The GTP-binding protein, YlqF, participates in the late step of 50S ribosomal subunit assembly in Bacillus subtilis

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Bacillus subtilis YlqF belongs to the Era/Obg subfamily of small GTP-binding proteins, and is essential for bacterial growth. Here we report that YlqF participates in the late step of 50S ribosomal subunit assembly. YlqF was co-fractionated with the 50S subunit, depending on the presence of non-cleavable GTP analogue. Moreover, the GTPase activity of YlqF was stimulated specifically by the 50S subunit in vitro. DMS footprinting analysis disclosed that YlqF binds to a unique position in 23S rRNA. Yeast two-hybrid data revealed interactions between YlqF and the B. subtilis L25 protein (Ctc). The interaction was confirmed by the pull-down assay of the purified proteins. Specifically, YlqF is positioned around the A-site and P-site on the 50S subunit. Proteome analysis of the abnormal 50S subunits that accumulated in YlqF-depleted cells showed that L16 and L27 proteins, located near the YlqF-binding domain, are missing. Our results collectively indicate that YlqF will organize the late step of 50S ribosomal subunit assembly.

The ribosome is the large RNA-protein complex composed of two subunits. In bacteria, the large 50S subunit is composed of 23S rRNA, 5S rRNA and some 30 additional proteins, whereas the small 30S subunit is composed of 16S rRNA and some 20 other proteins. Initially, each rRNA is synthesized as a single large precursor, and matures through a series of steps, including endo- and exonucleolytic cleavage into three rRNA precursors, sequential removal of additional sequences, and nucleotide modifications (1). Ribosomal proteins are assembled on rRNA in parallel with their synthesis and processing to yield the functional ribosomal subunits. In bacteria, functional subunits can be reconstituted from the isolated components in vitro (2, 3). However, this process requires long incubation times, high temperatures, and high ionic conditions, compared with the in vivo situation, suggesting that assembling factors are required for ribosome assembly in vivo. In eukaryotes, more than 100 factors participate in biogenesis of the ribosome (4, 5, 6), compared to only a few factors identified in bacteria. Depletion of RNA helicases, CsdA and SrmB, leads to slow-growth phenotype and accumulation of premature 50S particles at low temperatures in Escherichia coli (7, 8). The Obg/Era subfamily of small GTP-binding proteins has been suggested to participate in ribosomal biogenesis. This subfamily comprises a distinct group of small proteins originally characterized in bacteria, but later identified in genomes of eukaryote and archa (9).
Many of the bacterial members investigated so far are essential for cell growth (10, 11, 12, 13, 14, 15). Various phenotypic changes are observed in cells depleted of Obg or Era. However, numerous results suggest that the primary function of Obg/Era GTP-binding proteins is related to ribosome biogenesis or translation process, consistent with their evolutionary relationships with translation factors (16). Obg and Era are the most extensively characterized bacterial small GTP-binding proteins belonging to this family. Obg interacts with the 50S subunit in E. coli and Caulobacter crescentus (17, 18). In Bacillus subtilis, Obg co-fractionates with the 50S subunit, and specifically interacts with the ribosomal protein, L13 (19). In contrast, E. coli Era interacts with the 30S subunit (20, 21, 22). Furthermore, depletion of Era and Obg results in abnormal ribosome profiles in E. coli and C. crescentus, respectively (15, 23). E. coli Era functionally compensates for deletion of the gene encoding the cold-shock adaptation protein, RbfA (23), which is required for efficient processing of 16S rRNA (24). Another essential bacterial small GTP-binding protein, YjeQ, associates with the 30S subunit in E. coli. Its GTPase activity is specifically enhanced in the presence of 30S subunit (25, 26). However, the precise actions of GTP-binding proteins on the ribosome remain to be clarified. A recent three-dimensional cryo-electron microscopic map of the Thermus thermophilus 30S-Era complex (27) indicates that Era binds within the cleft between the head and platform of the 30S subunit. The binding site of Era overlaps with that of S1 in E. coli. Moreover, Era binding on the 30S subunit interferes with binding to the 50S subunit, suggesting that dissociation of Era and incorporation of S1 comprise the final step of the 30S subunit assembly, and that Era inhibits formation of the translation initiation complex on prematurely assembled 30S (27).

In an earlier report, we demonstrated that four additional GTP-binding proteins, YsxC, YlqF, YphC and YqeH, are indispensable for B. subtilis growth (11). Here we show that B. subtilis YlqF binds to 50S subunit, and its GTPase activity in vitro is completely dependent on interactions with the mature 50S subunit. Using DMS footprint analysis, we mapped the binding site of YlqF on helices 38, 81 and 85 of 23S rRNA. Finally, L16 and L27, located near the YlqF binding site on the 50S subunit at the final stage of assembly, were not detectable in the premature 50S subunit that accumulated in YlqF-depleted cells. The results collectively indicate that YlqF interacts with the premature 50S subunit, and monitors the completion of ribosomal subunit assembly.

EXPERIMENTAL PROCEDURES

Strain - B. subtilis 168 was used as the wild-type strain throughout. B. subtilis TMO208, an IPTG-dependent mutant, contains ylqF under the control of the IPTG-dependent spac promoter (11).

Buffer - The following buffers were employed: buffer A, 10 mM Tris-HCl, pH 7.6, 10 mM (CH3COO)2Mg, 100 mM CH3COONH4, 6 mM β-mercaptoethanol, and 2 mM PMSF; buffer B, 20 mM Tris-HCl, pH 7.6, 15 mM (CH3COO)2Mg, 1 M CH3COONH4, 6 mM β-mercaptoethanol, and 2 mM PMSF; buffer C, 10 mM Tris-HCl, pH 7.6, 1 mM (CH3COO)2Mg, 100 mM CH3COONH4, 6 mM β-mercaptoethanol, and 2 mM PMSF; binding buffer, 20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole; washing buffer, 0.5 M NaCl, 20 mM imidazole, 20 mM Tris-HCl, pH 7.9; hybridization buffer, 25 mM Tris-HCl, pH 8.3, 30 mM NaCl; PBS buffer, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, 137 mM NaCl, 27 mM KCl, pH 7.3; GST elution buffer, 50 mM Tris-HCl, pH 8.0, 10 mM glutathione.

Detection of GTP-binding proteins on the ribosome profile - Wild-type B. subtilis cells (168) were grown at 37°C in LB medium, and collected at the exponential phase (OD600=0.6). Cells were resuspended in buffer A containing GTPγS or GTP or GDP, and disrupted by passage through a French pressure cell at 8000 p.s.i. After the removal of cell debris, the supernatant was subjected...
to 10-40% sucrose density gradient centrifugation for 14 h at 90,000 × g. Sucrose gradients were separated into 18 fractions. During fractionation, absorbance at 254 nm was monitored. YlqF in each fraction was separated by SDS-PAGE on a 15% gel, and analyzed by immunoblotting with anti-YlqF antibody, according to a previous method (11).

**Preparation of crude ribosome - B. subtilis** 168 cells were grown in LB medium at 37°C, and collected at OD<sub>600</sub> of 0.6. 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, the supernatant was centrifuged for 100 minutes at 240,000 × g. The precipitate was dissolved in buffer B, and centrifuged at 40,000 × g for 60 minutes at 4°C. Next, 2 ml aliquots of supernatant were layered onto 2 ml buffer B containing a 30% (w/v) sucrose bed, and centrifuged at 240,000 × g for 3.5 h. The precipitate of ribosome was resuspended in buffer A, and stored at -80°C until use.

**Preparation of 50S and 30S ribosomal subunits -** Crude ribosome was subjected to 10-40% sucrose density gradient centrifugation in buffer C at 90,000 × g for 14 h. Gradients were fractionated, and fractions containing the 50S and 30S subunits were identified by peaks in absorbance at 260 nm. Magnesium acetate (1M) was employed to increase the concentration of magnesium ions to 20 mM, and the solution was mixed with an equal volume of ice-cold ethanol. After incubation for 1 h at -20°C, the precipitate was collected by centrifugation at 10,000 × g for 30 minutes. The pellet was resuspended in a small volume of buffer A, and stored at -80°C until use.

**Purification of YlqF-His<sub>6</sub>** - To express the YlqF with a histidine tag (His<sub>6</sub>) at the C terminus, the coding sequences of YlqF (from the initiation codon to the C-terminal amino acid codon, except the termination codon) were amplified by PCR and cloned into the pET29b plasmid (Novagen) to obtain pTM298. E. coli BL21(DE3)pLysS derivatives (Novagen) containing pTM298 was grown at 30°C in 500 ml LB medium and the his-tagged YlqF was purified according to the pET system protocol (Novagen). When the culture reached OD<sub>600</sub> of 0.6, IPTG was added to a final concentration of 1 mM. The cells were grown 3 hours more and then harvested by centrifugation. Collected cells were washed with binding buffer and resuspended in 20 ml of the same buffer. The cells were broken by sonication on ice and the lysate was centrifuged at 40,000 × g for 30 minutes at 4°C. The supernatant fraction was applied to a Ni<sup>2+</sup>-NTA resin column (Novagen), and the column was washed with 30 ml binding buffer containing 60 mM imidazole, and then was dialyze against buffer A. Purified YlqF-His<sub>6</sub> was stored at -80°C after addition of 10% (v/v) glycerol.

**Assay of GTPase activity -** The YlqF protein was purified using a procedure described in an earlier report (11). All reactions for assay of GTPase activity were performed at 37°C, and contained 20 mM Tris-HCl, pH 7.6, 100 mM CH₃COONH₄, 15 mM (CH₃COO)₂Mg, 100 µM [γ-<sup>32</sup>P]GTP (Perkin Elmer), YlqF, and ribosome and its subunit. Reactions were terminated by the addition of ice-cold water with 6% charcoal. Next, charcoal was pelleted by centrifugation at 10000 × g for 5 min, and the amount of free phosphate released by hydrolysis of [γ-<sup>32</sup>P]GTP was determined by Cerenkov counting of the supernatant.

**Binding of YlqF to the 50S ribosomal subunit -** Various concentrations of YlqF were incubated with 0.2 µM 50S ribosomal subunit in buffer A containing 100 µM GTP<sub>7</sub>S at 30°C for 10 min. The mixture was applied to Microcon 100 (Millipore), and centrifuged for 5 min at 3,000 × g. The micro-spin column was washed twice with the same buffer by centrifugation at 3,000 × g for 12 minutes. Buffer was added to the filter, and the column inverted after 1 min incubation at room temperature. The 50S
subunit-bound YlqF was recovered by centrifugation (1 min, 3,000 × g) of the inverted column, and each concentration of YlqF and 50S subunit determined by immunoblotting with anti-YlqF and anti-L3 antibodies, respectively.

**DMS modification** - The 50S subunit (8 pmol) was preincubated at 40°C for 10 min in 25 µl of buffer A. The reaction mixture was added to YlqF (80 pmol) in 50 µl of buffer A containing 100 µM GTPγS, and incubated at 30°C for 10 min. Chemical modification was initiated by the addition of DMS (1 µl; 1:4 dilution in ethanol), followed by incubation at 30°C for 15 min. Reactions were terminated using two volumes of cold ethanol, followed by rapid mixing. The modified 50S subunit was pelleted by centrifugation at 15,000 × g at 4°C for 20 min. The pellet was resuspended in buffer A, and extracted twice with phenol/chloroform/isoamyl alcohol (25/24/1 v/v/v). RNA was precipitated by the addition of 2.5 volumes of cold ethanol, and resuspended in buffer A. The position and extent of chemical modification were analyzed by primer extension with reverse transcriptase, followed by gel electrophoresis.

**Primer extension** - The primers specified in Table I were end-labeled with [γ-32P]ATP (Amersham Pharmacia) and T4 polynucleotide kinase (Takara Bio Inc). Labeled DNA primer (1 µl) (approximately 0.2 pmol) was added to modified RNA (0.5 pmol) in 20 µl of hybridization buffer. The mixture was heated to 90°C for 3 minutes, and slowly cooled to 42°C in 45 minutes. Reverse transcription was performed for 60 min at 42°C using AMV reverse transcriptase XL (Takara Bio Inc). The cDNA product was separated with a sequencing gel (8% polyacrylamide, 8 M Urea, 89 mM Tris-borate, pH 8.3, and 24 mM EDTA). After electrophoresis, the gel was dried, and exposed to an Imaging Plate (Fuji Film) for 16 h. Signals were detected using BAS2500 (Fuji Film).

**Analysis of the ribosome profile in YlqF-depleted cells** - Wild-type cells (168) were grown at 37°C in LB medium. Cells were collected at the exponential and stationary phases, respectively. An IPTG-dependent mutant of YlqF (TMO208) was grown to the mid-exponential phase at 37°C in LB with 5 µM IPTG. Cells were resuspended in buffer A, and disrupted by passage through a French pressure cell (Aminco) at 8000 p.s.i. After the removal of cell debris, the supernatant was subjected to 10-40% sucrose density gradient centrifugation for 14 h at 90,000 × g. The ribosome profile was monitored with UV at 254 nm.

**Yeast two-hybrid system** - Yeast strains PJ69-4A (MATa, trp1-901, leu2-3, 112ura3-52, his-200, gal4D, gal80D, LYS2::GAL1-HIS3, GAL2-AD2, met2::GAL7-lacZ) and PJ69-4a (MATa, trp1-901, leu2-3, 112ura3-52, his-200, gal4D, gal80D, LYS2::GAL1-HIS3, GAL2-AD2, met2::GAL7-lacZ) were kindly provided by Philip James (28). The pGBT9(TRP1)-ylqF and pGAD424(LEU2) harboring genes encoding each ribosomal protein were introduced into PJ69-4A and PJ69-4a, respectively, essentially according to the instructions of Bongiorni (29). Transformants were mated in the appropriate liquid media using flat bottom 96-well plates. After mating, cultures were collected, washed with sterilized water, and spotted on synthetic complete (SC) agar plates lacking leucine and tryptophan (SC-LW) for selection of LEU2 and TRP1 diploid cells. Next, cells were cultured in liquid SC-LW medium for one day, replicated on selection agar medium lacking histidine (SC-LEH), and supplemented with 5 mM 3-aminotriazazole (3-AT) to inhibit autoactivation of the HIS3 reporter gene.

**Pull-down assay** - To express ribosome protein L25, designated Ctc in *B. subtilis*, with a histidine tag at the N terminus and YlqF with a GST tag at the N terminus, the coding sequences of Ctc and YlqF were amplified by PCR and cloned into
the pDEST17 (Invitrogen) and pDEST15 (Invitrogen) using Gateway-Technology, to obtain pD17C and pD15L, respectively. GST was expressed in *E. coli* using pGEX2T (Novagen). *E. coli* BL21(DE3)pLysS derivatives (Novagen) containing pD17C, pD15L or pGEX2T were grown at 30°C in 500ml LB medium. His-tagged Ctc was purified using the procedure described in the section of purification of YlqF-His<sub>6</sub>. The cells expressing GST-YlqF or GST were broken in PBS buffer by sonication on ice and the lysate was centrifuged at 40,000 × g for 30 minutes at 4 ºC. The supernatant fraction was applied to a glutathione resin column (Novagen), and the column was washed with 10 ml PBS buffer. GST-YlqF or GST in the column was eluted with 1 ml GST elution buffer, and purified GST-YlqF and GST were stored at -80 °C after addition of 10% (v/v) glycerol.

To detect interaction between YlqF and Ctc, purified GST-YlqF and His<sub>6</sub>-Ctc (1µM each) were mixed in PBS buffer containing 250µM GTP, and incubated for 15 min at 30°C. The mixture was applied to a glutathione resin column, and the column was washed with PBS buffer. Proteins retained in the column was eluted with GST elution buffer, and separated by SDS-PAGE on a 4-20% gel, followed by silver staining.

**RESULTS**

YlqF is specifically associated with the 50S ribosomal subunit - Era, Obg, and YjeQ interact with ribosomal subunits in various bacteria (17, 18, 19, 20, 21, 22, 27). Accordingly, we examined whether the essential GTP-binding proteins (Era, Obg, YsxC, YlqF, YphC, and YqeH) in *B. subtilis* are associated with the ribosome. Cell lysates were separated by sucrose density gradient centrifugation, and GTP-binding proteins in each fraction were identified by immunoblotting with specific antibodies. Obg was detected in fractions containing the free 50S subunit in the presence of the non-hydrolysable GTP analog, GTP<sub>γ</sub>S (data not shown), as reported previously (Scott *et al.*, 2000). In addition we newly found that YlqF stably co-sedimented with the free 50S subunit in the presence of GTP<sub>γ</sub>S, but not GTP or GDP (Fig.1).

**GTPase activity of YlqF is stimulated by the 50S ribosomal subunit** - The GTPase activities of small bacterial GTP-binding proteins may be modulated by interactions with the ribosomal subunit. Recent studies show that the GTPase activity of *E. coli* YjeQ, which associates with 30S subunit, is enhanced in the presence of this subunit in vitro (25, 26). As YlqF bound to the free 50S subunit, but not 30S subunit, we next examined the activation of GTPase activity of YlqF by the 50S subunit. We prepared 30S and 50S subunit from *B. subtilis* cells by differential ultracentrifugation, as described in a previous report (30). YlqF was fused to a histidine tag, expressed in *E. coli* and purified by the Ni<sup>2+</sup>-NTA resin column (Fig.2A), as described in a previous report (11). Consistent with YlqF association with the 50S subunit, GTPase activity was stimulated in the presence of 50S subunit, but not 30S subunit (Fig.2B). No activity of YlqF with the 50S subunit treated RNaseA was observed (Fig.2B). Our results indicate that 50S is indispensable for the GTPase activity of YlqF.

**YlqF binds helices 38, 81 and 85 of 23S rRNA and the L25 protein on the 50S subunit** - To further characterize the interaction between YlqF and the 50S subunit in vitro, a filtration-based binding assay was performed to evaluate the stoichiometry of the YlqF-50S subunit complex. Upon application to the Microcon 100 column, YlqF without the 50S subunit passes straight through the filter (data not shown). In contrast, when YlqF was applied to the column in conjunction with the 50S subunit, the protein was recovered from the reservoir of the column. Recovered YlqF was saturated following the addition of 2-fold excess protein to the 50S subunit, indicating that two molecules of YlqF bind a 50S subunit (Fig.3).

DMS footprint analysis was employed to determine the interaction site of
YlqF and 23S rRNA in the 50S subunit (31, 32). The 50S subunit (8 pmol) and YlqF (80 pmol) were incubated at 30°C for 10 min, then chemical modification was initiated by the addition of DMS, followed by incubation at 30°C for 15 min. Modifications within the whole 23S rRNA sequence were investigated using a set of fifteen DNA primers for extension with reverse transcriptase, as described in Table I. Binding of YlqF led to protection of C928, C942, A2301, and A2354 (Fig.4A). According to the three-dimensional structure of the Deinococcus radiodurans 50S subunit (PDB code 1NKW, 34), C928 and C942 in *B. subtilis* lie in helix 38 (Fig.4B), that interacts with the elbow of tRNA in the A-site. A2301 and A2354 are present in helix 81 and 85, respectively (Fig.4C). Helices 81 and 85 are positioned near the L5 protein. The β-hairpin loop of L5 interacts with the elbow of tRNA in the P-site (33, 34). Based on the data, we propose that YlqF interacts with 23S rRNA around the A-site and P-site.

To examine the possibility of direct binding between YlqF and ribosomal proteins, we analyzed interactions using the yeast two-hybrid system. Fifty nine genes annotated as ribosomal protein gene in the *B. subtilis* genome sequence (BSORF; http://bacillus.genome.jp/) were cloned into pGAD424, and their interaction with YlqF cloned into pGBT9 were examined. The results revealed that YlqF interacts with ribosomal L25 protein (designated Ctc in *B. subtilis*, Fig.5A), but not with other ribosomal proteins (data not shown). To further confirm the direct interaction between YlqF and L25 (Ctc), we attempted a pull-down assay. We constructed the plasmids expressing GST-YlqF, His6-Ctc and GST in *E. coli*, and purified them to near homogeneity (Fig.5B). When purified GST-YlqF and His6-Ctc were incubated for 15 min at 30°C, and applied to a glutathione resin column. Although His6-Ctc itself did not bind to the column, it was retained in the column when incubated with GST-YlqF, but not with GST (Fig.5B). These data confirmed that YlqF has an ability to interact with L25 (Ctc).

In *E. coli*, L25 protein interacts with 5S rRNA (35), as well as helix 38 of 23S rRNA in the YlqF binding domain (33). However, the L25 protein has not been identified in the purified *B. subtilis* 50S subunit from exponentially growing cells (30, 36).

The results collectively provide evidence of interactions between YlqF and the 50S subunit at the central protuberance on the interface side (represented schematically in Figure 7).

**YlqF is possibly involved in assembly of the 50S ribosomal subunit** - The *E. coli* small GTP-binding protein, Era, interacts directly with the 30S subunit (27). The amounts of both 30S and 50S subunits relative to the 70S ribosome are increased in Era-depleted cells (23). In contrast, YlqF interacts with 50S subunit, triggering GTPase activity. Analogous to data obtained on interactions between Era and 30S, a ribosome profile may clarify defects of the 50S subunit in cells displaying YlqF mutations. Accordingly, we examined the ribosome profiles in YlqF-depleted cells, using a strain in which *ylqF* expression is regulated by the IPTG-inducible *spac* promoter (TMO208, 11). The rate of TMO208 cell growth was similar to that of wild-type cells in LB medium with 50 µM IPTG. In medium with 5 µM IPTG, the YlqF level in TMO208 cells was drastically decreased, and growth rate was approximately twice as slow as that of wild-type cells (Fig.6A, B). In TMO208 cells cultured with 5 µM IPTG, the ribosome profile was drastically altered, accompanied by accumulation of the premature 50S subunit and decrease in the amount of 70S ribosome (Fig.6C). SDS-PAGE of proteins in the premature 50S subunit fraction disclosed the loss of at least two bands in YlqF-depleted cells, compared to the normal profile of the 30S subunit (Fig.6D). We identified the absent proteins as L16 and L27 by mass spectrometric analysis of proteins in wild-type cells. L16 protein interacts with helix 38, while L27 protein interacts with helix 81 of 23S rRNA in the YlqF-binding region on the 50S subunit surface (Fig. 7, 34, 37, 38).
DISCUSSION

Recent studies have shown that the binding site of *E. coli* Era overlaps with that of S1 for the 30S subunit (27). Era and S1 proteins cannot coexist on the 30S subunit, and binding of Era to the 30S subunit inhibits its association with 50S, indicative of suppression of mRNA recruitment by the S1 protein (27). These findings imply that dissociation of Era and incorporation of S1 comprise the final step in maturation of the 30S ribosome, and trigger 70S ribosome assembly.

We propose that YlqF is involved in the late step of 50S subunit assembly in *B. subtilis*, in view of the following findings: 1) YlqF binds the free 50S subunit in the presence of GTPγS, and its GTPase activity is strongly stimulated by the 50S subunit *in vitro*, 2) YlqF interacts with helices 38, 81 and 85 of 23S rRNA, and L25 protein on the 50S subunit, 3) YlqF-depleted cells display a significant reduction in the concentration of the 70S ribosome and accumulation of premature 50S subunit, and 4) L16 and L27 proteins are absent in the accumulated premature 50S subunit.

Mapping of the YlqF binding domain as well as L16 and L27 on the *D. radiodurans* 50S subunit reveals that these proteins are located in close proximity to each other at the central protuberance on the interface side of 50S subunit (Fig.7). Both L16 and L27 proteins belong to the late assembly protein (37, 39, 40). Furthermore, L16 crosslinks with L27 in the mature 50S subunit (41, 42). Incomplete assembly of the 50S subunit without L16 leads to defects in peptidyl transferase activity (43, 44), peptidyl-tRNA hydrolysis activity (45), association with the 30S subunit (46), and binding of aminoacyl-tRNA (47). Thus, incorporation of L16 possibly triggers a significant conformational change and facilitates organization of the architecture of the functional site in the 50S subunit (48). On the other hand, deletion of *rpmA* encoding L27 in *E. coli* results in accumulation of the premature 40S subunit lacking L16, L20, L21 and L27, and inhibition of the peptidyl transferase activity of 70S ribosome (49). Incorporation of L16 into the 50S subunit depends on L25, which additionally interacts with YlqF, as evident from the *E. coli* 50S subunit assembly map (50). However, the L25 protein has not been identified in the purified *B. subtilis* 50S subunit from exponentially growing cells (30, 36). The *B. subtilis* L25 protein, designated ‘Ctc’, belongs to the general stress protein, and its synthesis is regulated by sigma B (36). Incorporation of L25 into the ribosome was observed under stress conditions (36). YlqF would associate the 50S subunit independent on L25 or dependent on the transient binding of L25 with 50S subunit in the normal growth condition.

Our results collectively indicate that GTP-bound YlqF interacts with the premature 50S subunit lacking L16 and L27 (Fig.8). Consistent with previous findings on L16 and L27 function, YlqF supports the organization of architecture for the 50S subunit and ensures peptidyl transferase activity through completion of its assembly. After 50S subunit assembly, YlqF dissociates from mature 50S through activation of GTPase (Fig.8). The YlqF binding site is located at the interface for association with the 30S subunit, suggesting that binding would interfere with 70S ribosome assembly. Therefore, YlqF may play a supplementary role in inhibiting formation of the translation initiation complex on prematurely assembled 50S, as proposed for *E. coli* Era in 30S subunit assembly. Under stress conditions, YlqF might bind to premature 50S after incorporation of the L25 protein, or facilitates integration of the L25 protein into the 50S subunit (Fig.8). Future studies should focus on whether this GTPase activity of YlqF is required for its dissociation from the mature 50S subunit, and the relationship between YlqF and L25 proteins under stress conditions.

YlqF has a typical and conserved motif in Gram-positive bacteria and eukaryotes, specifically, a circular permuted GTPase signature motif (9), and is thus classified as a YlqF/YawG protein type.
within the Era/Obg family. In this study, we report the characterization of YlqF in B. subtilis. Differences in bacterial small GTP-binding proteins may correspond to distinct functional checkpoints for ribosome assembly. Thus, functional analysis of these proteins in B. subtilis may facilitate our understanding of ribosome assembly in bacteria.

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NOTE ADDED IN PROOF

After completion of this work, we learned that Robert A Britton and his co-workers have reached a conclusion similar to ours regarding the role of YlqF in the late step of 50S ribosomal subunit assembly by examining the ribosome profile of the YlqF-depleted cells.

REFERENCES


**FOOTNOTES**
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FIGURE LEGEND

Fig. 1. YlqF is associated with the 50S ribosomal subunit in the presence of non-cleavable GTP analogue. B. subtilis 168 cells were grown to the exponential phase in LB medium, and harvested. Cell lysates were prepared in the presence of GTPγS or GTP or GDP, and sedimented through a 10-40% sucrose gradient at 90000 × g for 14 h. During fractionation, absorbance at 254 nm was monitored. YlqF in each fraction was separated by 15% SDS-PAGE, and analyzed by immunoblotting with anti-YlqF antibody.

Fig. 2. GTPase activity of YlqF is stimulated by the 50S ribosomal subunit. A, YlqF-His6 was purified as described in EXPERIMENTAL PROCEDURES. Protein standard marker (lane M), whole cell lysate (lane 1), soluble fraction (lane 2), insoluble fraction (lane 3), flow through fraction of Ni2+-NTA resin column (lane 4), and fraction bound to the column (lane 5) were loaded on a SDS-PAGE and stained with Coomassie Brilliant Blue after electrophoresis. B, The amount of free phosphate released by the hydrolysis of [γ-32P]GTP was measured in reaction mixtures containing 20 mM Tris-HCl, pH 7.6, 100 mM CH3COONH4, 15 mM (CH3COO)2Mg, 20 µM [γ-32P]GTP, 1 µM YlqF with 25 nM 50S subunit (●), 25 nM 30S subunit (○), 25 nM 50S subunit treated with RNase (■) or without ribosomal subunit(□). Reactions were performed at 37°C.

Fig. 3. Two YlqF molecules bind to one 50S ribosomal subunit. The number of YlqF molecules bound per ribosome was estimated by analysis of binding of different concentrations of the protein to the 50S subunit in vitro. YlqF (0.25, 0.5, 0.75, 1, 2, 3 and 4 µM) was incubated with 0.2 µM 50S subunit in buffer A with 100µM GTPγS at 30°C for 10 min, applied to Microcon 100 (Millipore), and centrifuged. The 50S-bound YlqF complex was recovered by centrifugation of the inverted column, and the amount measured by immunoblotting.

Fig. 4. Analysis of YlqF interaction sites on 23S ribosomal RNA in the 50S ribosomal subunit by DMS footprinting experiments. A, The 50S subunit was incubated with (lane +) or without (lane -) YlqF. Samples were treated with DMS. The position and extent of chemical modification were analyzed by primer extension with reverse transcriptase, followed by gel electrophoresis. Autoradiograms of two regions displaying protection against DMS modification as a result of YlqF binding were shown. Primers correspond to residues 1000-1020 (left panel) and 2400-2420 (right panel) in the B. subtilis 23S ribosome RNA sequence. Lanes U, A, C, and G contained sequence ladders obtained using the same primers. The regions protected against DMS modification due to YlqF binding are specified on the secondary structure of helices 38 (B), 81 (C) and 85 (D).

Fig. 5. YlqF binds to the ribosomal protein L25 (Ctc). A, ylqF and ctc were integrated in pGBT9 and pGAD424, respectively. pGBT9 and pGAD424 were employed as negative controls. pGBT9 and pGBT9-ylqF were introduced into PJ69-4A. pGAD424 and pDAD424-ctc were introduced into PJ69-4A. PJ69-4A and PJ69-4a derivatives were used for mating. The resultant diploid cells grew on selection agar medium lacking histidine (SC-LEH) supplemented with 5 mM 3-aminotriazole (3-AT) to inhibit autoactivation of the HIS3 reporter gene, as indicated. B, Purified GST-YlqF and His6-Ctc, GST and His6-Ctc, or His6-Ctc were applied to a glutathione column after incubation for 15 min at 30°C in PBS buffer containing 250µM GTP. Proteins retained in the column were eluted with GST elution buffer, and loaded on the SDS-polyacrylamide gel followed by silver staining. Purified GST-YlqF, His6-Ctc and GST were loaded on the gel as controls.

Fig. 6. Depletion of YlqF results in reduced levels of 70S ribosome and accumulation of the
premature 50S subunit lacking L16 and L27. A, Growth curves of wild-type cells (○) and IPTG-inducible mutant of \textit{ylqF} ( ) at 37°C. Mutant cells were grown in LB medium supplemented with 5 µM IPTG. B, Evaluation of the amounts of YlqF in wild-type cells and IPTG-inducible \textit{ylqF} mutant cells from immunoblotting data. C, Ribosome profile of wild-type cells (...) and IPTG-inducible \textit{ylqF} mutant cells grown in the presence of 5 µM IPTG (—), monitored at an absorbance at 254 nm. Cells were harvested at the exponential growth phase, and lysates prepared and sedimented through a 10-40% sucrose gradient at 90000 × g for 14 h. D, Analysis of components of the 50S and 30S subunits by SDS-PAGE, followed by staining with Coomassie Brilliant Blue. Lanes 1 and 2 were loaded with the 50S subunit in wild-type and IPTG-inducible \textit{ylqF} cells, respectively. Lanes 3 and 4 were loaded with the 30S subunit in wild-type and IPTG-inducible \textit{ylqF} mutant cells, respectively. The arrowheads indicate bands missing in the premature 50S subunit of the \textit{ylqF} mutant, L16 (upper) and L27 (lower).

\textbf{Fig. 7.} Mapping of the YlqF-binding domain, along with L16 and L27 on the \textit{D. radiodurans} 50S ribosomal subunit. The YlqF binding domain is identified by DMS footprinting and the yeast two-hybrid system, and encompasses bases 2301 and 2354 of 23S rRNA (2253 and 2306 in 23S rRNA of \textit{D. radiodurans}, respectively). L25, L16 and L27 proteins were mapped on the three-dimensional structure of the \textit{D. radiodurans} 50S ribosomal subunit (PDB code 1NKW) using software Cn3D (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml). Bases 928 and 942 in the helix 38 (895 and 909 in 23S rRNA of \textit{D. radiodurans}, respectively) could not be mapped, because bases 925-943 (892-910 in 23S rRNA of \textit{D. radiodurans}) were not determined in 1NKW file.

\textbf{Fig. 8.} A model of YlqF function in the late assembly stage of the 50S ribosomal subunit. GTP-bound YlqF binds to premature 50S lacking L16 and L27. Next, L16 and L27 are incorporated into the 50S subunit after YlqF induces conformational changes in the central protuberance at the interface side of 50S subunit. GTP-bound YlqF is replaced by the GDP-bound form through the stimulation of GTPase activity by the assembled 50S subunit, followed by dissociation from the mature 50S subunit. Under stress conditions, YlqF interacts with the premature 50S subunit after incorporation of L25 into the 50S subunit or facilitates L25 binding to 50S subunit.
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Figure 1.
Figure 2.

A

B

Hydrolysed GTP (pmol)

Time (min)
Figure 3.
Figure 4.
Figure 5.

A

B

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<tr>
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<td>+</td>
</tr>
<tr>
<td>GST</td>
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</table>

210 kDa
140 kDa
95 kDa
68 kDa
54 kDa
43 kDa
36 kDa
28 kDa
19.5 kDa
16.2 kDa
10.5 kDa

GST-YlqF
His₆-Ctc
GST
Figure 6.

A

B

D

C

Top  Bottom
Figure 7.
The GTP-binding protein, YlqF, participates in the late step of 50S ribosomal subunit assembly in bacillus subtilis
Yoshitaka Matsuo, Takuya Morimoto, Masayoshi Kuwano, Pek Chin Loh, Taku Oshima and Naotake Ogasawara

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