YqjD Is an Inner Membrane Protein Associated with Stationary-Phase Ribosomes in *Escherichia coli*

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Here, we provide evidence that YqjD, a hypothetical protein of *Escherichia coli*, is an inner membrane and ribosome binding protein. This protein is expressed during the stationary growth phase, and expression is regulated by stress response sigma factor RpoS. YqjD possesses a transmembrane motif in the C-terminal region and associates with 70S and 100S ribosomes at the N-terminal region. Interestingly, *E. coli* possesses two paralogous proteins of YqjD, ElaB and YgaM, which are expressed and bind to ribosomes in a similar manner to YqjD. Overexpression of YqjD leads to inhibition of cell growth. It has been suggested that YqjD loses ribosomal activity and localizes ribosomes to the membrane during the stationary phase.

In nature, bacteria survive in various environments, usually under stress conditions including nutrient starvation, temperature shock, osmolarity changes, and sudden changes in pH. For prolonged survival under such conditions, ordered expression of many stringent-response genes is required. The expression patterns of a large fraction of genes on the genome are altered by turning off or reducing expression of growth-related genes while switching on a set of genes that are required for adaptation to the specific stress condition. Changes in global patterns of gene expression in the stationary growth phase in organisms such as *Escherichia coli* involve drastic changes in cellular machineries, including changes in nucleoid conformation, transcription apparatus, and translation machinery (5, 9). From the variety of changes in the bacterial cell, we focused our attention on ribosomal changes in *E. coli* cells during transition from the exponential to the stationary phase (1, 14). Ribosomes are universally conserved ribonucleoproteins and are comprised of two asymmetric subunits. In bacteria, large (50S) and small (30S) subunits associate to form functional 70S ribosomes. Ribosomes can account for as much as 45% of the total mass of bacterial cells in organisms such as *E. coli* during the exponential phase and actively synthesize all of the cellular proteins required. However, in cells under stress conditions, such as starvation, it has been shown that ribosomal biosynthesis is repressed and that protein synthesis is also suppressed. These systems, which allow translational regulation, are very important for bacteria to survive in harsh environments.

In eukaryotes, it is known that phosphorylation of initiation factor 2α (eIF2α) is an adaptive mechanism for downregulating protein synthesis under stress conditions (7). In the *Gammaproteobacteria*, which includes *E. coli*, protein synthesis is mainly suppressed by the formation of 100S ribosomes (21). The 100S ribosome is a dimer of 70S ribosomes which is formed by the binding of ribosome modulation factor (RMF) to ribosomes (13). RMF is a small (M₉ of 6,507), basic (pI 11.3) protein, and its expression increases remarkably during transition from the exponential phase to the stationary phase. Another protein factor expressed during the stationary phase, hibernation promoting factor ([HPF] also known as YhbH), also binds to ribosomes and promotes 100S ribosome formation (14, 18). In previous studies, it has been shown that RMF inactivates ribosomes by covering the peptidyl transferase center and entrance of the peptide exit tunnel (22, 23).

This interconversion system between 70S and 100S ribosomes is an important strategy for bacterial survival under stress conditions. The ribosomal resting stage, the stage of 100S ribosome formation, is incorporated into the ribosome cycle and is called a hibernation stage (24). As seen above, the activity and conformation of ribosomes are altered by several protein factors in response to environmental stresses. However, the whole picture of ribosomal stress responses has not yet been elucidated.

In this study, we diligently search for novel *E. coli* proteins which associate to ribosomes during the stationary phase using two-dimensional gel electrophoresis. From the results, it was found that the YqjD protein (101 amino acids; M₉ of 11,052, pI 9.1), with unknown physiological functions, associates to ribosomes during the stationary phase. YqjD associates with 70S and 100S ribosomes at the N-terminal region, and expression is regulated by RNA polymerase sigma factor RpoS (σ⁷⁰) for transcription of stationary-phase-specific genes. Interestingly, overexpression of YqjD leads to growth inhibition, in a manner similar to that of RMF; hence, there is a possibility that this protein inactivates ribosomes. A predicted secondary structure for YqjD shows that this is a membrane binding protein having a transmembrane helix in the C-terminal region. In fact, YqjD was found in the inner membrane fraction separated by centrifugation. The experimental results of this study indicate that the *E. coli* YqjD protein is an inner membrane protein associated with stationary-phase ribosomes, which may localize a part of the ribosome to the membrane during the stationary phase.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *E. coli* W3110, a wild-type K-12 strain, was used in the main experiments. For mutational analysis, the deletion mutants of *yqjD* (∆yqjD) and *rpoS* (∆rpoS) (BW25113-derived...
strains) were obtained from the Keio collection (systematic knockout strain of *E. coli* K-12) (2). The coding sequences for full-length YqID (full-YqID; residues 1 to 101), YqID with a deletion of the C terminus (ΔC-YqID; residues 1 to 76), and YqID with a deletion of the N terminus (ΔN-YqID; residues 13 to 101), with and without N-terminal hexahistidine (His6) tags, were inserted into the *E. coli* expression vector pET30 (Novagen), which was transformed into *E. coli* BL21 cells. For prolonged culture, *E. coli* cells were grown in medium E containing 2% polypeptone and supplemented with 0.5% glucose at 37°C with shaking at 120 cycles per minute (19). Cells in the stationary phase were harvested after 2 days of incubation because the amounts of YqID in cells peaked at 2 days. For purification of overexpressed proteins, cells with plasmids were grown in Luria broth at 37°C and harvested 1 h after induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). His-tagged proteins were purified by a column filled with 1 ml of nickel-nitrioltriacetic acid–agarose (Ni-NTA Hi-Trap column; GE Healthcare) and then dialyzed against association buffer [*100 mM CH₃COONH₄, 15 mM (CH₃COO)₂Mg · 4H₂O, 20 mM Tris-HCl at pH 7.6, and 6 mM 2-mercaptoethanol*] for an *in vitro* assay and stored at −80°C until use.

**Preparation of ribosomes.** Each pellet of harvested cells was ground with an approximately equal volume of quartz sand and then suspended in association buffer. Homogenate was centrifuged at 15,000 rpm for 10 min at 4°C. The pellet was saved for use as a fraction of cell debris (CD). The supernatant was centrifuged at 4°C in a 55.2Ti rotor (Beckman) at 50,000 rpm for 1.5 h. The supernatant was saved for use as a fraction of the postribosomal supernatants (PRS). The pellet was resuspended in association buffer and saved for a fraction of the crude ribosomes (CR). The CR solution was layered onto a 5 to 20% linear sucrose density gradient in association buffer and centrifuged in an SW41Ti (Beckman) rotor at 40,000 rpm for 1.5 h at 4°C. The solution after centrifugation was fractionated, and absorbance at 260 nm was measured with a UV-1800 spectrophotometer (Shimadzu, Japan). The fraction containing ribosomes was passed through a filter (Amicon Ultracel-100K; Millipore) to eliminate sucrose, and the upper solution was saved for use as a ribosome fraction (RF). To obtain ribosomal 30S and 50S subunits, the buffer solution of RF was changed to dissociation buffer [*100 mM CH₃COONH₄, 1 mM (CH₃COO)₂Mg · 4H₂O, 20 mM Tris-HCl at pH 7.6, and 6 mM 2-mercaptoethanol*] and incubated at 37°C for 20 min; the solution was layered onto a 5 to 20% linear sucrose density gradient in dissociation buffer and centrifuged in the SW41Ti rotor at 40,000 rpm for 3 h at 4°C. The collection procedures of each fraction containing 30S and 50S were the same as described above.

**2D gel electrophoresis (RFHR method).** Proteins in each fraction (CD, CR, PRS, and RF) were extracted by the acetic acid method (6). One-tenth volume of 1 M MgCl₂ and two volumes of acetic acid were added to each fraction, and the mixture was stirred for 1 h at 4°C. After centrifugation at 20,000 × g for 10 min, the supernatant was dialyzed against 2% acetic acid three times. Proteins were lyophilized and stored at −80°C until use. Eighty micrograms of the lyophilized proteins was analyzed by radical-free and highly reducing (RFHR) two-dimensional gel electrophoresis (RFHR 2D-PAGE) (20). Protein spots on gels were scanned with a GS-800 calibrated densitometer (Bio-Rad Laboratories Inc.). The optical density values of proteins (YqID, YfA, HPF, RMF, and the stationary-phase-induced ribosome-associated protein [SRA]) were calculated as a function of their mass spectrometry, which was functionally unknown. The existence of YqID in the RF (Fig. 1A) suggests the possibility that this is a ribosome binding protein similar to RMF. YqID also existed in a fraction of cell debris (CD), as shown in Fig. 1B. Ribosomal proteins contained in CD are generally regarded as those from generating or degrading ribosomes because the amounts of ribosomal proteins on the gel were atypical. Insoluble and membrane proteins were also contained in this fraction, which suggests the possibility that YqID is a membrane binding protein. YqID was not contained in a fraction of the postribosomal supernatants (PRS), proteins in the cytoplasm without ribosomal proteins, as shown in Fig. 1C. This means that YqID does not exist in a free state. Figure 1D shows time variations of copy numbers (the molar ratio of the molecule binding to ribosomes) of YqID, YfA, HPF, RMF, and SRA. These proteins were not expressed during the exponential phase (cultured for 2.5 h) but increased concomitantly with the transition from the exponential phase to the stationary phase. The expression of YqID reached a maximum at 2 days, but expression levels of other proteins reached a maximum at 3 days. It is well known that the expression levels of many pro-
teins that increase from the early stationary phase are regulated by transcription sigma factor RpoS (8). Therefore, expression of YqjD in a mutant with a deletion of the \textit{rpoS} gene was examined by a comparison of 2D-PAGE gels (see Fig. S1 in the supplemental material). From the results, YqjD was not expressed in the \textit{rpoS} mutant. The above results suggest that \textit{E. coli} YqjD is a ribosome and membrane binding protein and that expression is regulated by RpoS.

\textbf{YqjD has a transmembrane motif in the C-terminal region.} Homologous proteins of YqjD were searched for using the BLAST program (http://www.genome.jp/tools/blast/). From the results, only YqjD of \textit{Shigella flexneri} was hit. It has been reported that \textit{S. flexneri} is closely related to \textit{E. coli} and belongs to the same genus (12). It has been speculated that YqjD is a protein that closely related organisms of \textit{E. coli} possess. On the other hand, two paralogous proteins of YqjD, ElaB (101 amino acids; M\textsubscript{r} of 11,306, pI 5.3) and YgaM (113 amino acids; M\textsubscript{r} of 12,288, pI 8.0), were picked up as paralogous proteins by the BLAST search, as shown in Fig. S2 in the supplemental material. Similar to YqjD, the functions of these paralogous proteins are unknown. The gene expression levels of these proteins were checked using a real-time PCR method. The results showed that the expression patterns of \textit{elaB} and \textit{ygaM} were found to be the same as expression of \textit{yqjD} (see Fig. S3). Although the spots of these proteins were not found on the 2D-PAGE gel, these paralogous proteins may start to be expressed from the early stationary phase in a similar manner to YqjD.

Interestingly, a program for the prediction of the secondary structure of proteins, the SOSUI system (http://bp.nuap.nagoya-u.ac.jp/sosui/), shows that YqjD has a transmembrane motif in the C-terminal region (residues 77 to 98) (marked with an asterisk Fig. S2 in the supplemental material). ElaB and YgaM also have transmembrane motifs in the C-terminal region (residues 78 to 99 and 89 to 110, respectively). Although the possibility that YqjD is a membrane binding protein is shown in Fig. 1C, the C-terminal region of YqjD may relate to a membrane binding function.

\textbf{YqjD binds to 70S and 100S ribosomes.} Figure 2A shows a ribosome profile of CR extracted from the W3110 strain during the stationary phase (cultured for 2 days) by sucrose density gradient centrifugation (SDGC). A peak of the 100S ribosome is seen in addition to the typical peaks of 30S, 50S, and 70S. The gradient was fractionated, and the proteins in each fraction were separated by Tricine-SDS-PAGE. YqjD in each fraction was detected by Western blotting using an antibody against YqjD, as shown in Fig. 2B (arrow). From the results, we see that YqjD existed in the fractions including the 70S and 100S ribosomes. This result indicates that YqjD binds to ribosomes.

Next, in order to examine whether YqjD binds to the 30S or 50S subunit, binding assays of YqjD to each subunit \textit{in vitro} were per-
YqjD is a membrane protein associated with ribosomes.

YqjD is an inner membrane binding protein. It has been speculated that YqjD is a membrane binding protein because YqjD exists in the CD fraction, as shown in Fig. 1B, and possesses the transmembrane motif in the C-terminal region, as shown in Fig. 2. In order to confirm this speculation, we examined whether YqjD was contained in an inner or outer membrane fraction. Membrane proteins prepared from the W3110 strain cultured for 2 days were separated into the inner and outer membrane fractions by successive centrifugation steps (see Materials and Methods). Figure 3A shows that inner and outer membrane proteins were contained in the upper and lower bands, respectively (4). Proteins contained in each fraction were resolved by Tricine-SDS-PAGE, and YqjD was detected by Western blotting using anti-YqjD, as shown in Fig. 3B. Membrane proteins extracted from ΔyqjD cells were also analyzed as controls. The results show that YqjD existed in the inner membrane fraction, as indicated by a red arrow, but not in the outer membrane fraction and in ΔyqjD cells. These results demonstrate that YqjD is an inner membrane binding protein.

YqjD inhibits cell growth by binding to ribosomes. To investigate the role of YqjD in the cell, we compared the features of the ΔyqjD mutant and the parental strain cultured for 2 days. The results of 2D-PAGE show that there were no significant differences in their growth rates, the shapes of cells observed by light microscopy, the ribosomal profiles by SDGC, or protein profiles except for the spot of YqjD (data not shown).

Next, cells that overexpressed the full-length YqjD protein (residues 1 to 101), ΔC-YqjD (residues 1 to 76), or ΔN-YqjD (residues 13 to 101) were cultivated on agar plates with several IPTG concentrations, as shown in Fig. 4. As a control, cells that overexpressed RMF were also cultivated. The results show that the growth of cells that overexpressed full-YqjD, ΔC-YqjD, and RMF was inhibited with an increase in IPTG concentrations but that of ΔN-YqjD was not inhibited. From the results shown in Fig. 2 and in Fig. S2 in the supplemental material, it can be suggested that the C-terminal and N-terminal regions of YqjD are involved in the functions of membrane binding and ribosome binding, respectively. Therefore, it can be interpreted that cell growth is inhibited by binding of YqjD to ribosomes at the N-terminal region. On the other hand, it has been reported that the overexpression of RMF inhibits E. coli cell growth by binding to the peptideyl transferase center in ribosomes (22, 23). The phenomenon of growth inhibition by overexpression of full-length YqjD or ΔC-YqjD, as shown in Fig. 4, is very similar to that of RMF. This fact supports a hypothesis that YqjD inactivates ribosomes by binding at the N-terminal region.

DISCUSSION

In this study, we provided the characterization of YqjD defined as an E. coli hypothetical protein as follows. (i) E. coli starts to express YqjD from the early stationary phase (Fig. 1D). The amount of YqjD peaks at 2 days of cell incubation and then decreases. In a previous study, it has been reported that yqjD was one of the RpoS-regulated genes identified by microarray analysis (15). In this study, it was demonstrated that the expression of this protein was regulated by the stress response sigma factor RpoS (see Fig. S1 in the supplemental material). (ii) It is suggested that YqjD binds to the 30S subunit in 70S and 100S ribosomes at the N-terminal region (Fig. 1A, 2, and 4). In a previous study, this protein was identified in proteomic data using mass spectrometric analysis as
one of the proteins existing in the 30S fraction (11). In this study, it was revealed that the paralogous proteins of YqjD, ElaB and YgaM, also bind to ribosomes (see Fig. S4). (iii) It was predicted that YqjD, ElaB, and YgaM possessed transmembrane motifs in their C-terminal regions (residues 77 to 98, 78 to 99, and 89 to 110, respectively) (see Fig. S2). It was demonstrated that YqjD bound to the inner membrane (Fig. 1B and 3). (iv) Overexpression of YqjD caused the inhibition of cell growth (Fig. 4). This phenomenon can be explained by assuming that YqjD inactivates ribosomes by binding at the N-terminal region.

The peak of binding numbers of YqjD per ribosome was about 0.2 copies, as shown in Fig. 1D. However, the amount of YqjD in cells must be quite large because YqjD in CD existed in relatively large quantities, as shown in Fig. 1B. Ribosomes probably cannot bind to all of the YqjD in the inner membrane because the affinity of YqjD to ribosomes is low enough that the protein is released from them in a high-salt buffer. Although YqjD bound to free ribosomes, as shown in Fig. 1A, this binding may have been caused by excess cell breakage.

To elucidate the function of YqjD, the features of the \( \Delta yqjD \) mutant and the parental strain have been examined. The results show that there were no significant differences in their phenotypes. The paralogous proteins of YqjD, ElaB and YgaM, may function complementarily because these proteins can be expressed during the stationary phase, can bind to the ribosome, and probably bind to the membrane in a manner similar to that of YqjD (see Fig. S3 and S4 in the supplemental material). If the assumption that the paralogous protein works as a backup is correct, the function of YqjD may be very important for cells. One of its important functions may be the inactivation of ribosomes. It is thought that inhibition of ribosomal activity by YqjD is reasonable during the stationary phase, the same as 100S ribosome formation. However, it is not clear why YqjD has to anchor a part of the ribosome to the inner membrane. The keywords “ribosome binding” and “membrane binding” remind us of the signal recognition particle (SRP). The SRP conveys ribosomes synthesizing inner membrane proteins to the SecYEG translocon in the plasma membrane (3). This raises the possibility that YqjD may be involved in the synthesis of some membrane proteins during the early stationary phase although YqjD itself may inactivate ribosomes.

In conclusion, YqjD is expressed under the regulation of RpoS.
YqjD Is a Membrane Protein Associated with Ribosomes

during the stationary phase and is set in the inner membrane at the C-terminal region. A part of the ribosome (70S and 100S) may be localized to the membrane by binding to the N-terminal region of YqjD. It is suggested that the ribosome binding with YqjD loses its translational activity. Future studies should aim to elucidate details of the biological function of YqjD.

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REFERENCES