Multiple rRNA operons are essential for efficient cell growth and sporulation as well as outgrowth in *Bacillus subtilis*

Koichi Yano,1† Tetsuya Wada,1 Shota Suzuki,1 Kazumi Tagami,1 Takashi Matsumoto,2 Yuh Shiwa,2 Taichiro Ishige,2 Yasuhiro Kawaguchi,1 Kenta Masuda,1 Genki Akanuma,3 Hideaki Nanamiya,4 Hironori Niki,5,6 Hirofumi Yoshikawa2,7 and Fujio Kawamura1‡

1Department of Life Science and Research Center for Life Science, College of Science, Rikkyo University, Toshima-ku Nishi-ikebukuro 3-34-1, Tokyo 171-8501, Japan
2Genome Research Center, Nodai Research Institute, Tokyo University of Agriculture, Setagaya-ku, Sakuragaoka 1-1-1, Tokyo 156-8502, Japan
3Department of Applied Chemistry, Faculty of Science and Engineering, Chuo University, Bunkyo-ku, Tokyo 112-8551, Japan
4Department of Biotechnology, Graduate School of Agricultural and Life Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan
5Microbial Genetics Laboratory, Genetic Strains Research Center, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan
6Department of Genetics, Graduate University for Advanced Studies, Sokendai, 1111 Yata, Mishima, Shizuoka 411-8540, Japan
7Department of Bioscience, Tokyo University of Agriculture, Setagaya-ku, Sakuragaoka 1-1-1, Tokyo 156-8502, Japan

The number of copies of rRNA (rrn) operons in a bacterial genome differs greatly among bacterial species. Here we examined the phenotypic effects of variations in the number of copies of rRNA genes in the genome of *Bacillus subtilis* by analysis of eight mutant strains constructed to carry from two to nine copies of the rrn operon. We found that a decrease in the number of copies from ten to one increased the doubling time, and decreased the sporulation frequency and motility. The maximum levels for transformation activity were similar among the strains, although the competence development was significantly delayed in the strain with a single rrn operon. Normal sporulation only occurred if more than four copies of the rrn operon were present, although ten copies were needed for vegetative growth after germination of the spores. This behaviour was seen even though the intracellular level of ribosomes was similar among strains with four to ten copies of the rrn operon. Furthermore, ten copies of the rrn operon were needed for the highest swarming activity. We also constructed 21 strains that carried all possible combinations of two copies of the rrn operons, and found that these showed a range of growth rates and sporulation frequencies that all fell between those recorded for strains with one or three copies of the rrn operon. The results suggested that the copy number of the rrn operon has a major influence on cellular processes such as growth rate and sporulation frequency.

**INTRODUCTION**

The bacterial ribosome, which is essential for protein biosynthesis, is composed of 3 rRNAs (23S, 16S and 5S rRNA) and more than 50 ribosomal proteins (Lecompte *et al.*, 2002). The three rRNA genes are located in the rRNA (rrn) operon, which contains the 16S, 23S and 5S rRNA genes in that order. Although most genes encoding ribosomal protein are present as a single copy, the number...
of rrn operons differs among bacterial species (Klappenbach et al., 2000, 2001). For example, Mycoplasma genitalium, a pathogenic bacterium with a very small genome (580,074 bp) (Fraser et al., 1995), contains only one rrn operon, whereas the genomes of Escherichia coli and Bacillus subtilis contain seven and ten rrn operons, respectively (Kunst et al., 1997; Klappenbach et al., 2001; Henkin, 2002). In general, organisms that possess multiple rrn operons grow faster than those that possess one or two operons. It has also been suggested that the evolution of multiple rrn operons enables bacterial species to cope with a variety of environmental conditions (Condon et al., 1992, 1995). This raises the possibility of functional differentiation among multiple rrn operons. However, characterization of the functional significance of heterogeneity among operons is difficult because, in general, such heterogeneity is limited and rrn operons are highly conserved (Nomura, 1999).

Several mutant strains of E. coli that contain multiple deletions of the rrn operon have been constructed (Asai et al., 1999a, b). Analyses of these strains showed that deletions of rrn operons reduced cell proliferation and protein synthesis (Asai et al., 1999a, b). To date, however, there is little information from B. subtilis on the effects of variation in the copy number of rrn operons.

B. subtilis is one of the best-characterized Gram-positive soil bacteria, and has been studied extensively as a model for unicellular differentiation because of its ability to form endospores (Schaefler et al., 1965; Losick & Stragier, 1992; Errington, 1993; Hoch, 1993; Grossman, 1995; Kunst et al., 1997; Errington et al., 2003). Recently, we sequentially deleted the ten rrn operons of B. subtilis, and constructed seven mutant strains that each possessed a single rrn operon (rrnA, B, D, E, I, J or O) in their genome (Nanamiya et al., 2010). The growth rates and sporulation frequencies of these mutants were reduced drastically compared with those of the wild-type strain (Nanamiya et al., 2010). These inhibitory effects appeared to be due to the reduction of the intracellular levels of ribosomes in the mutants (Nanamiya et al., 2010). These findings raised the question of how many copies of the rrn operon are necessary to enable the same growth rates and sporulation frequencies as in the wild-type strain. To test this, we constructed eight B. subtilis mutant strains with two to nine copies of the rrn operon in their genomes. In addition, we constructed strains with different combinations of two copies of the rrn operon, and monitored their growth rates and sporulation frequencies. Our analyses provide new insights into the requirement for multiple copies of the rrn operon in the genome of B. subtilis.

**METHODS**

**Bacterial strains and general methods.** The B. subtilis strains used in the study were isogenic with B. subtilis strain 168 and are listed in Table 1. Strain RIK539, which has a single copy of rrnA, was used as the parental strain because it has the best growth characteristics of the seven mutant strains that possess a single rrn operon (Nanamiya et al., 2010). The details of the construction of the successive markerless deletion mutants are described in the Supplementary Methods (Tables S1 and S2, available in Microbiology Online). Briefly, the process included: (i) construction of strains with an antibiotic-resistance gene in place of each rrn operon, and (ii) removal of the antibiotic-resistance marker via congression using chromosomal DNA from the strain that carried a deletion of the relevant rrn operon (Nanamiya et al., 2010). The media used included LB medium and LB agar (Sambrook & Russell, 2001), CI media (Ashikaga et al., 2000), minimal glucose agar supplemented with 0.05% amicase (MG-CH agar; Sigma; Rutberg, 1969), 2× Schaeffer’s sporulation medium supplemented with 0.1% glucose (2×SG; Leighton & Doi, 1971), and 2×LB supplemented with 0.5 mM L-Ala (Moeller et al., 2006). When required, antibiotics were added at the following concentrations: chloramphenicol, 5 μg ml⁻¹; erythromycin, 0.5 μg ml⁻¹; kanamycin, 5 μg ml⁻¹; and spectinomycin, 100 μg ml⁻¹.

**Southern blot hybridization analysis.** Southern blotting was performed as described previously with a slight modification in the amount of chromosomal DNA (4 μg) (Nanamiya et al., 2010). The template for the 23S-specific RNA probe was prepared from DNA from B. subtilis strain 168 by PCR using the primers rrnPro.F and rrnPro.R (Nanamiya et al., 2010).

**Measurement of doubling time.** For Table 2, B. subtilis cells were grown in LB medium at 37°C with shaking until the culture reached OD₆₀₀ ~0.2; the cultures were then diluted with fresh medium to OD₆₀₀ ~0.04 and incubated further. The doubling times were determined from the growth curve. For Fig. 3, cells were incubated in LB or competence-inducing medium at various temperatures with shaking, and the OD₆₀₀ of the cultures was automatically measured with a Bio-Photorecorder (TVS62CA; Advantec), and then doubling time was determined from the growth curve.

**Assay for sporulation.** B. subtilis cells were inoculated at OD₆₀₀ ~0.04 and grown in 2×SG medium for 24 h at 37°C with shaking. Heat-resistant spores were counted by heating the cells at 80°C for 10 min and then plating them on LB agar plates followed by incubation at 37°C for 24–36 h.

**Sucrose density gradient sedimentation analysis.** Cells were grown in 500 ml LB medium at 37°C with shaking; when they reached the early exponential phase (OD₆₀₀ ~0.2), they were harvested and then disrupted by passing through a French press (Aminco) at 55.2 MPa. Cell debris was removed by centrifugation as described previously (Natori et al., 2007), and the supernatant was used as the crude cell extract. Aliquots of the extract (A₂₆₀=10) were layered onto 10–40% sucrose density gradients and centrifuged at 4°C for 17.5 h at 65,000 g (Hitachi P400ST rotor). Absorbance profiles were monitored at 254 nm using a piston gradient fractionator (Biocomp) and a Bio-mini UV monitor (ATTO).

**Microscopic imaging.** Cells were grown in LB medium at 37°C with shaking to the exponential phase. FM4-64 (Invitrogen) at 10 μM and DAPI (Wako Pure Chemical Industries) at 5 μg ml⁻¹, final concentrations, respectively, were added to a 20 μl aliquot of the culture. The cell suspension was placed on a microscope slide covered with a thin film of 1% agarose in distilled water. Images were acquired with a SenSys-1401E air-cooled CCD camera (Roper Scientific) attached to an Olympus BX50 microscope equipped with a ×100 UPlanApo objective.

**Preparation of spores and spore germination.** Mature spores were prepared using a slight modification of the procedure described by Kodama et al. (1999). B. subtilis cells were grown in 2×SG medium for 24 h at 37°C with shaking. The spores were harvested by centrifugation, purified by washing in cold distilled water, and treated with 0.9% NaCl.
with lysozyme (0.2 mg ml⁻¹) at 25 °C for 15 min. They were then treated with DNase I (2 μg ml⁻¹) at 25 °C for 15 min, and washed three times in cold distilled water.

Germination of the spores was monitored using a slight modification of the method described by Moeller et al. (2006). The purified spores were heat activated at 65 °C for 15 min, suspended in 2 × LB medium supplemented with 0.5 mM L-alanine at OD₆₀₀ = 0.1, and incubated at 37 °C with shaking. Germination was monitored by measuring the OD₆₀₀ of the spore suspension every 15 min for up to 120 min.

**Motility assay.** The comparative motilities of strains with one to ten copies of the *rrn* operon were assayed on LB plates containing 0.4% Bacto agar (Difco). The strain with a single *rrn* operon was

### Table 1. *B. subtilis* strains used in the study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype (characteristics)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>168</td>
<td>trpC2 (10 <em>rrn</em>)</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>RIK218</td>
<td>trpC2 ΔrrnW:Δcat (9 <em>rrn</em>)</td>
<td>This study</td>
</tr>
<tr>
<td>RIK546</td>
<td>trpC2 ΔrrnW3 ΔrrnJ:Δcat (8 <em>rrn</em>)</td>
<td>This study</td>
</tr>
<tr>
<td>RIK1755</td>
<td>trpC2 ΔrrnHG1 ΔrrnD2 ΔrrnW2:Δcat ΔCEB1 (7 <em>rrn</em>)</td>
<td>This study</td>
</tr>
<tr>
<td>RIK1753</td>
<td>trpC2 ΔrrnHG1 ΔrrnD2 ΔrrnJ:Δspe ΔCEB1 (6 <em>rrn</em>)</td>
<td>This study</td>
</tr>
<tr>
<td>RIK1466</td>
<td>trpC2 ΔrrnHG1 ΔrrnD1 ΔrrnW2 ΔrrnJ:Δkan ΔCEB1 (5 <em>rrn</em>)</td>
<td>This study</td>
</tr>
<tr>
<td>RIK1463</td>
<td>trpC2 ΔrrnHG1 ΔrrnD1 ΔrrnB2 ΔrrnW2 ΔrrnJ:Δkan ΔCEB1 (4 <em>rrn</em>)</td>
<td>This study</td>
</tr>
<tr>
<td>RIK1437</td>
<td>trpC2 ΔrrnHG1 ΔrrnD1 ΔrrnE1 ΔrrnB2 ΔrrnW2 ΔrrnJ:Δspe ΔCEB1 (3 <em>rrn</em>)</td>
<td>This study</td>
</tr>
<tr>
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<td>trpC2 ΔrrnHG1 ΔrrnO1 ΔrrnD1 ΔrrnE1 ΔrrnB2 ΔrrnW2 ΔrrnJ:Δcat ΔCEB1 (2 <em>rrn</em>)</td>
<td>This study</td>
</tr>
<tr>
<td>RIK539</td>
<td>trpC2 ΔrrnHG1 ΔrrnO1 ΔrrnD1 ΔrrnE1 ΔrrnB2 ΔrrnW2 ΔrrnJ:Δcat ΔCEB1 (1 <em>rrn</em>)</td>
<td>Nanamiya et al. (2010)</td>
</tr>
<tr>
<td>RIK222</td>
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<td>This study</td>
</tr>
<tr>
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<td>This study</td>
</tr>
<tr>
<td>RIK224</td>
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<td>This study</td>
</tr>
<tr>
<td>RIK225</td>
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<td>This study</td>
</tr>
<tr>
<td>RIK226</td>
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<td>This study</td>
</tr>
<tr>
<td>RIK227</td>
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<td>This study</td>
</tr>
<tr>
<td>RIK228</td>
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<td>This study</td>
</tr>
<tr>
<td>RIK229</td>
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<td>This study</td>
</tr>
<tr>
<td>RIK230</td>
<td>trpC2 ΔrrnHG1 ΔrrnD1 ΔrrnE1 ΔrrnA1 ΔrrnB2 ΔrrnW2 ΔrrnJ:ΔCEB1 (IO)</td>
<td>This study</td>
</tr>
<tr>
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<td>This study</td>
</tr>
<tr>
<td>RIK232</td>
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<td>This study</td>
</tr>
<tr>
<td>RIK233</td>
<td>trpC2 ΔrrnHG1 ΔrrnB2 ΔrrnE1 ΔrrnA1 ΔrrnD2 ΔrrnW2 ΔrrnJ:ΔCEB1 (OD)</td>
<td>This study</td>
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<tr>
<td>RIK234</td>
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<tr>
<td>RIK235</td>
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<td>This study</td>
</tr>
<tr>
<td>RIK236</td>
<td>trpC2 ΔrrnHG1 ΔrrnD1 ΔrrnB2 ΔrrnA1 ΔrrnD2 ΔrrnW2 ΔrrnJ:ΔCEB1 (ED)</td>
<td>This study</td>
</tr>
<tr>
<td>RIK237</td>
<td>trpC2 ΔrrnHG1 ΔrrnD1 ΔrrnE1 ΔrrnA1 ΔrrnD2 ΔrrnW2 ΔrrnJ:ΔCEB1 (IA)</td>
<td>This study</td>
</tr>
<tr>
<td>RIK238</td>
<td>trpC2 ΔrrnHG1 ΔrrnO1 ΔrrnD1 ΔrrnA1 ΔrrnD2 ΔrrnW2 ΔrrnJ:ΔCEB1 (IB)</td>
<td>This study</td>
</tr>
<tr>
<td>RIK239</td>
<td>trpC2 ΔrrnHG1 ΔrrnO1 ΔrrnB2 ΔrrnA1 ΔrrnD2 ΔrrnW2 ΔrrnJ:ΔCEB1 (ID)</td>
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<tr>
<td>RIK240</td>
<td>trpC2 ΔrrnHG1 ΔrrnO1 ΔrrnD1 ΔrrnB2 ΔrrnA1 ΔrrnE1 ΔrrnW2 ΔrrnJ:ΔCEB1 (II)</td>
<td>This study</td>
</tr>
<tr>
<td>RIK241</td>
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<td>This study</td>
</tr>
<tr>
<td>RIK242</td>
<td>trpC2 ΔrrnHG1 ΔrrnO1 ΔrrnD1 ΔrrnB2 ΔrrnA1 ΔrrnW2 ΔrrnJ:ΔCEB1 (IE)</td>
<td>This study</td>
</tr>
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</table>

### Table 2. Doubling times and sporulation frequencies of strains with different numbers of *rrn* operons

The results shown are the means of three independent experiments (±SDs).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Doubling time (min)</th>
<th>Total (c.f.u. ml⁻¹)</th>
<th>Spores (c.f.u. ml⁻¹)</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIK539 (1 <em>rrn</em>)</td>
<td>65.8 (±3.5)</td>
<td>5.6 (±0.5) x 10⁸</td>
<td>6.2 (±4.2) x 10⁸</td>
<td>0.001±0.0008</td>
</tr>
<tr>
<td>RIK1754 (2 <em>rrn</em>)</td>
<td>35.3 (±3.5)</td>
<td>2.8 (±0.8) x 10⁸</td>
<td>1.5 (±0.5) x 10⁷</td>
<td>6.7±0.05</td>
</tr>
<tr>
<td>RIK1437 (3 <em>rrn</em>)</td>
<td>29.3 (±0.4)</td>
<td>5.6 (±0.2) x 10⁸</td>
<td>7.5 (±2.1) x 10⁷</td>
<td>31.0±0.03</td>
</tr>
<tr>
<td>RIK1463 (4 <em>rrn</em>)</td>
<td>26.7 (±1.0)</td>
<td>7.2 (±1.4) x 10⁸</td>
<td>4.0 (±0.2) x 10⁷</td>
<td>58.4±0.2</td>
</tr>
<tr>
<td>RIK1466 (5 <em>rrn</em>)</td>
<td>23.7 (±0.4)</td>
<td>8.2 (±0.4) x 10⁸</td>
<td>4.3 (±1.4) x 10⁷</td>
<td>53.0±0.2</td>
</tr>
<tr>
<td>RIK1753 (6 <em>rrn</em>)</td>
<td>23.2 (±0.8)</td>
<td>9.6 (±1.6) x 10⁸</td>
<td>5.4 (±1.4) x 10⁷</td>
<td>58.3±0.2</td>
</tr>
<tr>
<td>RIK1755 (7 <em>rrn</em>)</td>
<td>23.0 (±0.7)</td>
<td>6.3 (±5.2) x 10⁸</td>
<td>4.0 (±3.1) x 10⁶</td>
<td>63.9±0.09</td>
</tr>
<tr>
<td>RIK546 (8 <em>rrn</em>)</td>
<td>21.7 (±2.0)</td>
<td>7.3 (±1.5) x 10⁸</td>
<td>4.7 (±0.6) x 10⁶</td>
<td>67.0±0.2</td>
</tr>
<tr>
<td>RIK218 (9 <em>rrn</em>)</td>
<td>21.4 (±2.1)</td>
<td>5.7 (±4.2) x 10⁸</td>
<td>2.2 (±1.4) x 10⁶</td>
<td>45.9±0.09</td>
</tr>
<tr>
<td>168 (10 <em>rrn</em>)</td>
<td>18.3 (±0.8)</td>
<td>5.4 (±0.5) x 10⁸</td>
<td>4.4 (±0.9) x 10⁶</td>
<td>79.9±0.08</td>
</tr>
</tbody>
</table>
pre-cultured for 2 days on an LB plate; strains with two to ten \(rrn\) operons were pre-cultured overnight on LB plates. Colonies were picked and inoculated onto the centre of the assay plates. After incubation at 30 °C for 36 h, the size of the swarm colony was measured.

**Assay for transformation activity.** Trp\(^+\) transformation activity of cells with one to ten \(rrn\) operons was determined using 168W (trp\(^+\)) chromosomal DNA. Cells were grown in CI medium supplemented with 0.03% yeast extract instead of 0.06% amicase at 37 °C with shaking, and the \(OD_{660}\) was measured at the indicated times. An aliquot (0.1 ml) of the culture was withdrawn and mixed with the DNA solution at a final concentration of 2 \(\mu g\) ml\(^{-1}\). After incubation for 30 min at 37 °C with shaking, the cells were plated on minimal agar plates (Spizizen’s minimal glucose medium) supplemented with L-Arg, L-Gly, L-Phe, L-Ile, L-Ser, L-Thr, L-Val, L-Met, L-Lys, L-Leu, L-Ala, L-Pro, L-Asp, L-Asn, L-Glu and L-Gln (0.02 \(\mu g\) ml\(^{-1}\)), and L-Tyr and L-Cys (0.01 \(\mu g\) ml\(^{-1}\)). Trp\(^+\) transformants were counted after 2 days of incubation at 37 °C.

**RESULTS**

**Construction of multiple deletion mutants that carry two to nine copies of the \(rrn\) operon**

We constructed eight mutant strains that carried between two and nine copies of the \(rrn\) operon using deletion mutants with single copies of different \(rrn\) operons (Nanamiya *et al.*, 2010). The detailed procedures are described in the Supplementary Methods. The mutant strains are listed in Table 1. Deletion of the RNA genes in these strains was verified by Southern blot analysis using a riboprobe specific for the 23S rRNA gene. This analysis confirmed that each strain contained the expected \(rrn\) operons in its genome; a typical set of data is shown in Fig. 1.

During the current set of experiments, we noticed that ICEBs1, a mobile genetic element, was deleted from the strain carrying a single \(rrn\) operon (RIK539) (data not shown; Auchtung *et al.*, 2005; Lee *et al.*, 2007). We examined whether ICEBs1 might affect the phenotypes of strains carrying different copy numbers of the \(rrn\) operon.

**Growth rates in strains with different numbers of \(rrn\) operon**

The growth profiles of the eight deletion mutant strains with different copy numbers of the \(rrn\) operons were monitored in nutrient-rich (LB) medium at 37 °C, and were compared with those from the wild-type strain and strain RIK539. Strain RIK539 with a single \(rrn\) operon was constructed previously (Nanamiya *et al.*, 2010), and its growth profile was also monitored as a control. We found that strain RIK539 had the slowest growth rate, and that growth rates in the other strains increased with the increasing number of copies of the \(rrn\) operon (Fig. 2). The doubling times ranged from a mean of 65.8 min in the strain with one \(rrn\) operon to 21.4 min in the strain with nine \(rrn\) operons; the wild-type strain (ten \(rrn\) operons) had a mean doubling time of 18.3 min (Table 2). Thus, as the number of \(rrn\) operons increased, so did growth rate. Increasing the number of copies of the \(rrn\) operon from one to two had the greatest effect on growth rate, whereas the effect became modest when the copy number was more than five (Table 2). These results are consistent with previous reports in *E. coli* that deletion of a few copies of the \(rrn\) operon did not drastically affect the growth rate in rich and minimal media (Ellwood & Nomura, 1980). We also monitored growth rates in LB medium at 30 and 45 °C (Fig. 3). Although strain RIK539 grew at both temperatures, it had the slowest growth rate of the ten strains tested, as was the case at 37 °C. Growth rates in competence-inducing medium at 37 °C were also determined (Figs 3 and 9). The analysis showed that the increase in number of \(rrn\) operon copies from one to two had the greatest effect on growth rate, and that the \(rrn\) operon copy number had a lesser effect on growth rate at the lower temperature than at 37 °C and 45 °C.

A strain carrying ICEBs1 and a single \(rrn\) operon was constructed by transformation of strain RIK539, growth rates on nutrient-rich (LB) and poor (CI) media at 30, 37 and 45 °C were obtained, and the sporulation frequency at 37 °C was determined; the data were compared to those from strain RIK539. We found that growth rates and sporulation frequencies were similar in the two strains (data not shown). Therefore, we concluded that ICEBs1 had little effect on the growth rate and sporulation frequency in the strain with a single \(rrn\) operon. We assumed that ICEBs1 would also have little effect on strains carrying different copies of the \(rrn\) operon, and therefore decided to use the constructed strains described above for our analyses. Deletion of ICEBs1 was also checked by PCR in other strains carrying different copy numbers of the \(rrn\) operon. The analysis showed that ICEBs1 was deleted from strains with two (RIK1754), three (RIK1437), four (RIK1463), five (RIK1466), six (RIK1753) and seven (RIK1755) copies of the \(rrn\) operon. ICEBs1 was present in the strains that possessed eight (RIK546), nine (RIK218) and ten (wild-type) copies of the \(rrn\) operon (data not shown).

**Fig. 1.** Confirmation of the numbers of \(rrn\) operons using Southern blot analysis. BstI 107I digests were subjected to electrophoresis in a 0.8% agarose gel and probed with a 23S-specific RNA probe as described in Methods.
Strain RIK539 was found to have a morphological defect (see below). As it was possible that this defect might influence the sensitivity of the strain to high-salt conditions, we compared growth rates on LB medium containing 5% NaCl at 37°C for the ten strains with one to ten rrn copies (Fig. 3). The results of the analysis indicated that strain RIK539 was more sensitive to high-salt conditions than the strains with two to ten rrn operons. It has previously been proposed that many bacterial species have evolved multiple rrn operons to cope with variations in environmental conditions (Condon et al., 1992, 1995). Our results suggest that cells with a single rrn operon are less tolerant of stressful conditions.

**Sporulation frequencies in strains with one to ten rrn operons**

As *B. subtilis* characteristically forms endospores, we examined the effect of rrn operon copy number variation on the sporulation process as described in Methods. We found that the number of viable cells was comparable among the nine mutant strains and the wild-type strain. However, the strain that contained only one rrn operon exhibited the smallest number of spores (6.2 × 10³ spores ml⁻¹) and the lowest sporulation frequency (0.0011%) (Table 2). The data are consistent with those in our previous report (Nanamiya et al., 2010). In strain RIK1754, which carries two rrn operons, the numbers of spores increased dramatically compared to strain RIK539, and the sporulation frequency was enhanced 10³-fold. The number of spores formed by strains that carried three or more rrn operons was almost the same as for the wild-type strain. Thus, as found for growth rates, the presence of more than one copy of the rrn operon boosted the sporulation frequency, with the most marked effect being seen in the transition from one to two copies; in contrast, strains with four or more copies showed less of an effect (Table 2). To determine the stage at which sporulation was obstructed in the mutant strains, we carried out a microscopic analysis of cells grown in 2×SG for 24 h at 37°C. In strains RIK539 and RIK1754, which carry one and two rrn operons, respectively, refractile bodies were rarely observed, as were immature forespores and cells with an unequal septum; however, these cell types were frequently observed in strains with three or more rrn operons (data not shown). Furthermore, transcriptional activation of the spo0A Ps promoter was delayed and the maximal level was low in strain RIK539, as monitored using transcriptional fusion with the thermostable β-galactosidase-encoding gene, bgAB (data not shown; Nanamiya et al., 1998). Thus, it is very likely that cells carrying one or two rrn operons had a block at the initiation stage of sporulation. These observations are in good agreement with our previous results (Nanamiya et al., 2010).

**Intracellular ribosome levels in the strains with one to ten rrn operons**

One possible explanation for the reduced growth rates in *E. coli* following deletion of rrn operons is a reduction in the ribosome concentration (Asai et al., 1999b). Similarly, we previously suggested that the severe sporulation defects in strains with a reduced number of rrn operons might be due to a diminished level of cellular ribosomes (Nanamiya et al., 2010). Here, we sought to test this possible explanation by assessing the relationship between rrn...
operon copy number and intracellular ribosome levels. We determined the ribosome levels in strains with one to ten copies of the rrm operon using a sucrose density gradient sedimentation analysis. As shown in Fig. 4, the level of 70S ribosomes in the RIK539 strain, which has a single copy of the rrm operon, was the lowest among the ten tested strains. In strains with up to four copies of the rrm operon, the level of 70S ribosomes increased markedly with increasing copy number, with an especially large jump in level between strains with one and two copies. In contrast, in strains with five or more copies, the ribosome levels were similar. These results suggest that both growth rate and sporulation ability depend heavily on the level of intracellular ribosomes. Furthermore, they also suggest that rrm operon copy number is a major determinant of intracellular ribosome levels.

**Microscopic analysis of cells carrying one to ten copies of the rrm operon**

The results of the above experiments suggested that reduced levels of ribosomes might cause a lower production of total protein and thus affect cellular processes. To investigate the possible effect on cell morphology, we carried out a microscopic analysis of cells growing exponentially in LB medium at 37 °C with shaking. Surprisingly, cells with a single copy of the rrm operon exhibited a clear morphological defect, namely abnormal ‘bending’, whereas those that carried two or more copies showed no noticeable effect on morphology (Fig. 5). Asai and co-workers also observed pronounced morphological changes in an E. coli rrm Δ6 strain during the exponential phase (Asai et al., 1999b). Notably, the chromosomal DNA of the B. subtilis cells with a single rrm operon was distributed evenly in the cytosol, whereas in strains with two or more copies, the nucleoids tended to be condensed in a restricted region of the cell. Hence, a clear-cut effect of increasing rrm operon copy number from one to two was again observed.

**Growth rates and sporulation frequencies in strains with different combinations of two rrm operons**

The analyses above indicated that the most marked phenotypic effects were obtained when the rrm operon copy number increased from one to two. Each rrm operon differs with respect to its location on the bacterial chromosome, its expression level and the primary structure of the rRNA genes (Condon et al., 1992; Koga et al., 2006; Nanamiya et al., 2010). To examine the effect of the presence of multiple copies of specific rrm operons, we constructed strains that carried two different rrm operons in all possible combinations of rRNA, B, D, E, I, J and O; the growth rates and sporulation frequencies of the constructed strains were then analysed. Since the recipient strains used for construction of strains with two rrm operons had deletion of ICEBs1, the derived strains carried no ICEBs1. If the phenotypic effects described above are simply the result of the number of copies of the rrm operon, then we would expect that the growth rates and sporulation frequencies of the constructed strains should be the same as those obtained above. Data for strains with one or three copies of the rrm operon are shown for comparative purposes. We found that the growth rates and sporulation frequencies of the various strains with two copies of the rrm operon fell between those of strains with one or three copies (Fig. 6). Although remarkable differences in sporulation frequency were observed among strains containing two copies of the rrm operon, the maximum and minimum values nevertheless lay between those of the strains with one or three copies. Thus, it is likely that the observed effects of the rrm operon on cellular processes in B. subtilis, such as growth rate and sporulation, are primarily caused by differences in the copy number of the rrm operon, rather than by...
heterogeneity in promoter activity, rRNA gene sequences or the chromosomal locations of the various \textit{rrn} operons.

**Effect of \textit{rrn} operon copy number on germination and outgrowth**

Next, we monitored germination and outgrowth for the spores formed by strains that possessed three to ten \textit{rrn} operons in 2 \times LB medium supplemented with 0.5 mM L-Ala as a germinant (Moeller \textit{et al.}, 2006). Spores from the strains with one or two copies of the \textit{rrn} operon could not be monitored because it was too difficult to obtain sufficient numbers of spores from these strains. The spores were prepared as described in Methods. All the spores germinated within 30 min of inoculation, during which period the OD$_{600}$ was at its lowest level (Fig. 7). However, the strains tested showed differences in growth rates after germination of the spores, with those of the wild-type strain having the fastest growth rate. These results, together with the fact that similar numbers of spores were obtained from the mutant strains that carried more than four copies of the \textit{rrn} operon, indicate that the intracellular ribosome levels severely affect not only growth and sporulation but also vegetative growth after germination. They also suggest that substantially higher levels of ribosomes are required for growth after germination than for the completion of the sporulation process.

**Motility of cells carrying one to ten copies of the \textit{rrn} operon**

It was recently shown that the deletion of the \textit{rpsU} gene (encoding the S21 ribosomal protein) led to the formation of a small circle of a swarm colony (Akanuma \textit{et al.}, 2012). To assess the effect of \textit{rrn} copy number on motility, we measured the swarm circles for the ten strains on LB agar plates containing 0.4 % Bacto agar. Swarm activity in strain RIK539 was the lowest of the ten tested strains; moreover,

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**Fig. 4.** Ribosome sedimentation profiles of strains with different numbers of \textit{rrn} operons. Crude cell extracts were sedimented through a 10–40 % sucrose gradient as described in Methods. The areas of the 70S ribosome peaks were calculated for each strain. The values shown in parentheses are relative to the value for the 70S peak of wild-type cells, which was set at 1.00. The data profiles represent the means (±SD s) of three independent experiments. The 100S peaks, which are thought to include polysomes, are also indicated.

**Fig. 5.** Microscopic images of wild-type, 4 \textit{rrn}, 3 \textit{rrn}, 2 \textit{rrn} and 1 \textit{rrn} cells. Cells were grown to the exponential phase in LB medium at 37 °C and harvested. The cells were stained with DAPI and FM4-64, then examined by phase-contrast microscopy and by fluorescence microscopy. The cells of strains with five to nine \textit{rrn} operons were indistinguishable from each other and from the wild-type, and are not included in this figure. Bars, 5 μm. Arrowheads indicate sites of abnormal cell bending.
increasing rrrn copy number from one to two had a clear effect on motility (Fig. 8). Swarm activity increased as the number of rrrn operons increased; the motility of the wild-type was the highest in the ten tested strains. Our results suggest that ten copies of the rrrn operon are necessary for efficient swarming activity.

**Effect of varying rrrn operon copy number on competence development**

*B. subtilis* has the ability to acquire exogenous DNA through an uptake machinery, a phenomenon termed 'competence development'. In order to study the effect of variation in rrrn operon copy number on competence development, we compared the transformation activities of the ten strains. Cells were grown in competence-inducing medium at 37 °C with shaking, and the numbers of tryptophan-prototroph transformants were counted. Strain RIK539 showed delayed competence development compared with the other nine strains (Fig. 9). Development of competence was significantly faster in the strain with two rrrn operons compared to that with a single copy. A marked effect of rrrn operon copy number was observed (Fig. 9). Furthermore, we found that although a significant decrease in transformation activity was observed in the wild-type strain 6 h after inoculation, this decrease did not occur in strains with nine or fewer rrrn operons. The reason for this difference in transformation activity in strains with fewer than ten rrrn operons is unknown at present.

**DISCUSSION**

Deletion of a single rrrn operon has been reported to have little effect on cell growth rates or physiological processes in either *E. coli* or *B. subtilis* (Ellwood & Nomura, 1980; Widom *et al.*, 1988). Asai and co-workers observed reduced growth rates in *E. coli* strains in which the number of rrrn operon copies was reduced from seven to five (Asai *et al.*, 1999b). We recently constructed seven strains of *B. subtilis* that each carried a single individual rrrn operon and showed that the growth profiles and sporulation frequencies differed among these strains (Nanamiya *et al.*, 2010). In the present study, to analyse the effect of the rrrn operon copy number on specific physiological processes, we constructed eight strains that contained two to nine copies of the rrrn operon. We then characterized these strains and compared them to a strain with a single copy of the rrrn operon and to a wild-type strain. The slowest growth rate was observed in the strain that carried a single copy of the rrrn operon. As the copy number of the rrrn operon was increased, the growth rate increased, which was in good agreement with a previous report on *E. coli* in which the growth rate increased gradually as the number of operons increased (Asai *et al.*, 1999b). Since it is believed that intracellular ribosome levels determine cellular protein levels, the copy number effect could simply be caused by the amount of available protein. Asai and co-workers also reported that the magnitude of the effect on rRNA/protein levels was greater than that on growth rate as the number
of rrn operons in the E. coli genome was reduced (Asai et al., 1999b). It is likely that the same situation applies in the rrn deletion mutants in B. subtilis as in E. coli, although further experiments are needed to confirm this conclusion. We also determined the sporulation frequencies of the mutant strains and obtained similar results to those for growth rates, i.e. the lowest sporulation frequency was found in the strain with a single copy of the rrn operon, and increasing the copy number of the rrn operon increased the sporulation frequencies. Interestingly, the most marked effect was observed when a second rrn operon was introduced into the strain with a single copy of the rrn operon. When all possible combinations of two rrn operons were examined, the sporulation frequencies of these strains were 10²- to 10⁴-fold higher than that of the single copy rrn mutant, although there were differences in doubling times and sporulation frequencies among the different mutant strains. Our analyses of the effect of copy number variation on motility and competence development yielded similar results as for the effect on growth rates. Therefore, our observations here suggest that various physiological features including growth rate, sporulation frequency, competence development and motility are strongly affected by rrn operon copy number. The differences among the strains with different combinations of two rrn operons might be a consequence of sequence heterogeneity, the location of the operons on the chromosome, or the transcriptional activity of each rrn operon. It has been reported in E. coli that the promoter activity of an rrn operon depends on its distance from the replication origin (Condon et al., 1992), and different rrn operons are regulated differentially during the stringent response (Samarrai et al., 2011). Thus, the differences in sporulation frequency and growth rate of the strains that carry two copies of different rrn operons might result from differences in individual promoter activities and their regulation. Further experiments will be needed to resolve this issue.

It is generally assumed that prokaryotes evolved multiple rrn operons to cope with variations in environmental conditions (Condon et al., 1992, 1995). In the present study, we showed that many physiological features are controlled by the number of copies of the rrn operon. Although the mutant strains that carried more than four copies of the rrn operon had similar sporulation frequencies (Table 2), vegetative growth after germination of spores from strains with three to nine copies was not as rapid as that of spores from the wild-type strain (Fig. 7).

Fig. 7. Germination and outgrowth profiles of spores with three to ten copies of the rrn operon. Purified spores were heated at 65 °C for 15 min and then germinated in 2× LB containing L-Ala. OD600 was measured every 15 min after inoculation. The wild-type strain has ten rrn operons.

Fig. 8. Swarming activity of strains with different numbers of rrn operons. Strains with one to ten copies of the rrn operon were precultured on LB plates, and then colonies were picked and inoculated onto the centre of LB plates containing 0.4% Bacto agar. After incubation at 30 °C for 36 h, the size of the swarm colony was measured. The data obtained from three independent experiments are shown. SDs are indicated by the vertical bars.
Thus, in these mutants, it appears that the levels of ribosomes in the mother cells are sufficient to complete sporulation, but the levels in the spores are insufficient for normal growth after germination. Additionally, the transformation activity and motility of the strains with one to nine copies of the \textit{rrn} operon were not comparable to those of the wild-type strain, which has ten copies. Thus, our results suggest that transformation activity and swarming activity are also regulated by \textit{rrn} operon copy number. It is likely that the copy number of the \textit{rrn} operon has been subjected to evolutionary pressure in relation to various processes, such as vegetative growth, sporulation, germination, competence development and swarming. Further analyses should reveal more details about the evolution of rRNA, and expand our knowledge about the importance of copy number and heterogeneity in relation to the \textit{rrn} operon.

The chromosomal DNA in cells with a single copy of the \textit{rrn} operon was distributed evenly throughout the cytosol (Fig. 5). It has been reported that \textit{B. subtilis} with a null mutation of the \textit{smc} gene, which is homologous to the eukaryotic gene encoding SMC (structural maintenance of chromosome) protein, has a defective nucleoid structure (Britton \textit{et al.}, 1998). In the mutant, the nucleoid bodies did not have the condensed appearance seen in wild-type cells but often appeared decondensed, stretched and/or elongated. Additionally, genes located in ICE\textit{Bs1} are activated in the \textit{smc} mutant due to SOS induction (Britton \textit{et al.}, 2007). There appears to be a link between the effects of activation of ICE\textit{Bs1} in the \textit{smc} mutant and deletion of ICE\textit{Bs1} in the strain with a single \textit{rrn} operon. Together with previous reports, our data suggested that deletion of ICE\textit{Bs1} in the strain RIK539 might be due to SOS induction; further experiments are needed to confirm this conclusion.

Previously, we found that suppressor mutants that restored the growth rate and sporulation ability could be easily isolated for any of the strains that carried a single copy of the \textit{rrn} operon (Nanamiya \textit{et al.}, 2010). These suppressor mutants were found to contain tandemly duplicated \textit{rrn} operons, regardless of the specific \textit{rrn} operon that was present (unpublished data). These observations confirm the finding that increasing the number of copies of the \textit{rrn} operon decreases the growth rate and increases the sporulation frequency (Table 2, Figs 2 and 3), and together they strongly suggest that the major effects of the \textit{rrn} operon on growth rate and sporulation frequency depend upon copy number rather than the specific characteristics of different \textit{rrn} operons.

In conclusion, the present analysis of the physiological importance of \textit{rrn} operon copy number in \textit{B. subtilis}
indicated that the presence of multiple copies of the \textit{rrn} operon in the genome may have evolved to facilitate faster vegetative cell growth, growth after germination, acquisition of competence and formation of a larger swarm colony. However, we could not exclude completely the possibility that specific \textit{rrn} operons might have differential roles in other as-yet-untested physiological conditions, given that only specific combinations of \textit{rrn} operons were analysed in the present study. This might be clarified in the future by use of strains with other combinations of \textit{rrn} operons.

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