I-CeuI recognition sites in the *rrn* operons of the *Bacillus subtilis* 168 chromosome: inherent landmarks for genome analysis

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The *Bacillus subtilis* 168 circular chromosome yielded ten fragments on I-CeuI endonuclease digestion. I-CeuI recognizes a 26 bp sequence that is located within the gene encoding the 23S subunit of the rRNA in *Chlamydomonas eugametos*, *Escherichia coli* and *Salmonella typhimurium*. The precise locations of the I-CeuI sites of the *B. subtilis* chromosome were determined on a *NotI*–*SfiI* physical map by (i) double digestion analyses with I-CeuI and *SfiI*, (ii) comparison of mutant strains lacking a specific *rrn* operon, (iii) using an I-CeuI linking clone and (iv) analysis of nucleotide sequence data of some *rrn* operons. In conclusion, all the I-CeuI sites were located within the *B. subtilis* *rrn* operons and the I-CeuI sites were conserved in all the *B. subtilis* 168 derivatives tested. Thus, variations in size of the I-CeuI fragments must be due to genome alterations. A *B. subtilis* 168 strain was investigated with I-CeuI. We demonstrated that the aberrant structure was the outcome of the inversion of an ~ 1700 kb DNA segment.

Keywords: I-CeuI, *rrn* operons, *Bacillus subtilis*, inversion, genome analysis

INTRODUCTION

International cooperative sequencing projects for genomes of a few prokaryotes, *Escherichia coli*, *Mycobacterium leprae*, *Mycoplasma genitalium* and *Bacillus subtilis* are now in progress (Kunst *et al.*, 1995). The information of the entire nucleotide sequence for these microorganisms is expected to reveal many aspects of a unified as well as the diverse nature of the bacterial chromosome. In addition to understanding the genome structure of standard strains, studies on the variation of genomes within a given species are equally important.

A detailed physical map for a standard *B. subtilis* strain was constructed, namely a restriction map by two type II restriction endonucleases, *NotI* and *SfiI* (Itaya & Tanaka, 1991). Some DNA alterations, mostly deletion mutations, have been characterized based on the physical map (Itaya *et al.*, 1992; Itaya, 1993a).

A new class of sequence-specific endonucleases was recently discovered. These 'homing endonucleases' are classified as enzymes whose catalytic activity results in self-propagation. A number of such enzymes have been found and their biochemical and genetic characterization

has revealed their functional roles in species diversity and evolution (Mueller *et al.*, 1993).

Homing endonucleases have potential for analysis of large genomes because their unique recognition sequences are usually composed of more nucleotides than those for type II restriction endonucleases. For example, I-*SceI*, isolated from *Saccharomyces cerevisiae*, recognizes a unique 18 bp sequence (Mueller *et al.*, 1993). As the 4188 kb *B. subtilis* chromosome has no intrinsic I-*SceI* recognition sequences, the genome can be segmented on I-*SceI* digestion only when novel I-*SceI* sites have been created in the chromosome. Antibiotic resistance genes carrying an I-*SceI* site were designed to be integrated into the *B. subtilis* chromosome, resulting in creation of these sites (Itaya *et al.*, 1992; Itaya, 1992, 1993a, b, 1994). However, problems occur when the antibiotic resistance genes, namely the I-*SceI* sites, cannot be integrated into the genome of transformation-deficient *B. subtilis* strains. This is probably the case for other rare-cutting endonucleases.

As cognate inherent landmarks on the *B. subtilis* chromosome were desirable for in-depth genome analyses, we tested other homing endonucleases, I-*PpoI* (from *Physarum polycephalum*), VDE (*Sacc. cerevisiae*), I-*ThI* (*Thermococcus litoralis*), and I-CeuI (*Chlamydomonas eugametos*). Only I-CeuI cleaved the *B. subtilis* genome and yielded ten fragments reproducibly.

Abbreviation: CHEF, contour-clamped homogeneous electric field.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Reference
<i>B. subtilis</i>		
168 <i>trpC2</i> (= 1A1)	<i>trpC2</i>	Itaya (1993a)
BEST2076*	1A1 plus <i>terC::neo(I-SceI) recF::cat(I-SceI)</i>	Itaya (1993a)
BEST3015	<i>leuB7 arg Δ(rrnJ-rrnW)</i>	Itaya (1993c)
BD170	<i>trpC2 thr-5 Δ(rrnH-rrnG)</i>	Itaya (1993c)
60015	<i>metC7 trpC2</i>	Itaya (1993a)
60866	60015 plus <i>inv(C/S) Δigf Δ(rrnH-rrnG)</i>	Freese & Fortnagel (1967)
YF001	<i>trpC2 metB5 hisA1 leu-8 attSPB Δskin NotI(20N/21N)†</i>	Freese & Fortnagel (1967)
61656‡	YF001 plus <i>Δigf</i>	Fujita & Freese (1981)
<i>E. coli</i>		
JA221	F ⁻ <i>hsdR hsdM⁺ trp leu lacY recA1</i>	Fujita & Freese (1981)
<i>E. coli</i> plasmid§		
pCENT1	2.3 kb <i>EcoRI</i> carrying part of the <i>rrnG</i>	Itaya & Tanaka (1991)
pNEXT38	<i>NotI</i> -linking clone at 786 kb	This study
pNEXT49	<i>NotI</i> -linking clone at 140 kb	
pNEXT53	<i>NotI</i> -linking clone at 13 kb	
pNEXT59	<i>NotI</i> -linking clone at 483 kb	
pNEXT55	<i>NotI</i> -linking clone at 3142 kb	
pSOFT1	<i>SfiI</i> -linking clone at 1045 kb	
pSOFT8	<i>SfiI</i> -linking clone at 675 kb	Itaya & Tanaka (1991)
pSOFT11	<i>SfiI</i> -linking clone at 4055 kb	
pSOFT15	<i>SfiI</i> -linking clone at 2542 kb¶	

* I-*SceI* sites were integrated in the *recF* and *terC* region, resulting in two fragments, 2192 and 1996 kb, on I-*SceI* digestion (Itaya, 1993a).

† A *NotI* site between 20N and 21N was lost (Itaya & Tanaka, 1991).

‡ 61656 was isolated as a *purA* transductant to which the *igf* mutation was transmitted.

§ Location of the insert of the pNEXT and pSOFT series was previously described by Itaya & Tanaka (1991).

¶ Corrected position (unpublished data).

I-*CeuI* recognizes and cleaves a 26 nucleotide sequence, 5'-TAACTATAACGGTCCTAAGGTAGCGA-3', and is encoded by a group I intron in the large subunit rRNA gene of *C. eugametos* (Marshall *et al.*, 1994). Liu *et al.* (1993a, b) reported that I-*CeuI* recognized and cleaved the sites within the *rrn* operons of the genomes of *Salmonella typhimurium*, *E. coli* and other bacteria. The 26 bp sequence was found in the 23S rRNA gene of the *B. subtilis* *rrnB* operon (Green *et al.*, 1985), the only *rrn* operon whose nucleotide sequence had been determined when our work was initiated. We predicted that I-*CeuI* cleavage sites may be located in all ten *rrn* operons of the *B. subtilis* chromosome because the nucleotide sequences of *rrn* genes are highly conserved among distantly related species (Woese *et al.*, 1990).

In this paper we demonstrate experimentally that all *B. subtilis* *rrn* operons have an I-*CeuI* site, and this allowed the precise mapping of all the *rrn* genes on the *NotI*-*SfiI* physical map. We also report that the inherent I-*CeuI* sites are suitable for analysing DNA rearrangements particularly those of strains lacking transformability. In an

experiment using I-*CeuI* combined with *NotI* and *SfiI*, we showed that a previously unidentified DNA aberration is an ~ 1700 kb inversion.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *B. subtilis* 168 *trpC2* was from the *Bacillus* Genetic Stock Center (Columbus, OH, USA). The *SfiI*-*NotI* physical map has been previously reported (Itaya & Tanaka, 1991). Its brief description is given in the legend to Fig. 4. Preparation and transformation of *E. coli* JA221 competent cells were done by the method of Mandel & Higa (1970). LB broth (Miller, 1972) was used for growth of *E. coli* and *B. subtilis*. Nutritional requirements for *B. subtilis* were tested using Spizizen medium supplemented with appropriate amino acids (5 µg ml⁻¹; Spizizen, 1958). Bacteria were grown at 37 °C. All the plasmids were purified by CsCl/ethidium bromide ultracentrifugation.

In vitro DNA manipulations. Type II restriction enzymes and T4 DNA ligase were obtained from Toyobo, except for *SfiI* (New England Biolabs) and *NotI* (Takara Shuzo). Homing

Fig. 1. Separation of the I-CeuI fragments of the *B. subtilis* 168 *trpC2* chromosome. I-CeuI digests of about 2–3 µg DNA from *B. subtilis* 168 *trpC2* were separated by CHEF gel electrophoresis. Pulse time and running time adopted to obtain good resolution for different size ranges: left, 8 min and 44 h; middle, 3 min and 44 h; right, 18 s and 32 h. Other conditions were as described in Methods except for 4 V cm⁻¹ (right). I-CeuI fragments were designated by two capital letters with their estimated size in parentheses. Capital letters represent the last letter of the respective *rrn* operon (Figs 2, 4 and 6). The asterisked fragment was generated by partial digestion at a poorly cleavable I-CeuI site between GE and ED (see text for details). Intensities of larger bands in the left panel are small compared with their expected amounts, probably due to non-specific cleavage during DNA preparation and enzyme digestion. Size markers of 2192 and 1996 kb were the digests of BEST2076 with I-SceI (Table 1). Sizes of 730, 437 and 350 kb were the *Sfi*I digests of *B. subtilis* 168 *trpC2*. Size markers for 48.5–194 kb were concatemeric λ DNA oligomers. Size markers below 23 kb were from λ DNA digested with *Hind*III.

endonucleases I-SceI, I-CeuI, VDE, I-TliI and I-PpoI were purchased from NEB. DNA manipulations *in vivo* were done according to standard procedures (Maniatis *et al.*, 1982) or the manufacturers' instructions unless specified. DNA in gel blocks after digestion with I-CeuI was treated with proteinase K for 1 h at 37 °C to obtain sharp bands as suggested by the manufacturer.

Preparation of *B. subtilis* chromosomal DNA. *B. subtilis* chromosomal DNA for contour-clamped homogeneous electric field (CHEF) gel electrophoresis was prepared in agarose plugs as described previously (Itaya & Tanaka, 1991). Agarose gel (1.0%, w/v) in TBE solution [45 mM Tris/borate (pH 8.0), 1.0 mM EDTA] was used for CHEF gel electrophoresis under constant voltage of 3 V cm⁻¹ at 14 °C unless otherwise specified. Other running conditions are described in the figure legends. After electrophoresis, DNAs were stained in ethidium bromide solution (600 ng ml⁻¹) for 15 min, photographed and subjected to Southern analysis.

Two-dimensional CHEF gel electrophoresis. After CHEF gel electrophoresis, gel blocks containing *Sfi*I (or I-CeuI) fragments were excised by monitoring under 310 nm UV light and washed at least three times in 0.5 ml TE solution [10 mM Tris/HCl (pH 7.5), 1.0 mM EDTA] for 15 min. The gel blocks were immersed in the reaction buffer (0.2–0.8 ml) in 2.0 ml Eppendorf tubes, and digested with the second enzyme, I-CeuI (or *Sfi*I).

Isolation of an I-CeuI-linking clone. Plasmids carrying an I-CeuI site (I-CeuI-linking clone) were obtained from a *B. subtilis* *Eco*RI DNA library made on pBEST504 (Itaya & Tanaka, 1991). The DNA library (about 10 µg) was digested in 100 µl with one U of I-CeuI for 2 h. The digests were incubated at 65 °C for 10 min before loading in wells to denature ingredient proteins. Without denaturation, I-CeuI digests run with poor resolution (unpublished observations). Linearized plasmid fractions (equivalent to linearized I-CeuI linking clones) were separated and purified from closed circular plasmids by CHEF gel electrophoresis under the following conditions: 10 V cm⁻¹,

120 s pulse time, 3 h run at 14 °C in 2% low-melting-point agarose (type VII; Sigma) in TBE solution. The linearized DNAs were used to transform JA221 after ligation. Plasmid DNAs from the ampicillin- and chloramphenicol-resistant transformants were analysed. A linking clone was obtained and designated pCENT1. pCENT1 hybridized to all the I-CeuI fragments (data not shown) and it also hybridized to four *Sfi*I fragments (BS, CS, FS and MS) and five *Not*I fragments (7N, 9N, 13N, 24N and 27N) (data not shown). The *Sfi*I and *Not*I fragments were identical to those that hybridized with an *rrnO* probe in separate experiments (Itaya, 1993c), indicating that the 2.3 kb *Eco*RI insert of pCENT1 was derived from a *B. subtilis* *rrn* operon. Its size allowed us to assign it unequivocally to part of the *rrnG* operon as indicated in Fig. 2 (Rudner *et al.*, 1993). pCENT1 was used as a representative for I-CeuI linking clones in this study.

Southern hybridization and DNA probes. DNA in agarose gels was transferred onto nylon membranes (Nytran 13N; Schleicher & Schuell) by capillary blotting for 15–17 h (Southern, 1975). A non-radioactive labelling nucleotide, digoxigenin-11-dUTP, was used for preparing DNA probes. The random primer labelling technique, pre-hybridization and hybridization procedures and colour development were according to the protocol of the DNA Labelling and Detection Kit (Boehringer Mannheim).

RESULTS AND DISCUSSION

Digestion of genomic DNA of *B. subtilis* 168 *trpC2* with I-CeuI yielded ten fragments (Fig. 1). Preliminary double digestion analysis of the *B. subtilis* chromosome with *Sfi*I and I-CeuI indicated that I-CeuI sites were located on the BS, CS, MS, and FS fragments (data not shown). To determine the location of I-CeuI sites in these four *Sfi*I fragments, the respective *Sfi*I fragment was isolated and

Fig. 2. Location of I-CeuI sites on the *rrn* operons of the *B. subtilis* chromosome. Top: The 4188 kb *Sfi*I restriction map of the *B. subtilis* 168 *trpC2* strain (Itaya & Tanaka, 1991, 1993a) was drawn in linear form. Middle: The FS, BS, CS and MS fragments were enlarged with their sizes in the parentheses. Scales are not uniform. Positions of the *Sfi*I sites of interest are underlined. Fragments obtained by I-CeuI digestion from the respective *Sfi*I fragments are shown with their estimated sizes. Data are shown in Fig. 3. Locations for *Sfi*I-linking clones (pSOFT #) or *Not*I-linking clones (pNEXT #) are indicated with closed bars (see Table 1). They were used as probes to assign the fragments shown in Figs 3 and 5. Bottom: structure of the *rrnG* operon and its flanking chromosomal regions (closed area) redrawn from Widom *et al.* (1988). The 2.3 kb *Eco*RI insert (shaded box) cloned in pCENT1 includes most of the 23S gene, the 5S gene, and part of another chromosome area.

re-digested with I-CeuI. The strategy is presented in Fig. 2.

I-CeuI sites in the BS, CS and MS fragments

I-CeuI digestion of the isolated BS, CS or MS fragments gave two sub-fragments. In brief, BS (437 kb) was cleaved to a 431 and a 5.6 kb fragment (Fig. 3c), CS (350 kb) to a 300 and a 50 kb fragment (Fig. 3a), and MS (143 kb) to a 75 and a 68 kb fragment (Fig. 3e). The size of the sub-fragments should correspond to the distance from the nearest *Sfi*I site (Fig. 2). The order of the two sub-fragments was determined by using specific positional probes as indicated in Fig. 2: pSOFT10 and pSOFT8 for BS (Fig. 3d), pSOFT8, pNEXT38, and pSOFT1 for CS (Fig. 3b), and pNEXT55 for MS (Fig. 3f). The results are shown in Fig. 2. All the shortened fragments were hybridized with pCENT1 (data for the CS fragment only is shown in lane 3 of Fig. 3b).

The results were consistent with there being a I-CeuI site in the 23S gene of all *rrn* operons. The unequivocally determined positions of these I-CeuI sites were at a

distance of 669 kb (*rrnE* in the BS fragment), 975 kb (*rrnD* in the CS fragment) and 3195 kb (*rrnB* in the MS fragment) (Fig. 4).

Seven I-CeuI sites in the FS fragment

The isolated FS fragment yielded eight sub-fragments on I-CeuI digestion, a 33 kb and a 44 kb fragment in addition to the six originally found (Fig. 3h). This indicated that there were seven I-CeuI sites inside the FS fragment. Itaya (1993c) already demonstrated that seven *rrn* operons were located in the FS fragment. It was therefore most likely that these seven I-CeuI fragments belong to the seven *rrn* operons. This was demonstrated by the following experiments.

Two *B. subtilis* mutant strains that have lost a segment between two tandemly repeated *rrn* operons have been obtained. They are BEST3015 and BD170 (Table 1), having a deletion from *rrnJ* to *rrnW* and from *rrnG* to *rrnH*, respectively (Itaya, 1993a, c). Thus, I-CeuI digestion of the DNA of these mutants should reveal the loss of the respective I-CeuI fragments, and indeed this was the case

Fig. 3. Location of I-CeuI sites in *Sfil* fragments. Isolated *Sfil* fragments were digested with I-CeuI and the resulting fragments were separated by CHEF gel electrophoresis. Size markers for 48.5–463.5 kb were concatemeric λ DNA oligomers. Size markers below 23 kb were λ DNA digested with *Hind*III. Probes used are indicated in Fig. 2 and Table 1. (a) The 350 kb CS fragment was cleaved to a 300 and a 50 kb fragment indicated by arrows. Pulse and running times: 60 s and 20 h. (b) The 50 kb fragment was hybridized with pSOFT1 (lane 1). pNEXT38 hybridized to the 300 kb fragment (lane 2). pCENT1 (*rrnG*) hybridized with both fragments (lane 3). (c) The 437 kb BS fragment was cleaved to 431 and 5.6 kb. Pulse and running times: 12 s and 16 h. Voltage was constant at 4 V cm⁻¹. (d) The 5.6 kb fragment was hybridized with pSOFT8. The slight hybridization of pSOFT8 to the 431 kb fragment was due to partial digestion of this I-CeuI site (see text). (e) The 143 kb MS fragment (lane 1) was cleaved to a 75 and a 68 kb fragment (lane 2). Pulse and running times: 12 s and 40 h. Voltage was constant at 2.7 V cm⁻¹. Open-headed arrows indicate the LS fragment (143 kb) and a small amount of the NS fragment (133 kb) that were co-purified with the MS fragment and left unchanged in lane 2. (f) pNEXT55 hybridized to the 75 kb fragment. (g) Chromosomal DNA from BEST3015, 168 *trpC2* and BD170 was run after I-CeuI digestion. Pulse and running times: 12 s and 15 h. Voltage was constant at 4 V cm⁻¹. The 5.9 kb (JW) and 4.9 kb (HG) fragments were lost in BEST3015 and BD170, respectively. See text for details. (h) Lanes 1 and 2, the purified FS fragment cleaved to eight bands on I-CeuI digestion. Chromosomal DNA from strain 168 *trpC2* was run after I-CeuI digestion in lane 3. Pulse and running times: 12 s and 15 h. Voltage was constant at 4 V cm⁻¹. The internal six bands (AJ, WI, OA, JW, IH and HG) were unchanged in all lanes. Two new bands (44 kb and 35 kb) in lanes 1 and 2 were assigned to the distal ends of the FS fragment (Fig. 2) by using the specific probes pNEXT53 or pSOFT10 (data not shown).

(Fig. 3g). The smallest, 4.9 kb, fragment that was lost in BD170 was readily assigned to the segment between *rrnH* and *rrnG*. Similarly, the 5.9 kb fragment that was lost in BEST3015 was assigned to the segment between *rrnJ* and *rrnW*. The 5.6 kb fragment was automatically assigned to the segment between *rrnI* and *rrnH* (Widom *et al.*, 1988).

The location of the other fragments was determined with the position-specific probes shown in Fig. 2. The eight fragments were aligned (Fig. 2) and positioned on the physical map (Fig. 4).

The nucleotide sequence of the four *rrn* operons in the 9N fragment (*rrnO*, *rrnA*, *rrnJ*, and *rrnW*) was recently determined (Ogasawara *et al.*, 1994, 1995) and the I-CeuI

recognition sequence was found in all four 23S rDNA genes. The 26 bp of *rrnO*, *rrnA*, *rrnJ*, *rrnW* (Ogasawara *et al.*, 1994) and *rrnB* (Green *et al.*, 1985) are identical to the recognition site reported for the *rri* gene from *C. eugametos* (Marshall *et al.*, 1994).

It should be noted that complete cleavage at the I-CeuI site of *rrnE* could not be achieved in various digestion conditions; it resulted in small amounts of the uncleaved GE + ED fragment (see legend to Fig. 1). Poor cleavage sites were not found in *S. typhimurium* or in *E. coli* (Liu *et al.*, 1993a). Although the reason for a poor cleavage site at *rrnE* is not fully understood, it could be due to a degenerated sequence or to an as yet unidentified DNA modification in the recognition site (Mueller *et al.*, 1993).

Fig. 4. Location of the *rrn* operons on the *B. subtilis* *SfiI*–*NotI* physical map. The *NotI*–*SfiI* physical map (Itaya & Tanaka, 1991) was redrawn with minor modifications. Briefly, 26 *SfiI* fragments (AS–ZS) were aligned in the inner circle and 72 *NotI* fragments (1N–33N and smaller) in the outer circle. The *SfiI* site between fragments F5 and NS was designated as the zero point of the 4188 kb map. Physical distance (in kbp) of the *rrn* operons are in parentheses. I, II and III, spontaneous deletions previously characterized by Itaya (1993a). See text for details.

The *I-CeuI* recognition sequences are well-conserved in all *B. subtilis* 168 derivative strains tested to date. For example, three spontaneous DNA deletions frequently formed at three different regions of the *B. subtilis* 168 chromosome were analysed (Fig. 4). The smallest deletion, designated deletion I (Itaya, 1993a), was identical to that of the HG (*I-CeuI*) fragment observed in BD170. A 23 kb deletion that occurred in the BS (*SfiI*) fragment, designated deletion II, was found to be a 23 kb decrease in the GE (*I-CeuI*) fragment (data not shown). The mechanism for the formation of deletion II is not clear. An ~130 kb deletion associated with the excision of the SP β prophage occurring in the GS (*SfiI*) fragment (designated deletion III in Fig. 4) agrees with a 130 kb decrease in size of the DB (*I-CeuI*) fragment (data not shown). *I-CeuI* sites are, thus, inherent stable landmarks of the *B. subtilis* 168 chromosome and can be an immediate tool for structural analysis of the genome.

Use of *I-CeuI* to detect a chromosome DNA inversion

B. subtilis strain 60866, a sporulation- and transformation-deficient mutant, was isolated after ^{60}Co irradiation by Freese & Fortnagel (1967). Fujita & Freese (1981) later

demonstrated that the mutant did not exhibit fructose-bisphosphatase (*fdp*) activity. Fujita & Fujita (1983) characterized the mutation, designated *fdpA1*, as a deletion spanning a large region including the *iol*, *gnt* and *fdp* genes, and renamed it as Δ *igf*. Its parental strain, 60015, had a chromosome structure identical to that of 168 *trpC2* (Itaya, 1993a). In contrast, strain 60866 lacked four original *SfiI* fragments (CS, FS, NS and SS indicated in Fig. 5a, b) and at least three *NotI* fragments (14N, 19N and 24N) (data not shown, but the location of these *NotI* fragments is indicated in Fig. 4).

A decrease in size of the FS and 24N fragments was assigned to the 4.9 kb deletion between *rrnH* and *rrnG* (Figs 5b and 6).

A decrease in size of about 40 kb in the NS and 14N fragments (Fig. 5a) was assigned to the deletion including the *gnt* gene (Itaya & Tanaka, 1991). Thus, it was most likely that the 40 kb deletion corresponded to the Δ *igf* mutation suggested by Fujita & Fujita (1983).

As regards the CS and SS fragments, the size decrease for CS (350 to 270 kb), and the size increase for SS (38 to 107 kb) were identified in Southern hybridization experiments using pSOFT8 and pSOFT15 (data not shown).

Fig. 5. Altered fragments obtained from the *B. subtilis* strain having a chromosome DNA inversion. For all parts of the figure, chromosomal DNA from 168 *trpC2* (1) or 60866 (2) was loaded. (a) and (b) Separation of *Sfi*I fragments. Altered *Sfi*I fragments (CS, FS, NS and SS) are indicated with their new positions on the right. For details see text and Fig. 6. Pulse and running times were 60 s and 42 h for (a) and 24 s and 42 h for (b). *Sfi*I fragments were well-resolved above 200 kb in (a), and below 250 kb in (b). (c) and (d) Separation of I-CeuI fragments greater and less than 1000 kb, respectively. Fragments less than 62 kb were not detectable. Fragment designations in the 60866 lane are according to the structure of 60866 in Fig. 6. D', *rrnD'* operon of strain 60866 (see Fig. 6). Pulse and running times: 8 min and 44 h for (c) and 3 min and 44 h for (d). (e) and (f) I-CeuI fragments resolved in the first run (horizontal) were separated after *Sfi*I digestion in the second run (vertical). The procedure for the second *Sfi*I digestion is described in Methods. DB and BO fragments from 168 *trpC2* or ED' and BO fragments from 60866 were not well separated in the first run under the running conditions. Thus, *Sfi*I fragments from these two fragments appear to overlap in a single vertical lane in the right. Positions of the GE fragment from both strains and the D'B fragment from strain 60866 are indicated at the top. Four *Sfi*I fragments should be obtained from the D'B fragment predicted by the 60866 structure shown in Fig. 6. They are indicated by open arrowheads (from top): DS (310 kb), ES (268 kb), part of MS (75 kb) and an ~70 kb fragment. The 75 kb fragment is the same as that in lane 2 of Fig. 3(f). The last 70 kb fragment comprised the SS' and CS' shown in Fig. 6. Pulse and running times: 3 min and 14 h for the first run and 36 s and 36 h for the second run. Size markers of 2192 and 1996 kb in (c) were digests of BEST2076 with I-SceI (Table 1). Size markers for 48.5–463.5 kb were concatemeric λ oligomers.

The nature of these two changes remained unknown with little information from the *Not*I or *Sfi*I digestion analysis.

We examined the I-CeuI fragments obtained from the 60866 chromosome (Figs 5c, d). We observed three new fragments with altered sizes (ED', BO and D'B) and concomitant loss of four original fragments (DB, BO, ED, and HG). Loss of the 4.9 kb HG fragment was

consistent with the *rrnH-rrnG* deletion as described above in the *Not*I and *Sfi*I analysis (data not shown). An ~40 kb decrease in size of the BO fragment was also consistent with the Δigf mutation (Figs 5d and 6).

Results obtained from other altered I-CeuI fragments agreed only with the assumption that the alterations were due to an ~1700 kb inversion indicated in Fig. 6. The

Fig. 6. Inversion of a 1700 kb DNA segment in strain 60866: *Sfi*I physical maps for 168 *trpC2* and 60866. *rrn* operons are designated by capital letters at their locations except for *rrnD* for 168 *trpC2* or *rrnD'* for 60866. For 60866, the open box in the FS fragment is a 4.9 kb deletion between *rrnH* and *rrnG* designated as H/G and the open box in the NS fragment is an ~40 kb deletion identified as the *Δigf* mutation (see text and Figs 5b, d). The segment between two arrows in 168 *trpC2* was inverted in the 60866 strain (C/S-type inversion). One end of the inversion is indicated by the open arrow in the CS fragment, and the other end by the closed arrow in the SS fragment. The recombined ends are shown by half-closed arrows in strain 60866. The CS fragment was divided into regions CS' and CS'' at the end of the inversion. Similarly, the SS fragment was divided into SS' and SS''. These regions were recombined in the 60866 strain. Closed box, the *terC* region.

location of the *rrnD*, putatively designated as the *rrnD'* of 60866, resulted in a decrease in size of the DB fragment (2220 kb) to D'B (about 750 kb) and an increase in the size of the ED fragment (306 kb) to ED' (about 1750–1770 kb) (Figs 5c, d and 6).

If the C/S-type inversion was true, the D'B fragment had to include four *Sfi*I fragments. Three of them were the DS, ES and the 75 kb fragment from the MS. The fourth included the SS'' plus the CS'' region distal to *rrnD'* (Fig. 6). These predicted fragments were obtained from the isolated D'B fragment on *Sfi*I digestion as shown in Fig. 5(f). Similarly, the ED' fragment included the 13 *Sfi*I fragments predicted from the 60866 genome structure in Fig. 6 (data not shown).

One end of the inverted DNA region was located within the CS fragment, probably limited in the 19N fragment (805–901 kb), because the *Not*I fragment was altered. The other end was positioned within the SS fragment (*Sfi*I, 2481–2519) (Fig. 6). If the C/S-type inversion was caused by recombination at the two ends via equal crossing-over, the sum of the initial CS and SS fragments (350 + 38 = 388 kb) should be equal to the sum of the recombined fragments, CS' + SS' and SS'' + CS''. However, the estimated size of the CS' + SS' (268 kb) and SS'' + CS'' (107 kb) fragments (Fig. 5a, b) gave a sum of 375 kb, indicating that about 13 kb were lost in 60866. The precise location of both endpoints and the lost region(s) remain to be demonstrated.

Are the sporulation and transformation-deficient phenotypes of strain 60866 due to DNA rearrangements? First, the *Δigf* deletion mutation of 60866 was accurately transmitted to 61656 by PBS1-mediated transduction without the accompanying C/S-type inversion (Table 1; data not shown). As strain 61656 is sporulation- and competent-proficient, it is unlikely that the *Δigf* mutation alone was the cause. Second, deletion of *rrnG*–*rrnH* alone had no effect on either phenotype, it only resulted in nine *rrn* operons left intact per *B. subtilis* chromosome (Itaya, 1993c). Third, reversed orientation of the *terC* region, termination of DNA replication (Yoshikawa & Wake, 1993), seems detrimental to *B. subtilis*. Itaya (1994) reported a 1631 kb-long DNA inversion between the *met* (1255 kb) and *leuB* (2886 kb) regions (designated as the BEST2145-type inversion). The BEST2145-type inversion that overlaps about 70% with the C/S-type inversion also made the *terC* region inverted without causing sporulation and competence deficiency (Itaya, 1994). Thus, the reversed orientation of the *terC* region does not seem to be the cause. We suspect that the loss of about 13 kb in 60866 may be the reason, because the deletion(s) is specific for 60866.

Concluding remarks

The finding of the C/S-type inversion was preceded by that of another long inversion associated with *trpE26* mutation described by Anagnostopoulos and co-workers (designated here as the *trpE26*-type inversion: for a review see Anagnostopoulos, 1990). Jarvis *et al.* (1990) reported that the *trpE26*-type inversion was not induced

inversion is hereafter designated as the C/S-type inversion because its endpoints reside in the CS and SS fragments. The C/S-type inversion transferred the *rrnD* close to *rrnB* and made it distant from the *rrnE* (Fig. 6). The new

via homologous recombination between hypothetical homologous regions on the *B. subtilis* chromosome, rather it was likely formed during the repair process after γ irradiation. The C/S-type inversion likely occurred during the γ irradiation (Freese & Fortnagel, 1967) and, once established, was maintained stably in laboratory stocks. We think that long-range inversions do not occur normally (Itaya, 1993a), and speculate that unexpected inversion mutants could be found in strains isolated after γ irradiation.

The I-CeuI sites on the *B. subtilis* chromosome were characterized and demonstrated to be inheritable. I-CeuI is now a useful tool for *B. subtilis* genome analysis in addition to *Not*I, *Sfi*I and *I-Sce*I. Attempts to create new I-CeuI site(s) and/or elimination of I-CeuI sites from the *B. subtilis* chromosome are now being considered.

ACKNOWLEDGEMENTS

We thank Drs Naotake Ogasawara, Takehiko Shibata, Yasutaro Fujita and Yasuo Kobayashi for *B. subtilis* strains and discussions.

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Received 30 December 1994; revised 11 April 1995; accepted 25 April 1995.