Magnesium Suppresses Defects in the Formation of 70S Ribosomes as Well as in Sporulation Caused by Lack of Several Individual Ribosomal Proteins

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ABSTRACT Individually, the ribosomal proteins L1, L23, L36, and S6 are not essential for cell proliferation of *Bacillus subtilis*, but the absence of any one of these ribosomal proteins causes a defect in the formation of the 70S ribosomes and a reduced growth rate. In mutant strains individually lacking these ribosomal proteins, the cellular Mg^{2+} content was significantly reduced. The deletion of *YhdP*, an exporter of Mg^{2+} , and overexpression of *MgtE*, the main importer of Mg^{2+} , increased the cellular Mg^{2+} content and restored the formation of 70S ribosomes in these mutants. The increase in the cellular Mg^{2+} content improved the growth rate and the cellular translational activity of the $\Delta rplA$ (L1) and the $\Delta rplW$ (L23) mutants but did not restore those of the $\Delta rpmJ$ (L36) and the $\Delta rpsF$ (S6) mutants. The lack of L1 caused a decrease in the production of Spo0A, the master regulator of sporulation, resulting in a decreased sporulation frequency. However, deletion of *yhdP* and overexpression of *mgtE* increased the production of Spo0A and partially restored the sporulation frequency in the $\Delta rplA$ (L1) mutant. These results indicate that Mg^{2+} can partly complement the function of several ribosomal proteins, probably by stabilizing the conformation of the ribosome.

IMPORTANCE We previously reported that an increase in cellular Mg^{2+} content can suppress defects in 70S ribosome formation and growth rate caused by the absence of ribosomal protein L34. In the present study, we demonstrated that, even in mutants lacking individual ribosomal proteins other than L34 (L1, L23, L36, and S6), an increase in the cellular Mg^{2+} content could restore 70S ribosome formation. Moreover, the defect in sporulation caused by the absence of L1 was also suppressed by an increase in the cellular Mg^{2+} content. These findings indicate that at least part of the function of these ribosomal proteins can be complemented by Mg^{2+} , which is essential for all living cells.

KEYWORDS ribosome, ribosomal protein, magnesium, *Bacillus subtilis*, ribosomes

The bacterial 70S ribosome is a complex macromolecule that is composed of small (30S) subunit and large (50S) subunits. 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 (1, 2). Protein synthesis by the ribosome requires the coordinated action of these subunits. The small subunit associates with the mRNA and the anticodon stem-loop of the bound tRNA, and it engages in ensuring the fidelity of translation by checking for correct pairing between the codon and anticodon (3–7). 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

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and the nascent peptide chain bound to the tRNA in the P site (8, 9). The ribosomal proteins that constitute these subunits play an important role(s) in translation. For instance, ribosomal protein L1, which is localized to the stalk region near the E site (10, 11), plays a critical role in the translocation of the newly deacylated tRNA from the P to the E sites (12). Ribosomal protein L2 plays important roles in binding of the tRNA to the A and P sites, peptidyltransferase activity, and formation of the peptide bond (13–17). Therefore, the mature conformation of the 70S ribosomes is required for efficient translation activity.

Although the ribosomal proteins are important in the translation process, as well as in the association of the ribosomal subunits (13, 18, 19), several genes encoding ribosomal proteins can be deleted. In *Escherichia coli*, 22 of the 54 genes for ribosomal proteins are not individually essential for cell proliferation (20, 21). Similarly, in *Bacillus subtilis*, 22 of the 57 genes for ribosomal proteins can be individually deleted (22). The *rpmH* gene, encoding ribosomal protein L34, which is a component of the large subunit, is one of the nonessential genes. Mutants lacking L34 have a severe defect in the formation of the 70S ribosome and a reduced growth rate (22). However, we found that the defect in the formation of 70S ribosomes and the reduction in the growth rate could be suppressed by an increase in the Mg^{2+} content in the cell (23).

Magnesium ions are the most abundant divalent cations in living cells (24, 25), and are important for the maintenance of ribosome structure. Mg^{2+} is required for both stabilization of the secondary structure of rRNA and binding of the ribosomal proteins to the rRNA (26–28). The *in vitro* association of the 30S and 50S ribosomal subunits to form intact 70S ribosomes depends strongly on the concentration of Mg^{2+} (29–31). Therefore, we believe that Mg^{2+} can partly complement the L34 function by stabilizing both the conformation of the 50S subunit and the intersubunit bridges.

The goal of the present study was to elucidate whether Mg^{2+} can also complement mutant strains lacking ribosomal proteins other than L34. We examined the effect of increasing the Mg^{2+} content in mutant strains individually lacking ribosomal proteins L1, L23, L36, and S6 on the formation of 70S ribosomes, the growth rate, the cellular translational activity, and on sporulation.

RESULTS

Reduction in the cellular Mg^{2+} content caused by lack of ribosomal proteins was restored by disruption of *yhdP* and overexpression of *mgtE*. The defect in the formation of 70S ribosomes caused by the absence of L34 could be suppressed by increasing the cellular Mg^{2+} content (23). To investigate the generality of the partial complementation of ribosomal protein function by Mg^{2+} , disruptions of *yhdP* and the multicopy plasmid pDGMgtE, which can induce the overexpression of *mgtE*, were introduced into mutants lacking individual ribosomal proteins L1, L23, L36, and S6. Because we previously characterized the phenotypes of 19 mutants individually lacking ribosomal proteins and found that 70S ribosome formation by mutants individually lacking L1, L23, L36, and S6 was decreased (22), we chose these four mutants as the targets for analysis in this study.

MgtE is the main importer of Mg^{2+} (32), whereas YhdP is probably an exporter of Mg^{2+} in *B. subtilis* (23). We previously reported that the absence of L34 (RpmH) caused a decrease in the Mg^{2+} content in the cell, probably due to a reduced number of 70S ribosomes, and that the Mg^{2+} content in the $\Delta rpmH$ mutant was restored by disruption of *yhdP* and overexpression of *mgtE* (23). Similarly, the Mg^{2+} contents in the $\Delta rplA$ (L1), $\Delta rplW$ (L23) and $\Delta rpmJ$ (L36) mutants were also significantly reduced (Fig. 1). However, the Mg^{2+} content in these three mutants was restored, albeit incompletely, by disruption of *yhdP* and overexpression of *mgtE* (Fig. 1). In these experiments, the cellular Mg^{2+} concentration was calculated by dividing the amount of Mg^{2+} per cell by the cell volume. The cell volume of each mutant was estimated from the cell size, which was measured by microscopic analysis, as described in Materials and Methods. However, the cell size of the $\Delta rpsF$ (S6) mutant could not be defined because the cellular morphology of the $\Delta rpsF$ mutant was aberrantly filamentous (see Fig. S1 in the supplemental

FIG 1 Reduction in the Mg²⁺ content in mutant strains lacking individual ribosomal proteins and partial restoration by disruption of *yhdP* and overexpression of *mgtE*. The Mg²⁺ content per cell in exponential phase, which was measured as described in Materials and Methods, is shown. In the case of the *ΔrpsF* (S6) mutant, the relative amount of Mg²⁺ per cell is shown (see the text for details). White bars indicate the wild type and each mutant lacking individual ribosomal proteins. Gray bars indicate the results when *yhdP* was disrupted and *mgtE* was overexpressed. The means from three independent experiments are shown. Error bars indicate standard deviations.

material). Thus, in the *ΔrpsF* mutant, the relative Mg²⁺ amount per cell when the Mg²⁺ amount of a cell in the parental strain was defined as 1 is shown in Fig. 1. Although a comparison of the Mg²⁺ content in the *ΔrpsF* mutants with that in the wild type was difficult, the Mg²⁺ content in the *ΔrpsF* mutants was certainly increased by disruption of *yhdP* and overexpression of *mgtE*.

It should be noted that the Mg²⁺ concentrations that were measured in this study are total Mg²⁺ concentrations, including Mg²⁺ ions chelated in proteins and nucleic acids, and not only the free Mg²⁺ concentrations. Because the cells were completely disrupted by sonication and proteins were denatured by acid treatment, the Mg²⁺ ions that were chelated in ribosomes, other enzymes, and nucleic acids were also detected by this method. The Mg²⁺ ions that are contained in a cell are usually chelated by proteins and nucleic acids, and thus only 5% of the Mg²⁺ ions in a cell are present as free metal ions (33–35).

The effect of increasing the cellular Mg²⁺ content of mutants lacking individual ribosomal proteins on the formation of 70S ribosomes, the growth rate, and the cellular translational activity. As shown in Fig. 2, the lack of individual ribosomal proteins (L1, L23, L36, and S6) caused defects in the formation of 70S ribosomes that are consistent with our previous data for several mutants lacking individual ribosomal proteins (22). The defect in 70S ribosome formation observed in these mutants was suppressed to various degrees by disruption of *yhdP* and overexpression of *mgtE* (Fig. 2). In all of the mutants investigated here, the amount of 70S ribosomes relative to the amount of dissociated subunits was restored by increasing the cellular Mg²⁺ content. These results indicate that Mg²⁺ can suppress the defect in the formation of 70S ribosomes and/or in the reduction in the stability of the 70S complex caused by the absence of several individual ribosomal proteins.

We next investigated the effect of the cellular Mg²⁺ content on the growth rate of the mutants. We have reported that the slow growth observed for the *ΔrpmH* (L34) mutant was suppressed by an increase in the Mg²⁺ content, probably due to the restoration of the amount of 70S ribosomes (23). A reduction in the growth rate was

FIG 2 Defect in 70S ribosome formation in the absence of each ribosomal protein and its suppression by the disruption of *yhdP* and overexpression of *mgtE*. 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 individual profile. The term “/pDGmgtE” indicates the overexpression of *mgtE* in the mutant cells.

observed for the mutants lacking individual ribosomal proteins, which agrees with our previous results for several mutants lacking individual ribosomal proteins (22). As expected, in the $\Delta rplA$ (L1) and $\Delta rplW$ (L23) mutants, the growth rate was partially restored by disruption of *yhdP* and overexpression of *mgtE* (Fig. 3; Table 1). When only *mgtE* was overexpressed in the $\Delta rplA$ (L1) mutant, its effect on the growth rate was minimal (23). The combination of overexpression of *mgtE* and disruption of *yhdP*, however, increased the growth rate of the $\Delta rplA$ (L1) mutant. In contrast, the growth rates of the $\Delta rpmJ$ (L36) and $\Delta rpsF$ (S6) mutants were not significantly increased when the cellular Mg^{2+} content was increased (Fig. 3; Table 1).

To investigate whether the partial restoration of the growth rate of the $\Delta rplA$ (L1) and $\Delta rplW$ (L23) mutants by the increase in the cellular Mg^{2+} content was dependent on an increase in cellular translational activity, we measured the translational activity in cells lacking individual ribosomal proteins. As expected, the cellular translational activity of the $\Delta rplA$ (L1) and $\Delta rplW$ (L23) mutants was severely reduced (Fig. 4). In the $\Delta rplA$ (L1) and $\Delta rplW$ (L23) mutants, an increase in the cellular Mg^{2+} content partially restored the cellular translational activity, whereas in the $\Delta rpmJ$ (L36) and $\Delta rpsF$ (S6) mutants, the effect of increased cellular Mg^{2+} content on the translational activity was minimal. These results are correlated with the effect that the increased cellular Mg^{2+} content had on the growth rate. Therefore, the partial restoration of the cellular translational activity by the increased cellular Mg^{2+} content improved the growth rate of the $\Delta rplA$ (L1) and $\Delta rplW$ (L23) mutants.

The increase in the cellular Mg^{2+} content suppresses the defect in sporulation caused by the absence of ribosomal protein L1. We previously found that the absence of ribosomal protein L1 causes a defect in sporulation (22). It should be noted that this phenotype was not caused solely by the decreased growth rate, because the sporulation frequency of the $\Delta rpmH$ (L34) mutant, which also showed a severe growth

FIG 3 Effects of the increase in cellular Mg²⁺ content on the growth rate of the mutant strains lacking individual ribosomal proteins. Cells were grown in LB at 37°C, and the optical density at 660 nm was measured. Growth curves of the wild type and each mutant lacking individual ribosomal proteins are shown using black and gray solid lines, respectively. Growth curves of each mutant in which *yhdP* was disrupted and *mgtE* was overexpressed are shown by dotted lines.

defect similar to that of the $\Delta rplA$ (L1) mutant, was almost the same as that of the wild type (22). We therefore investigated whether the sporulation defect of the $\Delta rplA$ (L1) mutant could be suppressed by Mg²⁺. Consistent with our previous data, the $\Delta rplA$ (L1) mutant was severely defective in forming heat-resistant spores (the sporulation frequency was less than 0.01%), whereas the other mutants formed spores with frequencies of >70% (Table 2). However, the sporulation frequency of the $\Delta rplA$ (L1) mutant was partially restored by disruption of *yhdP* and overexpression of *mgtE* (Table 2). In

TABLE 1 Doubling times of mutants lacking ribosomal proteins

Strain description	Doubling time $\pm$ SD (min) ^a	
	Parental	$\Delta yhdP/pDGmgtE$
wt	21.4 $\pm$ 1.4	
$\Delta rplA$ (L1)	67.7 $\pm$ 1.7	54.6 $\pm$ 0.49
$\Delta rplW$ (L23)	56.7 $\pm$ 1.9	37.0 $\pm$ 2.4
$\Delta rpmJ$ (L36)	43.3 $\pm$ 1.3	41.2 $\pm$ 0.54
$\Delta rpsF$ (S6)	38.2 $\pm$ 1.1	36.4 $\pm$ 0.49

^aMeans from three independent experiments are shown.

FIG 4 Effects of the increase in cellular Mg^{2+} content on the cellular translational activity of the mutant strains lacking individual ribosomal proteins. The relative translational activity in mutant cells compared to that of the wild type is shown. White bars indicate the wild type and each mutant lacking individual ribosomal proteins. Gray bars indicate the results when *yhdP* was disrupted and *mgtE* was overexpressed. The means from three independent experiments are shown. Error bars indicate standard deviations.

addition, the growth rate of the $\Delta rplA$ (L1) mutant in sporulation medium was also restored by disruption of *yhdP* and overexpression of *mgtE* (Fig. 5A). These results indicate that increasing the cellular Mg^{2+} content can partially suppress not only the growth defect but also the sporulation defect in the $\Delta rplA$ (L1) mutant.

The restoration of spore formation by the $\Delta rplA$ (L1) mutant prompted us to identify which stage of sporulation was affected by the absence of L1 and restoration by Mg^{2+} . At the initiation stage of *B. subtilis* sporulation, cells divide asymmetrically, and chromosomal DNA is concentrated in the forespore (36). In fact, asymmetric septation and concentration of chromosomal DNA were detected in the wild-type cells 5 h after inoculation into sporulation medium (see Fig. S2 in the supplemental material). In contrast, in the $\Delta rplA$ (L1) cells, asymmetric septation was not observed, even 24 h after inoculation (Fig. S2). However, asymmetric septation and concentration of chromosomal DNA were detected in the $\Delta rplA$ (L1) cells 15 h after inoculation when *yhdP* was disrupted and *mgtE* was overexpressed (Fig. S2). We next examined the level of Spo0A in the $\Delta rplA$ (L1) mutant. Phosphorylation of Spo0A, the master transcriptional regulator of sporulation, governs the decision to initiate sporulation (37–39). In the wild type and mutants other than the $\Delta rplA$ (L1) mutant, the level of Spo0A increased 4 h and 6 to 8 h after inoculation into sporulation medium, respectively (Fig. 5B; see also Fig. S3 in the supplemental material). In contrast, Spo0A was barely detectible in $\Delta rplA$ (L1) cells even 10 h after inoculation (Fig. 5B). However, the disruption of *yhdP* and overexpression of

TABLE 2 Restoration of sporulation frequency of the $\Delta L1$ mutant by Mg^{2+}

Strain description	CFU · ml ⁻¹ ^a		Frequency (%) ± SD ^a
	Total	Spores	
wt	6.4×10^8	5.8×10^8	90 ± 7.9
$\Delta L23$	4.9×10^8	3.5×10^8	71 ± 5.8
$\Delta L36$	6.4×10^8	4.6×10^8	73 ± 13
$\Delta S6$	3.5×10^8	2.7×10^8	77 ± 12
$\Delta L1$	1.6×10^8	2.3×10^2	$(1.7 \pm 1.2) \times 10^{-4}$
$\Delta L1 \Delta yhdP/pDGmgtE$	3.1×10^8	7.5×10^7	25 ± 4.7

^aMeans from three independent experiments.

FIG 5 Reduction in the growth rate and in the production of Spo0A (caused by lack of L1) and their suppression by the disruption of *yhdP* and overexpression of *mgtE*. (A) Cells were grown in sporulation medium (2×SG) at 37°C, and the optical density at 660 nm was measured. Growth curves of the wild type and the $\Delta rplA$ (L1) mutant are shown using black and gray solid lines, respectively. The growth curve of the L1 mutant in which *yhdP* was disrupted and *mgtE* was overexpressed is shown by a dotted line. (B) Cells were grown in sporulation medium at 37°C and were collected at the indicated times. Crude cell extracts were subjected to Western blot analysis using antisera against Spo0A. The term “/pDGmgtE” indicates the overexpression of *mgtE* in the mutant cells.

mgtE in the $\Delta rplA$ (L1) cells increased the amount of Spo0A by 9 h after inoculation, although the level of Spo0A remained lower than that in the wild type (Fig. 5B). These results indicate that the defect in the initiation stage of sporulation caused by the absence of L1 can be at least partially suppressed by an increase in the Mg²⁺ content in the cell.

DISCUSSION

The cellular Mg²⁺ contents of the mutant strains individually lacking L1, L23, or L36 were reduced compared to that of the wild type, while that of the $\Delta rpsF$ (S6) mutant was difficult to calculate because of its filamentous cellular morphology (Fig. 1; also see Fig. S1 in the supplemental material). The reduction in the amount of Mg²⁺ was probably caused by the smaller amount of 70S ribosomes, which harbor more than 170 Mg²⁺ ions per complex (40), and by a decrease in the amount of protein and RNA other than ribosomes that can chelate Mg²⁺. In fact, we previously showed that the reduction in the cellular Mg²⁺ content correlated with the decrease in the amount of 70S ribosomes (23). On the other hand, the disruption of *yhdP* and overexpression of *mgtE* increased cellular Mg²⁺ content and restored the formation of 70S ribosomes in the mutants tested here that lacked individual ribosomal proteins (Fig. 2). Although the absence of L34 causes ribosomal protein L16 to dissociate from the 50S subunit, the increase in the Mg²⁺ content restores the binding of L16 to the 50S subunit, indicating that Mg²⁺ can stabilize the conformation of 50S subunits lacking L34 (23). Likewise, stabilization of the conformation of each subunit as well as bridges between the subunits by Mg²⁺ probably restored 70S formation and/or stability of 70S complexes in the mutants lacking individual ribosomal proteins tested here (L1, L23, L36, and S6).

The increase in 70S ribosome formation restored cellular translational activity and growth rates of the $\Delta rplA$ (L1) and $\Delta rplW$ (L23) mutants (Fig. 4, Fig. 3, and Table 1). However, the restoration of cellular translational activity and growth rates of these mutants was only partial. Possible reasons for these partial restorations are (i) an incomplete restoration of the normal amount of 70S ribosomes and (ii) functions of the ribosomal proteins other than in stabilizing the 70S ribosomes could not be complemented by Mg²⁺. Ribosomal protein L1 plays a critical role in the translocation of the newly deacylated tRNA from the P to the E site (12), while ribosomal protein L23, which

is located at the polypeptide exit channel of the large subunit, tethers trigger factor to the ribosome (41). Trigger factor, which is the first molecular chaperone interacting with newly synthesized polypeptides by the ribosome, promotes protein folding (42–44). The functions of these ribosomal proteins are probably essential for efficient growth. In contrast to mutants lacking L1 or L23, the cellular translational activity and growth rates of the $\Delta rpmJ$ (L36) and $\Delta rpsF$ (S6) mutants were not significantly increased when the Mg^{2+} content was increased, although 70S ribosome formation was restored to near wild-type levels (Fig. 2 and 3). Although the detailed functions of L36 and S6 in protein synthesis are unknown, their role(s) in translation or other function(s) does not appear to be complemented by Mg^{2+} . In addition, the filamentous morphology of cells caused by the absence of S6 was not suppressed by increasing cellular Mg^{2+} content (Fig. S2).

The increase in cellular Mg^{2+} content suppressed not only the defect of 70S ribosome formation but also the sporulation defect of the $\Delta rplA$ (L1) mutant (Table 2). Although the level of Spo0A, the master regulator of sporulation, was drastically reduced in the $\Delta rplA$ (L1) mutant, Spo0A levels were partially restored when cellular Mg^{2+} content was increased (Fig. 5B) and resulted in the restoration of sporulation frequency of the $\Delta rplA$ (L1) mutant. The phosphorylated form of Spo0A activates expression of sporulation genes, as well as its own gene, via a positive feedback loop (45, 46). Therefore, possible reasons why the level of Spo0A decreased in the $\Delta rplA$ (L1) mutant include inhibition of phosphorylation of Spo0A, which in turn causes reduction in the expression level of Spo0A, and/or simply a decrease in the stability of Spo0A. The increase in the cellular Mg^{2+} content in the $\Delta rplA$ (L1) mutant may suppress these defects by increasing the amount of 70S ribosomes and/or by increasing the cellular translational activity. However, the $\Delta rplW$ (L23) mutant, whose amount of 70S ribosomes and cellular translational activity were decreased similarly to those in the $\Delta rplA$ (L1) mutant, formed spores with an almost normal frequency (Table 2), implying a complex mechanism for the sporulation defect caused by the absence of L1.

In the present study, we demonstrated that the defect in the formation of 70S ribosomes as well as in sporulation caused by the absence of individual ribosomal proteins can be suppressed by increasing the cellular Mg^{2+} content. Mg^{2+} plays a crucial role not only in the ribosome but also in numerous biological processes and cellular functions, such as the activation and catalytic reactions of hundreds of enzymes, utilization of ATP, and maintenance of genomic stability (47, 48). Clarifying the relationship between the ribosome and Mg^{2+} , both of which are essential to living cells, is important for understanding cellular function. It has been suggested that the sizes of ribosomal proteins have increased during evolution to complement the function of the rRNA, which originally acted as a ribozyme (49, 50). From another point of view, increasing of the sizes of ribosomal proteins and/or binding of ribosomal proteins to the ribosome during evolution can be considered to complement the Mg^{2+} function in the ribosome, because in the ribosome, the relative abundance of Mg^{2+} is decreased, whereas that of ribosomal proteins is increased (50, 51). Further investigation to reveal the mechanism of complementation of the ribosomal protein function by Mg^{2+} may provide important information about the evolution of the ribosome.

MATERIALS AND METHODS

Media and culture conditions. LB medium (52), LB agar, and 2× Schaeffer's sporulation medium supplemented with 0.1% glucose (2×SG) (53) were used. The culture conditions and media for preparing competent cells have been described previously (54). When required, 5 $\mu g \cdot ml^{-1}$ chloramphenicol, 5 $\mu g \cdot ml^{-1}$ kanamycin, and 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) were added to the media. Growth curves of *B. subtilis* cells were generated by automatically measuring the optical density at 660 nm (OD_{660}) value of each culture every 5 min using a TVS062CA incubator (Advantec).

Bacterial strains. All of the *B. subtilis* strains used in this study are isogenic with *B. subtilis* strain 168 *trpC2*. The $\Delta rplA::cat$, $\Delta rplW::cat$, $\Delta rpmJ::cat$, and $\Delta rpsF::cat$ strains, which were constructed by replacing the open reading frame of each gene with a promoterless *cat* gene lacking an intrinsic terminator sequence, were described previously (22). Chromosomal DNA extracted from the $\Delta rplA::cat$, $\Delta rplW::cat$, $\Delta rpmJ::cat$, and $\Delta rpsF::cat$ strains was used to transform the strain harboring $\Delta yhdP::erm$ and the plasmid pDGmgtE, which carries the *mgtE* gene under the control of an IPTG-inducible Pspac promoter (23), and the transformants were selected on the basis of their chloramphenicol-resistant phenotype.

Measurement of the cellular Mg²⁺ content. The cellular Mg²⁺ content was measured as described previously (23). Briefly, *B. subtilis* cells were grown in LB medium to exponential phase and harvested. Simultaneously, viable cells were counted by plating the culture on LB agar plates. The cells were resuspended in lysis buffer and disrupted by sonication, and then the pH of the crude extract was adjusted to approximately 3.0 with hydrochloric acid to denature the proteins. The amount of Mg²⁺ in the cell lysate was measured with a Metallo assay kit for magnesium (Metallogenics). The Mg²⁺ content per cell was calculated by dividing the amount of Mg²⁺ in the crude extract by the number of viable cells. The concentration of Mg²⁺ was calculated by assuming that a *B. subtilis* cell is a cylinder. To measure the cell size (radius and length), microscopic images were analyzed by MicroJ, an ImageJ plug-in (55). The mean size of >30 cells was used for the calculation.

Sucrose density gradient sedimentation analysis. *B. subtilis* cells were grown in LB medium at 37°C with shaking to exponential phase (OD₆₀₀ ~0.4) and harvested. Sucrose density gradient sedimentation analysis was performed as described previously (22). Briefly, the 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, which were subjected to centrifugation at 4°C for 17.5 h at 65,000 × *g* (Hitachi P40ST rotor). Samples were collected with a Piston Gradient Fractionator (BioComp), and absorbance profiles were monitored at 254 nm using a Bio-Mini UV monitor (ATTO, Japan). When normalizing the applied volume by the total absorbance at 260 nm, 10 A₂₆₀ units of crude extract per tube were used.

Measurement of the cellular translational activity. *B. subtilis* cells were grown in LB medium at 37°C with shaking to exponential phase (OD₆₀₀ ~0.4) and harvested. The cellular translational activity was measured according to the manufacturer's instructions (Protein Synthesis assay kit; Cayman Chemical). Briefly, cells that were harvested from 100 μl of culture were incubated at 37°C with shaking for 30 min in 100 μl of *O*-propargyl-puromycin (OPP) working solution, which contained a 2-fold higher concentration of OPP than that recommended by the manufacturer. After incubation in the assay fixative and washing, cells were stained with 5-fluorescein amidite-azide (5-FAM-azide). The number of cells was normalized by the OD₆₀₀ value, and fluorescence was detected using a Typhoon FLA 9500 (GE Healthcare). The fluorescence intensities were calculated by ImageJ (56).

Sporeulation assay. *B. 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 the plates at 37°C for 24 h.

Microscopic imaging. *B. subtilis* cells were grown in 2×SG medium at 37°C with shaking. At the indicated times, 500 μl of the culture was removed and subjected to centrifugation at 12,000 × *g* for 1 min. The cell pellet was resuspended in 40 μl of culture supernatant and then FM 4-64 (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI) (Wako Pure Chemical Industries) were added to final concentrations of 10 μg/ml and 5 μg/ml, respectively. The cell suspension was mounted on a microscope slide and coated with poly-L-lysine to fix the cells, and differential interference contrast and fluorescence images were obtained with a confocal fluorescence microscope (LSM800; Carl Zeiss).

Western blot analysis. Western blot analysis was performed according to a previously described method (57). Aliquots (15 μg of protein) of crude cell extracts were loaded onto a sodium dodecyl sulfate polyacrylamide gel (12%) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Co., Japan). This membrane was then used in the Western blot assay, using antisera (1:10,000 dilution) against Spo0A (58).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00212-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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