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Essential Structure in the Cloned Transforming DNA That Induces Gene Amplification of the *Bacillus subtilis amyE-tmrB* Region

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***Bacillus subtilis* B7, a mutant which acquired gene amplification of the *amyE-tmrB* region, showed, as a result, hyperproductivity (about a 5- to 10-fold increase) of α-amylase and tunicamycin resistance. The mutational character was transferred to recipient cells by competence transformation. A 14-kilobase (kb) *Eco*RI chromosomal DNA fragment of strain B7 was found to have the transforming activity. We cloned a 6.4-kb *Eco*RI fragment on a phage vector λ Charon 4A through a spontaneous deletion of 7.6 kb from the 14-kb fragment and subcloned a 1.6-kb *Hin*dIII fragment on pGR71. The cloned 6.4-kb *Eco*RI and 1.6-kb *Hin*dIII fragments retained the transforming activity of inducing gene amplification of the *amyE-tmrB* region. At the junction point (J) of the repeating units (16 kb), the *tmrB* gene was linked to a DNA region (M) located 4 kb upstream of *amyE*. The essential structure of the cloned, transforming (gene amplification-inducing) DNA was deduced to be that around J. The subcloned 1.6-kb *Hin*dIII fragment that retained the transforming activity was shown to be almost solely composed of the *tmrB*-J-M region. In addition, the DNA sequence around J was determined.**

Bacillus subtilis B7, a *tmrA7* mutant, showed α-amylase hyperproductivity (about a 5- to 10-fold increase) and tunicamycin resistance (16). Recently it was revealed that amplification of a DNA sequence (about 16 kilobases [kb]), including the α-amylase structural gene (*amyE*) and the tunicamycin resistance gene (*tmrB*$^+$), occurred in the mutant and that the gene amplification resulted in α-amylase hyperproductivity (Amy^h) and tunicamycin resistance (Tm^r) (7). The mutational character of strain B7 can be transferred to recipient *B. subtilis* cells by competence transformation (13, 16). The *tmrA7* transformants acquired the gene amplified state in the *amyE-tmrB*$^+$ region (7). The *tmrA7* character-transforming (Tm^r Amy^h) DNA, therefore, is that which induces gene amplification of the *amyE-tmrB* region. Recently *B. subtilis tmrA7* transductants, obtained by the ρ11 prophage transformation technique (9), were also found to show amplification of the *amyE-tmrB* region at the ρ11 integrated position (4a).

We aimed to clone and characterize the *tmrA7* character-transforming DNA from strain B7 chromosomal DNA. In this report we describe the successful cloning of the *tmrA7* character-transforming DNA on λ Charon 4A and subcloning of it on pGR71 as a 1.6-kb *Hin*dIII fragment. Furthermore, we show the essential structure of the gene amplification-inducing DNA.

The characterization of the gene amplification-inducing DNA opens the route for generalization of gene amplification induction in any region of the *B. subtilis* chromosome.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The *B. subtilis* strains used in this study are listed in Table 1. *B. subtilis* B7 was one of the tunicamycin-resistant mutants isolated from *B. subtilis* NA64. An *Escherichia coli-B. subtilis* shuttle vector pGR71 (kanamycin-resistant [Km^r]) (6) was kindly provided by T. Kudo. pTM101 is a plasmid that was constructed by inserting the 0.8-kb *Hin*dIII fragment (*tmrB*) from strain B7 chromosomal DNA into the *Hin*dIII site of pGR71 (S. Harada, K. Yoda, M. Mori, M. Yamasaki, and G. Tamura, manuscript in preparation).

E. coli phage vector λ Charon 4A was used to clone chromosomal DNA of *B. subtilis*, and *E. coli* LE392 was used as the host.

Restriction enzymes and chemicals. Restriction enzymes *Ava*I and *Bcl*I were purchased from Boehringer GmbH (Mannheim, Federal Republic of Germany), and the other restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo Co. (Kyoto, Japan). They were used according to the recommendations of the manufacturers. Kanamycin was purchased from Sigma Chemical Co. (St. Louis, Mo.). Tunicamycin (lot no. 1) was prepared in this laboratory by a previously described method (19). [α-^{32}P]dCTP was purchased from Amersham Corp. (Buckinghamshire, England).

Preparation of chromosomal DNA. *tmrA7* transformants and strain B7 were pregrown on an nutrient agar plate containing 10 μg of tunicamycin per ml, and NA64 was pregrown on an nutrient agar plate. Strains that were tested were precultured in 2 ml of nutrient broth overnight. About 0.4 g of mid-log-phase cells cultured in 100 ml of nutrient broth were suspended in 4 ml of 20 mM Tris–0.5 mM EDTA buffer (pH 8.0) and treated with 4 mg of lysozyme (Seikagaku Kogyo Co., Tokyo, Japan) at 37°C for 10 min. Sodium dodecyl sulfate was added to the resultant cell lysates at a final concentration of 0.5%, and the lysates were clarified at 65°C. The cleared lysates were treated further with proteinase K (Boehringer) at 37°C for 1 h. The lysates were charged on the top of a discontinuous sucrose density gradient (from 5 to 10 to 15 to 20%) and centrifuged at 70,000 × *g* for 1 h. A small amount of the fractionated samples was electrophoresed to test for contamination with RNA. RNA-

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

B. subtilis strain	Genotype	Relevant phenotype	Source
NA64	*metB5 purB6 amyR2*	Amy^+	B. Maruo
B7	*metB5 purB6 amyR2 tmrA7*	Amy^h Tm^r	This laboratory
207-21	*metB5 lys-21 leuA8 aroI906 amyR2 amyE07 hsdR hsdM*		K. Yamane
207-25	*lys-21 leuA8 aroI906 amyR2 amyE07 hsdR hsdM recE4*	Rec^-	K. Yamane

free fractions were precipitated with ethanol and dissolved in the Tris-EDTA buffer. This method is recommended for the preparation of intact chromosomal DNA.

Fractionation of *Eco*RI-digested chromosomal DNAs by sucrose density gradient centrifugation. *B. subtilis* B7 chromosomal DNA was completely digested with *Eco*RI and charged on the top of the same discontinuous sucrose density gradient as described above for the preparation of chromosomal DNA. After centrifugation at 70,000 × *g* for 15 h, the sample was fractionated drop by drop. A small amount of the fractionated samples was monitored for the size distribution by agarose gel electrophoresis. The fractions containing 13- to 17-kb DNA fragments were precipitated with ethanol, suspended in distilled water, and used for the cloning experiment.

Construction of phage library. λ Charon 4A phage DNA was prepared by the method of Vande Woude et al. (21). The 13- to 17-kb *Eco*RI-digested fragments were ligated to *Eco*RI-cleaved λ Charon 4A DNA with T4 DNA ligase at 4°C overnight. The resultant recombinant molecules were packaged into phage coats by the in vitro method described by Blattner et al. (3).

Screening of recombinant phages. The 0.8-kb *Hin*dIII fragment (*tmrB*) from pTM101 was nick translated with [α−^{32}P]dCTP as described by Rigby et al. (14) and used as the screening probe.

Plaque hybridization (first screening). Recombinant phage plaques were blotted onto nitrocellulose filters by the procedure of Benton and Davis (2) and hybridized with the probe DNA by the procedure of Wahl et al. (22) with dextran sulfate.

Spot hybridization (second screening). After the first screening, the phage candidate clones were individually grown overnight in NZYM medium in wells of a 96-well microtiter plate. The lysates in the wells were replicated onto a plate inoculated with strain LE392. On the next day large phage plaques, the diameters of which were about 3 to 5 mm, have been formed. The large phage plaques were blotted onto nitrocellulose filters and hybridized with the probe DNA by the same method described above for plaque hybridization.

The recombinant phage DNAs which were positive in the second screening were prepared by a previously described method (11) and used to transform *B. subtilis* 207-21 by competence transformation.

Transformation. Competence transformation was performed by the method of Hass et al. (8), with the modification of Shibata and Saito (17). Tm^r transformants were selected on nutrient agar (Bacto-Agar [Difco Laboratories, Detroit, Mich.]) plates containing 10 μg of tunicamycin per ml. $AroI^+$ transformants were selected on plates without aromatic amino acids (containing 2% Bacto-Agar; 0.5% glucose; and 50 μg each of methionine, lysine, leucine, and threonine per ml in minimal medium). Amy^+ transformants were detected on nutrient agar plates containing 1% soluble starch by staining with iodine.

Protoplast transformation was performed by the method of Chang and Cohen (4).

α-Amylase assay. Tm^r transformants and strain B7 were pregrown on nutrient agar plates containing 10 μg of tunicamycin per ml, and strain NA64 was pregrown on an nutrient agar plate. The tested strains were precultured in 2 ml of nutrient broth overnight. The next day, they were seeded at 1% into 10 ml of nutrient broth and were incubated at 37°C for 15 h. After the cells were removed by centrifugation, α-amylase activity in the broth was measured by the method of Fuwa (5).

Construction of pTM201. pTM201 is a plasmid that was constructed by inserting the 1.6-kb *Hin*dIII fragment of phage clone 47 into the *Hin*dIII site of pGR71. The DNA of recombinant phage clone 47 was cleaved with *Hin*dIII. pGR71 DNA was cleaved with *Hin*dIII and treated with bacterial alkaline phosphatase (Takara Shuzo) at 60°C for 30 min. After phenol-chloroform (1:1) extraction and ethanol precipitation, the *Hin*dIII-cleaved pGR71 DNA was suspended in ligation buffer and ligated to the *Hin*dIII-cleaved DNA fragments from phage clone 47 with T4 DNA ligase overnight at 15°C. The ligated materials were used to transform *B. subtilis* 207-25 cells by protoplast transformation. Transformed cells were then spread on regeneration plates containing 100 μg of kanamycin per ml. Km^r colonies were replicated on nutrient agar plates containing 10 μg of tunicamycin per ml. Colonies resistant to both kanamycin and tunicamycin were picked and purified, and plasmid DNAs were isolated from them by a previously described method (10).

Southern hybridization analysis. Chromosomal DNA was completely digested with restriction enzymes and resolved by electrophoresis on a 0.8% agarose gel. DNA in the gel was transferred to a nitrocellulose filter and hybridized with nick-translated DNA probes by a previously described method (18). Nick-translated DNA probes were prepared with [α−^{32}P]dCTP and a nick translation kit from Amersham. The radioactive DNA had a specific activity of 5 × 10^6 to 5 × 10^7 cpm/μg.

DNA sequence analysis. Phage subclones were obtained by using both M13mp10 and M13mp11, and DNA sequences were determined on both strands by the dideoxy chain-termination method (15) with M13 sequencing kits (Takara Shuzo). The *Bcl*I-*Eco*RV fragment (see Fig. 7) from pTM201 was subcloned into the *Bam*HI-*Hin*cII regions of M13mp10 and M13mp11. Representative subclones were grown, single-stranded DNAs were extracted and purified, and these DNAs were used as templates for the dideoxy sequencing reactions. The sequencing mixtures were labeled with [α−^{32}P]dCTP, and the products were run on an 8% polyacrylamide sequencing gel.

RESULTS

Size determination of *tmrA7* character-transforming *Eco*RI fragment. To clone and analyze *tmrA7* character-transforming DNA, the chromosomal DNA of *B. subtilis* B7 (*tmrA7*) was completely digested with *Eco*RI and electrophoresed. In Fig. 1a the agarose gel electrophoresis patterns are indicated. A similarly prepared gel was cut into 2.5-mm blocks corresponding to the molecular weight. Each DNA sample that was extracted from the block was tested for the *tmrA7* and the $AroI^+$-transforming activity by using 207-21 (Tm^s

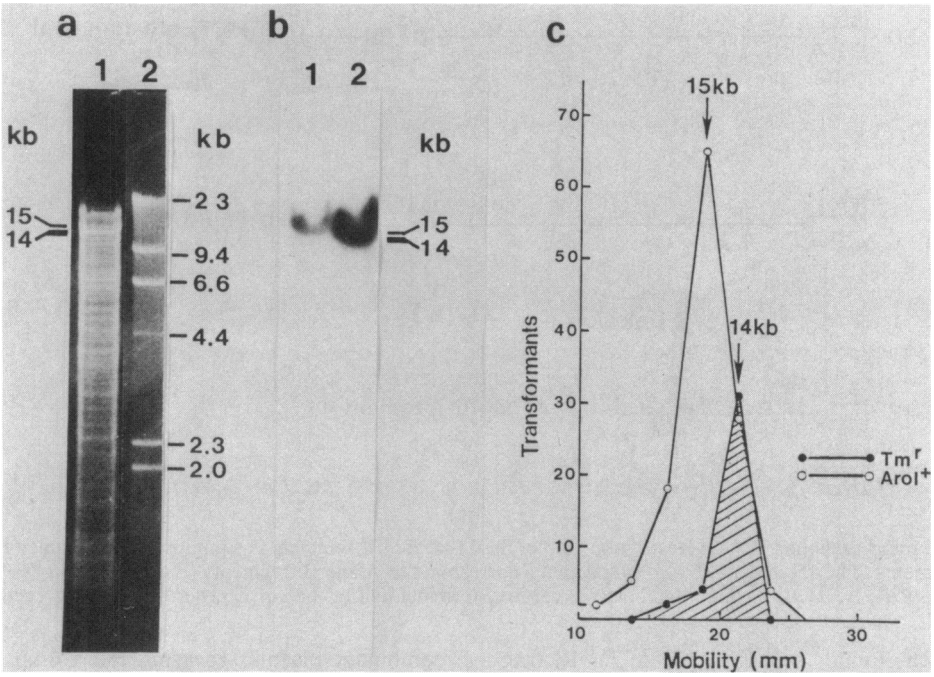

FIG. 1. Characterization of the Tm^r- and AroI$^+$-transforming *Eco*RI fragments. (a) Agarose gel electrophoresis patterns as visualized with ethidium bromide staining and UV illumination. Lane 1, strain B7 (*tmrA7*) chromosomal DNA digested with *Eco*RI; lane 2, λ *Hin*dIII marker. (b) Southern hybridization analysis of *Eco*RI-digested chromosomal DNAs with nick-translated *tmrB* probe (0.8-kb *Hin*dIII fragment; see Fig. 7). Lane 1, strain NA64 (wild type); lane 2, strain B7 (*tmrA7*). About 10 μg of chromosomal DNA was charged. (c) Size fractionation of Tm^r- and AroI$^+$-transforming *Eco*RI fragments from strain B7 chromosomal DNA by agarose gel electrophoresis.

amyE07 aroI906) as a recipient cell. *tmrA7* character-transforming activity was tested by determining the Tm^r-transforming activity. The size of the *tmrA7* character-transforming *Eco*RI fragment was estimated to be about 14 kb and that of AroI$^+$-transforming *Eco*RI fragment was estimated to be about 15 kb (Fig. 1c). It is noteworthy that the 14-kb band shown in Fig. 1a was more dense than any other bands, but the 14-kb dense band could not be detected in parental strain NA64 (see Fig. 4a, lane 6).

Cloning of the *tmrA7* character-transforming *Eco*RI fragment on λ Charon 4A phage vector. To clone the 14-kb *Eco*RI fragment of *B. subtilis* B7 DNA, λ Charon 4A was used as a vector. Strain B7 chromosomal DNA was completely digested with *Eco*RI, and 13- to 17-kb *Eco*RI fragments were purified by sucrose density gradient centrifugation and cloned into λ Charon 4A. About 1,300 recombinant plaques were obtained. The 0.8-kb *Hin*dIII fragment (*tmrB*$^+$) that was already cloned on pGR71 (Harada et al., in preparation) was used as a screening probe. The *aroI* and the *tmrB* genes are known to be located on the same *Eco*RI fragment. It was reasonable, therefore, to use the *tmrB*$^+$ gene as a probe for detecting the AroI$^+$-transforming 15-kb *Eco*RI fragment. In fact, only the 15-kb fragment was detected in the *Eco*RI digests of NA64 (wild-type) chromosomal DNA in Southern hybridization analysis when the nick-translated 0.8-kb *Hin*dIII fragment was used as a probe (Fig. 1b). However, a denser band of about 14 kb was detected in the *Eco*RI digests of B7 (*tmrA7* mutant) chromosomal DNA (Fig. 1b). The dense 14-kb band in the Southern hybridization pattern may correspond to the dense 14-kb band shown in Fig. 1a. We determined, therefore, that the 0.8-kb *Hin*dIII fragment (*tmrB*$^+$) should be used as a probe for detecting the *tmrA7* character-transforming 14-kb *Eco*RI fragment.

TABLE 2. Tm^r-, AroI$^+$-, and AmyE$^+$-transforming activities of recombinant phage DNAs

Donor phage DNA	No. of the following transformants[a]:			
	Tm^r	AroI$^+$	AmyE$^+$/Tm^r	AmyE$^+$/AroI$^+$
2	0	10^3		NT[b]
47	49	0	6/49	
50	0	10^3		3/120
55	145	0	5/18	

[a] *B. subtilis* 207-21 (*amyE07 aroI906* Tm^s) was used as the recipient cell. The number of Tm^r and AroI$^+$ transformants was given when each recombinant phage DNA prepared from 10 ml of phage lysate (10^7 PFU/ml) was used.
[b] NT, Not tested.

In the plaque hybridization and spot hybridization analyses, four positive clones were finally selected. Those positive phage clones were designated 2, 47, 50, and 55. When *B. subtilis* 207-21 (*amyE07*) was used as the recipient, phage 2 and 50 DNAs gave AroI$^+$ transformants, while phage 47 and 55 DNAs gave Tm^r transformants (Table 2). Next, *B. subtilis* NA64 was used as the recipient to test α-amylase productivity. Several hundred Tm^r transformants were obtained from clone 47 and 55 DNAs, and three Tm^r transformants of each were tested. All of them showed hyperproductivity of α-amylase. The result with the Tm^r transformants by phage 47 DNA is shown in Table 3. The phage DNAs of clones 47 and 55, therefore, had *tmrA7* character-transforming activity.

Restriction endonuclease analyses of cloned DNAs. Restriction maps of the inserted DNAs from phage clone 50 and 47 were determined (Fig. 2). Phage clone 50 carried the 15-kb *Eco*RI fragment which corresponded to the A fragment shown in Fig. 3a. The carboxy-terminal half of the *amyE* gene was located at the left terminus of the 15-kb *Eco*RI fragment, and *tmrB*$^+$-*aroI*$^+$ was located near the right terminus.

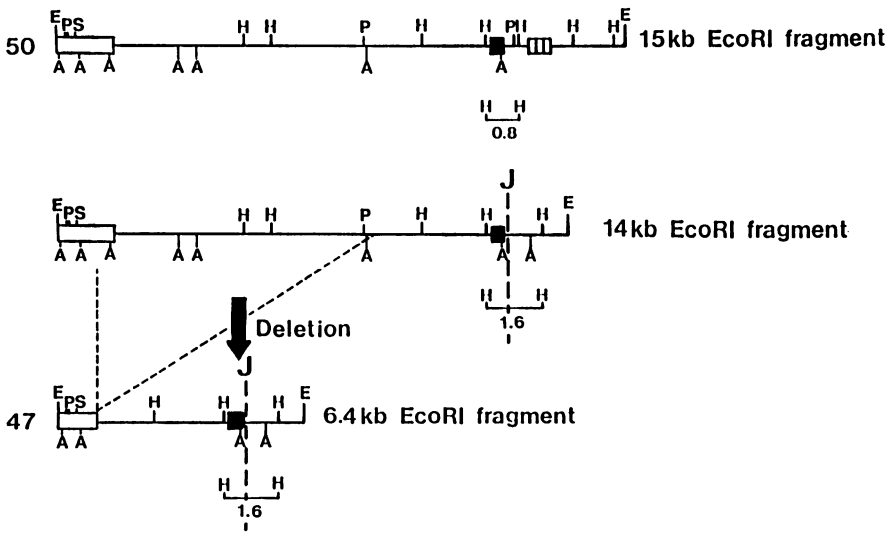

FIG. 2. Restriction maps of cloned DNAs. Restriction map of the 14-kb *Eco*RI fragment is a putative map assumed from those of the 6.4- and 15-kb *Eco*RI fragments. The 15- and 14-kb *Eco*RI fragments correspond to A and B fragments (Fig. 3a), respectively. Abbreviations: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; A, *Ava*I; S, *Sal*I. Symbols: □, carboxy-terminal half of the *amyE* gene; ■, the *tmrB*$^+$ gene; □, the *aroI* gene.

Phage clone 47 was found to carry a 6.4-kb *Eco*RI fragment derived from *B. subtilis*. Because phage 47 DNA could give AmyE$^+$ transformants when *B. subtilis* 207-21 was used as a recipient (Table 2), it is clear that at least the DNA sequence that complements *amyEO7* is present on the 6.4-kb DNA insert. Extensive analysis with several restriction endonucleases and comparison of the structure with that of the phage 50 insert revealed that the 6.4-kb *Eco*RI fragment was derived from the 14-kb *Eco*RI fragment (B fragment; Fig. 3a) through a spontaneous deletion of 7.6 kb of DNA. Even though the deletion occurred, the Tm^r transformants from clone 47 DNA showed α-amylase hyperproductivity (Table 3). It was proved that the 6.4-kb *Eco*RI fragment derived from the 14-kb *Eco*RI fragment in the amplified region had *tmrA7* character-transforming activity. In the 6.4-kb *Eco*RI fragment, the *tmrB*$^+$ gene was not present on a 0.8-kb *Hin*dIII fragment but was present on a 1.6-kb *Hin*dIII fragment. So the junction point of the repeating units should have been present on the 1.6-kb *Hin*dIII fragment.

Subcloning of the 1.6-kb *Hin*dIII fragment. Judging from the work of Trowsdale and Anagnostopoulos (20) and the review by Anderson and Roth (1), DNA fragments which have the junction point of the repeating units may serve as transforming DNAs that induce gene duplication (or the merodiploid state), because they facilitate unequal legitimate recombination between replicating sister chromatids. Therefore, we aimed to subclone the 1.6-kb *Hin*dIII fragment from the 6.4-kb *Eco*RI fragment to confirm the transforming activity of the *tmrA7* character, because the original event to gene amplification is gene duplication (1).

The 1.6-kb *Hin*dIII fragment prepared from the 6.4-kb *Eco*RI fragment was ligated to pGR71 which was cut at the unique *Hin*dIII site. It is known that the *tmrB*$^+$ gene at the multicopy state endows host cells with tunicamycin resistance (Harada et al., in preparation). The 1.6-kb *Hin*dIII fragment has the *tmrB*$^+$ gene, and pGR71 is a derivative of multicopy plasmid pUB110 (Km^r). The recombinant plasmid carrying the 1.6-kb *Hin*dIII fragment, therefore, is expected to give tunicamycin resistance to recipient *B. subtilis* cells by protoplast transformation.

Among 600 Km^r transformants, 13 Tm^r transformants were obtained. All the Tm^r transformants retained the recombinant plasmid carrying the 1.6-kb *Hin*dIII fragment. The structure of a representative recombinant plasmid pTM201, carrying the 1.6-kb *Hin*dIII fragment, is shown in Fig. 4.

***tmrA7* character-transforming activity of the cloned 1.6-kb *Hin*dIII fragment.** pTM201 DNA was tested by competence transformation for the *tmrA7* character-transforming activity after the linearization by cutting at the unique *Bgl*II site in the Km^r gene. In Table 3 the results of transformation are summarized. The linearized pTM201 DNA gave eight Tm^r transformants, all of which were kanamycin sensitive. They also showed α-amylase hyperproductivity to the same extent as that of the Tm^r transformants obtained from B7 chromosomal DNA and phage 47 DNA.

In another experiment, intact pTM201 DNA also gave *tmrA7* transformants, all of which were Km^s (data not shown), probably because the linearization occurred during transformation. Although linearized or intact pTM201 DNA gave Tm^r transformants, the 1.6-kb *Hin*dIII fragment itself did not exhibit Tm^r-transforming activity so far as we tested (data not shown). This fact suggests that the vector plasmid sequences connected to both ends of the 1.6-kb *Hin*dIII fragment would protect the essential *Hin*dIII fragment from nonspecific exonuclease attack.

Detection of induced gene amplification. Chromosomal

TABLE 3. The *tmrA7* character (Tm^r Amy^h) transforming activity

DNA source	No. of Tm^r transformants[a]/ μg of DNA	α-Amylase activity (U/ml)[b]
Strain B7 chromosomal	6×10^3	282
Phage 47	5×10^2	205
Linearized pTM201[c]	8	264
None		22[d]

[a] *B. subtilis* NA64 (Amy$^+$ Tm^s) was used as the recipient cell.

[b] α-Amylase activity was measured after 15 h of culture. In this condition, α-amylase activity of strain B7 (*tmrA7*) was 232 U/ml. Values are the average of three Tm^r transformants.

[c] pTM201 carrying the 1.6-kb *Hin*dIII fragment was linearized by *Bgl*II digestion.

[d] α-Amylase activity of strain NA64.

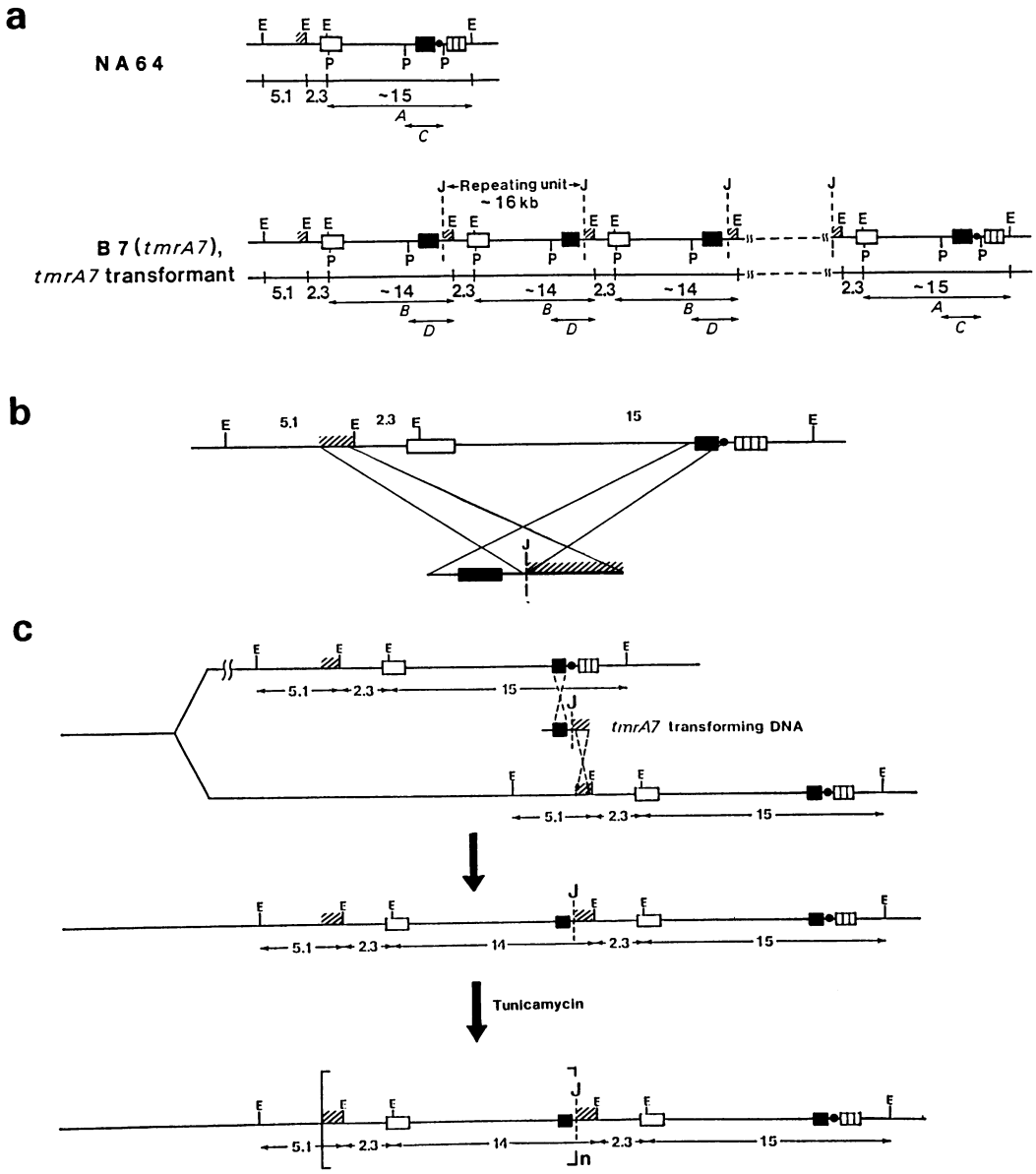

FIG. 3. (a) Chromosomal structure of *amyE-tmrB* and flanking regions in wild-type strain NA64, and gene-amplified strain B7 (*tmrA7*) and the *tmrA7* transformant. (b) Essential structures of the *tmrA7* character-transforming (gene amplification-inducing) DNA. (c) The proposed mechanism of transformation to gene duplication (or the merodiploid state) and to gene amplification induced by *tmrA7* character-transforming DNA. Abbreviations: E, *Eco*RI; P, *Pst*I. Symbols: □, the *amyE* gene; ■, the *tmrB* gene; ▨, the *aroI* gene; ▨, the M region; ●, the point on the 15-kb *Eco*RI fragment corresponding to J of the 14-kb *Eco*RI fragment. The A, B, C, and D fragments are described in the text.

DNAs were prepared from the *tmrA7* transformants (Table 3), digested with *Eco*RI and *Eco*RI-*Pst*I, and electrophoresed (Fig. 5a). All *tmrA7* transformants had the prominent bands corresponding to 14 and 2.3 kb (*Eco*RI cut) and 8.0, 5.6, and 2.3 kb (*Eco*RI-*Pst*I cut), as did the DNA from strain B7; they were absent from the DNA of wild-type strain NA64. Also, Southern hybridization analysis with the nick-translated *tmrB* probe (0.6-kb *Hin*dIII-*Bcl*I fragment; see Fig. 7) showed the gene amplification (Fig. 4b). The 15-, 14-, 3.9-, and 5.6-kb bands (Fig. 4b corresponded to the A, B, C, and D fragments, respectively (Fig. 3a). Only in the lanes of *tmrA7* transformants and B7 were detected 14- and 5.6-kb-thick bands. The above shows that the 1.6-kb *Hin*dIII fragment is essential for inducing gene amplification by competence transformation.

The copy number of the repeating units was estimated by densitometer scanning of the autoradiograph (Fig. 5b) and calculation of the ratio of the density of the 5.6-kb band (D fragment; Fig. 3a) to the 3.9-kb band (C fragment; Fig. 3a). The derived copy number of all of the Tm^r transformants (Fig. 5b, lanes 8 through 11) and B7 (Fig. 5b, lane 12) was about 10 (data not shown). This copy number is reasonable, keeping in mind that the α-amylase activities of those strains are about 10 times as much as that of strain NA64 (Table 3).

Southern hybridization analysis with the cloned 1.6-kb *Hin*dIII fragment as probe. In a previous report (7), we

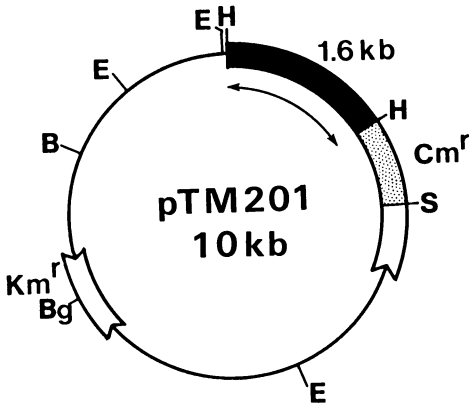

FIG. 4. Structure of pTM201. pTM201 is a plasmid that carries the 1.6-kb *Hin*dIII fragment derived from *B. subtilis* B7 on pGR71. Abbreviations: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; S, *Sal*I; Bg, *Bgl*II

proposed the structure of the repeating unit of the amplified region (Fig. 3a). If this structure is correct, *tmrB*$^+$ must be linked to the M region. We defined the M region as the region that was originally located on a 5.1-kb *Eco*RI fragment of the wild-type strain NA64 chromosome and on the left terminus

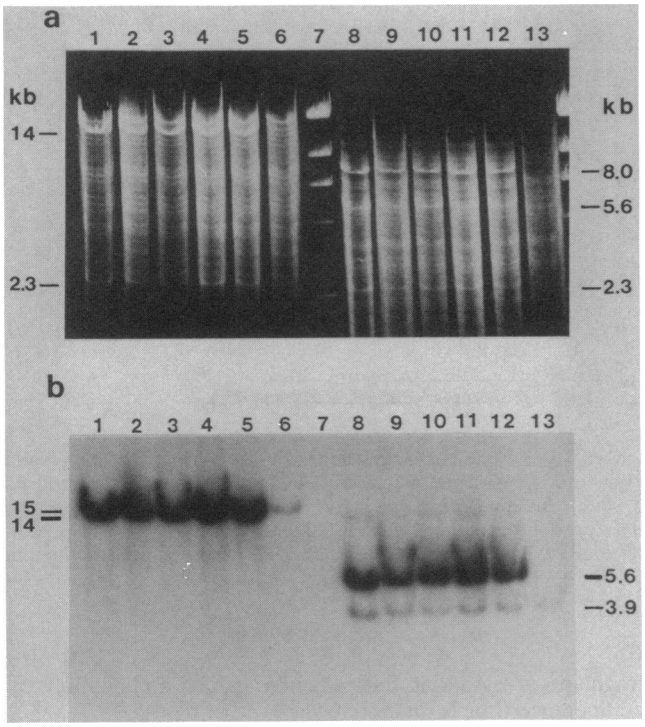

FIG. 5. Agarose gel electrophoresis (a) and Southern hybridization analysis (b) with the nick-translated *tmrB* probe (0.6-kb *Hin*dIII-*Bcl*I fragment; see Fig. 7) of *Eco*RI- and *Eco*RI-*Pst*I-digestedchromosomal DNAs. Chromosomal DNAs were prepared from *tmrA7* transformants which were transformed by strain B7 chromosomal DNA (lanes 1 and 8), phage 47 DNA (lanes 2 and 9), and linearlized pTM201 DNA (lanes 3, 4, 10, and 11). Chromosomal DNAs were preapred from strains B7 (lanes 5 and 12) and NA64 (lanes 6 and 13). Lanes 1 through 6, digested with *Eco*RI; lanes 8 through 13, double digested with *Eco*RI and *Pst*I; lane 7, λ *Hin*dIII marker. The 15-, 14-, 3.9-, and 5.6-kb bands correspond to the A, B, C, and D fragments (Fig. 3a), respectively.

of the repeating unit in this amplified model. The cloned 1.6-kb *Hin*dIII fragment should have carried almost exclusively the *tmrB*$^+$-J-M region, in which J is the junction point of the repeating units. The cloned 1.6-kb *Hin*dIII fragment, therefore, was expected to hybridize with the 5.1-kb *Eco*RI fragment on which the M region was originally located and also with 15- and 14-kb *Eco*RI fragments which corresponded to *tmrB*$^+$ carrying A and B fragments (Fig. 3a), respectively.

As expected, the cloned 1.6-kb *Hin*dIII fragment hybridized with 5.1-, 14-, and 15-kb *Eco*RI fragments (Fig. 6). It was confirmed that the 1.6-kb *Hin*dIII fragment really carried the M region which was derived from the 5.1-kb *Eco*RI fragment. This structure of the 1.6-kb *Hin*dIII fragment shows that the repeating unit is repeated tandemly in the amplified region and that the proposed model (Fig. 3a) is correct.

Determination of the position of J in the cloned 1.6-kb *Hin*dIII fragment. As mentioned above, the 0.8-kb *Hin*dIII fragment (*tmrB*$^+$) was already cloned on plasmid pGR71, and the entire DNA sequence of it was determined (Harada et al., in preparation). Restriction maps of the cloned 0.8-kb *Hin*dIII fragment and the 1.6-kb *Hin*dIII fragment cloned in this study are shown in Fig. 7. The restriction sites on the left half of the 1.6-kb *Hin*dIII fragment were exactly the same as those on 0.8-kb *Hin*dIII fragment (Fig. 7). The restriction sites on the right half of the *Bcl*I site, however, were different from each other. J was expected to exist in a 0.2-kb *Bcl*I-*Eco*RV fragment of the 1.6-kb *Hin*dIII fragment.

By the dideoxy chain-termination method, we determined the DNA sequence of the 0.2-kb fragment and compared it with the known corresponding sequence of 0.8-kb *Hin*dIII (*tmrB*$^+$) fragment. The DNA sequences from the *Bcl*I site to the base 26 were completely the same, but the sequences further on were completely different from each other (Fig. 8). It was confirmed that J exists between bases 26 and 27 from the *Bcl*I site.

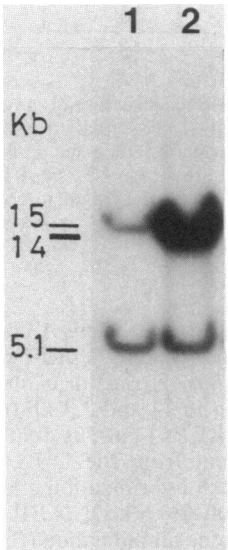

FIG. 6. Southern hybridization analysis of *Eco*RI-digested chromosomal DNAs, with the nick-translated 1.6-kb *Hin*dIII fragment from pTM201 used as a probe. Lane 1, strain NA64 (wild type); lane 2, strain B7 (*tmrA7*). About 10 μg of DNA was charged.

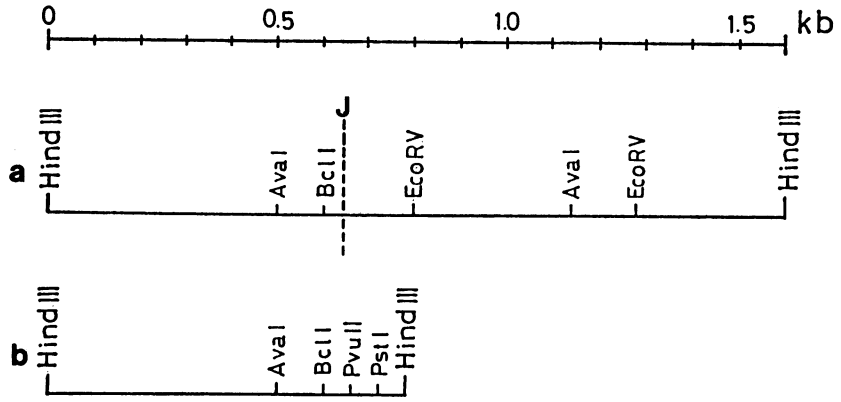

FIG. 7. Comparison of restriction sites. (a) Restriction sites of the 1.6-kb *Hin*dIII fragment. (b) Restriction sites of the 0.8-kb *Hin*dIII fragment.

DISCUSSION

It long has been an enigma why the *tmrA7* mutation endows *B. subtilis* cells with both α-amylase hyperproductivity and tunicamycin resistance. The mutational site of the *tmrA7* mutation was mapped previously just upstream of *amyR* (13). The *amyR* locus is currently identified as the promoter region of *amyE* (23). Recently, however, gene amplification was found to be the mechanism of expressing pleiotropy (7). The *tmrA7* mutation can be said to be the state of carrying the tandemly repeated structure of the *amyE-tmrB* region (7). Furthermore, Furusato et al. found that gene amplification is dependent on *recE* function (4a).

In this study we aimed to characterize the structure of *tmrA7* character-transforming DNA by cloning. At first, a 6.4-kb *Eco*RI fragment was cloned on λ Charon 4A phage vector (Fig. 2). The 6.4-kb fragment gave the transformants with the phenotype of both Amy^h and Tm^r (Table 3). We confirmed directly the gene amplification of the *amyE-tmrB* region in the transformants by Southern hybridization analysis (Fig. 4b, lanes 2 and 9). From the 6.4-kb *Eco*RI fragment we subcloned the *Hin*dIII fragment as short as 1.6 kb (Fig. 2 and 4), which was almost exclusively composed of the $tmrB^+$-J-M region (Fig. 3b). It was found that the *Hin*dIII fragment still retained the *tmrA7* character-transforming (Tm^r Amy^h) activity and induced gene amplification (Table 3 and Fig. 4).

The 1.6-kb *Hin*dIII fragment can be said to be the essential sequence for the transforming (gene amplification-inducing) activity (Fig. 3b). The sequence might serve as a helper DNA in competence transformation to facilitate the unequal legitimate recombination between replicating sister chromatids (Fig. 3c). The resultant *amyE-tmrB* merodiploid (or gene-duplicated) transformants might repeat the unequal legitimate recombination. Those descendants who could amplify the *amyE-tmrB* region might survive under the selective pressure of tunicamycin (Fig. 3c). An independent experiment revealed that the $tmrB^+$ gene only in the multicopy state gave *B. subtilis* cells tunicamycin resistance (Harada et al., in preparation). Theoretically, the transforming (or helper) DNA could be shorter than the 1.6-kb *Hin*dIII fragment because the minimum and essential structure is the J and neighboring sequence. For effective homologous recombination, however, a longer neighboring sequence is needed, because the DNA of phage clone 47 (containing the 6.4-kb *Eco*RI fragment) gave much higher transforming activity than the linearized pTM201 (containing the 1.6-kb *Hin*dIII fragment) even when both inserts were connected to vector DNA fragments long enough to protect them from nonspecific exonuclease attack (Table 3).

In the strain B7 mutant (*tmrA7*) there were about 5 to 10 copies of the repeating unit. Therefore, it produces about 5- to 10-fold larger amounts of α-amylase than the wild-type NA64 strain. The number of repeating units in strain B7 corresponded to the number of junction points on the chromosome, and each J is located just upstream of *amyE* (Fig. 3a). This arrangement explains the previous result about the mapping of the *tmrA7* mutational site (13). Considering our present finding that the minimum and essential structure of *tmrA7* character-transforming DNA is J and the neighboring sequence, the previously mapped mutational site corresponds to J. The *tmrA* gene is not present on the chromosome of *B. subtilis*. J and the neighboring sequence so far have behaved like the *tmrA7* gene. Strain B7 might

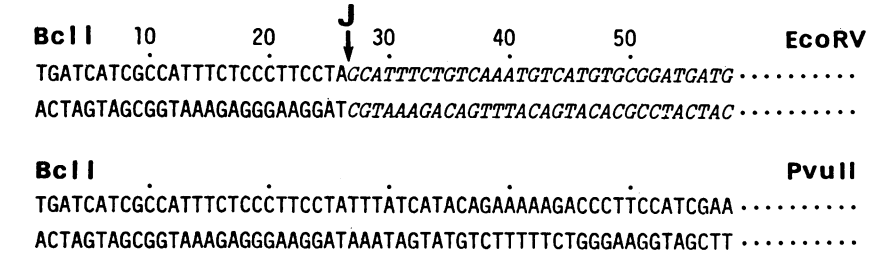

FIG. 8. Comparison of DNA sequences. (a) DNA sequence from the 1.6-kb *Hin*dIII fragment from the *Bcl*I site toward the *Eco*RV site. (b) DNA sequence from the 0.8-kb *Hin*dIII fragment from the *Bcl*I site toward the *Pvu*II site.

have originally mutated to give unequal illegitimate recombination at J. The pre-B7 mutant which obtained the merodiploid state in the region of *amyE-tmrB* (7) might follow the same path to gene amplification as in the case of *tmrA7* transformants. The *tmrA* locus in the genetic map of *B. subtilis* should be omitted in the next version so that it does not correspond to a gene.

In this study we determined exactly the position of J in the cloned 1.6-kb *Hin*dIII fragment. Recently we also determined the DNA sequence of the M region by cloning the 5.1-kb *Eco*RI fragment (Fig. 3a) from strain B7 (K. Hashiguchi, A. Tanimoto, S. Nomura, K. Yamane, T. Furusato, K. Yoda, S. Harada, M. Mori, A. Takatsuki, M. Yamasaki, and G. Tamura, Mol. Gen. Genet., in press). There was no sequence homology between the position at the left end of the M region and that at the right end of the $tmrB^+$ gene. This is the basis of our conclusion that unequal illegitimate recombination might occur as the first mutational event in parental strain NA64.

The characterization of the essential structure of *tmrA7* character-transforming DNA provides the generalization for induction of gene amplification at any chromosomal region. On the chromosome, imagine a gene array of -A-B-C-D-E-F-G- and imagine that genes A and G have been cloned. In vitro ligation of G to A, keeping the original direction of the genes, produces the sequence G-J-A, in which J is the junction point. If there is one gene in the A to G region which contributes to the survival at a multicopy state under a selective pressure, we may be able to amplify the region from A to G by competence transformation of recipient cells with the G-J-A DNA fragment under selective pressure by the same mechanism shown in Fig. 3c. The result of this type of an experiment will be published elsewhere.

Recently, Niaudet et al. (12) proposed a similar method to induce gene duplication, although the mechanism was explained differently.

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