Isolation and Characterization of a Dominant Negative Mutant of *Bacillus subtilis* GTP-binding Protein, YlqF, Essential for Biogenesis and Maintenance of the 50 S Ribosomal Subunit*

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The circularly permuted GTPase YlqF is essential for cell viability and is broadly conserved from Gram-positive bacteria to eukaryotes. We previously reported that YlqF participates in the late step of 50 S ribosomal subunit assembly in *Bacillus subtilis*. Here, we demonstrate that an N-terminal deletion mutant of YlqF (YlqFΔN10) inhibits cell growth even in the presence of wild-type YlqF. In contrast to the wild-type protein, the GTPase activity of this mutant was not stimulated by the 50 S subunit and did not dissociate from the premature 50 S subunit. Thus, YlqFΔN10 acts as a competitive inhibitor of wild-type YlqF. Premature 50 S subunit lacking ribosomal protein L27 and with a reduced amount of L16 accumulated in YlqFΔN10-overexpressing cells and in YlqF-depleted cells, suggesting that YlqFΔN10 binds to the premature 50 S subunit. Moreover, premature 50 S subunit from both YlqFΔN10-overexpressing and YlqF-depleted cells more strongly enhanced the GTPase activity of YlqF than the mature 50 S subunit of the 70 S ribosome. Collectively, our results indicate that YlqF is targeted to the premature 50 S subunit lacking ribosomal proteins L16 and L27 to assemble functional 50 S subunit through a GTPase activity-dependent conformational change of 23 S rRNA.

GTP-binding proteins are highly conserved and play critical roles in various cellular processes. Binding and hydrolysis of GTP results in reciprocal conformational changes of the GTP-binding proteins, and the GDP-bound forms define the active and inactive states, respectively (1). GTP-binding proteins are also crucial for bacterial growth. The genome of the Gram-positive spore-forming bacterium, *Bacillus subtilis*, encodes 21 GTP-binding proteins, 13 of which (IF-2, EF-Tu, EF-G, Ffh, FtsY, FtsZ, Era, Obg, YpHC, YxSC, YlqF, YqeH, and YloQ) are essential for cell viability (2, 3).

Interestingly, most of these GTP-binding proteins, except for Ffh and FtsY, which are involved in protein secretion, and FtsZ, which is involved in cell division, play important roles in ribosome function, including translation and biogenesis. Three proteins (IF-2, EF-Tu, and EF-G) are components of translation initiation and elongation factors, and their cellular functions are well characterized. The remaining seven belong to a distinct group of small GTP-binding proteins (3, 4) and are broadly conserved from bacteria to eukaryotes. These latter GTP-binding proteins have been reported to associate with the 30 S or 50 S subunit, and their depletion results in abnormal ribosome profiles in *B. subtilis* and other bacteria (5–14), suggesting that they are involved in the biogenesis of the 50 S or 30 S subunit; however, the precise actions of GTP-binding proteins on the ribosome remain to be clarified.

Obg interacts with the 50 S subunit in *Escherichia coli* and *Caulobacter crescentus* (11, 12). In *B. subtilis*, Obg co-fractionates with the 50 S subunit and interacts specifically with the ribosomal protein L13 (15). *B. subtilis* YphC and YxSC also bind to the ribosome, and when they are depleted from cells, premature 50 S subunit accumulates (7). In contrast, *E. coli* Era interacts with the 30 S ribosomal subunit (13, 16, 17). *E. coli* Era functionally compensates for deletion of the gene encoding the cold-shock adaptation protein RbfA (13), which is required for efficient processing of 16 S rRNA (18). *E. coli* YjeQ, an ortholog of *B. subtilis* YloQ, is also associated with the 30 S subunit in *E. coli*, and its GTPase activity is specifically enhanced in the presence of the 30 S subunit (19, 20). Finally, *B. subtilis* YqeH has been suggested to be involved in assembly of the 30 S subunit (21).

A recent report has revealed the molecular action of several of these GTP-binding proteins, notably *E. coli* Era (22). A three-dimensional cryoelectron microscopic map of the *Thermus thermophilus* 30 S-Era complex indicated that Era binds within the cleft between the head and platform of the 30 S subunit, which overlaps with the S1-binding site. Moreover, Era binding on the 30 S subunit interferes with association of 30 S and 50 S subunits, suggesting that dissociation of Era and incorporation of S1 compose the final step of 30 S subunit assembly and that Era inhibits formation of the translation initiation complex on prematurely assembled 30 S subunits. In contrast, we and others have demonstrated that *B. subtilis* YlqF is required for the assembly of the 50 S subunit (23, 24). In YlqF-depleted cells,

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incorporation of L16 and L27. Moreover, premature 50 S subunit in the presence of the nonhydrolyzable GTP free 50 S subunit in the absence of guanine nucleotide (23), it can bind stably to the mature 50 S subunit (45 S) and not to the mature 50 S subunit in the ribosome (23, 24). Although purified YlqF binds to the premature 50 S subunit lacking ribosomal proteins L16 and L27, it accumulates, and there is a decrease in the amount of 70 S premature 50 S subunit. Biogenesis of the 50 S subunit as a result of YlqF-induced conformational changes in the interface region of the premature 50 S subunit. Furthermore, we found that the mature 50 S subunit activates the GTPase activity of YlqF in low magnesium conditions. Based on these results, we propose a revised model of the role of YlqF in the biogenesis of the 50 S subunit.

Given these findings, we have proposed the following model (24). First, GTP-bound YlqF binds to the premature 50 S subunit lacking L16 and L27. Next, L16 and L27 are incorporated into the 50 S subunit as a result of YlqF-induced conformational changes in the interface region of the premature 50 S subunit. GTP-bound YlqF is converted to the GDP-bound form through the dissociation of YlqF from assembled 50 S subunit. However, how the GTPase activity of YlqF is stimulated has not yet been explored.

Regulation of the GTPase activity of translation factors such as EF-G, EF-Tu, and IF-2 plays an important role in the progression of each step of translation (25, 26). For example, GTP-bound EF-Tu forms a ternary complex with aminoacyl-tRNA and the ribosome. When a matching codon is recognized, the bound EF-Tu forms a ternary complex with aminoacyl-tRNA hydrolysis and releases aminoacyl-tRNA (26). Thereafter, GTP hydrolysis and releases aminoacyl-tRNA (26). Therefore, elucidation of the regulation of GTPase activity of YlqF is central to understanding how YlqF assists in the organization of the 50 S subunit.

We report here that an N-terminal deletion mutant of YlqF (YlqFΔN10) has a dominant-negative effect on the wild-type protein. The GTPase activity of YlqFΔN10 could not be activated by the 50 S subunit, so that the mutant protein could not dissociate from the premature 50 S subunit. Biogenesis of the 50 S subunit in YlqFΔN10-overexpressing cells stopped before the incorporation of ribosomal proteins L16 and L27, indicating that YlqFΔN10 can bind to the premature 50 S subunit but that it cannot assist in the reorganization of 50 S subunit for the incorporation of L16 and L27. Moreover, premature 50 S subunit from YlqFΔN10-overexpressing and YlqF-depleted cells greatly enhanced the GTPase activity of wild-type YlqF compared with mature 50 S subunit purified from 70 S ribosome particles. Unexpectedly, we also found that the free 50 S fraction separated by sucrose density gradient centrifugation caused a similar degree of YlqF GTPase activation as the premature subunits. Furthermore, we found that the mature 50 S subunit activates the GTPase activity of YlqF in low magnesium conditions. Based on these results, we propose a revised model of the role of YlqF in the biogenesis of the 50 S subunit.

**TABLE 1**

| Strain | Relevant genotype | Source |
|--------|------------------|--------|
| **B. subtilis** | | |
| 168 | trpC2 | Pasteur stock |
| YM01 | 168 Pspac-ylqF erm | This work |
| YM02 | 168 Pspac-ylqF erm amyE::Pspyl-ylqF cat | This work |
| YM03 | 168 Pspac-ylqF erm amyE::Pspyl-ylqFΔN10 cat | This work |
| **E. coli** | | |
| DH5α | supE44 ΔlacU169 (q80lacZΔM15) hsdR17 recA1 endA1 gylA96 thr-1 relA1 | Takara |
| BL21(DE3)pLysS | F- ompT hsdS2 (rB2 mB2 65T3) gal dcm | Novagen |

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† The abbreviations used are: GTPγS, guanosine 5′-O-(thio)triphosphate; IPTG, isopropyl 1-thio-β-D-galactopyranoside.

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**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—Bacterial strains, plasmids, and primers used in this study are listed in Table 1. *B. subtilis* cells were grown in LB medium at 37 °C. When necessary, various concentrations of IPTG, xylose, and/or antibiotics (chloramphenicol and erythromycin at 10 and 0.5 μg ml⁻¹, respectively) were added to the cultures. Transformation of *B. subtilis* cells was performed as previously reported (27). *E. coli* DH5α was used for plasmid construction and propagation. *B. subtilis* strains used in this study were derived from the wild-type strain 168. Strain YM01 (Pspac-ylqF) was constructed by transformation of strain 168 with the plasmid pTM208 (3) through a single-crossover recombination, resulting in the placement of the full-length *ylqF* gene behind the IPTG-inducible *spac* promoter (Pspac). YM02 (Pspac-ylqF, amyE::Pspyl-ylqF) and YM03 (Pspac-ylqF, amyE::Pspyl-ylqFΔN10) were constructed by transformation of strain YM01 with pYM01 and pYM02 plasmids, respectively, through double-crossover recombination. DNA fragments containing the Shine-Dalgarno sequence and the full-length *ylqF* gene (nucleotides 1–846) or the N-terminal-deleted *ylqF* gene (nucleotides 28–846) were amplified by PCR with primers 5′-AAAAAGCACGGCTCGAAAGGCGGTGGTTCGTTCAAC-3′/5′-AGAAAGCTTGGTTTACATCTGCGGTGGTTCAAA-3′ and 5′-ATGGCAAAAGCAAGAAGG-3′/5′-AGAAAGCTGGGTTTACATCTGCGGTGGTTCAAA-3′, respectively. These PCR products were cloned downstream of the xylose-inducible *xy1* promoter (Pxy1) on the plasmid pX (28) using Gateway technology (Invitrogen), creating pYM01 and pYM02, respectively. Plasmids pYM01 and pYM02 were transformed into strain YM01, and double-crossover recombination through *amyE*-up and *amyE*-down sequences flanking.
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PxyI and cat on pX resulted in the placement of PxyI-ylqF or PxyI-ylqFΔN10 into the amyE locus of the B. subtilis chromosome.

Buffers—The following buffers were used: buffer A, 10 mM Tris-HCl, pH 7.6, 10 mM Mg(CH3CO2)2, 100 mM NH4CH3CO2, 6 mM β-mercaptoethanol, and 2 mM phenylmethylsulfonyl fluoride (PMSF); buffer B, 20 mM Tris-HCl, pH 7.6, 15 mM Mg(CH3CO2)2, 1 mM NH4CH3CO2, 6 mM β-mercaptoethanol, and 2 mM PMSF; buffer C, 10 mM Tris-HCl, pH 7.6, 1 mM Mg(CH3CO2)2, 100 mM NH4CH3CO2, 6 mM β-mercaptoethanol, and 2 mM PMSF; buffer D, 20 mM Tris-HCl, pH 7.6, 15 mM Mg(CH3CO2)2, 500 mM NH4CH3CO2, and 6 mM β-mercaptoethanol; binding buffer, 20 mM Tris-HCl, pH 7.9, 0.5 mM NaCl, and 5 mM imidazole; washing buffer, 20 mM Tris-HCl, pH 7.9, 0.5 mM NaCl, and 20 mM imidazole.

Detection of GTP-binding Proteins on the Ribosome Profile—YM02 cells were grown at 37 °C in LB medium containing 30 μM IPTG with or without 2% xylose. YM03 cells were grown at 37 °C in LB containing 30 μM IPTG and 2% xylose. Each culture was collected during the exponential phase (A600 = 0.6) by centrifugation. Collected cells were resuspended in buffer A and disrupted by passage through a French pressure cell at 8000 p.s.i. After the removal of cell debris by centrifugation, the supernatant was subjected to 15–45% sucrose density gradient centrifugation for 3 h at 285,000 × g. Sucrose gradients were separated into 20 fractions. During fractionation, the absorbance at 254 nm was monitored. YlqF or YlqFΔN10 in each fraction was separated by SDS-PAGE on a 10% acrylamide gel and analyzed by immunoblotting with an anti-YlqF antibody as described previously (24).

Preparation of Mature 50 S Ribosomal Subunits from Wild-type Cells—Mature 50 S subunit in wild-type cells was prepared from a crude 70 S ribosome fraction as described previously (24).

Preparation of Free 50 S Subunit from YlqF-depleted, YlqFΔN10-overexpressing, and Wild-type Cells—YM01 cells and YM03 cells were grown at 37 °C in LB containing 5 μM IPTG and LB containing 30 μM IPTG and 2% xylose, respectively. Wild-type 168 cells were grown at 37 °C in LB. Cells were collected at A600 = 0.6, and free 50 S ribosome was purified as described previously (24).

Purification of YlqF-His6 and YlqFΔN10-His6—To express YlqF and YlqFΔN10 with a histidine tag (His6) at the C terminus, the YlqF coding sequence lacking the termination codon (nucleotides 1–843) and the N-terminal YlqF coding sequence (nucleotides 28–843) lacking the termination codon were amplified by PCR using primer pairs 5′-AAACCATGGATGA-CAATTCAATGGTCCCG-3′/5′-ATATCTCGACATGCGTGC- TGGTGTTCAATAAG-3′ and 5′-AAACCATGGATGCGC- AAAAGCGAAGAGGGA-3′/5′-ATATCTCGACATGCGTGTTCAATAAG-3′, respectively, and cloned into PET28b (Novagen) using Ncol and Xhol sites to obtain plasmids pYM03 and pYM04, respectively. E. coli BL21 (DE3)pLysS derivatives (Novagen) containing pYM03 or pYM04 were grown at 30 °C in 500 mL LB, and the His6-tagged YlqF and YlqFΔN10 were purified according to the pET system protocol (Novagen) as described previously (24).

Binding of YlqF to the 50 S Ribosomal Subunit—Various concentrations of 50 S subunit were incubated with 0.5 μM YlqF or YlqFΔN10 in buffer D containing 10 μM GTP at 25 °C for 10 min. The mixture was applied to a Microcon 100 (Millipore) and centrifuged for 5 min at 3000 × g. The column was washed 3 times with the same buffer by centrifugation at 3000 × g for 12 min. Buffer was added to the filter, and the column was inverted after a 1-min incubation at room temperature. The 50 S subunit-bound YlqF was recovered by centrifugation of the inverted column for 1 min at 3000 × g, and the concentration of YlqF and YlqFΔN10 was determined by immunoblotting with anti-YlqF antibodies.

Assay of GTPase Activity—All GTPase activity assays were performed at 37 °C and contained 20 mM Tris-HCl, pH 7.6, 100 mM NH4CH3CO2, 15 mM Mg(CH3CO2)2, 100 μM [γ-32P]GTP (PerkinElmer Life Sciences), YlqF, and 50 S ribosome subunit. The reactions were terminated by the addition of ice-cold water containing 6% charcoal. Next, charcoal was sedimented by centrifugation at 10,000 × g for 5 min, and the amount of free phosphate released by hydrolysis of [γ-32P]GTP was determined by Cerenkov counting of the supernatant.

Assay of GTPγS Binding—YlqF or YlqFΔN10 (2.5 μM) were incubated for 10 min in buffer A at 30 °C with various concentrations of [35S]GTPγS (PerkinElmer Life Sciences). Protein-bound [35S]GTPγS was collected onto nitrocellulose filters (0.45 μm; Millipore), and its radioactivity was measured with a scintillation counter.

RESULTS

N-terminal Deletion Mutant of YlqF Displays a Dominant-negative Phenotype—GTP-binding proteins have the characteristic amino acid sequence motifs G1 (G/IXXXGKT/S), G3 (DXXG), and G4 (NKXD) in the GTP binding domain that are required for the hydrolysis and binding of GTP (1). E. coli IF-2 G-motif mutants (V400G and H448E), which have a dominant-negative effect on growth, have been isolated (29). The GTPase activity of these mutants is not stimulated by ribosome binding, and they remain bound to the ribosome after formation of 70 S initiation complex, demonstrating that GTP hydrolysis is coupled with its dissociation from the 70 S initiation complex and is important for translation initiation and the recycling of IF-2. YlqF also has the GTP binding domain including the G1, G3, and G4 motifs. To isolate a dominant negative mutant of YlqF, we changed several conserved amino acids in this domain to alanine by site-directed mutation (Fig. 1A), but this did not have a dominant-negative effect.

The x-ray structure of YlqF (PDB code 1PJI) shows that YlqF consists of an N-terminal GTP binding domain (codons 1–177) and a C-terminal YlqF-specific domain (codons 178–282). We aligned multiple amino acid sequences of YlqF from B. subtilis and its orthologs from other Gram-positive bacteria using ClustalW. We found a newly conserved motif (residues 3–10; I(Q/N)W(F/Y)PGHM) in the N-terminal region (Fig. 1B). To examine whether this region (N10 region) is necessary for cell growth, we constructed a strain (YM103) in which the N-terminal deletion mutation of ylqF (ylqFΔN10) under the control of the xylose-inducible xyl promoter (Psxl) was placed at the amyE locus and wild-type ylqF was placed under the...
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Control of the IPTG-inducible _spac_ promoter (Pspac) at the native position. YMO3 (Pspac-ylqF, _amyE::PxyL-ylqF_ N10) grew on LB containing 25 μM IPTG but not on LB containing 2% xylose, indicating that YlqFΔN10 cannot support cell growth (Fig. 1C). Interestingly, these cells grew poorly on LB containing both IPTG and xylose. A control strain, YM02 (Pspac-ylqF, _amyE::PxyL-ylqF_), was able to grow well in all conditions. These results indicated that YlqFΔN10 inhibited cell growth even in the presence of wild-type YlqF.

**Overexpression of YlqFΔN10 Induces Accumulation of Premature 50 S subunit, and YlqFΔN10 Stably Associates with the Premature 50 S Subunit**—We next examined how YlqFΔN10 inhibits cell growth in the presence of wild-type YlqF. To investigate whether overexpression of YlqFΔN10 directly inhibited 50 S subunit biogenesis, we examined the ribosome profile of YlqFΔN10-overexpressing cells by sucrose density gradient centrifugation. YM02 and YM03 cells grown in LB containing 30 μM IPTG and 2% xylose were used as YlqF-overexpressing cells and YlqFΔN10-overexpressing cells, respectively. The level of YlqF increased about 7-fold in the YlqF-overexpressing cells, but their growth was normal compared with the control cells (Fig. 2, A and B). In contrast, in YlqFΔN10-overexpressing cells, the level of YlqFΔN10 increased about 10-fold, and their growth was significantly impaired.

Next, each of the cell cultures was collected during exponential phase (A600 = 0.6), and cell lysates were separated by sucrose density gradient centrifugation. The amount of YlqF or YlqFΔN10 in each fraction was determined by immunoblotting with an anti-YlqF antibody. In a previous report, we showed that YlqF co-fractionated with the free 50 S subunit in the presence of GTPγS but not in the presence of GTP or GDP (24). In the present experiments guanine nucleotide was not added to the buffer. In YlqF-overexpressing cells, the ribosome profile was normal compared with control cells, and most YlqF was present in the top fraction of the gradient (Fig. 2C). In contrast, the ribosome profile in YlqFΔN10-overexpressing cells was drastically altered, including a decrease in the amount of 70 S ribosome and accumulation of premature 50 S subunit with a decreased molecular weight. Furthermore, strong bands, possibly corresponding to YlqFΔN10, appeared in the premature 50 S subunit fraction, above the background of wild-type YlqF signals (Fig. 2C). These results indicated that YlqFΔN10 remained bound to premature 50 S subunit and interfered with the biogenesis of 50 S subunit.

**YlqFΔN10 Lacks GTPase Activity and Stably Binds the 50 S Subunit in Vitro**—We previously reported that GTPase activity of YlqF is activated by the 50 S subunit in _vitro_ and that YlqF is stably associated with the 50 S subunit when its GTPase activity is inhibited by GTPγS (24). The stable association of YlqFΔN10 with the premature 50 S subunit suggested that GTPase activity of YlqF was eliminated by deletion of the N terminus. To examine this possibility, we measured GTP binding and GTPase activities of YlqF and YlqFΔN10 in the presence and absence of the purified 50 S subunit. Although YlqFΔN10 retained the ability to bind GTPγS (Fig. 3A), as expected, the GTPase activity of YlqFΔN10 was not stimulated by the 50 S subunit (Fig. 3B). Furthermore, YlqFΔN10 was more stably associated with the 50 S subunit compared with wild-type YlqF _in vitro_, even in the presence of GTP (Fig. 3C).

Next, we examined the possibility that YlqFΔN10 interfered with stimulation of the GTPase activity of wild-type YlqF by 50 S subunit. Indeed, we found that YlqFΔN10 inhibited the stimulation of the GTPase activity of wild-type YlqF, depending on the ratio between YlqFΔN10 and YlqF (Fig. 3D), indicating that
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the YlqFΔN10 acted as a competitive inhibitor of wild-type YlqF in vitro.

Premature 50 S Subunit in YlqFΔN10-overexpressing Cells Lacks L27, and the Amount of L16 Is Reduced—We previously demonstrated that premature 50 S subunit lacking ribosomal proteins L16 and L27 accumulates in YlqF-depleted cells (24). Because there is insufficient expression of ylqF in these cells, biogenesis of the 50 S subunit stops before the YlqF binding step. In contrast, because YlqFΔN10 binds to the 50 S subunit but its GTPase activity is not stimulated, biogenesis of the 50 S subunit in YlqFΔN10-overexpressing cells should be blocked before the GTPase activation step. Therefore, to investigate the timing of YlqF GTPase activation during biogenesis of 50 S subunit, we measured the amount of premature 50 S subunit in YlqFΔN10-overexpressing cells. YM03 cells (Pspac-ylqF, amyE:Pxyl-ylqFΔN10) were grown in the LB supplemented with 30 μM IPTG or with a combination of 30 μM IPTG and 2% xylose. YM01 cells (Pspac-ylqF) were also grown with 5 μM IPTG to compare the level of premature 50 S subunit with that in YlqF-depleted cells. The cells were collected during exponential phase (A600 = 0.6), and cell lysates were separated by sucrose gradient centrifugation. The ribosome profile of YlqFΔN10-overexpressing cells was very similar to that of YlqF-depleted cells and the premature 50 S subunits sedimented in the same fraction (Fig. 4A).

Next, we isolated the premature 50 S subunit from YlqFΔN10-overexpressing cells or YlqF-depleted cells by ultracentrifugation. Premature 50 S subunit from the two cell types and from wild-type cells was compared by SDS-PAGE. As shown in Fig. 4B, the amount of ribosomal protein L16 was reduced, and L27 was absent in premature 50 S subunit from YlqFΔN10-overexpressing cells, the same finding as in YlqF-depleted cells. These results suggested that, contrary to our
previously proposed model (24), biogenesis of 50 S subunit in YlqFΔN10-overexpressing cells stops before the incorporation of L16 and L27.

**Premature and Free 50 S Subunits Strongly Enhance the GTPase Activity of YlqF**—We found that accumulated premature 50 S subunit in YlqFΔN10-overexpressing cells lacks ribosomal protein L27, and the amount of L16 is reduced. This suggested that GTP hydrolysis is activated by premature 50 S subunit lacking L16 and L27. To examine this hypothesis, we measured the GTPase activity of YlqF in the presence of premature 50 S subunit from YlqF-depleted and YlqFΔN10-overexpressing cells. As controls, we prepared free 50 S fraction from wild-type cells by the same method used to prepare premature subunit from mutant cells. In addition, we prepared mature 50 S subunit from purified 70 S ribosomes. We found that premature 50 S subunits from both YlqF-depleted and YlqFΔN10-overexpressing cells stimulated the GTPase activity of YlqF 3-fold more than 50 S subunit dissociated from the 70 S subunit (Fig. 5A), indicating that GTP hydrolysis is stimulated by premature 50 S subunit before the incorporation of L16 and L27. In addition, we observed that free 50 S subunit from wild-type cells also strongly activates YlqF GTPase activity (Fig. 5A).

These results point to significant differences between free 50 S subunit prepared directly from cell lysate and that prepared by dissociation from purified 70 S ribosome even though both are found in the same fraction in the ribosome profile.

Magnesium is known to be necessary for the formation of proper tertiary rRNA structure (30, 31). It also acts as a cofactor of the GTP-binding proteins for nucleotide binding (32). Therefore, we compared the extent of GTPase activation by the mature 50 S subunit in the 70 S ribosome at different magnesium concentrations. Interestingly, stimulation of the YlqF GTPase activity by the 50 S subunit prepared from 70 S ribosome was enhanced only when the magnesium concentration was decreased (Fig. 5B).

**DISCUSSION**

The bacterial ribosome consists of two subunits, 30 S and 50 S, which are composed of three rRNAs and more than 50 ribosomal proteins (33). Assembly of these ribosomal subunits occurs concomitantly with the synthesis of rRNA (34, 35). Both subunits assemble sequentially through various intermediates, and protein-dependent conformational changes seem to have a crucial role in their ordered assembly (33, 34). In eukaryotes, more than 100 non-ribosomal proteins are essential for the biogenesis of ribosomes (35, 36), whereas only a few factors have been identified in bacteria (37). Recently, multiple GTP-binding proteins have been implicated in the assembly of bacterial ribosomes (3–7, 19, 20, 23, 24, 38). In particular, *E. coli* Era binds to the interface region of 30 S and participates in the late assembly step of the 30 S subunit, and *B. subtilis* YlqF binds to the 50 S subunit and is involved in the late assembly of the 50 S subunit (22, 24). Despite these recent findings, the regulation and role of their GTPase activities during ribosome biogenesis have not been explored.

Here, we defined the YlqF binding and GTPase activation steps...
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During 50 S subunit biogenesis using a dominant-negative mutant of YlqF. An N-terminal deletion mutant of YlqF (YlqFΔN10) produced dominant-negative effects when overexpressed in wild-type cells. In vitro assays demonstrated that YlqFΔN10 retained GTP binding activity but lacked GTPase activity and interfered with 50 S subunit-dependent stimulation of the GTPase activity of wild-type YlqF. Furthermore, it could not dissociate from the 50 S subunit. These results indicated that GTP hydrolysis is required for dissociation of YlqFΔN10 from the 50 S subunit. The dominant-negative phenotype of YlqFΔN10 may be due to the inability of the mutant protein to dissociate from the 50 S subunit, thus inhibiting the interaction between wild-type YlqF and the 50 S subunit, although it is also possible that the mutant protein forms a non-functional heterodimer with wild-type protein. The deleted N terminus 10 amino acids (N10) sequence is highly conserved in YlqF from Gram-positive bacteria. Our results indicate that this sequence plays a critical role in the regulation of YlqF GTPase activity, although the molecular mechanism of this regulation is not yet clear.

We further showed that the incorporation of ribosomal proteins L16 and L27 into the 50 S subunit is impaired by the overexpression of YlqFΔN10, as it is in YlqF-depleted cells, suggesting that GTP hydrolysis is necessary for the incorporation of L16 and L27. In addition, premature 50 S subunit in YlqF-depleted cells and YlqFΔN10-overexpressing cells greatly enhanced the GTPase activity of YlqF compared with its activity in the mature 50 S subunit dissociated from 70 S ribosome. Given these results, we propose the following model of YlqF function (Fig. 6). Because premature 50 S subunit lacking ribosomal proteins L16 and L27 (premature form I) accumulates in YlqF-depleted cells, GTP-bound YlqF is incorporated into the 50 S subunit at this stage. Incorporation of L16 and L27 into the 50 S subunit is inhibited by overexpression of YlqFΔN10, and YlqF directly binds to the 23S rRNA (24), resulting in activation of the YlqF GTPase activity by the premature 50 S subunit lacking L16 and L27 (form II), suggesting that YlqF induces conformational changes in 23S rRNA in a GTP hydrolysis-dependent manner, leading to form III. L16 and L27 then bind to the premature 50 S subunit (form IV). We propose that YlqF is released after the incorporation of L16 and L27, completing the maturation of the 50 S subunit, although we have not experimentally examined the timing of YlqF dissociation.

Unexpectedly, we observed that free 50 S subunit from wild-type cells also strongly activates the YlqF GTPase activity compared with that in mature 50 S subunit dissociated from 70 S ribosome. The free 50 S subunit fraction may contain premature 50 S subunit. However, the GTPase-stimulating activity of free 50 S subunit was comparable with those of premature 50 S subunit accumulated in YlqF-depleted and YlqFΔN10-overexpressing cells, suggesting that there are significant differences between free 50 S subunit prepared from cell lysate and that prepared by dissociation from 70 S ribosomes. In addition, in contrast with the free 50 S subunit fraction from wild-type cells, stimulation of the YlqF GTPase activity by the 50 S subunit prepared from 70 S ribosome was enhanced only when the magnesium concentration was decreased. Magnesium ions are required for the formation of proper tertiary rRNA structure (30, 31). The reduction of magnesium ions may induce the misfolding or unfolding of rRNA in 50 S subunit, and such impaired 50 S subunits may activate the GTPase activity of YlqF even if they contain ribosomal proteins L16 and L27. The interface of free 50 S subunit for association with the 30 S subunit might be unstable, because this region is not covered with the ribosomal proteins. Therefore, free 50 S subunit fractions might often contain impaired 50 S subunit due to misfolding or unfolding of rRNA at the interface. However, magnesium ion is a cofactor of GTP-binding protein for nucleotide binding (32). Therefore, it is also possible that a high concentration of magnesium ion directly inhibited the GTPase activity of YlqF even if they contain ribosomal proteins L16 and L27. The interface of free 50 S subunit for association with the 30 S subunit might be unstable, because this region is not covered with the ribosomal proteins. Therefore, free 50 S subunit fractions might often contain impaired 50 S subunit due to misfolding or unfolding of rRNA at the interface. However, magnesium ion is a cofactor of GTP-binding protein for nucleotide binding (32). Therefore, it is also possible that a high concentration of magnesium ion directly inhibited the GTPase activity of YlqF even if they contain ribosomal proteins L16 and L27. The interface of free 50 S subunit for association with the 30 S subunit might be unstable, because this region is not covered with the ribosomal proteins. Therefore, free 50 S subunit fractions might often contain impaired 50 S subunit due to misfolding or unfolding of rRNA at the interface. However, magnesium ion is a cofactor of GTP-binding protein for nucleotide binding (32). Therefore, it is also possible that a high concentration of magnesium ion directly inhibited the GTPase activity of YlqF even if they contain ribosomal proteins L16 and L27. The interface of free 50 S subunit for association with the 30 S subunit might be unstable, because this region is not covered with the ribosomal proteins. Therefore, free 50 S subunit fractions might often contain impaired 50 S subunit due to misfolding or unfolding of rRNA at the interface. However, magnesium ion is a cofactor of GTP-binding protein for nucleotide binding (32). Therefore, it is also possible that a high concentration of magnesium ion directly inhibited the GTPase activity of YlqF even if they contain ribosomal proteins L16 and L27. The interface of free 50 S subunit for association with the 30 S subunit might be unstable, because this region is not covered with the ribosomal proteins. Therefore, free 50 S subunit fractions might often contain impaired 50 S subunit due to misfolding or unfolding of rRNA at the interface. However, magnesium ion is a cofactor of GTP-binding protein for nucleotide binding (32).

A recent report has shown similar accumulations of premature 50 S subunit lacking L16 and L27 in YsxC- and YphC-depleted cells (7). Moreover, the amount of accumulated premature 50 S subunit in YphC-depleted cells is almost equal to that in YlqF-depleted cells (7), suggesting that incorporation of ribosomal proteins L16 and L27 into 50 S subunit requires the formation of proper rRNA structure through the action of multiple GTP-binding proteins. We have been unable to show that
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overexpression of YphC in YlqF-depleted cells rescues cell growth, indicating that YlqF and YphC have distinct functions at the same step of 50 S subunit biogenesis. Therefore, in future studies we will distinguish the function of each GTP-binding protein to help elucidate the detailed molecular mechanisms of ribosome biogenesis in bacteria.

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