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Inactivation of Ribosomal Protein Genes in *Bacillus subtilis* Reveals Importance of Each Ribosomal Protein for Cell Proliferation and Cell Differentiation

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Among the 57 genes that encode ribosomal proteins in the genome of *Bacillus subtilis*, a Gram-positive bacterium, 50 genes were targeted by systematic inactivation. Individual deletion mutants of 16 ribosomal proteins (L1, L9, L15, L22, L23, L28, L29, L32, L33.1, L33.2, L34, L35, L36, S6, S20, and S21) were obtained successfully. In conjunction with previous reports, 22 ribosomal proteins have been shown to be nonessential in *B. subtilis*, at least for cell proliferation. Although several mutants that harbored a deletion of a ribosomal protein gene did not show any significant differences in any of the phenotypes that were tested, various mutants showed a reduced growth rate and reduced levels of 70S ribosomes compared with the wild type. In addition, severe defects in the sporulation frequency of the Δ*rplA* (L1) mutant and the motility of the Δ*rpsU* (S21) mutant were observed. These data provide the first evidence in *B. subtilis* that L1 and S21 are required for the progression of cellular differentiation.

The eubacterial ribosome (70S) is a complex macromolecule that is composed of a small (30S) subunit and a large (50S) subunit. The small subunit is comprised of the 16S rRNA and more than 20 proteins, whereas the large subunit is comprised of the 23S and 5S rRNAs and more than 30 proteins. Each ribosome has three binding sites for tRNA, namely, the A-site, which accepts the aminoacylated tRNA, the P-site, which binds the tRNA with the nascent peptide chain, and the E-site, which binds the deacylated tRNA before it leaves the ribosome (46, 47). The small subunit associates with the mRNA and the anticodon stem-loop of the bound tRNA and participates in ensuring the fidelity of translation by checking for correct pairing between the codon and anticodon (25, 40, 45, 60, 63). The large subunit associates with the acceptor arms of the tRNA and catalyzes the formation of a peptide bond between the amino acid attached to the tRNA in the A-site and the nascent peptide chain bound to the tRNA in the P-site (5, 38). The molecular mechanisms of translation have been elucidated in detail by the convergence of various approaches, including crystal structure analysis (20, 46, 47, 54, 62).

Most ribosomal proteins in eubacteria are highly conserved (42), and it has been proposed that several ribosomal proteins play essential roles in translation, as well as ribosome assembly (13, 17, 18, 32, 64). However, the detailed roles of most of the ribosomal proteins in cell proliferation, as well as the progression of cellular differentiation, have not been investigated fully. Mutation of the genes that encode the ribosomal proteins is one of the most effective ways to obtain further information about their function. In *Escherichia coli*, which is the best-characterized Gram-negative bacterium, several mutants in which specific ribosomal proteins are absent from the ribosome have been isolated (15, 16) and characterized (21, 31, 37, 51, 52). More recently, *E. coli* has been subjected to a systematic deletion of the genes encoding ribosomal proteins (49). Taken together with results that were obtained previously from a genome-wide analysis of gene inactivation (4), the results revealed that 22 of the 54 *E. coli* genes for ribosomal proteins could be deleted individually (49).

The Gram-positive bacterium *Bacillus subtilis* has been the subject of intensive scientific study because of its ability to sporulate, as well as to incorporate external DNA readily. Sequencing of the genome of the *B. subtilis* strain 168 was completed in 1997 (28). Systematic gene inactivation enabled us to clarify that, among 4,101 genes in the genome of *B. subtilis*, 271 genes are essential for cell proliferation at 37°C in LB medium (27). However, the 52 ribosomal protein genes that are present in the *B. subtilis* genome were excluded from the targets of the study on systematic gene inactivation because of their conservation among eubacteria. Despite the assumed importance of the ribosomal proteins, it was reported that in a *B. subtilis* mutant that harbored a mutation in *rplK*, which encodes ribosomal protein L11, L11 was absent from the ribosome (59). L11 is one of the ribosomal proteins that have been well investigated, because mutations in the *rplK* gene often result in thiostrepton resistance (9, 39, 59). The N-terminal domain of L11, which interacts with the elongation factor G and the antibiotic thiostrepton, is implicated in the termination of translation (8, 56). L11 is also required for the stringent response through the regulation of RelA activity, which synthesizes the signaling molecules GDP 3′-diphosphate (ppGpp) and GTP 3′-diphosphate (pppGpp), generally referred to as (p)ppGpp (11, 59, 61). Furthermore, L11 is involved in the acti-

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vation of σ^B, which regulates the general stress regulon of *B. subtilis* (65). Thus, it is most likely that, under adverse growth conditions, L11 contributes to cell survival by transmitting various signals that occur in the ribosome during translation to these regulatory proteins. These findings raised the question of whether the 52 ribosomal proteins in *B. subtilis* are actually all required for cell growth and, as is the case for L11, whether any others act as mediators that transmit signals from the ribosome to certain regulatory factors, and vice versa.

In the present study, we attempted to obtain a series of genetic deletion mutants that covered 50 ribosomal protein genes in the *B. subtilis* genome. The ribosomal protein homologues (Ctc, YpfD, YtiA, and YhzA), as well as the ribosomal proteins L11 and L31, the deletion mutants of which have been analyzed previously (34, 36, 50, 55), and S14, which is known to be essential (36), were not included among the targets of the study. Furthermore, the effects of the absence of each ribosomal protein on cell proliferation, as well as on 70S ribosome formation, sporulation, and motility, were examined to reveal phenotypes of deletions in individual ribosomal proteins in the Gram-positive, endospore-forming bacterium *B. subtilis*.

MATERIALS AND METHODS

Media and culture conditions. LB (44), LB agar, and 2× Schaeffer's sporulation medium supplemented with 0.1% glucose (2× SG) (29) were used. The culture conditions and media for preparation of competent cells have been described previously (3). When required, 5 μg ml^{-1} chloramphenicol or 5 μg ml^{-1} kanamycin was added to the media. Min-CH medium (43) is Spizizen's minimal glucose medium supplemented with 0.05% Amicase (Sigma).

Gene deletion experiments. All of the *B. subtilis* strains used in the study were isogenic with *B. subtilis* strain 168 *trpC2*. The ribosomal protein genes were replaced with a chloramphenicol acetyltransferase (*cat*) gene from pCBB31 (24). When the target gene was considered to be a monocistronic unit or was located at the end of the operon, a part of the open reading frame (ORF) was replaced with an amplified version of the *cat* gene that included its own promoter, which was obtained by PCR using the primers catF and catpt1R. In contrast, when the transcript of the target gene included downstream gene(s), a part of the ORF was replaced by an amplified version of the *cat* gene that lacked any promoter or Rho-independent terminator sequence (*cat*Δ*pt1*) (35), which was amplified with primers catpt1F and catpt1R. For the disruption, upstream and downstream regions of each target gene, at least 600 bp in length, were amplified by PCR using the appropriate primers (rpxXuF and rpxXuR for the upstream region, rpxXdF and rpxXdR for the downstream region). (For all primers used in the study, see Table S1 in the supplemental material.) The two resultant fragments and the *cat* (or *cat*Δ*pt1*) fragment were then ligated and amplified by PCR using the appropriate primers (rpxXuF and rpxXdR; see Table S1). The resultant amplified DNA fragment contained regions homologous to the genomic regions that flanked the target gene and, to disrupt the gene, was used to transform strain 168 at a final concentration of 5 μg ml^{-1}, which was considered to be a saturating DNA concentration (2). The transformants that harbored a deletion mutation of the target ribosomal protein gene as a result of a double-crossover event were selected for resistance to chloramphenicol at 30°C, 37°C, and 47°C. The development of competence was monitored by analysis of Trp$^+$ transformation using chromosomal DNA from strain 168W; in each analysis, the activity was higher than 10^5 Trp$^+$ transformants ml^{-1}. Each assessment of gene disruption was performed more than twice. Correct disruption was verified by PCR and two-dimensional (2-D) electrophoresis.

The Δ*fliE* mutant was constructed by replacing the *fliE* ORF with the kanamycin resistance gene of pET41b (Novagen), which was amplified by PCR using the primers fliEuRKmF and fliEdFKmR. The upstream and downstream regions of the *fliE* gene were amplified by PCR using the primers fliEuF and fliEuRKmF for the upstream region and fliEdFKmR and fliEdR for the downstream region (see Table S1 in the supplemental material). The two resulting fragments and the kanamycin resistance gene were then ligated and amplified by PCR using fliEuF and fliEdR. The *fliE* gene was then disrupted using the resulting product. Correct disruption was verified by PCR.

Sucrose density gradient sedimentation analysis. *Bacillus subtilis* cells were grown in LB medium at 37°C with shaking to early exponential phase (optical density at 600 nm [OD_{600}] of ~0.2) and harvested. The sucrose density gradient sedimentation analysis was performed as described previously (35). Briefly, cells were disrupted by passage through a French pressure cell, and cell debris was removed by centrifugation. Aliquots of extract were layered onto 10 to 40% sucrose density gradients and centrifuged at 4°C for 17.5 h at 65,000 × *g* (Hitachi P40ST rotor; 10 A_{260} units per tube). Samples were collected with a piston gradient fractionator (BioComP), and absorbance profiles were monitored at 254 nm (A_{254}) using a Bio-mini UV monitor (ATTO, Japan).

Preparation of crude ribosomes. Cells were grown in LB medium at 37°C and were harvested in early exponential phase (OD_{600} of ~0.2). Crude ribosomes were obtained as described previously (34). Radical free and highly reducing (RFHR) 2-D gel electrophoresis (57) was performed essentially in accordance with the published procedures (34).

Assay for sporulation. *Bacillus subtilis* cells were 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, plating them on LB agar plates, and then incubating them at 37°C for 24 h. Spores were observed by phase-contrast microscopy (Olympus BX50).

Microscopic imaging. Cells were grown in LB medium at 37°C with shaking to the exponential phase, and 500 μl of the culture was centrifuged at 12,000 × *g* for 1 min. The cell pellet was resuspended in 20 μl of the culture supernatant containing FM4-64 (10 μg ml^{-1}, Invitrogen) and DAPI (4′,6-diamidino-2-phenylindole; 5 μg ml^{-1}) (Wako Pure Chemical Industries). The cell suspension was mounted on microscope slides covered with a thin film of agarose (1.0%) in distilled water, and phase-contrast and fluorescence images were obtained with a SenSys-1401E air-cooled charge-coupled device (CCD) camera (Roper Scientific) attached to an Olympus BX50 microscope equipped with a 100× UPlanApo objective.

Motility assay. The motility of the ribosomal protein deletion strains was assayed on plates that contained 1% Bacto Tryptone (Difco) and 0.5% NaCl, which were solidified by the addition of 0.4% Bacto agar (Difco). An aliquot of a liquid culture (1 μl), which had been grown in LB medium at 37°C to early exponential phase (OD_{600} of ~0.2), was spotted onto the center of the plate. After incubation at 37°C for 24 h, the presence of swarm colonies was observed.

RESULTS

Systematic inactivation of genes encoding ribosomal proteins in the *B. subtilis* genome. *Bacillus subtilis* has the ability to take up exogenous DNA with high efficiency. When cells are grown in the appropriate minimal medium, a transformation efficiency as high as 10^6 transformants ml^{-1} can be obtained (2). We employed this procedure to obtain a set of mutants that each harbored a deletion mutation of a gene that encoded a ribosomal protein. Among the 57 genes that were annotated to encode ribosomal proteins in the genome of *B. subtilis*, 50 genes were chosen as targets. Six genes, which encoded Ctc, YpfD, YtiA, YhzA, L11, and L31, were not included in the present study, because successful attempts to obtain deletion mutants of these genes had already been reported (34, 36, 50, 55, 59). The *rpsN* gene encoding S14 was also excluded, because its essentiality has already been established (36).

In *B. subtilis*, most of the genes that encode ribosomal proteins

are located in the large S10-spc-α gene cluster, which contains 25 ribosomal protein genes (30). In the case of genes in this cluster, the target genes were replaced with an amplified version of the chloramphenicol resistance gene (*cat*) that lacked any promoter or Rho-independent terminator sequence (*cat*Δ*pt1*), to eliminate possible polar effects on downstream genes within the same operon. In these mutants, the *cat* gene was cotranscribed with the other genes that constitute the cluster, as a result of the gene replacement. In contrast, when the target gene was considered to be transcribed as a monocistronic message or was located at the end of the operon, the ORF of the target gene was replaced by an amplified version of the *cat* gene that included its own promoter. In either case, the DNA fragment that contained the *cat* (or *cat*Δ*pt1*) gene was ligated with at least 600 bp of the upstream and downstream regions of each target gene as described in Materials and Methods. The fragments obtained were used to transform *B. subtilis* strain 168 (*trpC2*). The transformants were selected for chloramphenicol resistance at three different temperatures (30°C, 37°C, and 47°C), because of the possibility that ribosomal protein deletion mutants will show high/low-temperature-sensitive phenotypes. In each experiment, to confirm that the recipient cells were competent to take up exogenous DNA, cells were exposed to chromosomal DNA extracted from strain 168W Trp^+ at a final concentration of 5 μg ml^{-1}, which was considered to give the maximum number of transformants (2), and conversion to the Trp^+ phenotype was monitored. Each assessment of gene disruption was performed more than twice.

Using this approach, it was possible to obtain deletion mutants for 16 genes that encoded ribosomal proteins (Table 1). Among these, three genes encoded ribosomal proteins found in the 30S subunit (S6, S20, and S21), and 13 genes encoded proteins in the 50S subunit (L1, L9, L15, L22, L23, L28, L29, L32, L33.1, L33.2, L34, L35, and L36). Disruption of the target genes was confirmed by PCR (data not shown). In addition, crude ribosomes were prepared from the obtained mutants, as well as from three mutants (the Δ*rpmE*, Δ*ytiA*, and Δ*yhzA* strains) that were isolated previously (34, 36), and RFHR 2-D gel electrophoresis of the crude ribosomes was performed to confirm the absence of the products of the deleted genes. As shown in Fig. 1 (see Fig. S1 in the supplemental material), the products of the deleted genes were not detected in the 2-D gels of crude ribosomes prepared from the mutants. However, it should be noted that L9, L33.2, and L36 could not be detected by the 2-D gel analysis of high-salt-washed ribosomes even from wild-type cells (34). Therefore, we did not expect to see any differences between the 2-D gel patterns of the wild-type strain and the Δ*rplI* (L9), Δ*rpmGB* (L33.2), and Δ*rpmJ* (L36) mutants. These results, in combination with previous reports (34, 36, 50, 55, 59), revealed that it is possible to delete 22 of the 57 ribosomal proteins, including ribosomal protein homologues, that are found in *B. subtilis* (Table 1).

Characterization of the deletion mutants. We attempted to characterize the phenotypes of the deletion mutants obtained above, together with those of the Δ*rpmE* (L31), Δ*ytiA*, and Δ*yhzA* mutants, which were constructed previously in our laboratory (1, 34, 36). Among the 19 mutants tested in the present study, seven mutants, which harbored deletions of the genes that encoded ribosomal proteins L1, L22, L23, L34, L36, S6, and S21, respectively, showed slow growth phenotypes in LB medium compared with the wild type (Fig. 2 and Table 2). In particular, all of these mutants grew more slowly than the wild type at the lowest temperature (32°C) (Fig. 2A and B and Table 2). In contrast, except for the mutant lacking L1 (Δ*rplA*), which showed severe growth defects at all temperatures tested, the growth rate of these mutants was largely restored at 45°C (Fig. 2E and F and Table 2). Interestingly, the growth of the Δ*rplV* (L22), Δ*rplW* (L23), and Δ*rpmH* (L34) mutants, which was extremely slow at 32°C, was markedly restored at the higher temperature. The three ribosomal proteins encoded by these genes might have a role in ribosomal assembly at low temperatures. The other 12 mutants did not show severe growth defects in LB medium at 37°C, even though some of the mutants (e.g., the Δ*rpmE* mutant, expressing L31) showed a slight decrease in growth rate (see Fig. S3 in the supplemental material). These results indicated that these gene products are dispensable for the growth in LB medium at 37°C.

Next, we assumed that, in the deletion mutants, the absence of each individual ribosomal protein might cause a defect in the formation of 50S or 30S subunits, and thus formation of the 70S ribosome might be impaired. To test for this, the formation of the 70S ribosome was monitored by sucrose density gradient sedimentation analysis (Fig. 3; see Fig. S3 in the supplemental material). Ribosomes from eight of the mutants (L1, L23, L29, L32, L34, L36, S6, and S21) showed notably unusual profiles compared with that of the wild type (Fig. 3). In particular, there were extremely high levels of 30S and 50S subunits in the Δ*rplA* (L1) and Δ*rpmH* (L34) mutants; the areas of the peaks that corresponded to the 50S subunit were larger in these mutants than that of the 70S ribosome. The growth of almost all of these mutants was inhibited significantly at 37°C (Fig. 2 and Table 2), and it is likely that defects in the assembly of the 70S ribosome affected their growth. However, the Δ*rplV* mutant (L22) showed a severe growth defect (Fig. 2 and Table 2), even though a sufficient amount of the 70S ribosome was detected (see Fig. S3). This result strongly suggests that L22 is not involved in the assembly of the 50S subunit or formation of the 70S ribosome, but rather is required for the efficient functioning of the 70S ribosomal complex.

The process of assembly of 30S and 50S ribosomal subunits in *E. coli* was investigated previously by reconstituting the subunits from purified rRNA and ribosomal proteins *in vitro* (23, 33). Given that the *in vitro* reconstitution of ribosomal subunits indicated that some ribosomal proteins are necessary for the binding of other ribosomal proteins to the ribosome, it was plausible that changes in the constitution of 70S ribosomes might occur in these mutants. If this was true, it should be possible to monitor these differences through 2-D gel analyses of the 70S ribosome. In fact, in 2-D gels of ribosomes prepared from the Δ*rplO* (L15) and Δ*rplV* (L22) mutants, significant reductions in L35 and L32, respectively, were detected (Fig. 1). However, 2-D gels of ribosomes prepared from the other mutants used in the study did not show any significant changes, except for the disappearance of the target proteins whose genes were deleted (see Fig. S1 in the supplemental material). These observations suggested strongly that the *in vivo* assembly of ribosomal proteins into the ribosome might differ, at least in part, from assembly *in vitro*.

Next, we studied the effects of the absence of each ribosomal protein on spore formation. Interestingly, although nearly all of the deletion mutant strains showed sporulation frequencies that were almost identical to that of the wild type, the sporulation frequencies of the Δ*rplA* (L1) and Δ*rplV* (L22) mutant strains were reduced markedly (Table 3). In particular, the sporulation frequency of the Δ*rplA* (L1) mutant was less than 0.01%. It was

TABLE 1 Summary of disruption of ribosomal protein genes

Gene	Protein	Transcriptional unit	Replacement[a]	Gene disruption in *B. subtilis*	Essentiality in *E. coli*[b]
rplA	L1	*rplK-rplA*	*catΔpt1*	Yes	Nonessential
rplB	L2	S10-spc-α	*catΔpt1*	No	Essential
rplC	L3	S10-spc-α	*catΔpt1*	No	Essential
rplD	L4	S10-spc-α	*catΔpt1*	No	Essential
rplE	L5	S10-spc-α	*catΔpt1*	No	Essential
rplF	L6	S10-spc-α	*catΔpt1*	No	Essential
rplI	L9	*yybS-yybT-rplI*	*cat*	Yes	Nonessential
rplJ	L10	*rplJ-rplL*	*cat*	No	Essential
rplK	L11	*rplK-rplA*		Yes[c]	Nonessential
rplL	L7/L12	*rplJ-rplL*	*cat*	No	Essential
rplM	L13	*rplM-rpsI*	*catΔpt1*	No	Essential
rplN	L14	S10-spc-α	*catΔpt1*	No	Essential
rplO	L15	S10-spc-α	*catΔpt1*	Yes	Nonessential
rplP	L16	S10-spc-α	*catΔpt1*	No	Essential
rplQ	L17	S10-spc-α	*cat*	No	Essential
rplR	L18	S10-spc-α	*catΔpt1*	No	Essential
rplS	L19	*rplS*	*cat*	No	Essential
rplT	L20	*infC-rpmI-rplT*	*cat*	No	Essential
rplU	L21	*rplU-ysxB-rpmA*	*cat*	No	Nonessential
rplV	L22	S10-spc-α	*catΔpt1*	Yes	Essential
rplW	L23	S10-spc-α	*catΔpt1*	Yes	Essential
rplX	L24	S10-spc-α	*catΔpt1*	No	Nonessential
ctc	L25 homologue	*ctc*		Yes[c]	Nonessential
rpmA	L27	*rplU-ysxB-rpmA*	*cat*	No	Nonessential
rpmB	L28	*rpmB*	*cat*	Yes	Essential
rpmC	L29	S10-spc-α	*catΔpt1*	Yes	Nonessential
rpmD	L30	S10-spc-α	*catΔpt1*	No	Nonessential
rpmE	L31	*rpmE*	*cat*	Yes[c]	Nonessential
ytiA	L31 homologue	*ytiA*	*cat*	Yes[c]	—
rpmF	L32	*rpmF*	*cat*	Yes	Nonessential
rpmGA	L33.1	*rpmGA*	*cat*	Yes[c]	Nonessential
rpmGB	L33.2	*rpmGB*	*cat*	Yes[c]	—
rpmH	L34	*rpmH*	*cat*	Yes	Nonessential
rpmI	L35	*infC-rpmI-rplT*	*catΔpt1*	Yes	Nonessential
rpmJ	L36	S10-spc-α	*catΔpt1*	Yes	Nonessential
ypfD	S1 homologue	*ypfD-cmk*		Yes[c]	Essential
rpsB	S2	*rpsB*	*cat*	No	Essential
rpsC	S3	S10-spc-α	*catΔpt1*	No	Essential
rpsD	S4	*rpsD*	*cat*	No	Essential
rpsE	S5	S10-spc-α	*catΔpt1*	No	Essential
rpsF	S6	*rpsF-ssb-rpsR*	*catΔpt1*	Yes	Nonessential
rpsG	S7	*rpsL-rpsG-fus*	*catΔpt1*	No	Essential
rpsH	S8	S10-spc-α	*catΔpt1*	No	Essential
rpsI	S9	*rplM-rpsI*	*cat*	No	Nonessential
rpsJ	S10	S10-spc-α	*catΔpt1*	No	Essential
rpsK	S11	S10-spc-α	*catΔpt1*	No	Essential
rpsL	S12	*rpsL-rpsG-fus*	*catΔpt1*	No	Essential
rpsM	S13	S10-spc-α	*catΔpt1*	No	Essential
rpsN	S14	S10-spc-α	*catΔpt1*	No	Essential
yhzA	S14 homologue	*yhzA*	*cat*	Yes[c]	—
rpsO	S15	*rpsO*	*cat*	No	Nonessential
rpsP	S16	*rpsP*	*catΔpt1*	No	Essential
rpsQ	S17	S10-spc-α	*catΔpt1*	No	Nonessential
rpsR	S18	*rpsF-ssb-rpsR*	*cat*	No	Essential
rpsS	S19	S10-spc-α	*catΔpt1*	No	Essential
rpsT	S20	*rpsT*	*cat*	Yes	Nonessential
rpsU	S21	*rpsU-yqeT*	*catΔpt1*	Yes	Nonessential

[a] Genes were replaced by the chloramphenicol resistance gene, either with its own promoter (*cat*) or lacking any promoter (*catΔpt1*).
[b] Essentiality in *E. coli* based on the studies by Baba et al. (4) and Shoji et al. (49). —, counterpart of this gene has not been found in *E. coli*.
[c] These genes have previously been reported to be nonessential (19, 34, 36, 41, 50, 55, 59).

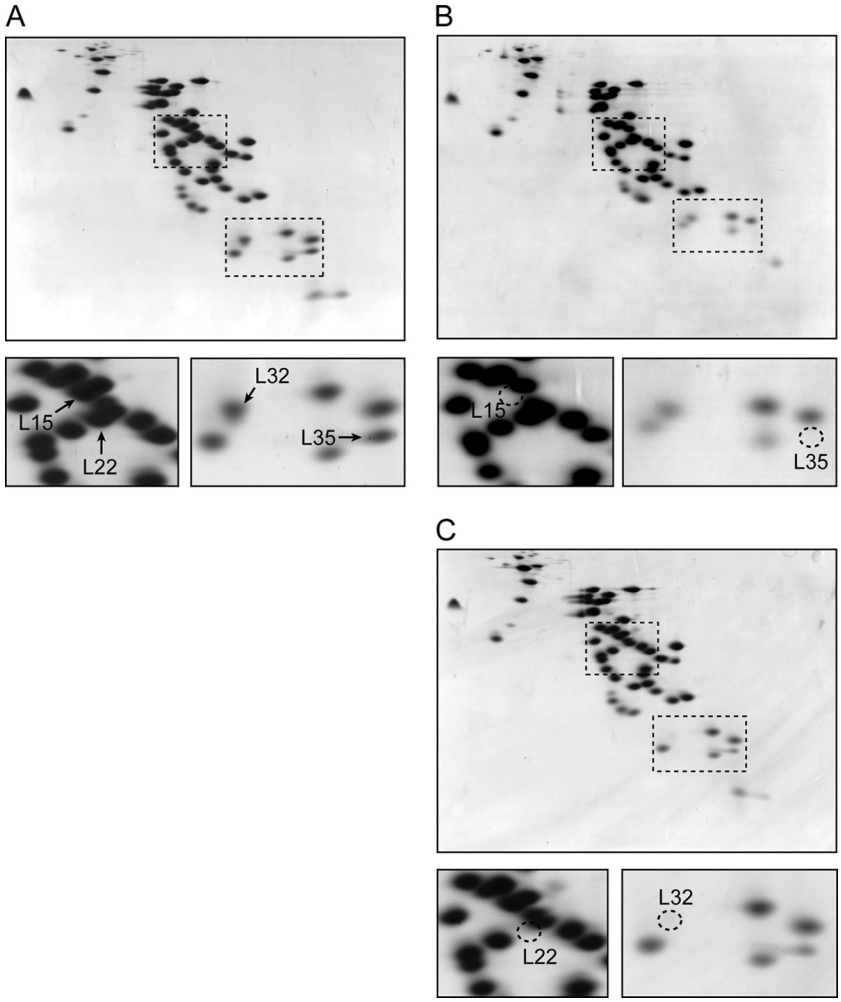

FIG 1 RFHR 2-D gel electrophoresis of ribosomal proteins prepared from deletion mutants of the ribosomal protein genes. Ribosomal proteins (750 μg) were prepared from cells in the early exponential phase (OD_{600} of ~0.2) of the wild type (wt) (A), Δ*rplO* (L15) mutant (B), or Δ*rplV* (L22) mutant (C), grown in LB medium at 37°C and were used for RFHR two-dimensional gel electrophoresis as described in Materials and Methods. The areas of the two-dimensional gels that contained the spots of the L15 and L22 or L32 and L35 proteins were extracted from the gel images. Arrows indicate each ribosomal protein spot (A). Circles with dotted lines indicate protein spots that have disappeared (B and C). The deletion of *rplO* and *rplV* was confirmed by the disappearance of spots that correspond to L15 and L22, respectively. Significant reductions in the amount of L35 and L32 proteins were observed in the ribosomes prepared from the Δ*rplO* (L15) and Δ*rplV* (L22) mutants, respectively.

assumed that this marked reduction in spore formation might be due to a combination of this mutant's severe growth defect (doubling time in LB at 37°C of 66.3 ± 4.3 min) and its abnormal profile for 70S formation. In contrast, although the Δ*rpmH* (L34) mutant also showed a severe growth defect (doubling time in LB at 37°C of 70.5 ± 1.4 min) and abnormal accumulation of ribosomal subunits (Fig. 2 and Fig. 3), the sporulation frequency of the Δ*rpmH* mutant was almost the same as that of the wild type (Table 3). The sporulation defect of the Δ*rplA* (L1) mutant was also confirmed by phase-contrast microscopy. Whereas refractile spores were observed with wild-type *B. subtilis* and the Δ*rpmH* (L34) mutant grown in 2× SG medium at 37°C for 24 h, virtually no spores were detected with the Δ*rplA* (L1) mutant (data not shown). Thus, it is unlikely that the sporulation defect of the Δ*rplA* mutant can be attributed to slow growth.

It was observed that some mutants, such as the Δ*rpsF* (S6) and Δ*rpsU* (S21) strains, formed heteromorphic colonies that included hard and/or predominant clumps (data not shown). This observation prompted us to monitor the cell morphology and the motility in the mutants tested in this study. Microscopic observation revealed that the Δ*rpsF* (S6) and Δ*rpsU* (S21) cells were significantly more filamentous than the wild-type cells in LB medium at 37°C, even though apparently normal septa were observed in each of the mutants (see Fig. S4 in the supplemental material). These results suggest that a late stage of cell division is impaired in the absence of proteins S6 and S21. Next, to investigate cell motility in these mutants, aliquots of cultures that had been grown to the exponential phase in LB medium at 37°C were spotted onto soft agar plates, and the presence of swarm colonies was observed after 24 h at 37°C. As shown in Fig. 4, swarm colonies of the Δ*rpsU* (S21) strain (with a doubling time in LB medium at 37°C of 34.4 ± 1.1 min) were barely detected, whereas the Δ*rpsF* (S6) strain, whose growth rate is same as that of the Δ*rpsU* (S21) strain (with a doubling time in LB medium at 37°C of 36.1 ± 0.1

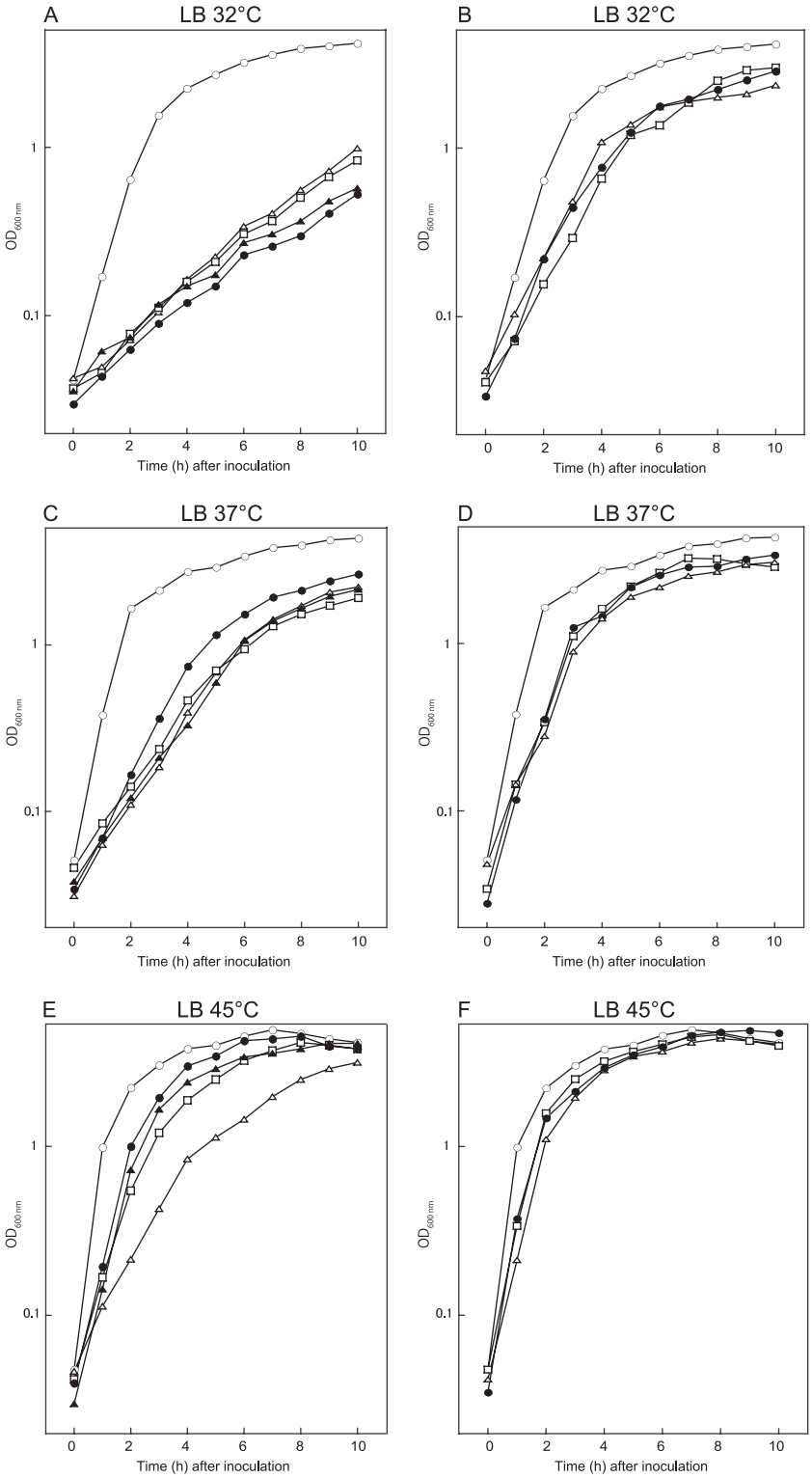

FIG 2 Growth characteristics of the deletion mutants. Cells were grown in LB medium at 32°C (A and B), 37°C (C and D), or 45°C (E and F), and the optical density at 600 nm (OD_{600}) was measured. Symbols in panels A, C, and E: ○, wild type; △, Δ*rplA* (L1) mutant; □, Δ*rplV* (L22) mutant; ●, Δ*rplW* (L23) mutant; ▲, Δ*rpmH* (L34) mutant. Symbols in panels B, D, and F: ○, wild type; △, Δ*rpmJ* (L36) mutant; □, Δ*rpsF* (S6) mutant; ●, Δ*rpsU* (S21) mutant. Results that are representative of three independent experiments are shown.

TABLE 2 Doubling times of ribosomal protein gene deletion strains at various temperatures

Strain	Doubling time (min) of strain at[a]:		
	32°C	37°C	45°C
Wild type	31.4 ± 0.9	19.9 ± 0.6	14.4 ± 0.7
Δ*rplA* (L1) mutant	122 ± 13	66.3 ± 4.3	52.1 ± 3.6
Δ*rplV* (L22) mutant	107 ± 9.8	78.4 ± 2.6	36.7 ± 0.5
Δ*rplW* (L23) mutant	111 ± 2.1	58.0 ± 3.8	32.7 ± 0.6
Δ*rpmH* (L34) mutant	111 ± 12	70.5 ± 1.4	31.8 ± 0.5
Δ*rpmJ* (L36) mutant	58.3 ± 6.5	46.2 ± 1.2	27.0 ± 1.3
Δ*rpsF* (S6) mutant	62.1 ± 0.4	36.1 ± 0.1	24.8 ± 1.2
Δ*rpsU* (S21) mutant	45.6 ± 2.1	34.4 ± 1.1	23.4 ± 1.0

[a] Means of three independent experiments ± standard deviations are shown.

TABLE 3 Sporulation of ribosomal protein gene deletion strains

Strain	Sporulation (CFU ml^{-1})[a]		
	Total	Spores	Frequency (%)[a]
Wild type	5.6×10^8	4.8×10^8	84 ± 3.9
Δ*rplA* (L1) mutant	1.5×10^8	1.5×10^2	$(1.1 \pm 1.3) \times 10^{-4}$
Δ*rplV* (L22) mutant	3.8×10^8	5.5×10^6	1.6 ± 0.5
Δ*rpmH* (L34) mutant	6.2×10^8	5.1×10^8	84 ± 7.4

[a] Means of three independent experiments (± standard deviation for percent sporulation frequency) are shown.

min), showed a small swarm circle compared with the wild type. These results suggest that S21 is necessary for cell motility.

DISCUSSION

In the present study, we constructed a set of deletion mutants of ribosomal proteins in the Gram-positive bacterium *B. subtilis*, which has high spontaneous transformation activity. We then characterized the fundamental properties of these mutants, including their involvement in cell growth, ribosome assembly, and cell development. Among the 53 genes that encode ribosomal proteins and the four homologues of ribosomal protein genes that are found in the genome of *B. subtilis*, we were able to obtain 16 mutants that each harbored a deletion mutation of a gene that encoded a ribosomal protein (L1, L9, L15, L22, L23, L28, L29, L32, L33.1, L33.2, L34, L35, L36, S6, S20, and S21) (Table 1). Given that deletion mutants for the genes that encode L11, L25 (Ctc), L31, YtiA, S1 (YpfD), and YhzA have already been isolated (34, 36, 50, 55, 59), the results revealed that 22 ribosomal proteins are not individually essential for growth under the conditions tested in the study. It was surprising that the ribosomal proteins L1, L15, L22, L23, and L29, which are conserved among all three domains of life (42), could be deleted. Many of the deletion mutants that were obtained in the present and previous studies (1, 19, 34, 36) were deletions of genes that encoded the smaller ribosomal proteins, with molecular masses of less than 10 kDa (L28, L29, L31, L32, L33.1, L33.2, L34, L35, L36, YtiA, S20, and S21). It has been suggested that the sizes of ribosomal proteins have increased during evolution to complement the function of the rRNA, which acted originally as a ribozyme (7). This leads us to suggest that the smaller ribosomal proteins may have been incorporated into the

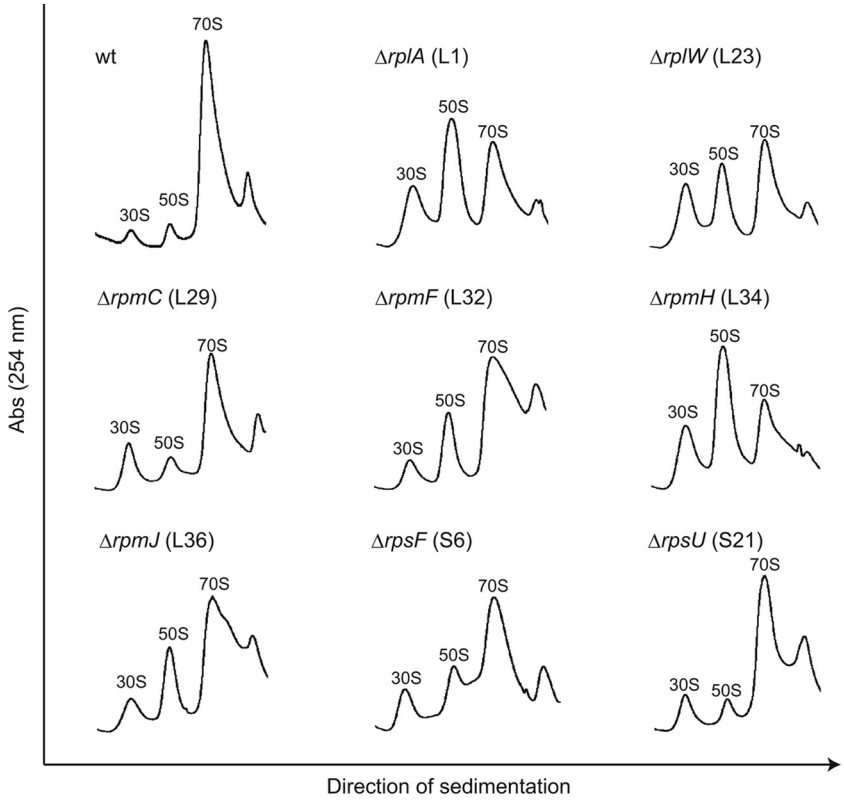

FIG 3 Ribosome sedimentation profiles for the deletion mutant strains. Crude cell extracts were sedimented through a 10 to 40% sucrose gradient as described in Materials and Methods. The 30S, 50S, and 70S peaks are indicated in each profile. Abs, absorbance.

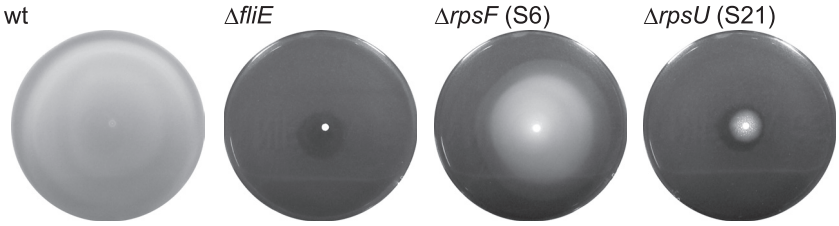

FIG 4 Disruption of *rpsU* resulted in a nonmotile phenotype. Motility plates showing the behavior of wild-type and Δ*fliE*, Δ*rpsF*, and Δ*rpsU* mutant cells, after 16 h at 37°C.

ribosome more recently than the larger proteins and that the role of these small, nonessential proteins is to enhance the activity of the ribosome beyond that simply of protein translation. Indeed, under conditions of zinc deficiency, the zinc-containing ribosomal protein L31 (7.3 kDa) is replaced on the ribosome by YtiA (9.4 kDa), which enables L31 to be degraded to provide the essential element zinc to the cell (1, 19, 34).

Whereas several mutants showed a defect in the formation of the 70S ribosome, ribosomes from almost all of the deletion mutants, except for the L15 and L22 mutants, did not lack any ribosomal proteins apart from the target protein (Fig. 1; see Fig. S1 in the supplemental material). Although this result was consistent with the observation that the composition of the ribosome did not change markedly in any ribosomal protein deletion mutants of *E. coli* (49), it was not consistent with the results of *in vitro* reconstitution of the *E. coli* ribosome (23, 33). Therefore, it is possible that assembly of the ribosome *in vitro* might differ from that *in vivo* in some aspects as reported previously (53). At present, we cannot exclude the possibility that immature 50S and 30S subunits, formed as the result of the absence of a particular ribosomal protein, might not be detected readily by our 2-D gel analyses, and this might explain the difference in the *in vitro* and *in vivo* results. Indeed, we reported previously that depletion of S14 causes the accumulation of immature 30S subunits in which S2 and S3 are decreased markedly (36). This result was in good agreement with previous reports based on *in vitro* studies (23, 33). Further investigation to clarify the composition of ribosomal proteins in the 50S and 30S subunits that accumulate abnormally in deletion mutants should provide more detailed information about the *in vivo* pathway of ribosome assembly.

The amount of L32 protein was decreased significantly in ribosomes from the Δ*rplV* (L22) mutant (Fig. 1). Thus, it is likely that the absence of both L22 and L32 is responsible for the severe effect on cell proliferation, as well as the reduction of sporulation frequency, in the Δ*rplV* mutant (Fig. 2 and Table 3). A previous study has reported that L22 is located close to L32 in the 50S ribosome of *Thermus thermophilus* (62). Cross-linking of L22 to L32 in *E. coli* has also been reported (58). In contrast, L22 protein could be detected at an appreciable level in the 2-D gel of ribosomal proteins prepared from the Δ*rpmF* (L32) strain (see Fig. S1 in the supplemental material). Taken together, these results suggested that L22 is required for binding of L32 to the ribosome, and thus that a lack of L22 causes structural alteration of the 50S subunit. Similarly, although the relationship between L15 and L35 had not been elucidated previously, a significant reduction of L35 in ribosomes of the Δ*rplO* (L15) mutant was observed (Fig. 1), whereas the amount of L15 in ribosomes from the Δ*rpmI* (L35) mutant was almost the same as that in wild-type ribosomes (see Fig. S1). Thus, it is most likely that L15 is required for binding of L35 to the ribosome.

The Δ*rplA* (L1) and Δ*rplV* (L22) mutants showed a reduction in sporulation frequency (Table 3). A previous study had shown that inactivation of *ctc*, which encodes a homologue of L25, causes a temperature-sensitive sporulation phenotype in *B. subtilis* (55). In addition, the sporulation frequency of an *rpmGB*::pMutinT3-rpmGB mutant, in which transcription of the *rpmGB* (L33.2) gene is under the control of an isopropyl-β-D-thiogalactoside (IPTG)-regulated promoter, decreased slightly at 47°C (41). However, the involvement of these ribosomal proteins in sporulation remains unclear.

The other observation that suggests a relationship between ribosomal proteins and cell development is the loss of motility of the S21 deletion mutant strain (Fig. 4). The expression of the genes that are involved in the motility of *B. subtilis* cells is directed mainly by σ^D, a sigma factor that activates the expression of the genes that encode the components of the flagellar hook and motor, and the flagellar filament protein (48). The activity of σ^D is controlled by an anti-sigma factor, FlgM, which binds to σ^D and inhibits σ^D-dependent gene expression (6, 10). In addition, SwrA activates the *fla-che* operon, which includes flagellum genes, genes involved in chemotaxis, and the gene for the σ^D, while SwrB is required for maximal σ^D-dependent gene expression (26). It is likely that S21 is involved in the sufficient expression of these factors via protein translation. Further research using the S21 mutant described herein should help elucidate the details of this relationship between the ribosomal protein and cell motility.

In the present study, we showed that 22 out of 57 genes that encode ribosomal proteins can be deleted. Among them, the genes that encode L22, L23, and L28 have been reported to be essential for cell proliferation in *E. coli* (Table 1) (4, 49). Although an L28 mutant, VT423, in which the altered L28 was unable to bind to the ribosome, had been isolated (14), L22 and L23 defective mutations were shown to be lethal in *E. coli* (49). Our strategy to obtain the deletion mutants was based on the replacement of the target gene with a chloramphenicol resistance gene, as described in Materials and Methods. During the course of the study, we carried out transformation experiments under various temperature conditions. Hence, these procedures differed from those used in the study of Shoji et al. (49), in which the essential nature of the genes was confirmed by gene complementation experiments. It is plausible that it might be possible to introduce deletions of the genes that encode L22 and L23 into *E. coli* at high temperature. Comparison of the functions of individual ribosomal proteins between *B. subtilis* and *E. coli* using two sets of ribosomal protein deletion mutants should help to elucidate the evolutionary processes that each ribosomal protein has undergone.

Although many of the strains that harbored a deletion mutation in a ribosomal protein exhibited various defective phenotypes with regard to cell proliferation, 70S formation, spore development, and cell motility, several mutants did not show any significant phenotype. It is possible that the phenotypes of the deletion mutants are masked by the vast number of ribosomes in the cell, because the *B. subtilis* genome contains 10 rRNA (*rrn*) operons. We have already constructed mutants that harbor only a single *rrn* operon (either *rrnA*, *-B*, *-D*, *-E*, *-I*, *-J*, or *-O*) in their genome and have confirmed a reduction in the number of ribosomes in these mutants (35). Novel phenotypes that have not been observed in the present study might appear if the deletion mutations of the ribosomal proteins are introduced into the strains that harbor a single *rrn* operon. It is known that some ribosomal proteins regulate the expression of their own genes. For example, in *B. subtilis*, the expression of the *infC-rpmI-rplT* operon, which encodes the translation factor IF3 and the ribosomal proteins L35 and L20, is regulated by L20 and the expression of *rpsD*, which encodes S4, is autoregulated (12, 22). However, the function of ribosomal proteins in cell development has not been elucidated. Further investigations, including genome-wide approaches such as a transcriptomic or proteomic analyses, are needed to clarify whether ribosomal proteins are involved in the adjustment to adverse environmental conditions.

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