Designed Gene Amplification on the \textit{Bacillus subtilis} Chromosome

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We previously reported the cloning of a 1.6 kb \textit{Hind}III fragment (containing the junction of the repeating unit) from chromosomal DNA of \textit{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 \textit{B. subtilis} chromosome, a gene amplification of the 22 kb repeating unit containing the \( \alpha \)-amylase structural gene (\textit{amyE}), the tunicamycin-resistance gene (\textit{tmrB}) and the shikimate kinase structural gene (\textit{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 \textit{B. subtilis} wild-type strain by this constructed plasmid. As a result, we succeeded in obtaining tunicamycin-resistant (\textit{Tm'}) transformants in which the designed gene amplification of 22 kb occurred on the chromosome. The \textit{Tm'} transformants showed high productivity of \( \alpha \)-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.

\textbf{INTRODUCTION}

Recently, many examples of chromosomal gene amplification have been discovered in prokaryotes. Amplifications of inherent chromosomal regions have been discovered in \textit{Escherichia coli} (Edlund \& Normark, 1981; Tlsty \textit{et al.}, 1984), \textit{Streptomycyes glaucescens} (Ono \textit{et al.}, 1982), \textit{Streptomycyes fradiae} (Fishman \& Hershberger, 1983), \textit{Streptomycyes lividans} (Altenbuchner \& Cullum, 1984), \textit{Bacillus subtilis} (Hashiguchi \textit{et al.}, 1986, Wilson \& Morgan, 1985) and \textit{Pseudomonas aeruginosa} (Deretic \textit{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 \textit{Streptococcus pneumoniae} (Vasseghi \& Claverys, 1983), \textit{Salmonella typhimurium} (Gutierrez \& Koshland, 1983) and \textit{B. subtilis} (Young, 1984; Albertini \& Galizzi, 1985; Jannikre \textit{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 \textit{B. subtilis} strain B7 (a \textit{tmrA7} mutant), which acquired tandem gene amplification of the \textit{amyE}–\textit{tmrB} region by nitrosoguanidine treatment, showed hyperproductivity (about a 5- to 10-fold increase) of \( \alpha \)-amylase and tunicamycin resistance (Hashiguchi \textit{et al.}, 1986; Sasaki \textit{et al.}, 1976). The length of the repeating unit was about 16 kb and the copy number was about 10. Amplification of the \textit{amyE}+ gene caused hyperproductivity of \( \alpha \)-amylase (\textit{Amyh}) and that of the \textit{tmrB}+ gene caused tunicamycin resistance (\textit{Tm'}) (Hashiguchi \textit{et al.}, 1986). The \textit{tmrB}+ gene endows host cells with tunicamycin resistance only in

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

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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 HindIII 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 (Tmr') mutants isolated from B. subtilis NA64. pAPT17 is a plasmid carrying the 1-2 kb HindIII 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 BclI 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 manufacturer's 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). Tmr transformants were selected on nutrient agar (Difco Bacto-Agar) plates containing 10 μg tunicamycin ml⁻¹. Amy⁺ 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. 2a and Fig. 3) were prepared as follows. Plasmid pKH11 (Hashiguchi et al., 1986), carrying the 5.1 kb EcoRI fragment on pBR327 (see Fig. 1), was doubly digested with EcoRI and HindIII, and the 0.6 kb HindIII-EcoRI fragment was prepared from a 0.8 kb HindIII fragment from the tmrB gene in pGR71 (Goldfarb et al., 1981) and constructed in our laboratory, was doubly digested with EcoRI and HindIII, and the 0-6 kb HindIII–EcoRI 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 HindIII–EcoRI 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 HindIII fragment containing the tmrB gene in pGR71 (Goldfarb et al., 1981) and constructed in our laboratory, was doubly digested with HindIII and BclI, and the 0.6 kb HindIII–BclI 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 HindIII–BclI fragment was used for probe C. About 1 μg of plasmid pAPT18, carrying the 1-6 kb HindIII fragment containing the aroL 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 hybridized 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 described above 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 ODs50 of 0.05, and incubated at 37 °C for
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Table 1. B. subtilis strains

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA64</td>
<td>metB5 purB6</td>
<td>B. Maruo</td>
</tr>
<tr>
<td>B7</td>
<td>metB5 purB6 tmrA7</td>
<td>This laboratory</td>
</tr>
<tr>
<td>APT139</td>
<td>metB5 leuA8 lys-21 hsrM hsmM</td>
<td>This laboratory</td>
</tr>
<tr>
<td>NMM13</td>
<td>metB5 purB6 (amyE+-tmrB+-aroI+),†</td>
<td>This study</td>
</tr>
<tr>
<td>NMM14</td>
<td>metB5 purB6 (amyE+-tmrB+-aroI+),†</td>
<td>This study</td>
</tr>
</tbody>
</table>

* 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+).

15 h. For strains B7, NMM13 and NMM14, tunicamycin (10 μg 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 μg adenine ml⁻¹ and 50 μg 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 OD₅₀₀ of 0.05, and incubated at 37 °C. For strains B7, NMM13 and NMM14, tunicamycin (10 μg ml⁻¹) was added to the medium at all times. Mid-exponential-phase cells (OD₅₀₀ = 0.5) were harvested by centrifugation, washed once with 5 ml 50 mM-Tris/HCl buffer (pH 8.0), and stored as a wet paste at −20 °C until used.

Cell-free extracts were prepared by treatment for 30 min with lysozyme (100 μg ml⁻¹; Seikagaku Kogyo Co.) and DNAase (20 μg 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 7.9, 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 μCi; 7.4 kBq); and DTT, 1 mM. Diluted cell-free extract (50 μl) 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 μl 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 EcoRI fragment to the right end of the 0.8 kb HindIII-ClaI fragment. If the designed amplification occurs, the resultant strain will show both the Amyh and the Tmr 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 HindIII–ClaI fragment and the 5.1 kb EcoRI fragment kept the original direction as shown in Fig. 1. In this plasmid, a 22 bp sequence derived from pBR327 was present between the ClaI and the EcoRI sites at the junction.

Transformation of B. subtilis with pKH81

We transformed B. subtilis NA64 by competence transformation with 10 μg linearized pKH81 DNA cut at the unique PstI site in the vector DNA region. Tmr transformants were selected on nutrient agar containing 10 μg tunicamycin ml⁻¹. Ten Tmr transformants were obtained, four of which (designated NMM13–NMM16) showed α-amylase hyperproductivity (Amyh) 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 arol (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 µg 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 µg or 10 µg tunicamycin ml⁻¹. As a result, 68 and 15 Tmr 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 Tmr 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 µg 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
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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.

<table>
<thead>
<tr>
<th>Prominent band (kb)</th>
<th>Hybrided probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>8.0</td>
</tr>
<tr>
<td>2.3</td>
<td>5.1</td>
</tr>
<tr>
<td>14.4</td>
<td>3.9</td>
</tr>
</tbody>
</table>

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 [14C]shikimate-5-phosphate produced per µg 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 µg tunicamycin ml⁻¹ at all times.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Final OD₅₅₀</th>
<th>Doubling time (min)</th>
<th>α-Amylase productivity (U ml⁻¹ per OD₅₅₀ unit)</th>
<th>Ratio</th>
<th>Shikimate kinase activity [c.p.m. (µg protein)⁻¹]</th>
<th>Ratio</th>
<th>Copy no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA64</td>
<td>4.5</td>
<td>50</td>
<td>22.5</td>
<td>5.0</td>
<td>1</td>
<td>232</td>
<td>1-0</td>
</tr>
<tr>
<td>B7</td>
<td>4.3</td>
<td>50</td>
<td>183</td>
<td>42.6</td>
<td>8.5</td>
<td>240</td>
<td>1-0</td>
</tr>
<tr>
<td>NMM13</td>
<td>1.2</td>
<td>100</td>
<td>108</td>
<td>90.0</td>
<td>18.0</td>
<td>954</td>
<td>4-1</td>
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<tr>
<td>NMM14</td>
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<td>100</td>
<td>145</td>
<td>111.5</td>
<td>22.3</td>
<td>968</td>
<td>4-2</td>
</tr>
</tbody>
</table>

(doublet) bands after EcoRI + PstI digestion (lanes 7 and 8); 9.9, 8.0 and 3.9 kb bands after PstI 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–imrB 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...
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 arol-containing 6-8 kb PstI fragment and multiple copies of the arol-containing 9-9 kb PstI fragment (see Fig. 2b-D, and Fig. 3 a, 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 α³²P-labelled DNA probe D (specific activity 2 × 10⁷ c.p.m. µg⁻¹). The ratio of the ³²P 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 ³²P radioactivity of the 13-7 kb band to that of the 5-1 kb band (see Fig. 2b-A, lane 1) using α³²P-labelled probe A (specific activity 3 × 10⁷ 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.
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Fig. 2(b). Southern hybridization patterns of the DNAs in Fig. 2(a). 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 arolgene 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 arol 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 pg 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 TmrAmyh 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 Tmr transformants were obtained from competent cells without DNA treatment.

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