JB
Journals.ASM.org

Efficient Assembly of Ribosomes Is Inhibited by Deletion of *bipA* in *Escherichia coli*

Promisree Choudhury, Ann M. Flower

Department of Basic Sciences, University of North Dakota School of Medicine and Health Sciences, Grand Forks, North Dakota, USA

ABSTRACT

The bacterial BipA protein belongs to the EF-G family of translational GTPases and has been postulated to be either a regulatory translation factor or a ribosome assembly factor. To distinguish between these hypotheses, we analyzed the effect of *bipA* deletion on three phenotypes associated with ribosome assembly factors: cold sensitivity, ribosome subunit distribution, and rRNA processing. We demonstrated that a Δ*bipA* strain exhibits a cold-sensitive phenotype that is similar to, and synergistic with, that of a strain with a known ribosome assembly factor, *deaD*. Additionally, the *bipA* deletion strain displayed a perturbed ribosome subunit distribution when grown at low temperature, similar to that of a *deaD* mutant, and again, the double mutant showed additive effects. The primary ribosomal deficiency noted was a decreased level of the 50S subunit and the appearance of a presumed pre-50S particle. Finally, deletion of *bipA* resulted in accumulation of pre23S rRNA, as did deletion of *deaD*. We further found that deletion of *rluC*, which encodes a pseudouridine synthase that modifies the 23S rRNA at three sites, suppressed all three phenotypes of the *bipA* mutant, supporting and extending previous findings. Together, these results suggest that BipA is important for the correct and efficient assembly of the 50S subunit of the ribosome at low temperature but when unmodified by RluC, the ribosomes become BipA independent for assembly.

IMPORTANCE

The ribosome is the complex ribonucleoprotein machine responsible for protein synthesis in all cells. Although much has been learned about the structure and function of the ribosome, we do not fully understand how it is assembled or the accessory proteins that increase efficiency of biogenesis and function. This study examined one such protein, BipA. Our results indicate that BipA either directly or indirectly enhances the formation of the 50S subunit of the ribosome, particularly at low temperature. In addition, ribosomes contain a large number of modified nucleosides, including pseudouridines. This work demonstrates that the function of BipA is tied to the modification status of the ribosome and may help us understand why these modifications have been retained.

Inquiry into protein function is often guided by a combination of observed mutant phenotypes and predicted protein activities based on homology comparisons to proteins with known functions. These tools do not, however, always provide clear directions for deciphering functions of unknown proteins, even in the well-studied model organism *Escherichia coli*, and this is particularly true in the study of nonessential proteins. One such protein whose function remains undefined is BipA. The *bipA* gene is conserved in a large number of bacterial species, as well as in at least one chloroplast genome (1, 2). The domain architecture of BipA demonstrates that it belongs to the elongation factor family of GTPases, which includes EF-G, EF-Tu, and LepA (EF-4) (3, 4), suggesting that BipA (and LepA) might be a translation factor(s). However, unlike EF-G and EF-Tu, BipA and LepA are dispensable for growth, as *bipA* and *lepA* deletion mutants do not display overt growth defects under standard conditions (5). Yet the conservation of *bipA* suggests that the function of the protein is important for optimal cell viability and that conditions exist that dictate retention of the gene.

Consistent with its similarity to EF-G, BipA has been shown to be a ribosome-binding GTPase, and the site of binding to the ribosome appears to overlap that of EF-G (6). BipA associates with 70S ribosomes only in the GTP-bound state, and hydrolysis of GTP to GDP dissociates BipA from ribosomes (7). The hydrolytic activity of BipA is stimulated in the presence of 70S ribosomes, tRNA, and mRNA (7), implying that this function is employed during active translation and supporting the proposal that BipA is a translation factor.

Genetic analyses have also yielded clues to the role of BipA. Mutations in *bipA* result in a diversity of phenotypes, including cold sensitivity (8), hypermotility (9), decreased capsule synthesis (10), increased chloramphenicol sensitivity (11), and decreased pathogenicity (12). These pleiotropic effects, combined with the similarity to elongation factors, led to the hypothesis that BipA is a translation factor that might regulate protein expression through an interaction with the ribosome that alters the efficiency of translation of target mRNAs (5, 7).

An alternate hypothesis, however, is that BipA is important for ribosome assembly. Supportive of this hypothesis, many known ribosome assembly factors, such as Era, EngA, and CgtAE, are also

Received 12 January 2015 **Accepted** 8 March 2015

Accepted manuscript posted online 16 March 2015

Citation Choudhury P, Flower AM. 2015. Efficient assembly of ribosomes is inhibited by deletion of *bipA* in *Escherichia coli*. J Bacteriol 197:1819–1827. doi:10.1128/JB.00023-15.

Editor: R. L. Gourse

Address correspondence to Ann M. Flower, ann.flower@med.und.edu.

doi:10.1128/JB.00023-15

TABLE 1 *E. coli* strains used in this study

Strain	Genotype[a]	Reference or source
JW2553	Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) λ$^-$ Δ*lepA738*::*kan rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	24
JW5531	Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) λ$^-$ Δ*deaD774*::*kan rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	24
KK30	TB28 Δ*bipA*<>*res*	5
KK33	TB28 Δ*bipA*<>*res* Δ*rluC*::*res-npt-res*	5
MG1655	*rph-1 ilvG rfb-50*	25
PC28	MG1655 Δ*bipA*::*res-npt-res*	This study
PC30	MG1655 Δ*bipA*<>*res*	This study
PC33	MG1655 Δ*rluC*::*res-npt-res*	This study
PC34	PC30 Δ*rluC*::*res-npt-res*	This study
PC95	MG1655 Δ*deaD774*::*kan*	This study
PC96	PC30 Δ*deaD774*::*kan*	This study
PC133	MG1655 Δ*lepA738*::*kan*	This study
S17-1λ*pir*(pJMSB8)	Tp^r Sm^r *recA thi hsdRM*$^+$ RP4::2-Tc::Mu::Km Tn*7* λ*pir*	26
TB28	MG1655 Δ*lacZYA*<>*frt*	27

[a] The symbols <>*res* and <>*frt* indicate that the kanamycin resistance gene cassette was replaced with the scar *res* or *frt* sequence after recombination with the ParA resolvase or FLP recombinase, respectively (26, 27). Km, kanamycin; Sm, streptomycin; Tp, trimethoprim.

GTPases (13–15), although they belong to a family of GTPases separate from the elongation factors (16). Additionally, like BipA, most of these ribosome assembly factors are dispensable for growth, exhibiting no obvious phenotypes under optimum growth conditions (17). However, mutations do often display cold-sensitive growth as well as ribosome assembly defects characterized by incorrectly assembled ribosomal subunits and impaired maturation of rRNA(s) (17–19). Finally, and again similarly to *bipA*, mutations in ribosome assembly factors also affect unrelated nonribosomal processes, including DNA replication, metabolism, or the cell cycle (17). The cold sensitivity and the diversity of phenotypes associated with *bipA* mutants are consistent with the hypothesis that BipA may be similar in function to these ribosome assembly factors.

Genetic analysis of mutations in potential ribosome assembly factor genes has provided insight into their involvement in ribosome maturation and assembly. In one study, deletion of *rhlE* exacerbated the cold-sensitive phenotype of Δ*deaD* mutants, while it alleviated the phenotype of Δ*srmB* strains (20). RhlE, DeaD, and SrmB are all DEAD box RNA helicases, and the latter two are involved in the biogenesis of the 50S ribosomal subunit. Based on these genetic data, it was proposed that RhlE is also a ribosome assembly factor, and subsequent analyses substantiated that hypothesis (20). Similarly, YjeQ was proposed to be a 30S subunit maturation factor when deletion of *rimM*, an essential 30S assembly factor, exacerbated the slow-growth phenotype of a Δ*yjeQ* strain (21). Thus, genetic interactions have facilitated characterization of proteins that are involved in the complex process of ribosome assembly.

A final factor in our consideration of BipA function was discovered in our previous search for suppressors of Δ*bipA* cold sensitivity. We found that deletion of *rluC*, a pseudouridine synthase coding gene, suppressed two phenotypes of Δ*bipA* mutants, cold sensitivity and decreased capsule synthesis (5). RluC is responsible for the insertion of three pseudouridines into the 23S rRNA, at positions 955, 2504, and 2580 (22). Although the role of rRNA modification is incompletely defined, it has been suggested that modifications are important structural determinants during the assembly process (23). Therefore, it is possible that RluC-modified ribosomes require BipA for complete and efficient assembly, at least under certain conditions such as low temperature. In the absence of modification, an alternate, BipA-independent pathway may function to assemble ribosomes. Alternately, it may be that the function of ribosomes is altered by pseudouridine modification and that BipA may affect translational efficiency. Again, the loss of modification would render the ribosomes independent of BipA (5).

The information provided thus far can support either of two hypotheses for BipA function: BipA may be a translation factor that regulates expression of specific mRNAs, or BipA might be involved in ribosome assembly (5). To distinguish between the two hypotheses, in this study we examined the involvement of BipA in ribosome assembly. As combinatorial genetics proved informative in defining RhlE and YjeQ as ribosome assembly factors, we created combinations of *bipA*, *deaD*, and *rluC* mutations to examine growth phenotypes, ribosome subunit distribution, and rRNA processing to determine the effect of BipA on these processes. Our results suggest that deletion of *bipA* adversely affects the efficiency of ribosome assembly and rRNA processing. The combination of *deaD* deletion with *bipA* deletion resulted in more profound effects on ribosomes, while deletion of *rluC* was able to suppress the effects of *bipA* deletion. Based on these results, we propose that the function of BipA is important for efficient assembly of ribosomes.

MATERIALS AND METHODS

Bacterial strains and media. *Escherichia coli* strains JW2553 and JW5531 (Table 1) were obtained from the *E. coli* Genetic Stock Center (24). All other bacterial strains used were derivatives of *E. coli* K-12 strain MG1655 and are listed in Table 1. Bacteria were grown in modified Luria Bertani (LB) media (28), except when M9 minimal medium (28) was used to select against the donor strain following conjugation. Ampicillin (125 mg/liter) and kanamycin (50 mg/liter) were used as indicated.

Strain constructions. Standard genetic techniques were used for strain constructions (28). Insertion-deletion mutations were introduced via P1 transduction. Where indicated, the *npt* cassette was removed by conjugation with *E. coli* S17-1 λ*pir*(pJMSB8) and subsequent homologous recombination (26).

Growth analysis. Growth curve analyses were performed using a Bioscreen C microbiology reader from Labsystems (Helsinki, Finland). Bacterial cultures were grown overnight in LB at 37°C in standard culture

TABLE 2 Probes used for rRNA analysis[a]

Probe	Sequence (5′–3′)	Temp (°C)	
		Hybridization	Stringent wash
16S-M	TACTCACCCGTCCGCCACTC	60	57
23S-U	CGCTTAACCTCACAAC	45	42
23S-M	AAGGTTAAGCCTCACGGTTC	60	58

[a] See reference 19 for details.

tubes and then diluted to an optical density at 600 nm (OD_{600}) of 0.02. A 300-μl volume of diluted culture was added in triplicate to each well of a honeycomb plate for use in the Bioscreen C reader. Growth was allowed to continue at either 37°C or 20°C for 40 h with continuous shaking, and OD_{600} was monitored every 30 min. Growth curves were generated by plotting OD_{600} versus time using GraphPad Prism. Due to the shorter path length of the honeycomb wells, the OD_{600} values obtained from a Bioscreen C reader are not directly comparable to those obtained from a standard spectrophotometer (5).

Ribosome profile analysis. Bacterial cells were grown either at 37°C or at 20°C to an OD_{600} of 0.5 to 0.7, and ribosome extracts were prepared as described previously (19). Briefly, chloramphenicol was added to the cultures to a concentration of 100 μg/ml and cells were collected by brief centrifugation. The pellets were resuspended in 1× buffer A (10 mM Tris-HCl [pH 7.5], 60 mM KCl, 10 mM $MgCl_2$) containing 0.5 mg/ml lysozyme and frozen overnight at −20°C. The samples were thawed on ice the next day, treated with buffer A containing 0.5% Brij 58, 0.5% deoxycholate, and 0.1 unit/μl RQ1 DNase (Promega), and incubated on ice. Following incubation, ribosomal extracts were prepared by collecting the supernatant after brief centrifugation. Approximately 20 A_{260} units of the ribosomal extracts were layered onto a 10% to 40% linear sucrose gradient prepared in buffer B (10 mM Tris-HCl [pH 7.5], 50 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM dithiothreitol [DTT]) and centrifuged at 22,000 rpm for 19 h at 4°C in an SW28 rotor (Beckman). Following centrifugation, ribosomal fractions were collected from the bottom of the centrifuge tubes to the top by piercing the bottom of the tube and allowing samples to drip into collection tubes. A_{260} values were monitored using a spectrophotometer (Biotek, Inc.) and Gene 5.2 software. Ribosome profiles were generated by plotting A_{260} values versus fraction numbers, using Microsoft Excel.

RNA analysis. Overnight cultures of bacterial cells were subcultured at 1:100 and grown at 20°C to an OD_{600} of 0.5 to 0.7. Whole-cell RNA was extracted using TRIzol reagent (Life Technologies) following the manufacturer's instructions. RNA was separated on 1.2% (23S rRNA analyses) or 2.0% (16S rRNA) agarose gels and transferred to nitrocellulose membranes (Micron Separations Incorporated) as described previously (15). Northern analysis was performed using the probes described in Table 2. The 16S-M probe was used to identify both precursor and mature 16S rRNAs, and the 23S-U and 23S-M probes were used to identify precursor and mature 23S rRNAs, respectively. The 16S-M probe was 5′ biotin tagged (purchased from Midland Oligos), and the 23S-M and 23S-U probes (purchased from IDT DNA) were labeled at the 5′ end using [γ-^{32}P]dATP (MP Biomedicals) (specific activity, 6,000 Ci/mmol) and T4 polynucleotide kinase (New England BioLabs). Following hybridization, the membranes were washed as described in Table 2 (19). Biotin signals were detected using a biotin detection kit (Thermo Scientific) and Immun-Star chemiluminescent substrate (Bio-Rad) following the manufacturer's instructions. Band intensities were quantified using Quantity One imaging software. The radioactive signals were detected using a Typhoon scanner (Amersham Biosciences) and quantified using Image J (NIH; http://imagej.nih.gov/ij/). Statistical significance was calculated by a one-way analysis of variance (ANOVA) and Dunnett's *post hoc* test. Statistical significance was set at $P < 0.05$.

RESULTS

The cold-sensitive phenotypes of the Δ*bipA* and Δ*deaD* strains are similar and are synergistic. Analysis of mutant phenotypes can often provide insights into the function of gene products (29, 30). In the case of ribosome assembly, the most common phenotypes associated with mutation of ribosome assembly factors are cold sensitivity, disruption of ribosome subunit distribution, and accumulation of precursor rRNAs (13, 18–20). To assess whether BipA might be involved in ribosome biogenesis, we examined these three phenotypes, compared the results to those seen with the known ribosome assembly factor *deaD*, and analyzed the double mutant Δ*bipA* Δ*deaD* to detect combinatorial effects.

We demonstrated previously that deletion of *bipA* results in a cold-sensitive growth phenotype (8). Now we compared the cold sensitivity of the Δ*bipA* strain to that of the Δ*deaD* strain. We monitored the growth of the wild-type (MG1655), Δ*bipA*, Δ*deaD*, and Δ*bipA* Δ*deaD* strains at 37°C and at 20°C (Fig. 1). At 37°C, all

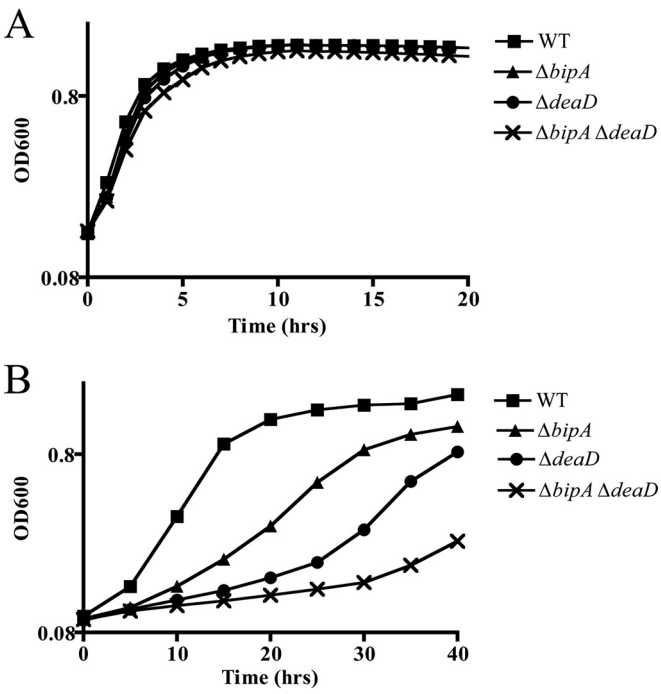


FIG 1 Cold sensitivity of Δ*bipA* and Δ*deaD* mutants. Growth analyses of wild-type (WT; MG1655), Δ*bipA* (PC28), Δ*deaD* (PC95), and Δ*bipA* Δ*deaD* (PC96) strains were performed at either 37°C (A) or 20°C (B). Optical density was measured every 30 min; data points are shown only at every hour (A) or 5 h (B) for clarity. Each strain was analyzed in triplicate, and experiments were repeated with three biological replicates. Representative examples for each strain are shown.

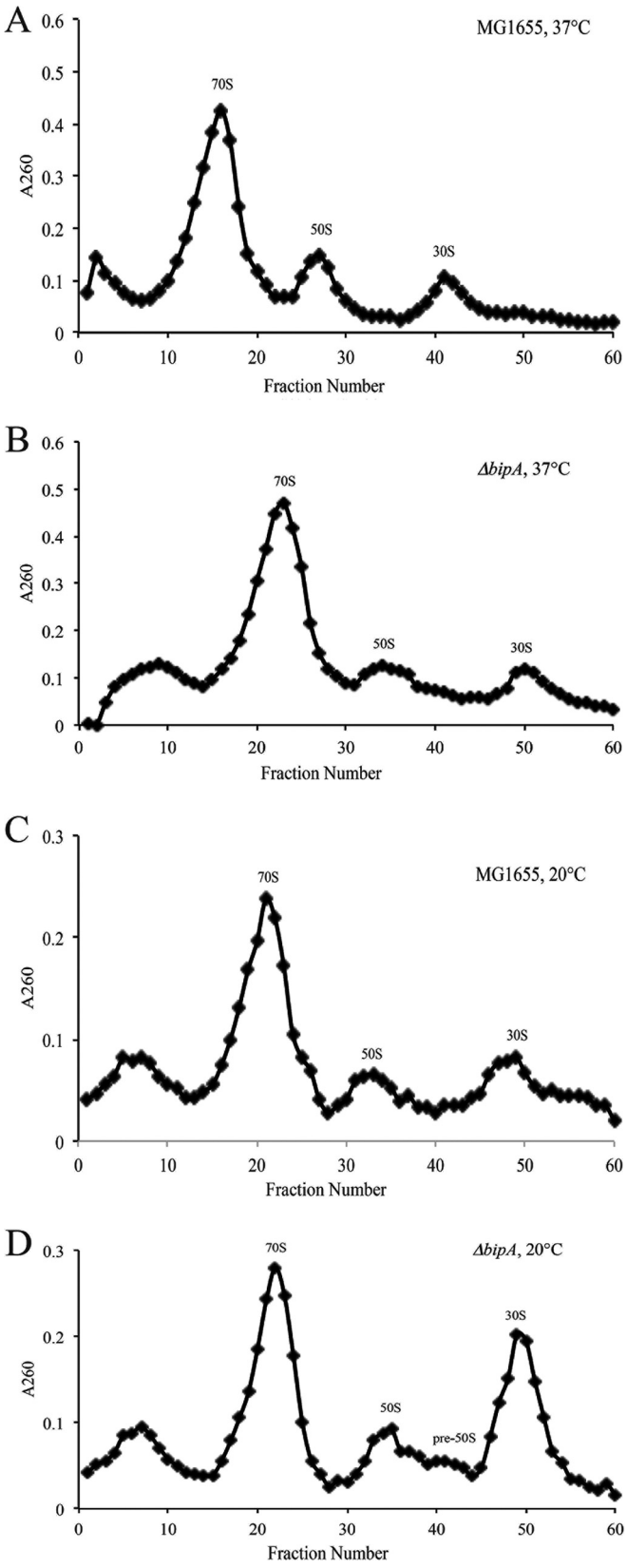


FIG 2 Deletion of *bipA* leads to altered ribosome profiles. Wild-type (MG1655) (A and C) and Δ*bipA* (PC28) (B and D) strains were grown either at 37°C (A and B) or at 20°C (C and D). Ribosomal subunits were separated as described in Materials and Methods, with fractions collected from the bottom of the sucrose density gradient to the top. Ribosome particles were identified by measuring the A_{260} of each fraction to identify rRNA. The peaks corresponding to the 30S and 50S subunits, 70S ribosomes, and the presumed pre-50S particle are indicated. All samples were analyzed from at least three biological replicates; representative examples are shown.

strains grew equally well (Fig. 1A); the single mutant strains had no detectable growth defect compared to the wild-type strain, and the double-mutant strain exhibited only a very slight decrease in its growth rate. At 20°C, both the Δ*bipA* and Δ*deaD* strains displayed a cold-sensitive phenotype, as previously reported (Fig. 1B) (8, 18). Both strains remained in lag phase for an extended period before entering exponential growth, with the lag for the Δ*deaD* strain noticeably longer than for the Δ*bipA* strain. Once the mutant strains entered the exponential-growth phase, they achieved comparable maximal rates of growth that were approximately half that of the wild-type strain (approximately 6 h doubling time for each single mutant compared to 3 h for the wild-type strain). However, the Δ*bipA* Δ*deaD* double-mutant strain exhibited a more severe cold-sensitive phenotype than either the wild-type strain or the single mutants. This defect manifested as a greatly protracted lag phase, and, once the exponential growth phase was reached, a maximal growth rate of approximately 13 h per doubling, approximately one-fourth the rate seen with the wild-type strain, was observed. This extremely slow growth indicates a multiplicative effect of the two mutations that may be due to a severe defect in ribosome assembly.

Deletion of *bipA* alters ribosome subunit distribution. To further examine the potential role for BipA in ribosome assembly, we analyzed the ribosome profile of a Δ*bipA* mutant to determine whether deletion of *bipA* results in altered proportions of 70S, 50S, and 30S ribosomal fractions and if precursor particles could be detected. Wild-type and Δ*bipA* strains were grown at either 37°C or 20°C, and ribosomes were extracted and fractionated by sucrose gradient centrifugation to separate the 70S ribosome, the 50S particle, and the 30S subunit. When grown at 37°C, the ribosome profile of the Δ*bipA* strain was similar to that of the wild-type strain (Fig. 2A and B). Additionally, ribosomes from the wild-type strain showed very similar profiles whether grown at 37°C or at 20°C (Fig. 2A and C). However, after growth at 20°C, ribosomes isolated from the Δ*bipA* strain were perturbed in the distribution of the subunits, exhibiting an increased ratio of 30S subunits to 50S subunits (Fig. 2D). Additionally, a small peak was observed between the 50S and 30S peaks that was suggestive of the accumulation of a precursor 50S ribosomal particle (19). It is also notable that the ratio of 30S and 50S subunits to the 70S monosomes was increased. These results indicate that BipA influences ribosomal subunit ratios, particularly that of the 50S ribosomal subunit, and suggest that BipA may be involved in the biogenesis of the 50S subunit.

Deletion of *rluC* relieves the ribosome defects of the Δ*bipA* mutant. We previously demonstrated that deletion of *rluC* alleviates the cold sensitivity and the capsule synthesis defect of a Δ*bipA* mutant (5). One hypothesis suggested by these findings was that ribosome assembly requires BipA but that ribosomes lacking the pseudouridines inserted by RluC are able to be assembled properly by an alternate, BipA-independent pathway. If that hypothesis is correct, we predicted that deletion of *rluC* would restore normal ribosome subunit profiles to the Δ*bipA* mutant. Accord-

ingly, a Δ*rluC* mutant and a Δ*bipA* Δ*rluC* double mutant were grown at 37°C or at 20°C, and the ribosomal profile of each was analyzed and compared to those of the wild-type and Δ*bipA* strains. The ribosome profiles of all three mutants were similar to that of the wild-type strain at 37°C (Fig. 2A and B and 3A and B). Following growth at 20°C, the Δ*rluC* mutant exhibited a ribosome profile similar to that of the wild-type strain, with no apparent defect in ribosome assembly (Fig. 3C). Strikingly, the Δ*bipA* Δ*rluC* double mutant also had no obvious defect in ribosome assembly when grown at 20°C (Fig. 3D), with subunit distributions that appeared to have been restored to wild-type levels. This observation is in accordance with our previous finding that deletion of *rluC* suppressed the cold sensitivity and capsule synthesis defects of a Δ*bipA* strain. Our results indicate that deletion of *rluC* also alleviates the ribosomal defect incurred by deletion of *bipA*.

Deletion of *bipA* and *deaD* affects ribosome assembly synergistically. Our results demonstrate that deletion of both *bipA* and *deaD* has a synergistic effect on cold sensitivity (Fig. 1), suggesting related functions for these two proteins. Therefore, we determined the effect of *deaD* deletion on the ribosome profile of a Δ*bipA* mutant. As seen in previous reports, the Δ*deaD* mutant was similar to the wild-type strain at 37°C (Fig. 4A) but at 20°C exhibited defective ribosome assembly characterized by an increased proportion of 30S subunits, decreased amounts of 50S subunits, and the presence of a precursor 50S ribosomal particle (Fig. 4C) (18). This profile is comparable to that of the *bipA* mutant, only more severe. The Δ*bipA* Δ*deaD* double mutant also displayed apparently normal ribosome profiles at 37°C (Fig. 4B). Strikingly, however, deletion of both *bipA* and *deaD* at 20°C resulted in a greatly exacerbated defect in ribosome subunit distribution. The ribosome profile of the Δ*bipA* Δ*deaD* double mutant displayed increased proportions of both 50S and 30S ribosomal subunits relative to 70S, decreased amounts of fully formed 70S ribosomes, and significant levels of a presumed precursor 50S particle (Fig. 4D). This finding is in concordance with the extremely slow growth exhibited by the Δ*bipA* Δ*deaD* double mutant at 20°C depicted in Fig. 1. Our results suggest that both BipA and DeaD are contributors to efficient and/or correct ribosome assembly.

Accumulation of precursor rRNAs in the Δ*bipA* mutant. Ribosome assembly defects are often characterized by the accumulation of precursor 23S rRNA (p23S) and/or precursor 16S rRNA (17S) (18, 19). Because our data suggest that deletion of *bipA* affects biogenesis of the 50S ribosomal subunit, we further characterized the ribosomal defects of a Δ*bipA* mutant by analyzing the conversion of p23S rRNA to mature 23S rRNA. Additionally, because it has been seen that defects in 50S biogenesis can indirectly impact 16S rRNA processing, as evident in strains lacking the 50S assembly factor SrmB (19), we monitored 16S rRNA processing as well. As before, we assessed the contribution of *rluC* or *deaD* deletion to 23S and 16S rRNA processing both alone and in combination with *bipA* deletion.

The p23S rRNA that accumulates in bacterial cells lacking ribosome assembly factors is either three or seven nucleotides longer at the 5′ end and seven to nine nucleotides longer at the 3′ end than the mature 23S rRNA (Fig. 5A) (18, 19). To analyze 23S rRNA processing in our strains, whole-cell RNA was isolated from bacterial cells grown at 20°C and 23S rRNA species were visualized by Northern blotting using two different probes. The first, 23S-U, anneals partially to the upstream sequence and partially to the

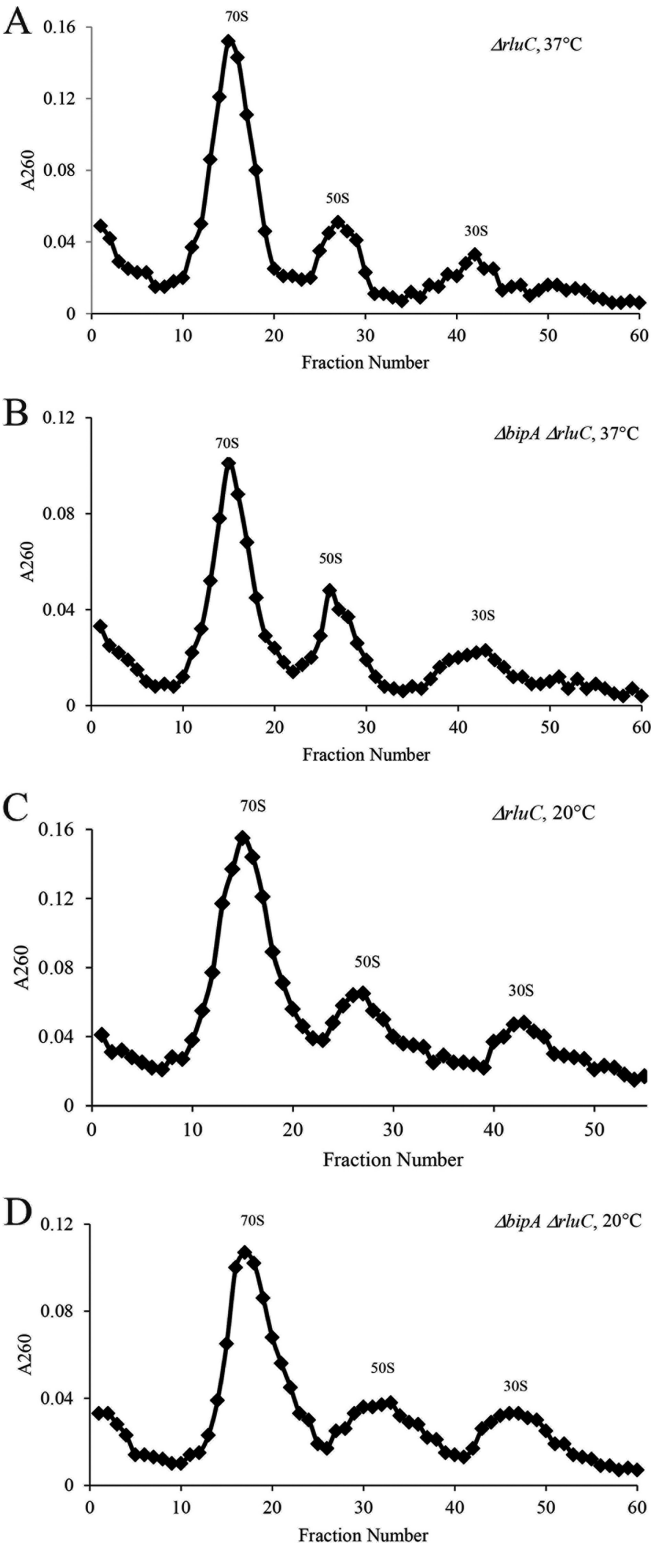


FIG 3 Deletion of *rluC* alleviates the ribosomal subunit distribution defects of a Δ*bipA* mutant. Δ*rluC* (PC33) (A and C) and Δ*bipA* Δ*rluC* (PC34) (B and D) strains were grown either at 37°C (A and B) or at 20°C (C and D). Ribosomal subunits were separated as described in Materials and Methods, with fractions collected from the bottom of the sucrose density gradient to the top. The peaks corresponding to the 30S and 50S ribosomal subunits and 70S ribosomes are indicated. All samples were analyzed from at least three biological replicates; representative examples are shown.

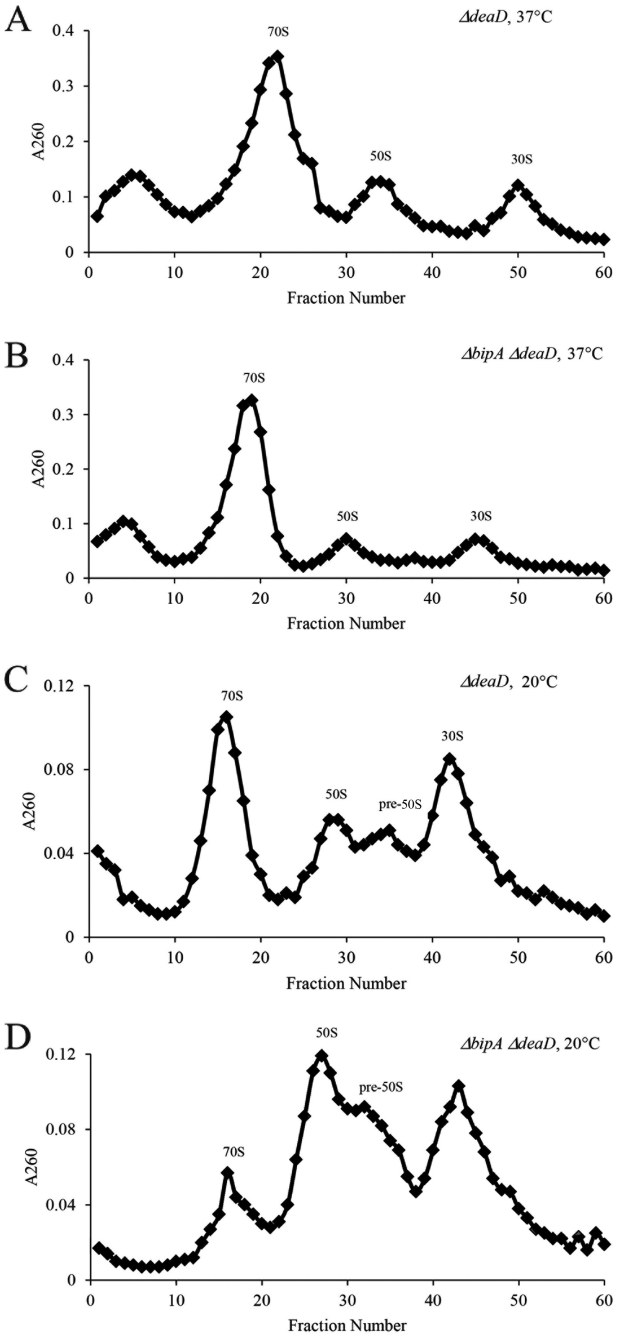
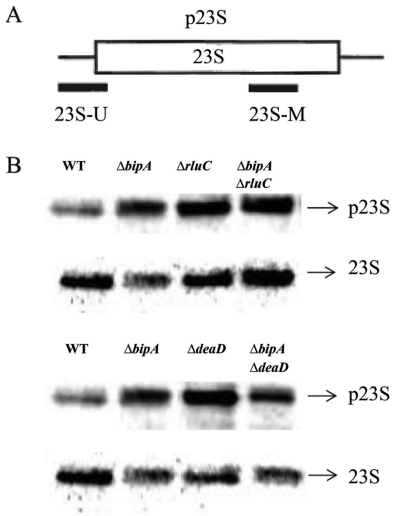


FIG 4 Deletion of *bipA* and *deaD* affects ribosome assembly synergistically. Δ*deaD* (PC95) (A and C) and Δ*bipA* Δ*deaD* (PC96) (B and D) strains were grown either at 37°C (A and B) or at 20°C (C and D). Ribosomal subunits were separated as described in Materials and Methods, with fractions collected from the bottom of the sucrose density gradient to the top. The peaks corresponding to the 30S and 50S ribosomal subunits, 70S ribosomes, and presumed pre-50S particle are indicated. All samples were analyzed from at least three biological replicates; representative examples are shown.



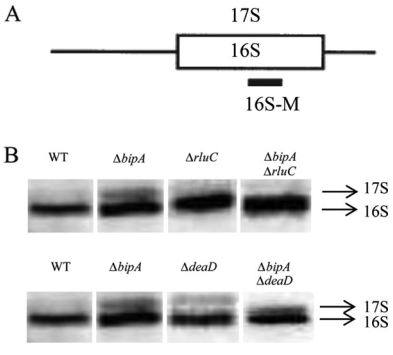
C

Strain	P/(P+M)
WT	0.31
Δ*bipA*	0.52*
Δ*rluC*	0.42*
Δ*bipA* Δ*rluC*	0.35
Δ*deaD*	0.62*
Δ*bipA* Δ*deaD*	0.54*

FIG 5 Analysis of 23S rRNA processing. (A) Schematic representation of the 23S rRNA, depicting the mature upstream and downstream regions along with the annealing sites for the 23S-U and 23S-M probes (19). (B) Northern blot analysis of whole-cell RNA isolated from the wild-type strain (MG1655) and the indicated mutant strains (PC28, PC33, PC34, PC95, and PC96). Precursor (p23S) and mature (23S) rRNA bands are indicated. The gels shown have been spliced for labeling purposes. (C) Band densities were determined by Image J quantification, and the extent of 23S rRNA processing was calculated as the amount of precursor divided by the sum of precursor and mature rRNAs. Data presented are from the image shown; repetition of the experiment resulted in similar values. Asterisks indicate values from mutant strains that are statistically different from the wild-type values with $P < 0.05$.

mature sequence of a 23S rRNA. Under the conditions used, the short region of complementarity between the probe and the mature 23S rRNA is insufficient to maintain the duplex; thus, the probe detects p23S rRNA only (Fig. 5A). The second probe, 23S-M, anneals specifically to mature 23S rRNA and therefore detects both species of rRNA. Therefore, the ratio between the signals from the two probes provides an indicator of the processing of p23S to 23S rRNA, described here as the ratio of the level of precursor rRNA (P) to the sum of the levels of precursor rRNA and mature rRNA (P + M), calculated as P/(P + M).

As observed previously by others, wild-type MG1655 accumulated small amounts of p23S rRNA when grown at 20°C (15) (Fig. 5B). The Δ*bipA* strain also accumulated p23S rRNA at 20°C and did so to a greater extent than the wild-type strain [P/(P + M) ratio, 0.52 versus 0.31]. While deletion of *rluC* resulted in a small amount of p23S accumulation (0.42), the Δ*bipA* Δ*rluC* double mutant restored wild-type levels of p23S (0.35), again demonstrating that deletion of *rluC* alleviates the defects imposed by deletion of *bipA*. The small amount of precursor accumulation in the *rluC* mutant is not surprising since RluC was previously re-



C

Strain	P/(P+M)
WT	0.23
Δ*bipA*	0.30
Δ*rluC*	0.23
Δ*bipA* Δ*rluC*	0.18
Δ*deaD*	0.25
Δ*bipA* Δ*deaD*	0.38*

FIG 6 Analysis of 16S rRNA processing. (A) Schematic representation of the 16S rRNA, depicting the mature upstream and downstream regions along with the annealing site for the 16S-M probe (19). (B) Northern blot analysis of whole-cell RNA isolated from the wild-type strain (MG1655) and the indicated mutant strains (PC28, PC33, PC34, PC95, and PC96). Precursor (17S) and mature (16S) rRNA bands are indicated. The gels shown have been spliced for labeling purposes. (C) Band densities were determined by Quantity One quantification, and the extent of 16S rRNA processing was calculated as the amount of precursor divided by the sum of precursor and mature rRNAs. Data presented are from the image shown; repetition of the experiment resulted in similar values. Asterisks indicate values from mutant strains that are statistically different from the wild-type values with $P < 0.05$.

ported to be associated with a pre-50S ribosomal particle, suggesting a function of RluC in 50S subunit biogenesis (15). The P/(P + M) ratio in the Δ*deaD* mutant (0.62) was higher than that in the wild-type strain or the *bipA* mutant strain (0.52) (Fig. 5B). This observation is consistent with our findings that the cold-sensitive phenotype of a Δ*deaD* mutant is more severe than that of a Δ*bipA* mutant and that the ribosome assembly defects in a Δ*deaD* mutant are more drastic than those in a Δ*bipA* mutant. However, p23S accumulation in the Δ*bipA* Δ*deaD* double mutant was not notably different than that in the individual mutants, even though the ribosomal profile of the double mutant exhibited severe defects. This may reflect a disparity between the effects of mutations on rRNA processing and on ribosome assembly.

16S rRNA results from RNase III cleavage of the 17S rRNA precursor (19). The 17S rRNA is 115 nucleotides longer than the mature 16S rRNA at the 5′ end and 33 nucleotides longer at the 3′ end (Fig. 6A). This difference is sufficient to separate the two species of rRNA on an agarose gel and distinguish them using a single probe corresponding to the mature portion (Fig. 6A).

Again, the degree of processing was determined by calculating the amount of precursor (17S) rRNA divided by the sum of precursor and mature (16S + 17S) rRNAs (Fig. 6B). Although the Δ*bipA* and Δ*deaD* mutants exhibited a slight increase in the P/(P + M) ratio (0.30 and 0.25, respectively) compared to a wild-type strain (0.23), there was no statistical difference between any of the mutant strains and the wild-type strain except for the Δ*bipA* Δ*deaD* strain. The slight processing defect observed in this double mutant may be an indirect effect of the severe impairment of 50S assembly.

To summarize, processing of 23S rRNA was impaired in a Δ*bipA* mutant, supportive of our proposal that BipA is important for ribosome assembly. We suggest that the 23S rRNA processing defect is related to defective biogenesis of the 50S ribosomal subunit.

Deletion of *lepA* does not result in ribosome assembly defects. The extensive homology between LepA and BipA suggested that they might perform similar functions. To determine whether deletion of *lepA* also impairs ribosome assembly, we analyzed ribosome profiles from a Δ*lepA* strain grown at 37°C (Fig. 7B) or at 20°C (Fig. 7D) and compared the results to the wild-type strain results (Fig. 7A and C). Although the 50S peak shows a small shoulder indicative of pre-50S accumulation, the profile perturbation was slight compared to the effect of *bipA* deletion, suggesting that LepA and BipA have distinct functions and that LepA is not a significant factor in ribosome assembly.

DISCUSSION

The process of protein production is elaborate and requires multiple steps and components, making it difficult to determine the role of each factor. Previous work indicated that BipA likely is important in translation, but elucidation of the specific step of the process and the mechanism has remained elusive. Protein domain analysis suggests that BipA is an elongation factor in the EF-G family (3, 4, 7); therefore, it was hypothesized by us as well as others that BipA confers selectivity on the ribosome such that mRNAs are translated preferentially in the presence or absence of BipA (5). However, an alternate hypothesis is that BipA is a ribosome assembly factor that is similar to known assembly factors such as DeaD. In this work, we sought to clarify the role of BipA by examining its effect on ribosome assembly.

The results presented here demonstrate that a Δ*bipA* mutant exhibits three phenotypes associated with defective ribosome assembly: (i) cold sensitivity, (ii) altered ribosome subunit distribution, and (iii) accumulation of precursor rRNA. The cold sensitivity of the Δ*bipA* mutant is similar to that of the Δ*deaD* mutant; each displayed a doubling time about half that of the wild type. The Δ*bipA* Δ*deaD* double mutant exhibited a multiplicative phenotype, with a doubling time one-quarter that of the wild type. Thus, this strain showed the double-mutant phenotype that would be expected when there is no genetic interaction, implying that the two gene products exert their effects independently of one another. These results led us to predict that if BipA is involved in ribosome assembly, it functions at a step distinct from DeaD.

The effect of *bipA* deletion on ribosome assembly was determined by separation of the 30S and 50S subunits from the 70S ribosome and qualitative analysis of the relative amounts of each. The Δ*bipA* mutant strain displayed an aberrant ribosome profile when grown at 20°C, with increased levels of 30S and decreased amounts of 50S relative to the wild type, as well as the accumulation of a presumed pre-50S particle. Additionally, the ratio of 30S and 50S subunits to 70S monosomes was increased, again indicat-

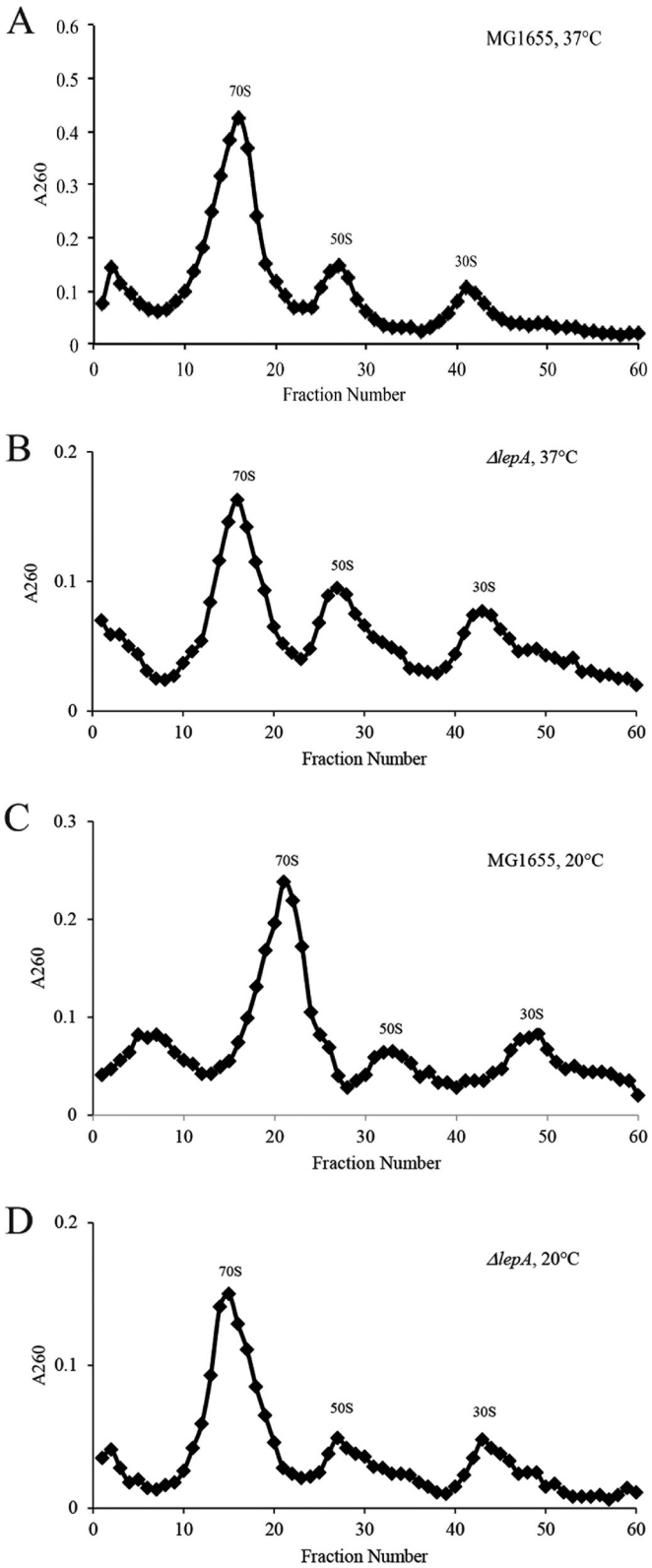


FIG 7 Deletion of *lepA* does not affect ribosome assembly at 20°C. Wild-type (MG1655) (A and C) and Δ*lepA* (PC133) (B and D) strains were grown either at 37°C (A and B) or at 20°C (C and D). Ribosomal subunits were separated as described in Materials and Methods, with fractions collected from the bottom of the sucrose density gradient to the top. The peaks corresponding to the 30S and 50S ribosomal subunits and 70S ribosomes are indicated.

ing a defect in ribosome assembly. The phenotype was similar to that displayed by the Δ*deaD* strain, and the double mutant again showed a combinatorial effect, although the extent could not be calculated. Finally, the accumulation of precursor 23S rRNA in the Δ*bipA* strain substantiated the results of the ribosome profiling, indicating that a defect in ribosome assembly led to accumulation of pre23S rRNA in a manner similar to that of the Δ*deaD* mutant.

The effects of *bipA* deletion in all three measures of defective ribosome assembly strongly indicate that BipA is important for efficient assembly of ribosomes when cells are grown at low temperatures. The effect on 50S subunit production, the presence of a pre-50S particle, and the accumulation of pre23S rRNA all suggest that BipA is primarily involved in the production of the 50S subunit. Although DeaD also is critical for 50S assembly (18), the genetic combinatorial phenotypes suggest that BipA and DeaD function independently of one another and are likely involved in separate assembly pathways.

Our findings also expand upon our previous work that demonstrated a relationship between *rluC* and *bipA* (5). Based on our results showing that deletion of *rluC* suppressed the cold-sensitive defect of Δ*bipA*, we had proposed that under certain conditions, such as low temperature, ribosomes are dependent on BipA. We further suggested that ribosomes unmodified by RluC become BipA independent. Our current data support and extend this hypothesis. Deletion of *rluC* suppressed not only the cold-sensitive phenotype but also the ribosome subunit distribution and rRNA processing defects that were exhibited by the Δ*bipA* strain, indicating that ribosome assembly was BipA independent when the 23S rRNA was unmodified by RluC. However, a *rluC* deletion was unable to suppress the cold sensitivity of the Δ*deaD* strain (data not shown), further suggesting that DeaD and BipA function independently of one another.

While it is clear from this work that deletion of *bipA* results in a ribosome assembly defect, these experiments are insufficient to distinguish whether this is a direct or indirect effect. BipA may be a bona fide assembly factor, with a direct function in ribosome assembly, probably driven by hydrolysis of GTP. It is also possible, however, that BipA is a ribosome translation factor and that deletion of *bipA* results in inefficient expression of a subset of proteins. Indeed, our unpublished results as well as the published results of others (7, 31, 32) support the hypothesis of a role for BipA in expression of some stress response proteins. It may be that deletion of *bipA* results in the inability of cells to respond properly to low-temperature stress and that ribosome assembly is impacted as an indirect result.

BipA and LepA have similar predicted protein structures, with the exception of their unique C-terminal ends (3), and the function of neither has been fully elucidated. One study demonstrated that LepA is a back-translocase, presumably enhancing the fidelity of translation by reversing miscoded posttranslocation ribosome complexes (33). However, more-recent studies have shown that deletion of neither *lepA* nor *bipA* resulted in detectable effects on the accuracy of translation, although both proteins, when overexpressed, inhibited transfer-messenger RNA (tmRNA) tagging of messages lacking stop codons, leading the authors to suggest that both LepA and BipA are translation factors (34). Subsequent studies demonstrated that deletion of *lepA* led to alterations in the frequency of translation initiation for many mRNAs (35). It was proposed that LepA functions during the late stages of ribosome biogenesis. We did not see a defect in ribosome assembly in a Δ*lepA* strain, even when the strain was grown at 20°C (Fig. 7). In

addition, Δ*lepA* strains are not cold sensitive, and the Δ*bipA* Δ*lepA* double mutant does not display any synthetic phenotype. For these reasons, we think that BipA and LepA, despite their structural similarities, are distinct in function and impact different aspects of the translational process.

ACKNOWLEDGMENTS

This work was supported by a seed grant from the Department of Microbiology and Immunology, UNDSMHS (A.M.F.), and a summer doctoral fellowship from the School of Graduate Studies, UND (P.C.).

We are grateful to Sergei Nechaev and members of the Nechaev laboratory for assistance and advice with Northern blots. We are also grateful for the thoughtful discussion with and advice from Thomas Hill, Catherine Brissette, members of the Brissette laboratory, and especially Lisa Burnette.

REFERENCES

1. **Margus T, Remm M, Tenson T.** 2007. Phylogenetic distribution of translational GTPases in bacteria. BMC Genomics **8:**15. http://dx.doi.org/10.1186/1471-2164-8-15.
2. **Wang F, Zhong NQ, Gao P, Wang GL, Wang HY, Xia GX.** 2008. SsTypA1, a chloroplast-specific TypA/BipA-type GTPase from the halophytic plant *Suaeda salsa*, plays a role in oxidative stress tolerance. Plant Cell Environ **31:**982–994. http://dx.doi.org/10.1111/j.1365-3040.2008.01810.x.
3. **Finn RD, Tate J, Mistry J, Coggill PC, Sammut SJ, Hotz HR, Ceric G, Forslund K, Eddy SR, Sonnhammer EL, Bateman A.** 2008. The Pfam protein families database. Nucleic Acids Res **36**(Database issue):D281–D288. http://dx.doi.org/10.1093/nar/gkm960.
4. **Qi SY, Li Y, Szyroki A, Giles IG, Moir A, O'Connor CD.** 1995. *Salmonella typhimurium* responses to a bactericidal protein from human neutrophils. Mol Microbiol **17:**523–531. http://dx.doi.org/10.1111/j.1365-2958.1995.mmi_17030523.x.
5. **Krishnan K, Flower AM.** 2008. Suppression of Delta*bipA* phenotypes in *Escherichia coli* by abolishment of pseudouridylation at specific sites on the 23S rRNA. J Bacteriol **190:**7675–7683. http://dx.doi.org/10.1128/JB.00835-08.
6. **Owens RM, Pritchard G, Skipp P, Hodey M, Connell SR, Nierhaus KH, O'Connor CD.** 2004. A dedicated translation factor controls the synthesis of the global regulator Fis. EMBO J **23:**3375–3385. http://dx.doi.org/10.1038/sj.emboj.7600343.
7. **deLivron MA, Robinson VL.** 2008. *Salmonella enterica serovar Typhimurium* BipA exhibits two distinct ribosome binding modes. J Bacteriol **190:**5944–5952. http://dx.doi.org/10.1128/JB.00763-08.
8. **Pfennig PL, Flower AM.** 2001. BipA is required for growth of *Escherichia coli* K12 at low temperature. Mol Genet Genomics **266:**313–317. http://dx.doi.org/10.1007/s004380100559.
9. **Farris M, Grant A, Richardson TB, O'Connor CD.** 1998. BipA: a tyrosine-phosphorylated GTPase that mediates interactions between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells. Mol Microbiol **28:**265–279. http://dx.doi.org/10.1046/j.1365-2958.1998.00793.x.
10. **Rowe S, Hodson N, Griffiths G, Roberts IS.** 2000. Regulation of the *Escherichia coli* K5 capsule gene cluster: evidence for the roles of H-NS, BipA, and integration host factor in regulation of group 2 capsule gene clusters in pathogenic E. coli. J Bacteriol **182:**2741–2745. http://dx.doi.org/10.1128/JB.182.10.2741-2745.2000.
11. **Duo M, Hou S, Ren D.** 2008. Identifying *Escherichia coli* genes involved in intrinsic multidrug resistance. Appl Microbiol Biotechnol **81:**731–741. http://dx.doi.org/10.1007/s00253-008-1709-6.
12. **Grant AJ, Farris M, Alefounder P, Williams PH, Woodward MJ, O'Connor CD.** 2003. Co-ordination of pathogenicity island expression by the BipA GTPase in enteropathogenic *Escherichia coli* (EPEC). Mol Microbiol **48:**507–521. http://dx.doi.org/10.1046/j.1365-2958.2003.t01-1-03447.x.
13. **Bharat A, Brown ED.** 2014. Phenotypic investigations of the depletion of EngA in *Escherichia coli* are consistent with a role in ribosome biogenesis. FEMS Microbiol Lett **353:**26–32. http://dx.doi.org/10.1111/1574-6968.12403.
14. **Inoue K, Alsina J, Chen J, Inouye M.** 2003. Suppression of defective ribosome assembly in a *rbfA* deletion mutant by overexpression of Era, an essential GTPase in *Escherichia coli*. Mol Microbiol **48:**1005–1016. http://dx.doi.org/10.1046/j.1365-2958.2003.03475.x.
15. **Jiang M, Datta K, Walker A, Strahler J, Bagamasbad P, Andrews PC, Maddock JR.** 2006. The *Escherichia coli* GTPase CgtAE is involved in late steps of large ribosome assembly. J Bacteriol **188:**6757–6770. http://dx.doi.org/10.1128/JB.00444-06.
16. **Verstraeten N, Fauvart M, Versees W, Michiels J.** 2011. The universally conserved prokaryotic GTPases. Microbiol Mol Biol Rev **75:**507–542. http://dx.doi.org/10.1128/MMBR.00009-11.
17. **Britton RA.** 2009. Role of GTPases in bacterial ribosome assembly. Annu Rev Microbiol **63:**155–176. http://dx.doi.org/10.1146/annurev.micro.091208.073225.
18. **Charollais J, Dreyfus M, Iost I.** 2004. CsdA, a cold-shock RNA helicase from *Escherichia coli*, is involved in the biogenesis of 50S ribosomal subunit. Nucleic Acids Res **32:**2751–2759. http://dx.doi.org/10.1093/nar/gkh603.
19. **Charollais J, Pflieger D, Vinh J, Dreyfus M, Iost I.** 2003. The DEAD-box RNA helicase SrmB is involved in the assembly of 50S ribosomal subunits in *Escherichia coli*. Mol Microbiol **48:**1253–1265. http://dx.doi.org/10.1046/j.1365-2958.2003.03513.x.
20. **Jain C.** 2008. The *E. coli* RhlE RNA helicase regulates the function of related RNA helicases during ribosome assembly. RNA **14:**381–389. http://dx.doi.org/10.1261/rna.80030.
21. **Campbell TL, Brown ED.** 2008. Genetic interaction screens with ordered overexpression and deletion clone sets implicate the *Escherichia coli* GTPase YjeQ in late ribosome biogenesis. J Bacteriol **190:**2537–2545. http://dx.doi.org/10.1128/JB.01744-07.
22. **Conrad J, Sun D, Englund N, Ofengand J.** 1998. The *rluC* gene of *Escherichia coli* codes for a pseudouridine synthase that is solely responsible for synthesis of pseudouridine at positions 955, 2504, and 2580 in 23 S ribosomal RNA. J Biol Chem **273:**18562–18566. http://dx.doi.org/10.1074/jbc.273.29.18562.
23. **Decatur WA, Fournier MJ.** 2002. rRNA modifications and ribosome function. Trends Biochem Sci **27:**344–351. http://dx.doi.org/10.1016/S0968-0004(02)02109-6.
24. **Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko K, Tomita M, Wanner B, Mori H.** 2006. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol **2:**2006.0008. http://dx.doi.org/10.1038/msb4100050.
25. **Guyer MS, Reed RR, Steitz JA, Low KB.** 1981. Identification of a sex-factor-affinity site in *E. coli* as γδ. Cold Spring Harbor Symp Quant Biol **45**(Pt 1):135–140. http://dx.doi.org/10.1101/SQB.1981.045.01.022.
26. **Kristensen CS, Eberl L, Sanchez-Romero JM, Givskov M, Molin S, Lorenzo VD.** 1995. Site-specific deletions of chromosomally located DNA segments with the multimer resolution system of broad-host-range plasmid RP4. J Bacteriol **177:**52–58.
27. **Bernhardt TG, deBoer PAJ.** 2004. Screening for synthetic lethal mutants in *Escherichia coli* and identification of EnvC (YibP) as a periplasmic septal ring factor with murein hydrolase activity. Mol Microbiol **52:**1255–1269. http://dx.doi.org/10.1111/j.1365-2958.2004.04063.x.
28. **Silhavy TJ, Berman ML, Enquist LW.** 1984. Experiments with gene fusions, p 215-300Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
29. **Baryshnikova A, Costanzo M, Myers CL, Andrews B, Boone C.** 2013. Genetic interaction networks: toward an understanding of heritability. Annu Rev Genomics Hum Genet **14:**111–133. http://dx.doi.org/10.1146/annurev-genom-082509-141730.
30. **Mani R, St Onge RP, Hartman JL, Giaever G, Roth FP.** 2008. Defining genetic interaction. Proc Natl Acad Sci U S A **105:**3461–3466. http://dx.doi.org/10.1073/pnas.0712255105.
31. **Freestone P, Trinei M, Clarke SC, Nyström T, Norris V.** 1998. Tyrosine phosphorylation in *Escherichia coli*. J Mol Biol **279:**1045–1051.
32. **Kiss E, Huguet T, Poinsot V, Batut J.** 2004. The *typA* gene is required for stress adaptation as well as for symbiosis of *Sinorhizobium meliloti* 1021 with certain *Medicago truncatula* lines. Mol Plant Microbe Interact **17:**235–244. http://dx.doi.org/10.1094/MPMI.2004.17.3.235.
33. **Qin Y, Polacek N, Vesper O, Staub E, Einfeldt E, Wilson DN, Nierhaus KH.** 2006. The highly conserved LepA is a ribosomal elongation factor that back-translocates the ribosome. Cell **127:**721–733. http://dx.doi.org/10.1016/j.cell.2006.09.037.
34. **Shoji S, Janssen BD, Hayes CS, Fredrick K.** 2010. Translation factor LepA contributes to tellurite resistance in *Escherichia coli* but plays no apparent role in the fidelity of protein synthesis. Biochimie **92:**157–163.
35. **Balakrishnan R, Oman K, Shoji S, Bundschuh R, Fredrick K.** 6 November 2014. The conserved GTPase LepA contributes mainly to translation initiation in *Escherichia coli*. Nucleic Acids Res http://dx.doi.org/10.1093/nar/gku1098.