Analysis of r-protein and RNA conformation of 30S subunit intermediates in bacteria

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ABSTRACT

The ribosome is a large macromolecular complex that must be assembled efficiently and accurately for the viability of all organisms. In bacteria, this process must be robust and tunable to support life in diverse conditions from the ice of arctic glaciers to thermal hot springs. Assembly of the Small ribosomal SUBunit (SSU) of *Escherichia coli* has been extensively studied and is highly temperature-dependent. However, a lack of data on SSU assembly for other bacteria is problematic given the importance of the ribosome in bacterial physiology. To broaden the understanding of how optimal growth temperature may affect SSU assembly, in vitro SSU assembly of two thermophilic bacteria, *Geobacillus kaustophilus* and *Thermus thermophilus*, was compared with that of *E. coli*. Using these phylogenetically, morphologically, and environmentally diverse bacteria, we show that SSU assembly is highly temperature-dependent and efficient SSU assembly occurs at different temperatures for each organism. Surprisingly, the assembly landscape is characterized by at least two distinct intermediate populations in the organisms tested. This novel, second intermediate, is formed in the presence of the full complement of r-proteins, unlike the previously observed RI* particle formed in the absence of late-binding r-proteins in *E. coli*. This work reveals multiple distinct intermediate populations are present during SSU assembly in vitro for several bacteria, yielding insights into RNP formation and possible antimicrobial development toward this common SSU target.

Keywords: ribosome; small subunit; in vitro assembly; *E. coli*; *G. kaustophilus*; *T. thermophilus*

INTRODUCTION

Studies of *Escherichia coli* 30S subunits, which have been ongoing for several decades, led to the current understanding of how this RNP (RiboNucleoProtein) particle is assembled and functions (Shajani et al. 2011). The ability of the *E. coli* Small ribosomal SUBunit (SSU) to assemble in vitro from purified 16S rRNA and either 20 individually purified r-proteins or Total Proteins from 30S subunits (TP30) has allowed for detailed analysis of SSU formation (Traub and Nomura 1968; Culver and Noller 1999). The discovery that in vitro assembly of SSUs occurs in an ordered, hierarchical manner (Mizushima and Nomura 1970) led to the development of an in vitro assembly map (Fig. 1A), which has been instrumental to many of the discoveries about form and function of SSUs and is largely consistent with new detailed in vitro and in vivo data (Gronddek and Culver 2004; Bunner et al. 2010; Chen et al. 2012; Gupta and Culver 2014).

In vitro assembly of *E. coli* SSUs is a highly temperature-dependent process. When 16S rRNA and TP30 are reconstituted in vitro at low temperature (4°C–15°C) an intermediate population termed RI (Reconstitution Intermediate) forms

(Fig. 1B). Both the r-protein composition and RNA conformation of RI have been examined (Held and Nomura 1973; Holmes and Culver 2004, 2005). The presence of similar RNPs in cold sensitive, ribosome biogenesis defective mutants of *E. coli* has lent credence to RI being related to SSU intermediates formed in vivo (Guthrie et al. 1969; Shajani et al. 2011). A second intermediate population termed RI* is formed when 16S rRNA is reconstituted in vitro with only early and mid-binding r-proteins at higher temperature (Fig. 1C; Traub and Nomura 1969). The RI* population has been studied and the rRNA conformation has been compared with both RI and SSUs (Holmes and Culver 2004, 2005).

A major question still exists as to whether these assembly intermediates are limited to *E. coli* or if similar intermediates are present during SSU assembly when using 16S rRNA and TP30 components from other bacteria. This question is especially intriguing given the temperature dependence of SSU assembly and the large range of temperatures and other environmental niches in which bacteria thrive.

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FIGURE 1. (A) In vitro determined assembly map of *E. coli* ribosomal Small Subunit. (B) SSU assembly cascade in *E. coli* using TP30, in which RI is formed at low temperature and contains a subset of r-proteins. Incubation at optimal reconstitution temperature allows for the addition of the remaining r-proteins and SSU formation. (C) Assembly cascade in *E. coli* using individually purified r-proteins in which both RI and RI* are formed. RI and RI* are formed in the absence of late-binding r-proteins. SSUs are formed upon the addition of late-binding proteins to the RI* complex.

To address this question, in vitro assembly of *E. coli* SSUs was compared with that of the thermophilic bacteria *Geobacillus kaustophilus* and *Thermus thermophilus*. These bacteria were chosen due to a drastically different optimum growth temperature from that of *E. coli* (37°C) and the range in optimal growth temperature (*G. kaustophilus* 55°C, *T. thermophilus* 68°C) as well as previous data suggesting that in vitro assembly of SSUs from these organisms is possible (Nomura et al. 1968, Agalarov et al. 1999). Not only do these bacteria differ in the optimal growth temperature, but they also represent diverse morphologic, metabolic, and physiologic groups of bacteria. *E. coli*, the model organism for studying bacterial translation, is a Gram-negative, facultative anaerobe, commonly found in the lower intestine of mammals (Bachmann 1972). In comparison, the Gram-negative bacteria *T. thermophilus* (HB8), isolated from thermal springs in Japan (68°C, pH 7.0) is an aerobic chemoorganotroph, able to grow aerobically or, in the presence of nitrate, anaerobically (Oshima and Imahori 1974). The Gram-posi-

tive, spore forming, aerobic bacterium *G. kaustophilus* was isolated from sea mud in the Mariana trench (55°C) (Takami et al. 1997). Given the differences between these organisms it is somewhat surprising they share a high degree of sequence conservation both in the rRNA sequences (>70% sequence identity) as well as composition of the r-proteins (average 54% sequence identity). Thus, these three bacteria represent a testable subset, which have both overlapping and unique characteristics.

Using these three bacterial systems we demonstrate that SSU assembly is highly temperature-dependent and optimum growth and in vitro reconstitution temperature of SSUs are correlated. These findings also suggest that the formation of stable intermediates is conserved and while 16S rRNA containing RNPs are distinct between the three bacteria, they share important similarities. Conserved intermediates present in multiple, diverse bacteria could be ideally suited for interrogation by novel antibiotics that target the forming ribosome.

RESULTS

Functional SSUs are formed in vitro in three distinct bacteria

To determine if components from the chosen thermophilic bacteria are able to assemble functional SSUs in vitro, reconstitution experiments using 16S rRNA and TP30 prepared from *G. kaustophilus*, *T. thermophilus* and for comparison, *E. coli* were performed. The formation of functional SSUs was monitored using sucrose-gradient sedimentation and mRNA-dependent tRNA binding (Nomura et al. 1969; Culver and Noller 2000). RNPs sedimenting coincidentally with SSUs purified from cell lysate (Fig. 2; *T. thermophilus* 16S rRNA and natural SSUs shown as a control; *E. coli* and *G. kaustophilus* 16S rRNA and SSUs look identical to that of *T. thermophilus* [data not shown]) and able to efficiently bind tRNA (Fig. 3A–C) were observed using components from all the organisms tested. Temperature-dependent assembly profiles were observed for each organism tested with incubation at suboptimal temperatures resulting in the formation of intermediate populations of RNPs for all three organisms (see below and Fig. 4).

Multiple temperature-dependent intermediates are formed at suboptimal temperature in vitro during 30S subunit formation

A single Reconstitution Intermediate (RI) is reported when *E. coli* 16S rRNA and TP30 are incubated at low temperature (4°C–15°C) (Fig. 1B; Traub and Nomura 1969). When temperature titrations were performed, surprisingly not one, but two intermediate populations were observed for all organisms tested. Statistical analysis in which the minimum number of Lorentzian distributions to fit the sedimentation

FIGURE 2. Sucrose-gradient profiles of reconstituted SSUs using *E. coli*, *G. kaustophilus*, and *T. thermophilus* components. The dotted and solid lines indicate where 16S rRNA and SSUs sediment in the sucrose gradient, respectively.

profiles of the 16S rRNA containing populations revealed multiple, discrete intermediate populations formed for each organism (Fig. 4). In all organisms, an intermediate population formed at low temperature (4°C–15°C) (Fig. 4, purple curves), which sediments further into the sucrose gradient (larger Svedberg value) than naked 16S rRNA, and is clearly distinct from SSUs was observed. Due to the complexity of these profiles and to allow for consistency between organisms, this intermediate will be referred to as Intermediate 1. Consistent with previous results, Intermediate 1 formed using *E. coli* components is unable to efficiently bind tRNA (Figs. 3A, 4 [*E. coli*], purple curves; Maki et al. 2002).

Similarly, Intermediate 1 formed using components from *G. kaustophilus* or *T. thermophilus* was unable to efficiently bind tRNA (Figs. 3B,C, 4 [*G. kaustophilus*, *T. thermophilus*, 4°C and 15°C, purple curves]).

A second 16S rRNA containing in vitro formed intermediate population, Intermediate 2, was formed in all organisms when components are incubated at higher, but still suboptimal temperatures (Fig. 4, blue curves). The temperature required for formation of Intermediate 2 was dependent on the organism from which the 16S rRNA and TP30 components had been derived. Intermediate 2 of *E. coli* showed little difference in tRNA binding assays relative to Intermediate 1 (Fig. 3A). *G. kaustophilus* and *T. thermophilus* Intermediate 2, however, showed a marked increase in binding tRNA relative to Intermediate 1, albeit far less than SSUs formed at the optimal reconstitution temperature (Fig. 3A–C). To test if this was a result of Intermediate 2 more efficiently binding tRNA or if the increase was due to the presence of SSUs, the relative amount of SSU formed in each RNP population was calculated. The amount of SSUs formed below the optimal reconstitution temperature (Fig. 4, *G. kaustophilus* 42°C, *T. thermophilus* 42°C, yellow curves) increased proportionally with relative tRNA binding (Fig. 3B,C). This, taken with enzymatic-probing data showing regions of 16S rRNA responsible for efficiently binding tRNA are not properly formed in Intermediate 2 (Fig. 5B,C), suggests the increase in tRNA binding is a result of SSUs being present and intermediate 2 lacks the ability to efficiently bind tRNA. Intermediate populations formed in *G. kaustophilus* are relatively stable as no change in the sucrose-gradient profile was evident upon incubation at 42°C for 3 h (data not shown). However, both Intermediate 1 and Intermediate 2 can transition to form SSUs if incubated at the optimal reconstitution temperature (data not shown). Previous studies have shown that RI formed in *E. coli* are also quite stable (Traub and Nomura 1969; Talkington et al. 2005).

Incubation at optimal reconstitution temperature (*E. coli* 42°C, *G. kaustophilus* 55°C and *T. thermophilus* 65°C) results in in vitro formed SSUs which cosediment with natural SSUs (Figs. 2, 4, yellow curves) and efficiently bind tRNA (Fig. 3). Previous studies indicate that incubation of RNPs above the optimal reconstitution temperature abolish SSU formation and activity (Nomura et al. 1968). For the three organisms tested, sedimentation profiles of RNPs formed above the optimal reconstitution temperature showed a marked decrease in RNP populations co-sedimenting with natural SSUs (data not shown) and in tRNA binding efficiency (Fig. 3).

The presence of two in vitro assembly intermediates had not been previously reported when reconstitution studies using 16S rRNA and TP30 were undertaken. More automated and precise techniques, along with statistical peak analysis have been instrumental in identification and validation of this additional intermediate in a complex mixture of RNP populations. It should be noted for all organisms,

FIGURE 3. Percent relative tRNA binding with bar graph colors indicating the temperature of reconstitution (15°C: purple, 25°C: blue, 37/42°C: light green/green, 55°C: orange, 65°C: red, and 75°C: brown) and area under SSU peaks (line graph). Both percent tRNA binding and percent area under SSU peak were calculated based on reconstitution at optimal temperature for each organism. (A) *E. coli*, (B) *G. kaustophilus*, (C) *T. thermophilus*.

reconstitution at suboptimal temperature produces a mixture of intermediate populations with distinct sedimentation profiles on sucrose gradients. While both Intermediates 1 and 2 can clearly be seen within a single temperature-dependent reconstitution reaction of *G. kaustophilus* and *T. thermophilus* (see Fig. 4, 25°C), this is not the case for the intermediate populations formed in *E. coli*. Lorentzian distributions of *E. coli* intermediates formed at 4°C and 25°C can, however, identify two distinct peaks if not restricted to identify the lowest possible number of peaks (data not shown). When a combination of both Intermediates 1 and 2 were run on a single

sucrose gradient significant overlap of the intermediate populations occur, causing classification as a single intermediate. For this reason, in vitro SSU reconstitutions at suboptimal temperature are labeled based on the predominant intermediate population present, but are not considered a homogeneous population. These analyses do not eliminate the possibility that Intermediate 2 observed as a defined peak when *E. coli* components are reconstituted at 25°C, is not a heterogeneous mixture of Intermediate 1 and SSUs that result in an apparent shift in the sucrose-gradient sedimentation profile.

Reconstitution intermediates have distinct rRNA conformations

Proper folding of the rRNA backbone is essential for SSU function and X-ray crystal structures of ribosomes from both *E. coli* and *T. thermophilus* yield intricate detail about the end point of SSU biogenesis and the assembly process. Though the specific functional residues may differ slightly in each organism, rRNA secondary structure and functionally important regions are likely conserved between the three surveyed organisms (Cannone et al. 2002). Therefore, for this study, helices containing nucleotides (nt) known to be important for SSU function, such as central pseudoknot formation, tRNA binding, and intersubunit bridges, have been examined.

To determine how the rRNA conformation of each intermediate population is related to one another as well as naked 16S rRNA or SSUs, particles of interest were isolated from sucrose gradients and subjected to enzymatic-probing using the enzyme RNase T1. Using this enzyme, which cleaves single stranded RNA at the 3' end of

guanosine residues, along with primer extension (PE) analysis revealed cleavage events which were quantified and expressed relative to in vitro formed SSUs, and these data have been plotted onto 16S rRNA secondary structure (Cannone et al. 2002) for each organism (Fig. 5A–C; Supplemental Table 1A–C).

RNase T1 cleavage events revealed temperature-dependent conformational changes of the rRNA backbone in functionally important regions of 16S rRNA. Changes in relative intensity of cleavage between two 16S rRNA containing

FIGURE 4. QtiPlot analyzed sucrose-gradient profiles of *E. coli*, *G. kaustophilus*, and *T. thermophilus*. Sucrose-gradient sedimentation scans are shown in the background with coloring the same as in Figure 3 with the addition of 4°C in gray. Lorentzian fit curves of sucrose-gradient sedimentation profiles are shown in black dashed lines with Lorentzian distribution curves color coded indicating Intermediate 1: purple, Intermediate 2: cyan, SSUs: yellow, and undefined peaks: light gray. The vertical dotted lines represent where 16S rRNA sediments in the gradient while the solid vertical lines indicate the location of 30S subunits. Sucrose-gradients presented are representative of those observed in multiple experiments; however, only six gradients were compared at any one time. Hashed lines in boxes indicate temperatures not tested in the given experiment.

populations have been presented in Supplemental Table 1A–C as No Change (NC) where little or no difference in cleavage intensity is present at a particular nucleotide (defined here as less than twofold difference). Nucleotides greater than twofold more available for RNase T1 cleavage are listed as Enhancements (E) and nucleotides which are greater than twofold less available for action by RNase T1 are labeled as Protections (P), which can occur by more stably base-pairing, binding of r-proteins or conformational rearrangements which would act to bury the region of rRNA internally into the RNP making it less likely for cleavage by RNase T1.

In *E. coli*, 58 RNase T1 cleavage events were identified and quantified (Figs. 5, 6A; Supplemental Table 1A). Of those 58 nt, 19 nt were constant (NC) across all RNPs while two (Ec 837 and 839) (Ec indicates nucleotide numbering from *E. coli*) nucleotides showed changes in reactivity in all three transitions (from naked 16S rRNA to Intermediate 1, Intermediate 1 to Intermediate 2, and from Intermediate 2 to SSUs). Nine nucleotides have altered RNase T1 activity in two of the transitions with 28 nt showing reactivity changes in one transition (Table 1). The transition from naked 16S rRNA to Intermediate 1 involved protection of 10 nt (17%) all of which are located toward the 3' end of 16S rRNA sequence, in the head and neck region of the SSU. Enhanced cleavage of 16 nt (27%) occurs in the middle of 16S rRNA sequence located in the shoulder region of the SSU. The transition from Intermediate 1 to Intermediate 2 involved 6 nt (10%) being protected and 2 nt (3%) showing enhanced cleavage. The final transition from Intermediate 2 to SSUs involved protection of 15 nt (26%) and enhancement in 3 nt (5%). The final two transitions involve changes in RNase T1 cleavage intensity spread along the length of 16S rRNA with enhancements and protections occurring in close sequence to one another (Fig. 6A).

G. kaustophilus had 81 RNase T1 cleavage events which were quantified in this study (Figs. 5, 6B; Supplemental Table 1B). There were 38 nt, which were constant (NC) across all of the 16S rRNA containing populations while 1 nt (Gk 581) (Gk indicates nucleotide numbering from *G. kaustophilus*) showed changes in all three of the transitions. Nine nucleotides showed reactivity changes in two of the transitions and 33 nt showed a change of reactivity in one transition (Table 1). The transition from 16S rRNA to Intermediate 1 involved protection of 11 nt (14%) and enhanced cleavage in 9 nt (11%). All of the protections occurred in the 3' end of 16S rRNA, while the enhancements were toward the 5' end. The transition from Intermediate 1 to Intermediate 2 involved protection of 20 nt (25%) which clustered in the middle of the 16S rRNA sequence and enhanced cleavage in 2 nt (2%) located in the 3' end. The transition from Intermediate 2 to SSUs involved protection of 12 nt (15%) spread out along the length of 16S rRNA (Figs. 5, 6B; Supplemental Table 1B).

Cleavage events from RNase T1 probing have been quantified for 101 nt in *T. thermophilus* (Figs. 5, 6C; Supplemental Table 1C). There are 21 nt, which had constant levels of cleavage (NC) in all of the 16S rRNA containing populations while 10 nt (Tt 620, 895, 981, 1035, 1037, 1176, 1277, and 1281–1283) (Tt indicates nucleotide numbering from *T. thermophilus*) had altered reactivity in all three of the transitions. Another 45 nt showed reactivity changes in two of the transitions and 25 nt showed a change of reactivity in one transition (Table 1). The transition from naked 16S rRNA to Intermediate 1 involved protection of 23 nt (23%) and enhanced cleavage in 25 nt (25%). The transition from Intermediate 1 to Intermediate 2 involved protection of 34 nt (34%) and enhanced cleavage in 30 nt (30%). The

transition from Intermediate 2 to SSUs involved protection of 26 nt (26%) and enhancement in 7 nt (7%). Unlike the other organisms tested, *T. thermophilus* protections and enhancements occurred along the entire length of 16S rRNA in each of the transitions.

Of the 25 helices that show RNase T1 specific cleavage events, 9 helices (H2, 21, 24, 26, 27, 33, 36, 37, and 39) are cleaved in the three organisms tested. Five of these helices reside in the head of the SSU, three are in the platform and one is in the body. RNase T1 cleavage events in three helices are shared between *E. coli* and *G. kaustophilus*, with helix 18 re-

siding in the body, helix 23 in the platform and helix 28 being part of the head. Interestingly, all three helices reside near the active center of the SSU. Helix 25, which resides in the platform, shows cleavage in both *E. coli* and *T. thermophilus*. Five helices (H22, 31, 32, 34, 40) show cleavage events in *G. kaustophilus* and *T. thermophilus*. Helix 22 resides in the platform of the SSU while the others (H31, 32, 34, and 40) reside in the head and have been shown to be in close proximity to the late-binding r-proteins (S3, S10, S14) (Powers and Noller 1995). No pattern based on r-protein or rRNA conservation is immediately clear

when comparing the helices that have shared cleavage events. However, very few RNase T1 cleavage events were identified in the 5' domain. This is consistent with other data showing the 5' domain folds early and in the absence of r-proteins (Powers et al. 1993; Adilakshmi et al. 2005), most likely occluding cleavage by RNase T1.

The vast majority of nucleotides in 16S rRNA are not available for cleavage by RNase T1 and many of those that do show RNase T1 dependent cleavage appear not to undergo large conformational rearrangements in the transitions from naked 16S rRNA to in vitro formed SSUs. This may not be surprising given the secondary structure of 16S rRNA would limit the availability of single stranded rRNA regions, which can be cleaved by RNase T1. However, RNase T1-dependent cleavage events do yield information about the multiple conformational rearrangements that must occur throughout the transition from 16S rRNA to SSUs.

Functional regions of 16S rRNA are not appropriately structured in intermediate RNPs

The central pseudoknot, formed when nucleotides Ec9–Ec13 base-pair with Ec21–Ec25 (helix 1) and Ec17–Ec19 base-pair with Ec916–Ec918 (helix 2), is vital to assembly of all three domains and proper function of SSUs (Brink et al. 1993; Poot et al. 1998). A conformation like that observed in SSUs for this vital region is not obtained in *E. coli* or *G. kaustophilus* (Ec 918, Gk 923, and 924) intermediate populations 1 and 2. The conserved nucleotide Tt 896 (Ec 918) is similarly protected from RNase T1 cleavage in all *T. thermophilus* 16S rRNA

FIGURE 5. RNase T1 cleavage intensities plotted onto 2° structures of 16S rRNA. Gray circles indicate cleavage events similar to SSUs (0.45- to 1.44-fold). Enhancements in RNase T1 cleavage events relative to SSUs are shown as light orange circles (1.45- to 4.44-fold), dark orange circles (4.45- to 9.44-fold), and red circles (9.45- to >100-fold). Protections from RNase T1 cleavage events are shown as blue circles (0.44- to 0.224-fold), blue-green circles (0.225- to 0.106-fold), and green circles (0.105- to >0.01-fold). (A) *E. coli* rRNA conformational differences plotted onto the 2° structure of *E. coli* for 16S rRNA, intermediate 1 (4°C) and intermediate 2 (25°C). Circles represent relative intensity of primer extension bands to the optimal reconstitution temperature (42°C). (B) *G. kaustophilus* rRNA conformational differences plotted onto the 2° structure of the closely related organism *Bacillus subtilis* (due to no 2° structure being available for *G. kaustophilus*, with nucleotide changes as necessary) for 16S rRNA, intermediate 1 (4°C) and intermediate 2 (37°C). Circles represent relative intensity of primer extension bands to the optimal reconstitution temperature (55°C). (C) *T. thermophilus* rRNA conformational differences plotted onto the 2° structure of *T. thermophilus* for 16S rRNA, intermediate 1 (4°C) and intermediate 2 (42°C). Circles represent relative intensity of primer extension bands to the optimal reconstitution temperature (62°C). (Figure continues on following pages.)

containing populations, while Tt 895 remained dynamic in the intermediate populations. This data, while limited, suggests that the central pseudoknot is not properly formed in intermediate RNP populations.

The SSU must be able to efficiently bind tRNA to properly function in translation. The helices required for tRNA binding are known (Yusupov et al. 2001) and between the three organisms tested six are represented (helices 18, 23, 24, 28, 29, 31, and 34). A conformation like SSUs is not obtained in the intermediate populations in any of the organisms (Fig. 5A–C; Supplemental Table 1A–C). This, along with the tRNA binding data (Fig. 3) shows that these intermediate populations are incapable of efficiently binding tRNA and therefore translation.

The SSU must interact appropriately with the ribosomal Large SUBunit (LSU) to form the ribosome, allowing for translation to occur. The regions of 16S rRNA, which play a role in forming these intersubunit bridges are known in both *E. coli* and *T. thermophilus* from biochemical and crys-

tallographic methods with many of the bridge regions shared (Chapman and Noller 1977; Noller et al. 2001; Schuwirth et al. 2005), therefore it is assumed for this work that subunit bridge regions are similar in all organism tested. Four helices known to be important for subunit bridge formation, helices 20, 23, 24, and 27 (helix numbers from Sykes and Williamson [2009]), show RNase T1 cleavage patterns in at least one of the organisms tested. In *E. coli* three helices (23, 24, and 27) and *G. kaustophilus* four helices (20, 23, 24, and 27), show RNase T1 cleavage events with only helix 27 obtaining a conformation similar to SSUs upon formation of Intermediate 2 (Fig. 5A,B; Supplemental Table 1A,B). Neither helix 24 or 27 obtained a conformation similar to SSUs in the intermediate populations of *T. thermophilus* (Fig. 5C; Supplemental Table 1C). This data indicate that in all three organisms tested, neither Intermediate 1 nor 2 would be capable of subunit association and therefore translation.

B

FIGURE 5. (Continued.)

E. coli SSU Intermediate 1 and Intermediate 2 have distinct r-protein composition

The r-protein composition of RI from *E. coli* has previously been reported; therefore as comparison proteomic studies of Intermediate 1 as well as Intermediate 2 and in vitro formed SSUs from *E. coli* were undertaken. RNPs were purified from sucrose gradients and analyzed by LC MS–MS with the quantity of each r-protein based on spectral counts (Searle 2010) relative to SSUs (Fig. 7, yellow bars). These data showed *E. coli* Intermediate 1 was composed entirely of early and mid-binding proteins, with several mid-binding proteins drastically under-represented compared with SSUs (Fig. 7 [purple bars], see S5, S12, and S19) and lacked any measurable amount of the late-binding r-proteins (Fig. 7 [purple bars], see S2, S3, S10, S14, and S21). Previous data shows RI and 17S rRNA containing intermediates (21S) formed in cold sensitive *E. coli* mutants are lacking late-binding r-proteins and are also lacking S5 and S9 and show reduced amounts of S19 (Nashimoto et al. 1971; Held and Nomura 1973; Gupta and Culver 2014). Upon formation of *E. coli* Intermediate 2, all r-proteins were observed in the mass spectrometry analysis (Fig. 7, cyan bars); yet proper stoichiometry was not achieved until components were incubated at the optimal reconstitution temperature allowing for formation

of SSUs in vitro (Fig 7, green bars). These data are suggestive that there is a distinct Intermediate 2 that is formed when *E. coli* components are used in reconstitution at suboptimal temperatures. However, it cannot be definitely determined that the observed *E. coli* Intermediate 2 r-protein composition is not due to the presence of SSUs and Intermediate 1 both contributing to this protein composition.

Detailed proteomic analysis of Intermediate 1 and 2 from *G. kaustophilus* and *T. thermophilus* were not undertaken due to a significant proportion of both intermediate populations being present at suboptimal reconstitution temperatures. This along with limited information is available concerning the association of the r-proteins at suboptimal temperature made such analysis untenable.

DISCUSSION

In vitro temperature-dependent SSU assembly is common to several bacteria

Previous studies have identified a low temperature in vitro assembly intermediate using *E. coli* TP30 (as an r-protein source) and natural 16S rRNA (Fig. 1B). This intermediate population, referred to as RI, has been studied and the r-protein components as well as rRNA architecture have been reported (Held and Nomura 1973; Holmes and Culver 2004). Reconstitution studies using individually purified *E. coli* r-proteins have also revealed a second intermediate, RI*, which forms when 16S rRNA is reconstituted with only the early and

mid-binding r-proteins at elevated temperature (Fig. 1C; Held and Nomura 1973). The differences between RI and RI* are largely conformational rearrangements as the r-protein content is virtually unchanged (Held and Nomura 1973; Tam and Hill 1981; Holmes and Culver 2004). However, until now, a second intermediate had never been reported using 16S rRNA and TP30. Intermediate 1 from *E. coli* and RI formed in previous studies appear to be highly related; based on the incubation temperature at which they form and while the technique used to probe rRNA structures differs, the regions shown to undergo conformational changes are similar (Fig. 5A). Also, Intermediate 1 from *E. coli* shares the same r-protein composition (Fig. 7, purple bars) to that reported of RI (Held and Nomura 1973; Holmes and Culver 2004). Thus, not surprisingly, our identified Intermediate 1 appears the same as RI.

A second intermediate is observed in this study; formed at elevated, but suboptimal temperature for reconstitution of SSUs. Proteomic studies of Intermediate 2 formed in *E. coli* show that some proportion of the early, mid, and late-binding r-proteins are present upon formation of Intermediate 2. This is a difference between Intermediate 2 and the previously reported RI*, which is formed in the absence of late-binding r-proteins. There are regions of rRNA in Intermediate 2 that resemble the conformation of RI*, but others that are clearly different. The observance of Intermediate 1 and Intermediate 2 along with Intermediate 2 and SSUs in sucrose-gradient analysis of *G. kaustophilus* and *T. thermophilus*

FIGURE 5. (Continued.)

clearly suggest the presence of multiple distinct intermediates. However, in *E. coli*, sucrose-gradient analysis by QtiPlot and determination of Lorentzian distributions shows only a single peak formed at 4°C and another formed at 25°C. It seems likely that distinct Intermediate populations classified as Intermediate 1 and Intermediate 2 are also present in *E. coli*, however, the possibility remains that the sedimentation of Intermediate 2 is caused by a mixture of Intermediate 1 and SSUs within the reconstitution reaction.

Previous studies have shown that SSUs from thermophilic bacteria could be reconstituted in vitro (Nomura et al. 1968; Agalarov et al. 1999), but it was unknown if these bacteria undergo similar temperature-dependent 16S rRNA containing intermediate formation as documented for *E. coli*. This study demonstrates reconstitution of functional SSUs in vitro from the thermophilic bacteria *G. kaustophilus* and *T. thermophilus* and reveals that both these bacteria form at least two distinct temperature-dependent intermediates (Intermediates 1 and 2) during the assembly process. The rRNA conformation of these intermediates as well as 16S rRNA and SSUs reconstituted in vitro was studied using partial RNase T1 digestion followed by primer extension. This work represents the first study of *G. kaustophilus* rRNA conformation and the most comprehensive analysis of *T. thermophilus* rRNA assembly intermediates to date.

The presence of nonfunctional reconstitution intermediates is shared in the three organisms tested, suggesting that this may be a general theme of SSU reconstitution and possibly biogenesis. However, there are distinct RNase T1 cleavage patterns as well as rRNA conformational changes between the intermediates formed in the different organisms indicating that they are structurally distinct (Figs. 5, 6A–C; Supplemental Table 1A–C). The presence of both conserved and distinct pathways may prove useful in future studies of RNP formation and function in bacteria.

Intermediate populations are incapable of translation

Intermediate populations formed in *E. coli*, *G. kaustophilus*, and *T. thermophilus* lack the proper rRNA conformation in regions responsible for central pseudoknot formation, tRNA binding or

intersubunit bridge formation, indicating that neither Intermediate 1 nor 2 are capable of efficiently binding tRNA or interacting with 50S subunits to form 70S ribosomes and enter into the translation cycle. The two thermophilic bacteria used in this study show two distinct 16S rRNA containing intermediate populations, neither of which is structurally similar to SSUs as shown by both sucrose-gradient sedimentation and partial RNase T1 cleavage patterns. Conservation of late forming important functional regions of the SSU is not surprising. Evidence from other work shows that maturation of ribosomal components are tightly regulated in the cell to ensure only properly formed subunits enter the translation cycle (for review, see Woodson 2008).

FIGURE 6. Changes in RNase T1 cleavage intensity greater than twofold for each nucleotide are plotted linearly with Enhancements indicated by orange triangles, Protections indicated by blue triangles, and No Change shown in gray circles for (A) *E. coli*, (B) *G. kaustophilus*, and (C) *T. thermophilus*.

TABLE 1. Number of nucleotides showing reactivity changes for the three organisms tested in zero, one, two, or all three transitions

	One change			Two changes			Three changes	No change
	16S to I1	I1 to I2	I2 to SSU	16S to I1 and I1 to I2	16S to I1 and I2 to SSU	I1 to I2 and I2 to SSU		
<i>E. coli</i>	17	3	8	1	6	2	2	19
<i>G. kaustophilus</i>	10	18	5	3	6	0	1	38
<i>T. thermophilus</i>	14	11	0	22	2	21	10	21

The number of nucleotides which change in only one transition (16S to Int. 1, Int. 1 to Int. 2, or Int. 2 to SSU) is indicated. The number of nucleotides which change in two transitions (16S rRNA to Int. 1 and Int. 1 to Int. 2, Int. 1 to Int. 2 and Int. 2 to SSU, or 16S rRNA to Int. 1 and Int. 2 to SSUs) is also indicated. The number of nucleotides showing changes in all three transitions is also shown. Finally, the number of nucleotides in each organism that show no significant change in all three transitions is indicated.

CONCLUSIONS

This work highlights both conserved and species-specific aspects of SSU assembly. The presence of temperature-dependent, nonfunctional intermediates in the assembly process of three organisms tested imply there may be an underlying conservation to these processes. However, differences between the organisms tested can also be seen. The optimal reconstitution temperature for each organism is quite different. Also, the temperatures at which the predominant intermediate forms in each organism differ. The sedimentation profiles of each intermediate also differ somewhat as do specific rRNA contacts, meaning the intermediate populations formed in each organism are distinct. However, it appears that each of the differences observed in SSU assembly may be a result of these bacteria thriving in different environmental conditions. Optimum growth temperature as well as other physiological differences may require differences in rRNA, r-protein, and assembly factors that cause SSU assembly to proceed differently. However, the end result of SSU assembly and biogenesis, a functional 30S subunit must be maintained

for cell survival. The presence of structurally distinct non-functional assembly intermediates could be manipulated to produce antimicrobials to stall SSU assembly during multiple stages and produce RNPs incapable of translation.

MATERIALS AND METHODS

Bacterial strains and growth conditions

E. coli strain MRE600 was grown in 2xYT (1.6% Tryptone, 1% Yeast extract, 0.5% NaCl [w/v]) medium at 37°C using baffled flasks and agitated at 200RPM. *Geobacillus kaustophilus* (ATCC # 8005) was grown at 55°C in LB (1% Tryptone, 0.5% Yeast Extract, 1% NaCl [w/v]) or 2xYT medium, using baffled flasks and agitated at speeds from 200 to 250 RPM. *T. thermophilus* (ATCC # 27634) was grown in 697 *Thermus* medium (0.4% Yeast extract, 0.8% polypeptone, 0.2% NaCl [w/v] at pH to 7.5) at 65°C–70°C using baffled flasks and shaken at speeds from 200 to 250 RPM. All bacterial cell growths for purification of RNP components were started using saturated cultures and grown to an OD₆₀₀ of 0.6–0.8. Cells were then washed as described in Cameron et al. (2004) and stored at –80°C.

Purification of 16S rRNA, 30S, 50S, 70S ribosomes and TP30

Preparation of *E. coli*, *G. kaustophilus*, and *T. thermophilus* 30S and 50S ribosomal subunits and 70S ribosomes were prepared as described (Cameron et al. 2004). 16S rRNA was prepared as described (Moazed et al. 1986) or by LiCl/Urea extraction (Talkington et al. 2005) and followed by purification described in Moazed et al. (1986). TP30 was prepared as detailed in Nierhaus (1990).

Reconstitution reaction

16S rRNA in Recon A– buffer (80 mM K⁺-HEPES [pH 7.6], 20 mM MgCl₂ and 0.01% Nikkol) was incubated at 42°C for 10 min. TP30 was added to a volume of Recon A– buffer and β-mercaptoethanol (BME) to obtain final concentrations of 330m KCl and

FIGURE 7. *E. coli* r-protein ratio of in vitro formed RNPs relative to natural 30S subunits (yellow bars) for intermediate 1 (purple bars) (formed at 4°C), intermediate 2 (blue bars) (formed at 25°C), and in vitro formed 30S subunits (green bars) (formed at 42°C).

6mM BME. The TP30 mix was then incubated at room temperature for 10 min. Both the 16S rRNA mix and TP30 mixes were then placed on ice for a minimum of 5 min after which the 16S rRNA mix, TP30 mix, and Recon A+ buffer (80 mM K⁺-HEPES 7.6, 20 mM MgCl₂, 330 mM KCl, and 0.01% Nikkol) were combined to yield a concentration of 16S rRNA of 0.4 pmol/μL. The mix was then incubated at the appropriate temperature for a minimum of 30 min. The reconstitution mix was then placed on ice prior to being loaded onto sucrose gradients for sedimentation analysis (Culver and Noller 1999) or subjected to tRNA binding assay (see below).

Sucrose-gradient formation, sedimentation, fractionation, and analysis by QtiPlot

Sucrose gradients were formed as described in Culver and Noller (1999) with the gradient of 10%–40% being formed using the BioComp gradient master, tubes were allowed to cool for at least 2 h before samples were added. Samples were loaded to the top of the gradient and spun at 31,000 rpm for 16.5 h. Fractionation was performed using the BioComp piston fractionator system. The software QtiPlot was used to analyze sucrose-gradient profiles. The profiles were fitted to the minimum number peaks to establish a good fit of the profile with the Cauchy–Lorentzian distribution. The software determined the area under each peak and the relative percent area to SSUs formed at the optimal reconstitution temperature was calculated.

Transfer RNA binding reaction

Transfer RNA binding was performed as previously described (Culver and Noller 1999) with the following modifications. A volume of 25–100 μL of reconstitution mix was added to tRNA binding buffer A (80 mM K⁺-HEPES (pH 7.6), 20 mM MgCl₂) containing a twofold excess of tRNA^{Phe} and Poly-Uridine (1 pmol tRNA:1 μg Poly-Uridine) and a final concentration of 100 mM KCl. The mix was then incubated at 15°C for 30 min followed on ice for at least 10 min. Reactions were spotted onto nitrocellulose paper using the Millipore 1225 sampling manifold, washed with 30 mL of tRNA binding buffer B (20 mM MgCl₂, 100 mM KCl, 80 mM K⁺-HEPES [pH 7.6]) and counted by scintillation counter.

T1 digestion and primer extension analysis

RNPs were formed in at least triplicate, at the temperature indicated, followed by sucrose-gradient sedimentation and fractionation. Fractions corresponding to the RNP of interest were collected, concentrated, and buffer exchanged into RA+ (see above) using ultrafiltration with a 100,000 molecular weight cutoff. Partial RNase T1 digestion (enzymatic-probing) was performed using 0.2 Units RNA Fermentas RNase T1 per picomol of RNA. Enzymatic-probing was performed in the presence of 2 M Urea and 20 mM Tris similar to Morgan and Brimacombe (1972) on ice for 15 min. RNA was precipitated using 2.5 volumes of RNA precipitation mix (85% EtOH, 100 mM NaOAc, 625 μg/μL glycogen), stored at –80°C for 15 min, briefly thawed on ice and then spun at 13,000 rpm for 15 min. RNA was resuspended in 50 μL of RNA resuspension buffer containing 1 mg/mL Proteinase K as described in Khaitovich et al. (1999) and agitated at 4°C for 1 h. Following Proteinase K digestion, RNA extrac-

tion, and primer extension was done essentially as described by Merryman and Noller (1998).

Primer extension reactions were run on multiple gels for varying lengths of time, followed by gels being exposed to X-ray film for an appropriate length of time prior to being developed. Images of representative films were then obtained using the Versadoc system by BioRad and images quantified using Image Lab by BioRad.

Proteomics analysis

Reconstituted RNPs were separated by sucrose-gradient sedimentation and fractionated using BioComp piston fractionator. Peaks of interest were concentrated and buffer exchanged into Protein Storage Buffer (1 M KCl, 20 mM MgCl₂, 80 mM K⁺-HEPES [pH 7.6]) using ultrafiltration units with a 3000 molecular weight cutoff ensuring little to no specific r-protein loss and the retentate precipitated. Samples were then dialyzed into 100 mM ammonium bicarbonate and dried using a vacuum concentrating system at room temperature. Three biological triplicates were submitted to University of Rochester Proteomics facility where LC/MS/MS was performed in technical triplicate (nine total samples for each RNP formed). Data analysis was performed using Scaffold software. R-protein values are shown relative to natural SSUs with a maximum value of 1.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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