

# Mutagenesis of the peptidyltransferase center of 23S rRNA: the invariant U2449 is dispensable

Michael O'Connor\*, Wyan-Ching Mimi Lee, Anuj Mankad, Catherine L. Squires<sup>1</sup> and Albert E. Dahlberg

J. W. Wilson Laboratory, Department of Molecular and Cellular Biology and Biochemistry, Brown University, Providence, RI 02912, USA and <sup>1</sup>Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA 02111, USA

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## ABSTRACT

**U2449 is one of many invariant residues in the central loop of domain V of 23S rRNA, a region that constitutes part of the peptidyltransferase center of the ribosome. In *Escherichia coli*, this U is post-transcriptionally modified to dihydrouridine (D) and is the only D modification found in *E.coli* rRNAs. To analyze the role of this base and its modification in ribosomal function, all three base substitutions were constructed on a plasmid copy of the *rrnB* operon and assayed for their ability to support cell growth in a strain of *E.coli* lacking chromosomal *rrn* operons. Both purine substitution mutations were not viable. However, growth and antibiotic sensitivity of cells expressing only the mutant D2449C rRNA was indistinguishable from wild type. We conclude that while a pyrimidine is required at position 2449 for proper ribosomal function, the D modification is dispensable.**

## INTRODUCTION

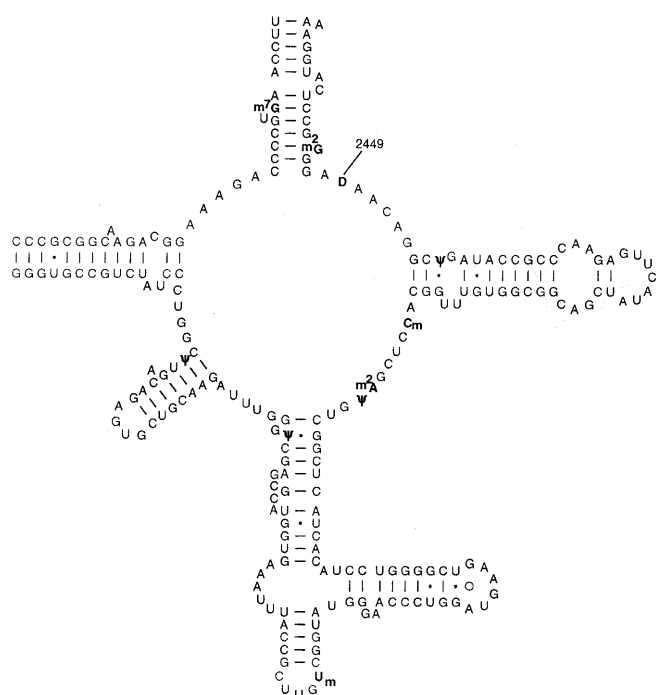
The regions of large and small subunit rRNAs that comprise the functional centers of the ribosome contain many invariant residues, and at least some of these residues are post-transcriptionally modified in all organisms (1,2). The localization of such modifications to conserved, functionally important regions of rRNA has given rise to the notion that these modifications must somehow be important for ribosome assembly and/or function (3). In agreement with this idea, *in vitro* reconstitution of *Escherichia coli* 50S subunits from the individual RNA and protein components was found to require the presence of one or more modifications contained within an ~80 nt segment of 23S rRNA (positions 2445–2523; 4). However, reconstitution of 50S subunits and peptidyltransferase activity from *Thermus aquaticus* (5) or *Bacillus stearothermophilus* (6) ribosomal proteins and *in vitro* transcribed rRNA was accomplished without the need for any modifications, albeit with lower activity than that obtained with fully modified rRNAs. These data may indicate a difference

between mesophilic and thermophilic organisms with respect to their requirement for rRNA modifications, and suggest that post-transcriptional modifications may be dispensable for catalytic function.

Only a minority of the genes encoding rRNA methylases have been identified (7,8) and disruption of many of these methylase genes has little effect on cell growth or ribosome function (2). However, in yeast mitochondrial ribosomes, loss of G2251 modification leads to defects in subunit assembly (9). Lack of pseudouridylation of several residues in the 1916 loop in domain IV of 23S rRNA in *E.coli* has substantial effects on cell growth (10) while lack of ribose modification of U2552 affects subunit–subunit interactions (11).

The central loop of domain V of 23S rRNA is the site of peptidyltransferase activity and this region of rRNA is also rich in post-transcriptional modifications (Fig. 1). In addition, this region of rRNA is one of the sites of ribosomal interaction with antibiotics that inhibit peptidyltransferase activity and growth of peptide chains (12). In *E.coli*, the invariant U2449 is modified to dihydrouridine (D) and, in contrast to its abundance in tRNAs, this is the only D modification found in *E.coli* rRNAs (13). Studies with tRNAs have suggested that D allows conformational flexibility of RNA molecules (14). This fact, together with the importance of the 2449 region of 23S rRNA in antibiotic binding and peptidyltransferase function prompted us to investigate the importance of the D modification at this position in *E.coli* 23S rRNA. In the absence of any knowledge concerning the location and nature of the rRNA-specific dihydrouridine synthetase, our approach has been to construct all three base substitution mutations at position 2449 on a plasmid-borne copy of the *rrnB* operon and attempt to express these mutant rRNAs in a strain of *E.coli* lacking all chromosomal *rrn* operons. Our findings were that while D2449A and D2449G transversion mutants were not viable under these conditions, the D2449C transition mutant was without any obvious phenotype. Our conclusions are that despite its extreme conservation, a U at position 2449 is not essential for ribosomal function. Moreover, since D2449 could be replaced with another (unmodified) pyrimidine without apparent consequence, we also conclude that the D modification at 2449 is dispensable.

\*To whom correspondence should be addressed. Tel: +1 401 863 3652; Fax: +1 401 863 1182; Email: michael\_o'connor@brown.edu



**Figure 1.** Sequence and secondary structure of the central loop region of domain V of *E. coli* 23S rRNA. The 10 post-transcriptional modifications are indicated in bold.

## MATERIALS AND METHODS

### Bacterial strains and plasmids

Plasmids pMO10 and pKK3535 are derived from plasmids pSC101 and pBR322, respectively, and contain the intact *rrnB* operon under the control of the native  $P_1P_2$  promoters (15). In plasmid pLK35, the *rrnB* operon is transcribed from the inducible  $\lambda P_L$  promoter and, in the presence of the thermolabile  $\lambda cI$  repressor, transcription of the *rrnB* operon is induced by a temperature shift to 42°C. Plasmid pts1192U was constructed by ligating the *Bam*HI fragment containing the intact *rrnB* operon (and carrying the spectinomycin resistance C1192U mutation) from plasmid pKK1192U (16) to *Bam*HI-cleaved pMAK705 (17). Strains MC242 ( $\lambda^-$  Hfr *relA1 spoT1 metB1*) or MC140 [*F^-*  $\Delta(lac-pro)$  *thi^- recA^- srl^-*] transformed with pLG857 (encoding the temperature-sensitive  $\lambda cI_{857}$  repressor) were used as hosts for pLK35-derived plasmids. The  $\Delta 7$  prn strain MC227 ( $\Delta rrnE \Delta rrnB \Delta rrnA \Delta rrnH \Delta rrnG::lacZ \Delta rrnC::cat \Delta rrnD::cat \Delta recA/pTRNA66$ , pts1192U; 18) was constructed by replacing the *rrnC*-containing plasmid pHKrrnC in strain AVS69009 ( $\Delta rrnE \Delta rrnB \Delta rrnA \Delta rrnH \Delta rrnG::lacZ \Delta rrnC::cat \Delta rrnD::cat \Delta recA/pTRNA66$ , pHKrrnC; 19) with pts1192U, selecting for spectinomycin resistance and screening for neomycin-sensitive transformants. Strain TA542 ( $\Delta rrnE \Delta rrnB \Delta rrnA \Delta rrnH \Delta rrnG::cat \Delta rrnC::cat \Delta rrnD::cat \Delta recA56/pTRNA66$  pHKrrnC) was used as a host for pKK3535 and its mutant derivatives. The neomycin-resistant *rrnB*-containing plasmid, pHKrrnC, in strain TA542 was replaced with ampicillin-resistant, pKK3535-derived plasmids by transforming TA542 with the ampicillin-resistant plasmids and growing the transformants in

the absence of selection for pHKrrnC. Loss of pHKrrnC was monitored by the appearance of neomycin-sensitive clones after three to four cycles of overnight growth and dilution into fresh medium in the absence of neomycin selection. Plasmid prrnS12 was constructed by inserting the *rrnB* operon from plasmid pKK3535 and the wild-type *rpsL* gene from plasmid pNO1523 (20) into plasmid pLG339 (21). MC230 is  $\Delta rrnE \Delta rrnB \Delta rrnH \Delta rrnG::cat \Delta rrnA \Delta rrnD::cat \Delta rrnC::cat \Delta recA56/pts1192U$  p70. MC250 is a derivative of MC230 containing the *rpsL121* streptomycin resistance mutation and carrying plasmid prrnS12 as the sole source of rRNA genes.

The pSG and pBRlac series of *lacZ* mutant plasmids contain nonsense or frameshift mutations in the 5'-end of the *lacZ* coding region (22,23).

### Mutagenesis

Site-directed mutagenesis was performed as described by Kunkel *et al.* (24), using an M13 clone carrying the *Eco*RI-*Bam*HI fragment of plasmid pLK35, encoding the 3' half of 23S rRNA as a template. Mutant M13 clones were digested with *Sph*I and *Bam*HI and ligated to *Sph*I/*Bam*HI/*Ceu*I-treated pLK35. DNA sequencing of the intact plasmids confirmed the presence of the desired mutations. The D2449C mutation was transferred to plasmid pKK3535 by fragment exchanges using *Bgl*II and *Ceu*III. The same mutation was constructed in plasmid pMO10 by ligating the *Bam*HI fragment from pKK2449C with *Bam*HI-treated pLG339 (21).

### Isolation and analysis of rRNA

Total RNA was extracted from detergent-treated cells as described (23). Ribosomes were extracted from logarithmically growing cells following a 150 min temperature shift to induce transcription of mutant rRNA. 30S and 50S subunits were separated from 70S ribosomes and polysomes on sucrose gradients as described (25). The amounts of plasmid-encoded rRNA in each gradient fraction were determined by primer extension as described by Sigmund *et al.* (26) using an oligonucleotide complementary to bases 2470–2450. The bands corresponding to plasmid- and chromosomally-encoded rRNA extension products were quantitated using a Fuji phosphor-imager. Growth rates and  $\beta$ -galactosidase assays were carried out as described previously (23,27).

## RESULTS

### Construction and expression of mutations at position 2449 in 23S rRNA

All three base substitution mutations at position D2449 were constructed by site-directed mutagenesis. Despite repeated attempts, only the D2449C mutation could be expressed from the strong constitutive  $P_1P_2$  promoters in plasmid pKK3535. Accordingly, mutant rRNAs were expressed from the inducible  $\lambda P_L$  promoter in plasmid pLK35 in strains also expressing the  $\lambda$  temperature-sensitive repressor. In this expression system, ~50–60% of the total rRNA is plasmid-encoded upon induction of transcription from  $P_L$  (Table 1; 15,23). After induction of mutant rRNA synthesis, cells expressing the D2449G rRNA displayed a slightly prolonged doubling time while expression of the D2449A mutant rRNA increased the doubling time substantially (Table 1). In contrast, expression of the D2449C

**Table 1.** Effects of 2449 mutations on growth rate, the accuracy of decoding and distribution of plasmid encoded rRNA in 50S subunits, 70S ribosomes and polysomes

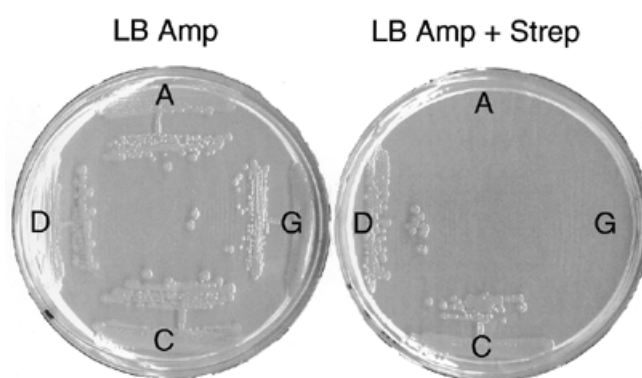
rRNA plasmid	Doubling time (min)	Readthrough and frameshifting levels in <i>lacZ</i> mutants					% distribution of mutant rRNA in gradient fractions		
		pSG12-6 (UAG)	pSG34-11 (UGA)	pSG12DP (-1 FS)	pSGlac7 (+1 FS)	PSG163 (UAG)	50S	70S	Polysomes
pLK35 wt	43 ± 4	13 ± 1	27 ± 3	79 ± 5	27 ± 1	21 ± 1	ND	ND	ND
pLK2449A	59 ± 3	18 ± 1	49 ± 2	152 ± 7	34 ± 1	46 ± 3	66 ± 5	57 ± 6	50 ± 3
pLK2449C	41 ± 3	14 ± 1	29 ± 2	86 ± 4	28 ± 2	21 ± 1	73 ± 1	64 ± 7	66 ± 8
pLK2449G	48 ± 3	17 ± 1	34 ± 2	92 ± 10	25 ± 1	28 ± 1	70 ± 3	62 ± 6	48 ± 1

All plasmids for growth rate determinations and ribosome preparations were maintained in strain MC242 co-transformed with pLG857, encoding the temperature-sensitive  $\lambda$ C<sub>I857</sub> repressor. Growth rates were measured following induction of plasmid-encoded rRNA transcription at 42°C. Ribosomes were harvested from cells grown at 42°C for 150 min. The relative proportions of plasmid and chromosomally encoded rRNAs were determined by primer extension assays on RNA from three independent ribosome preparations. ND, not determined. Values for stop codon readthrough and frameshifting are expressed in Miller units of  $\beta$ -galactosidase (29).  $\beta$ -Galactosidase activities were measured after induction of plasmid-encoded rRNA transcription at 42°C for 150 min. Each value represents the mean  $\pm$  SE of three to five independent measurements.

mutant had no effect on growth rate. Taken together, the inability to express the D2449G and D2449A mutant rRNAs from the P<sub>1</sub>P<sub>2</sub> promoters, and the slower growth rates after induction from  $\lambda$  P<sub>L</sub>, suggested that the purine base substitutions at D2449 might be deleterious to ribosome function.

The recent development of an *E.coli* strain that lacks all seven chromosomal *rrn* operons and expresses plasmid encoded rRNA exclusively ( $\Delta 7$  prn; 18) has facilitated the analysis of rRNA mutations. Construction of strains expressing only mutant rRNA involves transformation of  $\Delta 7$  prn with the relevant mutant *rrn* plasmid and subsequent displacement of the resident wild-type *rrn* plasmid. Strain TA548 was transformed with pLK35 and each of the D2449 mutant derivatives. Growth of these transformants on medium containing ampicillin but lacking neomycin led to loss of the resident *rrn* plasmid, pHKrrnC, in strains expressing wild-type or 2449C rRNAs. However, after three cycles of overnight growth and dilution into fresh medium, no neomycin-sensitive transformants were obtained with strains expressing 2449G or 2449A rRNAs, suggesting that these mutant rRNAs were unable to supply the total protein synthetic needs of the cell.

The plasmid displacement system described above relies on passive loss of the resident *rrn* plasmid in the absence of antibiotic selection. To facilitate displacement of the resident *rrn* plasmid by mutant derivatives and to allow recovery of deleterious rRNA mutants, we have developed a system that actively selects for displacement of the resident *rrn* plasmid. This system relies on the observation that resistance to streptomycin caused by mutations in *rpsL* is recessive (28). Derivatives of the  $\Delta 7$  prn strain, such as MC250, carrying the *rpsL121* mutation on the chromosome are sensitive to streptomycin because they also contain plasmid prnS12 carrying an intact *rrnB* operon and a wild-type *rpsL* gene. Resistance to streptomycin can be recovered when prnS12 is displaced from this strain by another *rrn* plasmid that does not contain the *rpsL* gene. Strain MC250 was, therefore, transformed with pLK35 and each of the mutant D2449 derivatives and ampicillin-resistant transformants were streaked on plates containing ampicillin and streptomycin. MC250 cells transformed with either the wild-type or D2449C mutant plasmid were able to grow on streptomycin-containing plates and these transformants were subsequently



**Figure 2.** Displacement of the resident *rrn* plasmid, prnS12, from MC250 by pLK35 (D, wild type at position 2449) and each of the D2449 mutant derivatives (A, C and G). The MC250 transformants were streaked on LB ampicillin (left) and on LB ampicillin + streptomycin plates (right). Loss of prnS12 allows expression of *rpsL121*-associated streptomycin resistance and growth on streptomycin. Streptomycin-resistant colonies were subsequently shown to have lost the prnS12-associated neomycin resistance.

shown to have lost the prnS12-associated neomycin resistance. In contrast, the pLK2449A and pLK2449G transformants remained streptomycin sensitive (Fig. 2). This indicated that both purine substitutions at position 2449 had deleterious effects on ribosome function whereas the pyrimidine substitution was viable. While we have not been able to analyze unmodified U2449 rRNAs, we believe that D2449C ribosomes are functionally equivalent to such unmodified ribosomes and thus conclude that the D modification itself is not essential for ribosome function.

#### Lethal mutations at position 2449

The inability to construct strains expressing the D2449A or D2449G mutant rRNAs exclusively necessitated their further analysis in an inducible expression system in a strain carrying intact chromosomal *rrn* operons. Our previous work had shown that several mutations in the peptidyltransferase center, including mutations at G2447,  $\Psi$ 2504 (M.O'Connor and

**Table 2.** Growth rates and decoding fidelity of strains expressing wild-type or D2449C rRNA exclusively

rRNA mutant	Doubling times (min)			Readthrough and frameshifting levels in <i>lacZ</i> mutants			
	30°C	37°C	42°C	p240	p415	p12-6	plac7
Wild-type	74 ± 4	60 ± 2	80 ± 6	11 ± 1	392 ± 27	38 ± 3	41 ± 7
D2449C	82 ± 4	64 ± 4	76 ± 3	9 ± 1	384 ± 30	38 ± 5	36 ± 7

Growth rates were determined for pKK3535 and pKK2449C-containing derivatives of the  $\Delta 7$ prn strain, TA542, where all rRNA is plasmid encoded.  $\beta$ -Galactosidase measurements were performed on TA542 derivatives expressing wild-type or D2449C rRNA from plasmids pMO10 and pMO2449C, respectively; *lacZ* constructs were carried on pBR322-derived plasmids. Values for stop codon readthrough and frameshifting are expressed in Miller units of  $\beta$ -galactosidase (29). All values represent the mean  $\pm$  SE of three to five independent determinations.

A.E.Dahlberg, unpublished data) and G2458 (29) affected the accuracy of translation. The effects of the D2449 mutations on stop codon readthrough and frameshifting were examined using a series of *lacZ* reporter gene constructs. Cells expressing the D2449A mutation showed a 1.3–2.2-fold increase in the levels of stop codon readthrough and frameshifting (Table 1). Some very modest, but reproducible increases in readthrough and frameshifting were also observed with the less deleterious D2449G mutation, whereas the D2449C mutant was indistinguishable from wild type. Apart from some under-representation of D2449G and D2449A in the polysome fractions, ribosomes isolated from induced cells showed normal levels of subunits, 70S ribosomes and polysomes on sucrose gradients (data not shown) and the mutant rRNA was well-represented in all ribosome fractions (Table 1). These results showed that while both of the deleterious purine substitution mutations decreased the accuracy of decoding, none of the D2449 mutations had dramatic effects on 50S subunit assembly, 70S ribosome formation or entry into the polysome pool.

#### Characterization of the D2449C mutation

The effects of the D2449C substitution on cell growth and translational fidelity were examined in  $\Delta 7$  prn strains that expressed the mutant rRNA exclusively from the native, growth-regulated  $P_1P_2$  promoters in plasmid pKK3535 rather than from the  $\lambda P_L$  promoter in the pLK35 plasmids used above. Successful replacement of the resident wild-type pts1192U *rrn* plasmid by pKK2449C in the  $\Delta 7$  prn strain MC227 was assessed both by loss of pts1192U-associated spectinomycin resistance and by primer extension of total RNA (26; data not shown). No difference in growth rate was observed between strains expressing only wild-type or the D2229C rRNAs under any of the conditions used (Table 2). Moreover, both wild-type and mutant strains grew at equivalent rates on solid, minimal and rich media at 25, 30, 37 and 42°C (data not shown). The influence of the D2449C mutation on recovery from heat stress was addressed by incubating mutant or wild-type cells for varying lengths of time at 52°C and measuring the number of colony forming units in the cultures before and after heat treatment. After 25 min incubation at high temperature, both wild-type and mutant strains displayed the same 100-fold loss of viability. A further 25 min incubation at 52°C led to an additional 2-fold decrease in the viability of both cultures. The effect of the D2449C mutation on low temperature survival was assessed by incubating

diluted, mutant and wild-type cultures at 15°C and measuring the increases in OD<sub>600</sub> and the numbers of viable cells in the cultures during a 60-h period after transfer to 15°C. No difference in low temperature survival and recovery was observed between the wild-type and D2449C mutant strains (data not shown). Analysis of ribosomes on sucrose gradients revealed no differences between mutant and wild-type in ribosome assembly or subunit–subunit interactions (data not shown). When assayed under conditions where only mutant rRNA was expressed, the D2449 mutation had no effect on stop codon readthrough or frameshifting (Table 2). Many mutations in the central loop of domain V of 23S rRNA have been associated with altered sensitivity to antibiotics that inhibit peptide bond formation or growth of peptide chains (12). We were unable to examine any effects of the D2449C mutation on chloramphenicol resistance since the  $\Delta 7$  prn strain contains multiple chloramphenicol acetyltransferase (CAT) cassettes inserted in chromosomal *rrn* operons. However, the D2449C alteration did not affect sensitivity of the strain to the antibiotics tylosin, spiramycin, oleandomycin, erythromycin, clindamycin, tetracycline, paromomycin, kasugamycin or fusidic acid (data not shown). Thus, in summary, it appeared that substitution of the D modification by a C at position 2449 had no detectable effect on cell growth, heat or cold shock survival, translational fidelity or antibiotic sensitivity.

#### DISCUSSION

Substantial biochemical data as well as the recently solved crystal structure of the 50S ribosomal subunit indicate that the central loop of domain V of 23S rRNA forms part of the peptidyltransferase center of the ribosome (30,31). In keeping with its central role in protein synthesis, many of the rRNA residues in this region of large subunit rRNAs are invariant. The clustering of modified bases in the central loop has given rise to several models in which modified bases play essential roles in translation (32). The observation that D in tRNAs promotes conformational flexibility has led to the suggestion that its presence in a functional center of 23S rRNA may be necessary to accommodate the dynamic, conformational changes that must occur in this region of the ribosome (14). The enzyme(s) responsible for D modification at position 2449 in *E.coli* has not yet been identified, preventing a direct analysis of unmodified, U2449 ribosomes. However, our observation that the D2449C mutation is without apparent phenotype indicates that D modification is not essential for

ribosome function, and thus the role of this modification remains unclear. Consistent with our data is the finding that although the rRNA modifications of *Aeromonas hydrophilia* are very similar to those found in *E.coli*, D is conspicuously absent (33). Analysis of the published rRNA sequences indicates that a U is always found at position 2449 in large subunit rRNAs. However, at least in the context of the *E.coli* ribosome, a C appears to function as well as D and thus neither the modification nor the uridine residue itself appears to be essential for ribosome function. The possibility remains that the D modification aids in ribosome assembly or ribosome function under particular conditions not revealed by the laboratory growth conditions utilized here.

A majority of the mutations that have been made in the central loop of domain V of 23S rRNA have deleterious effects on ribosome function (34–36). While effects on peptidyltransferase activity and antibiotic sensitivity have been documented for many of these deleterious mutations, a more surprising finding is that several of the mutations that have been linked with peptide bond formation also affect the fidelity of decoding, an activity associated with the small ribosomal subunit. Such a phenotype is observed with the D2449A and D2449G mutations studied here. Similar effects on decoding have also been observed with mutations at the adjacent positions G2447 and  $\Psi$ 2504 (M.O'Connor and A.E.Dahlberg, unpublished data) as well as at several positions in helix 89 (29). These findings highlight the interconnectedness of the central ribosomal activities of decoding, translocation and peptide bond formation. However, the effects of the D2449A and D2449G mutations on decoding are very modest in comparison to other (viable) ribosomal mutations (37) and are thus unlikely to account for the lethality of these base substitutions in the  $\Delta 7$  prrn strain. The distribution of D2449A and D2449G rRNAs in the subunit, 70S ribosome and polysome (Table 1) indicates that the mutant RNAs are fully assembled into 50S particles, competent to interact with 30S subunits and initiate translation. However, their inability to support the total protein synthetic needs of the cell indicates that one or more of the 50S-associated elongation functions is compromised in the mutant ribosomes. The recently published crystal structure of the 50S ribosomal subunit of *Haloarcula marismortui* indicated that residues at positions 2444 and 2446 participate in a base triple interaction with U2449 (31). The phenotypic effects of purine substitutions at position 2449 may thus reflect their inability to participate in an equivalent base triple interaction in the *E.coli* ribosome. In addition, the crystal structure of the catalytic center of the 50S subunit as well as an anomalous pKa associated with A2451 has been interpreted to indicate that A2451 may play an essential role in peptide bond formation (31,38). This raises the possibility that the defects associated with the D2449A and D2449G mutants may be due to alterations in the efficiency of the peptidyltransferase reaction.

Finally, we have developed a method that facilitates replacement of the resident *rrn* plasmid in  $\Delta 7$  prrn strains with other mutant *rrn* plasmids. This method permits recovery of deleterious rRNA mutations and should allow rapid screening and analysis of rRNA mutations.

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