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CspA, the Major Cold Shock Protein of *Escherichia coli*, Negatively Regulates Its Own Gene Expression

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When the gene for CspA, the major cold shock protein of *Escherichia coli*, was disrupted by a novel positive/negative selection method, the Δ*cspA* cells did not show any discernible growth defect at either 37 or 15°C. By two-dimensional gel electrophoresis, total protein synthesis was analyzed after temperature downshift in the Δ*cspA* strain. The production of the CspA homologs CspB and CspG increased, and the duration of their expression was prolonged, suggesting that both CspB and CspG compensate for the function of CspA in the absence of CspA during cold shock adaptation. Interestingly, the production of the 159-base 5′-untranslated region (5′-UTR) of *cspA* from the chromosomal *cspA::cat* gene, detected by primer extension, failed to be repressed after cold shock. When an independent system to produce CspA was added to the Δ*cspA* strain, the 5′-UTR production for the *cspA::cat* gene was significantly reduced compared to that of the Δ*cspA* strain. By examining the expression of translationally fused *cspA* and *cspB* genes to *lacZ* in the Δ*cspA* strain, it was found that *cspA* is more strongly regulated by CspA than *cspB* is. We showed that the increased expression of the 5′-UTR of the *cspA* mRNA in the Δ*cspA* strain occurred mainly at the level of transcription and, to a certain extent, at the level of mRNA stabilization. The mRNA stabilization in the Δ*cspA* strain was observed for other mRNAs, supporting the notion that CspA functions as an mRNA chaperone to destabilize secondary structures in mRNAs.

When *Escherichia coli* cell cultures are transferred from 37°C to 15°C, the cells exhibit an adaptive response to the temperature downshift which includes induction of a set of proteins defined as cold shock proteins (15, 16). The cold shock response occurs during a lag period of cell growth called the "acclimation phase" immediately after temperature downshift. The synthesis of cold shock proteins becomes repressed once cells resume growth after the acclimation phase. A number of cold shock proteins, such as RbfA (18), CsdA (17), and CspA (14), have been shown to play important roles in protein synthesis at low temperature.

CspA, the major cold shock protein of *E. coli*, is dramatically induced upon cold shock, comprising more than 10% of the total protein synthesis (7). CspA is composed of 70 amino acid residues and shares 43% identity with the cold shock domain of the Y-box protein family in eukaryotes, which has been shown to be involved in gene regulation and mRNA masking (35). Interestingly, *E. coli* contains a large CspA family consisting of eight homologous proteins from CspB to CspH (7, 9, 19, 23, 25, 37). Among them, only CspA, CspB, and CspG have been shown to be cold shock inducible (7, 19, 23).

The three-dimensional structure of CspA has been determined to have a β-barrel structure composed of five antiparallel β-strands containing two RNA binding motifs, RNP1 and RNP2, on the β2 and the β3 strands, respectively (24, 30). CspA binds to the unusually long 5′-untranslated region (5′-UTR) of its own mRNA to destabilize secondary structures of the mRNA (14). This indicates that CspA functions as an RNA chaperone to prevent the formation of stable secondary structures in RNA molecules at low temperature and thus facilitates translation of cellular mRNAs at low temperatures (16).

During the acclimation phase, the *cspA* expression is tightly regulated, and its production is transient. This regulation is controlled at the level of mRNA stability as well as at the levels of transcription and translation (1, 5, 6, 22, 32). Even if a heterologous promoter was used for the expression of *cspA*, CspA was still induced at low temperature, indicating that cold shock induction of *cspA* is not dependent on its own promoter (1, 5).

Interestingly, the overproduction of the 5′-UTR of the *cspA* mRNA at 15°C caused the synthesis of not only CspA but also CspB and CspG to be no longer transient and delayed the induction of the cold shock adaptive response (12). However the coproduction of CspA together with its 5′-UTR suppressed the effects of overproduction of the 5′-UTR described above (12). In addition, deletion analysis of the *cspA* 5′-UTR revealed that the unusually long 5′-UTR is responsible for its extreme instability at 37°C as well as for the transient expression of *cspA* during the acclimation phase (22). Taken together, these findings suggest that the 5′-UTR of the *cspA* mRNA plays a crucial role in the expression of *cspA* and that CspA is involved in the regulation of its own gene expression.

At this point, the construction of a Δ*cspA* strain is valuable not only to further examine the regulation mechanism of the *cspA* expression, but also to study its role in cold shock. Here, we report the construction of WB002, a Δ*cspA* strain, by a novel counterselection method (33). It was found that when the *cspA* deletion strain was cold shocked, the production of the CspA homologs CspB and CspG was increased and prolonged, but no growth defect was observed. This suggests that expression of *cspB* and *cspG* increases to compensate for the absence of CspA. Since in the Δ*cspA* mutant only the open reading frame was deleted, transcription of *cspA* can still be monitored by detecting the production of the 5′-UTR of *cspA*. It was found that in the Δ*cspA* strain not only was *cspA* tran-

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scription stimulated, but also the half-life of the 5′-UTR of the *cspA* mRNA became longer than that in the wild-type strain. Furthermore the mRNAs for CspB and CspG became stabilized at low temperature in the deletion strain compared to those in the wild-type strain. Here, we demonstrate genetic evidence that CspA negatively regulates its own gene expression at the levels of transcription and mRNA stability.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* JM83 was used as a host for plasmid constructions (38). JC7623 (*galK2 rpsL31 recB21 recC22 sbcB15*) (20), an *E. coli* K-12 strain, was used to construct WB002, a deletion strain of *cspA*. *E. coli* AR137 (MC4100 but *pcnB80*) (10) was used in the assay for β-galactosidase activity. P1*vir*-mediated transduction was carried out to make WB012, the Δ*cspA* mutant in AR137, as described previously (21).

pIEkgdA^{ts} was constructed as follows: the 2.2-kb *Hin*dIII fragment encompassing the region downstream of the *cspA* coding region was cloned from pJJG101 (8), and cloned into the *Hin*dIII site of pUC19 to generate pADM1. The 0.6-kb fragment encompassing the region upstream of the *cspA* coding region (bases −454 to +166) (7) was obtained by PCR with pJJG101 as template with the following primers: 6041 (5′-CAGGTACCGCTTCGATGCAATTC-3′) (bases −454 to −440; the sequence underlined represents a *Kpn*I site added to the PCR fragment) and 6042 (5′-CTCTGCAGGATCCGACATAGTGTATTA C-3′) (bases +152 to +166; the sequence underlined represents a *Pst*I site added to the PCR fragment). The PCR product was digested with *Kpn*I and *Pst*I and cloned into pADM1, generating pADM2. The chloramphenicol resistance gene (*cat*) was used as a marker for gene disruption and was inserted between the 5′ upstream region and the 3′ region of pADM2 at the unique *Pst*I site of pADM2, yielding pADM3. Subsequently, the 5.6-kb fragment containing the *cspA* gene disrupted with the *cat* gene was generated by digestion with *Eco*RI and cloned into the *Eco*RI site of pIEkg^{ts}. pIEkg^{ts} was constructed as follows. A 3-kb fragment containing both Km^r and *galK* under the Km^r promoter was released by *Bam*HI and *Nde*I digestion of pK01Km (33). The resultant fragment was treated with the Klenow enzyme and subcloned into the *Sma*I site of pUC19SmaI, creating pUC7kg. The Km^r plus *galK* fragment was again released by digestion of pUC7kg with *Pst*I and subcloned into the *Pst*I site of pIE^{ts}, creating pIEkg^{ts}.

Plasmid pMM016 contained the *cspA* upstream region and the first 13 codons of *cspA* fused translationally to *lacZ* on the pRS414 vector, as described previously (22). Plasmid pCspB-LacZ contained the *cspB* upstream region and the first 13 codons of *cspB* fused translationally to *lacZ* on the pRS414 vector, as described previously (19).

Integration of the disrupted *cspA* gene into the chromosome. Gene disruption was carried out by using a novel positive and negative selection method developed in our laboratory. In short, an *E. coli* fragment with a *cspA::cat* gene was inserted into pIEkgdA^{ts} (Km^r) carrying the *E. coli galK*$^+$ gene (28). Note that the vector plasmid replicates only at the permissive temperature (30°C) but not at nonpermissive temperatures because of the temperature-sensitive origin of replication. Transformation of JC7623 (*galK* mutant) cells with this plasmid at 42°C generated 2-dG^s Cm^r Km^r cointegrates. These cells were then grown at 37°C in L broth medium containing 1% 2-Deoxygalactose (2-dG) and chloramphenicol (30 μg/ml) for 6 h and then were diluted and plated on the L agar plate containing 1% 2-dG and chloramphenicol (30 μg/ml). After an overnight incubation at 37°C, cells isolated from a single colony were tested for kanamycin sensitivity to generate strain WB002, which was 2-dG^r Km^s Cm^r. The strain was further characterized by Southern blot analysis.

Southern blot analysis. Chromosomal DNA was isolated from JC7623 and WB002. *Pst*I (or *Pvu*II)-digested chromosomal DNA was separated on a 1% agarose gel and transferred to a nitrocellulose membrane. Prehybridization was carried out at 42°C for 2 h. A 5′ upstream fragment (−454 to +166) of *cspA* was labeled with [α-^{32}P]dCTP through nick translation. The hybridization was carried out as described by Sambrook et al. (29).

Protein labeling experiment. The pulse-labeling experiments were carried out as described previously (11). Extracts were prepared from JC7623, the parent strain, and WB002, the Δ*cspA* strain, and processed by two-dimensional gel electrophoresis as described previously (34). Spot intensities on the autoradiograms were quantified with a Bio-Rad imaging densitometer (model GS-670), and mean values were calculated from at least two individual experiments. EF-Tu was used as a standard for the quantification (18).

Primer extensions and analysis for mRNA stability. Total RNA was isolated immediately before the temperature downshift and at various time points after the temperature downshift. At each time point, RNA was extracted, and 4 μg of RNA was used in each primer extension experiment as described previously (2, 11). All of the primer extension assays were carried out at least two times. Primer 4592 (5′-GTGCACTACGAGGGGTATCA-3′) corresponds to the sequence from base +82 to +63, with the *cspA* transcription initiation defined as +1 (7). Primer 6617 (5′-TCAAAGAAGGAATAA-3′) corresponds to the sequence from base +85 to +71 of the *cspB* transcripts (4). Primer 7388 (5′-TGGCAAC CTTAGAGC-3′) corresponds to the sequence from +220 to +206 of the *cspG* transcripts (23). The primer was labeled at the 5′ end by [γ-^{32}P]ATP with T4 kinase, and primer extension assays were carried out as described previously (11).

Bacterial strains JC7623 and WB002 were grown at 37°C to an optical density at 600 nm of 0.6. Rifampin was then added to a final concentration of 200 μg/ml. Samples taken at different time points were subjected to primer extension analysis. The band intensities on the autoradiograms were quantified with a Bio-Rad imaging densitometer (model GS-670). The mean values of the half-lives of mRNA were calculated based on the results from at least two individual experiments.

Assay for β-galactosidase activity. Strains AR137 and WB012 were transformed with pMM016 (22) and pCspB-LacZ (19), respectively. Cells were grown in Luria-Bertani medium or M9 medium to reach the mid-log phase and sampled before and after cold shock for the measurement of β-galactosidase activities as described previously (21).

RESULTS

Construction of WB002, a Δ*cspA* strain. The chromosomal *cspA* gene was disrupted by homologous recombination by a positive/negative selection method developed in this laboratory as described in Materials and Methods. pIEkgdA^{ts} (*cspA*::*cat*), which carries a temperature-sensitive origin of replication (Fig. 1A), was integrated into the *E. coli* chromosome by using kanamycin and chloramphenicol for positive screening. This was carried out by transformation of JC7623, the parent strain, with the plasmid at 42°C. 2-dG was then used for negative screening to isolate disruptants (2-dG^r Km^s Cm^r) from cointegrates (2-dG^s Km^r Cm^r), because 2-dG, an unmetabolizable analog of galactose, is phosphorylated by galactokinase exerting cell growth toxicity (28). The integrated Km^r-*galK* fragment should be looped out through homologous recombination so that cells can survive in the presence of 2-dG (Fig. 1B), resulting in *cspA* deletion strains which are 2-dG^r Km^s and Cm^r.

In order to confirm the chromosomal deletion of *cspA*, Southern blot analyses were carried out as shown in Fig. 1C. Because *Pst*I sites are located on both ends of the chloramphenicol resistance gene (Fig. 1B), the chromosomal DNAs of the mutant and the parent strain were digested with *Pst*I. With the 5′ upstream region (−454 to +166) of *cspA* used as a probe, a 1.5-kb fragment was detected from the chromosomal digest of WB002, one of the deletion mutant strains (lane 2, Fig. 1C), while an 11-kb fragment was detected from the wild-type strain (lane 1, Fig. 1C). In addition, the *Pvu*II digestion showed the size difference of the restriction fragments (1.8 kb in the wild-type cells and 4.6 kb in the mutant cells, as shown in lanes 3 and 4 of Fig. 1C, respectively; also see Fig. 1B), since the *Pvu*II site within the *cspA* coding region was lost as a result of replacement of the *cspA* coding region with the *cat* gene. These results confirmed that the *cspA* gene was disrupted in strain WB002.

Characterization of the Δ*cspA* strain. Strain WB002 did not show any discernible growth defect at 37 and 15°C compared with the parent strain, JC7623 (data not shown). Subsequently, by two-dimensional gel electrophoresis, the patterns of total protein synthesis in the Δ*cspA* strain were analyzed at least twice at each time point. At 37°C, CspA was hardly detectable in the wild-type strain (Fig. 2A). However, at 30 min after the temperature downshift from 37°C to 15°C, CspA (protein 1) was dramatically induced with the concomitant production of CspB and CspG (Fig. 2B). In contrast, in the Δ*cspA* strain only, CspB (protein 3) and CspG (protein 2) were produced, and their production was increased 1.3 ± 0.1-fold compared to that of the wild-type strain (Fig. 2F).

At 3 h after cold shock, in the wild-type strain there was little production of CspB and CspG, but there was still detectable production of CspA (7, 15). In contrast, in the Δ*cspA* strain, CspB and CspG were still being produced at the same level as the amount of CspA produced in the wild-type strain at the same time point (Fig. 2G). However, 16 h after cold shock,

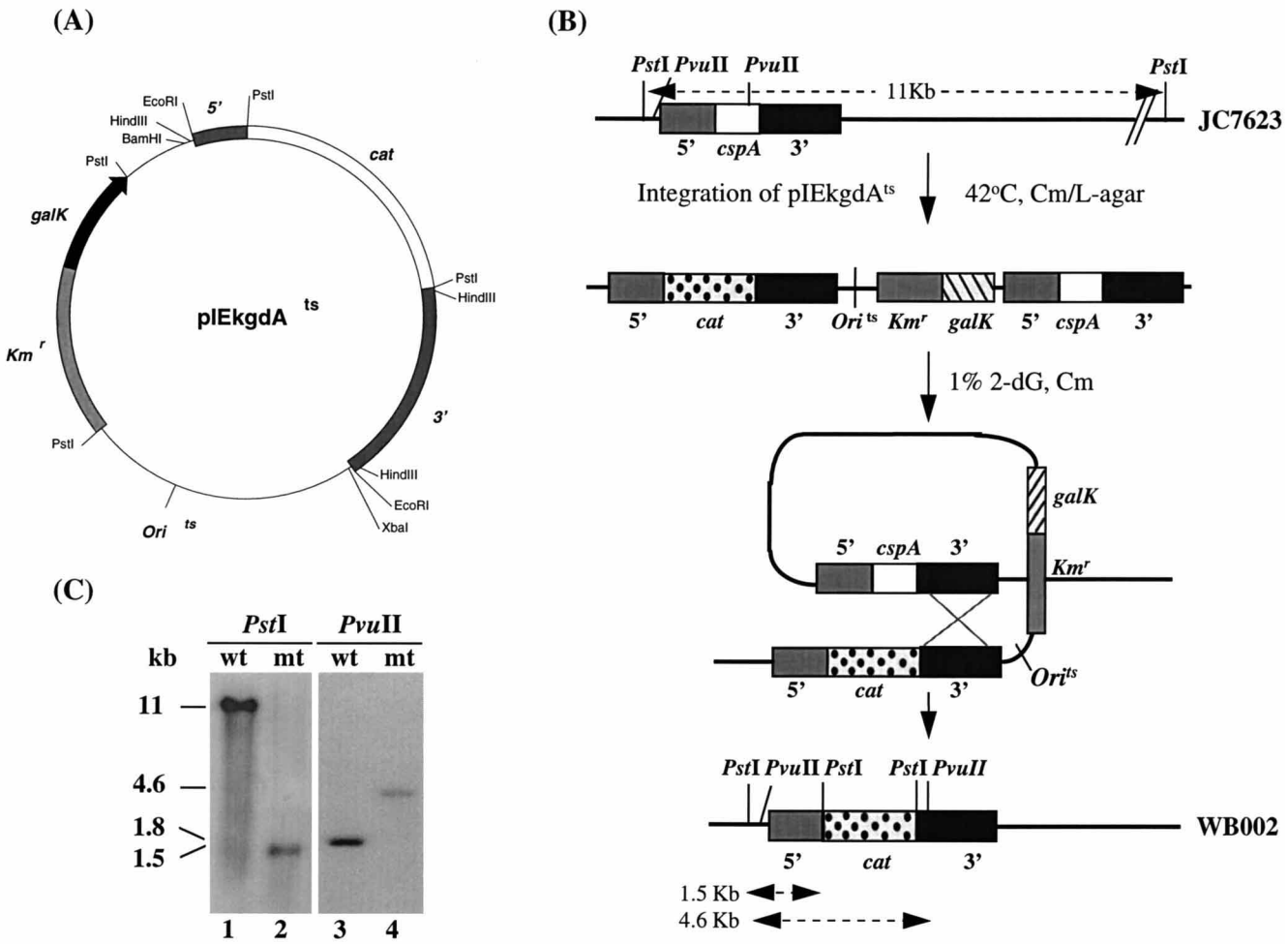

FIG. 1. (A) Plasmid map of pIEkgdA^{ts}, the plasmid used to construct WB002, the Δ*cspA* strain. The PCR product and the restriction fragment were used for the 5′ upstream fragment of *cspA* and the 3′ downstream fragment of *cspA*, respectively. The *cat* gene was replaced with the *cspA* coding region. The *galK* gene, which is in an operon driven by the control of the Km^r gene promoter, was used as the counter selection marker. The temperature-sensitive origin of replication makes cells sustain this plasmid only at the permissive temperature. The construction of pIEkgdA^{ts} is described in Materials and Methods. (B) Construction of WB002, the Δ*cspA* strain. JC7623 (*galK* mutant) was transformed with pIEkgdA^{ts} at the nonpermissive temperature and plated on L agar plates containing chloramphenicol (30 μg/ml). The resulting cointegrates (Cm^r Km^r 2-dG^s) were further grown in L medium containing chloramphenicol (30 μg/ml) and 2-dG (1%) to force the second crossover, resulting in the chromosomal deletion of the *cspA* gene, such as that in WB002 (Cm^r Km^s 2-dG^r). The position of possible crossover in the 3′ downstream region of *cspA* is indicated on the assumption that the integration of pIEkgdA^{ts} occurred through the homologous recombination in the 5′ upstream region of *cspA*. The *Pst*I and *Pvu*II sites are indicated for the Southern blot analysis in panel C. (C) Southern blot analysis to confirm the chromosomal deletion of the *cspA* gene. The chromosomal DNAs isolated from the wild-type strain (JC7623) and the Δ*cspA* strain (WB002) were digested with *Pst*I and *Pvu*II, respectively, separated on a 1% agarose gel, and hybridized to a probe, the 5′ upstream fragment of *cspA*, which comprises the region from −454 to +166, with the *cspA* transcription initiation site defined as +1 (shown in Fig. 1B). Sizes are shown on the left in kilobases.

when cells were adapted to the new growth condition, both CspB and CspG were no longer detectable in the Δ*cspA* strain (Fig. 2H), demonstrating that repression of *cspB* and *cspG* in the Δ*cspA* strain can occur in a delayed fashion.

When the culture temperature was downshifted from 37°C to 30°C, the production of CspA but not that of CspB or CspG was induced. *cspA* expression and *cspB* and *cspG* expression are known to be differentially regulated in response to temperature (4). Interestingly, in the Δ*cspA* strain, CspB and CspG became detectable at 30°C at a level similar to that of the CspA produced in the wild-type strain (data not shown).

In addition, in the Δ*cspA* strain, the production of CspE (protein 4) increased more than twofold. CspE is a homolog of CspA that is constitutively expressed at 37°C (unpublished results). Interestingly, the *cspA* expression at both 37 and 15°C was derepressed in a Δ*cspE* strain (unpublished results), suggesting that CspA and CspE may mutually regulate the gene expression of one another.

Expression of 5′-UTR of the *cspA* mRNA in WB002. It has been suggested that CspA either directly or indirectly regulates its own gene expression (12, 13). Thus, we attempted to examine whether the Δ*cspA* mutation affects the expression of its own gene. Note that in the Δ*cspA* strain, only the coding region of *cspA* was deleted. Therefore, the *cspA* transcription in the deletion strain can be still examined by detection of the 5′-UTR of the *cspA* mRNA in the primer extension experiments.

At 37°C, the expression of the 5′-UTR was undetectable for both the Δ*cspA* strain and the wild-type strain (Fig. 3A) because of its extreme instability. On the other hand, the *cspA* mRNA becomes stabilized immediately after cold shock (1, 5–7). Interestingly, compared with the wild-type strain, the production of the 5′-UTR in the Δ*cspA* strain at 30 min after

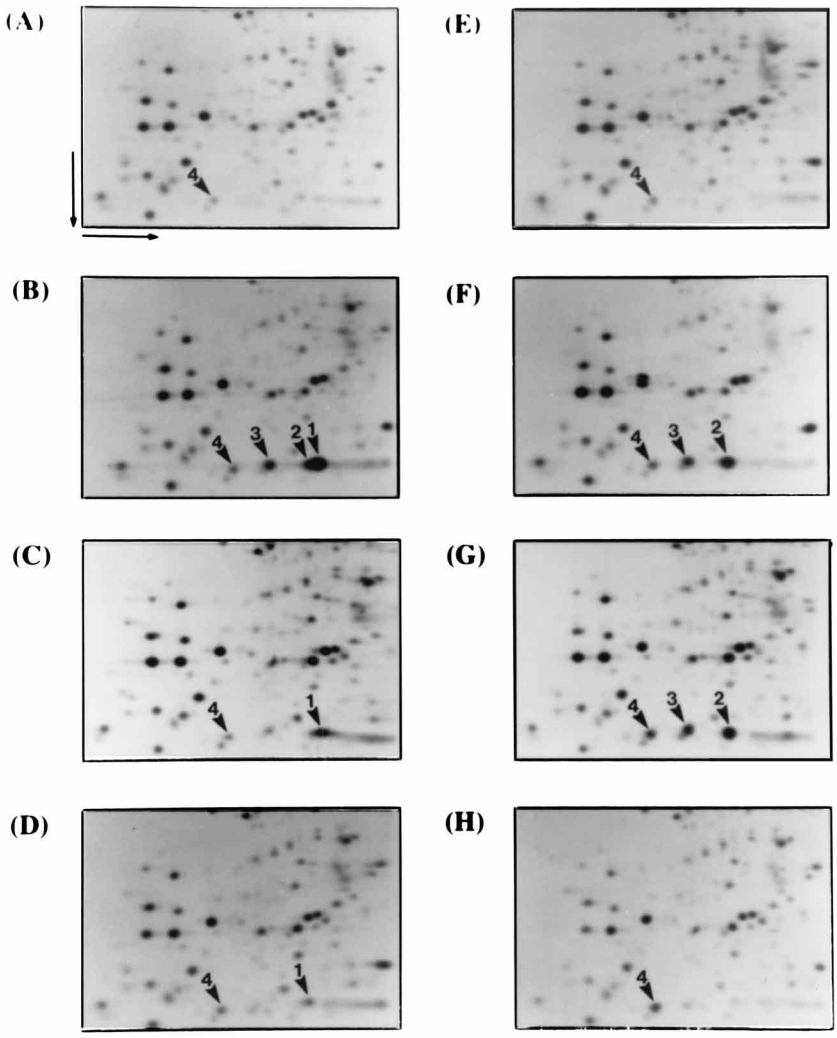

FIG. 2. Prolonged production of CspA homologs CspB and CspG in the Δ*cspA* strain. Pulse-labeling experiments and two-dimensional gel electrophoresis were carried out to compare the total protein synthesis of the Δ*cspA* strain with that of the wild-type strain. Cultures of JC7623 (*cspA*$^+$) (A to D) and WB002 (Δ*cspA*) (E to H) were labeled at an optical density at 600 nm of 0.5 at 37°C (A and E) and then were shifted to 15°C. Cells were labeled at 0.5 (B and F), 3 (C and G), and 16 (D and H) h after temperature downshift. In panel A, the horizontal arrow points from basic pH to acidic pH of the isoelectric focusing gel, and the vertical arrow shows the direction of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Arrows 1 to 4 correspond to CspA, CspG, CspB, and CspE, respectively.

cold shock was much higher (an approximately threefold increase), as shown in Fig. 3A. Three hours after cold shock, although the level of the 5′-UTR in strain WB002 was reduced by more than 50%, its production was still much higher (approximately 4.5-fold) than that in the wild-type strain (compare lane 6 with lane 5). This high level of the 5′-UTR in the Δ*cspA* strain was maintained even at 7 h after temperature downshift, when cells were already well adapted to low temperature (lane 8). These results indicate that the disruption of *cspA* causes derepression of the production of the 5′-UTR of the *cspA* mRNA at low temperature.

To examine whether the increased level of the *cspA* mRNA in the Δ*cspA* strain is specific for *cspA* or not, the *cspB* expression was measured by primer extension with the same RNA preparation used for Fig. 3A. At 30 min after cold shock, the level of *cspB* mRNA in the Δ*cspA* strain slightly increases to approximately 1.4-fold over that of the parent strain (compare lane 4 with lane 3 in Fig. 3B). The level of the *cspB* mRNA appears to be a little higher at 3 and 7 h after cold shock in the Δ*cspA* strain than that in the wild-type strain (compare lanes 6 and 8 with lanes 5 and 7, respectively, in Fig. 3B). This result agrees with the level of CspB production 30 min after cold shock in WB002, where its production is slightly increased (1.3 ± 0.1-fold increase [Fig. 2F]) compared with that of the wild-type strain (Fig. 2B). These results indicate that CspA more specifically regulates its own gene and the *cspB* gene at a much lower level.

Repression of the 5′-UTR of the *cspA* mRNA by reintroduction of CspA in the Δ*cspA* strain. The results shown above suggest that *cspA* expression is negatively regulated by its own product, CspA. In order to further prove this notion, we reintroduced a CspA production system into the Δ*cspA* strain by inserting a *cspA* mutant gene having a 22-base deletion (+3 to +24) in the cold-box region (12) into the *lpp-28* gene of strain WB002. In this strain, termed WB003, the production of CspA is comparable to that of CspA in the wild-type strain 30 min after cold shock. However, CspA production was poorly repressed at the end of the acclimation phase, and the level of CspA was maintained at a reasonably high level after the acclimation phase (unpublished results). The construction of WB003 will be described elsewhere.

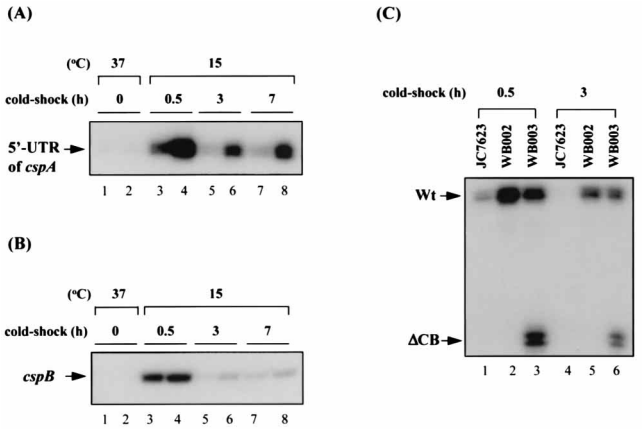

FIG. 3. Expression pattern of the 5′-UTR of *cspA* and *cspB* mRNA. (A) Derepression of the 5′-UTR of the *cspA* gene in the Δ*cspA* strain. Total RNAs were prepared from JC7623 cells (lanes 1, 3, 5, and 7 [wild type]) and from WB002 (lanes 2, 4, 6, and 8 [Δ*cspA*]) at 37°C and after the temperature downshift to 15°C at different time points. Reverse transcription was carried out with a primer corresponding to the sequence from base +82 to base +63 of *cspA*, with the transcription initiation site defined as +1. (B) Primer extension analysis to estimate the expression of *cspB*. The same RNA preparation as that in Fig. 3A was used and analyzed. Reverse transcription was carried out with a primer corresponding to the sequence from base +85 to base +71 of *cspB*. (C) Reintroduction of CspA in the Δ*cspA* strain showed repression of the 5′-UTR of *cspA*. WB003 was constructed through an integration of a conditional plasmid (temperature sensitivity), which carries the *cspA* gene with the deletion of the cold box (+5 to +25) in the 5′-UTR of *cspA*, into the *Ipp-28* gene of the chromosome of WB002. Because of the deletion of the cold box, RNA from WB003 showed two reverse transcription products: the band marked Wt is from the original *cspA* gene, and the band marked ΔCB is from the newly integrated *cspA* gene with the Δcold box.

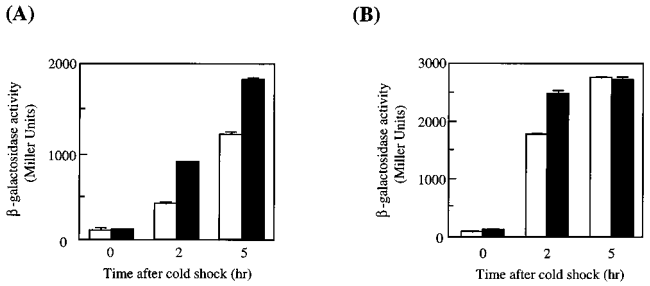

FIG. 4. Effects of the *cspA* deletion on β-galactosidase activity of the translational *lacZ* fusion constructs of *cspA* and *cspB*. (A) Strain AR137 (*cspA*$^+$) and WB012 (Δ*cspA*) harboring pMM016 (in which *lacZ* was fused at the 13th codon of the *cspA* gene) were grown in Luria-Bertani medium or in the M9-Casamino Acids containing ampicillin (50 μg/ml) at 37°C until mid-log phase. Cells were sampled before and after temperature downshift for the measurement of β-galactosidase activities. Two independent β-galactosidase assays were carried out. The open bars show β-galactosidase activities obtained for the wild-type cells, and the shaded bars show those obtained for the Δ*cspA* cells. (B) Strains AR137 and WB012 harboring pCspB-LacZ, in which *lacZ* was fused at the 13th codon of the *cspB* gene, were used for the β-galactosidase assay. Other descriptions are same as those in panel A.

Using WB003, the *trans* effect of CspA on the 5′-UTR production from *cspA*::*cat* was examined. Figure 3C shows the RNA products from WB003 as well as WB002 and JC7623 30 min and 3 h after the temperature downshift. Note that the size of the primer extension products from JC7623 and WB002 is approximately 85 bases long (shown in Fig. 3C by the "Wt" arrow), while RNAs from WB003 contain a new product at 63 bases (shown in Fig. 3C by the "ΔCB" arrow) in addition to the 85-base product. The 65-base product is derived from the *cspA* gene carrying 22-base deletion at the cold-box region. The production of the 5′-UTR of the *cspA* mRNA from the original *cspA* gene was clearly reduced in WB003 (compare lanes 2 and 5 with lanes 3 and 6, respectively, in Fig. 3C). These results are consistent with the notion that the *cspA* gene is negatively regulated by CspA. However, it is not known at present why the *cspA* expression (lanes 3 and 6, Fig. 3C) was still substantially higher than the levels found in the parent cells (lanes 1 and 4, Fig. 3C) 0.5 and 3 h after cold shock, respectively.

In addition, even in the Δ*cspA* strain, the expression of the 5′-UTR of the *cspA* mRNA become repressed to a certain extent after the acclimation phase (compare lane 4 with lane 6 in Fig. 3A), suggesting that there exists another mechanism responsible for the regulation of the *cspA* gene expression besides CspA, a negative factor.

Expression of the *cspA-lacZ* fusion construct in the Δ*cspA* strain. The results obtained above indicate that *cspA* is negatively regulated by CspA at the level of transcription. In order to further confirm this conclusion, we carried out an experiment with a *cspA-lacZ* translational fusion construct, pMM016 (22). Strains AR137, which carried *pcnB* to keep the copy number of plasmids low (10), and WB012, an AR137 isogenic strain with the *cspA* deletion mutation, were transformed with pMM016. pCspB-LacZ (19), a translational fusion of *cspB* and *lacZ*, was also used as a control. The β-galactosidase activities of these fusion constructs have been shown to be dramatically induced upon cold shock (19, 22).

After reaching the mid-log phase, cells harboring pMM016 and pCspB-LacZ were treated by cold shock at 15°C. Two hours after cold shock, the level of β-galactosidase activity of the *cspA-lacZ* fusion in the Δ*cspA* strain was more than two times higher than that in the wild-type strain, and it was 1.5 times higher 5 h after cold shock (Fig. 4A), while 2 h after cold shock, the level of β-galactosidase activity of the *cspB-lacZ* fusion in the Δ*cspA* strain was only 1.4 times higher than that in the wild-type strain, and little difference was observed for this construct between the Δ*cspA* and wild-type strains 5 h after cold shock (Fig. 4B). These results are consistent with the earlier notion that CspA negatively regulates its own gene expression and, to a lesser extent, *cspB* gene expression at low temperature.

Stability of the 5′-UTR of the *cspA* mRNA and the *cspB* mRNA in the Δ*cspA* strain. In order to study the mechanism that is responsible for the increased expression of the 5′-UTR of the *cspA* mRNA in the Δ*cspA* strain, we measured the stability of the *cspA* transcripts in the Δ*cspA* strain. The stability of mRNA was examined at 30 min and 3 h after cold shock by measuring the levels of the *cspA* mRNA by primer extension analysis in both the wild-type and Δ*cspA* strains (Fig. 5A and B, respectively). Again, the *cspA* mRNA was produced at a much higher level in the Δ*cspA* strain than in the wild-type strain. Thirty minutes after cold shock, the *cspA* mRNA from the Δ*cspA* strain was approximately three times higher than that from the wild-type cells (compare lane 1 of Fig. 5B with lane 1 of 5A). The amounts of the *cspA* mRNA were densitometrically quantitated and plotted as shown in Fig. 5C. The mean values from three independent experiments are listed in Table 1. The half-lives of the *cspA* mRNAs were almost identical, with both strains having half-lives of longer than 60 min. This result demonstrates that at the early cold shock time point, the increased amount of the *cspA* mRNA in the Δ*cspA* strain is mainly due to the changes at the level of transcription rather than at the level of mRNA stability.

However, the mRNA stability dramatically changed during the acclimation phase. Three hours after cold shock, at the end

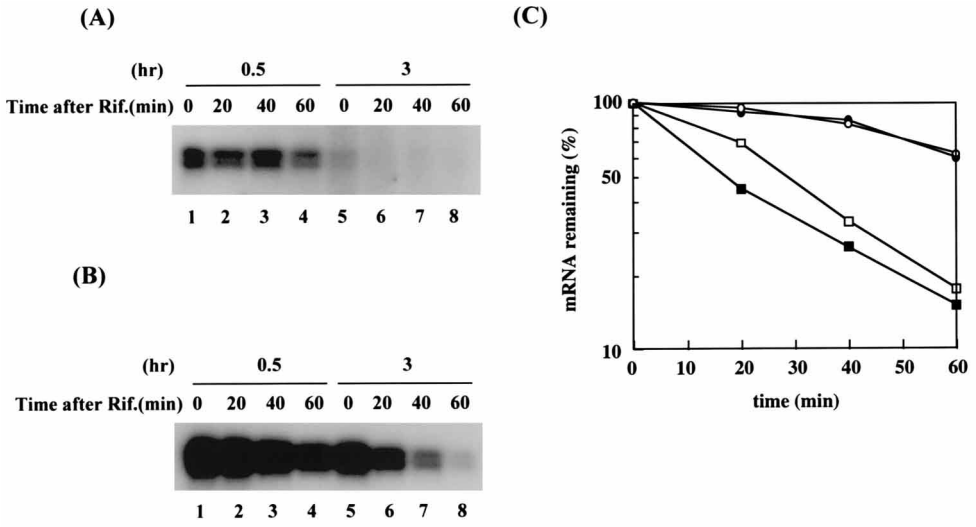

FIG. 5. Analysis of the mRNA stability of the 5′-UTR of *cspA*. Primer extension analyses of the 5′-UTR of *cspA* from the wild-type strain (A) and from the Δ*cspA* strain (B) were carried out as described in the legend to Fig. 3A. Rifampin (Rif. [final concentration, 200 μg/ml]) was added to the culture at either 0.5 or 3 h after the temperature downshift. RNAs were extracted at different time points and analyzed by the primer extension method as described in Materials and Methods. Lanes 1 to 4 show results at 0.5 h and lanes 5 to 8 show results at 3 h after the temperature downshift to 15°C. Lanes: 1 and 5, 0 min; 2 and 6, 20 min; 3 and 7, 40 min; 4 and 8, 60 min (after the addition of rifampin). (C) Graphic presentation of the results shown in panels A and B. The radioactivity of each band was quantitated and plotted on the assumption that the intensity of the band at the 0 time point is 100%. ●, JC7623 0.5 h after temperature downshift; ○, WB002 0.5 h after temperature downshift; ■, JC7623 3 h after temperature downshift; □, WB002 3 h after temperature downshift.

of the acclimation phase, the half-life of the *cspA* transcript became much shorter than that 30 min after cold shock in both the wild-type strain and the Δ*cspA* strain (lanes 5 to 8, Fig. 5A and B, respectively); the half-life for the wild-type strain was reduced to 13.3 ± 4.7 min, while that for the Δ*cspA* strain was reduced to 25 ± 5.2 min. Similar mRNA stability changes during cold shock adaptation have been reported (6). It should be noted that the *cspA* mRNA in the Δ*cspA* strain 3 h after cold shock was substantially more stable than that in the wild-type strain.

We also tested the mRNA stability for the *cspB* mRNA as shown in Fig. 6. Again the mRNA stability was dramatically altered during the acclimation phase; the half-lives of the *cspB* mRNA in the Δ*cspA* strain were 52.5 ± 0.7 min at 30 min after cold shock and 14 ± 2.8 min at 3 h. In the wild-type strain, the half-lives changed from 41 ± 1.41 min at 30 min to 8.3 ± 0.35 min at 3 h. Clearly, in the absence of CspA, the *cspB* mRNA became more stabilized. This is consistent with the notion that CspA functions as an RNA chaperone which appears to destabilize not only its own mRNA but also other cellular mRNAs (14). It seems that the increased production of CspB in the Δ*cspA* strain immediately after cold shock (an approximately 40% increase over that of the wild-type strain [Fig. 2F]) can be accounted for by the mRNA stabilization. The prolonged synthesis of CspB in the Δ*cspA* strain even after the acclimation phase (at 3 h [Fig. 2G]) is also likely to be due to the mRNA stabilization (Fig. 6C). However, one cannot rule out the possibility that in addition to the mRNA stabilization, the transcription of *cspB* may be increased.

TABLE 1. Half-lives of the 5′-UTR of *cspA* mRNA as well as *cspB* mRNA during the cold shock response

Strain	Half-life (min) at time after cold shock[a]			
	5′-UTR of *cspA* mRNA		*cspB* mRNA	
	0.5 h	3 h	0.5 h	3 h
JC7623 (wild type)	>60	13.3 ± 4.7	41 ± 1.41	8.3 ± 0.35
WB002 (Δ*cspA*)	>60	25 ± 5.2	52.5 ± 0.7	14 ± 2.8

[a] Results are mean values of at least two assays and were measured based on the results from primer extension experiments in the presence of rifampin. The cold shock was a drop from 37°C to 15°C.

The half-lives of the *cspG* mRNA were estimated and were found to be almost identical to those of the *cspB* mRNA 30 min and 3 h after cold shock (data not shown), consistent with the previous proposal that *cspB* and *cspG* are regulated by the same mechanism (4).

DISCUSSION

CspA, the major cold shock protein of *E. coli*, is dramatically induced upon temperature downshift. It was proposed to function as an RNA chaperone preventing RNA from forming stable secondary structures for efficient translation of mRNAs at low temperature (14, 16). Here, we constructed WB002, a Δ*cspA* strain, by using a novel positive/negative selection method. The Δ*cspA* strain exhibited unusual cold shock induction of the CspA homologs CspB and CspG even at 30°C, and their production was prolonged at low temperature even after the acclimation phase. The Δ*cspA* strain did not show any discernible growth defect at any temperature tested, suggesting that CspB and CspG, which have high sequence homology (80%) with CspA, may compensate for the absence of CspA. We are currently constructing double- and triple-deletion strains of *cspA*, *cspB*, and *cspG* in order to elucidate the exact cellular function of the CspA protein family, especially at low temperature.

In the present report, we have also showed that the disruption of the *cspA* gene caused, an approximately threefold increase in the expression of the 5′-UTR of the *cspA* mRNA 30 min after cold shock without a significant effect on mRNA stability, indicating that the increased expression of the 5′-UTR of the *cspA* mRNA was caused at the level of transcription. This could be due to the lack of production of CspA,

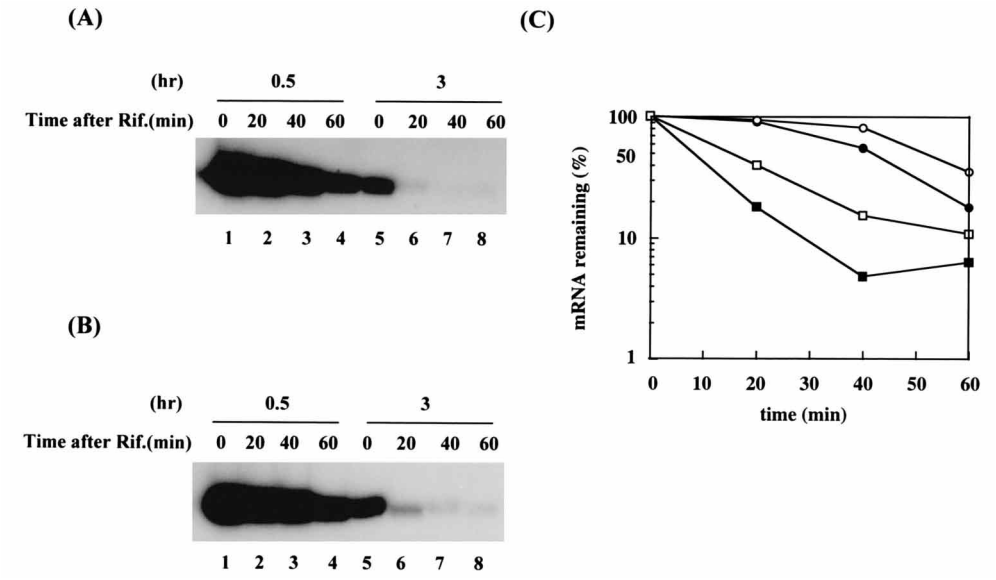

FIG. 6. Analysis of the mRNA stability of the *cspB* transcript. Primer extension analyses of the *cspB* mRNA from the wild-type strain (A) and from the Δ*cspA* strain (B) were carried out as described in the legend to Fig. 3B. (C) Graphic presentation of the results shown in panels A and B. Autoradiograms presented in panels A and B were exposed longer than those in Fig. 5A and B. Other descriptions are the same as those in the legend to Fig. 5.

which autoregulates its gene expression at the level of transcription. When CspA was produced in the Δ*cspA* strain, it was able to repress the *cspA* expression by approximately 50%. This is consistent with the notion that CspA autoregulates its gene. However, it appears that the *cspA* autoregulation in *trans* is not as tight as that in *cis* (Fig. 3C). This could be due to the fact that transcription is closely coupled with translation in *E. coli*. After the acclimation phase, not only the *cspA* mRNA but also the *cspB* mRNA became more stable in the Δ*cspA* strain than in the wild-type strain. These in vivo results are consistent with the in vitro results showing that the addition of CspA, which functions as an RNA chaperone, stimulated RNA hydrolysis by preventing the formation of RNase-resistant secondary structures (14).

How does CspA affect its own gene transcription? The unusually long 5′-UTR of the *cspA* mRNA contains a unique sequence called the "cold box," highly conserved in other cold shock genes (12), which is a presumed transcriptional pausing site and which is involved in repression of *cspA* expression. The stem-loop structures of the 5′-UTR of the *cspA* mRNA (14) are reminiscent of the structure of the transcriptional pausing site and the rho-independent intrinsic terminators (27). The cold box is also composed of a stem-loop structure. CspA has been shown to be an RNA chaperone (14), which may be involved in transcriptional termination/antitermination control, as are other RNA-binding proteins (3). It is conceivable that RNA polymerase somehow bypasses the pausing site in the *cspA* mRNA immediately following a temperature downshift. However, as the cellular CspA concentration increases during the acclimation phase, CspA starts to bind its own mRNA to destabilize the elongation complex of RNA polymerase, resulting in attenuation of transcription. It is interesting that NusA, which is involved in the elongation-termination step of transcription (3), is also a cold shock protein (16).

In the present report, we also show that mRNAs for cold shock proteins such as CspA and CspB are significantly destabilized during the cold shock response as previously reported (1, 6), indicating that mRNA destabilization is also involved in repression of *cspA* during and after the acclimation phase. This mRNA destabilization may be caused by cold shock-inducible RNases and/or RNA chaperones, such as CspA and its homologs. On the other hand, ribosomes have been suggested to be a physiological sensor of cold shock as well as heat shock (18, 34). It has been demonstrated that upon cold shock, ribosomes become nonfunctional for cellular mRNAs except for mRNAs for cold shock proteins (18). Recently, it has been shown that the downstream box existing downstream of the translation initiation codon (31, 36) confers higher translational efficiency to the mRNAs for class I cold shock proteins, such as CspA, CspB, CspG, CsdA, and RbfA, with cold-unadapted ribosomes (22). After the acclimation phase, however, cold-unadapted ribosomes are converted to cold-adapted ribosomes by acquiring cold shock ribosomal factors, such as RbfA and CsdA, which are produced during the acclimation phase (17, 18). These cold-adapted ribosomes regain the ability to translate non-cold shock mRNAs, and this may result in destabilizing the mRNAs for cold shock proteins. The decay rate of mRNA is closely associated with the translation mechanism (26). Therefore, the status of ribosomes during the cold shock response may be the presumable cause of changes in the stability of mRNAs for cold shock proteins, such as CspA, CspB, and CspG.

This Δ*cspA* strain provides an ideal system with which to study not only the mechanism of its own gene expression but also the role of CspA during cold shock stress. It remains to be elucidated to what extent CspA regulates genes for cold shock-inducible CspA homologs, such as CspB and CspG, and whether CspB and CspG are functional homologs of CspA or have their own specific function under cold stress.

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