

Escherichia coli RNase R Has Dual Activities, Helicase and RNase^{∇†}

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In *Escherichia coli*, the cold shock response occurs when there is a temperature downshift from 37°C to 15°C, and this response is characterized by induction of several cold shock proteins, including the DEAD-box helicase CsdA, during the acclimation phase. CsdA is involved in a variety of cellular processes. Our previous studies showed that the helicase activity of CsdA is critical for its function in cold shock acclimation of cells and that the only proteins that were able to complement its function were another helicase, RhIE, an RNA chaperone, CspA, and a cold-inducible exoribonuclease, RNase R. Interestingly, other major 3'-to-5' processing exoribonucleases of *E. coli*, such as polynucleotide phosphorylase and RNase II, cannot complement the cold shock function of CsdA. Here we carried out a domain analysis of RNase R and showed that this protein has two distinct activities, RNase and helicase, which are independent of each other and are due to different domains. Mutant RNase R proteins that lack the RNase activity but exhibit the helicase activity were able to complement the cold shock function of CsdA, suggesting that only the helicase activity of RNase R is essential for complementation of the cold shock function of CsdA. We also observed that *in vivo* deletion of the two cold shock domains resulted in a loss of the ability of RNase R to complement the cold shock function of CsdA. We further demonstrated that RNase R exhibits helicase activity *in vitro* independent of its RNase activity. Our results shed light on the unique properties of RNase R and how it is distinct from other exoribonucleases in *E. coli*.

When exponentially growing cells of *Escherichia coli* are shifted from 37°C to a low temperature, such as 15°C, a cold shock response is elicited. This response is characterized by a transient arrest of cell growth termed the acclimation phase, followed by resumption of growth at the low temperature. During the acclimation phase there is severe inhibition of general protein synthesis. However, several cold shock proteins are induced during this phase, including CspA (19) and its homologues, such as CspB (26), CspG (34), and CspI (55), polynucleotide phosphorylase (PNPase) (15, 43, 44), RNA helicase CsdA (49, 50), initiation factor IF2 (20), transcription factor NusA (18), RecA (54), histone-like protein H-NS (14), DNA gyrase (23), and ribosome-binding factor RbfA (12).

CsdA is a DEAD-box protein that belongs to the large family of putative RNA helicases. Members of this family are conserved in organisms from bacteria to humans (29) and play important roles in many cellular processes, such as processing, transport, or degradation of RNA or ribosome biogenesis (for a review, see reference 21). CsdA has been identified as a multifunctional protein, and it has been proposed that this protein participates in a variety of processes, such as ribosome

biogenesis, mRNA decay, translation initiation, and gene regulation. CsdA is essential at low temperatures, and deletion of its gene impairs growth when there is a cold shock (8, 24). On the other hand, it is dispensable at 37°C. Previously, we showed that the helicase activity of CsdA is pivotal in its role at low temperature (3). Our *in vivo* genetic screening of an *E. coli* strain revealed that another DEAD-box RNA helicase, RhIE, can compensate for the CsdA function at low temperature. This finding is also consistent with another report, which showed that RhIE can complement the cold shock function of CsdA and proposed that this protein is involved in ribosome assembly (22). We also observed that although not detected in our genetic screen, an exonuclease, RNase R, can also complement the cold shock function of CsdA (3).

RNase R is one of the three major 3'-to-5' processing exoribonucleases in *E. coli*; the other two are PNPase and RNase II. These enzymes are involved primarily in RNA metabolism in *E. coli*. Both PNPase (25, 61) and RNase R (7), but not RNase II, are induced by cold shock. It has been suggested that PNPase and RNase R are the universal degraders of structured RNA *in vivo* (11, 28). However, RNase R was the only RNase that was able to complement the helicase activity of CsdA (3). This observation led us to hypothesize that complementation of the cold shock function of CsdA by RNase R is not merely due to its ability to degrade secondary structures in RNAs but that it may also possess helicase activity.

RNase II and RNase R belong to the RNR family, and their secondary structures exhibit approximately 60% similarity.

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TABLE 1. Sequences of substrates

Substrate	Sequences
pds10-3'U ₂₀	5'-A GUU ACA GCC-3', 3'-U ₂₀ -AUC UGG UGU CAA UGU CGG-5'
pds18-3'U ₂₀	5'-UAG ACC ACA GUU ACA GCC-3', 3'-U ₂₀ -AUC UGG UGU CAA UGU CGG-5'
pds18-5'U ₂₀	5'-U ₂₀ -UAG ACC ACA GUU ACA GCC-3', 3'-AUC UGG UGU CAA UGU CGG-5'
ds18.....	5'-UAG ACC ACA GUU ACA GCC-3', 3'-AUC UGG UGU CAA UGU CGG-5'

However, RNase R is larger than RNase II (10). The crystal structures of *E. coli* RNase II and a catalytic site mutant of it in a complex with single-stranded RNA (ssRNA) have been solved (17, 32, 62). RNase R consists of 813 amino acids. Based on sequence analysis and comparison with the RNase II structure, RNase R contains a central nuclease domain, two cold shock (CSD) domains near the N terminus of the protein, an S1 domain, and a highly basic region near the C terminus of the protein (52). Here we carried out an *in vivo* domain analysis of RNase R and showed that this protein also has helicase activity and that this activity is essential for complementation of the cold shock function of CsdA. Mutant RNase R proteins lacking the RNase activity were still able to complement the cold shock function of CsdA. On the other hand, the CSD2 domain, which is not essential for the RNase activity of RNase R, was found to be important for its helicase activity. We also showed that the RNase R protein exhibits helicase activity *in vitro*, which is independent of its RNase activity. Our results shed light on the unique properties of RNase R and how it is distinct from other 3'-to-5' processing exoribonucleases in *E. coli*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strain JM83 [F⁻ *araΔ(lac-proAB) rpsL(Str^r)*] (60) was used as the wild-type strain. The *ΔcsdA*-JM83 deletion strain has been described previously (59). The *Δmr*-JW5741 strain was obtained from the Keio collection, Japan. *ΔcsdA* and *Δmr* single and double deletions were created in strain JM83 by P1 transduction after the kanamycin cassette was removed from each strain by the method described by Datsenko and Wanner (13). The bacterial cultures were grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl). The antibiotics ampicillin and kanamycin (50 μg ml⁻¹) were added to media when required.

Plasmids pINIII-*csdA*, pINIII-*ppp*, pINIII-*mb*, and pINIII-*mr* were constructed by cloning the regions corresponding to the *csdA*, *ppp*, *mb* and *mr* genes, respectively, using NdeI and BamHI and have been described previously (2, 3). pINIII plasmids containing different domains of RNase R were created using NdeI and BamHI (see Fig. 2A). The constructs encoding D272N RNase R, D278N RNase R, and D280N RNase R were cloned in the pINIII vector. The genes encoding the wild-type protein, the three mutant RNase R proteins, and CsdA-ΔC (3) were also cloned in plasmid pET28a to create N-terminal His₆-tagged versions of the proteins.

Isolation of polysomes and sucrose density gradient sedimentation. Polysomes were prepared and resolved as described previously (46), with minor modifications. *E. coli* cells were grown at 37°C in LB medium to log phase. When an appropriate culture density (optical density at 600 nm [OD₆₀₀], 0.5; 100-ml culture) was reached, polysomes were trapped by addition of chloramphenicol to the culture to a final concentration of 0.1 mg ml⁻¹. After an additional 4 min of incubation, cells were harvested by centrifugation. The cell pellet was resuspended in 1 ml of buffer BP (20 mM Tris-HCl [pH 7.6], 10 mM MgCl₂, 100 mM NH₄Cl, 5 mM β-mercaptoethanol). The cell suspension was placed in a Beckman ultracentrifuge tube. The cells were then lysed by immersing the tube in a liquid nitrogen bath for 1 min and then thawing it in a water bath at room temperature until no traces of ice remained. This freeze-thaw cycle was repeated two more times, and the lysate was subsequently subjected to centrifugation at 100,000 × *g* for 10 min in a Beckman TLA100.3 rotor. Polysomes were resolved by applying 0.2-ml portions of the supernatants to a 5 to 40% linear sucrose density gradient

(10 ml) in buffer BP, which was followed by ultracentrifugation at 4°C in a Beckman SW41 rotor for 2.5 h at 35,000 rpm.

The ribosome profile was analyzed at 254 nm, and rRNA was detected. Peaks corresponding to polysomes and 70S, 50S, and 30S ribosomes were obtained. The 40S peak was identified based on its position relative to the positions of the 30S and 50S peaks and by comparison with previously published gradient profiles showing the 40S peak (8). The peak adjacent to the 30S ribosomal peak consists of small RNAs, including small mRNAs, tRNAs, etc.

Note that the temperature sensitivity of the *ΔcsdA Δmr* strain is more evident on solid medium since in order to form a visible colony, a single cell has to divide more than 20 times. For our ribosome (polysome) analysis, the cells grown overnight in LB medium at 37°C were diluted in fresh LB medium and grown until the OD₆₀₀ was 0.5. The cells were then diluted using fresh LB medium and incubated at 30°C, 25°C, or 20°C. Diluted cells were able to divide at least two or three times at 25°C or 20°C during 24 h of incubation in the liquid medium. Therefore, we were able to obtain enough cells for the ribosome analysis.

Purification of proteins. Wild-type and mutant RNase R and CsdA-ΔC proteins that were tagged with His₆ at the N-terminal end were expressed in strain BL21(DE3) carrying pET-28 plasmids with corresponding constructs. Proteins were then purified using Ni-nitrilotriacetic acid (NTA) agarose and the method suggested by the manufacturer (Qiagen) by first binding the His-tagged proteins in the cell lysate to the agarose and then eluting them with imidazole and dialyzing the preparation against 50 mM NaH₂PO₄ buffer containing 50 mM NaCl (pH 7.5). The purity of the proteins was checked by SDS-PAGE analysis and Coomassie blue staining.

Helicase assay. The RNA substrates used to characterize the helicase activity of RNase R were either 10 bp or 18 bp long. The 18-bp substrates had either no overhang, a 3'-U₂₀ overhang, or a 5'-U₂₀ overhang. The 10-bp substrate had a 28-nucleotide 3' overhang. The RNA oligonucleotides were purchased from Dharmacon Inc. (now Thermo Scientific, Lafayette, CO) and were deprotected according to the manufacturer's instructions. The concentration of RNA was determined by measuring the absorbance at 260 nm in 8 M urea. The sequences of the duplex RNA substrates (substrate 1 with a 3' overhang [pds10-3'U₂₀], substrate 2 with a 3' overhang [pds18-3'U₂₀], substrate 3 with a 5' overhang [pds18-5'U₂₀], and blunt-ended substrate 4 [ds18]) used in this study are shown in Table 1.

The unwinding activities of wild-type RNase R and active site mutants of this protein were assessed by mixing 2.5 nM RNA substrate and 1 μM enzyme and initiating the reaction with 5 mM ATP and an ssRNA trap at a concentration of 2 μM for the times indicated below in a buffer containing 50 mM Tris-Cl (pH 7.5) and 5 mM MgCl₂ at 37°C. The single-stranded RNA trap consists of the same strand as the labeled strand of the duplex and prevents reannealing of the two strands of the substrate during unwinding. The only source of NaCl in the reaction mixture was the enzyme storage buffer. Since the stock enzyme concentrations were different for the different enzymes used in this study, some reaction mixtures were supplemented with NaCl so that the final concentration of NaCl in all reaction mixtures was 17 mM. The reactions were quenched with 100 mM EDTA and 10% SDS, and the mixtures were analyzed on a 20% native Tris-borate-EDTA polyacrylamide gel. The control reaction was carried out by pre-quenching the enzyme and then adding the ATP and trap to the reaction mixture.

Analysis of unwinding data. The unwinding data were quantified using the ImageQuant software (Molecular Dynamics, GE Life Sciences, NJ). A zero correction to account for any single-stranded RNA generated due to spontaneous dissociation under the reaction conditions was carried out using the following equation:

$$F(t) = [(SS \times DS_0) - (DS \times SS_0)] / [DS_0 - (DS + SS)] \quad (1)$$

where *t* is time, *F* is the fraction unwound, SS is the total amount of single strand generated, SS₀ is the amount of single strand present in the prequenched reaction mixture, DS is the total amount of duplex, and DS₀ is the total amount of

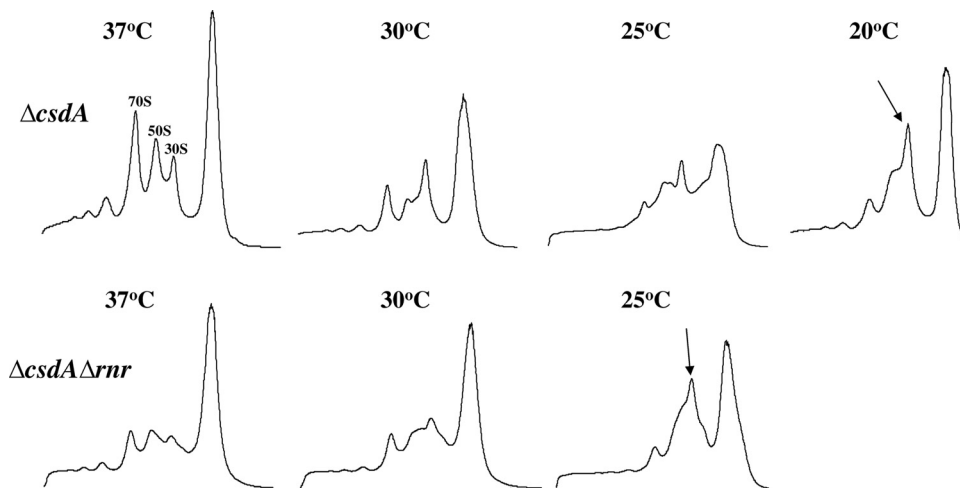


FIG. 1. Comparison of ribosome profiles of $\Delta csdA$ and $\Delta csdA \Delta rn r$ cells. $\Delta csdA$ and $\Delta csdA \Delta rn r$ cells were grown in LB medium with kanamycin at 37, 30, 25, and 20°C until the OD_{600} was 0.5. Polysomes were isolated and resolved as described in Materials and Methods.

duplex present in the prequenched reaction mixture. The fraction unwound obtained in this way was then plotted as a function of time. The data were then fitted to the exponential equation to obtain the unwinding amplitude (A) and the unwinding rate (k). The initial rate of unwinding was obtained from $A \times k$.

$$Y = A \cdot e^{-kt} \quad (2)$$

RESULTS AND DISCUSSION

The role of CsdA in mRNA decay was demonstrated by its association with the cold shock degradosome (41) and its involvement in degradation of certain mRNAs at low temperature (59). RNA structures are stabilized upon a temperature downshift, and therefore, the helicase activity of CsdA may be important for unwinding the mRNAs for degradation by RNases. The essentiality of CsdA upon cold shock may be due to its role in mRNA decay, in which its helicase activity plays a crucial role. Our previous screening of an *E. coli* genomic library for an *in vivo* counterpart of CsdA that can compensate for the absence of this protein at low temperature revealed only one protein, another DEAD-box RNA helicase, RhlE (3). We also observed that although not detected in our genetic screen, an exonuclease, RNase R, was also able to complement the cold shock function of CsdA. RNase R is a cold shock-inducible exonuclease that has recently been shown to be associated with a DEAD-box RNA helicase, RhlE, in the cold-adapted bacterium *Pseudomonas syringae* (42). RNase R is a processive, 3'-to-5' hydrolytic exoribonuclease that together with polynucleotide phosphorylase is thought to play an important role in the degradation of structured RNAs. However, RNase R differs from other exoribonucleases in that by itself it can degrade RNAs with extensive secondary structure provided that a single-stranded 3' overhang is present (53).

We observed that interestingly, an absence of CsdA and RNase R leads to increased sensitivity of cells to even moderate temperature downshifts. $\Delta csdA \Delta rn r$ cells transformed with the vector pINIII alone and streaked on LB medium plates containing ampicillin were able to grow at 37°C but not at 20°C or 15°C, while $\Delta csdA$ cells can grow at 20°C (3). $\Delta csdA \Delta rn r$ cells expressing CsdA or RNase R were able to grow at 20 or 15°C, although the cells expressing RNase R grew a bit slower

at 15°C. This suggests that either CsdA or RNase R is required when cells experience even a modest temperature downshift. This observation is especially interesting in light of the fact that RNase R, although cold shock inducible, is not essential at low temperature and CsdA is essential only at low temperature. This suggests that there may be a functional overlap between CsdA and RNase R and that at least one of these proteins must be present for cell growth even after a modest temperature downshift. Importantly, this function seems to be specific to RNase R, and other 3'-to-5' processive exoribonucleases, such as RNase II and PNPase, did not complement the cold-sensitive phenotype of the $\Delta csdA \Delta rn r$ cells even at 20°C.

During ribosome biogenesis in *E. coli*, a single rRNA precursor is concomitantly synthesized, processed into 23S, 16S, and 5S rRNAs, and assembled with the ribosomal proteins to form mature 30S and 50S ribosomal subunits (35, 48). Based on the observation that significantly more time is required for *in vitro* assembly of ribosomal subunits using isolated components than for *in vivo* assembly, it was suggested that nonribosomal factors, such as RNA helicases or chaperones, may assist ribosomal assembly *in vivo* (22, 35, 57). As RNA structures are more stable at low temperatures, the rRNA rearrangements that accompany ribosome biogenesis are probably more dependent on these RNA chaperone or helicase activities at low temperatures. It was shown previously that CsdA is involved in the biogenesis of the 50S ribosomal subunits and that deletion of the *csdA* gene leads to a deficit in free 50S subunits and accumulation of a 40S-like particle. Charollais et al. also showed that CsdA associates with 50S precursors at low temperature (8). We analyzed whether the increased sensitivity of the $\Delta csdA \Delta rn r$ cells to modest downshifts in temperature affects the ribosome biosynthesis in a temperature-dependent manner. $\Delta csdA$ and $\Delta csdA \Delta rn r$ cells were grown at 37, 30, 25, and 20°C until the OD_{600} was 0.5, and then samples were removed for polysome profile analysis. Cell lysates were subjected to 5 to 40% sucrose density gradient fractionation as described in Materials and Methods. As shown in Fig. 1, in the *csdA* single-deletion cells, accumulation of 40S-like particles (indicated by an arrow) was apparent at 20°C, while in the

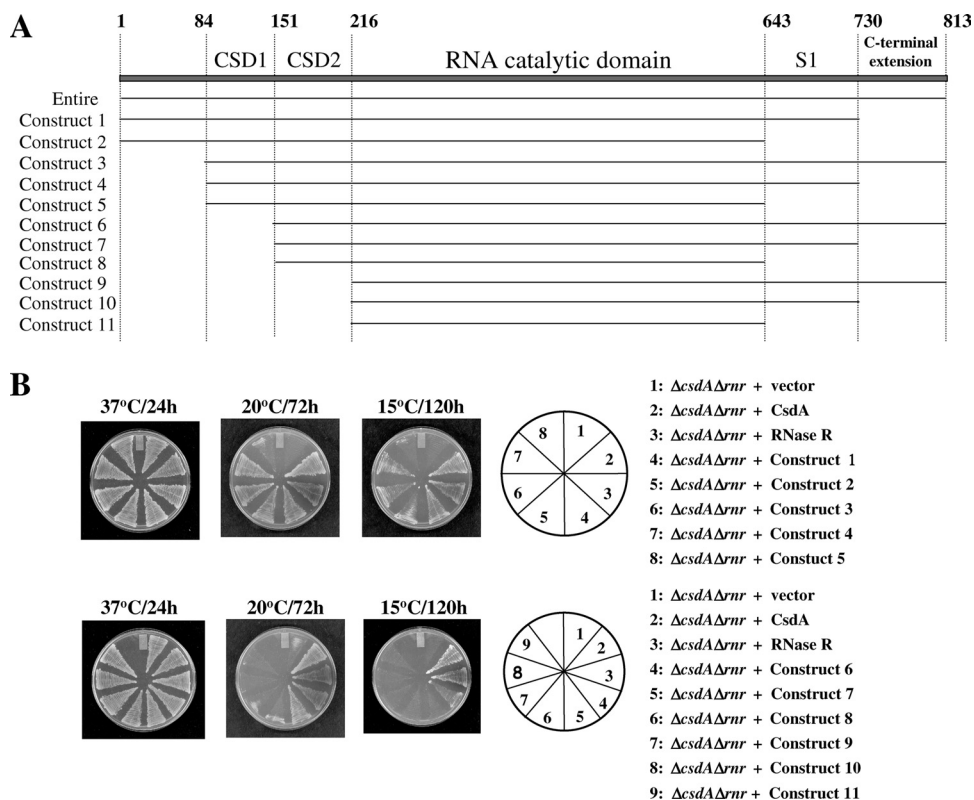


FIG. 2. Domain analysis of RNase R with respect to its ability to complement the cold shock function of CsdA. (A) Schematic diagram of domains of RNase R and plasmids that were constructed. Different domains of RNase R were cloned in the pINIII vector using NdeI and BamHI sites, as indicated. (B) *E. coli* $\Delta csdA \Delta rnr$ cells were transformed with plasmid pINIII alone or plasmid pINIII containing CsdA, RNase R, or different domains of RNase R. The plasmids carrying different domains of RNase R are designated as shown in panel A. The cells were streaked on LB medium plates containing ampicillin ($50 \mu\text{g ml}^{-1}$) and incubated at 37°C, 20°C, and 15°C for the times indicated.

$\Delta csdA \Delta rnr$ cells, the synthesis of 50S ribosomal particles seemed to be affected even at 30°C and there was a significant accumulation of 40S-like particles at 25°C. These observations are consistent with the growth defect of the $\Delta csdA \Delta rnr$ cells observed after a moderate temperature downshift. The $\Delta csdA \Delta rnr$ cells cannot grow at 25°C, and their growth is severely inhibited even at 30°C. Note that the temperature sensitivity of the RNase R and CsdA double-deletion strain is more evident on solid medium since in order to form a visible colony, a single cell has to divide more than 20 times. On the other hand, cells were able to divide at least two or three times at 25°C or 20°C during 24 h of incubation in the liquid medium. Therefore, we were able to obtain enough cells for the ribosome analysis.

Analysis of individual domains of RNase R with respect to its ability to complement the cold shock function of CsdA. The observation that only RNase R, and not RNase II or PNPase, was able to complement the cold-sensitive phenotype of the $\Delta csdA$ cells suggests that the degradation of the secondary structure in RNAs due to the RNase activity of RNase R may not be primarily responsible for the function of this enzyme as a protein analogue of CsdA and that it may possess helicase activity. To test this possibility, we carried out a systematic analysis of different domains of RNase R to determine which of them contribute to complementation of the cold shock func-

tion of CsdA and if indeed the RNase activity of RNase R is not important for its ability to complement the CsdA function.

RNase R consists of 813 amino acids. Based on the sequence analysis and comparison with the RNase II structure, RNase R contains a central nuclease domain, two cold shock (CSD) domains (CSD1 and CSD2) near the N terminus of the protein, an S1 domain, and a highly basic region near the C terminus (52). It should be noted that the structure of the CSD domains is very similar to the structure of the S1 domain. On the basis of this similarity, the CSD and S1 domains are grouped into the OB (oligomer binding) fold (33), and the S1 domain proteins have been shown to exhibit *in vivo* and *in vitro* activities similar to those of CSD domain proteins (38, 56, 58). Therefore, it can be concluded that RNase R actually contains three CSD domains. Figure 2A shows a schematic diagram of RNase R. We created several constructs in which one or more domains of RNase R were deleted, as shown Fig. 2. These constructs were cloned in the pINIII vector. $\Delta csdA \Delta rnr$ cells expressing these proteins were streaked on LB medium plates containing ampicillin and incubated at 37, 20, and 15°C. $\Delta csdA \Delta rnr$ cells containing the vector alone or expressing CsdA or the entire RNase R enzyme were also streaked as controls. As shown in Fig. 2B, the $\Delta csdA \Delta rnr$ cells expressing CsdA or the entire RNase R protein could grow at all temperatures, as expected, while the $\Delta csdA \Delta rnr$ cells containing the vector

alone could not grow at 20 or 15°C even after prolonged incubation. The plates streaked with constructs 1 to 5 (Fig. 2B, upper panel) show that deletion of the C-terminal region (construct 1), the S1 domain and the C-terminal region (construct 2), the N-terminal region (construct 3), or the N-terminal and C-terminal regions (construct 4) did not affect the ability of RNase R to complement the cold-sensitive phenotype of the $\Delta csdA \Delta rnr$ cells. However, deletion of the N-terminal and C-terminal regions together with the S1 domain (construct 5) resulted in loss of the RNase R activity that is essential for complementation of the cold shock function of CsdA. Also, the growth of cells expressing construct 2, in which the S1 domain was deleted together with the C-terminal domain, was slow compared to the growth of the other cells. The plates streaked with constructs 6 to 11 (Fig. 2B, lower panel) showed that $\Delta csdA \Delta rnr$ cells expressing RNase R in which the N-terminal region is deleted along with the CSD1 domain (construct 6) could grow at 20 and 15°C. Cells expressing RNase R with the N-terminal and C-terminal regions deleted together with the CSD1 domain (construct 7) were able to grow at 20°C but not at 15°C, while cells expressing RNase R with the N-terminal and C-terminal regions deleted together with the CSD1 and S1 domains (construct 8) were not able to grow at 20 or 15°C. Deletion of the CSD2 domain together with (i) the N-terminal region and the CSD1 domain (construct 9), (ii) the N-terminal and C-terminal regions and the CSD1 domain (construct 10), and (iii) the N-terminal and C-terminal regions and the CSD1 and S1 domains (construct 11) resulted in loss of the ability of RNase R to support growth of $\Delta csdA \Delta rnr$ cells at 20 and 15°C. In summary, the CSD2 domain seems to be absolutely essential for the ability of RNase R to complement the cold shock function of CsdA, and the presence of an additional CSD domain (either the CSD1 or S1 domain) is required for optimum helicase activity. The N-terminal and C-terminal regions and the CSD1 and S1 domains of RNase R can be deleted singly without affecting this activity.

Next we analyzed the effect of mutations in the RNA catalytic domain, which consists of 427 amino acids. Examination of the effects of mutations of highly conserved residues in the catalytic site of *E. coli* RNase II revealed that replacement of Asp209 by Asn (D209N) leads to complete loss of RNase activity without affecting the RNA-binding ability of the protein (1, 5). A similar mutation in the yeast RNase II homologue Dis3/Rrp44 also totally abolished its RNase activity without reducing substrate binding (16, 47). The *E. coli* RNase II proteins with the D201N and D207N mutations exhibit 0.2 and 12% of the activity of the wild-type protein (5). A comparison of the structural models of RNase II and RNase R showed that residues D272, D278, and D280 in RNase R correspond to the D201, D207, and D209 residues in RNase II, respectively. This conclusion was also supported by a structure analysis of *E. coli* RNase R (unpublished data), which showed that these amino acids in RNase R are critical for its catalytic activity. A recent report showed that the D280 residue in RNase R is crucial for its RNase activity (31). Plasmids expressing three mutant RNase R proteins with an Asp-to-Asn substitution at residue 272, 278, or 280 were constructed, and the proteins were expressed and purified as described in Materials and Methods. As described below (see Fig. 4A [compare the effects of addition of the purified mutant and wild-type RNase R proteins on

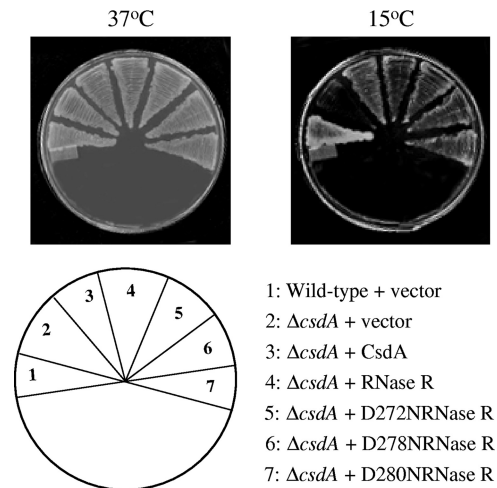


FIG. 3. RNase activity of RNase R is not required for the ability to complement the helicase activity of CsdA that is essential for its cold shock function. *E. coli* wild-type cells were transformed with plasmid pINIII as a control, and $\Delta csdA$ cells were transformed with plasmid pINIII alone as a control or plasmid pINIII expressing CsdA, RNase R, or RNase R having mutations in the RNA catalytic domains, as indicated. The cells were streaked on LB medium plates containing ampicillin ($50 \mu\text{g ml}^{-1}$). The results for plates incubated at 37°C for 24 h and at 15°C for 120 h are shown.

the substrate RNA]), the mutations did result in either a complete (D272N and D280N) or significant (D278N) loss of the RNase activity of RNase R. We created RNase R constructs with these mutations in the pINIII vector. The $\Delta csdA$ cells expressing these proteins were streaked on LB medium plates containing ampicillin and incubated at 37 and 15°C. The $\Delta csdA$ cells containing the vector alone or expressing CsdA or wild-type RNase R were also streaked as controls. As shown in Fig. 3, the $\Delta csdA$ cells expressing CsdA or wild-type RNase R were able to grow at all temperatures, as expected, while the $\Delta csdA$ cells containing the vector alone were not able to grow at 15°C. Interestingly, all of the cells expressing various mutant RNase R proteins were also able to grow at 15°C. This result suggests that the RNase activity of RNase R is not essential for its role in complementing the cold shock function of CsdA and that it possesses helicase activity which is independent of its RNase activity.

RNase R exhibits helicase activity *in vitro*. In order to test if RNase R indeed possesses helicase activity, we carried out *in vitro* helicase assays using purified mutant RNase R proteins. Purified CsdA- Δ C and wild-type RNase R proteins were used as controls. The proteins were expressed with an N-terminal His tag using the pET28a vector and were purified using Ni-NTA agarose as described in Materials and Methods. The purity of the protein preparations was confirmed by SDS-PAGE analysis and protein staining. We used CsdA with its C-terminal region deleted since it is easier to purify and it has been shown that the C-terminal region of CsdA is not required for its helicase activity *in vivo* and *in vitro* (3, 6). As it has been reported that CsdA exhibits helicase activity with short RNA duplexes with 3' or 5' extensions (50), we first tested the helicase activity of the proteins using a substrate (substrate 1 [pds10-3'U₂₀]) with a 10-bp duplex region. The substrate and

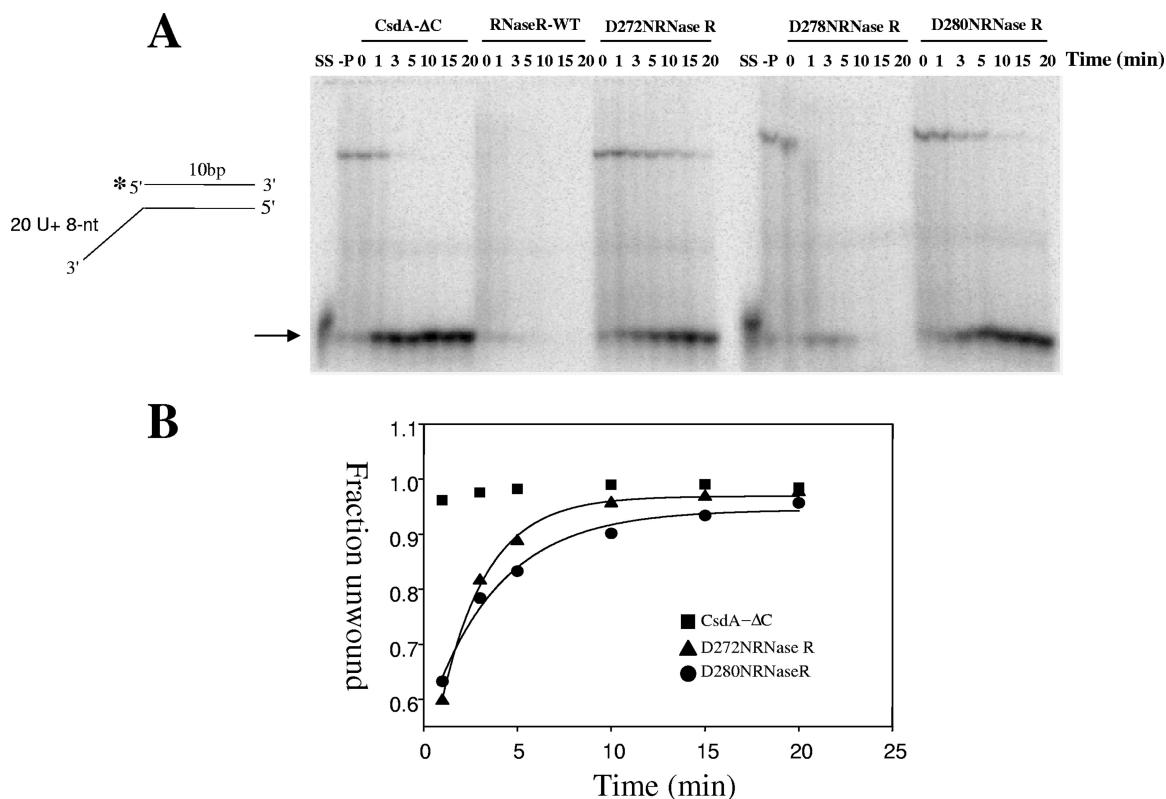


FIG. 4. RNase R exhibits helicase activity *in vitro*. (A) Helicase assay carried out at 37°C as described in Materials and Methods using substrate 1 (pds10-3'U₂₀). The products were analyzed by native PAGE followed by phosphorimaging analysis. Lanes SS, heat-denatured substrate showing ssRNA; lanes -P, control reaction without added protein; CsdA-ΔC, reactions carried out with CsdA-ΔC; RNaseR-WT, reactions carried out with wild-type RNase R; D272NRNase R, reactions carried out with D272N RNase R; D278NRNase R, reactions carried out with D278N RNase R; D280NRNase R, reactions carried out with D280N RNase R; lanes 0, 1, 3, 5, 10, 15, and 20, reaction mixtures incubated for 0, 1, 3, 5, 10, 15, and 20 min, respectively. The position of the single-stranded RNA is indicated by an arrow. The labeled strand is indicated by an asterisk. The experiment was repeated two times. (B) Results of a quantitative analysis of the helicase activity of the CsdA-ΔC and RNase R proteins corresponding to the gel shown in panel A.

the helicase assay are described in Materials and Methods. The reaction products were analyzed using a 20% native Tris-borate-EDTA polyacrylamide gel, followed by phosphorimaging analysis. The data were quantified using the ImageQuant software (Molecular Dynamics, GE Life Sciences, NJ). As shown in Fig. 4 and Table 2, the CsdA-ΔC and D272N and D280N RNase R proteins showed very efficient and rapid unwinding activity with this substrate. The wild-type RNase R degraded the substrate, as expected. The D278N RNase R protein degraded the RNA substrate used, but to a significantly lesser extent than the wild-type protein. This finding is consis-

tent with the report that the corresponding *E. coli* RNase II mutant protein exhibits 12% of the RNase activity (5). Thus, the unwinding rates were not calculated for this protein. Taken together, these results suggest that the D272N RNase R and D280N RNase R proteins do not have the RNase activity but do exhibit the helicase activity, so that these proteins are able to complement the cold shock function of CsdA.

We also analyzed the unwinding activity of these proteins using an RNA substrate with a longer duplex region (substrate 2 [pds18-3'U₂₀]). The results are shown in Fig. 5A, and data are also shown in Fig. 5B and Table 2. CsdA-ΔC unwound the pds18-3'U₂₀ substrate, although its activity was less than its activity with the 10-bp substrate described above. Wild-type RNase R degraded the substrate, as expected. D272R RNase R and D280N RNase R also showed helicase activity with this substrate, albeit to a lesser extent than CsdA-ΔC. Importantly, consistent with the results seen with the 10-bp substrate, neither the D272R RNase R protein nor the D280R RNase R proteins degraded the substrate RNA, supporting the notion that the mutations in these mutants result in a complete loss of RNase activity. The lower unwinding activity of the CsdA and RNase R mutant proteins observed with the RNA substrate containing a longer (18-bp) duplex region is consistent with the

TABLE 2. Kinetic parameters of RNA unwinding by CsdA-ΔC and RNase R mutants

Substrate	Enzyme	Initial rate of unwinding (duplex/min)
pds10-3'U ₂₀	CsdA-ΔC	Too fast
	D272N RNase R	0.23
	D280N RNase R	0.11
pds18-3'U ₂₀	CsdA-ΔC	0.015
	D272N RNase R	0.005
	D280N RNase R	0.005

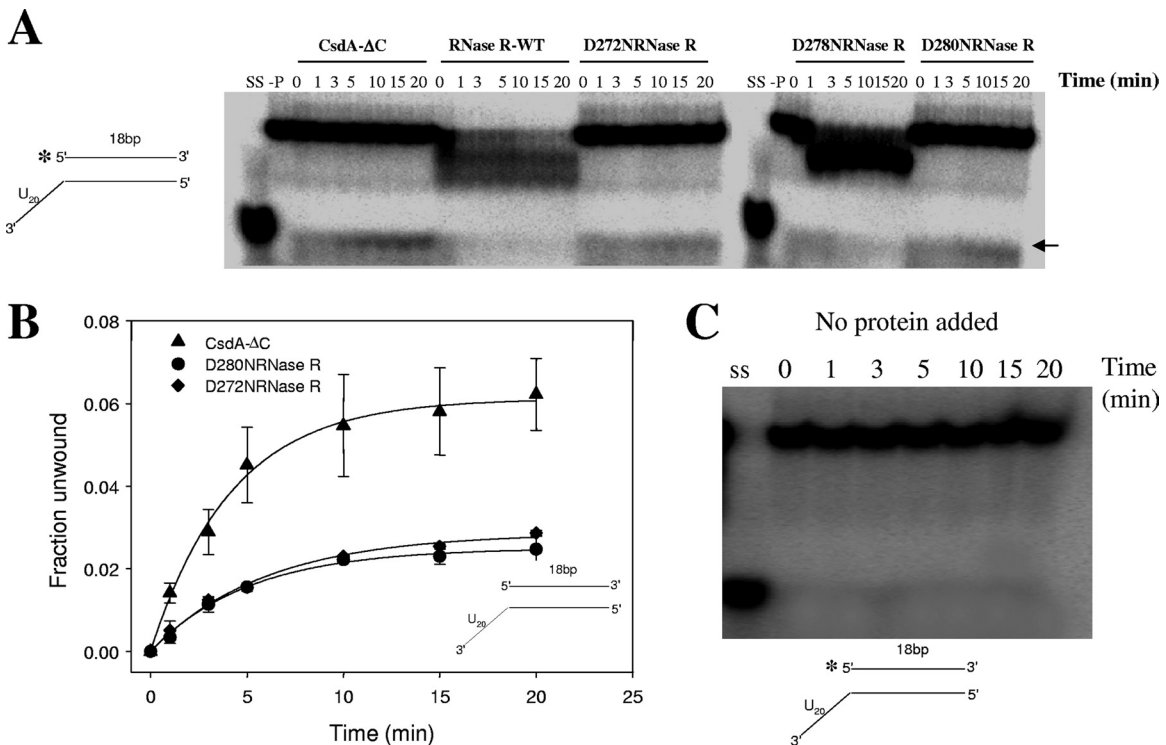


FIG. 5. *In vitro* helicase activity of RNase R with an 18-bp RNA substrate. (A) *In vitro* helicase assay carried out as described in Materials and Methods using substrate 2 (pds18-3'U₂₀). The products were analyzed by native PAGE followed by phosphorimaging analysis. Lanes SS, heat-denatured substrate showing ssRNA; lanes -P, control reaction without added protein; CsdA-ΔC, reactions carried out with CsdA-ΔC; RNaseR-WT, reactions carried out with wild-type RNase R; D272NRNase R, reactions carried out with D272N RNase R; D278NRNase R, reactions carried out with D278N RNase R; D280NRNase R, reactions carried out with D280N RNase R; lanes 0, 1, 3, 5, 10, 15, and 20, reaction mixtures incubated for 0, 1, 3, 5, 10, 15, and 20 min, respectively. The position of the single-stranded RNA is indicated by an arrow. Labeled strands are indicated by an asterisk. (B) Results of a quantitative analysis of the helicase activity of the CsdA-ΔC and RNase R proteins corresponding to the gel shown in panel A. The experiment was repeated three times. (C) Assay carried out like the assay described above for panel A, but without proteins. The position of ssRNA is indicated. The labeled strands is indicated by an asterisk.

data described previously for other DEAD-box helicases. Many DEAD-box RNA helicases have been shown to have extremely low unwinding processivity (9, 27, 45). These enzymes have been demonstrated to exhibit little or no unwinding with substrates longer than 12 to 15bp.

In order to exclude the possibility that the ssRNA product obtained in the helicase assays was due to the ssRNA trap used, we carried out the same assay without protein in the reaction mixture. As shown in Fig. 5C, no ssRNA was detected, supporting the notion that the ssRNA products obtained in the assays described here were due to the unwinding activity of proteins. To further confirm that the D272N and D280N RNase R mutants lack RNase activity, we carried out assays using the substrate pds18-3'U₂₀ in which the strand carrying the 3'-U₂₀ overhang was radiolabeled with [γ -³²P]ATP (pds18-3'U₂₀-b) (as opposed to the short 5' strand of the substrate used in the experiments whose results are shown Fig. 5A) and monitored the unwinding activity of the proteins. The rationale behind this approach was that since the enzyme is a 3'-5' exoribonuclease, if it digests the strand as it unwinds it, then single strands of different lengths would be generated in the reaction as the RNA strand is radiolabeled at the 5' end. If the protein lacks RNase activity, only ssRNA would be generated due to unwinding of the substrate. As expected, addition of

CsdA-ΔC or the D272N or D280N RNase R protein led to generation of ssRNA with no digestion of the double-stranded RNA (see Fig. S1 in the supplemental material). On the other hand, addition of D278N RNase R resulted in partial degradation of the substrate, while addition of the wild-type protein resulted in complete degradation.

We also tested the helicase activity of these proteins with two more RNA substrates, a substrate with the 5' overhang (pds18-5'U₂₀ [substrate 3]) and a blunt-ended substrate (ds18 [substrate 4]). CsdA showed some activity with the substrate with the 5' overhang and weaker activity with the blunt-ended substrate (data not shown). This is consistent with the results of a previous study which analyzed the helicase activity of CsdA (50). The RNase R mutant proteins did not exhibit activity with either of these substrates. One reason for this may be that RNase R is a 3'-5' exoribonuclease and requires the presence of a 3' overhang in its substrate.

We next analyzed the helicase activity of RNase R at 20°C using the 10-bp substrate. We used the wild-type and D272N RNase R proteins for this assay along with the CsdA-ΔC protein (see Fig. S2 in the supplemental material). Both the CsdA-ΔC and D272N RNase R proteins showed helicase activity at 20°C. However, the level of the activity was lower for both proteins (for CsdA-ΔC, 0.11 duplex/min; for D272N

RNase R, 0.096 duplex/min). Wild-type RNase R also showed RNase activity at 20°C, as the RNA substrate was completely degraded. Turner et al. (50) reported that CsdA shows significantly less helicase activity in *in vitro* assays at 15°C. We also observed that CsdA exhibited less unwinding activity (0.03 duplex/min) at 15°C, and similarly, the levels of helicase activity of the RNase R proteins were also reduced at 15°C (data not shown).

Recently, a systematic study was carried out with a series of truncated RNase R proteins and a wide range of specific RNA substrates (52). It was observed that the nuclease domain alone is sufficient for RNase R to bind and degrade RNAs, including structured RNAs. Another report by the same group substantiated this finding (51). Our study shows that the RNase activity of RNase R is not required for its helicase activity and the CSD domains (especially CSD2) are essential for helicase activity. Thus, it is apparent that RNase R possesses two distinct activities which are independent of each other and are due to different domains. RNase R has three cold shock domains, the CSD1, CSD2, and S1 domains. Our previous study showed that CSD domains can act as nucleic acid chaperones and unwind RNAs (4, 36–40). Based on the *in vivo* data, the CSD2 domain seems to be critical for the helicase activity of RNase R and either the CSD1 or S1 domain is required for optimal activity. The CSD2 domain may be essential for the helicase activity of RNase R due to its location (5, 30) in the RNase R molecule since its deletion may lead to changes in the structure of the protein resulting in diminished helicase activity. It should be noted that compared to the results for CsdA, the complementation of $\Delta csdA \Delta mr$ cells by RNase R is weaker. Thus, the presence of at least two CSD domains may be essential for effective helicase activity of RNase R.

Temperature downshift leads to stabilization of several RNAs, and helicase activity of CsdA was shown to be critical for RNA metabolism to facilitate cell adaptation and growth at low temperature (3). In the absence of CsdA, RNase R is important as it is cold shock inducible and can complement the helicase activity of CsdA. Our results also emphasize the finding that RNase R is unique in that it is the only RNase that can complement CsdA. It is also interesting that RNase R has two distinct activities which are independent of each other and are due to different domains. Further studies, especially a structural analysis, should elaborate on the essentiality of the CSD2 domain for the helicase activity of RNase R.

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