Designed Gene Amplification on the *Bacillus subtilis* Chromosome

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We previously reported the cloning of a 1.6 kb *Hind*III fragment (containing the junction of the repeating unit) from chromosomal DNA of *Bacillus subtilis* strain B7 in which tandem amplification of a 16 kb region occurred, and the induction of B7-type gene amplification by competence transformation with this cloned fragment. Based on this result, we designed, on the *B. subtilis* chromosome, a gene amplification of the 22 kb repeating unit containing the α -amylase structural gene (*amyE*), the tunicamycin-resistance gene (*tmrB*) and the shikimate kinase structural gene (*aroI*). We cloned only two short DNA fragments from both termini of the 22 kb region, constructed a junction structure of the designed repeating unit on pBR327 and transformed a *B. subtilis* wild-type strain by this constructed plasmid. As a result, we succeeded in obtaining tunicamycin-resistant (Tm^r) transformants in which the designed gene amplification of 22 kb occurred on the chromosome. The Tm^r transformants showed high productivity of α -amylase and shikimate kinase. The copy number of the repeating unit was estimated to be 10–20. This system may provide an effective means of amplifying long (> 20 kb) DNA regions on the chromosome.

INTRODUCTION

Recently, many examples of chromosomal gene amplification have been discovered in prokaryotes. Amplifications of inherent chromosomal regions have been discovered in *Escherichia coli* (Edlund & Normark, 1981; Tlsty *et al.*, 1984), *Streptomyces glaucescens* (Ono *et al.*, 1982), *Streptomyces fradiae* (Fishman & Hersherberger, 1983), *Streptomyces lividans* (Altenbuchner & Cullum, 1984), *Bacillus subtilis* (Hashiguchi *et al.*, 1986; Wilson & Morgan, 1985) and *Pseudomonas aeruginosa* (Deretic *et al.*, 1986). They have usually been found in drug-resistant mutants. Other gene amplifications have been induced artificially by integrating plasmid DNAs which were then amplified on the chromosome, in *Streptococcus pneumoniae* (Vasseghi & Claverys, 1983), *Salmonella typhimurium* (Gutterson & Koshland, 1983) and *B. subtilis* (Young, 1984; Albertini & Galizzi, 1985; Jannire *et al.*, 1985; Sargent & Bennett, 1985). The repeating unit of this latter type of gene amplification is limited to less than about 10 kb. In this report, we will describe another type of artificial gene amplification suitable for amplifying long (> 20 kb) DNA regions.

We reported previously that *B. subtilis* strain B7 (a *tmrA*7 mutant), which acquired tandem gene amplification of the *amyE*–*tmrB* region by nitrosoguanidine treatment, showed hyperproductivity (about a 5- to 10-fold increase) of α -amylase and tunicamycin resistance (Hashiguchi *et al.*, 1986; Sasaki *et al.*, 1976). The length of the repeating unit was about 16 kb and the copy number was about 10. Amplification of the *amyE*⁺ gene caused hyperproductivity of α -amylase (Amy^h) and that of the *tmrB*⁺ gene caused tunicamycin resistance (Tm^r) (Hashiguchi *et al.*, 1986). The *tmrB*⁺ gene endows host cells with tunicamycin resistance only in

Abbreviations: DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; SSC, saline sodium citrate (0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0).

the multi-copy state (S. Harada, K. Yoda, A. Tanimoto, M. Mori, M. Yamasaki & G. Tamura, unpublished results). The *tmrA7* mutational character was transferred to recipient cells by competence transformation (Nomura *et al.*, 1978; Sasaki *et al.*, 1976). We succeeded in cloning the essential 1.6 kb *Hind*III fragment from the *B. subtilis* B7 chromosome, which induced the same gene amplification as in the B7 strain by competence transformation under the selective pressure of 10 µg tunicamycin ml⁻¹. This fragment was derived from the junction region of the repeating unit (Mori *et al.*, 1986). Based on the junction structure (or 'cross structure') described in the previous report (Mori *et al.*, 1986), we constructed a new junction structure by *in vitro* ligation of two DNA fragments and challenged to induce a 'designed' gene amplification.

METHODS

Bacterial strains and plasmids. *B. subtilis* strains used in this study are listed in Table 1. *B. subtilis* B7 (Sasaki *et al.*, 1976) was one of the tunicamycin-resistant (Tm^r) mutants isolated from *B. subtilis* NA64. pAPT17 is a plasmid carrying the 1.2 kb *Hind*III fragment (see Fig. 1) on pBR327 and constructed in our laboratory. Other plasmids are described under 'Preparation of biotin-labelled DNA probes'.

Restriction enzymes and chemicals. Restriction enzyme *Bcl*I was purchased from Boehringer, and the other restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo Co. They were used according to the manufacturers' recommendations. Tunicamycin (lot no. 1) was prepared in this laboratory by a previously described method (Takatsuki *et al.*, 1970). [α -³²P]dCTP was purchased from Amersham, shikimic acid from Nakarai Chemicals, and [G-¹⁴C]shikimic acid from New England Nuclear.

Preparation of chromosomal DNA. Chromosomal DNAs were prepared as previously described (Mori *et al.*, 1986).

Transformation. Competence transformation was done by the method of Hass & Yoshikawa (1969). Tm^r transformants were selected on nutrient agar (Difco Bacto-Agar) plates containing 10 µg tunicamycin ml⁻¹. Amy^h transformants were detected on nutrient agar plates containing 1% (w/v) soluble starch by staining with iodine.

Preparation of biotin-labelled DNA probes. Plasmid DNAs and DNA fragments were nick-translated by the BRL nick-translation system using biotin-11-dUTP (BRL), according to the manufacturer's recommendations. DNA probes A, B, C and D (see Fig. 2*b* and Fig. 3) were prepared as follows. Plasmid pKH11 (Hashiguchi *et al.*, 1986), carrying the 5.1 kb *Eco*RI fragment on pBR327 (see Fig. 1), was doubly digested with *Eco*RI and *Hind*III, and the 0.6 kb *Hind*III-*Eco*RI fragment was prepared from a 5% (w/v) polyacrylamide gel as described by Maniatis *et al.* (1982). About 0.1 µg of the 0.6 kb *Hind*III-*Eco*RI fragment was used for probe A. About 1 µg of plasmid pTUB4 (Yamazaki *et al.*, 1983), carrying the 2.3 kb *amyE* fragment on pUB110, was used for probe B. Vector pUB110 DNA did not hybridize with *B. subtilis* chromosomal DNA under the conditions used in Fig. 2(*b*) (data not shown). Plasmid pTM101 (S. Harada and co-workers, unpublished), carrying the 0.8 kb *Hind*III fragment containing the *tmrB* gene in pGR71 (Goldfarb *et al.*, 1981) and constructed in our laboratory, was doubly digested with *Hind*III and *Bcl*I, and the 0.6 kb *Hind*III-*Bcl*I fragment (Mori *et al.*, 1986) was prepared from a 5% polyacrylamide gel by the same method as was used for probe A. About 0.1 µg of the 0.6 kb *Hind*III-*Bcl*I fragment was used for probe C. About 1 µg of plasmid pAPT18, carrying the 1.6 kb *Hind*III fragment containing the *aroI* gene on pBR327 and constructed in our laboratory, was used for probe D. Vector pBR327 DNA did not hybridize with *B. subtilis* chromosomal DNA under the conditions used in Fig. 2(*b*) (data not shown).

Southern hybridization. About 4 µg of chromosomal DNA was completely digested with restriction endonucleases, subjected to electrophoresis in 0.8% vertical agarose gels and transferred to a nitrocellulose filter by the method of Southern (1975). Four filters were blotted in the same way. Blotted filters were prehybridized for 2 h at 65 °C in a solution containing 6 × SSC, 1 × Denhardt's solution and 50 µg denatured salmon sperm DNA ml⁻¹. The hybridization buffer was prepared as above plus 0.5% SDS and denatured biotin-labelled DNA probe. Blotted filters were hybridized overnight at 65 °C, then washed twice with 2 × SSC/0.1% SDS for 3 min and then twice with 0.2 × SSC/0.1% SDS for 3 min at room temperature. Then, biotinylated DNAs on the nitrocellulose filters were detected using the BRL DNA detection system, according to the manufacturer's recommendations.

Determination of the copy number of the amplified strains. Chromosomal DNA (1 µg, 0.5 µg and 0.25 µg) of each strain was completely digested with restriction endonuclease and was blotted on a nitrocellulose filter as described above. Southern hybridization analysis was done as above but using α -³²P-labelled DNA probes. After autoradiography, two hybridized bands that existed in the chromosome in only one copy and in multiple copies, respectively, were excised from the nitrocellulose filter and their radioactivity measured by liquid scintillation counting. The ratio of the counts of the two bands was assumed to correspond to the copy number of the repeating unit. The values listed in Table 3 are the means of three ratios obtained from the experiments using 1 µg, 0.5 µg and 0.25 µg of each chromosomal DNA.

α -Amylase assay. Strains were pregrown on nutrient agar plates and precultured in 2 ml nutrient broth overnight. The next day, they were seeded into 10 ml nutrient broth at an OD₅₅₀ of 0.05, and incubated at 37 °C for

Table 1. *B. subtilis* strains

Strain*	Genotype	Source
NA64	<i>metB5 purB6</i>	B. Maruo
B7	<i>metB5 purB6 tmrA7</i>	This laboratory
APT139	<i>metB5 leuA8 lys-21 hsrM hsmM</i>	This laboratory
NMM13	<i>metB5 purB6 (amyE⁺-tmrB⁺-aroI⁺)_n†</i>	This study
NMM14	<i>metB5 purB6 (amyE⁺-tmrB⁺-aroI⁺)_n†</i>	This study

* All these strains have the *amyE*⁺ gene which has the upstream *amyR2* region that confers hyperproduction of α -amylase (Yamazaki *et al.*, 1983).

† The markers in parentheses are tandemly amplified. Using this term, *tmrA7* can be expressed otherwise as (*amyE*⁺-*tmrB*⁺)_n.

15 h. For strains B7, NMM13 and NMM14, tunicamycin (10 μ g ml⁻¹) was added to the medium at all times. After removal of the cells by centrifugation, α -amylase activity in the broth was measured by the method of Fuwa (1954).

Shikimate kinase assay. Strains were precultured in nutrient broth as for the α -amylase assay. The cells were harvested by centrifugation and washed once with fresh culture medium [containing 50 μ g adenine ml⁻¹ and 50 μ g ml⁻¹ each of 17 amino acids excluding three aromatic amino acids (phenylalanine, tyrosine and tryptophan) in Spizizen's minimum medium (Anagnostopoulos & Spizizen, 1961)]. The bacteria were then seeded into 50 ml of the culture medium at an OD₅₅₀ of 0.05, and incubated at 37 °C. For strains B7, NMM13 and NMM14, tunicamycin (10 μ g ml⁻¹) was added to the medium at all times. Mid-exponential-phase cells (OD₅₅₀ = 0.5) were harvested by centrifugation, washed once with 5 ml 50 mM-Tris/HCl, 1 mM-MgCl₂ buffer (pH 7.5), and stored as a wet paste at -20 °C until used.

Cell-free extracts were prepared by treatment for 30 min with lysozyme (100 μ g ml⁻¹; Seikagaku Kogyo Co.) and DNAase (20 μ g ml⁻¹; Sigma) in 50 mM-Tris/HCl, 1 mM-MgCl₂ buffer (pH 7.5).

Shikimate kinase was assayed by the procedure of Nakatsukasa & Nester (1972) with slight modification. The reaction mixture consisted of (final concn): Tris/HCl (pH 7.5), 50 mM; ATP, 1 mM; MgCl₂, 5 mM; KF, 5 mM; unlabelled shikimic acid, 0.09 mM; [¹⁴C]shikimic acid, 0.01 mM (0.2 μ Ci; 7.4 kBq); and DTT, 1 mM. Diluted cell-free extract (50 μ l) was added and the 0.5 ml reaction mixture was incubated for 15 min at 37 °C. Protein was removed by centrifugation at room temperature after precipitation with 50 μ l 50% (w/v) TCA. Then 0.4 ml of the supernatant was pipetted into 0.5 ml saturated Ba(OH)₂ and a drop of saturated (NH₄)₂SO₄ was added as a coprecipitant. The precipitate of barium shikimate-5-phosphate was collected on a glass filter (Whatman GF/C), dried, suspended in 10 ml scintillant and its radioactivity measured in a liquid scintillation counter.

Shikimate kinase must associate with the bifunctional complex of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthetase and chorismate mutase in order to function to any significant degree (Nakatsukasa & Nester, 1972). Under the conditions used, the assay was linear with respect to bacterial protein concentration measured by the Lowry method, suggesting that the shikimate kinase complex was not dissociated.

RESULTS

Construction of pKH81

The restriction map of the *amyE*-*tmrB* region of *B. subtilis* wild-type strain NA64 is shown in Fig. 1. We wished to amplify the 22 kb region, from the left end of the 5.1 kb *Eco*RI fragment to the right end of the 0.8 kb *Hind*III-*Cla*I fragment. If the designed amplification occurs, the resultant strain will show both the Amy^h and the Tm^r phenotype by the gene-dosage effect of *amyE* and *tmrB* as in *B. subtilis* B7.

First we constructed pKH81; the construction strategy is shown in Fig. 1. In pKH81, the 0.8 kb *Hind*III-*Cla*I fragment and the 5.1 kb *Eco*RI fragment kept the original direction as shown in Fig. 1. In this plasmid, a 22 bp sequence derived from pBR327 was present between the *Cla*I and the *Eco*RI sites at the junction.

Transformation of *B. subtilis* with pKH81

We transformed *B. subtilis* NA64 by competence transformation with 10 μ g linearized pKH81 DNA cut at the unique *Pst*I site in the vector DNA region. Tm^r transformants were selected on nutrient agar containing 10 μ g tunicamycin ml⁻¹. Ten Tm^r transformants were obtained, four of which (designated NMM13-NMM16) showed α -amylase hyperproductivity (Amy^h) when replicated on nutrient agar containing 1% starch. To increase the transformation

Fig. 1. Construction of pKH81. Above, the chromosomal structure of *amyE-tmrB* and flanking regions in wild-type strain NA64 (Hashiguchi *et al.*, 1986). Numbers refer to size in kb. The open box represents the *amyE* (α -amylase structural) gene, the filled box represents the *tmrB* (tunicamycin-resistance) gene and the striped box represents the *aroI* (shikimate kinase structural) gene. The long stippled box at the top of the figure shows the 22 kb repeating unit designed in this study. Below, details of plasmid construction. The 0.8 kb *HindIII-ClaI* fragment and the 5.1 kb *EcoRI* fragment were connected on pBR327, keeping the direction shown by thick arrows. Numbers refer to size in kb. Thin lines in the plasmid diagrams denote pBR327-derived regions. The short thin line between the 0.8 kb *HindIII-ClaI* fragment and the 5.1 kb *EcoRI* fragment is also derived from pBR327 and is 22 bp long. E, *EcoRI*; H, *HindIII*; C, *ClaI*; P, *PstI*; A, *AvaI*.

efficiency, we transformed strain APT139 (see Table 1) with 5 μ g of intact pKH81 in the presence of 40 mM-MgCl₂, using the modified transformation method of Tanaka & Sakaguchi (1978) and selected on plates containing 5 μ g or 10 μ g tunicamycin ml⁻¹. As a result, 68 and 15 Tm^r transformants were obtained, respectively, and 65 and 10 of them showed α -amylase hyperproductivity. Restriction patterns of the latter 10 chromosomal DNAs were analysed; they were identical to those of NMM13 and NMM14 (data not shown). As a control experiment, it was shown that no Tm^r transformants were obtained from competent cells of strains NA64 and APT139 without DNA treatment (data not shown).

Detection of the designed gene amplification

Strains NMM13 and NMM14, selected as representatives, were cultured in the presence of 10 μ g tunicamycin ml⁻¹, and chromosomal DNA was prepared from them and digested with restriction enzymes. As expected, several prominent bands were detected after electrophoresis (Fig. 2a): 14.4, 5.1 and 2.3 kb bands after *EcoRI* digestion (lanes 3 and 4); 8.0, 5.1, 3.9 and 2.3 kb

Table 2. Mapping the amplified DNA of strains NMM13 and NMM14 detected by Southern hybridization after agarose gel electrophoresis

Prominent bands detected in Fig. 2(a) (lanes corresponding to DNA from strains NMM13 and NMM14) and the probes that hybridized to these bands are shown on the basis of Fig. 2(b). Probes A–D correspond to those shown in Fig. 3.

<i>Eco</i> RI-cut		<i>Eco</i> RI + <i>Pst</i> I-cut		<i>Pst</i> I-cut	
Prominent band (kb)	Hybridized probe	Prominent band (kb)	Hybridized probe	Prominent band (kb)	Hybridized probe
5.1	A	8.0	B	9.9	A, B, D
2.3	B	5.1	A	8.0	B
14.4	B, C, D	3.9	C	3.9	C
		2.3	B		
		2.3	D		

Table 3. α -Amylase productivities, shikimate kinase activities and copy numbers of the repeating unit of control and amplified bacteria

α -Amylase activities in the broth after 15 h culture were measured by the method of Fuwa (1954) as described in Methods. The assay was repeated three times; representative values are shown. Shikimate kinase activities were measured with mid-exponential-phase cells and repeated twice; mean values are shown. Shikimate kinase activity is shown as the amount of [14 C]shikimate-5-phosphate produced per μ g bacterial protein during a 15 min reaction; details of the assay are given in Methods. For the estimation of the copy number (see text for details), the same chromosomal DNA samples as those shown in Fig. 2 were used, prepared from mid-exponential-phase cells grown at 37 °C. Strains B7, NMM13 and NMM14 were precultured and cultured in medium containing 10 μ g tunicamycin ml $^{-1}$ at all times.

Strain	Final OD ₅₅₀	Doubling time (min)	α -Amylase productivity			Shikimate kinase activity		Copy no.
			(U ml $^{-1}$)	(U ml $^{-1}$ per OD ₅₅₀ unit)	Ratio	[c.p.m. (μ g protein) $^{-1}$]	Ratio	
NA64	4.5	50	22.5	5.0	1.0	232	1.0	1
B7	4.3	50	183	42.6	8.5	240	1.0	10
NMM13	1.2	100	108	90.0	18.0	954	4.1	17
NMM14	1.3	100	145	111.5	22.3	968	4.2	12

(doublet) bands after *Eco*RI + *Pst*I digestion (lanes 7 and 8); 9.9, 8.0 and 3.9 kb bands after *Pst*I digestion (lanes 11 and 12). Summing the sizes of the prominent bands of each lane, an amplified unit of 22 kb was detected. No prominent (amplified) bands were detected in DNA from the wild-type strain NA64 (lanes 2, 6 and 10); several prominent bands were detected in DNA from strain B7 as described previously (Hashiguchi *et al.*, 1986): 13.7 and 2.3 kb bands (lane 1); 8.0, 5.6 and 2.3 kb bands (lane 5); 8.0 kb (doublet) band (lane 9).

To confirm that the prominent bands in DNA from strains NMM13 and NMM14 detected in electrophoresis were derived from the designed 22 kb region, Southern hybridization analyses were performed using fragments A, B, C and D (see Fig. 3) as probes (Fig. 2b). The restriction map of *amyE*–*tmrB* and flanking regions in strain NA64 and that of the repeating unit detected in strain B7 have been already determined as shown in Fig. 3(a, b) (Hashiguchi *et al.*, 1986). All of the bands detected in lanes of strains NA64 and B7 in Fig. 2(b) can be identified from the maps. As for strains NMM13 and NMM14, the relationships between the prominent bands detected in electrophoresis and the probes hybridized to those bands are listed in Table 2. Based on Table 2, we constructed a restriction map of the repeating unit detected in strains NMM13 and NMM14 (Fig. 3c). This map showed that the detected repeating unit exactly corresponded to the designed repeating unit shown in Fig. 1. The weak bands detected in Fig. 2(b), 12.0 kb (lanes 11 and 12, probes A and B), 6.8 kb (lanes 11 and 12, probe D) and 3.0 kb (lanes 7 and 8, probe D), also exist in DNA from the parental strain NA64. These fragments are derived from

Fig. 2(a). Agarose gel electrophoresis patterns of chromosomal DNA of *B. subtilis* strains completely digested with *EcoRI* (lanes 1–4), *EcoRI* + *PstI* (lanes 5–8) and *PstI* (lanes 9–12). Lanes 1, 5 and 9, strain B7 (*tmrA7* mutant); lanes 2, 6 and 10, strain NA64 (wild-type); lanes 3, 7 and 11, strain NMM13 (see text); lanes 4, 8 and 12, strain NMM14 (see text) Fig. 2(b) appears on the facing page.

the flanking regions located adjacent to the amplified region and exist as only one copy in the genome.

Another restriction analysis using *ClaI* showed that the *ClaI* site was located at the junction of each repeating unit (data not shown).

Estimation of the number of copies of the amplified segment in strains NMM13 and NMM14

We measured the copy numbers of the repeating units in the presence of 10 μg tunicamycin ml^{-1} . Because strains NMM13 and NMM14 have one copy of the *aroI*-containing 6.8 kb *PstI* fragment and multiple copies of the *aroI*-containing 9.9 kb *PstI* fragment (see Fig. 2b-D, and Fig. 3a, c), the copy number of the repeating unit of 22 kb corresponds to the ratio of the number of molecules of the 9.9 kb *PstI* fragment to that of the 6.8 kb *PstI* fragment. We performed Southern hybridization analysis as in Fig. 2(b) but using α - ^{32}P -labelled DNA probe D (specific activity 2×10^7 c.p.m. μg^{-1}). The ratio of the ^{32}P radioactivity of the 9.9 kb band to that of the 6.8 kb band (see Fig. 2b-D, lanes 11 and 12), measured by liquid scintillation counting, was calculated. The copy number of the designed repeating unit of 22 kb calculated in this way was 17 in strain NMM13 and 12 in strain NMM14. Similarly, the copy number of the repeating unit of 16 kb in the mutant strain B7 was estimated to be 10, calculated by the ratio of the ^{32}P radioactivity of the 13.7 kb band to that of the 5.1 kb band (see Fig. 2b-A, lane 1) using α - ^{32}P -labelled probe A (specific activity 3×10^7 c.p.m. μg^{-1}).

α -Amylase productivity

We measured the α -amylase productivities of the strains containing amplified DNA (Table 3). Strains NMM13 and NMM14, in which designed amplification occurred, showed hyperproductivity of α -amylase but their growth rates at the exponential phase were only about half those of strains NA64 (wild-type) and B7 (*tmrA7* mutant). The poor growth of strains NMM13 and NMM14 may be caused by keeping too long an amplified region on the chromosome. Alternatively, a gene which has a growth-inhibitory effect in the multi-copy state might be located on the 22 kb repeating unit. The reason why the α -amylase productivity of NMM14 is higher than that expected from the copy number is unknown.

Fig. 2(b). Southern hybridization patterns of the DNAs in Fig. 2(a) using biotin-labelled fragments A, B, C and D (see Fig. 3) were used as probes, corresponding to Fig. 2(b) parts A-D, respectively.

Fig. 3. (a) Restriction map of *amyE-tmrB* and flanking regions detected in strain NA64 (wild-type) (Hashiguchi *et al.*, 1986). Fragments A, B, C and D were used as probes for Southern hybridization analyses (see Fig. 2b). (b) Restriction map of the repeating unit detected in strain B7 (Hashiguchi *et al.*, 1986). (c) Restriction map of the repeating unit detected in strains NMM13 and NMM14. The *EcoRI* site located at the right terminus of the repeating unit of 22 kb corresponds to the left terminus of the next repeating unit. Numbers refer to size in kb. Symbols for genes are the same as in Fig. 1. The short zig-zag line in (c) denotes a 22 bp sequence derived from pBR327 (see Fig. 1). E, *EcoRI*; H, *HindIII*; P, *PstI*; C, *ClaI*; B, *BclI*.

Shikimate kinase assay

Since the *aroI* gene was amplified to about 12–17 copies in strains NMM13 and NMM14, it was expected that the activity of shikimate kinase, which is encoded by the *aroI* gene, would be elevated. We measured the shikimate kinase activities of strains NMM13 and NMM14 and compared them with those of strains NA64 (wild-type) and B7 (Table 3). Strains NMM13 and NMM14 showed activities per μg bacterial protein about four times higher than those of NA64 and B7.

DISCUSSION

In this study, we aimed to amplify a predetermined 22 kb chromosomal region of the *B. subtilis* chromosome *in situ* without cloning the whole 22 kb region but using instead only two short DNA fragments from both termini of the 22 kb region. We constructed pKH81 *in vitro* and succeeded in inducing the designed gene amplification with pKH81 by competence transformation. At first, only four amplified clones were obtained in the transformation experiment with 10 μg linearized pKH81, but by using a modified transformation method we obtained 10 and 65 Tm^r Amy^h transformants with 5 μg intact pKH81 under selective pressure with 10 μg and 5 μg tunicamycin ml^{-1} , respectively. Designed gene amplification occurred in these transformants (see Results), showing that linearization of pKH81 was not essential to induce designed gene amplification. Furthermore, complete coincidence of the designed repeating unit (Fig. 1) and the detected repeating unit (Fig. 3c) proved that this amplification was not caused by a spontaneous mutation but by the aimed method. No Tm^r transformants were obtained from competent cells without DNA treatment.

We had expected that all Tm^r transformants would be Amy^h ; however, in fact only 4 out of 10, 10 out of 15 and 65 out of 68 Tm^r transformants showed the Amy^h character (see Results). We analysed eight Tm^r transformants that were not Amy^h as representatives; no amplification of

Fig. 4. Proposed mechanism of transformation to gene duplication (a) and to gene amplification (b) induced by pKH81 DNA. Numbers refer to size in kb. Symbols for genes are the same as in Fig. 1. Bold lines show the 22 kb region; zig-zag lines show the DNA sequence derived from pBR327. E, *EcoRI*; H, *HindIII*; C, *ClaI*.

their chromosomal regions was detected (data not shown). In these cases, the Tm^r character might be caused by a mutation on *tmrB* locus. We reported previously that four independent Tm^r mutations generated by NTG treatment mapped in the *tmrB* locus (Nomura *et al.*, 1978).

A mechanism for the induction of designed gene amplification by pKH81 DNA is explained in Fig. 4, by analogy with the work of Trowsdale & Anagnostopoulos (1975) and the review by Anderson & Roth (1977), which presented a model for the induction of gene duplication by transforming DNA fragments. The pKH81 DNA might serve as a helper DNA in competence transformation to facilitate the unequal illegitimate recombination between replicating sister chromosome arms (Fig. 4a). The resultant merodiploid (or gene-duplicated) transformants might repeat the unequal legitimate recombination. Those descendants that amplified the 22 kb region sufficiently might survive under the selective pressure of tunicamycin, because of the gene dosage effect of the *tmrB* gene (Fig. 4b). Based on this model, if transforming DNA has a longer homology around the junction, a higher transformation frequency will be attained. Furusato *et al.* (1986) found that an amplification of the *amyE*-*tmrB* region under the selective pressure of tunicamycin is dependent on *recE* function.

In strains NMM13 and NMM14, the copy number of the repeating unit was determined as 12–17. We have already found, in *B. subtilis* strain B7, that the copy number of the repeating unit of 16 kb is constant under selective pressure with $10 \mu\text{g}$ tunicamycin ml^{-1} (unpublished data). By analogy, the amplification levels of strains NMM13 and NMM14 in all experiments in this

paper are expected to remain approximately constant because 10 µg tunicamycin ml⁻¹ was added to the medium.

The α -amylase and shikimate kinase activities of strains NMM13 and NMM14 were higher than those of the wild-type strain NA64 (Table 3). The reason why shikimate kinase activity in NMM13 and NMM14 strains was only four times as high as that of strain NA64 and was not in proportion to the copy numbers of the *aroI* gene may be explained as follows. It is known that shikimate kinase must associate with the bifunctional complex of DAHP synthetase and chorismate mutase in order to function to any significant degree (Nakatsukasa & Nester, 1972). Therefore, overproduction of shikimate kinase might not be sufficient to promote an elevation of its activity; the lack of a similar increase in the activity of the other two enzymes might prove limiting. Alternatively, shikimate kinase might not be overproduced in direct proportion to the copy number of the *aroI* gene.

Recently, Niaudet *et al.* (1985) reported an induction of gene duplication in *B. subtilis* by a similar rearranged fragment. Taking our work and that of Niaudet *et al.* (1985) into consideration, it may be possible to amplify long (> 20 kb) chromosomal regions of *B. subtilis* in the presence of tunicamycin, by cloning only two short DNA fragments located on both termini of the region, constructing a 'cross structure' and inserting the *tmrB* gene at the junction of those two fragments on a plasmid, and then transforming a *B. subtilis* wild-type strain with this plasmid by competence transformation. The *tmrB* gene may be replaced by another gene which contributes to the survival in the multi-copy state under selective pressure. This system may provide an effective means of amplifying long (> 20 kb) DNA regions on the chromosome, for example, when a gene cluster can not be stably maintained on multi-copy plasmids. Furthermore, if this system is applicable to other organisms, the similar amplification of any chromosomal region may be possible.

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REFERENCES

- ALBERTINI, A. M. & GALIZZI, A. (1985). Amplification of a chromosomal region in *Bacillus subtilis*. *Journal of Bacteriology* **162**, 1203–1211.
- ALTENBUCHNER, J. & CULLUM, J. (1984). DNA amplification and an unstable arginine gene in *Streptomyces lividans* 66. *Molecular and General Genetics* **195**, 134–138.
- ANAGNOSTOPOULOS, C. & SPIZIZEN, J. (1961). Requirements for transformation in *Bacillus subtilis*. *Journal of Bacteriology* **81**, 741–746.
- ANDERSON, R. P. & ROTH, J. R. (1977). Tandem genetic duplications in phage and bacteria. *Annual Review of Microbiology* **31**, 473–505.
- DERETIC, V., TOMASEK, P., DARZINS, A. & CHAKRABARTY, A. M. (1986). Gene amplification induces mucoid phenotype in *rec-2 Pseudomonas aeruginosa* exposed to kanamycin. *Journal of Bacteriology* **165**, 510–516.
- EDLUND, T. & NORMARK, S. (1981). Recombination between short DNA homologies causes tandem duplication. *Nature, London* **292**, 269–271.
- FISHMAN, S. E. & HERSHBERGER, C. L. (1983). Amplified DNA in *Streptomyces fradiae*. *Journal of Bacteriology* **155**, 459–466.
- FURUSATO, T., YAMANE, K., HASHIGUCHI, K., TANIMOTO, A., HARADA, S., MORI, M., YODA, K., YAMASAKI, M. & TAMURA, G. (1986). *recE*-dependent gene amplification induced by a tunicamycin resistant mutation (*tmrA7*) in *Bacillus subtilis*. *Agricultural and Biological Chemistry* **50**, 1771–1775.
- FUWA, H. (1954). A new method for microdetermination of amylase activity by the use of amylose as the substrate. *Journal of Biochemistry* **41**, 583–603.
- GOLDFARB, D. S., DOI, R. H. & RODRIGUEZ, R. L. (1981). Expression of Tn9-derived chloramphenicol resistance in *Bacillus subtilis*. *Nature, London* **24**, 309–311.
- GUTTERSON, N. I. & KOSHLAND, D. E., JR (1983). Replacement and amplification of bacterial genes with sequences altered *in vitro*. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 4894–4898.
- HASHIGUCHI, K., TANIMOTO, A., NOMURA, S., YAMANE, K., YODA, K., HARADA, S., MORI, M., FURUSATO, T., TAKATSUKI, A., YAMASAKI, M. & TAMURA, G. (1986). Amplification of the *amyE*–*tmrB* region on the chromosome in tunicamycin-resistant cells of *Bacillus subtilis*. *Molecular and General Genetics* **204**, 36–43.
- HASS, M. & YOSHIKAWA, H. (1969). Defective bacteriophage PBSH in *Bacillus subtilis*. 2. Intracellular development of the induced prophage. *Journal of Virology* **3**, 248–260.
- JANNIÈRE, L., NIAUDET, B., PIERRE, E. & EHRLICH,

- S. D. (1985). Stable gene amplification in the chromosome of *Bacillus subtilis*. *Gene* **40**, 47–55.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MORI, M., TANIMOTO, A., YODA, K., HARADA, S., KOYAMA, N., HASHIGUCHI, K., OBINATA, M., YAMASAKI, M. & TAMURA, G. (1986). Essential structure in the cloned transforming DNA that induces gene amplification of the *Bacillus subtilis* *amyE-tmrB* region. *Journal of Bacteriology* **166**, 787–794.
- NAKATSUKASA, W. M. & NESTER, E. W. (1972). Regulation of aromatic amino acid biosynthesis in *Bacillus subtilis* 168. *Journal of Biological Chemistry* **247**, 5972–5979.
- NAUDET, B., JANNIÈRE, L. & EHRLICH, S. D. (1985). Integration of linear, heterologous DNA molecules into the *Bacillus subtilis* chromosome: mechanism and use in induction of predictable rearrangements. *Journal of Bacteriology* **163**, 111–120.
- NOMURA, S., YAMANE, K., SASAKI, T., YAMASAKI, M., TAMURA, G. & MARUO, B. (1978). Tunicamycin-resistant mutants and chromosomal locations of mutational sites in *Bacillus subtilis*. *Journal of Bacteriology* **136**, 818–821.
- ONO, H., HINTERMANN, G., CRAMERI, R., WALLIS, G. & HUTTER, R. (1982). Reiterated DNA sequences in a mutant strain of *Streptomyces glaucescens* and cloning of the sequence in *Escherichia coli*. *Molecular and General Genetics* **186**, 106–110.
- SARGENT, M. G. & BENNETT, M. F. (1985). Amplification of a major membrane-bound DNA sequence of *Bacillus subtilis*. *Journal of Bacteriology* **161**, 589–595.
- SASAKI, T., YAMASAKI, M., MARUO, B., YONEDA, Y., YAMANE, K., TAKATSUKI, A. & TAMURA, G. (1976). Hyperproductivity of extracellular α -amylase by a tunicamycin resistant mutant of *Bacillus subtilis*. *Biochemical and Biophysical Research Communications* **70**, 125–131.
- SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503–517.
- TAKATSUKI, A., ARIMA, K. & TAMURA, G. (1970). Tunicamycin, a new antibiotic. I. Isolation and characterization of tunicamycin. *Journal of Antibiotics* **24**, 215–223.
- TANAKA, T. & SAKAGUCHI, K. (1978). Construction of a recombinant plasmid composed of *B. subtilis* leucine genes and a *B. subtilis* (natto) plasmid: its use as cloning vehicle in *B. subtilis* 168. *Molecular and General Genetics* **165**, 269–276.
- TLSTY, T. D., ALBERTINI, A. M. & MILLER, J. H. (1984). Gene amplification in the *lac* region of *E. coli*. *Cell* **37**, 217–224.
- TROWSDALE, J. & ANAGNOSTOPOULOS, C. (1975). Evidence for the translocation of a chromosome segment in *Bacillus subtilis* strains carrying the *trpE26* mutation. *Journal of Bacteriology* **122**, 886–898.
- VASSEGGHI, H. & CLAVERYS, J. P. (1983). Amplification of a chimeric plasmid carrying an erythromycin-resistance determinant introduced into the genome of *Streptococcus pneumoniae*. *Gene* **21**, 285–292.
- WILSON, C. R. & MORGAN, A. E. (1985). Chromosomal-DNA amplification in *Bacillus subtilis*. *Journal of Bacteriology* **163**, 445–453.
- YAMAZAKI, H., OHMURA, K., NAKAYAMA, A., TAKEICHI, Y., OTOZAI, K., YAMASAKI, M., TAMURA, G. & YAMANE, K. (1983). α -Amylase genes (*amyR2* and *amyE*⁺) from α -amylase-hyperproducing *Bacillus subtilis* strain: molecular cloning and nucleotide sequences. *Journal of Bacteriology* **156**, 327–337.
- YOUNG, M. (1984). Gene amplification in *Bacillus subtilis*. *Journal of General Microbiology* **130**, 1613–1621.