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Osmolytes stimulate the reconstitution of functional 50S ribosomes from in vitro transcripts of *Escherichia coli* 23S rRNA

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ABSTRACT

Functional *Escherichia coli* 50S ribosomal subunits can be reconstituted from their natural rRNA and protein components. However, when the assembly is performed with in vitro-transcribed 23S rRNA, the reconstitution efficiency is diminished by four orders of magnitude. We tested a variety of chemical chaperones (compounds that are typically used for protein folding), putative RNA chaperones (proteins) and ribosome-targeted antibiotics (small-molecule ligands) that might be reasoned to aid in folding and assembly. Addition of the osmolyte trimethylamine-oxide (TMAO) and the ketolide antibiotic telithromycin (HMR3647) to the reconstitution stimulates its efficiency up to 100-fold yielding a substantially improved system for the in vitro analysis of mutant ribosomes.

Keywords: in vitro reconstitution; ribosome assembly; RNA folding; telithromycin; trimethylamine oxide (TMAO)

INTRODUCTION

The large subunit of the eubacterial ribosome has a molecular weight of 1.6 million Da and is composed of two RNAs (23S and 5S) and approximately 30 different proteins. The assembly of this ribonucleoprotein particle in vivo depends on the ordered interaction of proteins with the rRNA as transcription occurs (Nierhaus, 1991). Assembly of the large subunit in vitro from its natural rRNA and protein components is not only possible but remarkably efficient (about 40% of the 23S rRNA is incorporated into a functional particle; Fahnestock et al., 1974; Dohme & Nierhaus, 1976; Londei et al., 1986). Although the general features of this assembly pathway in vitro must mimic the in vivo pathway, the time scales differ considerably. The protocol for *Escherichia coli* large subunit reconstitution involves a two-step incubation, first at low temperature (44 °C) and low magnesium concentration (4 mM), followed by higher temperature (50 °C) and magnesium concentration (20 mM) over a time period of more than an hour (Dohme & Nierhaus, 1976). That such a simple protocol can promote efficient reconstitution implies that strong determinants for ordered assembly are found in the various ribosomal components.

Despite the robust reconstitution observed with natural ribosomal components, several years ago we observed that the substitution of in vitro-transcribed 23S rRNA for the natural transcript (lacking the naturally occurring posttranscriptional modifications) decreased the efficiency of the reconstitution by up to four orders of magnitude. Further experiments suggested that the limitations of in vitro transcripts in the *E. coli* system could be localized to an 80-nt region of domain V of 23S rRNA containing six different posttranscriptional modifications (Green & Noller, 1996). Though these modified nucleotides are not among those that appear to be most conserved across phylogeny (Sirum-Connolly et al., 1995), these data have been interpreted as consistent with a critical role for posttranscriptional modification in ribosome assembly and/or function.

The function of modified nucleotides in ribosomal RNA is not fully understood, though a growing body of evidence suggests that they play a structural rather than catalytic role. Nucleoside pseudouridylations in RNA can increase the rigidity of an RNA structure by enhancing base stacking (Auffinger & Westhof, 1998). The observations that the archeal hyperthermophile *Sulfolobus solfataricus* has an exceptionally high number of modified nucleosides, and that modification levels can vary in response to growth temperature (Buck

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& Ames, 1984; Dalluge et al., 1997; Noon et al., 1998), are consistent with the idea that modifications stabilize structure in extreme conditions. A recent solution structure of the A loop of *E. coli* 23S rRNA in the absence and the presence of a ribose methylation modification at U2552 demonstrates that the presence of the modification within the loop restricts and stabilizes a particular conformation (Blanchard & Puglisi, 2001).

More recently, large subunit ribosomal particles have been efficiently assembled using in vitro-transcribed 23S rRNA in two different thermophilic organisms, *Bacillus stearothermophilus* and *Thermus aquaticus* (Green & Noller, 1999; Khaitovich et al., 1999), indicating that posttranscriptional modifications are not fundamental to the basic functions of the ribosome. From this, we can speculate that the poor reconstitution efficiency observed with in vitro transcripts of *E. coli* 23S rRNA is fundamentally a folding problem. On the one hand, RNA has the well-documented tendency to form stable folding intermediates that compete with productive assembly pathways (Herschlag, 1995; Pan et al., 1997; Pan & Sosnick, 1997). RNA chaperones or mild denaturants can increase the sampling by the RNA species of alternate conformations, thus minimizing the effects of stable folding intermediates (Coetzee et al., 1994; Pan & Sosnick, 1997; Rook et al., 1998; Clodi et al., 1999). Alternatively, the unmodified 23S rRNA in vitro transcript may have lost some structural stability that was critical in determining its assembly pathway. Agents that stabilize RNA structure (either by specific or general interactions) might be expected to minimize these effects. A priori, either (or a combination) of these two very different problems might be responsible for the profound assembly defects observed in the *E. coli* system. Here we ask whether any of a number of classes of reagent have a positive effect on the in vitro assembly of functional large subunits from unmodified, in vitro-derived 23S rRNA.

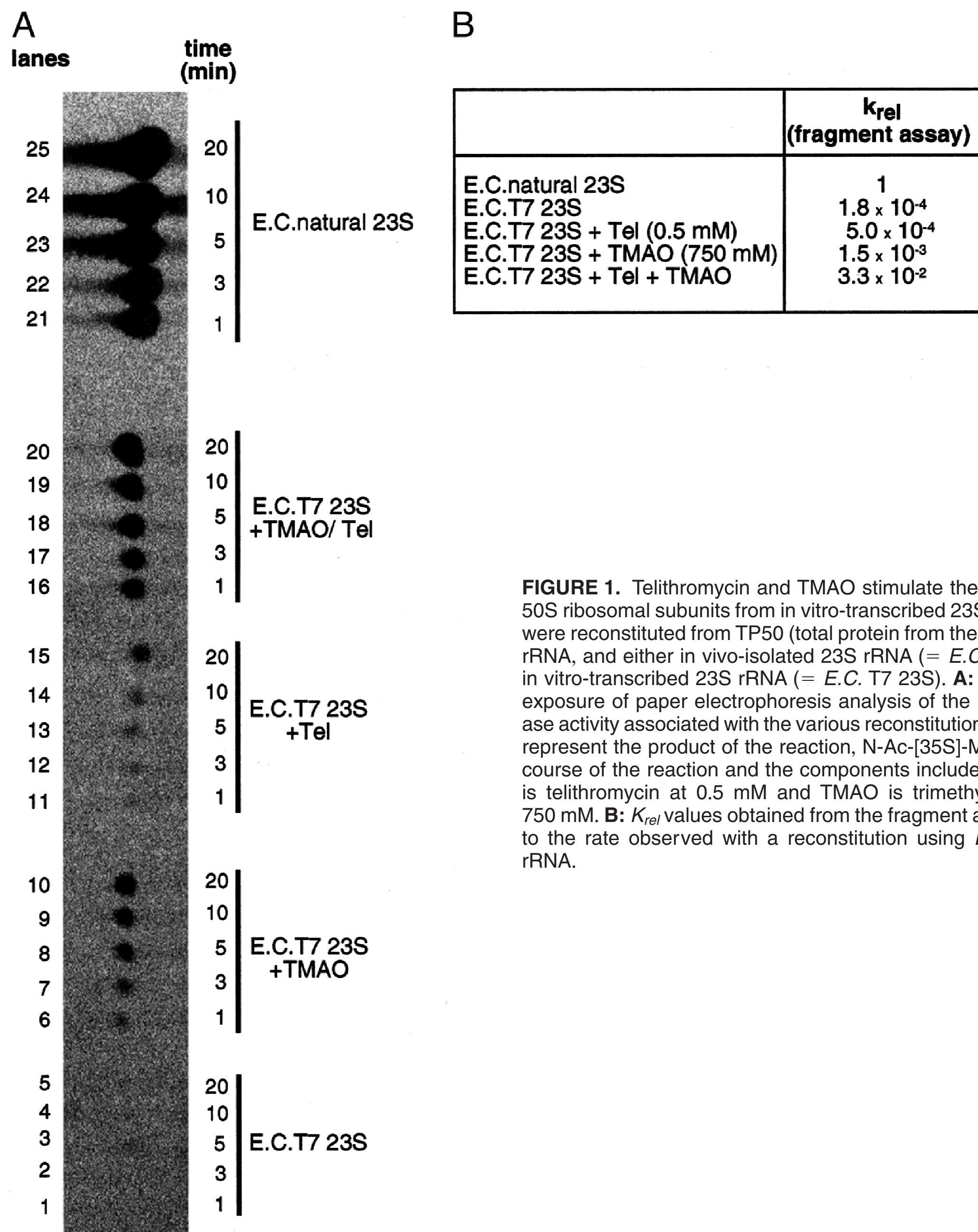
RESULTS

Two different classes of compounds increase the reconstitution efficiency of in vitro-transcribed *E. coli* 23S rRNA into functional 50S particles

The reconstitution of functional *E. coli* 50S ribosome particles from in vitro-transcribed 23S rRNA (*E.C.* T7 23S) is not efficient. Using standard procedures, the reconstitution reaction with in vitro-transcribed 23S rRNA is four orders of magnitude diminished in peptidyl transferase (PT) activity (as measured in the fragment reaction) relative to the same reconstitution reaction with natural (in vivo-derived) 23S rRNA (Fig. 1A, lanes 1–5 and lanes 21–25; Green & Noller, 1996). Here we have screened for compounds that increase the overall efficiency of the in vitro reconstitution reaction. Briefly, a standard reconstitution reaction containing natural or in vitro-transcribed 23S rRNA, 5S rRNA, and TP50 (total proteins from the 50S subunit) was incubated with a collection of reagents (including antibiotics, RNA chaperones, chemical chaperones, and denaturants) at varying concentrations (see Table 1). Where possible, the added reagent was removed from the reconstitution reaction by dialysis overnight prior to assessing the reconstitution efficiency.

Overall activity of the reconstituted 50S ribosomal particles was assessed using two different peptidyl transferase assays. The fragment reaction utilizes minimal tRNA substrates (CAACCA-N-Ac-Methionine and puromycin) to follow the formation of a single peptide bond on isolated 50S subunits, independent of the 30S subunit or added mRNA template (Monro & Marcker, 1967). The products of the reactions were resolved by paper electrophoresis and quantitated as previously described (Kim & Green, 1999). A second assay uses intact aminoacylated tRNA (N-Ac-Phe-$tRNA_{Phe}$) as a peptidyl tRNA substrate and radioactively labeled [^{32}P]-CPm (cytidyl-puromycin) as a minimal aminoacyl substrate. These substrates are incubated with 30S subunits programmed with poly-uridine mRNA and the reconstituted 50S subunit. The formation of the dipeptidyl product was monitored by polyacrylamide gel electrophoresis (Kim & Green, 1999). The addition of peptidyl hydrolase to the intact tRNA assay did not alter the reaction rate, suggesting that the reaction is measuring a single turnover event catalyzed by the reconstituted ribosome population (data not shown; Kim et al., 2001). By contrast, in the fragment reaction, the presence of peptidyl-hydrolase did diminish the reaction rate, suggesting that there is significant substrate (tRNA fragment) dissociation from the reconstituted 50S subunit during the time course of the reaction. These results are consistent with known binding affinities of intact tRNA (nM; Lill et al., 1986) and minimal tRNA substrates (μM; R. Green, unpubl. observations). Overall, the fragment reaction is expected to be the most sensitive to irregularities in the reconstituted particles due to the low binding affinity of the minimal substrates. In the intact tRNA assay, binding interactions with the natural 30S subunit by the anticodon end of the P-site substrate can compensate for some of the binding deficiencies of a reconstituted 50S particle.

Two different classes of compound tested substantially increased the peptidyl transferase activity of the reconstituted ribosomes made from in vitro-transcribed 23S rRNA. First, the ketolide antibiotic telithromycin (HMR3647) stimulated the reconstitution reaction by approximately threefold as determined by the fragment peptidyl transferase assay (Fig. 1A, lanes 11–15 vs. lanes 1–5 and Fig. 1C). When 5S rRNA is excluded from the reconstitution reaction, the ketolide antibiotic is not able to stimulate the reconstitution (data not shown) as had been previously observed in the *T. aquat-*



	k_{rel} (fragment assay)
E.C.natural 23S	1
E.C.T7 23S	1.8×10^{-4}
E.C.T7 23S + Tel (0.5 mM)	5.0×10^{-4}
E.C.T7 23S + TMAO (750 mM)	1.5×10^{-3}
E.C.T7 23S + Tel + TMAO	3.3×10^{-2}

FIGURE 1. Telithromycin and TMAO stimulate the reconstitution of 50S ribosomal subunits from in vitro-transcribed 23S rRNA. Particles were reconstituted from TP50 (total protein from the 50S subunit), 5S rRNA, and either in vivo-isolated 23S rRNA (= *E.C.* natural 23S) or in vitro-transcribed 23S rRNA (= *E.C.* T7 23S). **A:** Phosphorimager exposure of paper electrophoresis analysis of the peptidyl transferase activity associated with the various reconstitution reactions. Spots represent the product of the reaction, N-Ac-[35S]-Met-Pm. The time course of the reaction and the components included are shown. Tel is telithromycin at 0.5 mM and TMAO is trimethylamine oxide at 750 mM. **B:** K_{rel} values obtained from the fragment assay normalized to the rate observed with a reconstitution using *E.C.* natural 23S rRNA.

icus system (Khaitovich & Mankin, 1999). Telithromycin did not affect the activity of reconstitution reactions containing natural (in vivo-isolated) 23S rRNA (data not shown). Other antibiotics targeting the peptidyl transferase active site (chloramphenicol or sparsomycin) or locations critical for translocation (viomycin) had no apparent effect on the efficiency of the reconstitution reaction (Table 1).

A second class of compounds known as osmolytes also significantly increased the activity of the reconstitution reaction from in vitro transcripts. In this class, two particular compounds, trimethylamine-oxide (TMAO) and betaine (both containing a trimethylamine moiety), were most effective in stimulating the reconstitution reaction (Fig. 1A, lanes 6–10; Table 1). As measured in the fragment reaction, these compounds stimulated the reconstitution by 5- to 10-fold. Other related compounds such as sarcosine and taurine had no stimulatory effects. TMAO and betaine had no detectable effect on the already efficient reconstitution reaction with natural 23S rRNA transcripts.

The antibiotic telithromycin and the osmolyte TMAO, both increase reconstitution efficiency (see Fig. 1 and Table 1) though their modes of action are likely different. We asked if the effects of these compounds were additive in the reconstitution reaction. Using the frag-

TABLE 1. List of all compounds that were tested for their effect on in vitro reconstitution of *E. coli* 50S particles with in vitro-transcribed 23S rRNA.[a]

Compound	Titrated range or optimal concentration	Effect on reconstitution of *E.C.* T7 23S	Effect on reconstitution of *E.C.* natural 23S
HMR 3647 (telithromycin)	0.1 mM–5 mM (0.5 mM)	3× increase	no effect
Viomycin	0.05 mM–0.2 mM	no effect	no effect
Chloramphenicol	0.05 mM–0.2 mM	no effect	no effect
Sparsomycin	0.1 mM–0.2 mM	no effect	no effect
StpA (*E. coli* protein, 15.3 kDa)	35 nM–14 μM (14 μM)	3× increase	no effect
HNS	2.2 μM–14 μM	no effect	no effect
Glycerol	1%–30%	no effect	no effect
Polyvinylalcohol	1%–10%	no effect	no effect
PEG	1%–10%	no effect	no effect
TMAO $(CH_3)_3-N=O$	0.5 mM–1 M (750 mM)	10× increase	no effect
Betaine $(CH_3)_3-N^+-CH_2-COO^-$	500 mM–1 M (1 M)	5–10× increase	no effect
Sarcosine $CH_3-NH-CH_2-COOH$	500 mM–1 M	no effect	no effect
Sorbitol	500 mM–1 M	no effect	no effect
Mannitol	250 mM–500 mM	no effect	no effect
Taurine	100 mM–500 mM	no effect	no effect
Urea	57 mM–125 mM	no effect	decrease in activity
Guanidine-hydrochloride	45 mM–75 mM	no effect	n.d.*
CHAPS	0.1 mM–200 mM	no effect	decrease in activity
PBA (4-phenylbutyric acid)	100 mM	no effect	decrease in activity
2-methyl-pentanediol	10%–50%	no effect	decrease in activity
Triton X-100	0.04%–4%	no effect	n.d.*
Tri-fluoro-ethanol	1%–10%	no effect	n.d.*
Hexa-fluoro-isopropanol	2.5%–7.5%	no effect	n.d.*

[a]Column 2 shows the range of concentrations that were tested for the respective compounds and, in the case of an increase in reconstitution efficiency, the concentration that gave highest PT activity of the resulting particles is shown in parentheses. When reconstitution efficiency was enhanced, the increase in PT activity is shown in *x*-fold increase compared to *E.C.* T7 23S particles reconstituted without the compound (column 3). The tested compounds are listed group-wise starting from antibiotics (rows 1–4), RNA-chaperones (rows 5–6), osmolytes (rows 7–15), denaturants (rows 16, 17), and miscellaneous chemical chaperones and fluorinated alcohols (rows 18–23).

*n.d.: not determined.

ment reaction, we found that the combined activity of telithromycin and TMAO on the reconstitution efficiency was substantially higher (approximately 200-fold stimulated relative to the reconstitution reaction without either compound) than anticipated based on their individual properties (3-fold and 10-fold). None of the other tested compounds had any significant effect on the reconstitution reaction containing both TMAO and telithromycin.

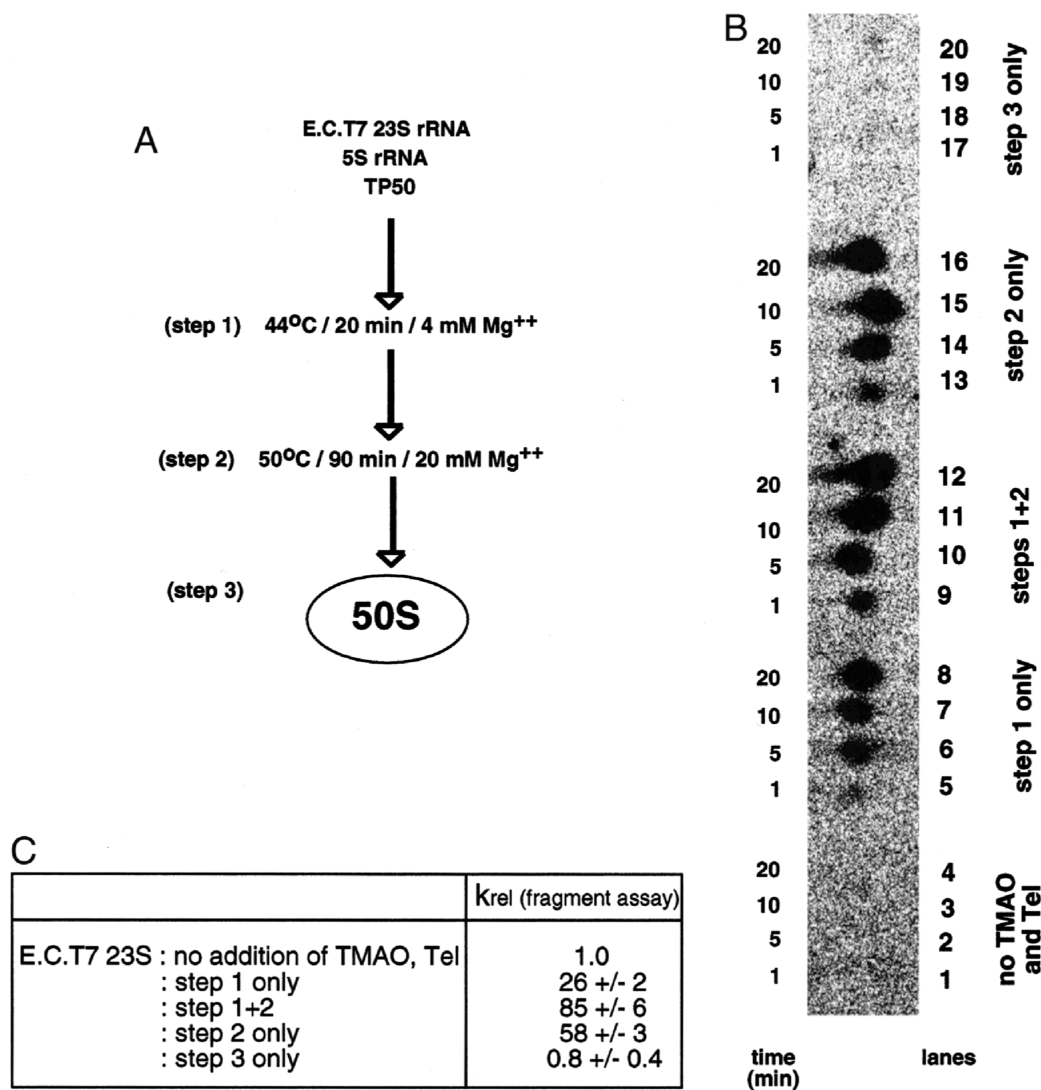
Modest increases (approximately threefold) in the activity of the reconstituted particles were observed with the RNA chaperone StpA. However, because its presence did not further stimulate the reaction containing TMAO and telithromycin, we did not further pursue this effect. Several other classes of compounds were tested for stimulation of reconstitution including the poly-ol osmolytes (glycerol, sorbitol, etc.), fluorinated alcohols, and denaturants such as urea and guanidinium chloride (Table 1). None of these compounds had any positive effect on reconstitution, even when combined with the stimulating reagents TMAO and telithromycin.

As a further test of the effects of TMAO and telithromycin on reconstitution efficiency, we looked at the activity of the treated reconstitution reaction using the intact tRNA peptidyl transferase assay (described above). In this assay, the peptidyl transferase activity associated with the reconstitution reaction using in vitro-transcribed 23S rRNA (without TMAO and telithromycin) is only 10-fold lower than that associated with the reaction using natural 23S rRNA. TMAO and telithromycin had no obvious stimulatory effects on the activity of the reconstitution reactions with in vitro transcripts as assessed by the intact tRNA assay (data not shown).

TMAO and telithromycin influence assembly during both steps of reconstitution

The standard reconstitution protocol for *E. coli* 50S subunits involves a first incubation step at low temperature (44 °C) and low magnesium (4 mM), which is then followed by incubation at high temperature (50 °C) and

high magnesium (20 mM; Dohme & Nierhaus, 1976). To investigate at which step telithromycin and TMAO affect the assembly process, the reagents were added at various points during the reconstitution protocol (Fig. 2A). First, TMAO and telithromycin were added to the reconstitution reaction at the start of the reconstitution so that the compounds were present throughout the entire protocol (Steps 1 and 2). Next, the compounds were added at the start but dialyzed after the first step of reconstitution (Step 1 only). Next, both compounds were added after the first and before the second step of incubation (Step 2 only). Finally, both compounds were added at the completion of the incubations to samples sitting at 4 °C (Step 3 only). All of the reconstitution reactions were dialyzed to remove the stimulatory agents prior to assaying the peptidyl transferase activity. As controls, we confirmed that the compounds have no effect on reconstitution with a natural transcript or on peptidyl transferase activity with natural 50S subunits. The k_{rel} values calculated from the experiment (Fig. 2C) indicate that the greatest increase in reconstitution efficiency was obtained when telithromycin and TMAO were present during the entire reconstitution procedure (85-fold stimulation), though the effects were only modestly reduced if the compounds were only included in the second step (58-fold



	krel (fragment assay)
E.C.T7 23S : no addition of TMAO, Tel	1.0
: step 1 only	26 +/- 2
: step 1+2	85 +/- 6
: step 2 only	58 +/- 3
: step 3 only	0.8 +/- 0.4

FIGURE 2. Telithromycin and TMAO most effectively stimulate reconstitution when present throughout the reconstitution protocol. **A:** Schematic describing the reconstitution protocol and the stages when the stimulatory compounds were added. **B:** Phosphorimager exposure of paper electrophoresis analysis of the peptidyl transferase activity associated with the various reconstitution reactions. Spots represent the product of the reaction, N-Ac-[35S]-Met-Pm. The time course of the reaction, the components included, and the stage when they were added are detailed. **C:** K_{rel} values obtained from the fragment assay normalized to the rate observed with a reconstitution using *E.C.* T7 23S rRNA with no telithromycin and TMAO added.

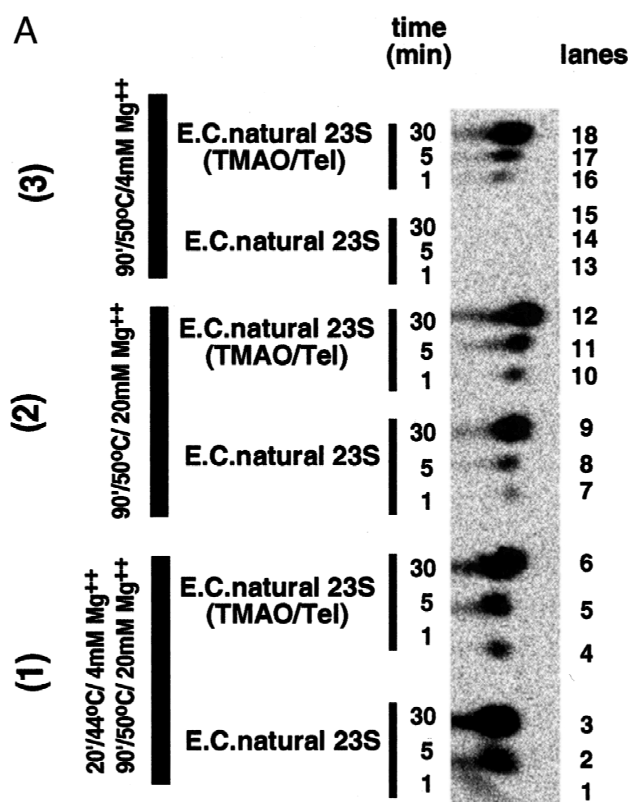
stimulation). Indeed, the milder stimulation (26-fold) observed when the compounds were only included in the first incubation step may be the result of residual reagent left by incomplete dialysis. When the compounds were added after completion of the reconstitution protocol, no stimulatory effect was seen.

TMAO and telithromycin partly compensate for low levels of magnesium during reconstitution of natural 23S rRNA-containing particles

The efficient reconstitution of in vivo derived (natural) 23S rRNA into 50S particles is not affected by the addition of TMAO and telithromycin. We next asked whether the obligate two-step reconstitution protocol might be simplified by the addition of telithromycin or TMAO. As seen in Figure 3, reconstitution solely under the conditions of Step 2 (50 °C and 20 mM Mg^{2+}) with natural 23S rRNA reduces the reconstitution efficiency by threefold whereas reconstitution solely under conditions of 50 °C and 4 mM Mg^{2+} reduces the efficiency by at least four orders of magnitude. Interestingly, the addition of telithromycin and TMAO to these reconstitution reactions has little effect on the normal two-step protocol (Fig. 3A, lanes 1–3 vs. 4–6) or the single Step 2 only protocol (Fig. 3A, lanes 7–9 vs. 10–12), but has an enormous effect on the modified protocol with 50 °C and 4 mM Mg^{2+} (Fig. 3A, lanes 13–15 vs. 16–18). In this case, addition of the compounds stimulates the reconstitution efficiency nearly to the levels observed in the two-step protocol (nearly four orders of magnitude).

In vitro reconstitution of 23S rRNA transcripts in the presence of TMAO and telithromycin provides an in vitro system for studying rRNA mutants

The addition of TMAO and telithromycin to the ribosome reconstitution reactions using in vitro transcripts substantially increases the level of associated PT activity allowing for the analysis of mutant ribosomal populations. Mutations in the 23S rRNA were generated at three different positions $C_{2499}U$, $C_{2452}U$, and $A_{2058}G$ where previous reports had implicated these positions in resistance to the antibiotics sparsomycin, chloramphenicol, and clindamycin, respectively (Fig. 4; Slott et al., 1983; Harris et al., 1989; Tan et al., 1996). Mutant rRNAs were transcribed and incorporated by reconstitution into 50S particles and then assessed by peptidyl transferase assays. The Sparso® mutant ($C_{2499}U$) had slightly increased levels of PT-activity (almost twofold) in the fragment reaction, whereas $C_{2452}U$ (CAM®) showed decreased (two- to threefold) activity in both assays compared to the wild type (*E.C.* T7 23S) particles (see Fig. 5). Interestingly, $A_{2058}G$ had no detectable activity in the fragment reaction and yet had substantial activity (within twofold of wild type) in the intact tRNA assay. These data are consistent with the idea discussed earlier suggesting that the fragment reaction is more sensitive to structural perturbations than the intact tRNA assay. Only the $C_{2452}U$ (CAM) mutant showed some resistance to the relevant antibiotic (chloramphenicol) in the peptidyl transferase assay (Fig. 5C). The $C_{2499}U$ and $A_{2058}G$ ribosomes were not resistant to sparsomycin and clindamycin as had been predicted from previous in vitro experiments.



B

Protocol for E.C.natural 23S		k_{rel} (fragment assay)
2-step	(no addition)	1
2-step	(+ TMAO / Tel)	0.83
1-step, 20 mM Mg++	(no addition)	0.33
1-step, 20 mM Mg++	(+ TMAO / Tel)	0.26
1-step, 4 mM Mg++	(no addition)	8×10^{-5}
1-step, 4 mM Mg++	(+ TMAO / Tel)	0.26

FIGURE 3. Telithromycin and TMAO stimulate the reconstitution of 50S ribosomal subunits from natural 23S rRNA under suboptimal reconstitution conditions. **A:** Phosphorimager exposure of paper electrophoresis analysis of the peptidyl transferase activity associated with the various reconstitution reactions. Spots represent the product of the reaction, N-Ac-[35S]-Met-Pm. The time course of the reaction, the components included, and the incubation conditions are detailed. **B:** K_{rel} values obtained from the fragment assay normalized to the rate observed with the standard reconstitution protocol with natural 23S rRNA and no telithromycin and TMAO added.

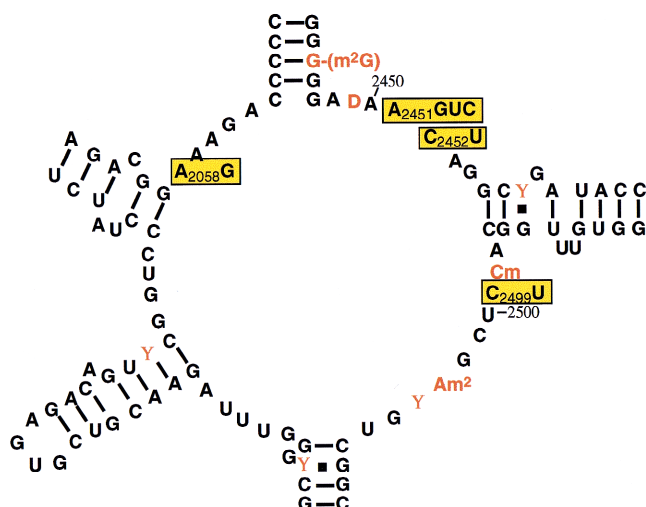
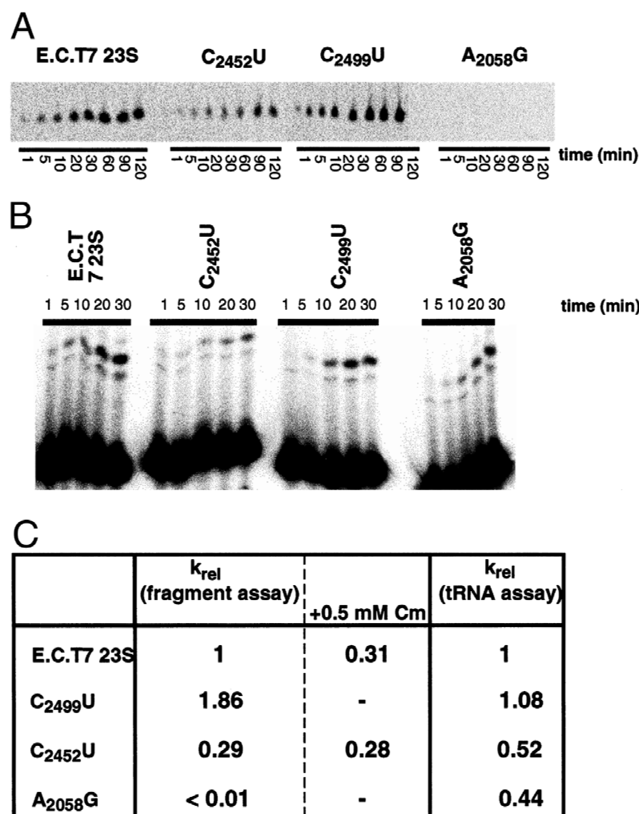


FIGURE 4. Peptidyl transferase loop from domain V of *E. coli* 23S rRNA. The nucleotide positions that were mutated and tested in the modified in vitro reconstitution system are highlighted in yellow. Post-transcriptionally modified nucleotides in this region are shown in red.



	k_{rel} (fragment assay)	+0.5 mM Cm	k_{rel} (tRNA assay)
E.C.T7 23S	1	0.31	1
$C_{2499}U$	1.86	-	1.08
$C_{2452}U$	0.29	0.28	0.52
$A_{2058}G$	< 0.01	-	0.44

FIGURE 5. Site-directed mutations in domain V of 23S rRNA (*E. coli* numbering) alter reconstitution efficiency. Wild-type (*E.C.* T7 23S) and mutant 23S rRNAs were reconstituted in the presence of TMAO and telithromycin and tested in the fragment assay (**A**) and the intact tRNA assay (**B**). All reconstitutions were performed in the presence of TMAO and telithromycin. **C:** K_{rel} values for the mutant 23S rRNAs relative to the wild-type sequence (*E.C.* T7 23S) obtained from the two different PT assays. The effect of chloramphenicol (Cm at 0.5 mM) was compared for wild-type and the $C_{2452}U$ mutant rRNA in the fragment assay.

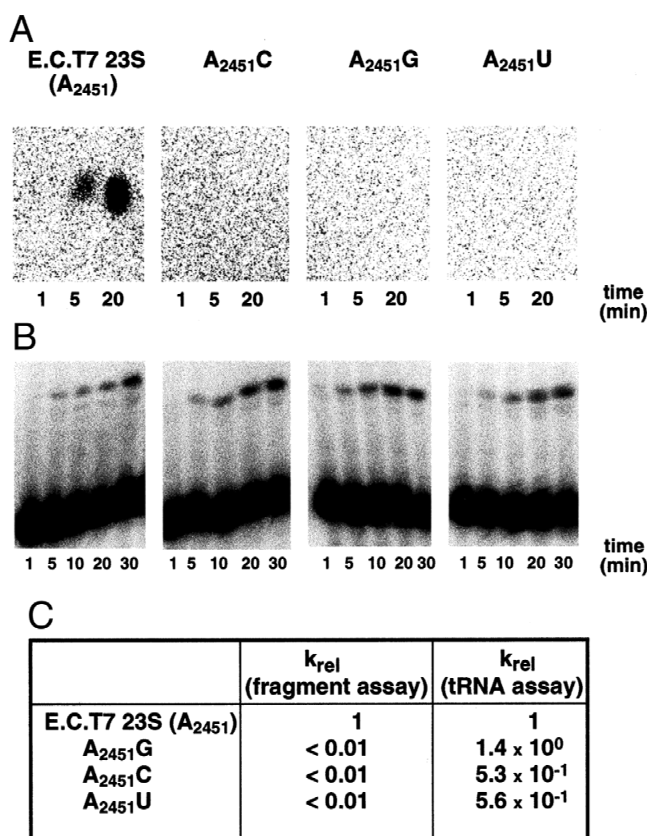
Analysis of mutations in the peptidyl transferase active site (A_{2451})

In the atomic resolution structure of the large subunit of the ribosome, nucleotide A_{2451} in domain V of 23S rRNA is found proximal to a bound transition state analog and has been proposed to play a direct role in the catalysis of peptide bond formation (Ban et al., 2000). We made a series of mutations in the *E. coli* reconstitution system (A_{2451} to C, G, and U) and compared the results to what had been observed in the *B. stearothermophilus* and *T. aquaticus* systems (Polacek et al., 2001; Thompson et al., 2001). In the fragment reaction, none of the three mutants (A2451C, G, or U) showed any detectable activity when compared to wild type (*E.C.* T7 23S) particles (Fig. 6A,C). However, in the intact tRNA assay, the activity of each mutant was comparable to that of the wild-type reconstitution (Figs. 6B,C). In a final set of experiments, we asked whether A2451C mutant ribosomes displayed any resistance to the antibiotic chloramphenicol as had previously been observed (Thompson et al., 2001). As previously reported, the mutant ribosomes are moderately resistant to chloramphenicol relative to the wild-type version (data not shown).

DISCUSSION

The in vitro assembly of the large ribosomal subunit from its individual RNA and protein components occurs with reasonably high efficiency in a number of bacterial systems (Fahnestock et al., 1974; Dohme & Nierhaus, 1976; Londei et al., 1986). Whereas the standard *E. coli* 50S subunit in vitro reconstitution protocol depends on two different incubation steps (Step 1: 44 °C and 4 mM Mg^{2+}, and Step 2: 50 °C and 20 mM Mg^{2+}), in a number of other organisms a two-step protocol is not required (e.g., Londei et al., 1986). Precise variation of the divalent metal ion concentration in this protocol is consistent with the critical role of Mg^{2+} in establishing higher order RNA structure (Brion & Westhof, 1997). The two-step incubation protocol must somehow prevent the accumulation of stable off-pathway intermediates in the early assembly steps of the 50S subunit. Such stable RNA intermediates have been described in a number of simpler systems (Treiber & Williamson, 2001). There are likely to be numerous mechanisms in vivo for favoring the correct folding pathway, including an ordered assembly, precise milieu, and the presence of a variety of RNA chaperones (Herschlag, 1995).

Somewhat surprisingly, in *E. coli*, whereas in vitro assembly is efficient with natural posttranscriptionally modified RNA, unmodified in vitro transcripts are incorporated with poor efficiency (Green & Noller, 1996). Reconstitutions of the large subunit from in vitro-



	k_{rel} (fragment assay)	k_{rel} (tRNA assay)
E.C.T7 23S (A_{2451})	1	1
A_{2451}G	< 0.01	1.4×10^{0}
A_{2451}C	< 0.01	5.3×10^{-1}
A_{2451}U	< 0.01	5.6×10^{-1}

FIGURE 6. Effect of mutation of A_{2451} in the catalytic center of the ribosome. **A:** Wild-type (*E.C.* T7 23S) and mutant A_{2451} to G, C, or U 23S rRNA reconstitution reactions were tested in the fragment assay (**A**) and the intact tRNA assay (**B**). All reconstitutions were performed in the presence of TMAO and telithromycin. **C:** K_{rel} values for the mutant 23S rRNAs relative to the wild-type sequence (*E.C.* T7 23S) obtained from the two different PT assays.

transcribed 23S rRNA from the thermophilic organisms *T. aquaticus* and *B. stearothermophilus* are significantly more robust (Green & Noller, 1999; Khaitovich et al., 1999). Because the *E. coli* ribosome has been well characterized both biochemically and genetically, we have been interested in improving the assembly of *E. coli* large subunits from in vitro transcripts to allow for more thorough in vitro mutant analyses. We have also been interested in understanding why unmodified transcripts are such poor substrates for the in vitro assembly pathway. The precise requirements of the *E. coli* reconstitution procedure suggest that this 23S rRNA might be particularly troubled by off-pathway stable folding intermediates. We reasoned that the addition of small molecules or protein chaperones that either loosen or stabilize RNA structure might substantially improve the reconstitution reaction with in vitro-transcribed 23S rRNA.

In this study, we screened for compounds that increase the reconstitution efficiency of *E. coli* 23S rRNA in vitro transcripts. Briefly, a variety of compounds were added directly to the standard *E. coli* reconstitution reaction and the efficiency of the reconstitution reaction was assessed by two different peptidyl transferase assays. Because of its high sensitivity to structural imperfections, we routinely use the fragment reaction as a stringent test of the efficiency of a reconstitution reaction. Here we identify two different classes of compound that efficiently stimulate the *E. coli* reconstitution reaction with in vitro-derived transcripts (and in certain protocols with in vivo-derived transcripts). First, the osmolyte trimethylamine oxide significantly stimulates (approximately 10-fold) the efficiency of the reconstitution reaction as assessed by the fragment reaction (Fig. 1A). The antibiotic telithromycin also significantly stimulated the reaction (approximately threefold). When added together, the two compounds stimulated the reaction by almost 300-fold.

The assembly of the large ribosomal subunit is a highly complex process. An explicit understanding of how TMAO and telithromycin function to stimulate its assembly is beyond the scope of this work. We can, however, attempt to characterize the properties of these species and compare them with those that had little or no effect in this system (see Table 1). For example, the *E. coli* protein StpA had modest (at most threefold) effects on reconstitution (in the absence of other small compounds), consistent with its previously described role as an RNA chaperone (Zhang et al., 1995). General denaturants such as urea and guanidine hydrochloride that have been effective in other RNA systems, presumably by minimizing the effects of stable folding intermediates, were also not effective at a range of concentrations (Rook et al., 1998). Poly-ols such as glycerol, sorbitol and mannitol, commonly used for protein folding, had no effect (Shelanski et al., 1973; Gekko & Timasheff, 1981). Similarly, volume excluding reagents such as polyethylene glycol (PEG) and polyvinylalcohol (PVA), fluorinated alcohols, detergents, and other small molecules that have stimulated folding or reconstitution in other systems had no discernible effects (Cleland et al., 1992; Konno et al., 2000).

Telithromycin is a ketolide antibiotic (MW 812) that interacts with domains II and V of 23S rRNA and has been shown to stimulate the reconstitution of *T. aquaticus* large ribosomal subunits in the absence of 5S rRNA (Khaitovich & Mankin, 1999). Small molecules that specifically interact with the large subunit of the ribosome might stimulate (or stabilize) the adoption of a critical conformation during the folding process. None of the other large subunit specific antibiotics tested had similar effects (including chloramphenicol, viomycin, or sparsomycin). The stimulation observed here by telithromycin is likely related to that observed in previous experiments with *T. aquaticus* (Khaitovich & Mankin, 1999) though 5S rRNA was present in all of the reconstitution reactions.

Trimethylamine-oxide likely acts on the large subunit reconstitution in a much more general manner. TMAO

is a low molecular weight (MW 75 Da) compound belonging to a class known as the osmolytes (Santoro et al., 1992). This class of compounds is thought to function in protein folding by excluding water and predisposing species to adopt the most stable folded structural core (Yancey et al., 1982). Interestingly, such compounds can be found at extremely high levels in certain organisms (e.g., cartilaginous fishes and coelacanths) that concentrate urea (to 400 mM) in their systems (Lin & Timasheff, 1994). A simplistic view of this unusual finding is that these two compounds, urea and TMAO, have opposite effects on a system: Urea is overall destabilizing for macromolecular structure and TMAO balances these effects. Betaine is a related osmolyte with a trimethylamine core, that displayed similar properties in the in vitro reconstitution reaction. Our efforts to identify a ratio of denaturant (urea) and stabilizer (TMAO) that would effectively stimulate the reconstitution were not successful (data not shown).

It is not clear why TMAO and telithromycin synergistically improve the reconstitution efficiency of the large ribosomal subunit with in vitro transcripts. In vitro transcripts are missing posttranscriptional modifications that at least partially specify the appropriate folding pathway in the RNP assembly (Green & Noller, 1996). In such a compromised system (where in vitro transcripts are four orders of magnitude less efficiently incorporated than natural transcripts), large effects can be observed. We would propose that these two classes of compounds act by distinct mechanisms—TMAO by a general effect on RNA/protein structure and telithromycin by a specific effect in organizing the rRNA domains. The identification of unanticipated synergy between the two classes of compounds in this complex system is intriguing. Though the properties of the osmolytes are well studied in the protein folding field (Santoro et al., 1992), we believe that this is the first time that this class of compound has been observed to have substantial effects on the assembly or folding of RNA.

The combined action of TMAO and telithromycin provides us with a powerful tool for the analysis of *E. coli* mutant ribosome populations. Mutant transcripts are readily generated by standard approaches and can be incorporated into 50S subunit particles that can be functionally analyzed as in other systems (Green & Noller, 1999; Khaitovich et al., 1999). Previously, it was necessary to construct *E. coli* 23S rRNA from two pieces (one natural and one in vitro derived) to avoid complications associated with the extremely low reconstitution efficiency of intact in vitro-transcribed 23S rRNA (Samaha et al., 1995).

Here we constructed site-directed mutants in 23S rRNA at three sites previously associated with antibiotic resistance in the 23S rRNA (Slott et al., 1983; Harris et al., 1989; Tan et al., 1996) and at one site in the catalytic core of the ribosome proposed to be critical for peptide bond formation (Nissen et al., 2000). Using the optimized reconstitution system, the peptidyl transferase activity of the mutant ribosomes was assessed in the fragment reaction and in the intact tRNA peptidyl transferase assay. Whereas mutations at C2499 and C2452 had relatively minor effects on catalysis in either assay, mutation of A2058 to a G resulted in the complete loss of activity (at least 100-fold) in the fragment reaction and modest (2-fold) effects in the intact tRNA assay (Fig. 4). Similarly, mutation of the active site residue A2451 resulted in substantial (greater than 100-fold) effects on PT activity as assessed by the fragment reaction and only modest (2-fold) effects assessed by the intact tRNA assay (Kim & Green, 1999; Fig. 5). The implications of similar results obtained in the *B. stearothermophilus* and *T. aquaticus* systems have been discussed in detail elsewhere (Kim et al., 2001; Polacek et al., 2001; Thompson et al., 2001). The different results yielded by these PT assays provide a starting point for the dissection of the specific deficiencies of these mutant ribosomes. Interestingly, telithromycin effectively stimulated the reconstitution of A2058G mutant ribosomes, though the identity of this nucleotide is thought to be a critical binding determinant for this ketolide antibiotic (Khaitovich & Mankin, 1999). We anticipate that the system described here will allow for the detailed analysis of mutant ribosome populations that will be critical in reconciling models based on current high resolution X-ray crystallographic views of the ribosome (Ban et al., 2000; Yusupov et al., 2001).

MATERIALS AND METHODS

Site-directed mutagenesis and in vitro transcription

Oligonucleotide-directed mutations were constructed in the plasmid pCW1 (Weitzmann et al., 1990) using the QuikChange protocol (Stratagene) and were confirmed by sequencing. In vitro-transcribed 23S rRNA was generated with T7 RNA polymerase from linearized plasmid pCW (digested with *Afl*II) and its derivatives as previously described (Green & Noller, 1999). Natural 23S rRNA was prepared as previously described (Dohme & Nierhaus, 1976).

In vitro reconstitution of *E. coli* 50S subunits

The in vitro reconstitution of 50S subunits was performed essentially as described (Dohme & Nierhaus, 1976) using either natural 23S rRNA or in vitro-transcribed 23S rRNA from *Afl*II-digested plasmid pCW1 with the following modifications: 1.25 A_{260} of 23S rRNA (natural or in vitro-derived) and 0.05 A_{260} of 5S rRNA were incubated with 1 e.u. of TP50 in 20 mM Tris-HCl, 7.4, 4 mM MgAc, 400 mM NH_4Cl, 0.2 mM EDTA, and 5 mM β-mercaptoethanol for 20 min at 44 °C. Then the magnesium concentration was raised to 20 mM and the reactions were further incubated at 50 °C for 90 min. Various compounds were added to the reconstitution reac-

tions at the concentrations defined in the text (see Table 1). In Figure 2, TMAO and telithromycin were added at different stages during the incubation protocol. After the high-temperature incubations, the reconstitution reactions were dialyzed overnight against 20 mM Tris-HCl, 7.4, 20 mM MgAc, 400 mM NH_4Cl, 0.2 mM EDTA, and 5 mM β-mercaptoethanol. In the reactions where TMAO was added at different stages of the reconstitution protocol, reactions were dialyzed for 30 min.

Peptidyl transferase reactions

The fragment reaction was performed essentially as previously described (Green & Noller, 1996). The intact tRNA assay was performed essentially as described (Thompson et al., 2001) where the reactions were incubated at 37 °C.

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