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Studies of the Interaction of *Escherichia coli* YjeQ with the Ribosome In Vitro

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***Escherichia coli* YjeQ represents a conserved group of bacteria-specific nucleotide-binding proteins of unknown physiological function that have been shown to be essential to the growth of *E. coli* and *Bacillus subtilis*. The protein has previously been characterized as possessing a slow steady-state GTP hydrolysis activity ($8\ h^{-1}$) (D. M. Daigle, L. Rossi, A. M. Berghuis, L. Aravind, E. V. Koonin, and E. D. Brown, Biochemistry 41:11109-11117, 2002). In the work reported here, YjeQ from *E. coli* was found to copurify with ribosomes from cell extracts. The copy number of the protein per cell was nevertheless low relative to the number of ribosomes (ratio of YjeQ copies to ribosomes, 1:200). In vitro, recombinant YjeQ protein interacted strongly with the 30S ribosomal subunit, and the stringency of that interaction, revealed with salt washes, was highest in the presence of the nonhydrolyzable GTP analog 5′-guanylylimidodiphosphate (GMP-PNP). Likewise, association with the 30S subunit resulted in a 160-fold stimulation of YjeQ GTPase activity, which reached a maximum with stoichiometric amounts of ribosomes. N-terminal truncation variants of YjeQ revealed that the predicted OB-fold region was essential for ribosome binding and GTPase stimulation, and they showed that an N-terminal peptide (amino acids 1 to 20 in YjeQ) was necessary for the GMP-PNP-dependent interaction of YjeQ with the 30S subunit. Taken together, these data indicate that the YjeQ protein participates in a guanine nucleotide-dependent interaction with the ribosome and implicate this conserved, essential GTPase as a novel factor in ribosome function.**

The YjeQ protein from *Escherichia coli* represents a family of orthologous proteins that are broadly conserved in bacteria and absent in eukaryotes. YjeQ and its ortholog (YloQ) from *Bacillus subtilis* have been shown to be essential in their respective organisms (4). Sequence analysis and homology modeling of YjeQ have revealed all diagnostic motifs of the P-loop GTPases, albeit in an unusual arrangement (9). YjeQ and its orthologs exhibit an altered connectivity described by the G4–G1–G3 pattern of motifs as opposed to the consensus G1–G3–G4 pattern seen in most GTPases. By using purified, recombinant protein, it has been shown that YjeQ is an unusual GTPase that catalyzes rapid hydrolysis of GTP ($100\ s^{-1}$) despite having a low steady-state turnover of $8\ h^{-1}$ (9). The low turnover is consistent with a slow, rate-limiting release of its products, GDP and phosphate. The kinetic disconnect between the chemical and product release steps of YjeQ is consistent with a role for the protein in transduction of the energy of hydrolysis of GTP into signal generation or mechanical work. Sequence analysis of YjeQ and its orthologs has also revealed an N-terminal, S1-like, OB-fold domain, found in various RNA-binding proteins such as translation factors and regulators of mRNA metabolism (3, 9). Given the presence of this domain and the fact that many GTPases, particularly those that are highly conserved during evolution, function in translation, we reasoned that YjeQ and its orthologs may be factors with a role in ribosome function (9).

Here we present the first experimental evidence that YjeQ associates with the ribosome. We report that YjeQ copurified with ribosomes from *E. coli* extracts. Using recombinant, purified protein, we have revealed a 160-fold stimulation of the GTPase activity of YjeQ through interaction with the 30S ribosomal subunit. Using N-terminal truncation variants, we have delineated a role for the OB-fold region of YjeQ and for amino acids 1 to 20 in the interaction with the ribosome. The work is thus consistent with the hypothesis that YjeQ has a role in ribosome function, and it provides a solid foundation for ongoing studies to further explore this theory.

MATERIALS AND METHODS

Materials. Tris, HEPES, and dithiothreitol (DTT) were from Bioshop Canada Inc. (Burlington, Ontario, Canada). 2-Mercaptoethanol, 5′-guanylylimidodiphosphate (GMP-PNP), diethyl pyrocarbonate, Triton X-100, Malachite green, and ammonium molybdate were from Sigma-Aldrich (Oakville, Ontario, Canada). RNase-free DNase I and Complete EDTA-free protease inhibitor cocktail were from Roche Diagnostics (Laval, Quebec, Canada). Recombinant tobacco etch virus (TEV) protease was a kind gift from Murray Junop (McMaster University). Rabbit polyclonal anti-YjeQ(21-350) antibodies were produced at McMaster University and affinity purified by standard methods (5). The secondary antibody, horseradish perixidase (HRP)-conjugated donkey anti-rabbit immunoglobulin G (IgG), was from Jackson ImmunoResearch, a branch of BIO/CAN (West Grove, Pa.). GTP was from Amersham Biosciences (Baie d'Urfe, Quebec, Canada). The Gateway Recombination Cloning and Expression kits were from Invitrogen-Life Technologies (Carlsbad, Calif.).

Construction of overexpression clones. Previous work characterizing recombinant YjeQ protein from *E. coli* showed that the expressed protein lacked the first 20 amino acids (9). Full-length YjeQ [YjeQ(1-350)] was produced with Gateway Recombination technology using a TEV protease-cleavable N-terminal His_6 affinity purification tag. The gene was PCR amplified from *E. coli* MG1655 genomic DNA by using Vent DNA polymerase (New England Biolabs, Beverly, Mass.) and primers P1 (5′-G GGG ACA AGT TTG TAC AAA AAA GCA GGC TTA GAT TAC GAT ATC CCA ACG ACC GAA AAC CTG TAT TTT CAG * GGC AGT AAA AAT AAA CTC TCC AAA GGC-3′) and P2 (5′-G GGG ACC ACT TTG TAC AAG AAA GCT GGG TCT CAG TCA TCC GTA TCA GAA AAG TTT TTA CGC G-3′) (the protease cleavage site is marked

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with an asterisk; coding sequences are underlined). Cleavage of this protein with recombinant TEV protease yielded full-length YjeQ(1-350) where the initiator Met was replaced by Gly. YjeQ(21-350) and YjeQ(21-350) S221A were constructed and purified as previously described (9). YjeQ(114-350) was PCR amplified from plasmid pLR-1 (9) with primers P3 (5′-G GGG ACA AGT TTG TAC AAA AAA GCA GGC TTA GAA GGA GAT AGA ACC ATG GAC GGC GTA AAA CCT ATT GCC GCC-3′) (coding sequences are underlined) and P2. The PCR-amplified products were cloned into plasmid pDest14 (native) or pDest17 (His_6 tagged) by using Invitrogen-Life Technologies' Gateway Cloning and Expression kits. All plasmid constructs were confirmed by sequencing (MOBIX; McMaster University).

Purification of YjeQ(1-350) and variants. To purify YjeQ(1-350), 4 liters of *E. coli* BL21(DE3)/pDest17YjeQ-TEV was grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.3 and was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM for 3 h at 37°C (final OD_{600}, ~1.2). Cells were harvested by centrifugation at 15,000 × *g* for 20 min. The cells were resuspended in buffer A (20 mM sodium phosphate [pH 7.2]–15 mM imidazole) containing 1× Complete EDTA-free protease inhibitor cocktail (Roche Diagnostics) and lysed by three consecutive passes through a French pressure cell at 20,000 lb/in^2. Cell debris was pelleted by centrifugation at 40,000 × *g* for 1 h at 4°C. The clarified lysate was loaded onto a HiTrap metal chelation column (bed volume, 5 ml) (Amersham Biosciences) charged with 5 ml of 100 mM nickel sulfate and preequilibrated with buffer A. A linear gradient of buffer B (buffer A plus 350 mM imidazole) over 40 column volumes was used to elute the protein. His_6-YjeQ(1-350) eluted between 200 and 250 mM imidazole. Fractions containing His_6-YjeQ were pooled and concentrated to 2 ml by using Amicon Ultra (Fisher Scientific, Nepean, Ontario, Canada) centrifugal concentrators (molecular size cutoff, 15 kDa), and buffer was gradually changed by buffer removal and replacement with 100 mM Tris (pH 8). The His_6 tag was removed by digestion with purified recombinant TEV protease under the following conditions: 260 mg of YjeQ was digested with 6.5 mg of TEV protease for 2 h at 16°C in 50 mM Tris (pH 8)–0.5 mM EDTA–100 mM NaCl–0.25 mM DTT. The proteolyzed sample was dialyzed to remove DTT and EDTA and was rechromatographed over the HiTrap metal chelation column. YjeQ(1-350) lacking the His_6 tag was located in the column flowthrough and concentrated to 2 ml. The sample was dialyzed in buffer C (50 mM HEPES [pH 7.5], 1 mM DTT), loaded onto a Q Sepharose (Amersham Biosciences) anion-exchange column (2.6 by 20 cm), and eluted with a linear gradient of buffer D (buffer C plus 1 M NaCl) over 35 column volumes. Fractions containing YjeQ eluted between 300 and 350 mM NaCl and were pooled and concentrated to 1.2 ml, followed by gel filtration chromatography on a Sephacryl S-200 column (1.6 by 70 cm) preequilibrated in buffer C. Fractions containing YjeQ were pooled, concentrated, and purified further by a second anion-exchange step on a Mono Q (Amersham Biosciences) column (bed volume, 1 ml) preequilibrated in buffer C. By use of a linear gradient of buffer D, pure YjeQ(1-350) (eluting between 320 and 340 mM NaCl and judged to be >99% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] and staining with Coomassie brilliant blue R250)—was pooled, concentrated, dialyzed in storage buffer (50 mM HEPES [pH 7.5], 0.5 mM DTT, 50 mM NaCl, 15% [vol/vol] glycerol), and stored at −80°C.

Untagged truncation variants of YjeQ, namely, YjeQ(21-350), YjeQ(21-350) S221A, and YjeQ(114-350), were purified as described previously (9). Protein concentrations of all variants were determined by a Bradford assay (8) and verified by the method of Gill and von Hippel (10).

Rapid isolation of ribosomes from *E. coli* cell extracts to determine the localization of YjeQ. Crude and rapid isolation of ribosomes from *E. coli* cell extracts was achieved by centrifugation at 40,000 × *g* for 1 h. The supernatant was further clarified by ultracentrifugation at 150,000 × *g* for 2 h. Stringency washes, first with 0.5% Triton, then with 60 mM NH_4Cl, and finally with 1 M NH_4Cl, were performed for 2 h at 4°C, according to standard methods (13). Immunoblotting using SDS–15% polyacrylamide gels was performed as described in the legend to Fig. 1.

Isolation of ribosomes and subunits depleted of YjeQ. Highly purified ribosomes and ribosomal subunits, containing no YjeQ detectable by Western blotting, were prepared using multiple, lengthy centrifugations over sucrose cushions and gradients as previously described (16). Four liters of Luria broth (LB) was inoculated with 40 ml of a saturated overnight culture of *E. coli* MG1655, grown to an OD_{600} of 0.8, and slowly cooled to 15°C to produce runoff ribosomes, free of mRNA (16). Cells were harvested by centrifugation at 8,500 × *g* for 15 min, resuspended, and washed with buffer A (20 mM Tris-HCl [pH 7.5 at 4°C], 10.5 mM magnesium acetate, 100 mM NH_4Cl, 0.5 mM EDTA, 3 mM 2-mercaptoethanol). All subsequent steps were performed at 4°C. The cell suspension was lysed by three consecutive passes through a French pressure cell at 10,000 lb/in^2 followed by the addition of 500 U of RNase-free DNase I (Roche Diagnostics) to the extract. An S30 fraction was generated by centrifugation of the extract at 30,000 × *g* for 1 h. The top three-fourths of the S30 supernatant was recovered and overlaid onto an equal volume of 1.1 M sucrose cushions made up in buffer B (20 mM Tris-HCl [pH 7.5 at 4°C], 10.5 mM magnesium acetate, 500 mM NH_4Cl, 0.5 mM EDTA, 3 mM 2-mercaptoethanol). Samples were centrifuged at 100,000 × *g* for 15 h to produce sucrose- and salt-washed ribosomes (16). The clear ribosomal pellet was separated from the flocculent brownish material above it, gently washed with buffer A, and then resuspended in buffer A by gentle agitation for 1 h. The sucrose–salt washing step described above was repeated. The clear ribosomal pellet was washed, and sucrose was removed by two consecutive resuspensions in buffer C (10 mM Tris-HCl [pH 7.5 at 4°C], 10.5 mM magnesium acetate, 500 mM NH_4Cl, 0.5 mM EDTA, 7 mM 2-mercaptoethanol) followed by centrifugation at 100,000 × *g* for 16 h.

To obtain 70S ribosomes, the ribosomal pellet was resuspended in buffer D (10 mM Tris-HCl [pH 7.5 at 4°C], 5.25 mM magnesium acetate, 60 mM NH_4Cl, 0.25 mM EDTA, 3 mM 2-mercaptoethanol) and separated by centrifugation on 10-to-30% (wt/vol) linear sucrose gradients made up in buffer D. The gradients were centrifuged at 48,000 × *g* for 15 h. The gradients were fractionated by upward displacement using 60% (vol/vol) glycerol, and the fractions (200 μl) were analyzed by absorbance at 260 nm as well as by SDS–15% PAGE to pool the appropriate subunits or 70S ribosomes based on their protein complement. To ensure high purity of the ribosomal subunits, fractions overlapping the A_{260} peak absorbance for the subunits and those judged by SDS-PAGE and Coomassie brilliant blue R250 staining to be impure were eliminated as described previously (2). Fractionation of the gradients was followed by mixing of the 70S pool with an equal volume of buffer E (10 mM Tris-HCl [pH 7.5 at 4°C], 10 mM magnesium acetate, 60 mM NH_4Cl, 3 mM 2-mercaptoethanol) and centrifugation at 56,000 × *g* for 24 h. The 70S ribosomes were resuspended in buffer E and stored at a concentration of 1,000 A_{260} units/ml at −80°C (1 A_{260} unit is equal to 23 pmol of 70S ribosomes in buffer E). The A_{260}/A_{280} ratio was determined to be 1.98.

Similarly, to obtain 30 and 50S subunits, the ribosomal pellet was resuspended in buffer F (10 mM Tris-HCl [pH 7.5 at 4°C], 1.1 mM magnesium acetate, 60 mM NH_4Cl, 0.1 mM EDTA, 2 mM 2-mercaptoethanol). Fifty A_{260} units of the subunit suspension was layered onto 10-to-30% (wt/vol) sucrose gradients made up in buffer F, followed by centrifugation at 43,000 × *g* for 16 h. The gradients were fractionated as described above. The individual subunit pools were recovered by pelleting at 200,000 × *g* for 12 h. The pellets for both 30 and 50S ribosomes were resuspended in buffer E, clarified by centrifugation at 15,000 × *g* for 15 min, and stored at −80°C as described above for 70S ribosomes. Quantitation of subunits was determined by absorbance at 260 nm (1 A_{260} unit is equivalent to 69 or 34.5 pmol of 30 or 50S ribosomes, respectively).

RESULTS

YjeQ copurifies with ribosomes from cell extracts. Purification of ribosomes from wild-type *E. coli* and Western blotting with anti-YjeQ antibodies revealed that nearly all of the YjeQ in the cell copurified with ribosomes (Fig. 1). In these experiments we employed simple detergent and salt washes followed by ultracentrifugation to simply isolate ribosomes from *E. coli* extracts and assess the localization of YjeQ. Indeed, the interaction was stable to detergent and high salt wash conditions, conventionally used to prepare ribosomes that are substantially free from translation factors (13). Furthermore, quantitative Western blotting revealed that YjeQ was of low abundance in *E. coli*, possessing a protein copy number of ~100 copies/cell and consequently in a substoichiometric association with ribosomes (YjeQ/ribosome ratio, 1:200) (data not shown).

Production of YjeQ(1-350) and its variants. Figure 2 shows a scaled diagram and SDS-PAGE analysis of full-length YjeQ and variants that were purified for this work. We produced full-length YjeQ(1-350) and N-terminal truncation variants lacking either the first 20 amino acids [YjeQ(21-350)] or the N-terminal OB-fold region [YjeQ(114-350)]. We also prepared an S221A variant previously characterized as having a significant impairment of the chemical hydrolytic steps in catalysis

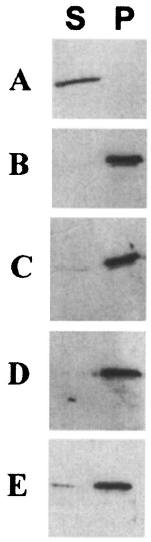

FIG. 1. Colocalization of YjeQ with ribosomes from *E. coli* MG1655 visualized by immunoblotting. Wild-type *E. coli* (4 liters) was grown in LB to an OD_{600} of 0.8, harvested by centrifugation at 8,500 × *g* for 15 min, and lysed by three consecutive passes through a French pressure cell at 10,000 lb/in^2. (A) The lysate was clarified by centrifugation at 40,000 × *g* for 1 h, and both pellet (P) and supernatant (S) fractions were kept for analysis. (B) The supernatant was further clarified by ultracentrifugation at 150,000 × *g* for 2 h. (C through E) Subsequent washing and pelleting steps first with 0.5% Triton (C), then with 60 mM NH_4Cl (D), and finally with 1 M NH_4Cl (E) were performed by standard methods (13). At each step, pellets were resuspended in volumes identical to those of the supernatants for analysis. Immunoblotting employed SDS–15% polyacrylamide gels with a rabbit polyclonal antibody specific for YjeQ(21-350) as the primary antibody and HRP-conjugated donkey anti-rabbit IgG as the secondary antibody. Blots were developed by using the Western Lightning Chemiluminescence Reagent Plus kit (Perkin-Elmer, Boston, Mass.).

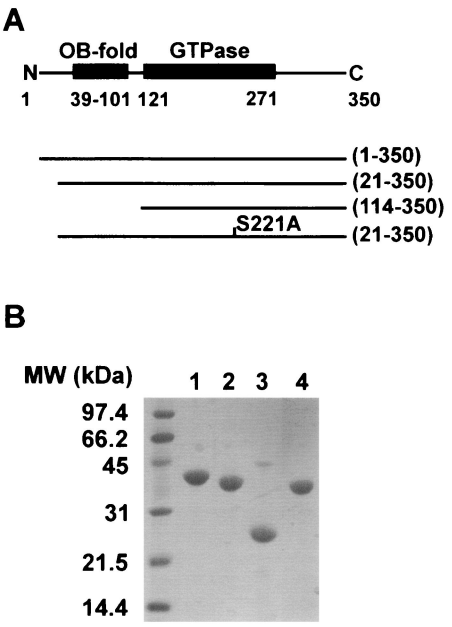

FIG. 2. YjeQ variants constructed and purified in this study. (A) Scaled diagram showing the locations of motifs in YjeQ and the deletion variants constructed. (B) Five micrograms of the purified proteins was prepared by boiling in Laemmli buffer (14) containing 8% 2-mercaptoethanol prior to SDS–15% PAGE. The gels were visualized by staining with Coomassie brilliant blue R250. YjeQ variants characterized in this study are as follows: YjeQ(1-350) (39.1 kDa) (lane 1), YjeQ(21-350) (36.8 kDa) (lane 2), YjeQ(114-350) (27.9 kDa) (lane 3), and YjeQ(21-350) S221A (36.8 kDa) (lane 4).

with only a minor impact on steady-state turnover (9). Full-length YjeQ(1-350) was produced by engineering a TEV protease cleavable N-terminal His_6 tag to prevent proteolysis of the N terminus. Overexpression and purification of untagged YjeQ(1-350) from *E. coli* resulted in isolation of the truncated protein YjeQ(21-350) lacking the first 20 residues, as described previously (9). All proteins were isolated to high purity (Fig. 2B), and all the truncation variants of YjeQ possessed similar steady-state GTPase activities (Table 1).

Binding of YjeQ(1-350) to 30, 50, and 70S ribosomes. To further characterize the interaction of YjeQ with ribosomes, it was necessary to produce ribosomes free of the YjeQ protein. We turned to a ribosome purification procedure involving multiple and lengthy sedimentations through sucrose cushions and gradients for the preparation of 70S ribosomes as well as 30 and 50S subunits (16). Western blotting of these preparations revealed that all forms were free of YjeQ (data not shown). Figure 3 shows the results of an in vitro pelleting assay in which the YjeQ-ribosome incubations are pelleted through sucrose cushions to test the interaction of full-length YjeQ(1-350) with the ribosome and its subunits under a variety of conditions (with or without GDP, GTP, or GMP-PNP). YjeQ pelleted, to some extent, with all forms of the ribosome but showed the most extensive interaction with the 30S subunit. In the presence or absence of GTP or GDP, YjeQ was distributed equally between the pellet and supernatant fractions when incubated with the 30S subunit. In the presence of saturating levels of GMP-PNP, nearly all of the protein was found associated with the ribosomal pellet (Fig. 3A). While not tested explicitly here, this finding is consistent with a stronger association of YjeQ with the 30S subunit in the presence of GMP-PNP, where a lower off-rate (i.e., rate of protein release from the ribosome) for YjeQ would be manifest in more complete pelleting over the 3-h ultracentrifugation run. This observation suggests that the binding affinity of YjeQ for 30S ribosomal subunits might be modulated by substrate hydrolysis and characterized by maximal affinity for 30S subunits when GTP is found in the active site.

Binding of YjeQ to 50 and 70S ribosomes was also observed, but to a lesser extent, and appeared to be independent of the presence of GMP-PNP (Fig. 3B and C). Interaction with the 50S subunit was slightly increased in the presence of saturating levels of GDP and may have some functional significance. Western blotting in these experiments revealed a doublet of protein bands cross-reactive with anti-YjeQ polyclonal antibodies. We believe that this doublet is a result of proteolytic activity contaminating these ribosomal preparations. Indeed, the contaminating proteolytic activity may be the source of the previously observed phenomenon where the overexpression of the recombinant untagged YjeQ in *E. coli* resulted in a cleavage product, lacking the first 20 amino acids (9). In fact, the protein that copurified with ribosomes from wild-type *E. coli* and was detected by Western blotting (Fig. 1) was found to

TABLE 1. Stimulation of YjeQ GTPase activity by ribosomal subunits[a]

Protein	Activity[b] (fold change)			
	Intrinsic	With 30S ribosomal subunit	With 50S ribosomal subunit	With 70S ribosomes
YjeQ(1–350)	3.1 ± 0.4	502.3 ± 13.0 (160)	40.7 ± 3.2 (13)	300.3 ± 3.2 (96)
YjeQ(21–350)	4.6 ± 0.3	463.0 ± 34.5 (100)	35.0 ± 10.9 (7.5)	165.6 ± 7.3 (36)
YjeQ(114–350)	3.7 ± 0.3	10.1 ± 3.0 (2.8)	12.6 ± 3.5 (3.4)	7.8 ± 3.0 (2.1)
YjeQ S221A	1.8 ± 0.4	5.6 ± 0.4 (3.1)	ND	ND

[a] GTPase stimulation by ribosomal subunits was assessed by measurement of inorganic phosphate produced using a previously described Malachite green–ammonium molybdate colorimetric assay (9). Reaction mixtures (50 μl) consisted of 200 nM YjeQ and saturating levels of GTP (2.5 mM), in the presence or absence of ribosomes (200 nM), in 50 mM HEPES (pH 7.5)–10 mM $MgCl_2$ for 1 h at 30°C. Values are averages from quadruplicate determinations ± standard deviations.

[b] Expressed in k_{cat} per hour and corrected for background GTPase originating from ribosomes. Background GTPase levels derived from ribosomes were low relative to the intrinsic GTPase of YjeQ for both 70 and 50S ribosomes and were undetectable with the 30S subunit. ND, not determined.

comigrate with full-length YjeQ(1-350) (data not shown).

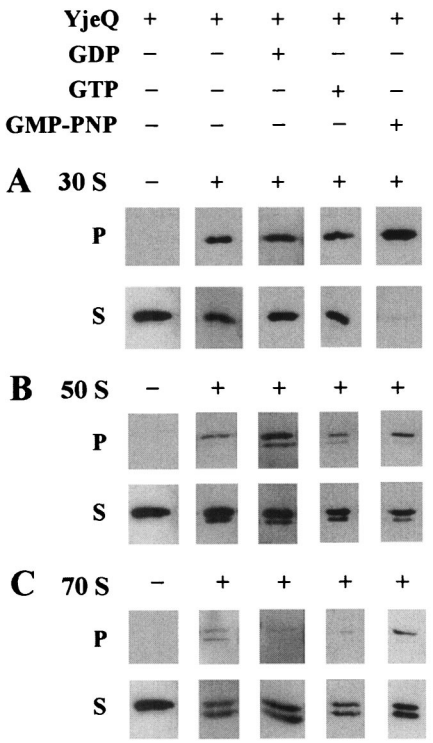

FIG. 3. Binding of YjeQ to 70S ribosomes and ribosomal subunits revealed by immunoblotting. Full-length YjeQ(1-350) was tested for the ability to interact with 70S ribosomes and ribosomal subunits following a 1-h incubation in 20 mM Tris-HCl (pH 7.5)–10.5 mM magnesium acetate–60 mM NH_4Cl–3 mM 2-mercaptoethanol at 30°C in the presence or absence of GDP, GTP, or GMP-PNP (2 mM). Reactions consisted of YjeQ and ribosomes, each at 2 μM. Samples (50 μl) were overlaid onto 20% (wt/vol) sucrose cushions (bed volume, 150 μl) and pelleted by ultracentrifugation at 513,000 × *g* in a Beckman Optima Max ultracentrifuge with a TLA 120.1 rotor for 1.5, 2, or 3 h for 70, 50, or 30S subunits, respectively. The pellets were resuspended in an equivalent volume (200 μl) of assay buffer, and supernatant (S) and pellet (P) fractions were mixed with 40 μl of sixfold-concentrated SDS-polyacrylamide gel electrophoresis loading buffer and separated by SDS–15% PAGE. Western blotting used a rabbit polyclonal antibody raised against YjeQ as the primary antibody and donkey anti-rabbit IgG coupled to HRP as the secondary antibody. (A) Binding of YjeQ to the 30S ribosomal subunit. (B) Binding of YjeQ to the 50S ribosomal subunit. (C) Binding of YjeQ to 70S ribosomes.

Thus, the cleavage product seen in Fig. 3 upon incubation with 50 and 70S ribosomes is presumed not to have a physiological function.

To further substantiate the binding of YjeQ to ribosomal subunits, and to confirm that results obtained by the pelleting assay reflected ribosomal interaction and not simply YjeQ precipitation in the presence of ribosomes, a complementary in vitro binding experiment was devised. YjeQ was incubated with GMP-PNP and 70S ribosomes under conditions (lower magnesium acetate concentration, 1.1 instead of 10.5 mM) that result in dissociation of 70S ribosomes to 50 and 30S ribosomal subunits (16). The sample was subsequently separated on a 10-to-30% sucrose gradient instead of being pelleted through sucrose cushions. In this gradient system, all of the YjeQ was found to comigrate with the separated ribosomal subunits, with the majority (more than 75%) comigrating with the 30S material (Fig. 4).

Stringency of the interaction between YjeQ and 30S ribosomal subunits. The stringency of the association of YjeQ(1-350) with the 30S ribosomal subunit in the presence of GDP or GMP-PNP was evaluated by using the pelleting assay with increasing concentrations of either KCl or NH_4Cl (Fig. 5) as previously described (11). At lower salt concentrations (100 and 250 mM KCl), the binding of YjeQ to the 30S subunit was essentially complete with GMP-PNP and about 50% complete with GDP, in agreement with the experiments for which results are presented in Fig. 3. Addition of 500 mM NH_4Cl (and 250 mM KCl) reduced binding to about 50% in the presence of GMP-PNP and completely abolished binding in the presence of GDP. One-third of the YjeQ protein remained associated with the 30S subunit at the highest salt concentration tested (1 M NH_4Cl). Thus, the stringency experiments support the conclusion that the GMP-PNP-bound form of YjeQ has a stronger association with the 30S ribosomal subunit.

The GTPase activity of YjeQ is stimulated by the 30S ribosomal subunit. The intrinsic GTPase levels observed with YjeQ were strongly stimulated by the presence of ribosomes, particularly the 30S ribosomal subunit, with which we observed a 160-fold increase in k_{cat} (Table 1). We further confirmed, by using the catalytically impaired (Ser221Ala) variant of YjeQ, that the GTPase activity upon stimulation originated from YjeQ. We previously showed that the Ser221Ala variant had an impairment in the chemical steps of GTP hydrolysis, and we show here that no stimulation of GTPase activity by the 30S subunit was observed with this YjeQ variant (Table 1). Figure

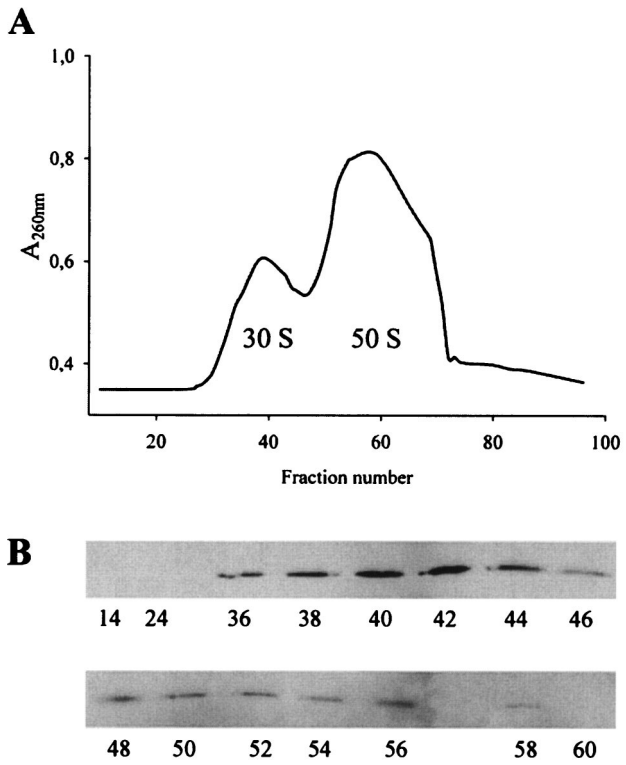

FIG. 4. YjeQ binding to both the 30 and 50S ribosomal subunits in the presence of saturating levels of GMP-PNP (2 mM) analyzed by 10-to-30% (wt/vol) sucrose gradient ultracentrifugation. The reaction mixture consisted of 70S ribosomes (8 A_{260} units) purified by sucrose gradient ultracentrifugation (as described in Materials and Methods) and YjeQ, each at 3.7 μM. The sample (50 μl) was overlaid onto a 5-ml 10-to-30% (wt/vol) sucrose gradient and separated by ultracentrifugation at 43,000 × *g* in a Beckman Optima Max ultracentrifuge with an MLS 50 rotor for 16 h. (A) The gradient was fractionated as described in Materials and Methods, and fractions were analyzed by absorbance at 260 nm. (B) Selected fractions were separated by SDS–15% PAGE and analyzed by immunoblotting for YjeQ (as described in the legend to Fig. 3).

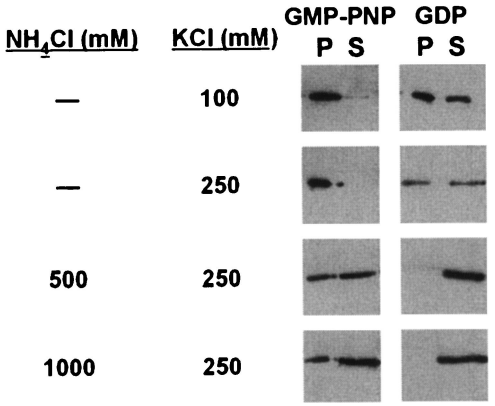

FIG. 5. Salt stringency of the interaction of YjeQ(1-350) with the 30S ribosomal subunit. Immunoblotting of SDS–15% polyacrylamide gels separating pellet (P) and supernatant (S) fractions from the ribosomal pelleting assay (described in the legend to Fig. 3) was performed with increasing salt concentrations (KCl and NH_4Cl) and with saturating (2 mM) levels of GMP-PNP or GDP as indicated.

6 shows the dependence of the GTPase stimulation of YjeQ on the amount of the 30S subunit present and reveals a plateau corresponding to a stoichiometry of 1 YjeQ copy to 1 30S subunit. As was the case for binding, the level of stimulation of GTPase activity by 70S ribosomes was lower (96-fold) than that with 30S subunits (Table 1). The 50S subunit showed considerably less stimulation (13-fold) of intrinsic GTPase levels of YjeQ (Table 1).

Impact of N-terminal truncations on ribosome binding and ribosome-stimulated GTPase activity. Ribosome binding and GTPase stimulation were measured in experiments conducted to assess the contribution of the N-terminal region of YjeQ to the ribosome interaction. Figure 7 shows the results of ribosome pelleting assays with N-terminal truncation variants performed in the presence of GMP-PNP. YjeQ(21-350), which lacks the N-terminal 20 amino acids, demonstrated an affinity for both 50 and 70S ribosomes that was similar to that seen in Fig. 3 for full-length YjeQ(1-350). In contrast, where nearly all of the full-length YjeQ(1-350) protein was associated with the 30S subunit in the presence of GMP-PNP, the 20-amino-acid truncation variant [YjeQ(21-350)] was found equally distributed between the pellet and supernatant fractions, suggesting that this variant had lost significant GMP-PNP-dependent binding affinity for the 30S subunit. YjeQ(114-350), lacking the first 113 amino acids, which encompass the OB-fold domain of the protein, was unable to bind to any ribosome form (Fig. 7). Similar experiments yield identical findings when incubations are carried out with GDP or in the absence of nucleotide (data not shown).

Table 1 details the ribosome-stimulated GTPase activities of the N-terminal truncation variants. Stimulation of the GTPase of the 20-amino-acid truncation variant [YjeQ(21-350)] was slightly less than that of full-length YjeQ (e.g., 100- versus 160-fold stimulation by the 30S subunit for the 20-amino-acid truncation variant and the full-length protein, respectively). Loss of the OB-fold of YjeQ(114-350) resulted in the near-elimination of stimulation of GTPase by the ribosome and its subunits (e.g., only 2.8-fold stimulation by the 30S subunit).

Taken together, the effects on ribosome binding and GTPase stimulation observed with the N-terminal truncation variants suggest that the OB-fold is critical for ribosome association and associated GTPase stimulation, while the N-terminal sequence (residues 1 to 20) of YjeQ appears to be essential in imparting GMP-PNP-dependent binding affinity for the 30S ribosomal subunit.

DISCUSSION

Daigle et al. previously presented steady-state and pre-steady-state kinetic characterizations of recombinant YjeQ to show that the protein was an unusual GTPase enzyme where the chemical steps of catalysis were 45,000-fold faster than those of product release (9). They likewise presented sequence analysis of YjeQ and its orthologs to reveal an unusual circular permutation in the GTPase domain of the protein and the presence of an N-terminal S1-like OB-fold domain (9), typical of proteins that interact with RNAs (3). The unusual kinetics associated with the GTPase function is consistent with a role for YjeQ in signal or energy transduction. In the work pre-

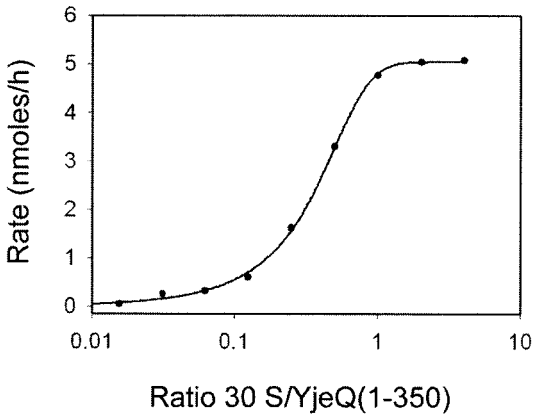

FIG. 6. Maximal stimulation of YjeQ GTPase at 1:1 stoichiometry with ribosomes. The GTPase activitiesof YjeQ and its variants were assessed by monitoring the steady-state release of phosphate from the enzyme by using the Malachite green-ammonium molybdate colorimetric assay described previously (9). All reactions were carried out at 30°C for 1 h, and reaction mixtures contained 200 nM YjeQ and saturating (2.5 mM) levels of GTP. The concentration of 30S ribosomes was varied from 3 to 800 nM. Sample data points are averages of duplicate reactions. The data were fit to a sigmoidal four-parameter equation by using SigmaPlot (version 8.0) to generate the curve shown.

sented here, we characterize a selective interaction between YjeQ and the ribosome, mediated by the OB-fold domain, modulated by the nonhydrolyzable GTP analog GMP-PNP, and with a stimulatory impact on the GTPase activity of YjeQ. The results are intriguing and consistent with a role for YjeQ in ribosome function.

Our work began with the routine isolation of ribosomes from wild-type *E. coli*. To our surprise, isolation of 70S ribosomes from *E. coli* and Western blotting with anti-YjeQ antibodies

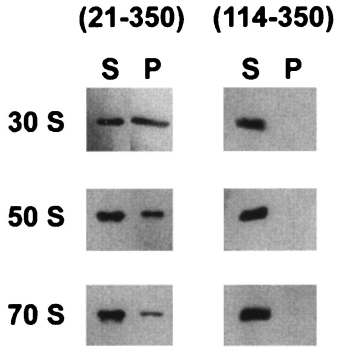

FIG. 7. Ribosomal association by YjeQ N-terminal variants. The abilities of the YjeQ N-terminal deletion variants to bind various forms of the ribosome were assayed by a ribosomal pelleting assay (described in the legend to Fig. 3). Reaction components (50 μl) were separated following a 1-h incubation at 30°C of 100 pmol of YjeQ variant and 100 pmol of ribosome in 20 mM Tris-HCl (pH 7.5)–10.5 mM magnesium acetate–60 mM NH_4Cl–3 mM 2-mercaptoethanol containing 2 mM GMP-PNP. Identical binding behavior was observed when assays contained 2 mM GDP instead of GMP-PNP (data not shown). The samples were overlaid onto 20% (wt/vol) sucrose cushions (bed volume, 150 μl) and pelleted by ultracentrifugation. The pellets (P) were resuspended in a volume (200 μl) equivalent to that of supernatants (S), and both fractions were separated by SDS–15% PAGE followed by immunoblot analysis (described in the legend to Fig. 3).

revealed that nearly all of the YjeQ in the cell was associated with ribosomes. Indeed, the interaction was stable to wash conditions that are typically used to remove translation factors from ribosome preparations (13). Quantitative Western blotting put the copy number of YjeQ at about 100 copies per cell, in a stoichiometry of about 1 for every 200 ribosomes, consistent with the fact that this protein has not been reported in ribosome preparations previously. In speculating on a role for this protein in ribosome function, the low copy number of the protein is noteworthy. The celebrated translational GTPases, EF-Tu, EF-G, and IF2, for example, are abundant and in near-stoichoimetry with ribosomes. Elongation factor P, on the other hand, has a relatively low copy number (EF-P/ribosome ratio, 1:30) (1) and functions in stimulating the peptidyltransferase to enhance peptide bond formation only in certain dipeptides (2). It is conceivable that YjeQ also has a critical but narrow role in a subset of translating ribosomes.

Some technical hurdles are noteworthy in the work presented here. Meticulous sucrose gradient density sedimentation procedures (16) were required to prepare highly purified ribosomes and subunits depleted of YjeQ. That material was critical to our examinations of the YjeQ-ribosome interaction in vitro by use of a ribosome pelleting assay. We also elected to troubleshoot the purification of full-length recombinant YjeQ for these experiments. It was noted previously that an N-terminal truncation variant [YjeQ(21-350)] was consistently generated upon overexpression and purification of the untagged protein (9). Here, full-length YjeQ(1-350) was produced by engineering a TEV protease cleavage site to remove an N-terminal polyhistidine tag from affinity-purified YjeQ protein. Apparently, the N-terminal tag protected YjeQ from the proteolysis that beset the native protein upon overexpression.

While YjeQ bound to all forms of the ribosome in our pelleting assay, the extent of binding, judged by the fraction of YjeQ that pelleted with a stoichiometric amount of ribosomes, varied and was the highest with the 30S ribosomal subunit, where about one-half of the YjeQ protein copelleted. Addition of GDP or GTP to the pelleting assay had no impact on the extent of binding, while the nonhydrolyzable GTP analog GMP-PNP resulted in complete copelleting of full-length YjeQ(1-350) with the ribosomal subunit. The identical outcome with GTP, GDP, and no added nucleotide is consistent with the fact that YjeQ is purified in a form bound by 0.6 equivalent of GDP and rapidly hydrolyzes GTP to GDP with a rate constant of 100 s^{-1} (9). The fact that the GMP-PNP-bound form of YjeQ had a higher affinity for the 30S subunit than the GDP-bound form was also evident in the stringencies of the respective interactions to increasing salt concentrations.

We speculate that we have probed, in these experiments, a physiologically relevant modulation of the 30S subunit binding activity of YjeQ in its GTP- and GDP-bound states, where the GTP analog GMP-PNP facilitates the production of a static mimic of the GTP-bound form. Such modulations are paradigmatic of signal and energy transducing G-proteins and are frequently associated with an impact on GTPase function. Thus, it follows in this work that we have also noted a significant stimulation of the steady-state GTPase activity of YjeQ by the ribosome, in particular by the 30S subunit. Also remarkable is the fact that maximum stimulation of GTPase activity occurs at a 1:1 stoichiometry of YjeQ with the ribosome. This

implies that despite the low copy number of YjeQ, it is capable of a functionally significant and stoichiometric interaction with the 30S subunit.

Low intrinsic GTPase activity is not uncommon among prokaryotic translational GTPases, including EF-Tu and EF-G, which require interaction with the ribosome for maximal activity (6, 7). Interestingly, Era, another prokaryotic GTPase possessing low intrinsic activity, is stimulated by 16S rRNA and has recently been discovered to be a factor involved in the maturation of 16S rRNA (12). Full-length YjeQ's steady-state GTPase activity of 3.1 h^{-1} is comparable to intrinsic GTPase levels observed with EF-Tu (1.8 h^{-1}) (15, 17). Stimulation of EF-Tu by unprogrammed ribosomes (lacking an mRNA template and associated translation factors) is about to 2- to 20-fold, while the binding of EF-Tu to programmed ribosomes results in 100,000-fold stimulation of the GTPase (to 500 s^{-1}). Thus, the 160-fold stimulation seen here with YjeQ and unprogrammed ribosomes has a precedent. In the case of YjeQ, the pre-steady-state kinetic analysis described a rapid GTP hydrolysis step (100 s^{-1}) followed by a much slower steady-state turnover, apparently limited by-product release (9). It seems likely that the interaction of YjeQ with the ribosome impacts primarily on product release kinetics. We will test this hypothesis in due course.

The ribosome binding experiments performed with the N-terminal truncation variants of recombinant YjeQ, presented here, have revealed the importance of the first 20 amino acids and the OB-fold for YjeQ function on the ribosome. Typically composed of a five-stranded closed β-barrel structure and often capped by an alpha helix, OB-folds form a binding surface employed for binding oligosaccharides, proteins, and most often oligonucleotides (3). We found low but significant sequence similarity between the OB-fold in YjeQ and its orthologs and the OB-fold of the protein translation factor eIF-1A (9). Truncation of the OB-fold in the YjeQ(114-350) variant abolished binding and GTPase stimulation by the ribosome or its individual subunits. Truncation of the first 20 amino acids in YjeQ(21-350), on the other hand, showed little effect on ribosome binding or on ribosome stimulation of the GTPase activity of YjeQ. This variant exhibited behavior comparable to that of the full-length enzyme, except that its 30S binding activity was no longer modulated by the nonhydrolyzable GTP analog GMP-PNP. Given the critical role of the N-terminal 20 amino acids in the latter phenomenon, it is worth emphasizing our previous finding that this peptide is proteolytically sensitive to removal and is absent when the untagged protein is overexpressed and purified from *E. coli* (9). Thus, its role is likely critical to a fully functional YjeQ protein.

Information gathered in this study provides further support for the hypothesis that the YjeQ protein from *E. coli* and its orthologs are bacteria-specific factors with a role in ribosome function. We have concentrated in this work on the interaction of YjeQ with the ribosome and the impact of that interaction on YjeQ function. Work to address the impact of YjeQ on the function of the ribosome is ongoing.

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