Enhanced expression of *Bacillus subtilis yaaA* can restore both the growth and the sporulation defects caused by mutation of *rplB*, encoding ribosomal protein L2

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A temperature-sensitive mutation in *rplB*, designated *rplB142*, encodes a missense mutation at position 142 [His (CAT) to Leu (CTT)] of *Bacillus subtilis* ribosomal protein L2. The strain carrying the mutation grew more slowly than the wild-type, even at low temperatures, probably due to the formation of defective 70S ribosomes and the accumulation of incomplete 50S subunits (50S* subunits). Gel analysis indicated that amounts of L2 protein and also of L16 protein were reduced in ribosomes prepared from the *rplB142* mutant 90 min after increasing the growth temperature to 45 °C. These results suggest that the assembly of the L16 protein into the 50S subunit requires the native L2 protein. The H142L mutation in the defective L2 protein affected sporulation as well as growth, even at the permissive temperature. A suppressor mutation that restored both growth and sporulation of the *rplB142* mutant at low temperature was identified as a single base deletion located immediately upstream of the *yaaA* gene that resulted in an increase in its transcription. Furthermore, genetic analysis showed that enhanced synthesis of YaaA restores the functionality of L2 (H142L) by facilitating its assembly into 50S subunits.

**INTRODUCTION**

The eubacterial ribosome (70S) is a complex macromolecule that is composed of a small (30S) subunit and a large (50S) subunit. The small subunit comprises the 16S rRNA and more than 20 proteins, whereas the large subunit comprises the 23S and 5S rRNAs and more than 30 proteins (Nomura, 1970; Kurland, 1972). *Bacillus subtilis* ribosomal protein L2, which is composed of 277 amino acids, is encoded by the *rplB* gene and is the largest ribosomal protein in the 50S subunit. Due to the high degree of conservation and its functional importance, L2 has been considered to be one of the most evolutionarily ancient of the ribosomal proteins (Tanaka et al., 2000). Biochemical and genetic analyses, including reconstitution analysis, footprinting and mutational analysis, have demonstrated that L2 is a primary RNA-binding protein that recognizes helix 66 of the 23S rRNA and plays an important role in ribosome assembly (Nomura & Erdmann, 1970; Herold & Nierhaus, 1987; Gulle et al., 1988; Romero et al., 1990; Eggebjerg et al., 1991; Hayashi et al., 2002; Kitahara et al., 2007). Furthermore, crystal structure analyses have revealed that L2 is localized to the intersubunit space of the 70S ribosome and that amino (N)- and carboxy (C)-terminal extensions of L2 are proximal to the peptidyltransferase centre in the 23S rRNA (Ban et al., 2000; Nissen et al., 2000; Yusupov et al., 2001). The functional activities of L2, such as the assembly of the ribosomal subunits, tRNA binding to the A- and P-sites, peptidyltransferase activity and peptide-bond formation, have been demonstrated in several investigations, including the use of reconstituted 50S subunits lacking L2 (Schulze & Nierhaus, 1982; Uhlein et al., 1998; Khaitovich et al., 1999; Diedrich et al., 2000; Willumeit et al., 2001). Although the essential role that L2 plays in ribosome biogenesis and translation has been known for some time, more recently this subunit has also been shown to be involved in transcription by interacting with the x-subunit of RNA polymerase and in...
Fig. 1. Growth characteristics and ribosome sedimentation profiles of the rplB142 mutant, and its suppressor (rplB142 srb1) strain. (a) Cells were grown on LB plates at 30, 37 or 45 °C for 18 h. (b) Cells were grown in LB at 30 °C with shaking, and at the indicated times the OD _600_ of the cultures was measured. (c) Crude cell extracts were sedimented through a 10–40% sucrose gradient as described in the Supplementary Methods.

Table 1. Sporulation of rplB142 and rplB142 srb1 mutant strains

Values are means (±SD) of three independent experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>c.f.u. ml⁻¹</th>
<th>Frequency (%)</th>
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<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Spores</td>
</tr>
<tr>
<td>32 °C Wild-type</td>
<td>7.8 (±1.0) x 10⁸</td>
<td>6.4 (±0.6) x 10⁸</td>
</tr>
<tr>
<td>rplB142</td>
<td>4.8 (±0.7) x 10⁸</td>
<td>2.5 (±1.5) x 10⁴</td>
</tr>
<tr>
<td>rplB142 srb1</td>
<td>5.0 (±0.4) x 10⁸</td>
<td>4.4 (±1.1) x 10⁸</td>
</tr>
<tr>
<td>37 °C Wild-type</td>
<td>8.0 (±1.1) x 10⁸</td>
<td>7.3 (±1.1) x 10⁸</td>
</tr>
<tr>
<td>rplB142</td>
<td>5.9 (±0.8) x 10⁸</td>
<td>1.0 (±1.3) x 10⁵</td>
</tr>
<tr>
<td>rplB142 srb1</td>
<td>4.0 (±0.3) x 10⁸</td>
<td>1.9 (±0.9) x 10⁷</td>
</tr>
</tbody>
</table>
(a) wt

OD_{600} (32 °C vs 45 °C)

Time (h) after inoculation

(b) rplB142

OD_{600} (32 °C vs 45 °C)

Time (h) after inoculation

(c) wt

Direction of sedimentation

(d) rplB142

Direction of sedimentation

(e) wt 70S 45 °C 90 min

(f) rplB142 70S 45 °C 90 min
replication by inhibiting oligomer formation of DnaA (Rippa et al., 2010; Chodavarapu et al., 2011). Taken together, the experimental data indicate that ribosomal protein L2 is a multifunctional protein.

Although ribosomal subunits can be reconstituted from isolated rRNA and ribosomal proteins in vitro, this process requires long incubation times, high temperatures and high ionic strength, compared with the physiological conditions in prevailing in the cell (Dohme & Nierhaus, 1976). Thus, there is no doubt that there are proteins in the cell that facilitate ribosome assembly. In Escherichia coli, the RNA helicases CsdA, DbpA and SmrB have been proposed to be involved in ribosome biogenesis (Jones et al., 1996; Diges & Uhlenbeck, 2001; Charollais et al., 2003), whereas in B. subtilis, CshA, CshB and YfmL have been suggested to participate in this process (Lehnik-Habrink et al., 2013). Several GTPases also facilitate 50S subunit assembly, namely RgaB, YscC and YphC in B. subtilis and Der and ObgE in E. coli (Hwang & Inouye, 2006; Jiang et al., 2006; Matsuo et al., 2006; Schaefer et al., 2006; Uicker et al., 2006; Wicker-Planquart et al., 2008). In addition, using a proteomic approach, several candidate ribosome-maturation factors have been identified (Jiang et al., 2007). Therefore, it is clear that a large number of proteins participate in ribosome biogenesis.

Recently, we generated a set of mutants containing deletions in the 57 annotated ribosomal protein-encoding genes of B. subtilis and found that at least 22 of these genes were not individually essential for cell proliferation (Akanuma et al., 2013). Using this method, we introduced single mutations into the rplC and rpsJ genes, which encode the essential ribosomal proteins L3 (RplC) and S10 (RpsJ), respectively, and found that these mutations affect growth rate as well as sporulation (Akanuma et al., 2013).

In the present study, we isolated a temperature-sensitive rplB142 mutant that carried a missense mutation in the gene coding for the essential ribosomal protein L2, and provide evidence indicating that a step in the assembly of the 50S subunit was inhibited in the mutant at high temperature. Furthermore, enhanced expression of yaaA was found to suppress the rplB142 mutant phenotype at low temperature, probably at a step in the assembly of the 50S subunit.

**METHODS**

**Bacterial strains and plasmids.** All of the B. subtilis 168 strains and oligonucleotide primers used in the study are listed in Tables S1 and S2 (available in the online Supplementary Material).

**Media.** The media used included Luria–Bertani (LB) medium and LB agar (Sambrook & Russell, 2001), CI and CII media (Ashikaga et al., 2000), minimal glucose agar supplemented with 0.05% ampicillin (Sigma; Rutberg, 1969), and 2× Schaeffer’s sporulation medium supplemented with 0.1% glucose (Leighton & Doi, 1971). When required, antibiotics were added at the following concentrations: chloramphenicol, 5 μg ml⁻¹; erythromycin, 0.5 μg ml⁻¹.

**70S ribosome formation experiments.** Native 30S and 50S ribosomal subunits and the incomplete 50S* subunits were partially purified from wild-type and rplB142 mutant cells grown in LB medium at 32 °C with shaking, respectively. The preparation of purified small and large subunits and association experiments were performed as described by Natori et al. (2007) with a slight modification. Briefly, the crude ribosomes from wild-type cells cultivated to vegetative phase (OD600 of ~0.2) in 3.5 litres of LB medium were centrifuged at 65 000 g at 4 °C for 17 h in a 10–40% sucrose gradient in buffer IV [20 mM Tris/HCl, pH 7.6, 1 mM (CH3COO)2Mg, 100 mM CH3COONH4, 6 mM β-mercaptoethanol and 2 mM PMSF] in a Hitachi P28SA rotor to separate the 30S and 50S subunits. The crude ribosomes from rplB142 mutant cells cultivated to vegetative phase (OD600 of ~0.2) in 3.5 litres of LB medium were centrifuged at 65 000 g at 4 °C for 17 h in a 10–40% sucrose gradient in buffer I (Natori et al., 2007) in a Hitachi P28SA rotor to separate the 30S, and 50S subunits and 70S ribosomes. 50S subunits were diluted (1 : 2) with buffer I, sedimented at 65 000 g for 21 h at 4 °C in a Hitachi P40ST rotor and suspended in buffer I. The partially purified 50S subunits of the wild-type cells and incomplete 50S* subunits of the rplB142 mutant cells contained approximately 5.5% of 70S ribosomes and 6.4% of 30S subunits, respectively (Fig. S3). The purified small subunit (A60=1.5) and large subunit (A260=4) were mixed in buffer I. The mixture was centrifuged at 55 000 g for 17.5 h at 4 °C in a 10–40% sucrose gradient in buffer I after incubation for 15 min at 37 °C or in buffer IV without incubation in a Hitachi P40ST rotor. Absorbance profiles were monitored at 254 nm using a Piston Gradient Fractionator (Biocomp) and a Bio-mini UV Monitor (ATTO).

**Other methods.** Other methods, such as primer extension, sucrose gradient centrifugation, preparation of ribosomes, 2D gel electrophoresis and assay for sporulation, are described in the Supplementary Methods.
RESULTS

Isolation and characterization of a temperature-sensitive L2 mutant and its suppressor mutant

To delineate the functions of ribosomal proteins whose genes are located in the S10 gene cluster, we isolated a temperature-sensitive mutant that carried three base changes in the rplB gene encoding the ribosomal protein L2. Only a single amino acid, at position 142 (CAT for His to CTT for Leu), was altered in the mutant, given that the other two base changes, at positions 160 (ACA to ACG; both Ser) and 186 (TCT to TCA; both Thr), were silent mutations. The rplB142 mutant did not grow on LB medium at 45°C but did grow at significantly slower rates than the wild-type at 30 and 37°C (Fig. 1a, b and Fig. S1a). In addition, the sporulation frequencies of this mutant at 32 and 37°C were also significantly lower than those of the wild-type (Table 1).

As shown in Fig. 1(b), a spontaneously isolated suppressor mutant had a similar growth rate to that of the wild-type at 30°C and the mutation was designated srb1 (suppressor of rplB). The sporulation frequency of the rplB142 mutant at 32°C was also restored to that of the wild-type by the srb1 suppressor mutation, although growth at 45°C was not restored (Fig. 1a and Table 1).

Ribosomal protein L2 is one of the early assembling proteins of the 30S subunit (Herold & Nierhaus, 1987) and the region between amino acids 60 and 201 is required for binding to the 23S rRNA (Nakagawa et al., 1999). As the rplB142 mutation is in the centre of this region, it is possible that the H142L mutation results in a defect in the assembly of the 50S subunit. To test this we first monitored the ribosome profile of the rplB142 mutant at 30°C by sucrose density gradient sedimentation analysis and compared it with that of wild-type (Fig. 1c). The rplB142 mutant contained a smaller proportion of 70S ribosomes than the wild-type, whereas the rplB142 mutant harbouring the srb1 suppressor mutation contained proportions of 70S ribosomes that were comparable with that of the wild-type (Fig. 1c). The relative proportions of 70S ribosomes, including polysomes, in the wild-type, rplB142 and rplB142 srb1 strains were 0.90, 0.53 and 0.68, respectively [where the total amount of 30S subunit + 50S subunit including 50S* subunit + 70S ribosomes including polysomes (≥70S) in each strain was set at 1.0]. It is therefore likely that the increased level of 70S ribosomes in the suppressor mutant was responsible for restoring efficient growth and sporulation at 30°C. As shown, the peak areas for ~47S particles (‘50S* subunit’) in the rplB142 mutant strains were much greater than that for 70S ribosomes (Fig. 1c). However, in the rplB142 and rplB142 srb1 mutant strains, the peak area for 30S subunits was greater than that in the wild-type. These results suggest that assembly of the 50S subunit was partially defective in the rplB142 mutant because of a conformational change(s) at or near the L2 binding site and/or a defect in the maturation of the 23S rRNA. As a result, this mutant accumulates an incomplete 50S subunit (50S* subunit).

Analysis of 70S ribosome formation and ribosomal proteins in the rplB142 mutant at high temperature

To investigate the effects of the H142L substitution in the L2 protein on growth and 70S ribosome formation at high temperature, we first monitored the kinetics of growth and cell morphology in LB medium after a temperature upshift from 30 to 45°C. As shown in Fig. 2(b), the OD_{600} of a culture of the rplB142 mutant increased about fourfold during the initial 90 min of growth at 45°C. The number of viable cells (c.f.u.) in the culture is also shown in Fig. 2(b). Whereas the OD_{600} increased until 4.5 h after temperature upshift to 45°C, an increase in the number of viable cells was observed in the first 60 min after temperature upshift and then decreased, indicating that the ability of the mutant to form colonies was reduced without cell lysis (Fig. 2b and Fig. S1a). These results suggested that growth of the rplB142 mutant at high temperature resulted in a filamentous cell morphology, and this was confirmed by light microscopy (Fig. S1b).

We next compared the relative amounts of 70S ribosomes, and 30S and 50S subunits in the rplB142 mutant and wild-type before and after temperature upshift by sucrose density gradient sedimentation analysis (Fig. 2c, d) and the amounts of each ribosomal protein in the 70S ribosome by radical free and highly reducing (RFHR) 2D gel electrophoresis as described in the Supplementary Methods (Wada, 1986; Nanamiya et al., 2004, 2006) (Fig. 2e, f). When the rplB142 mutant was grown at 32°C in LB medium, the ribosomal profile showed the accumulation of an approximately 47S particle (designated ‘50S* subunits’) compared with that of the wild-type (Fig. 2c). Compared with those at 32°C, the relative peak areas corresponding to the 50S* subunit increased and that of the 70S ribosomes decreased in the rplB142 mutant 90 min after the temperature upshift to 45°C (Fig. 2d). In contrast, a dimer of 70S ribosomes was formed in the wild-type cells after a 90 min temperature upshift to 45°C (Fig. 2d). Significantly, the YvyD protein, which is essential for dimerization of the 70S ribosome (Tagami et al., 2012), was detected in the ribosome fraction of the wild-type 90 min after the temperature upshift but not in the rplB142 mutant (Fig. 2e, 2f), and the amounts of L2 and also L16 were greatly reduced (Fig. 2e, 2f). These results suggest that the slow growth of the rplB142 mutant at low temperature was caused by a partial defect in 50S subunit formation, and inhibition of growth at high temperature was due to a severe defect in 50S subunit formation.

The incomplete 50S* subunit synthesized in the rplB142 mutant cells is not able to form 70S ribosomes

To examine the 50S* subunit formed in the rplB142 mutant at 45°C, a partially purified 50S* subunit fraction from the rplB142 mutant 90 min after temperature upshift was analysed by RFHR 2D gel electrophoresis and compared
Fig. 3. Association activity of the incomplete 50S* subunit prepared from the rplB142 mutant strain to form 70S ribosomes in vitro. (a) Incomplete 50S* subunit from the rplB142 mutant and small subunit (30S) and large subunit (50S) from the wild-type were prepared, and association experiments were performed as described in Methods. (b, c) The ribosomal proteins of the wild-type 50S subunits and 50S* subunits from the rplB142 mutant strain cultivated at 32 °C were further analysed by RFHR 2D gel electrophoresis.
(a) DNA sequence and gene expression diagram.

(b) Gel electrophoresis image showing DNA fragments.

(c) Graph showing OD600 and LacZ activity over time.
with the 50S subunit produced in the wild-type under the same conditions (Fig. S2). The amount of L2 in the 50S* subunit in the rplB142 mutant grown at low temperature was about 30% of that in the 50S subunit in the wild-type grown at the same temperature (Fig. S2a, b, e). The amount of L2 in the rplB142 mutant was drastically reduced when grown at the higher temperature (Fig. S2b, d, e). Only about 15% of the amount of L2 found in the 50S subunit of wild-type grown at low temperature was observed in the rplB142 mutant 90 min after temperature upshift (Fig. S2a, d, e). The amount of L16 was also greatly reduced in the 50S* mutant 90 min after temperature upshift (Fig. S2a, d, e). The amount of L16 was also greatly reduced in the 50S* mutant cells is due to the inability to form active mature 50S subunits, as indicated by the accumulation of incomplete 50S* subunits lacking the L2 and L16 proteins.

We next examined whether the incomplete 50S* subunits formed in the rplB142 mutant at 32 °C have the ability to form 70S ribosomes in vitro with 30S subunits prepared from the wild-type (Fig. 3). It is clear from Fig. 3(a) that the 30S subunit of the wild-type could form 70S ribosomes with the 50S subunit from the wild-type but not with the 50S* subunits from the rplB142 mutant. These results indicate that the incomplete 50S* subunit that lacks L2 and L16 does not have the ability to bind to the 30S subunit; this results in the inability to translate mRNA and leads to the observed growth defect.

**Genetic mapping and identification of the suppressor mutation srb1 and the actual suppressor gene, yaaA**

We mapped the srb1 suppressor mutation using transducing phage PBS1 (Takahashi, 1961, 1963), and found a linkage with the ybaC::cat marker at a frequency of 60%. Linkages were also demonstrated with rrrO2::catpt1 and rrrA2::catpt1 at co-transformation frequencies of 66 and 4%, respectively. These results indicate that the srb1 mutation is located near the dnaA region. Various DNA segments located near dnaA were amplified by PCR using chromosomal DNA from the strain carrying the srb1 mutation as the template and the products were used to transform the rplB142 mutant strain, monitoring for the presence of the srb1 suppressor mutation. Results showed that only the amplified DNA fragments containing the region from dnaN to yaaA suppressed the rplB142 mutation at low temperature. DNA sequencing analysis showed that the srb1 mutation was a one base deletion of a single T base from a 9T cluster upstream of the yaaA gene (Fig. 4). To examine the transcription of the yaaA gene, primer extension was performed and two distinct bands were detected (Fig. 4b). One transcript was initiated from UTP by the σ70-like promoter within the yaaA coding region, and the other was initiated from ATP just upstream of the yaaA gene. The transcript from within the yaaA coding region was reduced in both rplB142 and its suppressor mutant strains, whereas the transcript initiated 26 bp upstream of the start codon for the YaaA protein was significantly enhanced by the srb1 mutation, compared with the wild-type (Fig. 4b). Two other transcripts were found approximately 74 bp upstream of the initiation codon for the YaaA protein. As we were unable to identify any characteristic promoter sequences upstream of these transcripts, it is possible that the transcripts are cleavage products of the mRNA that was transcribed from an upstream promoter. To confirm the upregulation of transcription from the promoter just upstream of yaaA in the rplB142 srb1 strain, we integrated PyaaAwt–lacZ and PyaaAsrb1–lacZ fusion genes, which contained this promoter (−63/−64 to +26 bp in Fig. 4a), into the amyE locus. As expected, significantly higher LacZ activity was observed in the strain harbouring PyaaAsrb1–lacZ than in the strain harbouring PyaaAwt–lacZ (Fig. 4c). It is likely that the PyaaAsrb1 promoter is recognized by the major sigma, σA, judging from its sequence and transcription activity pattern as shown in Fig. 4(c). We also determined the transcript of the yaaA gene by S1 hybridization analysis (Fig. S4a). The level of yaaA transcript in the suppressor mutant (rplB142 srb1) was approximately 1.5-fold that in the wild-type. These results indicate that enhanced expression of yaaA and downstream genes, including the recF, yaaB, gyrB and gyrA genes, can restore the defect of the altered L2 protein in the rplB142 mutant at low temperature.

To identify which of the above genes is responsible for the suppression of the rplB142 mutation, we first constructed a deletion of the yaaA gene and examined its effect on the rplB142 mutation. As shown in Fig. 5(a), deletion of the yaaA gene in the rplB142 srb1 mutant not only removed the suppressor effect of srb1 on the rplB142 mutation but also led to a more severe inhibition of growth. Moreover, the presence of the Psrb1-yaaA gene integrated at the aprE locus restored the growth rate of the rplB142 mutant to almost that of the wild-type, although the introduction of
the Pwt-yaaA gene did not (Fig. 5b). The ribosome profiles of the strains carrying Pwt-yaaA or Psrb1-yaaA genes were monitored and compared with those from the wild-type, and the rplB142 and rplB142 srb1 mutant strains (Fig. 5c). The relative amounts of 70S ribosomes in the wild-type, and rplB142, srb1 rplB142, aprE::Pwt-yaaA rplB142 and aprE::Psrb1-yaaA mutants were 0.92, 0.55, 0.67, 0.52 and 0.63, respectively, indicating that the 70S ribosomes, which actively synthesize proteins, decreased in the rplB mutant and increased in the rplB142 srb1 suppressor mutant. By contrast, accumulation of the incomplete 50S* subunits was reduced in the suppressor mutant compared with that in the rplB142 mutant (Figs 1c and 5c). Introduction of the Psrb1-yaaA gene resulted in a phenotype similar to that of the srb1 mutant (Fig. 5b, c). In contrast, the introduction of ΔyaaA into either the rplB142 or the rplB142 srb1 mutant significantly reduced their growth rates as well as levels of 70S ribosomes, but had no apparent effect in the wild-type (Fig. 5a, d and Fig. S4b). We further examined the 70S ribosomes formed in the rplB142 srb1 suppressor mutant at 32 °C by RFHR 2D gel electrophoresis, and found that the amounts of L2 and also L16 were slightly increased compared with that observed in rplB142 mutant cells (Fig. 6c). The ratios of L2 to L1 in the wild-type, and rplB142 and rplB142 srb1 mutant strains were 1.06 (Fig. 6a), 0.65 (Fig. 6b) and 0.68 (Fig. 6c), respectively. Moreover, the amount of L2H142L and L16 were significantly increased in the 50S* subunit in rplB142 srb1 mutant cells compared with that observed in rplB142 mutant cells (Fig. 6d). The ratio of L2 to L1 in the wild-type, and rplB142 and rplB142 srb1 strains were 1.15 (Fig. 3b), 0.36 (Fig. 3c) and 0.51 (Fig. 6d), respectively. These results indicate that the 50S subunits containing L2H142L interact with 30S subunits to form 70S ribosome as rapidly as those of the wild-type, indicating that the rplB142 mutation reduces the affinity of L2 to 23S rRNA, but has little effect on the other functions of this protein. It is thus clear that increased amounts of YaaA do not suppress the defects of the rplB142 mutation by carrying out the same function as L2. These results revealed that enhanced expression of the yaaA gene almost completely suppressed the defects in both growth and sporulation in the rplB142 mutant at low temperature, most likely by assisting the binding of altered L2 to the 23S rRNA during formation of the 50S subunit.

**DISCUSSION**

In the present study, we isolated a temperature-sensitive mutant, rplB142, which harboured an H142L substitution in the L2 ribosomal protein. This mutant could not grow at high temperature (45 °C) and showed a reduced growth rate even at low temperatures (30 or 32 °C), probably due to reduced translation activity caused by the defective formation of 70S ribosomes (Fig. 1 and Fig. S1). Indeed, a significant reduction in 70S ribosomes and the accumulation of incomplete 50S* subunits were observed in the rplB142 mutant (Fig. 1c). In particular, after a temperature upshift to 45 °C, only a few 70S ribosomes were detected in the rplB142 mutant cells (Fig. 2d). The defect in 70S ribosome formation may be caused by the reduced affinity of the L2 protein harbouring the H142L substitution for the 50S subunit. L2 binds to 23S rRNA via the N-terminal domain (positions 60–130), OB-fold, and the C-terminal domain (positions 131–201), SH3-like barrel (Nakagawa et al., 1999). Because His142 is located within the SH3-like barrel of the L2 protein, the H142L mutation probably affects the conformation of this domain and reduces the affinity for 23S rRNA. In addition, given that histidine is a positively charged amino acid, it is likely that His142 recognizes the negatively charged phosphate backbone of the 23S rRNA. Thus, the change in charge caused by the replacement of His142, a basic amino acid, by the neutral amino acid leucine is one of the reasons for the reduced affinity of the L2 protein for 23S rRNA. In fact, it has been reported that the replacement of Arg86 or Arg155 (basic amino acid) by glutamine (neutral amino acid) in the B. subtilis L2 protein reduces the binding affinity for the 23S rRNA (Harada et al., 1998). A significant reduction of the amount of L2 protein in the incomplete 50S* subunits was also observed in this study (Fig. S2). The 50S* subunit, the so-called L2-lacking large subunit, was incapable of forming 70S ribosomes even with the wild-type 30S subunit in vitro (Fig. 3). It has been shown that L2 localizes to the intersubunit space of the 70S ribosome and is involved in the association of the ribosomal subunits (Diedrich et al., 2000; Yusupov et al., 2001). It has also been shown that a deletion mutation in L2 (Δ222–228) causes a complete lack of L16 and reduced amounts of L28, L33 and L34 in 48S particles that failed to associate with 30S subunits in E. coli (Romero et al., 1990) (Fig. 7b). Similarly, as illustrated in Fig. 7, we could not detect L16 in the incomplete 50S* subunits in the rplB142 mutant strain, indicating that as in E. coli, assembly of L16 into the 50S subunits requires L2 in vivo in B. subtilis (Fig. 7c). Our observations suggest that His142 of the L2 protein plays an important role in it binding to the 23S rRNA and confirmed that L2 is a key component for the formation of an active 70S ribosome complex.

We also identified a mutation that suppressed the defective phenotypes caused by the H142L substitution in the L2 protein, including the reduced growth rate and sporulation frequency at low temperature. This suppressor mutation was found to be a one-base deletion of a single T from a 9T
cluster in the promoter region of \textit{yaaA}, which is probably recognized by $\sigma^A$. Because the transcription of \textit{yaaA} was increased in the \textit{rplB142 srb1} mutant and only the \textit{yaaA} gene with a promoter containing the one-base deletion of T could suppress the defects observed in the \textit{rplB142} mutant, there is little doubt that upregulation of YaaA can suppress
the phenotypic effect of the H142L mutation in the L2 protein. In the rplB142 srb1 mutant, the amount of 70S ribosomes increased, whereas that of incomplete 50S* subunits decreased, compared with the rplB142 mutant (Fig. 5c). Moreover, the introduction of ΔyaaA into either the rplB142 or the rplB142 srb1 mutant significantly reduced their growth rates as well as the levels of 70S ribosomes (Fig. 5a, d). In addition, the amounts of both L2 and L16 in the ribosome were improved in the rplB142 srb1 mutant compared with the rplB142 mutant (Fig. 6). These results suggest that YaaA assists in the binding of the mutant L2 protein harbouring the H142L mutation (L2H142L) to the 50S subunit, which leads to the binding of L16 to the 50S subunit, followed by the association of the 30S and mature 50S subunits. It is thus plausible that YaaA is constitutively expressed even in the wild-type, because the yaaA gene is located in an operon containing essential genes such as dnaA, dnaN, gyrB and gyrA. In fact, a transcript containing yaaA was detected in the wild-type by S1 nuclease assays (Fig. S4a). Therefore, it seems likely that YaaA may assist in the assembly of 50S subunits in wild-type cells. However, as a yaaA-disrupted mutant grew normally, it is possible that the formation of 70S ribosomes in the yaaA-disrupted mutant is affected only under the harsher conditions. The upregulation of yaaA suppressed the defects observed in the rplB142 mutant at low temperature, but not at high temperature. Analysis of ribosomes in the rplB142 srb1 mutant by RFHR 2D electrophoresis showed that YaaA improves binding of not only L2H142L but also L16 into 50S subunits. These results suggest strongly that YaaA facilitates the association of L2H142L with the 50S subunit, which leads to binding of L16 to the 50S subunit, as illustrated in Fig. 7(d). These results also indicated that the L2H142L protein is fully functionally active once it is assembled to the 50S subunit. The H142L mutation in the L2 protein affects growth rate as well as

Fig. 7. Incomplete 50S subunit formation in the mutant cells carrying altered L2 protein. (a) Wild-type strain, (b) rplB deletion (L2Δ222-228) mutant (Romero et al., 1990), (c) rplB142 mutant strain, (d) rplB142 srb1 mutant strain. See text for details.
sporulation and cell morphology (Fig. 1, Table 1 and Fig. S1). Previously, we found that several mutants harbouring deletions or point mutations in genes encoding certain ribosomal proteins, such as a ArpI (L1) mutant, showed sporulation defects and that cells of the ArpsF (S6) and ArpsU (S21) mutants were significantly more filamentous than the wild-type (Akanuma et al., 2012, 2013). Although reduced translation activity in these mutants probably affects sporulation and cell morphology, further investigations are needed to understand why the lack of L2 in the ribosome affects sporulation and cell morphology and how YaA participates in assembly of the 50S subunit.

ACKNOWLEDGEMENTS

We thank T. Fukuda for primer extension analysis. We also thank C. R. Harwood for critical reading of the manuscript. This work was supported in part by the Ministry of Education, Culture, Sports, Science, and Technology of Japan through a Grants-in-Aid for Scientific Research (C) (F.K.) and a Strategic Research Foundation Grant-aided Project for Private Universities (S1201003) (F.K. and Y.S.). In addition, this work was partly supported by Research Grants from the Noda Institute for Scientific Research (NISR) and the Institute for Fermentation, Osaka, to F. K.

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Edited by: J. Stülke