Cryo-electron microscopy structure of the 30S subunit in complex with the YjeQ biogenesis factor

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ABSTRACT

YjeQ is a protein broadly conserved in bacteria containing an N-terminal oligonucleotide/oligosaccharide fold (OB-fold) domain, a central GTPase domain, and a C-terminal zinc-finger domain. YjeQ binds tightly and stoichiometrically to the 30S subunit, which stimulates its GTPase activity by 160-fold. Despite growing evidence for the involvement of the YjeQ protein in bacterial 30S subunit assembly, the specific function and mechanism of this protein remain unclear. Here, we report the costructure of YjeQ with the 30S subunit obtained by cryo-electron microscopy. The costructure revealed that YjeQ interacts simultaneously with helix 44, the head and the platform of the 30S subunit. This binding location of YjeQ in the 30S subunit suggests a chaperone role in processing of the 3' end of the rRNA as well as in mediating the correct orientation of the main domains of the 30S subunit. In addition, the YjeQ binding site partially overlaps with the interaction site of initiation factors 2 and 3, and upon binding, YjeQ covers three inter-subunit bridges that are important for the association of the 30S and 50S subunits. Hence, our structure suggests that YjeQ may assist in ribosome maturation by preventing premature formation of the translation initiation complex and association with the 50S subunit. Together, these results support a role for YjeQ in the late stages of 30S maturation.

Keywords: ribosome assembly; 30S subunit; YjeQ protein; RsgA protein; GTPase

INTRODUCTION

The 70S ribosome is composed of two distinct subunits of unequal size (Ramakrishnan 2002). The smaller subunit, termed 30S, is formed by 21 ribosomal proteins (designated S1 to S21) and one RNA molecule, the 16S rRNA. The 50S, or large, subunit is made of two RNA molecules, the 23S and 5S rRNAs, and 34 proteins (Moazed et al. 1995). Despite the size and complexity of this macromolecule, *Escherichia coli* efficiently assembles the ribosome assisted by more than 50 *trans*-acting factors (Wilson and Nierhaus 2007). These include RNases that perform the cleavage of the precursor rRNAs (Misra and Apirion 1979; Li et al. 1999a,b) and factors executing rRNA and protein modifications (Connolly and Culver 2009). In addition, there is a subset of factors for which identification of a particular functional role has been

problematic. This list of “enigmatic factors” includes the putative maturation factors RimM, RimP, and RbfA, as well as the highly conserved GTPases Era and YjeQ (also known as RsgA) (Brown 2005; Karbstein 2007). All of these proteins have been shown to interact with the 30S subunit, suggesting that they participate in late steps of 30S subunit assembly, facilitating the action of RNases or the binding of late r-proteins (Shajani et al. 2011).

The YjeQ protein represents a subfamily of GTPases whose defining structural feature is a circular permutation of the GTPase domain (Leipe et al. 2002). The protein is broadly conserved in bacteria but absent in eukaryotes and has a unique architecture with an N-terminal oligonucleotide/oligosaccharide fold (OB-fold), a central GTPase domain with a classical P-loop fold, and a C-terminal TAZ2-like zinc-finger domain (Levdikov et al. 2004; Shin et al. 2004; Nichols et al. 2007). YjeQ is dispensable for growth but critically important for the overall fitness of *E. coli*, *Bacillus subtilis*, and *Staphylococcus aureus* (Himeno et al. 2004; Campbell et al. 2005, 2006).

YjeQ catalyzes the rapid hydrolysis of GTP (100 sec⁻¹) with a low steady-state turnover rate, 9.4 h⁻¹ (Daigle et al.

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2002). Recombinant YjeQ binds tightly and stoichiometrically to the 30S subunit in the presence of a nonhydrolyzable GTP analog, GMP-PNP. Binding to the 30S subunit stimulates the YjeQ GTPase activity by 160-fold (Daigle and Brown 2004). Previous studies have suggested that YjeQ interacts with the 30S subunit through the A-site and P-site in the decoding center of the ribosome. This is evidenced by two separate studies demonstrating that A-site-targeting antibiotics such as neomycin inhibit the ribosome stimulation of YjeQ GTPase activity (Himeno et al. 2004; Campbell et al. 2005). More recently, chemical footprinting studies (Kimura et al. 2008) suggested that binding of YjeQ to the 30S subunit induces conformational changes around the A-site, P-site, and helix 44. Accordingly, it was further shown that YjeQ promotes dissociation of tRNA but has the capacity to coexist with mRNA in the 30S subunit (Kimura et al. 2008).

There is a growing body of evidence implicating a role for YjeQ in the late stages of 30S biogenesis. Fractionation of ribosomal subunits from strains lacking *yjeQ* revealed accumulation of free 30S and 50S subunits (Himeno et al. 2004; Campbell et al. 2005). Accumulated 30S subunits are nonfunctional and contain immature 17S rRNA species (Himeno et al. 2004; Jomaa et al. 2011). A three-dimensional (3D) reconstruction by cryo-electron microscopy (cryo-EM) of these 30S subunits (Jomaa et al. 2011) revealed that the presence of precursor sequences in the rRNA induces a severe distortion in the 3' minor domain of the subunit, which is involved in the decoding of mRNA and interaction with the large ribosomal subunit. These findings suggest that YjeQ has an indirect role in processing the precursor 16S rRNA, either by directly facilitating the recruitment of RNases to the processing site or by stabilizing the 30S subunit in a specific conformation that ensures efficient processing. YjeQ is presumably not mediating this process by itself but in conjunction with other *trans*-acting factors including Era, RimM, RimP, and RbfA (Bylund et al. 1998; Inoue et al. 2003, 2006; Goto et al. 2011).

To further understand the involvement and mechanism of action of YjeQ in the late stages of the 30S subunit assembly, we obtained the co-structure of the 30S subunit in complex with YjeQ by cryo-EM. The 3D reconstruction shows the small ribosomal subunit with an additional density covering part of the platform domain and the upper region of helix 44. The atomic structure of the YjeQ protein fit unambiguously into this density. Contacts between the protein OB-fold domain and the 16S rRNA in the platform mediate the interaction between YjeQ and the 30S subunit. At the C-terminal end of the protein, the zinc-finger domain sits in the upper region of helix 44, inducing a displacement of this helix in the 30S subunit. In this location, YjeQ might be optimally placed to assist in late maturation events, including processing of the 3' end of the 17S rRNA and mediating the correct orientation of the main domains of the small subunit. In addition, YjeQ binding also covers

three inter-subunit bridges in the 30S subunit that are important for its association with the 50S subunit. This finding structurally explains biochemical data indicating that YjeQ induces the dissociation of the 70S ribosome (Himeno et al. 2004).

RESULTS

Cryo-EM structure of the 30S subunit in complex with YjeQ

Attempting to maximize the occupancy level of YjeQ in the 30S:YjeQ complex, we found that a fivefold excess of freshly purified YjeQ protein in the presence of GMP-PNP, incubated with a 1 μ M preparation of 30S subunits purified from *E. coli*, produced an occupancy of $\sim 70\%$ (Fig. 1). Higher excesses of YjeQ in the assembly reaction did not significantly increase the occupancy of the protein in the complex.

A diluted sample from this mixture was imaged using cryo-EM (Supplemental Fig. S1). Dilution of the specimen was necessary to obtain a suitable concentration of 30S subunits for cryo-EM imaging. To guard against a decrease in the occupancy level of YjeQ in the 30S:YjeQ complex upon dilution, we experimented with including free YjeQ in the dilution buffer. A fivefold molar excess of YjeQ protein with respect to the 30S subunits maximized the proportion of

FIGURE 1. Assembly of the 30S:YjeQ complex. Purified YjeQ was incubated with 30S subunits in ratios of 1:1 (lanes 6,7) and 5:1 (lanes 8,9) in the presence of 1 mM GMP-PNP. The concentration of 30S subunits in these reactions was 1 μ M. Following incubation, samples were overlaid onto a sucrose cushion and pelleted by ultracentrifugation. Pelleted material was resuspended, and both the supernatant (S) and pellet (P) were resolved by SDS-PAGE and stained with Coomassie brilliant blue. The lanes are as follows: (lane 1) molecular weight marker; (lanes 2,3) YjeQ + GMP-PNP; (lanes 4,5) 30S + GMP-PNP; (lanes 6,7) YjeQ + GMP-PNP + 30S (1:1); (lanes 8,9) YjeQ + GMP-PNP + 30S (5:1); (lanes 10–13) known quantities of YjeQ (1–12.5 pmol). The position of YjeQ in the gel is indicated. The occupancy of the YjeQ–30S complex was assessed by quantifying the amount of YjeQ that co-pelleted with ribosomes (2.5 pmol) in each reaction using the lanes with known quantities of YjeQ (lanes 10–13). For this calculation the fraction of YjeQ that pellets in the absence of ribosomes (25%) (lane 3) was subtracted from that quantified in the reaction pellet (lanes 7,9). Using the previously determined binding ratio of 1:1, occupancy for both 1:1 and 5:1 incubations was found to be $\sim 70\%$.

30S:YjeQ complexes and minimized excessive background that came with higher excesses of YjeQ.

Cryo-EM images were collected, and the two-dimensional (2D) projections in the electron micrographs were selected and subjected to a supervised classification approach with the aim of eliminating particles representing 30S subunits that were not bound to YjeQ (Fig. 2). This classification approach produced four classes of particles from which corresponding 3D reconstructions were calculated. The SC 1 class (Fig. 2, bottom panel) produced a reconstruction with all of the recognizable landmarks of the 30S subunit and represents the particles that were not bound to YjeQ. The other three classes, SC 2–SC 4 (Fig. 2, bottom panel), produced a similar 3D reconstruction but with different amounts of additional density attached to the upper domain of helix 44 and the nearby region of the platform. The reconstruction from SC 4 particles (Fig. 2, bottom panel) showed the largest density and was produced from a subpopulation with the highest proportion of 30S subunits bound to YjeQ. This reconstruction represents the structure of the 30S:YjeQ complex (Fig. 3A, left panel), was refined to 16.5 Å resolution (Supplemental Fig. S2), and was used in our subsequent analysis. Reconstructions SC 2 and SC 3 with smaller amounts of additional density represent mixed subpopulation containing both free 30S subunits and 30S:YjeQ complexes. These two classes suggested that the classification approach was effective in separating only a fraction of the 30S:YjeQ complexes present in our images. A second round of supervised classification using SC 1 (as a free 30S subunit reference) and SC 4 (as a reference for the 30S:YjeQ complex) did not improve the efficiency of the classification. Similarly, we also attempted to sort out our particles using a maximum-likelihood-based classification technique (Scheres et al. 2005a,b, 2007), but we did not obtain an improved separation of the two populations existing in the sample (data not shown).

In addition to a 3D reconstruction of the 30S:YjeQ complex, we also imaged free 30S subunits, and a control cryo-EM map was calculated. This structure was used as one of the references in the supervised classification (Fig. 2) and for the difference map analysis of the 30S:YjeQ structure (see below). In addition, the control cryo-EM map from free 30S subunits was a necessary aid for unequivocally assigning the extradensity in the cryo-EM map of the complex to the YjeQ protein. In particular, it was important to rule out that initiation factors 1 (IF1), 2 (IF2), and 3 (IF3) that bind in an area partially overlapping with the location of the additional density observed in the 30S:YjeQ complex (Moazed et al. 1995; Dallas and Noller 2001; Allen et al. 2005) were not contaminating the purified 30S subunits used to assemble the complex. The presence of IF1 and IF2 in the 30S subunit preparation was discarded. iTRAQ (isobaric tag for relative and absolute quantification) analysis (Ross et al. 2004) of a sample of 30S subunits purified with the same procedure in a previous study from our laboratory (Jomaa et al. 2011)

FIGURE 2. Supervised classification of the projections representing the 30S:YjeQ complex. Projections of 30S:YjeQ complexes were classified using two references: the control cryo-EM structure obtained from free 30S subunit low-pass filtered to 25 Å resolution (reference #1) and the 3D reconstruction obtained from all the particle images in the data set (reference #2). Reference #2 already showed an extra mass of density attached to the upper domain of helix 44 and the nearby region of the platform. All images in the data set were aligned to the projections generated from each of the references, and each image was assigned to the projection view from each reference that yielded the highest cross-correlation coefficient, obtaining two cross-correlation coefficients per particle image (CC1 and CC2). Then, we plotted the distribution of particles against the difference between the two cross-correlation coefficients ($\Delta CC = CC2 - CC1$). The particles were classified following a unimodal distribution. This histogram was divided into four classes (from SC 1 to SC 4) using the P_x values indicated by the dashed lines. The 3D map for each subset of particles was calculated using projection matching. The number of particles classified in each group is indicated in the figure. The reference map used to refine the four structures was the X-ray structure of the *E. coli* 30S subunit (PDB ID: 2AVY) (Schuwirth et al. 2005) low-pass filtered to 25 Å resolution. The estimated resolution for each map is noted in the figure below the number of particles assigned to each class. The group of particles most similar to reference #2 (SC 4) formed the 3D cryo-EM map of the 30S:YjeQ complex.

revealed that IF1 and IF2 were not present in the 30S subunit preparation. However, the sample did contain initiation factor 3 (IF3). The interaction site of the N-terminal domain of IF3

FIGURE 3. Cryo-EM structure of the 30S:YjeQ complex and docking of the YjeQ structure. (A) Cryo-EM map of the 30S:YjeQ complex side by side with a control reconstruction obtained from free 30S subunits. The head, platform, body, and helix 44, which are main landmarks in the 30S subunit, are indicated as well as the inter-subunit bridges B2a, B3, and B7a covered upon YjeQ binding. The additional density (red) represents the YjeQ protein binding site. (B) The four panels provide different views of the additional density covering the upper domain of helix 44 and nearby region in the platform in the cryo-EM map of the 30S:YjeQ complex. The additional density is represented as a mesh with the docked X-ray structure of the YjeQ protein. The X-ray structure of the YjeQ protein from *S. typhimurium* (PDB ID 2RCN) is shown with the OB-fold domain (dark gray), the GTPase domain (yellow), and the zinc-finger domain (green). As an aid for orientation, each panel shows the cryo-EM map of the 30S:YjeQ complex in a matching view. The first residue described in the YjeQ structure (L43) and residues N245 and T259 flanking a sequence not described by the atomic coordinates are labeled. Residues H285 and A345 are indicated to mark the first and last α -helices at the C-terminal zinc-finger domain of YjeQ. Areas of the density not filled by the X-ray structure of YjeQ are labeled with numbers from 1 to 4.

with the 30S subunit has been mapped near the E-site and proteins S7 and S11 (Dallas and Noller 2001), which is a different location from the one proposed here for binding of YjeQ. But the C-terminal domain of IF3 interacts with helices

23, 24, and 45 (Dallas and Noller 2001). This is the area of the platform covered by additional density in the cryo-EM map of the 30S:YjeQ complex. Nevertheless, the control cryo-EM map from free 30S subunits showed all the recognizable landmarks of the 30S subunit but did not show any additional density, including in the area occupied by the C terminus of IF3. Therefore, the control reconstruction indicates that this factor in our preparation is either dissociated from the 30S subunits or in substoichiometric amounts.

Under the high-salt conditions used to purify the 30S subunits in our study, only small amounts of S1 protein were expected to remain associated to rRNA (Spedding 1990). However, SDS-PAGE analysis of the purified 30S subunits used to assemble the 30S:YjeQ complex revealed a band at ~ 70 kDa (Fig. 1) that we identified as the S1 protein by mass spectrometric analysis (see Materials and Methods). When bound to the 30S subunit, S1 fills the cleft region between the head and platform (Sengupta et al. 2001), but its binding site does not overlap with the area occupied by the additional density observed in the 30S:YjeQ complex. In any case, the control cryo-EM map obtained from free 30S subunits did not show any density in the area occupied by S1 (Fig. 3A, right panel), suggesting that even though S1 is present in the preparation, it probably dissociated from the 30S subunits during the cryo-EM sample preparation. This was not surprising because it has been described that association of S1 to the small subunit is weak and reversible (Subramanian and van Duin 1977). Taken together, these data enabled us to confidently attribute the additional density observed in the cryo-EM structure to the YjeQ protein.

Location of YjeQ in the structure of the 30S:YjeQ complex

The X-ray structures of YjeQ from *Salmonella typhimurium* (PDB ID 2RCN), *Thermotoga maritima* (PDB ID 1UOL), and *Aquifex aeolicus* (PDB ID 2YV5) have been determined (Shin et al. 2004; Nichols et al. 2007). These proteins share different degrees of sequence homology with the *E. coli* YjeQ used to form the 30S:YjeQ complex in our study. In particular, *S. typhimurium* YjeQ has a 92% sequence identity and a 94% sequence similarity with *E. coli* YjeQ (Supplemental Fig. S3), whereas *A. aeolicus* and *T. maritima* only have 50% sequence similarity. In spite of their sequence diversity, the structure of these three proteins is remarkably conserved (Supplemental Fig. S4). The superimposition of the X-ray structure of *A. aeolicus* and *T. maritima* onto that of *S. typhimurium* returned root mean square deviations of 1.79 (244 residues superimposed) and 1.51 Å (247 residues superimposed), respectively. Nevertheless, due to the high degree of sequence homology, the X-ray structure of *S. typhimurium* YjeQ was used for our docking experiments.

To locate the YjeQ protein in the complex structure, the cryo-EM map obtained as a control from free 30S subunits (Fig. 3A, right panel) was subtracted from the cryo-EM

structure of the 30S:YjeQ complex (Fig. 3A, left panel). The resulting difference map showed a density clearly resembling the X-ray structure of YjeQ covering the upper domain of helix 44 and nearby region of the platform in the 30S subunit (Fig. 3B). The *S. typhimurium* YjeQ atomic coordinates (Nichols et al. 2007) fit remarkably well and unambiguously into this density with a cross-correlation coefficient (CCC) value of 0.90. This was consistent with previous biochemical data indicating that only one molecule of YjeQ binds to the 30S subunit at a time (Daigle and Brown 2004). Only small translational and rotational movements of each domain independently were necessary (Supplemental Fig. S5) to optimize the fitting (Fig. 3B). A CCC value of 0.91 was obtained after optimization. The finding that only small adjustments in the relative position of the YjeQ domains were necessary to optimize the fitting suggests that the three domains may not undergo a large rearrangement upon binding to the 30S subunit.

The difference map analysis to locate the YjeQ protein in the 30S:YjeQ cryo-EM map was repeated using two other X-ray structures of the *E. coli* 30S subunit (PDB IDs: 2AW7 and 2AVY) (Schuwirth et al. 2005) filtered to match the resolution of our 30S:YjeQ cryo-EM map. These two X-ray structures only differ by a 6° rotation of the head around the neck region; however, helix 44 and the platform are in the same conformation. The additional density isolated using these two difference maps was very similar to the one obtained when the cryo-EM map of the free 30S subunit (Fig. 3, right panel) was used for the difference map analysis (data not shown). These results suggested that our method to locate the YjeQ protein in the cryo-EM map of the complex was not biased by the 30S structure used for the difference map analysis.

Some small parts of the additional density covering helix 44 and the platform of the 30S:YjeQ complex were not filled by the crystal structure of YjeQ. This may be due to the fact that some sequence motifs in YjeQ are not represented in the *S. typhimurium* YjeQ X-ray structure (Nichols et al. 2007). In particular, YjeQ in *S. typhimurium* contains a 42-amino-acid N-terminal stretch that is not described by the atomic coordinates (Supplemental Fig. S4, upper panel). This sequence is also present in *E. coli*, although it is a slightly shorter sequence of 34 amino acids (Supplemental Fig. S3). Interestingly, the additional density in the 30S:YjeQ complex extends beyond the docked N-terminal OB-fold domain of YjeQ toward the tip of the platform (Fig. 3B, density labeled as 1). This additional density may well encompass these residues. Another void in the density attributed to YjeQ was proximal to the GTPase domain and extended upward, contacting helix 31 and the C terminus of S13 in the head of the subunit (Fig. 3B, density labeled as 2). Here again, residues Asp 246 to Thr 258 (Asp 238 to Thr 250 in *E. coli* YjeQ) are not described in the X-ray structure of *S. typhimurium* YjeQ (Supplemental Figs. S3, S4) and may be filling this void (Fig. 3B, density labeled as 2).

We noticed that the first and last α -helices of the C-terminal zinc-finger domain of YjeQ protruded partially from the cryo-EM density of the 30S:YjeQ structure. However, in both cases, we found a nearby void space in the additional mass attached to the 30S:YjeQ complex that could easily accommodate these two α -helices (Fig. 3B, densities labeled as 3 and 4), suggesting that the conformation of these two motifs in YjeQ may be slightly different in the unbound and bound states of the protein. Indeed, the distal part of the C-terminal α -helix in the homologous *A. aeolicus* structure is oriented differently than in the *S. typhimurium* (Nichols et al. 2007) and *T. maritima* (Shin et al. 2004) structures (Supplemental Fig. S4), indicating that this α -helix is flexible and capable of adapting alternative conformations.

Next, we superimposed the obtained difference map containing the docked X-ray structure of YjeQ into the cryo-EM structure of the 30S:YjeQ complex to locate YjeQ in the map. Subsequent docking of the X-ray structure of the 30S subunit (PDB ID: 2AVY) (Schuwirth et al. 2005) in the remaining density of the cryo-EM map allowed us to produce a pseudo-atomic model of the 30S:YjeQ complex (Fig. 4A). In this model, we noticed that part of the density corresponding to the upper domain of helix 44 and helix 45 in the cryo-EM map of the 30S:YjeQ complex was very weak or completely absent, suggesting that these two regions became displaced upon YjeQ binding (Fig. 4B). Interestingly, the cryo-EM map of the 30S:YjeQ complex exhibited a density covering the r-protein S12 and adjacent to the segment of helix 44 missing in the cryo-EM map. It is plausible that these two regions move from their native location in the 30S subunit to this new site upon YjeQ binding (Fig. 4C). However, this density is large enough to house the missing segment of helix 44 but not helix 45, suggesting that parts of these two helices became flexible or unfolded and thus invisible in the cryo-EM structure. The displacement of parts of helices 44 and 45 in the structure of the 30S:YjeQ complex is consistent with these two helices establishing a limited number of contacts with r-proteins and being stabilized by non-sequence-specific backbone interactions with the rest of the 16S rRNA (Whitby et al. 2000).

Refinement and validation of the 30S:YjeQ structure

The pseudo-atomic model of the 30S:YjeQ complex obtained by manual placement of the X-ray structure of the 30S subunit and YjeQ protein into the cryo-EM map of the 30S:YjeQ complex was refined with flexible fitting using the YUP.scx refinement method within the YUP program (Tan et al. 2008). The model was initially placed into the cryo-EM map in a position equivalent to our manual placement and was then divided into 26 distinct units. Five fragments were the 16S rRNA structural domains (head, body, platform, and 3' minor domain that was broken up into helices 44 and 45). YjeQ and the ribosomal proteins constituted the remaining 21 fragments. The model was then

FIGURE 4. Pseudo-atomic model of the 30S:YjeQ complex and displacement of the upper domain of helix 44 in the cryo-EM map of the 30S:YjeQ complex. (A) Pseudo-atomic model of the 30S:YjeQ complex. The backbone of the 16S rRNA (ribbon in cyan); ribosomal proteins (dark blue); the YjeQ protein (black). (B) Back view of the 30S:YjeQ complex (see orientation aid in panel above) after a clipping plane orthogonal to the direction of view was applied to the cryo-EM map removing the blocking densities on the back. The panel shows the density map of the 30S:YjeQ complex as a mesh. The OB-fold (gray), GTPase (yellow), and zinc-finger (green) domains of the YjeQ structure are shown fitted into this density. (Purple) The nucleotides in helices 44 and 45 for which a corresponding density in the 30S:YjeQ complex EM map does not exist. (C) Top view of a slab of the cryo-EM map of the 30S:YjeQ complex. The slab was cut orthogonal to the longitudinal axis of helix 44 (see orientation aid in panel above). Binding of YjeQ to the 30S subunit causes a displacement of the upper domain of helix 44. The location of this motif in the free 30S subunit (purple) and in the 30S:YjeQ complex (light purple) is shown. (Black arrow) The direction of the shift.

refined to the cryo-EM density map of the 30S:YjeQ complex by simulated annealing with molecular dynamics. The energy function contains terms for scoring the quality of the fit of the model to the density map, restraint energies for a Gaussian Network Model that represents the all-atom structure, and volume exclusion terms.

The modeled structure fit the density well and the placement of the 30S subunit and YjeQ differed minimally from the placement obtained by manual rigid-body docking (Fig. 4A; Supplemental Fig. S6A), except for the OB-fold domain. This domain was placed rotated with respect to the manual placement and toward the strip of density connecting the head and platform (Supplemental Fig. S6B). However, we favor the orientation predicted by the manual placement because it is consistent with other OB-fold-containing proteins that interact with RNA (Supplemental Fig. S7) (see below). In addition, the domains of the 16S rRNA were in slightly different orientations when compared with the X-ray structure, which suggests that the relative orientation of these 30S subunit domains change slightly upon YjeQ binding (Supplemental Fig. S6C).

To determine whether refinements using different starting models converge to the same solution, the process was repeated using the same manually constructed pseudo-atomic model but having removed the helix 45 of the 16S rRNA, which lacks a corresponding density in the cryo-EM map of the 30S:YjeQ complex. The refinement converged to an identical solution. Subsequently, we used a starting model with YjeQ bound in the same orientation but displaced laterally toward the platform. During refinement of this model, YjeQ drifted toward the previous solution and reached the same location. Starting models with YjeQ bound in drastically different orientation from our manual placement diverged to dissimilar solutions. However, these models were then subjected to subsequent refinements, and divergent orientations were often found, which suggests that these placements were far from optimal. Any of these solutions were consistent with our manually constructed pseudo-atomic model of the 30S:YjeQ complex (data not shown). In addition, they left large regions of the density assigned to YjeQ unoccupied and regions of the YjeQ structure protruding from the cryo-EM map. Therefore, all of these unstable solutions were discarded.

In conclusion, the convergence of refinements with multiple starting models to a solution very similar to our manually constructed pseudo-atomic model of the 30S:YjeQ complex validates our placement of the YjeQ and 30S subunit X-ray structures in the cryo-EM map.

Interactions of YjeQ with the 30S subunit

The manually constructed pseudo-atomic model of the 30S:YjeQ complex was used to study the interactions of the YjeQ protein with the 30S subunit. Bound YjeQ interacts with the 3' minor, the 3' major (head), and the central (platform)

domains in the 30S subunit, (Fig. 3A, left panel). In this location, YjeQ covers the inter-subunit bridge B2a at the top of helix 44 (Fig. 3A, right panel), which is an essential region to maintain the association of the small and large subunits in the 70S ribosome (Maivali and Remme 2004). In addition, the inter-subunit bridges B3 and B7a are also covered. The bridge B3, located in the upper domain of helix 44, serves as the pivot point during the ratcheting motion of the ribosome, and the bridge B7a is at the apex of the platform (Fig. 3A, right panel). The steric blockage of these inter-subunit bridges in the 30S:YjeQ complex provides a structural explanation to previous biochemical data indicating that YjeQ induces the dissociation of the 70S ribosome (Himeno et al. 2004).

In the region of the 30S subunit to which YjeQ binds, rRNA occupies most of the contact areas, except for the point of interaction in the head that is near the S13 r-protein (Fig. 5). The most prominent interaction of YjeQ with the 30S subunit involves the N-terminal OB-fold domain that contacts helices 23b and 24a in the platform of the subunit (Fig. 5A), which correlates with a previous biochemical study of truncated YjeQ revealing that the N-terminal OB-fold region is essential for ribosome binding (Daigle and Brown 2004). The interaction between the OB-fold domain and these two helices occurs through its binding face and encompasses loop 1 and β -strands 2 and 3. The binding mechanism is very similar to r-proteins S12 or S17, which also have an OB-fold domain and bind RNA through this conserved structural motif (Supplemental Fig. S7). Consistently, the electrostatic surface potential of YjeQ shows a continuous surface with positive potential covering the binding face of the OB-fold that interacts with helices 23b and 24a (Fig. 5C).

The C-terminal zinc-finger domain at the other end of YjeQ sits in the upper domain of helix 44 in its ascending way to the decoding center (Fig. 5B). It is interesting that negatively charged residues in the surface of the loop region between the 3¹⁰-helix and H5 α -helix in the zinc-finger domain face the negatively charged phosphate-oxygen backbone of nucleotides of helix 44 in the native 30S subunit (Fig. 5B,C, right panel). This charge distribution supports the necessity of a shift in the helix 44 motif to avoid electrostatic repulsion. In addition, we noted a steric clash of a stretch of helix 44 (nucleotides U1481–G1486) with the C-terminal α -helix of the zinc-finger domain in the manual placement of the YjeQ protein (Fig. 5B). Indeed, during the flexible fitting refinement, this domain slightly moved outward and partially came out of the cryo-EM density (Supplemental Fig. S6D, asterisk), minimizing the steric clash with helix 44. Therefore, we hypothesized that YjeQ dislocates helix 44 from its mature conformation upon binding to the 30S subunit through the negatively charged residues in the surface of the zinc-finger domain loop. Consistent with this, the obtained cryo-EM map of the 30S:YjeQ complex lacked any density matching the upper domain of helix 44 in the mature 30S subunit

FIGURE 5. Interactions of YjeQ with the 30S subunit. (A) Interactions of the OB-fold and GTPase domain of YjeQ with the 16S rRNA helices 24a and 23b in the platform and helix 45 in the 3' minor domain of the 30S subunit. Important loops and β -strands in YjeQ for the interaction with the 30S subunit are labeled. The OB-fold (gray), GTPase (yellow), and zinc-finger (green) domains of the YjeQ structure are shown. The extra density attributed to YjeQ is displayed as a red semitransparent surface. The rRNA (cyan) except for the nucleotides (purple) for which a corresponding density in the 30S:YjeQ complex EM map does not exist. (B) Interactions of the zinc-finger domain of YjeQ with the 16S rRNA helix 44. As an aid for orientation, each panel shows the cryo-EM map of the 30S:YjeQ complex in a matching view. (C) Electrostatic surface potential of *S. typhimurium* (PDB ID 2RCN) YjeQ. Zones are negatively charged (red), positively charged (blue), and uncharged (white). (Left panel) The surface of YjeQ that is not in contact with the 30S subunit; (right panel) the opposite face of the protein contacting the 16S rRNA.

structure (Fig. 4B). As a consequence of the displacement of helix 44, the surface of the GTPase domain facing the 30S subunit did not make significant contact with the rRNA. However, a small number of interactions with helix 45, involving residues from the strands $\beta 6$ and $\beta 7$ that connect this domain with the OB-fold domain, were observed (Fig. 5A,B).

DISCUSSION

Binding site of YjeQ in the 30S subunit

The cryo-EM structure of 30S:YjeQ complex presented here shows that YjeQ binds in the platform domain and in the upper region of helix 44 in the 30S subunit. Interestingly, the area of helix 44 bound by YjeQ is distorted in immature particles purified from an *E. coli* strain lacking the *yjeQ* gene (Jomaa et al. 2011). The cryo-EM reconstruction obtained from these immature 30S subunits revealed that the upper segment of helix 44 was displaced outwardly, protruding from the interface of the 30S subunit that contacts the large 50S subunit. The fact that immature particles accumulated in a strain lacking the *yjeQ* gene contain a distortion in the same area where YjeQ binds suggests that YjeQ may be involved in the correct folding of the upper domain of helix 44.

The binding location of YjeQ established by our cryo-EM structure is consistent with previous footprinting studies (Kimura et al. 2008), indicating that the chemical reactivity of residues around the A-site and P-site (G530, A790, G925, G926, G966, C1054, G1339, and G1405) was reduced in the presence of YjeQ (bound to a nonhydrolyzable GTP analog) (Fig. 6). The footprinting data suggested that YjeQ binding protects this region of the 30S subunit, which is near the YjeQ binding site established by cryo-EM here. In addition, these experiments found increased reactivity of nucleotides in helix 44 (A1408, A1468, and A1483) (Fig. 6), suggesting a conformational change on YjeQ binding that increases the accessibility of these nucleotides to footprinting reagents. This finding is in accordance with the distortion of the upper domain in helix 44 observed in our 30S:YjeQ structure (Fig. 4B,C). Furthermore, the footprinting experiments revealed that GDP-bound YjeQ produced significantly fewer changes or no changes in the reactivity of most of the residues mentioned above, consistent with the lower affinity of YjeQ for the 30S subunit in its GDP-bound state (Daigle and Brown 2004; Kimura et al. 2008).

Conformational changes in YjeQ

GDP-bound *S. typhimurium* YjeQ structure (Nichols et al. 2007) fits remarkably well in the additional density observed in our cryo-EM map of the 30S:YjeQ complex that was assembled in the presence of GMP-PNP. This nucleotide mimics the GTP state of YjeQ. This observation suggests that the protein does not undergo a large interdomain rearrangement upon binding

FIGURE 6. Comparison of the interaction model of YjeQ with the 30S subunit from cryo-EM and footprinting studies. The backbone of the 16S rRNA of the 30S subunit (a ribbon in cyan). Nucleotides whose chemical reactivity was decreased (black) or increased (red) by the addition of YjeQ with a nonhydrolyzable GTP analog (Kimura et al. 2008). The area covered by YjeQ according to the 30S:YjeQ cryo-EM structure presented here is shown as a pink semitransparent density. The 30S:YjeQ map suggests that a region (dark blue) of helix 44 is displaced upon YjeQ binding.

and GTP hydrolysis. Only small translational and rotational movements of each domain independently were necessary to optimize the fitting (Supplemental Fig. S5). The displacement of the N-terminal OB-fold with respect to the GTPase domain was larger than the one applied to the C-terminal zinc-finger domain. This is consistent with the higher flexibility predicted for the OB-fold domain with respect to the GTPase and zinc-finger domains (Shin et al. 2004). These small conformational differences observed between free and bound YjeQ contrast with the large interdomain rearrangements that Era undergoes upon binding to the 30S subunit (Sharma et al. 2005).

Association of YjeQ with the 30S subunit results in a 160-fold stimulation of YjeQ GTPase activity (Daigle and Brown 2004). The most prominent interactions between the protein and the 30S subunit appear to occur through the N-terminal OB-fold domain, whereas the GTPase domain establishes comparatively very few contacts. Thus, the OB-fold domain may have a central role in this stimulation by contacting the 30S subunit and propagating conformational changes through the large area of interaction between the OB-fold and GTPase domains ($\sim 700 \text{ \AA}^2$) (Shin

et al. 2004). Likewise, conformational changes in the GTPase domain caused by GTP hydrolysis can also be easily transmitted to the OB-fold domain to induce dissociation from the 30S subunit. This interpretation of the structure is consistent with previous finding indicating that the OB-fold region is essential for ribosome binding and GTPase stimulation (Daigle and Brown 2004).

Functional interplay of YjeQ with other ribosomal assembly factors

The KsgA protein is a universally conserved methyltransferase that dimethylates both A1518 and A1519 in helix 45 of the 16S rRNA (Sparling 1970; Helser et al. 1972). It has been proposed that KsgA also binds the 30S subunit at late stages of assembly and these two dimethylations could act as a “mark” for fully assembled 30S subunits (Xu et al. 2008). Therefore, it is plausible that KsgA and YjeQ may both be bound to the 30S subunit at some point during assembly. However, concurrent binding of these two factors has not been described. Footprinting experiments mapped the interaction of KsgA with the 30S subunit mainly in the lower domain of helix 44 and helices 11, 27, and 45 (Xu et al. 2008), which is near but not overlapping with the YjeQ binding site. KsgA also contacts helix 24, which is the only interaction motif overlapping with the YjeQ binding site. Therefore, concurrent interaction of KsgA and YjeQ with the 30S subunit is not inconsistent with available structural data.

In addition, previous studies suggest a functional interplay between YjeQ and other biogenesis factors, especially Era, RbfA, and RimM (Inoue et al. 2003, 2006; Campbell and Brown 2008; Goto et al. 2011). The principal known function of both YjeQ and Era is to assist the processing of the 3′ end of the precursor 17S rRNA. The binding sites of Era and YjeQ to the 30S subunit do not overlap, thus simultaneous binding of the two proteins to the 30S subunit to perform this function is possible. Era sits in the cleft region between the head and platform and directly contacts the 3′ end of the rRNA through its C-terminal domain (Sharma et al. 2005). The OB-fold domain in YjeQ binds in the immediate vicinity of Era’s binding position, but it does not contact the 3′ end directly. In these locations, both proteins may mediate the processing of the 3′ end by either recruiting the RNase or by inducing a specific conformation on this end that is recognized by the RNase. Alternatively, YjeQ may assist the processing of the 3′ end by covering inter-subunit bridges and simply preventing premature association with 50S subunits, which, in turn, could block access of the RNases to the cleavage site. YjeQ also promotes the release of RbfA from the mature 30S subunit in a GTP-dependent manner (Goto et al. 2011). RbfA binds in the neck region of the 30S subunit. Binding of RbfA in this position induces a shift in the upper domain of helices 44 and 45, moving these motifs over RbfA and locking the protein deeply within the cleft between the head and body. YjeQ also produces a distortion of helices 44 and 45 upon

binding to the 30S subunit. Therefore, it is possible that movement of these two helices induces the release of RbfA from the 30S subunit. In any case, plausibly the conformational changes induced by YjeQ in the context of these multicomplexes, where several biogenesis factors bind simultaneously to the 30S subunit, may be different from the ones described here. Structures of these multicomplexes are necessary to establish how the functional interplay between these factors occurs.

The 30S:YjeQ complex in this study was assembled with mature 30S subunits, which bind YjeQ with much greater affinity than immature 30S subunits (Goto et al. 2011). However, it is possible that the rRNA precursor sequences in immature 30S subunits may modify the binding mechanism of YjeQ and the conformational changes induced upon binding. Therefore, it is plausible that the binding mode described herein and associated conformational states induced by YjeQ with mature subunits may be different from those associated with YjeQ upon binding to immature subunits.

Mechanisms of YjeQ to assist maturation of the 30S subunit

Each of the 30S subunit large rRNA domains (body, head, and platform) can fold separately *in vitro* (Agalarov et al. 1998, 1999) driven by binding of r-proteins (Sykes and Williamson 2009; Shajani et al. 2011). Interdomain interactions occur late in the assembly process to properly orient the domains in the mature structure. Our structure reveals that YjeQ binds the 30S subunit simultaneously, contacting the head, platform, and helix 44. This binding places YjeQ in a position to assist in the process of domain orientation during the final stages of the 30S subunit assembly (Poot et al. 1998). Interestingly, RbfA and Era biogenesis factors also bind at the junction between the 30S subunit domains and may also assist in this process.

YjeQ distorts the binding site for IF1, which binds the 30S subunit in the cleft between helix 44 and protein S12 (Carter et al. 2001). In addition, the YjeQ binding site partially overlaps with the interaction site of IF2 (Simonetti et al. 2008) and the C-terminal domain of IF3 (Moazed et al. 1995; Dallas and Noller 2001). Hence, our structure suggests that YjeQ might assist in ribosome maturation by preventing premature formation of the translation initiation complex. Curiously, Era also inhibits formation of the translation initiation complex by contacting the conserved anti-Shine-Dalgarno nucleotides of the 30S subunit (Sharma et al. 2005), providing an additional example of partially overlapping functions between ribosome biogenesis factors.

MATERIALS AND METHODS

Construction of YjeQ overexpression clone

Gateway Recombination Cloning technology (Invitrogen) was used to construct full-length YjeQ featuring an N-terminal His₆

affinity purification tag cleavable by TEV protease. Briefly, the *yjeQ* gene was amplified from *E. coli* MG1655 genomic DNA using the polymerase chain reaction. PCR was performed using VENT DNA polymerase (New England Biolabs) and the following primers: 5'-GGGGGACAAGTTTGTACAAAAAAGCAGGCTTA GATTACGATATCCCAACGACCGAAAAACCTGTATTTTCAG*G GCAGTAAAAATAAACTCTCCAAAGGC-3' and 5'-CGCGGAT CCTCAGTCATCCGTATCAGAAAAG-3', where the recombination sites, coding sequence, and cleavage sites are denoted by bold letters, underlining, and an asterisk, respectively. The amplified product was inserted into the pDONR201 (Invitrogen) vector as per the manufacturer's protocol and subsequently cloned into the pDEST17 (Invitrogen) destination vector that encodes six histidine residues at the N terminus of the insert. The resulting construct pDEST17-YjeQ was validated by sequencing (MOBIX, McMaster University).

Purification of YjeQ protein

YjeQ protein was expressed as an N-terminal His₆-tagged protein in *E. coli* BL21-AI competent cells transformed with the expression vector pDEST17-YjeQ (see above). One liter of cells was grown in LB medium at 37°C to OD₆₀₀ = 0.6, and expression was induced with 0.2% L-arabinose. Cells were induced for 3 h at 37°C and harvested by centrifugation at 3700g for 10 min. The cell pellet was washed with 1× PBS buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.76 mM KH₂PO₄ at pH 7.4) and resuspended in 20 mL of lysis buffer (50 mM Tris-HCl at pH 8.0, 10% [w/v] sucrose, 100 mM NaCl). The cell suspension was passed through a French pressure cell at 20,000 lb/in² three consecutive times to lyse the cells. The lysate was spun at 30,000g for 45 min to clear cell debris. NaCl was added to bring the concentration to 0.5 M, and the lysate was filtered with a 0.45-μm filter and added to a HiTrap Metal Chelating column (GE Healthcare Life Sciences) equilibrated with 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, and 5% (v/v) glycerol. Unspecifically bound proteins were washed with increasing concentrations of imidazole up to 90 mM. YjeQ was eluted with 240 mM imidazole. Purity of the fractions was monitored by SDS-PAGE. Fractions containing pure protein were pooled and dialyzed against 50 mM Tris-HCl (pH 8.0), 10% (v/v) glycerol.

The N-terminal His₆-tag was removed by digestion with purified tobacco etch virus (TEV) protease. An amount of 0.74 mg of TEV was added to the pooled and dialyzed fractions containing YjeQ (40 mg). The total volume of the reaction was 10 mL, and the reaction mixture was incubated for 4 h at 16°C. The mixture was added to 1 mL of Ni-NTA agarose resin (QIAGEN) previously equilibrated with 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, and 5% (v/v) glycerol, and incubated in a rocking platform for 1 h at 4°C. The mixture was spun at 1400g for 5 min, and the supernatant containing the untagged YjeQ protein was recovered, dialyzed against 50 mM Tris-HCl (pH 8.0), 10% (v/v) glycerol, and concentrated using a 10 kDa-cutoff filter (Amicon). The protein was frozen in liquid nitrogen and stored at -80°C.

Purification of the 30S ribosomal subunits and preparation of the 30S:YjeQ complex

Purified 30S subunits from BW25113 of *E. coli* were prepared using centrifugations over sucrose cushions and gradients (Daigle and Brown 2004). One liter of Luria broth (LB) was inoculated with 10 mL

of a saturated overnight culture. Cultures were grown at 37°C to an OD₆₀₀ of 0.4 and cooled down to 4°C with all subsequent steps performed at this temperature. Harvesting of the cultures was done by centrifugation at 8500g for 15 min, and the cell pellet was resuspended in buffer A (20 mM Tris-HCl at pH 7.5, 10.5 mM magnesium acetate, 100 mM NH₄Cl, 0.5 mM EDTA, and 3 mM 2-mercaptoethanol). Cell lysis was performed by three consecutive passes of the cell suspension through a French pressure cell at 20,000 lb/in². The cell lysate was spun at 30,000g for 45 min to clear cell debris. Recovered supernatant (S30 fraction) was overlaid on an equal volume of 1.1 M sucrose cushion made up in buffer B (20 mM Tris-HCl at pH 7.5, 10.5 mM magnesium acetate, 500 mM NH₄Cl, 0.5 mM EDTA, and 3 mM 2-mercaptoethanol) and centrifuged at 100,000g for 16 h. The ribosomal pellet was gently washed, and sucrose was removed by resuspension in buffer C (10 mM Tris-HCl at pH 7.5, 10.5 mM magnesium acetate, 100 mM NH₄Cl, 0.5 mM EDTA, and 7 mM 2-mercaptoethanol). The crude ribosomes were pelleted at 100,000g for 16 h. To obtain the 30S fraction, the crude ribosome pellet was resuspended in buffer F (10 mM Tris-HCl at pH 7.5, 1.1 mM magnesium acetate, 60 mM NH₄Cl, 0.5 mM EDTA, and 2 mM 2-mercaptoethanol) (dissociating conditions). A portion of the subunit suspension (50–60 A₂₆₀ units) was layered onto a 32-mL 10%–30% (w/v) sucrose gradient made up in buffer F and centrifuged at 43,000g for 16 h using a Beckman SW32 Ti rotor. Gradients were fractionated using an AKTApurification system (GE Healthcare), and the elution peaks corresponding to 30S and 50S particle peaks were monitored by absorbance at A₂₆₀. The 30S ribosomal subunits were then recovered by centrifugation at 100,000g for 16 h, and the pellet was resuspended in buffer E (10 mM Tris-HCl at pH 7.5, 10 mM magnesium acetate, 60 mM NH₄Cl, 3 mM 2-mercaptoethanol), and stored at -80°C until further use. Quantification of the 30S subunits was accomplished by absorbance at 260 nm (1 A₂₆₀ unit is equivalent to 69 pmol of 30S).

To determine the occupancy level of YjeQ in the 30S:YjeQ complex, a fivefold excess of freshly purified YjeQ protein was incubated with a 1 μM preparation of purified 30S subunits for 1 h at 30°C in the presence of 1 mM GMP-PNP in buffer E. The volume of the reaction was 50 μL. The reaction mixture was layered onto a 1.1 M sucrose cushion (volume 150 μL) made up in buffer E. The ribosomal subunits were pelleted by ultracentrifugation at 330,000g for 4 h using a Beckman TLA-120.1 rotor, and the pellet was resuspended in 200 μL of buffer E. The supernatant and resuspended pellet of each sample (12-μL load volume) were resolved by SDS-PAGE and stained with Coomassie brilliant blue. The stained gel was subsequently photographed using a FluorChem FC3 system (Alpha Innotech). The occupancy level of YjeQ in the 30S:YjeQ complex was estimated by quantifying the amount of YjeQ that co-pelleted with ribosomes (2.5 pmol) in each reaction. The integrated pixel density of each YjeQ band was calculated using Imagequant, version 5.2 (Molecular Dynamics), with the local average of the region surrounding the band used for background correction. This yielded an optical density for each band that was used to estimate the proportion of YjeQ found in the supernatant and pellet of each reaction. YjeQ protein (25%) was seen to pellet in the absence of ribosomes. Thus, to determine the amount of YjeQ that co-pelleted with 30S subunits, it was necessary to subtract this fraction from the YjeQ observed in the reaction pellets (lanes 7,9). The previously established 1:1 stoichiometry for the 30S:YjeQ

complex (Daigle and Brown 2004) was assumed to determine the occupancy level.

To assemble the complex for cryo-EM, a 50- μ L reaction mixture in buffer E with 2 mM GMP-PNP was prepared by adding 30S subunits to a concentration of 1 μ M and a fivefold excess of freshly purified YjeQ protein. The reaction was incubated for 1 h at 30°C and then diluted 100 times in buffer E containing 2 mM GMP-PNP before applying it to the EM grids.

Nanoscale microcapillary liquid chromatography electrospray ionization tandem mass-spectrometry (LC-MS/MS)

The protein gel bands were destained with 50 mM ammonium bicarbonate and water until clear, then rinsed with water several times to remove all color. The band was reduced with 10 mM DTT for 30 min at 56°C and alkylated with 100 mM iodoacetamide for 15 min at room temperature in the dark. Protein in the band was digested in situ with 30 μ L of (13 ng/ μ L) trypsin (Promega) in 50 mM ammonium bicarbonate overnight at 37°C, followed by peptide extraction with 30 μ L of 5% formic acid, then 30 μ L of acetonitrile. The pooled extracts were concentrated to <5 μ L on a SpeedVac spinning concentrator and then brought up in 0.1% formic acid and 5% acetonitrile for protein identification by micro-flow liquid chromatography electrospray tandem mass spectrometry (microLC-ESI-MS/MS) using a ThermoFisher LTQ-XL Orbitrap Hybrid Mass Spectrometer (ThermoFisher) coupled to an Eksigent nanoLC-2D HPLC system (Eksigent). Chromatography was performed using 0.1% formic acid in both the A solvent (98% water, 2% acetonitrile) and B solvent (10% water, 80% acetonitrile, 10% isopropanol), and a 5% B to 95% B gradient over 30 min at 5 μ L/min through an Eksigent capillary (CSP-3 C18 -100, 0.3 m $\times$ 100 mm) column.

The instrument method consisted of one MS full scan (200–2000 m/z) in the Orbitrap mass analyzer, an automatic gain control target of 500,000 with a maximum ion injection of 500 msec, one microscan, and a resolution of 60,000. Three data-dependent MS/MS scans were performed in the linear ion trap using the three most intense ions at 35% normalized collision energy. The MS and MS/MS scans were obtained in parallel fashion. In MS/MS mode automatic gain control targets were 10,000 with a maximum ion injection time of 100 msec. A minimum ion intensity of 1000 was required to trigger an MS/MS spectrum. The dynamic exclusion was applied using a maximum exclusion list of 500 with one repeat count with a repeat duration of 30 sec and exclusion duration of 45 sec. The lock-mass option was enabled for the FT full scans using the ambient air polydimethylcyclsiloxane (PCM) ion of $m/z = 445.120024$ or a common phthalate ion $m/z = 391.284286$ for real-time internal calibration.

For database searching, tandem mass spectra were extracted by Proteome Discoverer 1.2. All MS/MS samples were analyzed using SEQUEST (ThermoFinnigan; version 27, revision 12) and X! Tandem (The Global Proteome Machine Organization; version 2006.04.01.2). Both search engines were set up to search the *E. coli* database, assuming trypsin digestion, allowing two missed cleavages, and using a fragment ion mass tolerance of 0.5 Da and a parent ion tolerance of 0.02 Da. The iodoacetamide derivative of cysteine was specified as a fixed modification. Deamidation of asparagine and glutamine and oxidation of methionine were

specified in X! Tandem as variable modifications. Oxidation of methionine was specified in SEQUEST as a variable modification.

Cryo-electron microscopy, image classification, and 3D reconstruction

For cryo-EM, holey carbon grids (400 mesh copper) containing an additional continuous thin (5–10 nm) layer of carbon were previously washed with acetone vapor for 15 min and glow discharged in air for 30 sec (Aebi and Pollard 1987). Then, 3.5- μ L aliquots of sample were applied to the grid for 1 min. Grids were blotted for 7 sec and vitrified by rapidly plunging into liquid ethane at -180°C with a Vitrobot (FEI). Data acquisition was performed under low dose conditions ($\sim 15 \text{ e}^-/\text{\AA}^2$) on a JEOL 2010F FEG microscope operated at 200 kV with a Gatan 914 side-entry cryo-holder and at a nominal magnification of 50,000 $\times$. A total number of 121 electron micrographs were collected. The defocus range of the images was from -0.65 to $-3.9 \mu\text{m}$. The micrographs were digitized with a step size of 12.7 μm in a Nikon Supercool Scan 9000 producing images with a sampling value of 2.54 $\text{\AA}/\text{pixel}$.

Particle projections from the electron micrographs were picked using Boxer (Ludtke et al. 1999). Contrast transfer function of the micrographs was estimated using CTFFIND software (Mindell and Grigorieff 2003) and corrected using the Xmipp software package (Scheres et al. 2008). Image classification was performed using supervised methods (Valle et al. 2002; Gao et al. 2004). Detailed procedures regarding the classification method are included in the figure legend for Figure 2. From the total 73,896 projections collected, 16,228 showed a clear YjeQ density in our image classification procedure, and only those particles were subsequently used for the reconstruction of the 30S:YjeQ complex. The 3D reconstruction of the 30S:YjeQ complex was calculated using 3D projection alignment procedures as implemented in the Xmipp software package (Scheres et al. 2008). The reference map used to refine the 3D reconstruction was the X-ray structure of the *E. coli* 30S subunit (PDB ID: 2AVY) (Schuwirth et al. 2005) low-pass filtered to 25 $\text{\AA}$ resolution. In each refinement, sets of projections were calculated from the reference map using an angular spacing that ranged from 15° to 2° during the multiple cycles of refinement.

The correct handedness of the structures was imposed by the X-ray crystal structure of the 30S subunit from *E. coli* (PDB ID: 2AVY). Resolution of the cryo-EM maps was estimated by calculating two maps following the last cycle of refinement from the even- and odd-numbered particles. Then Fourier shell correlation was calculated between both maps, and the resolution was estimated using an FSC value of 0.5. These values were used to low-pass filter the refined cryo-EM maps.

Construction of a pseudo-atomic model of the 30S:YjeQ complex

To locate the YjeQ protein in the cryo-EM map of the 30S:YjeQ complex, a difference map between the control cryo-EM structure of the free 30S subunit (Fig. 3A, left panel) (low-pass filtered at the same resolution) and the YjeQ-bound 30S subunit was calculated. To this end, we scaled the two maps relative to each other by normalizing their average density and standard deviation values (σ) to zero and one, respectively. Then, the two maps were superimposed by maximizing the correlation coefficient and subtracted. The resulting difference map had an average density equal to zero and a σ of 0.6, and in the rendering only significant values ($>2\sigma$)

were displayed. The atomic structure of *S. typhimurium* YjeQ (PDB ID 2RCN) (Nichols et al. 2007) was then docked as a single rigid body into the density of the obtained difference map covering the upper domain of helix 44 and platform in the 30S subunit. Subsequently, we applied small translational and rotational movements to each domain independently to optimize the fitting (Supplemental Fig. S5). Overlapping the obtained difference map containing the docked X-ray structure of YjeQ into the cryo-EM structure of the 30S:YjeQ complex and subsequent docking of the X-ray structure of the 30S subunit (PDB ID: 2AVY) (Schuwirth et al. 2005) in the remaining density of the cryo-EM map allowed us to produce a pseudo-atomic model of the 30S:YjeQ complex. The same difference map analysis was repeated using two *E. coli* 30S subunit X-ray structures (PDB IDs: 2AW7 and 2AVY) (Schuwirth et al. 2005). In these two cases, the structures were first low-pass filtered to match the resolution of our 30S:YjeQ cryo-EM map and then fitted into the cryo-EM map of the 30S:YjeQ complex to perform the map subtraction and calculate the difference map. The CCC values to measure the fitting of the X-ray coordinates of YjeQ into the cryo-EM density were determined after conversion of the fitted atomic coordinates into a density map.

Flexible fitting refinement

The starting model for the flexible fitting refinement was the atomic structures of the 30S subunit (PDB ID: 2AVY) and YjeQ (PDB ID: 2RCN) manually placed in the cryo-EM density of the 30S:YjeQ complex (see above). This assembly was divided into 26 distinct units (five 16S rRNA structural domains including the head, body, platform, helix 44, and helix 45 along with 20 ribosomal proteins and YjeQ) that were refined with a flexible fitting algorithm using the YUP.scx module (Tan et al. 2008) of YUP (Tan et al. 2006). The potential energy function included terms for the all-atom structures of the 30S:YjeQ complex that were represented as a Gaussian Network Model, terms for scoring the quality of the fit of the model to the density map, and volume exclusion terms. Specifically, the Gaussian Network was created from the initial structure, taking in any atom pairs that were within the cutoff distance of 4 Å. This prevented motions that would otherwise distort the starting structure, while allowing for flexibility between more isolated domains. The molecular dynamics simulation was carried out with a time step of 5 fsec. After an initial energy minimization, the model was heated from a low temperature to 10 K over 5000 steps and then held at 10 K over 20,000 steps. After equilibration, the annealing process was performed by reducing the temperature from 10 K to 1 K over ~50,000 steps, and the model was subjected to a final round of energy minimization. The final placement provides a pseudo-atomic model with stereochemical restraints that fits into the electron density map optimally.

Visualization of structures

Visualization of the fitted YjeQ and 30S atomic structures and cryo-EM maps was done with UCSF Chimera software (Pettersen et al. 2004). The electrostatic surface potential shown for YjeQ was also created using this software.

Accession numbers

The EM map and coordinates of the pseudo-atomic model of the 30S:YjeQ complex have been deposited in the Electron Microscopy Data Bank (EMDB: 1895) and Protein Data Bank (PDB: 4a2i).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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NOTE ADDED IN PROOF

While this paper was under review another study describing the cryo-EM structure of the YjeQ in complex with the 30S ribosomal subunit was published (Guo et al. 2011).

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