Coevolution of RtcB and Archease created a multiple-turnover RNA ligase

KEVIN K. DESAI,[1] AMANDA L. BELTRAME,[1] and RONALD T. RAINES[1,2]

[1]Department of Biochemistry, [2]Department of Chemistry, University of Wisconsin–Madison, Madison, Wisconsin 53706, USA

ABSTRACT

RtcB is a noncanonical RNA ligase that joins either 2′,3′-cyclic phosphate or 3′-phosphate termini to 5′-hydroxyl termini. The genes encoding RtcB and Archease constitute a tRNA splicing operon in many organisms. Archease is a cofactor of RtcB that accelerates RNA ligation and alters the NTP specificity of the ligase from *Pyrococcus horikoshii*. Yet, not all organisms that encode RtcB also encode Archease. Here we sought to understand the differences between Archease-dependent and Archease-independent RtcBs so as to illuminate the evolution of Archease and its function. We report on the Archease-dependent RtcB from *Thermus thermophilus* and the Archease-independent RtcB from *Thermobifida fusca*. We find that RtcB from *T. thermophilus* can catalyze multiple turnovers only in the presence of Archease. Remarkably, Archease from *P. horikoshii* can activate *T. thermophilus* RtcB, despite low sequence identity between the Archeases from these two organisms. In contrast, RtcB from *T. fusca* is a single-turnover enzyme that is unable to be converted into a multiple-turnover ligase by Archease from either *P. horikoshii* or *T. thermophilus*. Thus, our data indicate that Archease likely evolved to support multiple-turnover activity of RtcB and that coevolution of the two proteins is necessary for a functional interaction.

Keywords: Archease; RNA ligation; RtcB

INTRODUCTION

The RNA ligase RtcB catalyzes the GTP and Mn(II)-dependent joining of either 2′,3′-cyclic phosphate or 3′-phosphate termini to 5′-hydroxyl termini (Englert et al. 2011; Popow et al. 2011; Tanaka and Shuman 2011; Tanaka et al. 2011). RtcB is an essential enzyme for the ligation of tRNAs in metazoa (Popow et al. 2011), and possibly archaea (Englert et al. 2011; Sarmiento et al. 2013), upon intron removal by the tRNA splicing endonuclease (Abelson et al. 1998; Popow et al. 2012). RtcB is also essential for the ligation of XBP1 exons in metazoa upon intron removal by IRE1, which initiates the unfolded protein response during endoplasmic reticulum stress (Jurkin et al. 2014; Kosmaczewski et al. 2014; Lu et al. 2014). RtcB-catalyzed RNA ligation proceeds through three nucleotidyl transfer steps, with 2′,3′-cyclic phosphate termini being hydrolyzed to 3′-p termini in a step that precedes 3′-p activation with GMP (Tanaka et al. 2011; Chakravarty and Shuman 2012; Chakravarty et al. 2012). In the first nucleotidyl transfer step, RtcB reacts with GTP to form a covalent RtcB–histidine–GMP intermediate and release PP_i; in the second step, the GMP moiety is transferred to the RNA 3′-p; in the third step, the 5′-OH from the opposite RNA strand attacks the activated 3′-p to form a 3′,5′-phosphodiester bond and release GMP.

In many bacteria and archaea, the genes encoding RtcB and Archease are localized in an operon (Desai et al. 2014). Archease is a cofactor of RtcB that accelerates RNA ligation and alters the NTP specificity of the *Pyrococcus horikoshii* enzyme such that ligation proceeds efficiently with both GTP and ATP (Desai et al. 2014). Like RtcB, Archease is critical for tRNA splicing and XBP1 splicing in metazoa (Jurkin et al. 2014; Popow et al. 2014). Archease is a small (~16 kDa) protein with an anionic surface charge, suggesting that it might bind in the cationic RNA-binding cleft of RtcB. A crystal structure of Archease has revealed a metal-binding site, located on the exterior of the protein, which is essential for its activity (Desai et al. 2014). RtcB and Archease are conserved across all three domains of life; though, Archease is not widely distributed in bacteria. Thus, many bacterial taxa encode RtcB but not Archease.

We sought to characterize the differences between Archease-dependent and Archease-independent RtcBs from the bacterial domain of life. An understanding of these

Corresponding authors: rtraines@wisc.edu, kkdesai@wisc.edu

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differences could explain the selective pressures that existed to drive the evolution of Archease and provide further insight into Archease function. Here we report on the Archease-dependent RtcB from *Thermus thermophilus* and the Archease-independent RtcB from *Thermobifida fusca*. Our studies show that RtcB from *T. thermophilus* can catalyze multiple turnovers only in the presence of Archease and that the *T. fusca* ligase is a single-turnover enzyme. In addition, we find that Archease from the archaeon *P. horikoshii* can activate RtcB from *T. thermophilus*, despite low sequence identity among the Archeases from these two organisms. In contrast, RtcB from *T. fusca* is unable to be converted into a multiple-turnover enzyme by Archease from either *P. horikoshii* or *T. thermophilus*, demonstrating that Archease and RtcB must coevolve to produce a multiple-turnover RNA ligase.

RESULTS

The Archease-dependent RtcB from *T. thermophilus*

The genome of the bacterium *T. thermophilus* encodes both RtcB and Archease, suggesting that RtcB from this organism is susceptible to activation by Archease and thus Archease-dependent. The genes encoding *T. thermophilus* RtcB and Archease were synthesized using codons optimized for expression in *Escherichia coli* and the proteins were purified to homogeneity (see Materials and Methods section). First, we titrated Archease into ligation reactions with RtcB to see if Archease does indeed activate RtcB from *T. thermophilus* and to determine the concentration of Archease required for maximal activation. The ligation substrate we used in the current study is a 20-nt RNA with a 5′-OH, 3′-p, and an internal 6-carboxyfluorescein fluorophore (Fig. 1A). Upon reaction of this RNA (1 μM) with RtcB (5 μM) and the cofactors GTP and Mn(II), we observe a cyclic ligation product. During incubation of the reaction at 70°C for 1 min, in the absence of Archease, the reaction only goes to 5.4% completion. Remarkably, when the Archease concentration is increased to 2 μM we observe that the reaction now goes to completion during the same incubation time (Fig. 1A). Thus, RtcB from *T. thermophilus* displays a strong dependency on Archease for maximal activity.

Next, we performed single-turnover kinetics of *T. thermophilus* RtcB in the absence and in the presence of Archease to

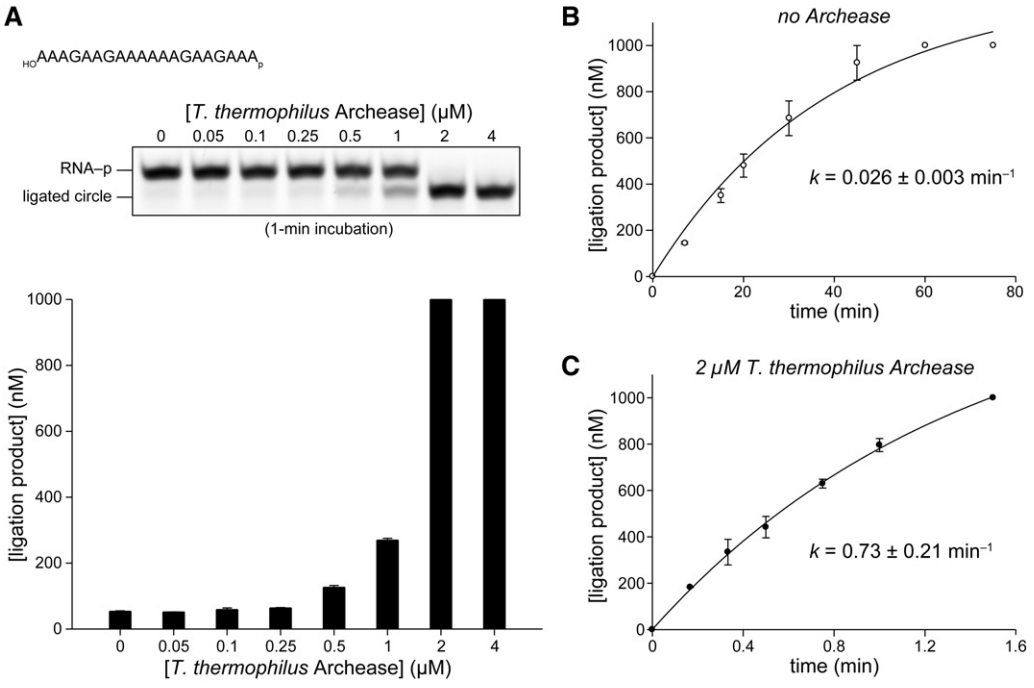

FIGURE 1. Archease titration of *T. thermophilus* RtcB and single-turnover ligation kinetics. (*A*) *T. thermophilus* RtcB-catalyzed RNA ligation reactions titrated with increasing concentrations of *T. thermophilus* Archease, as specified. Reactions were performed in 50 mM Tris–HCl buffer (pH 7.4) containing NaCl (300 mM), $MnCl_2$ (0.5 mM), GTP (0.1 mM), RtcB (5 μM), and RNA (1 μM). (RNA substrate is shown at *top* and has an internal 6-carboxyfluorescein label.) Reaction mixtures were incubated at 70°C for 1 min, and quenched with an equal volume of RNA gel-loading buffer (5× TBE containing 7 M urea, 20% v/v glycerol, and 15 mg/mL blue dextran). Reaction products were resolved by electrophoresis through an 18% w/v urea–polyacrylamide gel and visualized by fluorescence scanning of the 6-carboxyfluorescein label. (*B*) Single-turnover kinetics of catalysis by *T. thermophilus* RtcB in the absence of Archease. Reactions were performed in 50 mM Tris–HCl buffer (pH 7.4) containing NaCl (300 mM), $MnCl_2$ (0.5 mM), GTP (0.1 mM), RtcB (5 μM), and RNA (1 μM). Reaction mixtures were incubated at 70°C, and aliquots were removed and quenched at the indicated times by adding an equal volume of RNA gel-loading buffer. Ligation product formation over time is plotted and fitted to a single-exponential equation. (*C*) Single-turnover kinetics of catalysis by *T. thermophilus* RtcB in the presence of *T. thermophilus* Archease (2 μM). Ligation product formation over time is plotted and fitted to a single-exponential equation. Values are the mean ± SD for two separate experiments.

determine the extent of Archease activation. Reaction mixtures containing 5 μM RtcB alone and including 2 μM Archease were incubated at 70°C, and aliquots were removed and quenched at various time intervals. Plots of the concentration of ligation product formed over time were fitted to a single-exponential to obtain apparent rate constants of (0.026 ± 0.003) min^{-1} for RtcB alone and (0.73 ± 0.21) min^{-1} upon inclusion of Archease (Fig. 1B,C). Thus, 2 μM Archease accelerates RtcB ligation by 28-fold under our reaction conditions.

We had shown previously that RtcB and Archease from *P. horikoshii* can function in tandem to allow RNA ligation to proceed efficiently with ATP (Desai et al. 2014). Yet, in assays with *T. thermophilus* RtcB and Archease, we were unable to observe RNA ligation using ATP as a cofactor (data not shown), suggesting that dual GTP/ATP cofactor usage by RtcB and Archease might be unique to archaea or perhaps exclusive to *P. horikoshii*.

Archease proteins are interchangeable

Archease proteins across domains of life share low sequence identity; however, the two essential aspartates in the metal-binding site are strictly conserved (Desai et al. 2014). To discern if Archease proteins are interchangeable, we determined if Archease from *P. horikoshii* can activate RtcB from *T. thermophilus*, despite the Archeases from these two organisms having only 36% sequence identity. When Archease from *P. horikoshii* was titrated into ligation reactions with *T. thermophilus* RtcB, we observed an increase in ligation product upon Archease addition with maximal activation observed at an Archease concentration of 8 μM (Fig. 2A). A single-turnover kinetic experiment was performed to obtain an apparent rate constant of (0.12 ± 0.05) min^{-1} upon inclusion of 8 μM Archease (Fig. 2B). Thus, *P. horikoshii* Archease is able to activate *T. thermophilus* RtcB by 4.6-fold under our reaction conditions. This finding suggests that whereas the overall sequence identity of Archease proteins across the domains of life is low, that part of Archease critical for recognition and activation of RtcB might be the highly conserved metal-binding site (Desai et al. 2014). Although *P. horikoshii* Archease can accelerate the rate of ligation by *T. thermophilus* RtcB, we were unable to observe an effect on cofactor usage when testing for RNA ligation with ATP (data not shown).

In a test for binding of *P. horikoshii* RtcB to *P. horikoshii* Archease, we mixed N-terminal hexahistidine-tagged Archease with native RtcB and tested for coelution of the two proteins from a nickel column (data not shown). The hexahistidine-tagged Archease is as active as the native protein, suggesting that the tag would not interfere with protein binding. However, we were unable to detect a physical interaction between the two proteins, suggesting that they interact only transiently. The finding of Archease interchangeability, despite low sequence identity, is consistent with a functional interaction between RtcB and Archease not requiring tight binding.

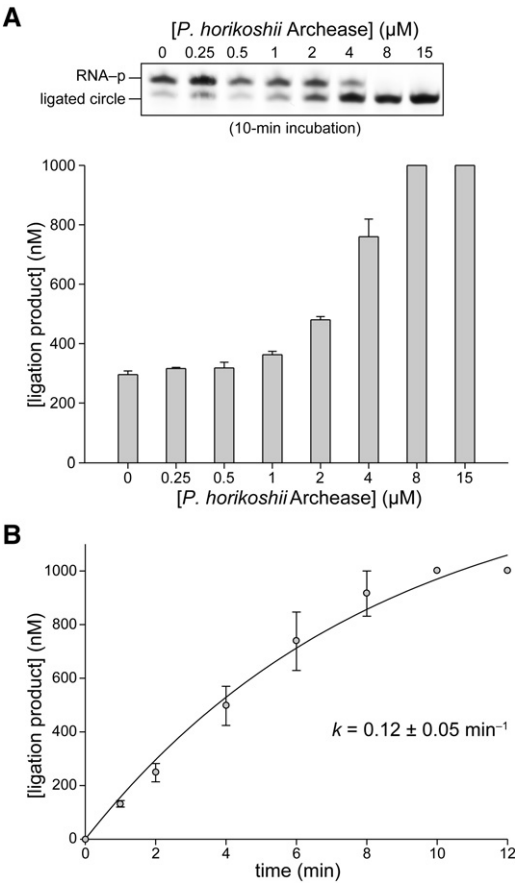

FIGURE 2. Activation of *T. thermophilus* RtcB by *P. horikoshii* Archease. (*A*) *T. thermophilus* RtcB-catalyzed RNA ligation reactions titrated with increasing concentrations of *P. horikoshii* Archease, as specified. Reactions were performed in 50 mM Tris–HCl buffer (pH 7.4) containing NaCl (300 mM), $MnCl_2$ (0.5 mM), GTP (0.1 mM), RtcB (5 μM), and RNA (1 μM). Reaction mixtures were incubated at 70°C for 10 min, and quenched with an equal volume of RNA gel-loading buffer. (*B*) Single-turnover kinetics of catalysis by *T. thermophilus* RtcB in the presence of *P. horikoshii* Archease (8 μM). Reactions were performed in 50 mM Tris–HCl buffer (pH 7.4) containing NaCl (300 mM), $MnCl_2$ (0.5 mM), GTP (0.1 mM), RtcB (5 μM), and RNA (1 μM). Reaction mixtures were incubated at 70°C, and aliquots were removed and quenched at the indicated times by adding an equal volume of RNA gel-loading buffer. Ligation product formation over time is plotted and fitted to a single-exponential equation. Values are the mean ± SD for two separate experiments.

The Archease-independent RtcB from *T. fusca*

The genome of the bacterium *T. fusca* encodes RtcB but not Archease; therefore, we refer to RtcB from *T. fusca* as "Archease-independent." The gene encoding *T. fusca* RtcB was synthesized using codons optimized for expression in *E. coli*, and the enzyme was purified to homogeneity (see Materials and Methods section). The RNA ligation assay conditions used for *T. fusca* RtcB were identical to those for

T. thermophilus RtcB, except for the incubation temperature. *T. fusca* is moderately thermophilic, and ligation reactions were thus performed at 45°C rather than the 70°C used for the hyperthermophilic *T. thermophilus* proteins. A single-turnover kinetic experiment with 5 μM RtcB and 1 μM RNA gave an apparent rate constant of (0.084 ± 0.012) min^{-1} (Fig. 3A). In reactions with *T. fusca* RtcB, we observed the appearance of the activated RNA intermediate, which migrates slightly above the substrate RNA (Fig. 3A), indicating that phosphodiester bond synthesis is rate limiting for *T. fusca* RtcB under our reaction conditions.

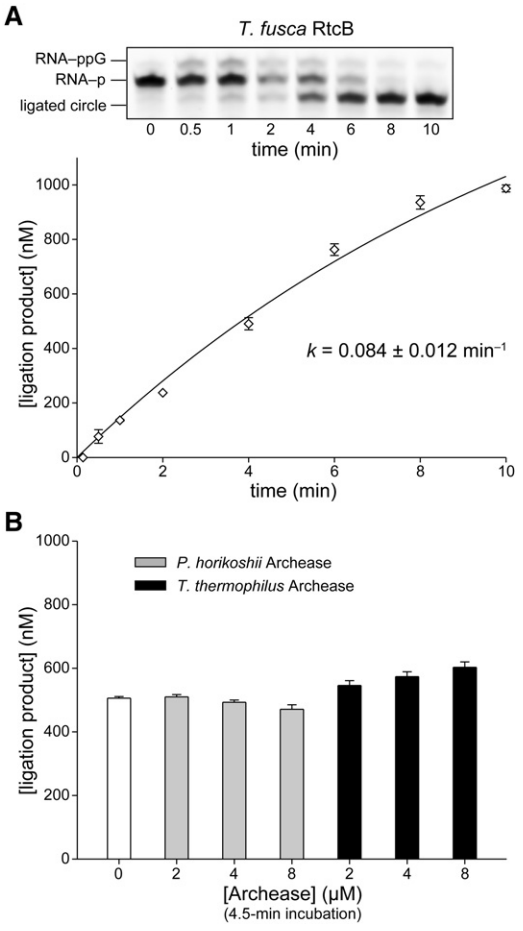

FIGURE 3. Single-turnover ligation kinetics of catalysis by *T. fusca* RtcB. (*A*) Urea–polyacrylamide gel depicting ligation product formation and a plot of product formation over time fitted to a single-exponential equation. Reactions were performed in 50 mM Tris–HCl buffer (pH 7.4) containing NaCl (300 mM), $MnCl_2$ (0.5 mM), GTP (0.1 mM), RtcB (5 μM), and RNA (1 μM). Reaction mixtures were incubated at 45°C, and aliquots were removed and quenched at the indicated times by adding an equal volume of RNA gel-loading buffer. (*B*) Plot of ligation product formation during catalysis by *T. fusca* RtcB in reaction mixtures that included the indicated concentration of either *P. horikoshii* or *T. thermophilus* Archease. Reactions were performed in 50 mM Tris–HCl buffer (pH 7.4) containing NaCl (300 mM), $MnCl_2$ (0.5 mM), GTP (0.1 mM), *T. fusca* RtcB (5 μM), and RNA (1 μM). Reaction mixtures were incubated at 45°C for 4.5 min. Values are the mean ± SD for two separate experiments.

Having demonstrated that Archease proteins are interchangeable using an Archease-dependent RtcB, we sought next to determine if an Archease-independent RtcB is susceptible to Archease activation. Ligation reactions with *T. fusca* RtcB (5 μM) and *P. horikoshii* or *T. thermophilus* Archease (2, 4, and 8 μM) were performed (Fig. 3B). At 8 μM, *P. horikoshii* Archease inhibited the reaction by 7% and *T. thermophilus* Archease activated the reaction by 19%. In marked contrast, *T. thermophilus* RtcB was activated 28-fold and 4.6-fold by *T. thermophilus* and *P. horikoshii* Archease, respectively. That Archease has a very minimal effect on catalysis by *T. fusca* RtcB suggests that RtcB and Archease must coevolve to enable a functional interaction between the two proteins.

RNA ligation reactions under multiple-turnover conditions

Next, we performed ligation reactions under multiple-turnover conditions using 0.5 μM RtcB and 2 μM RNA. In ligation reactions that did not include Archease, we were unable to observe product formation with *T. thermophilus* RtcB after incubation for 30 min (Fig. 4A). Yet, upon the inclusion of 2 μM Archease, *T. thermophilus* RtcB catalyzed four turnovers within 6 min. Thus, *T. thermophilus* RtcB is a multiple-turnover enzyme only in the presence of Archease. Ligation reactions with 0.5 μM *T. fusca* RtcB and 2 μM RNA were only able to maximally produce ~0.5 μM of ligation product even after an extended incubation of 75 min (Fig. 4B). Thus, *T. fusca* RtcB produced an amount of ligation product equal to the RtcB concentration, which demonstrates that *T. fusca* RtcB is a single-turnover RNA ligase (Fig. 4C). Neither *P. horikoshii* nor *T. thermophilus* Archease was able to convert *T. fusca* RtcB into a multiple-turnover enzyme, demonstrating that RtcB and Archease must coevolve to produce a multiple-turnover RNA ligase (Fig. 4D).

DISCUSSION

In the current study, we sought an understanding of the selective pressure that existed to enable the evolution of Archease. RtcB and Archease are a unique system for studying selective pressure because nature has not selected for Archease-dependent RtcBs in all organisms. Accordingly, we were able to report findings on Archease-dependent and Archease-independent RtcBs. Our data suggest that Archease evolved due to its ability to convert RtcB into a multiple-turnover enzyme. An organism with a multiple-turnover RtcB would need to synthesize fewer molecules of RtcB and use fewer Mn(II) ions to produce the same amount of ligated RNA as would an organism with a single-turnover RtcB. Additionally, because the Archease-independent RtcB from *T. fusca* is not susceptible to Archease action, our work demonstrates that RtcB and Archease must coevolve to produce a functional interaction. The ability for *P. horikoshii* Archease

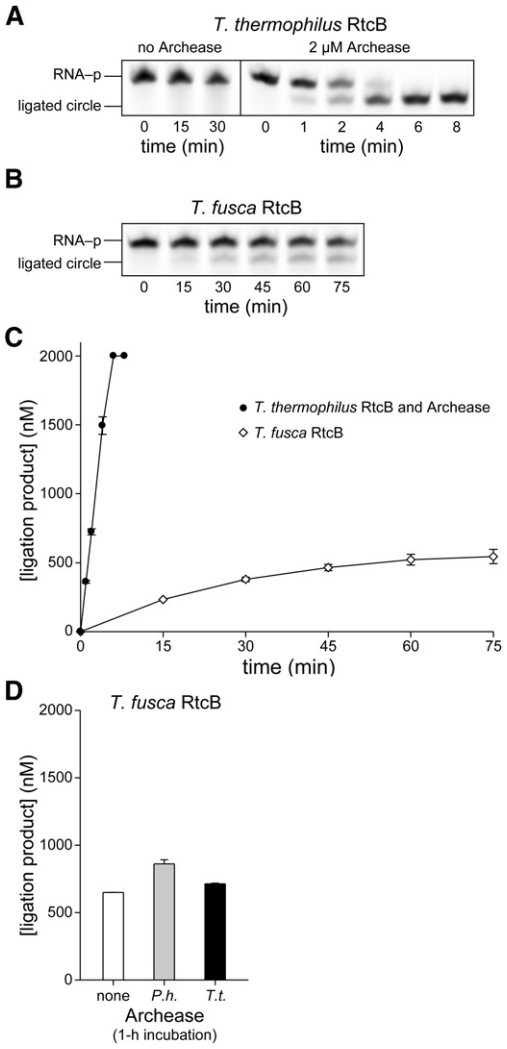

FIGURE 4. Ligation reactions under multiple-turnover conditions. (*A*) Urea-polyacrylamide gel depicting ligation product formation during catalysis by *T. thermophilus* RtcB under multiple-turnover conditions in the absence and presence of *T. thermophilus* Archease (2 μM). Reactions were performed in 50 mM Tris–HCl buffer (pH 7.4) containing NaCl (300 mM), $MnCl_2$ (0.5 mM), GTP (0.1 mM), RtcB (0.5 μM), and RNA (2 μM). Reaction mixtures were incubated at 70°C, and aliquots were removed and quenched at the indicated times by adding an equal volume of RNA gel-loading buffer. (*B*) Urea–polyacrylamide gel depicting ligation product formation during catalysis by *T. fusca* RtcB under multiple-turnover conditions. Reactions were performed in 50 mM Tris–HCl buffer (pH 7.4) containing NaCl (300 mM), $MnCl_2$ (0.5 mM), GTP (0.1 mM), RtcB (0.5 μM), and RNA (2 μM). Reaction mixtures were incubated at 45°C, and aliquots were removed and quenched at the indicated times by adding an equal volume of RNA gel-loading buffer. (*C*) Plots of ligation product formation during catalysis by *T. thermophilus* RtcB in the presence of *T. thermophilus* Archease (closed circles) and ligation product formation during catalysis by *T. fusca* RtcB (open diamonds) under multiple-turnover conditions. Plotted values were obtained from the above gels. Values are the mean ± SD for two separate experiments. (*D*) A plot of ligation product formation by *T. fusca* RtcB under multiple-turnover conditions in reactions that included either *P. horikoshii* or *T. thermophilus* Archease (8 μM). Reactions were performed in 50 mM Tris–HCl buffer (pH 7.4) containing NaCl (300 mM), $MnCl_2$ (0.5 mM), GTP (0.1 mM), *T. fusca* RtcB (0.5 μM), and RNA (2 μM). Reaction mixtures were incubated at 45°C for 1 h. Values are the mean ± SD for two separate experiments.

to activate *T. thermophilus* RtcB, despite low sequence identity between the Archeases from these two organisms, suggests that the highly conserved metal-binding site of Archease is critical for both RtcB recognition and activation (Desai et al. 2014).

Archease-independent RtcBs are commonly found in bacteria but not archaea or eukarya. Bacteria might not need the additional RtcB ligation activity endowed by Archease because bacterial tRNAs have self-splicing group 1 introns (Reinhold-Hurek and Shub 1992), whereas archaea and eukarya require more RtcB ligation activity to sustain tRNA splicing. Indeed, *E. coli* with a deletion in the *rtcB* gene has no apparent growth defects in rich media (Baba et al. 2006). In contrast, *rtcB* in the archaeon *Methanococcus maripaludis* has been annotated as possibly essential for growth in rich media based on a whole-genome transposon mutagenesis study (Sarmiento et al. 2013). Likewise, RtcB is required for tRNA splicing in humans (Popow et al. 2011). Moreover, RtcB activity is also required for XBP1 splicing during the unfolded protein response in metazoa (Jurkin et al. 2014; Kosmaczewski et al. 2014; Lu et al. 2014). Attesting to the importance of Archease activating RtcB in mammals, a decrease in the concentration of Archease has been shown to impair both tRNA splicing and XBP1 splicing (Jurkin et al. 2014; Popow et al. 2014). Thus, RtcB alone is unable to support these fundamental eukaryotic processes. In addition to facilitating RtcB turnover, it is possible that Archease evolved to function as a "switch" that regulates RtcB activity by turning on activity only at the appropriate time, potentially preventing erroneous RNA ligation events.

What causes the RtcB ligation reaction to stall after a single turnover? Classical nucleic acid ligases that join 5′-p and 3′-OH termini are multiple-turnover enzymes (Lohman et al. 2011), highlighting a peculiar aspect of catalysis by RtcB. Studies on human RtcB and Archease suggest that Archease facilitates the guanylylation of RtcBq after a single turnover, enabling another round of catalysis (Popow et al. 2014). RtcB uses a two-manganese mechanism during catalysis (Desai et al. 2013), which is analogous to the two-magnesium mechanism used by classical ligases (Cherepanov and de Vries 2002). The presence of an essential metal-binding site on the exterior of Archease at a tip, has led us to suggest that it might function to reach into and position Mn(II) ions within the RtcB active site. This scenario is analogous to the function of the RNA polymerase transcription factor GreB, which is also a small protein with a metal-binding site at its tip (Sosunova et al. 2003).

Investigations into how Archease and RtcB interact will likely be important for understanding the mechanism by which Archease activates RtcB. Currently, there is no structure available of an Archease-independent RtcB, but there are crystal structures of RtcB from *P. horikoshii* and *T. thermophilus*. Solving a structure of *T. fusca* RtcB and comparing its active site architecture to the Archease-dependent RtcBs could help to explain why it is not susceptible to

Archease action. Further, it is interesting to speculate about the possibility of engineering Archease dependency into the *T. fusca* RtcB by retracing its evolutionary trajectory into an Archease-dependent ligase. Here we have demonstrated that Archease endows RtcB with multiple-turnover activity and that RtcB and Archease must coevolve to create a functional interaction.

MATERIALS AND METHODS

Protein production and purification

The *T. thermophilus rtcB* and *archease* genes and the *T. fusca rtcB* genes were synthesized on gBlocks by Integrated DNA Technologies using codons optimized for expression in *E. coli*. *T. thermophilus* RtcB was encoded without a tag, whereas the *T. thermophilus* Archease and *T. fusca* RtcB genes encoded an N-terminal hexahistidine-tag. The genes were assembled into plasmid pET32 via Gibson assembly. The plasmid encoding the *P. horikoshii* Archease gene was described previously, and a version encoding the N-terminal hexahistidine-tagged protein was used (Desai et al. 2014). Proteins were produced in BL21 cells by growing in Terrific Broth medium at 37°C to an OD_{600} of 0.6 and inducing gene expression with IPTG (0.5 mM). Growth was continued at 37°C for 3 h to produce *T. thermophilus* RtcB and Archease and *P. horikoshii* Archease, but for *T. fusca* RtcB production, the temperature was decreased to 18°C for overnight growth. Cells were harvested by centrifugation and resuspended at 6 mL per gram of wet pellet in buffer A. For *T. thermophilus* RtcB purification, buffer A was 50 mM MES–NaOH (pH 5.6) containing NaCl (45 mM). For *T. thermophilus* Archease and *P. horikoshii* Archease purification, buffer A was 50 mM Tris–HCl (pH 7.7) containing NaCl (300 mM) and imidazole (20 mM). For *T. fusca* RtcB purification, buffer A was 50 mM Tris–HCl (pH 7.7) containing NaCl (300 mM), imidazole (40 mM), glycerol (5% v/v), and DTT (0.5 mM). Cells were lysed by passage through a cell disruptor (Constant Systems) at 20,000 psi, and the lysate was clarified by centrifugation at 20,000*g* for 30 min. Purification of the hyperthermophilic *T. thermophilus* and *P. horikoshii* proteins included a heat-kill step to remove host proteins by incubating the lysate at 70°C for 25 min followed by centrifugation at 20,000*g* for 20 min. *T. thermophilus* RtcB lysate was then loaded onto a 5-mL HiTrap HP SP cation-exchange column (GE Healthcare). The column was washed with 50 mL of buffer A, and RtcB was eluted with a gradient of NaCl (45 mM–1.0 M) in buffer A over 20 column volumes. The histidine-tagged proteins were purified by loading the lysate on a 5-mL HisTrap column (GE Healthcare). The HisTrap column was washed with 100 mL of buffer A, followed by a wash with 100 mL of buffer A containing an additional 25 mM imidazole. Pure proteins were eluted with buffer A containing 250 mM imidazole. Fractions containing purified protein were dialyzed overnight at 4°C against 2 L of 10 mM HEPES–NaOH buffer (pH 7.5), containing NaCl (200 mM); and *T. fusca* RtcB was dialyzed against 20 mM Tris–HCl buffer (pH 7.5) containing NaCl (250 mM), glycerol (10% v/v), DTT (0.5 mM), and $MnCl_2$ (25 μM). Proteins were flash-frozen in liquid nitrogen and stored at −80°C. The activity of *T. fusca* RtcB was observed to decrease significantly upon freezing; hence, all data reported for this enzyme were obtained using freshly purified protein. Protein concentrations were calculated from the A_{280} value and calculated (ExPASy) extinction coefficients of 41,830 $M^{-1}cm^{-1}$ for *T. thermophilus* RtcB, 8480 $M^{-1}cm^{-1}$ for *T. thermophilus* Archease, 53,400 $M^{-1}cm^{-1}$ for *T. fusca* RtcB, and 19,940 $M^{-1}cm^{-1}$ for *P. horikoshii* Archease.

RNA ligation assays

The RNA substrate was a 20-nt RNA with a 5′-OH, 3′-p, and an internal 6-carboxyfluorescein label (Fig. 1A). Ligation reactions were performed in 50-μL solutions consisting of 50 mM Tris–HCl buffer (pH 7.4) containing NaCl (300 mM), $MnCl_2$ (0.5 mM), and GTP (0.1 mM). For single-turnover reactions, the RtcB concentration was 5 μM and the RNA concentration was 1 μM. For multiple-turnover reactions, the RtcB concentration was 0.5 μM and the RNA concentration was 2 μM. Reactions with *T. thermophilus* RtcB were incubated at 70°C, whereas reactions with *T. fusca* RtcB were incubated at 45°C. Reactions were quenched by the addition of an equal volume of RNA gel-loading buffer (5× TBE containing 7 M urea, 20% v/v glycerol, and 15 mg/mL blue dextran). Reaction products were separated on an 18% w/v urea–polyacrylamide gel, and the RNA was visualized by fluorescence scanning with a Typhoon FLA9000 imager (GE Healthcare). Product quantification was performed using ImageQuant TL (GE Healthcare).

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