Bacillus subtilis mutants harbouring a single copy of the rRNA operon exhibit severe defects in growth and sporulation

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The number of copies of rRNA genes in bacterial genomes differs greatly among bacterial species. It is difficult to determine the functional significance of the heterogeneity of each rRNA operon fully due to the existence of multiple rRNA operons and because the sequence heterogeneity among the rRNA genes is extremely low. To overcome this problem, we sequentially deleted the ten *rrn* operons of *Bacillus subtilis* and constructed seven mutant strains that each harboured a single *rrn* operon (either *rrnA*, *B*, *D*, *E*, *I*, *J* or *O*) in their genome. The growth rates and sporulation frequencies of these mutants were reduced drastically compared with those of the wild-type strain, and this was probably due to decreased levels of ribosomes in the mutants. Interestingly, the ability to sporulate varied significantly among the mutant strains. These mutants have proved to be invaluable in our initial attempts to reveal the functional significance of the heterogeneity of each rRNA operon.

INTRODUCTION

Most rRNA (*rrn*) operons in bacterial cells are composed of three rRNA genes, which encode 16S, 23S and 5S rRNA in that order (Klappenbach *et al.*, 2000). The copy number of rRNA operons differs among many bacterial genomes, although most of the genes that encode ribosomal proteins are present as a single copy (Klappenbach *et al.*, 2001). For example, *Mycoplasma genitalium*, a pathogenic bacterium whose genome size is very small (580 074 bp) (Fraser *et al.*, 1995), contains only one rRNA operon in its genome, whereas the genomes of *Escherichia coli* and *Bacillus subtilis* contain seven and ten *rrn* operons, respectively (Klappenbach *et al.*, 2001). In general, organisms that possess multiple *rrn* operons are considered to be able to grow faster than those that possess one or two operons. However, it is possible to delete one rRNA operon in either *E. coli* or *B. subtilis* without having a major effect on cell growth rate or physiology (Ellwood & Nomura, 1980; Widom *et al.*, 1988), which suggests that the full number of

copies of the rRNA operon is not required for rapid growth in these two organisms. Instead, it is assumed that many prokaryotes evolved multiple rRNA operons to cope with a variety of environmental conditions (Condon *et al.*, 1992, 1995). This raises the possibility that a functional differentiation exists among multiple rRNA operons. However, it is difficult to elucidate the functional significance of the heterogeneity among these operons because, in general, this heterogeneity is limited and the rRNA operons are highly conserved (Nomura, 1999).

B. subtilis, which is the best-characterized Gram-positive bacterium, has been studied extensively as a model for unicellular differentiation because of its ability to form dormant spores (Schaeffer *et al.*, 1965; Hoch, 1993; Grossman, 1995). Ten *rrn* operons (*rrnO*, *A*, *J*, *W*, *I*, *H*, *G*, *E*, *D*, *B*), which include the *rrnJ*–*rrnW* and *rrnI*–*rrnH*–*rrnG* clusters, have been identified in the genome of *B. subtilis* (Widom *et al.*, 1988; Kunst *et al.*, 1997; Henkin, 2002) (Fig. 1a). Clusters of tRNA genes are located downstream of the 5S rRNA genes in *rrnJ*, *I*, *E*, *D* and *B*, upstream of the 16S rRNA gene in *rrnE*, and in the spacer regions between the 16S rRNA and 23S rRNA genes in *rrnO* and *rrnA* (Fig. 2). To our knowledge, there have been few studies that have focused on the heterogeneity of rRNA sequences among the ten *rrn* operons in *B. subtilis* and the functional significance of this heterogeneity. Therefore, we sequentially deleted the *rrn* operons in *B. subtilis* strain 168 and constructed seven mutant strains, each of which

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Supplementary methods describing the construction of the strains and a supplementary table showing the oligonucleotide primers used in this study are available with the online version of this paper.

Fig. 1. (a) Location of the *rrn* operons in *Bacillus subtilis*. The numbers shown at the position of each *rrn* operon indicate the distances (kbp) between the origin of replication and the bases that correspond to the 5' end of the mature 16S rRNA in *rrnO*, *A*, *J*, *I*, *D*, *E* and *B*. Heterogeneity of 16S (b), 23S (c), and 5S rRNA (d) sequences between the different *rrn* operons in *B. subtilis*. The numbers shown above the alignments are the positions (in both *B. subtilis* and *E. coli* numbering) at which differences were found between the rRNAs; the 5' ends of mature 16S, 23S and 5S rRNA genes were each designated 1.

harboured a single *rrn* operon (either *rrnA*, *B*, *D*, *E*, *I*, *J* or *O*) in their genome. In this study, the growth profiles, ribosome content and sporulation frequency of the seven mutant strains were monitored to examine whether or not the function of each individual rRNA operon was equivalent.

METHODS

Strains and media. All the *B. subtilis* strains used in this study were isogenic with *B. subtilis* strain 168 and are listed in Table 1. The details of their construction are available in the supplementary material available with the online version of this paper. The media used included LB and LB agar (Sambrook & Russell, 2001), CI and CII media (Ashikaga *et al.*, 2000), minimal glucose (MG) agar (Rutberg, 1969) supplemented with 0.05 % ampicillin (Sigma) (MG-CH agar), and 2 × Schaeffer's sporulation medium supplemented with 0.1 % glucose (2 × SG; Leighton & Doi, 1971). When required, antibiotics were added at the following concentrations: 5 µg chloramphenicol ml⁻¹, 0.5 µg erythromycin ml⁻¹ and 5 µg kanamycin ml⁻¹.

Southern blot hybridization analysis. One microgram of chromosomal DNA from each of the wild-type and mutant strains was cut with *Bst*1107I (*Sna*I; Takara) and then the digested DNA was electrophoresed through a 0.8 % agarose gel in 1 × TAE buffer (Sambrook & Russell, 2001). After the gel had been soaked in 0.25 M HCl for 5 min with gentle shaking, it was soaked twice in denaturing buffer (0.5 M NaOH, 1.5 M NaCl) for 15 min and twice in neutralization buffer (0.5 M Tris/HCl pH 7.5, 3 M NaCl) for 15 min. Next, the digested DNA was blotted onto a Hybond-N⁺ membrane (GE Healthcare) by capillary transfer. After the membrane had been baked at 120 °C for 30 min, it was soaked in hybridization

buffer [5 × saline sodium citrate (SSC), 50 % deionized formamide, 0.02 % SDS, 0.1 % *N*-lauroylsarcosine and 2 % Blocking Reagent (Roche Diagnostics)] that contained a 23S-specific RNA probe at a final concentration of 100 ng ml⁻¹, and incubated at 40 °C for 16 h. Binding of the probe was detected using NBT/BCIP as the substrate for alkaline phosphatase according to the manufacturer's protocol (Roche Diagnostics). The template for the 23S probe was prepared from *B. subtilis* strain 168 by PCR using the primers *rrnPro.F* and *rrnPro.R* (Supplementary Table S1).

Assay for sporulation. *B. subtilis* cells were grown in 2 × SG medium for 24 h at 37 °C with shaking, and heat-resistant spores were counted by heating the cells at 80 °C for 10 min and then plating them on LB agar plates.

Microscopic imaging. Cells were grown in 2 × SG medium in the presence of 5 µg ml⁻¹ of FM4-64 (Invitrogen) for 20–22 h at 37 °C with shaking. A 0.1 ml portion of the culture was centrifuged at 12 000 g for 1 min at 25 °C. The cell pellet was resuspended in 30 µl of the culture supernatant and mounted on microscope slides covered with a thin film of 1 % agarose in distilled water. All images were acquired with a SenSys-1401E air-cooled CCD camera (Roper Scientific) attached to an Olympus BX50 microscope equipped with an 100 × UPlanApo objective.

Sucrose density gradient sedimentation analysis. Cells were grown in LB medium at 37 °C with shaking to early exponential phase (OD₆₀₀ ~0.2), harvested and then disrupted by passing them through a French press (Aminco) at 55.2 MPa. The cell debris was then removed by centrifugation as described previously (Natori *et al.*, 2007) and the supernatants were used as crude cell extracts. The OD₆₀₀ of each sample was measured before (designated *D*_b) and after (designated *D*_a) passage through the French press, and the efficiency of cell lysis (designated *E*) for each sample was calculated from the following equation: $E = D_a/D_b$.

Fig. 2. Sequence alignment of seven *B. subtilis* mutants, each of which contained the *rrnO*, *A*, *J*, *I*, *E*, *D*, or *B* operon. Detailed protocols for the construction of these mutants are described in the supplementary material available with the online version of this paper. rRNA genes and tRNA genes are shown by closed and open boxes, respectively. The -10 regions of the promoters and the transcriptional start sites (indicated in boldface) are shown by rectangles and bars with arrows, respectively. The junction points created by the introduction of the deletion are shown by the bars connected to dotted lines, and the dotted lines indicate the regions that were deleted. The additional restriction sites created by the introduction of the deletion mutation of *rrnHG*, as well as *rrnW3*, at the junction points, are shown with an open arrowhead. The 5S rRNA sequences that remained in each deletion mutant are boxed.

The OD units (designated *U*) of each sample that had been prepared from 500 ml of LB medium were calculated as $U = 500 \times L \times E$, where *L* is the OD₆₀₀ value for each culture at harvesting. Next, the A₂₆₀ of

the extract from the wild-type cells was measured and the volume of wild-type extract (designated *A*_{wt}) that contained 10 A₂₆₀ units was calculated. This corresponded to the volume of extract used for the

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

Strain	Genotype (characteristics)	Source or reference
168	<i>trpC2</i> (wild-type)	Laboratory stock
RIK539	<i>trpC2</i> Δ <i>rrnHG1</i> Δ <i>rrnO1</i> Δ <i>rrnD1</i> Δ <i>rrnE1</i> Δ <i>rrnB2</i> Δ <i>rrnI2</i> Δ <i>rrnW2</i> Δ <i>rrnJ1::cat</i> (Δ 9 <i>rrnA</i> ⁺)	This study
RIK540	<i>trpC2</i> Δ <i>rrnHG1</i> Δ <i>rrnO1</i> Δ <i>rrnD1</i> Δ <i>rrnE1</i> Δ <i>rrnA1</i> Δ <i>rrnI2</i> Δ <i>rrnW2</i> Δ <i>rrnJ1::cat</i> (Δ 9 <i>rrnB</i> ⁺)	This study
RIK541	<i>trpC2</i> Δ <i>rrnHG1</i> Δ <i>rrnO1</i> Δ <i>rrnE1</i> Δ <i>rrnB2</i> Δ <i>rrnA1</i> Δ <i>rrnI2</i> Δ <i>rrnW2</i> Δ <i>rrnJ1::cat</i> (Δ 9 <i>rrnD</i> ⁺)	This study
RIK542	<i>trpC2</i> Δ <i>rrnHG1</i> Δ <i>rrnO1</i> Δ <i>rrnD1</i> Δ <i>rrnE1</i> Δ <i>rrnB2</i> Δ <i>rrnA1</i> Δ <i>rrnW2</i> Δ <i>rrnJ1::cat</i> (Δ 9 <i>rrnI</i> ⁺)	This study
RIK543	<i>trpC2</i> Δ <i>rrnHG1</i> Δ <i>rrnD1</i> Δ <i>rrnE1</i> Δ <i>rrnB2</i> Δ <i>rrnA1</i> Δ <i>rrnI2</i> Δ <i>rrnW2</i> Δ <i>rrnJ1::cat</i> (Δ 9 <i>rrnO</i> ⁺)	This study
RIK545	<i>trpC2</i> Δ <i>rrnHG1</i> Δ <i>rrnO1</i> Δ <i>rrnD1</i> Δ <i>rrnB2</i> Δ <i>rrnA1</i> Δ <i>rrnI2</i> Δ <i>rrnW2</i> Δ <i>rrnJ1::cat</i> (Δ 9 <i>rrnE</i> ⁺)	This study
RIK551	<i>trpC2</i> Δ <i>rrnHG1</i> Δ <i>rrnO1</i> Δ <i>rrnD1</i> Δ <i>rrnE1</i> Δ <i>rrnB2</i> Δ <i>rrnA1</i> Δ <i>rrnI2</i> Δ <i>rrnW3</i> (Δ 9 <i>rrnJ</i> ⁺)	This study

sucrose density gradient sedimentation analysis. Then, the appropriate volume of extract for each mutant (designated pA_{rrnX}) for sucrose density gradient sedimentation analysis was determined according to the following equation: $pA_{rrnX} = A_{wt} \times (U_{wt}/U_{rrnX})$, where U_{wt} and U_{rrnX} are the OD units of the wild-type sample and each mutant sample, respectively.

To normalize the applied volumes of the samples further, each strain was cultured in 1 ml LB medium at 37 °C with shaking and harvested at an OD_{600} of ~0.2. Chromosomal DNA was then extracted from these samples. The amounts of chromosomal DNA extracted from the cells were quantified by spectrophotometric assay and agarose gel electrophoresis. Then the ratio of DNA content of each mutant strain to that of the wild-type (designated R) was calculated using the following equation, which normalized the values with respect to the ratio of OD_{600} values for the mutant and wild-type cultures: $R_{rrnX} = (OD_{wt}/OD_{rrnX}) \times (C_{wt}/C_{rrnX})$, where OD_{wt} and OD_{rrnX} are the OD_{600} values for the wild-type and mutant cultures, respectively, and C_{wt} and C_{rrnX} are the total amounts of chromosomal DNA extracted from the wild-type and mutant cells, respectively. Finally, the volume of extract for each mutant (designated A_{rrnX}) to be used for sucrose density gradient sedimentation analysis was determined from the following equation: $A_{rrnX} = pA_{rrnX} \times R_{rrnX}$.

The aliquots of extract were layered onto 10–40 % sucrose density gradients and centrifuged at 4 °C for 17.5 h at 65 000 g (HITACHI P40ST rotor). Absorbance profiles were monitored at 254 nm using a Piston Gradient Fractionator (Biocomp) and a Bio-mini UV Monitor (ATTO).

RESULTS

Construction of seven mutant strains harbouring a single *rrn* operon (*rrnA*, *B*, *D*, *E*, *I*, *J* or *O*)

Before we sequentially deleted the rRNA operons in the genome of *B. subtilis* strain 168, we resequenced all ten *rrn* operons. The complete genome sequence of *B. subtilis* was obtained originally using strain 168, which is used as the parental strain in our laboratory. We identified sequence heterogeneity at nine, eight and eight locations in the 16S, 23S and 5S rRNA genes, respectively (Fig. 1b, c, d). It was noteworthy that, of the ten 5S genes, only the 5S gene in the *rrnD* operon differed substantially from the other 5S genes. This suggested that the 5S rRNA expressed from the *rrnD* operon might have a different role in the function of ribosome to 5S rRNAs encoded by the other operons.

To determine whether the function of all the rRNA operons was equivalent, we decided to construct a range of mutant strains, each of which carried only one of the rRNA operons in their genome. Some laboratory strains of *B. subtilis* contain only nine *rrn* operons due to spontaneous deletions within *rrnW*, *H* or *G* (Widom *et al.*, 1988). This indicates that these operons are dispensable for growth in *B. subtilis* in the presence of the other *rrn* genes. Therefore, we focused on the other seven *rrn* operons, i.e. the first operons of the contiguous *rrnJ*–*rrnW* and *rrnI*–*rrnH*–*rrnG* clusters (*rrnJ* and *rrnI*), and the noncontiguous operons *rrnO*, *A*, *E*, *D* and *B*. We constructed seven mutant strains

that each harboured a single *rrn* operon (either *rrnA*, *B*, *D*, *E*, *I*, *J* or *O*) in their genome (Fig. 2).

Multiple deletions of *rrn* operons have been constructed previously in *E. coli* by the sequential deletion of the 16S and 23S rRNA genes; however, the 5S rRNA genes were left intact (Asai *et al.*, 1999a, b). On the other hand, as described above, it is assumed that 5S rRNA expressed from the *rrnD* operon in *B. subtilis* has a different role in the function of ribosome to 5S rRNA encoded by the other operons (Fig. 1d). In order to investigate the function of 5S rRNA expressed from *rrnD*, it was desirable to construct a strain which carries multiple deletion mutations of 16S, 23S and 5S rRNA genes. Therefore, we attempted to delete the 5S rRNA sequences in each *rrn* operon. To obtain the PCR products, which were used as donor DNAs to delete the rRNA operons, the upstream and downstream regions of each rRNA operon were first amplified by PCR. The two fragments obtained were then used simultaneously as the template for the next PCR amplification to generate the DNA fragments covering the deletion of the rRNA, as described in the supplementary material. During these processes, we used the forward primer *rrnXDF* (Supplementary Table S1), which was designed to be used commonly to amplify the downstream regions of each rRNA operon, to cover the bases between +62 and +84 (+1 denotes the 5' end of the mature 5S rRNA) of each 5S rRNA gene. Therefore, the 3' portion of the 5S rRNA gene (bases +62 to +116) was included in the deletion mutants of *rrnO*, *I*, *E*, *D* and *B* (Fig. 2). In addition, given that *rrnADF*, which was used for amplifying the downstream region of *rrnA*, was designed in the same manner as *rrnXDF*, the deletion mutant of *rrnA* also contained the 3' portion of the 5S rRNA gene (Fig. 2). On the other hand, the deletion mutants of *rrnW* ($\Delta rrnW3$), *G* and *H* did not contain any portion of the 5S rRNA gene (Fig. 2), even though a *Bam*HI site (ggatcc), which was created via the cloning procedures as described in the supplementary material, was included in each deletion (Fig. 2). In addition, the deletion mutant of *rrnW* ($\Delta rrnW2$), as well as the replacement of *rrnJ* with the *cat* gene (*rrnJ1::catpt1*), did not contain any portion of the 5S rRNA gene (Fig. 2).

There are various tRNA genes in each operon (Fig. 2). It has not been clarified, so far, whether these tRNA genes are actually required for cell proliferation. Therefore, most of these tRNA genes, including the *trnJ*, *trnI*, *trnE*, *trnD* and *trnB* clusters, remained intact after the introduction of the deletion mutations into the rRNA operons (Fig. 2). On the other hand, two tRNA genes, tRNA^{Ile} and tRNA^{Ala}, are located between the 16S and 23S rRNA genes in both the *rrnO* and *rrnA* operons (Loughney *et al.*, 1982; Ogasawara *et al.*, 1983). Given that the DNA sequences of these two genes are the same, the tRNA genes in the *rrnA* operon were left intact, but the equivalent tRNA genes in *rrnO* were deleted, as shown in Fig. 2. In addition, given that many of these tRNA genes are co-expressed from the

promoters of each rRNA operon, none of the *rrn* promoters was deleted.

The $\Delta 9$ *rrnJ*⁺ (RIK551) mutant, which harboured a single *rrnJ* operon, contained the Δ *rrnW*3 mutation, whereas the other mutants that harboured a single rRNA operon contained the Δ *rrnW*2 mutation (Fig. 2, Table 1). During the construction of the $\Delta 9$ *rrnJ*⁺ strain, transformants that carried a tandem duplication of *rrnJ* were obtained. It is likely that this duplication of the *rrnJ* operon could be generated by unequal crossing-over via the promoter region of *rrnW* (from bases -166 to -50; +1 denotes the 5' end of the mature 16S rRNA of *rrnW*), which was present in the Δ *rrnW*2 deletion mutant. Therefore, we deleted the entire *rrnW* operon, which included the region between -166 nt from the 5' end of the mature 16S rRNA and the 3' end of the mature 5S rRNA in *rrnW*, and named this deletion Δ *rrnW*3 (Fig. 2).

The detailed protocols for the construction of the mutant that carried multiple deletions of the *rrn* operons are described in the supplementary material. The deletion of the rRNA genes in these strains was verified by Southern blot analysis using a riboprobe that was specific for the 23S rRNA gene. This confirmed that each strain contained a single *rrn* operon in its genome; a typical result is shown in Fig. 3.

Growth rates and intracellular ribosome levels differ among the mutant strains

First we monitored the growth profiles of the seven mutant strains, which each harboured only one rRNA operon, in a nutrient-rich medium. All of the strains that carried a single rRNA operon grew more slowly than the wild-type strain. This result is consistent with a previous report in which the introduction of multiple deletions of rRNA

Fig. 3. Confirmation of the multiple deletion of the *rrn* operons by Southern blot analysis. *Bst*1107I digests were electrophoresed through a 0.8% agarose gel and probed with a 23S-specific RNA probe as described in Methods. Given that the *Bst*1107I site located between *trnJ* and *rrnW* was deleted simultaneously with the sequence of *rrnW*, the size of the band derived from the *rrnJ* 23S rRNA in the strain $\Delta 9$ *rrnJ*⁺ (approx. 9.6 kb) differed from that of the wild-type (approx. 3.0 kb).

operons in *E. coli* caused a decrease in the growth rate (Asai *et al.*, 1999b). However, the growth rates of the *B. subtilis* mutants differed: the doubling times of the strains that harboured *rrnO* ($\Delta 9$ *rrnO*⁺), *rrnA* ($\Delta 9$ *rrnA*⁺), *rrnJ* ($\Delta 9$ *rrnJ*⁺), *rrnI* ($\Delta 9$ *rrnI*⁺), *rrnE* ($\Delta 9$ *rrnE*⁺), *rrnD* ($\Delta 9$ *rrnD*⁺) and *rrnB* ($\Delta 9$ *rrnB*⁺) were 69 min, 58 min, 57 min, 66 min, 62 min, 79 min and 80 min, respectively, whereas that of the wild-type strain was 23 min (Fig. 4). One plausible explanation for these observations is that the expression of rRNA and/or the intracellular ribosome concentration is decreased significantly in the $\Delta 9$ *rrnD*⁺ and $\Delta 9$ *rrnB*⁺ strains. To test this hypothesis, the intracellular concentration of 70S ribosomes was monitored by sucrose density gradient sedimentation analysis. The volume of each sample that was applied to the gradient was calculated by determining the ratio of chromosomal DNA between wild-type and mutant cells as described in Methods. Each of the strains that harboured a single rRNA operon contained a lower level of 70S ribosomes than that found in the wild-type cells (Fig. 5). The relative levels of 70S ribosomes in the $\Delta 9$ *rrnI*⁺, $\Delta 9$ *rrnE*⁺, $\Delta 9$ *rrnD*⁺ and $\Delta 9$ *rrnB*⁺ strains were 0.28, 0.3, 0.20 and 0.23, respectively (where the level of 70S ribosomes in the wild-type cells was set at 1.0), whereas in the $\Delta 9$ *rrnO*⁺, $\Delta 9$ *rrnA*⁺ and $\Delta 9$ *rrnJ*⁺ strains the levels were 0.34, 0.29 and 0.35, respectively (Fig. 5). It was assumed that, during the exponential growth phase, the level of ribosomes in each mutant that harboured a single rRNA operon was, at least in part, dependent on the location of each operon in the genome. Namely, as the distance between the origin of DNA replication and the location of the rRNA operon in the genome increased, the level of 70S ribosomes decreased (Figs 1a and 5). Given that multiple replication forks of the chromosomal DNA are found in exponentially growing cells, the copy numbers of *rrnO*, *rrnA* and *rrnJ*, which are

Fig. 4. (a, b) Growth characteristics of various $\Delta 9$ *rrn* strains that harboured a single rRNA operon. Cells were incubated in LB medium at 37 °C with shaking and the OD₆₀₀ of the cultures was measured at the indicated times after inoculation. The curves for the wild-type strain are shown in both (a) and (b).

Fig. 5. Ribosome sedimentation profiles from the strains that harboured a single rRNA operon. Crude cell-extracts were sedimented through a 10–40 % sucrose gradient as described in Methods. Representative results of the ribosome profiles obtained by three independent experiments are shown. The areas of the 70S ribosome peaks were measured for each strain. The values shown in parentheses are relative to the value for the 70S peak in wild-type cells, which was set at 1.0. The values are the means $\pm$ SD of three independent experiments.

located near the origin of replication, would be relatively higher than those of *rrnD* and *rrnB*, which are located approximately 950 kb and 1000 kb, respectively, from the origin of replication. Therefore, the expression of rRNA genes in $\Delta 9$ *rrnO*⁺, $\Delta 9$ *rrnA*⁺ and $\Delta 9$ *rrnJ*⁺ would be expected to be higher than in $\Delta 9$ *rrnD*⁺ and $\Delta 9$ *rrnB*⁺, thereby resulting in larger amounts of ribosomes in $\Delta 9$ *rrnO*⁺, $\Delta 9$ *rrnA*⁺ and $\Delta 9$ *rrnJ*⁺ cells as compared with those in $\Delta 9$ *rrnD*⁺ and $\Delta 9$ *rrnB*⁺ cells. Together with the observation that the growth rates of $\Delta 9$ *rrnD*⁺ and $\Delta 9$ *rrnB*⁺ were significantly lower than those of $\Delta 9$ *rrnA*⁺ and $\Delta 9$ *rrnJ*⁺ (Fig. 4a), these results suggest that the slower growth rates observed in the strains that harboured a single rRNA operon might be due to a reduction in the intracellular levels of ribosomes.

Sporulation frequencies differ among the strains that harbour a single rRNA operon

The sporulation frequencies of the strains containing a single rRNA operon were determined as described in Methods. Among the mutant strains, $\Delta 9$ *rrnE*⁺ yielded the highest number of spores (1.7×10^6 ml⁻¹) at 24 h after inoculation in $2 \times$ Schaeffer's sporulation medium (Table 2). However, this number was two orders of magnitude lower than that obtained from the wild-type strain (6.8×10^8 ml⁻¹) (Table 2). In addition, the numbers of spores obtained from $\Delta 9$ *rrnI*⁺ and $\Delta 9$ *rrnB*⁺ (3.1×10^3 ml⁻¹ and 6.5×10^2 ml⁻¹, respectively) were five and six orders of magnitude lower than that obtained for the wild-type (Table 2). Moreover, the number of spores produced by the $\Delta 9$ *rrnD*⁺ strain was below the detection limit (Table 2). Therefore, it is clear from these results that sporulation efficiency differed significantly among the mutant strains. To determine the stage of sporulation that is blocked in the mutant strains harbouring a single *rrn* operon we next carried out fluorescence microscopy analysis using the vital membrane stain FM 4-64. When cells were incubated in $2 \times$ Schaeffer's sporulation medium at 37 °C for 20–22 h, most of the wild-type cells were observed as free spores (Fig. 6a). However, the mutant cells, except for $\Delta 9$ *rrnE*⁺ cells (Fig. 6b), were not able to

form spores and were blocked at the early stages of sporulation, where the apparent asymmetrical septum, seen in wild-type cells, was not observed (Fig. 6c, d show a representative result obtained with $\Delta 9$ *rrnB*⁺ cells). These results strongly suggested that a single rRNA operon was not sufficient to support efficient spore development. On the other hand, refractile bodies were observed in $\Delta 9$ *rrnE*⁺, although the number was apparently low when compared with that of the wild-type strain (Fig. 6b). This result was coincident with the fact that $\Delta 9$ *rrnE*⁺ exhibited the highest number of spores among the mutant strains (Table 2).

Next we examined the efficiency of the mutant strains to develop the ability to take up exogenous DNA. The induction of the developmental pathways for competence was delayed in all of the strains that contained a single rRNA operon (data not shown). This could be a result of the slow-growth phenotypes. Given that all of the mutant strains were able to develop competence, although with a lower frequency than the wild-type strain, these strains should be very useful for genetic analyses and for investigating the functions of each rRNA gene.

Table 2. Sporulation of the strains harbouring a single rRNA operon

Strain	C.f.u. ml ⁻¹ *		Frequency (%)*
	Total	Spores	
168 (wild-type)	8.1×10^8	6.8×10^8	84.0 ± 3.4
RIK543 ($\Delta 9$ <i>rrnO</i> ⁺)	7.6×10^8	4.4×10^4	$5.6 (\pm 2.5) \times 10^{-3}$
RIK539 ($\Delta 9$ <i>rrnA</i> ⁺)	5.1×10^8	5.6×10^3	$10 (\pm 5.4) \times 10^{-4}$
RIK551 ($\Delta 9$ <i>rrnJ</i> ⁺)	6.9×10^8	5.5×10^3	$6.5 (\pm 4.7) \times 10^{-4}$
RIK542 ($\Delta 9$ <i>rrnI</i> ⁺)	4.9×10^8	3.1×10^3	$5.8 (\pm 3.4) \times 10^{-4}$
RIK545 ($\Delta 9$ <i>rrnE</i> ⁺)	6.7×10^8	1.7×10^6	$2.5 (\pm 2.2) \times 10^{-1}$
RIK541 ($\Delta 9$ <i>rrnD</i> ⁺)	4.1×10^8	<10	<0.000003
RIK540 ($\Delta 9$ <i>rrnB</i> ⁺)	5.2×10^8	6.5×10^2	$1.4 (\pm 1.1) \times 10^{-4}$

*Means of three independent experiments ($\pm$ SD for sporulation frequency).

Fig. 6. Microscopic observation of wild-type (a), $\Delta 9$ *rrnE*⁺ (b), and $\Delta 9$ *rrnB*⁺ (c, d) cells. Cells grown in 2×Schaeffer's sporulation medium at 37 °C for 20–22 h were collected and phase-contrast images (a, b, c) and a fluorescence image using FM4-64 (d) were obtained. A refractile body found in $\Delta 9$ *rrnE*⁺ (b) is indicated by an arrow. Bars, 5 μ m.

DISCUSSION

An *E. coli* strain that lacks all seven *rrn* operons has been constructed previously (Asai *et al.*, 1999a, b). In this strain, which carried no chromosomal copy of an intact rRNA operon, rRNA molecules were expressed from a multicopy plasmid that contained a single rRNA operon. This strain has contributed to the identification and characterization of novel mutations in the rRNA genes that confer resistance to antibiotics (Recht & Puglisi, 2001). It has also contributed to the characterization of the functional domain in the rRNA molecules that is required for translation (Sergiev *et al.*, 2005; Hirabayashi *et al.*, 2006). Similar genetic systems have also been developed using *Synechococcus* PCC 7942 (Monshupanee *et al.*, 2006) and *Mycobacterium smegmatis* (Sander *et al.*, 1996; Hobbie *et al.*, 2006). However, at least to our knowledge, there have been no other reports of studies that clarify the functional differentiation among multiple rRNA operons, with the exception of a study in *Plasmodium*, in which two distinct genes that encode 18S rRNA were shown to be expressed differentially in different stages of the life cycle (Gunderson *et al.*, 1987). Therefore, we constructed seven different novel strains of *B. subtilis*, each of which harboured a different single rRNA operon.

One notable difference among the strains that harboured a single rRNA operon was with respect to spore formation. All of the mutant strains grew more slowly than the wild-type during the exponential growth phase (Fig. 4). In addition, we found that the expression of *spo0A* from the *P_s* promoter, which is recognized by the sporulation initiation sigma factor, σ^H (Chibazakura *et al.*, 1991;

Predich *et al.*, 1992), was hardly detected in the $\Delta 9$ *rrnO*⁺ strain (data not shown). Also, microscopic observations revealed that most of the mutant cells were blocked at the early stages of sporulation, where no obvious asymmetrical septum was found (Fig. 6). These results strongly suggested that the reduced sporulation frequencies seen in the mutant strains were caused by a delay in the entry into the initiation of sporulation, which could be due to the reduced growth rates caused by a decrease in the intracellular levels of ribosomes during the exponential growth phase (Fig. 5).

On the other hand, sporulation frequency differed considerably among these strains (Table 2). It was noteworthy that the sporulation frequencies of $\Delta 9$ *rrnD*⁺ and $\Delta 9$ *rrnB*⁺ were extremely low when compared with that of the other mutant strains (Table 2). The difference in spore formation might also be explained by the heterogeneity of the rRNA sequences. For example, in the 23S rRNA expressed from eight of the *rrn* operons in *B. subtilis*, the nucleotide at position 327 (in *B. subtilis* numbering) is G. However, in the 23S rRNA expressed from *rrnD* or *rrnB* this nucleotide is replaced by A (Fig. 1). Nucleotide 327 is located within a 39 nt sequence that is inserted near helix 16–18 in domain I of the *B. subtilis* 23S rRNA but is not present in the *E. coli* sequence. This additional sequence is commonly found in *Bacillus* species, which suggests that this region could play an important role in sporulation. Although the molecular details remain unclear, the G327A substitution found in *rrnD* and *rrnB* might result in a reduction in the numbers of spores produced. However, it could not simply be concluded that the variation in sporulation ability among these mutants was due to the

heterogeneity among the rRNA genes. Rather, as described above, growth rates, as well as intracellular levels of ribosomes, in each mutant during exponential growth would strongly affect the initiation of spore formation. In fact, the intracellular levels of ribosomes in strains $\Delta 9$ *rrnD*⁺ and $\Delta 9$ *rrnB*⁺ were particularly low among the mutant strains that harboured a single rRNA operon, and the doubling times of $\Delta 9$ *rrnD*⁺ and $\Delta 9$ *rrnB*⁺ were relatively longer than those of the other mutant strains during exponential growth (Figs 4 and 5). It is thus strongly suggested that a drastic reduction in the sporulation frequency of the $\Delta 9$ *rrnD*⁺ and $\Delta 9$ *rrnB*⁺ is due to severe growth defects during the exponential growth phase.

In conclusion, the deletion mutants that we have constructed have proved invaluable in the initial attempts described herein to clarify the functional significance of each rRNA operon. However, at present, we cannot fully explain the significance of the chromosomal location of each rRNA operon using these strains. To resolve this issue, mutant strains that harbour a single rRNA operon should be constructed in which each individual rRNA operon is introduced into the same chromosomal location and transcribed from the same promoter. Given that the molecular genetic system in *B. subtilis* has been well established due to the ability of the bacterium to develop natural genetic competence, these seven strains, which each harbour a single rRNA operon, should provide novel insights into the significance of the presence of multiple rRNA operons in bacteria in the near future.

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