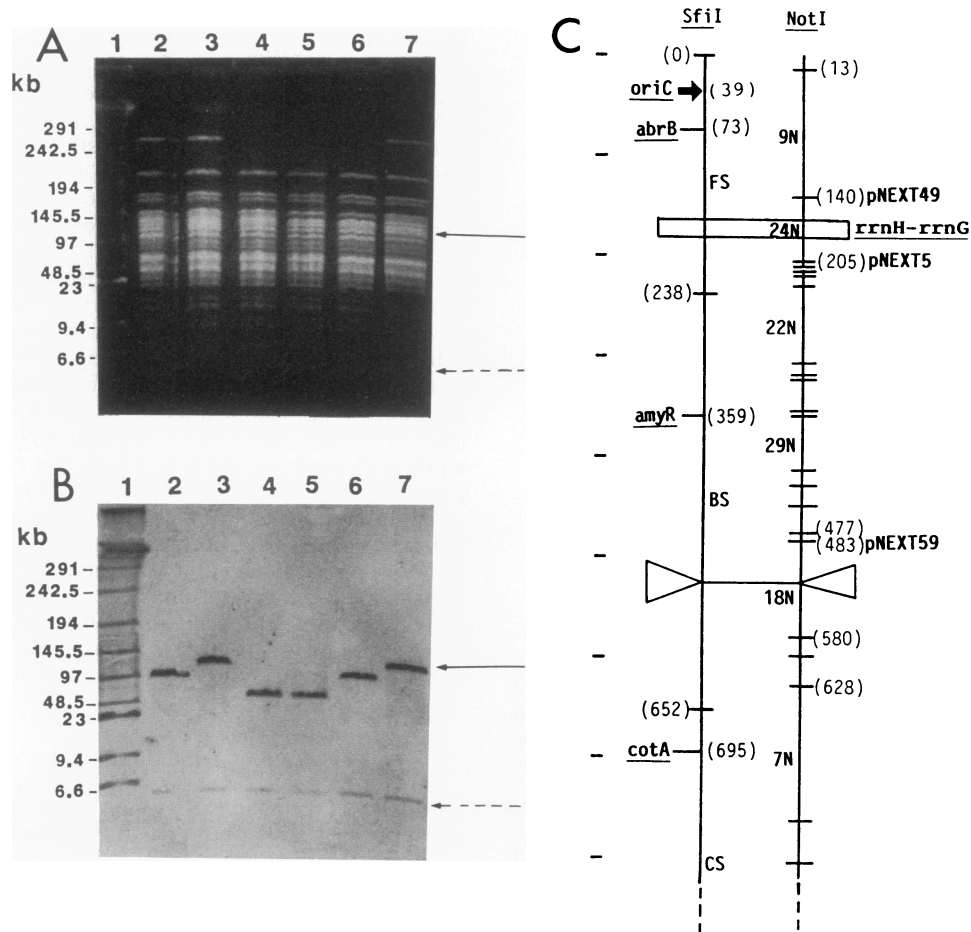


FIG. 4. Location of deletions I and II. The region from 0 to 800 kb on the *SfiI*-*NotI* map of the *B. subtilis* chromosome is redrawn on the right (C) (22). The physical distances (in kilobases) for genes *oriC*, *abrB*, *amyR*, and *cotA* are indicated in parentheses between the two vertical lines. The short horizontal bars indicate *NotI* or *SfiI* sites. The *NotI*-linking clones (pNEXT49, pNEXT5, and pNEXT59) are located at 140, 205, and 483 kb, respectively. The region of the deletion between operons *rmG* and *rmH* (deletion I) is enclosed in a box, and the deletion in the 18N fragment (deletion II) is indicated by open triangles. (A) Ethidium bromide-stained gel. Lane 1 contained a concatemeric lambda DNA segment plus lambda DNA digested with *HindIII*; sizes (in kilobase pairs [kb]) are indicated on the left. Approximately 4 μ g of chromosomal DNA from strain CU741 (lane 2), 168 *trpC2* (lane 3), YS11 (lane 4), RM125 (lane 5), MI112 (lane 6), or BD224 (lane 7) was loaded onto the gel after *NotI* digestion. The running conditions were as follows: 1% agarose, TBE solution (14°C), 18-s pulse time, 4 V cm^{-1} , 36-h running time. (B) pNEXT59 was labeled and used as a hybridization probe after being cohybridized with labeled lambda DNA. The position of the 18N fragment in strain 168 *trpC2* is indicated by an arrow, and the position of an adjacent 6-kb fragment (477 to 483 kb) is indicated by a broken arrow.

tigators should be aware that such differences may exist among various derivatives of *B. subtilis* 168.

Several reports have indicated that *B. subtilis* tolerates large DNA rearrangements, including (i) formation of an inversion or merodiploid state in the genome, resulting in the *trpE26* mutation (5), (ii) amplification of the DNA segment at the tetracycline resistance determinant (*tetBS908*) locus accompanying polyethylene glycol treatment (3) or by multiple unequal crossing-over recombination induced by the presence of tunicamycin at the *tmr* locus (16), and (iii) formation of a *sigK* gene fusion by excision of 43 kb of an intervening DNA segment in a mother cell during sporulation (33, 37). In addition, mutants selected for deletions in a specific region on the chromosome have also been reported (for example, at the *gnt* locus [15] or the *terC* region [18]); however, these mutants were all induced under certain specific environmental stress or were selected. Thus, the extents and locations of the mutations are distinct from those found in this study. The

results of this survey do not rule out changes such as 1-bp differences at sites other than sites in the *NotI* and *SfiI* sequences or changes too small to be resolved by the methods used in this study. Also, it is possible that the strains analyzed in this study merely represent the average of different populations in *B. subtilis* cultures, and other potential genome rearrangements in different locations may occur.

Chromosomal instabilities in other bacteria result from the presence of insertion sequences (38), gene duplications (6), and illegitimate recombination (2, 14). For *B. subtilis*, little is known about spontaneous gene duplications. The presence of reiterated sequences at various loci does not affect stability (41). No recombinational hot spot has been reported, and repeated transformation had no observable effect on genome stability (22). No insertion sequence element has been described, and few examples of illegitimate recombination in the *B. subtilis* chromosome have been reported previously

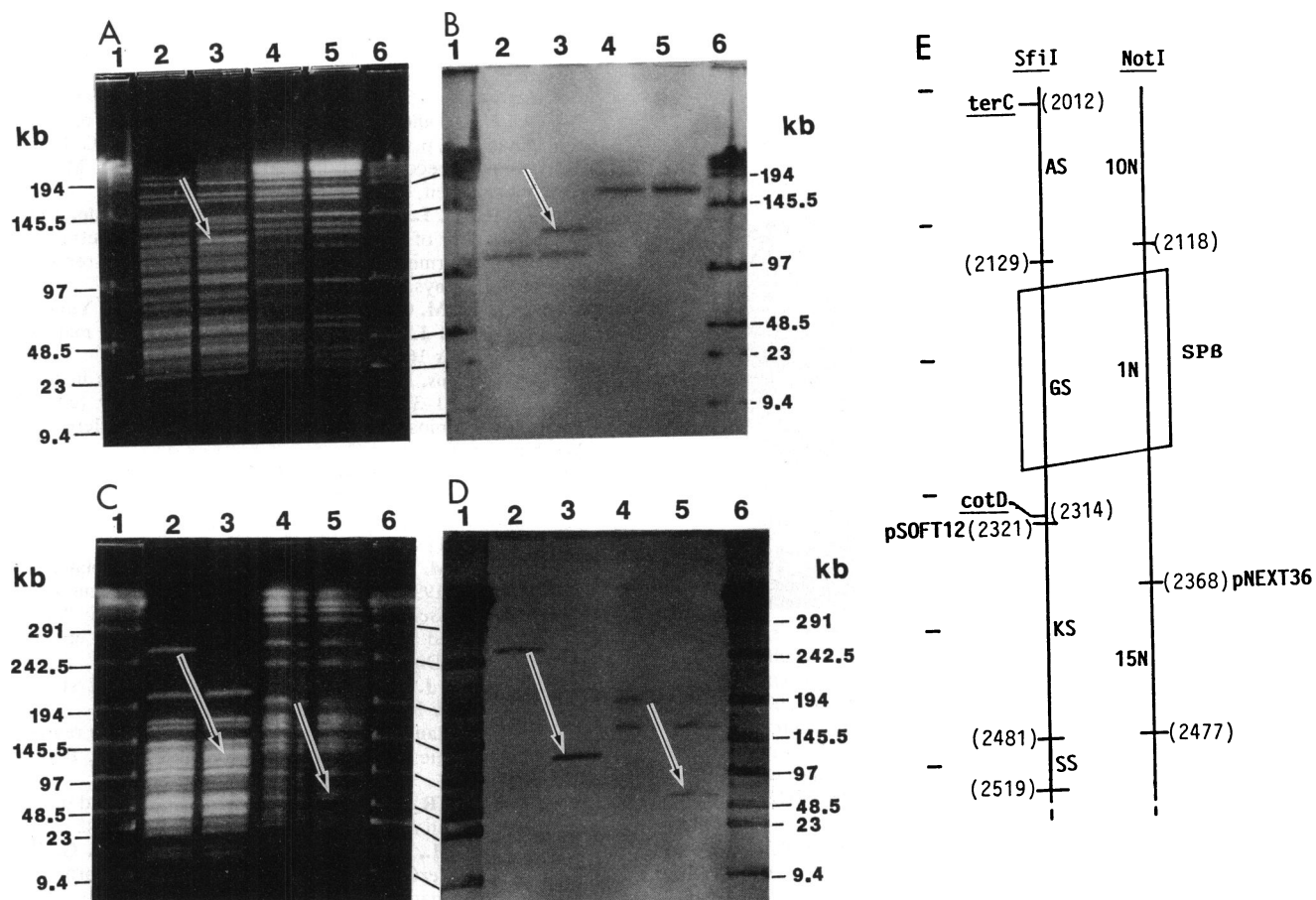


FIG. 5. Deletion at the SPB integration locus (deletion III). The region from 2,000 to 2,500 kb on the *Sfi*I-*Not*I map of the *B. subtilis* chromosome is redrawn on the right (E) (22). The short horizontal bars across the chromosome indicate the *Sfi*I and *Not*I sites; the positions of these sites (in kilobases) are indicated in parentheses. pSFT12 and pNEXT36 are linking clones that were used for the hybridization experiment. *terC*- and *cotD*-carrying clones pWS10 or pBD54 were also used for the hybridization experiment (see text). The region of deletion III (130 kb) is enclosed in a box. In panels A through D lanes 1 and 6 contained concatemeric lambda DNA segments plus lambda DNA digested with *Hind*III; the sizes of marker DNAs (in kilobase pairs [kb]) are indicated on the right and left. Approximately 4 μ g of chromosomal DNA from strain 168 *trpC2* (lanes 2 and 4) or BG126 (lanes 3 and 5) was loaded after *Not*I or *Sfi*I digestion. The positions of shortened fragments identified by Southern analysis are indicated by arrows. (A and B) Labeled pNEXT36 was used as the probe, and labeled lambda DNA was cohybridized. The running conditions were the same as those described in the legend to Fig. 1, except that the pulse time was 14 s. (C and D) Labeled pSFT12 was used as the probe, and labeled lambda DNA was cohybridized. The running conditions were the same as those described in the legend to Fig. 1, except that the pulse time was 18 s.

(5). Thus, the overall structure of the *B. subtilis* 168 genome seems to be very stable under normal growth conditions, and large DNA rearrangements take place at particular loci on the genome spontaneously or can be induced by specific treatments.

The relatively stable pattern of *Sfi*I- and *Not*I-generated DNA fragments for *B. subtilis* 168 derivatives can be useful for defining strains in pedigree trees starting from *B. subtilis* Marburg (10). The use of I-*Sce*I nuclease may facilitate the analysis. Similarly, the relationships among several *Bacillus* species can be examined in more detail (1). For example, DNAs isolated from the closely related organisms *B. subtilis* W23 and *Bacillus natto* produced different fragment patterns (unpublished data).

Characterization of SPB phage integration. Spancake and Hemphill described a restriction map of the SPB prophage, whose size was estimated to be about 120 kb (36). A 130-kb deletion was observed in this study and was attributed to the loss of an SPB prophage. It is not surprising that no recog-

nition sequence for *Not*I (GCGGCCGC) or *Sfi*I (GGCCNNN NNGGCC) is present in the 130-kbp interval (Fig. 5), since the G+C content of SPB DNA is only 31 mol% (32) and is distinct from the G+C content of the chromosome (43 mol%) (25).

Asymmetry of the *B. subtilis* genome. *B. subtilis* has unique regions for initiation (*oriC*) (27, 28) and termination (*terC*) (39) for chromosome DNA replication. This feature is characteristic for *B. subtilis* and differs from the situation in *E. coli*, which has multiple termination sites (17). Both *oriC* and *terC* sites were located precisely on the physical map (21, 22). The length for the clockwise direction is 1,996 kb, and in the counterclockwise direction the length is 2,192 kb. It is clear that the genome of *B. subtilis* 168 (4,188 kb) is somewhat asymmetrical with regard to DNA replication (Fig. 3 and 6). Wake and coworkers have shown that the clockwise DNA replication fork reaches the termination site first and is arrested at the *terC* site until the counterclockwise fork approaches (39). Change in the relative distance

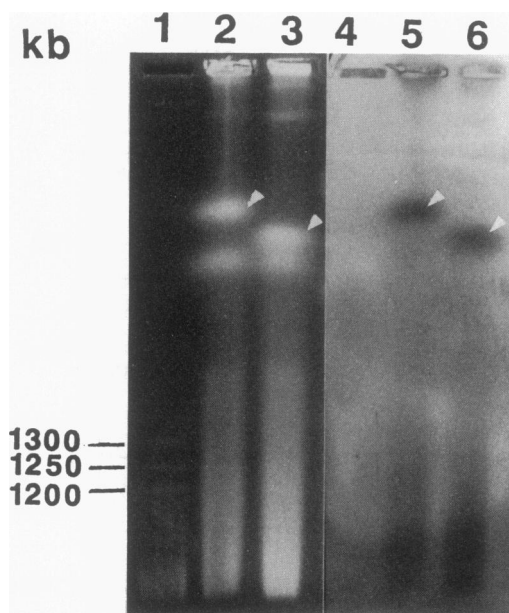


FIG. 6. Separation of two fragments from the *B. subtilis* 168 chromosome by I-SceI digestion. The *B. subtilis* chromosome (approximately 5 μ g) was digested with I-SceI from strain BEST 2076 (derived from strain 168 *trpC2*) (lane 2) or BEST2078 (derived from strain RM125) (lane 3). Lane 1 contained *Saccaromyces cerevisiae* DNA as size markers; the sizes of the DNA markers (in kilobase pairs [kb]) are indicated on the left. The bands identified in a Southern hybridization experiment in which pBS275HE (*hut* gene carrier) (see Fig. 3 and the text) was used as the probe are indicated by arrowheads. The estimated size of the larger band was 2,192 kb in lane 2 and 2,062 kb in lane 3. The size of the smaller band was 1,996 kb in lane 2 and 1,956 kb in lane 3. The size difference between the larger bands is more exaggerated than the size difference between the smaller bands under the running conditions used. The smears on the bottom half of the gel are degraded chromosomal DNA, most of which was degraded during I-SceI digestion. The running conditions were as follows: 1.5% agarose, TBE solution (14°C), 480-s pulse time, 3 V cm^{-1} , 65-h running time.

between the loci of *oriC* and *terC* can be achieved by deletion or insertion in either half of the *B. subtilis* chromosome. However, the asymmetrical feature was unchanged in all of the chromosomes that exhibited size alterations (Table 1 and Fig. 3 and 6). The slightly longer counterclockwise length agrees with observations based on the assumption that fork movement occurs at equal rates on both parts of the chromosome. *B. subtilis* can tolerate a degree of naturally occurring imbalance in chromosome length since the *terC* region can be displaced closer to *oriC* in the clockwise part of the chromosome by up to about 1,000 kbp without affecting the viability of the cells (11, 18). It is not yet clear whether the deletions found in this study influence subtle regulation of the fork movement and arrest at the *terC* region. None of the *B. subtilis* 168 strains listed in Table 1 showed significant growth impairment on rich medium (Penassay or LB medium).

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