INTRODUCTION

Gene amplification occurs spontaneously during replication in all organisms (Carginale et al., 2004; Gaines et al., 2010; Hashiguchi et al., 1986; Gijzen et al., 2006; Huang & Campbell, 1995; Lengauer et al., 1998; Sandegren & Andersson, 2009). The mechanism of gene amplification is believed to consist of two steps, duplication and expansion. First, gene duplication occurs by recombination through either RecA-dependent or RecA-independent mechanisms (Sandegren & Andersson, 2009). The mechanism of gene amplification is believed to occur largely through RecA-independent recombination. The correlation between the tetB copy number and the MIC values for tetracycline indicated that more than three copies of tetB were required for tolerance to 10 μg tetracycline ml⁻¹. Thus, the RecA-dependent expansion step appears to be necessary for developing significant tetracycline tolerance mediated by tetB amplification.

Tetracycline tolerance mediated by gene amplification in Bacillus subtilis

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Bacillus subtilis can acquire a higher tolerance to tetracycline by increasing the gene dosage of its resistance gene tetB. In this study, we estimated the multiplication effect of tetB on tetracycline tolerance. Cells harbouring multiple copies of tetB were found to comprise approximately 30% of the total tetracycline-resistant cell population when selected on medium containing 10 μg tetracycline ml⁻¹. Disruption of recA resulted in a significant decrease in the frequency of tetB amplification. Although four direct repeats exist around tetB, the majority of tetB amplicons were found to be flanked by non-homologous sequences, indicating that the initial duplication of tetB can occur largely through RecA-independent recombination. The correlation between the tetB copy number and the MIC values for tetracycline indicated that more than three copies of tetB were required for tolerance to 10 μg tetracycline ml⁻¹. Thus, the RecA-dependent expansion step appears to be necessary for developing significant tetracycline tolerance mediated by tetB amplification.

Abbreviation: Tc, tetracycline.

Two supplementary tables are available with the online Supplementary Material.
replication. In fact, *B. subtilis* has a Tc resistance gene, *tetB*, encoding a Tc metal/H+ antiporter in this region of the genome (Amano *et al.*, 1991). We therefore selected *tetB* as a model system to study the gene amplification-mediated adaptation mechanism in *B. subtilis*. In this study, we demonstrated that *B. subtilis* can develop a higher tolerance to Tc by increasing the copy number of *tetB*. Our results also suggested that RecA (formerly RecE) contributed to the expansion step rather than the initial duplication in *B. subtilis*.

**METHODS**

**Bacterial strains, plasmids, and growth conditions.** All *B. subtilis* strains used in this study were derived from strain 168. Cells were grown on L medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, per litre). The spontaneous Tc-resistant mutants were obtained by spreading cells onto solid L medium containing 10 μg Tc ml⁻¹.

Strain RIK1476 (trpC2 recA::erm) was constructed as follows. Both flanking regions of *recA* were amplified using PCR with the primer pair recA-erm-pt1-UF and recA-erm-pt1-UR or recA-erm-pt1-DF and recA-erm-pt1-DR. The erythromycin resistance gene was also amplified, using the chromosomal DNA from RIK900 (Nanamiya *et al.*, 2008) as a template with primers erm-pt1-F and erm-pt1-R. These PCR fragments were mixed and used as a template for the second round of PCR, in which primers recA-erm-pt1-UF and recA-erm-pt1-DR were used. The resulting fragment was used to transform *B. subtilis* 168 with selection for erythromycin resistance. All oligonucleotides used for strain construction are listed in Table S1.

**Genomic DNA preparation.** Genomic DNA was prepared by two methods. Genomic DNA used for quantitative PCR (qPCR) analysis was prepared using InstaGene Matrix as per the manufacturer's instructions (Bio-Rad). To prepare the genomic DNA used in identifying the *tetB* amplicon, the general phenol/chloroform extraction method was used.

**qPCR.** To determine the frequency of *tetB* amplification, the genomic DNA of strains with *tetB*-associated Tc resistance was prepared as described above and then analysed using the 7300 real-time PCR system (Applied Biosystems) and Thunder Bird SYBR qPCR Mix (Toyobo). The genomic DNA of *B. subtilis* WT strain 168 prepared using the phenol/chloroform extraction method was used as the standard DNA. The *rpsJ* amplicon was used as an internal control. Oligonucleotides used for qPCR are listed in Table S2.

**Identification of the *tetB* amplicon.** The *tetB* amplicon was identified by PCR analysis with a series of outward-facing primers and sequence analysis of the PCR product using appropriate primers (Tables S1 and S2). Sequence analysis was conducted using the ABI PRISM 310 Genetic Analyzer and BigDye Terminators v3.1 Cycle Sequencing kit (Applied Biosystems).

**RESULTS AND DISCUSSION**

**Inducing Tc tolerance by *tetB* amplification in *B. subtilis***

The MIC of Tc in *B. subtilis* 168, which has a single copy of *tetB* in its genome, was 5 μg ml⁻¹. We first determined the occurrence of Tc-resistant cells by selecting with 10 μg Tc ml⁻¹. As shown in Fig. 1, the Tc-resistant cells emerged at a high frequency (approx. 1 × 10⁻⁷ c.f.u. ml⁻¹). This frequency was nearly constant during growth. We then estimated the *tetB* copy number in these Tc-resistant cells by comparing with *rpsJ* amplification as an internal control. qPCR analysis showed that 37% (26 of 70) of Tc-resistant colonies displayed an increased copy number of *tetB*, ranging from 2.4 to 230. This result suggested that the *tetB* copy number in *B. subtilis* can randomly fluctuate in a Tc concentration-independent manner. Because a mutation in *rpsJ*, which encodes the ribosomal protein S10, confers Tc resistance (Akanuma *et al.*, 2013; Williams & Smith, 1979), we analysed the nucleotide sequence of *rpsJ* in these Tc-resistant strains. Sequence analysis revealed that approximately 11% of cells carried mutant *rpsJ* (8 of 70). All *rpsJ* mutants had the same single-base substitution at position 173 (A to G), causing an amino acid substitution at position 58 (Tyr to Cys). Recently, a Tc-resistant *rpsJ* mutant carrying a single amino acid substitution at position 56 (His to Arg) was found to grow extremely slowly (Akanuma *et al.*, 2013). It is thus possible that we did not obtain this mutation because of its growth defect. No significant difference in the *tetB* copy number was observed among these *rpsJ* mutants. The Tc resistance...
mechanisms of the remaining Tc-resistant colonies (36 of 70) were not identified.

**Effect of RecA on tetB amplification**

To examine the effect of RecA on tetB amplification, we constructed a recA-disrupted strain, RIK1476, by replacing recA with the erythromycin resistance gene (see Methods). We then compared the frequency of tetB amplification between the ΔrecA mutant and its WT strain (Table 1). The frequency of tetB amplification (tetB/rpsJ \(\geq 2.0\)) in the WT (recA\(^+\)) strain was 34 % (65 of 191), which was close to that described above (37 %, 26 of 70). In contrast, the frequency of tetB amplification in the ΔrecA mutant was much lower than that in the WT (recA\(^+\)) strain. All isolated tetB-amplified strains derived from the ΔrecA mutant were found to carry duplicated copies of tetB, indicating that RecA is dispensable for the initial duplication. We could not isolate any recA-derivative strain carrying more than three copies of tetB. Our results indicate that RecA has an important role in the development of Tc tolerance in *B. subtilis*.

### Identification of the amplified DNA region

Our results suggest that RecA-dependent recombination is involved in tetB amplification in *B. subtilis*. Amano & Shishido (1995) previously reported that three direct repeats, which we designated DR1–3, are located around tetB. DR1 and DR2 overlap the open reading frames yyaL and yyaO, respectively (Fig. 2a). DR2 is nearly identical (95.5 %, 171/179) to the DR1 sequence, with a one base gap (Fig. 2b). DR3 is 79.3 % (142/179) identical to the DR1 sequence, and it is located in the internal region between tetB and yyaP (Fig. 2a). Furthermore, we found an additional direct repeat, which is 49.7 % (89/179) identical to the DR1 sequence (Fig. 2b), which we designated DR4. DR4 is located in the internal region between yyaQ and yyaR (Fig. 2a). It is possible that tetB amplification is caused by homologous recombination between these direct repeats. We thus sequenced the junction of the DNA amplicons in 59 tetB-amplified strains. To predict the amplified regions, we quantified the copy numbers of the tetB-surrounding genes (Fig. 3a). We then identified all amplicons by PCR analysis using outward-facing primers and sequence analysis. Both flanking sequences of all

<table>
<thead>
<tr>
<th>Effect of RecA on tetB amplification</th>
<th>recA(^+)</th>
<th>ΔrecA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Tc(^{+}) cells tested</td>
<td>191</td>
<td>262</td>
</tr>
<tr>
<td>Number of tetB-amplified cells(^{\ast})</td>
<td>65</td>
<td>3</td>
</tr>
<tr>
<td>Frequency of tetB amplification(^{\dagger})</td>
<td>34 %</td>
<td>1.1 %</td>
</tr>
<tr>
<td>Range of tetB copy number</td>
<td>2.0–230</td>
<td>2.0–2.2</td>
</tr>
</tbody>
</table>

\(^{\ast}\)The tetB-amplified cells are defined as tetB/rpsJ \(\geq 2.0\).

\(^{\dagger}\)Frequencies are calculated as the number of tetB-amplified cells divided by the number of total Tc\(^{+}\) cells tested.

**Fig. 2.** Direct repeats in the tetB region. (a) Organization of the tetB region. Thick black bars indicate direct repeats, DR1–4. (b) Comparison of the nucleotide sequence of the four direct repeats. Asterisks (*) denote sequence identity relative to DR1. Bars represent the gaps.
identified amplicons are listed in Table 2. The identified amplicons ranged from approximately 0.7 to 115 kb in length. qPCR analysis showed that only five strains could possess an amplicon flanked by direct repeats (Fig. 3b, Groups 14–16). Among them, one strain categorized under Group 16 was found to have a duplicated copy of the tetB promoter region flanked by non-homologous sequences (Table 2, Amplicon 21). As a result, unlike the case in Salmonella enterica (Reams et al., 2012), only four strains (of 59) had an amplicon flanked by direct repeats (Amplicons 18–20). All other strains (55) carried an amplicon flanked by non-homologous or micro-homologous sequences. Three strains carrying the amplicon 1 showed micro-homology with matches for 12/14 bp. The most frequently observed amplification (Amplicon 5) made use of a 5 bp repeat with matches for 7/12 bp. Thus, the initial duplication in B. subtilis tetB amplification is thought to occur largely through RecA-independent non-homologous recombination, suggesting that the RecA-dependent expansion step is required for tolerance to 10 μg Tc ml⁻¹.

**Figure 3.** Quantitative analysis of the tetB-surrounding genes. (a) Schematic diagram of the tetB region is shown. The quantified PCR regions are shown with each gene name. Four direct repeats are shown as thick black bars. (b) The quantified genes are indicated as black (multiple copy) or white boxes (single copy). All tested strains were categorized into 16 groups on the basis of the quantitative PCR analysis results. The number of strains categorized is also shown.

**Relationship between the tetB copy number and Tc tolerance**

To clarify the relationship between the tetB copy number and MIC, we isolated 51 other Tc-resistant strains carrying multiple copies of tetB (tetB/rpsJ ≥ 2.0). These strains were categorized into four groups on the basis of their MIC values (10, 20, 30 or 40 μg ml⁻¹). Then, the mean tetB copy number in each group was calculated. The results showed a clear correlation between the tetB copy number and MIC (Fig. 4a). The mean copy number of tetB in
Table 2. Nucleotide sequences of the flanking regions of each amplicon

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>tetB upstream*</th>
<th>tetB downstream*</th>
<th>Size (bp)</th>
<th>No. of cells‡</th>
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<tr>
<td></td>
<td>Gene†</td>
<td>Sequence</td>
<td>Gene†</td>
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<td>1</td>
<td>recR</td>
<td>GATCCCTGCGAATTTCATAA</td>
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<td>2</td>
<td>yyaL</td>
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<td>yycR</td>
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<tr>
<td>3</td>
<td>dck</td>
<td>CGATCATATAACATAATTTG</td>
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</tr>
<tr>
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<tr>
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<td>AAGATTTGAGGGAAGAATATT</td>
<td>16158</td>
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<tr>
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<tr>
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<td>16</td>
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<td>17</td>
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<tr>
<td>20</td>
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<td>1997</td>
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<tr>
<td>21</td>
<td>DR2-tetL§</td>
<td>TTTTTTTTATGAAAACAGTAG</td>
<td>GGTATTTTATTTTTACGTTATATT</td>
<td>728</td>
</tr>
</tbody>
</table>

*Twenty base pair nucleotide sequences of the flanking region are indicated. Bold letters denote the terminal sequences of the amplicons. Underlined sequences denote the matching bases between both flanking regions.

†The flanking genes of each amplicon.

‡Number of cells isolated in this study.

§These junction sites are located in the intergenic region between the indicated genes.

Fig. 4. Correlation between the tetB copy number and Tc resistance. (a) The Tc-resistant colonies were isolated by spreading B. subtilis strain 168 cells onto L medium containing 10 µg Tc ml⁻¹. The tetB copy numbers of the isolated Tc-resistant colonies were analysed by quantitative PCR analysis. Fifty-one tetB-amplified Tc-resistant strains were found. These strains were categorized into four groups on the basis of their Tc MICs (10, 20 or 40 µg ml⁻¹). The mean tetB copy number in each group was calculated and plotted on the graph. (b) Change in the tetB copy number in the absence of Tc. Four tetB-amplified strains were serially passaged three times on the solid L medium lacking Tc. At each passage, colonies were picked from these plates for preparing the genomic DNA, and their tetB copy numbers were analysed. The ratio of the copy number at each passage was calculated as the copy number after passage divided by the initial copy number. The initial tetB copy number in each strain tested was 85.4, 73.3, 29.0 and 16.1, respectively.
TetB amplification in *B. subtilis*

parental strain 168, which has a MIC of 5 µg ml⁻¹, was 1.04 ± 0.0412 (n = 30). Although tetB copy numbers in the groups were 3.06 ± 0.462, 8.19 ± 2.67, 16.5 ± 5.64 and 25.3 ± 5.32 for MICs of 10 (n = 17), 20 (n = 16), 30 (n = 10) and 40 (n = 8) µg ml⁻¹, respectively, it is most likely that the duplicated copies of tetB insufficiently confer full tolerance to 10 µg Tc ml⁻¹. In fact, all three isolated recA-derivative strains carrying duplicated copies of tetB exhibited slow growth in liquid L medium containing 10 µg Tc ml⁻¹ (data not shown).

### Instability of the amplified tetB region

To monitor the change in the tetB copy number under the non-selective condition, four tetB-amplified strains were serially passaged three times on solid L medium lacking Tc. At each passage, colonies were picked from these plates for preparing the genomic DNA, and their tetB copy numbers were analysed. The results demonstrated that the copy number was reduced by approximately 40% during the first cultivation and thereafter gradually decreased (Fig. 4b). After 15 subcultures under the non-selective condition, the tetB copy numbers in all strains were reduced to a single copy (data not shown). These results indicate that the amplified tetB is unstable under non-selective conditions.

### Concluding remarks

Gene amplification is considered an important adaptation mechanism in bacteria. In this study, we demonstrated that the multiplication of the tetB locus is one of the factors conferring resistance to *B. subtilis* at high concentrations of Tc. Disruption of recA significantly decreased the frequency of tetB amplification (Table 1). Sequence analysis of the junctions revealed that the initial duplication in tetB amplification occurred largely through RecA-independent mechanisms (Table 2). Thus, the RecA-dependent expansion step is required by tetB for significant Tc tolerance, which is mediated by tetB amplification.

## REFERENCES


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