

## Mutational Analysis of Two Highly Conserved UGG Sequences of 23 S rRNA from *Escherichia coli*\*

(Received for publication, July 2, 1996, and in revised form, September 12, 1996)

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The 23 S-type rRNA contains two phylogenetically conserved UGG sequences, which have the potential to bind the universal CCA-3'-ends of tRNAs at the ribosomal peptidyltransferase center by base pairing. The first two positions, UG, of these sequences at the helix-loop 80 (U2249G2250) and helix-loop 90 (Ψ2580G2581) and some related nucleotides were tested by site-directed mutagenesis for their involvement in ribosomal function, i.e. peptidyltransferase. The plasmid-derived mutated 23 S rRNA comprised about 50% of the total 23 S rRNA. None of the single mutations caused an assembly defect, and all 50 S subunits carrying an altered 23 S rRNA could freely exchange with the pools of 70S ribosomes and polysomes. The mutations at the helix-loop 80 region hardly affected bacterial growth. However, mutations at the helix 90 caused severe growth effects and severely impaired the *in vitro* protein synthesis, showing that this 23 S rRNA region is of high importance for ribosomal function.

The central enzymatic activity of ribosomes is the formation of peptide bonds. The corresponding peptidyltransferase (PTF)<sup>1</sup> center is located on the large ribosomal subunit (1, 2). Reconstitution analyses have identified the ribosomal proteins L2, L3, and L4, and the 23 S rRNA as PTF candidates in *Escherichia coli* ribosomes (3–5). A complex derived from the large subunit of *Thermus aquaticus* ribosomes consisting of 23 S rRNA and only 3–8 proteins had significant PTF activity (6, 7). This observation underscores the possible involvement of 23 S rRNA in this activity.

An impressive wealth of data points to a distinct feature of the secondary structure map of 23 S rRNA, the so-called “peptidyltransferase ring” of domain V (8, 9) as a component at or near the PTF center. The PTF ring comprises about 40 nucleotides and represents a cluster of universally conserved nucleotides (10, 11). It is satisfying that a highly divergent array of methods correspondingly identifies the same region of 23 S rRNA, methods such as cross-linking studies with substrates of the PTF center (12–15), mutations of the 23 S rRNA gene conferring resistance against inhibitors of the PTF activity (for review, see Refs. 16 and 17), and protection studies (18–20).

Some studies aimed to identify nucleotides at the PTF center. The nucleotides G2252 and G2253, which are at the helix-loop 80, were protected against kethoxal by the 3'-terminal

CCA of P-site bound tRNA (19). Mutation of G2252 generated a dominant lethal phenotype (21). A double mutant having both Gs altered was lethal and showed a reduced activity of peptide bond formation by approximately 50% (22). A recent analysis presented evidence that G2252 is involved in canonical base pairing with C74 at the acceptor end of tRNA, thus obviously playing a role in the binding of the donor substrate at the P site region of the PTF center (23).

Another study applied a random mutagenesis of the “Southern half” of the PTF ring (residues 2493–2606). With an elegant screening procedure, 21 mutations of 18 positions were found. Mutations at three positions blocked growth, one of which was universally conserved (Ψ2580). The observation that a mutation of Ψ2580 caused a 90% inhibition of the PTF activity led the authors to suggest that the sequence ΨGG2582 might be involved in the binding of the CCA-3'-end of tRNAs at the P site (24).

Here we pursue a different strategy. Base pairing of the tRNA CCA-3'-end with a complementary UGG sequence of 23 S rRNA is the most likely explanation for experimental data gained with minimal substrates (25, 26) and with tRNAs mutated in the CCA-3'-end (27) as substrates of the P-site region of the PTF center. The 23 S rRNA contains 55 UGG sequences, only two of which are universally conserved in non-mitochondrial 23 S-type rRNA, and are therefore candidates for binding the universal CCA-3'-end of tRNAs, possibly via canonical base pairing. One of these UGG sequences is U2249 to G2251 at the helix-loop 80, and the other is Ψ2580 to G2582 at helix 90, adjacent to the PTF ring. The U (Ψ) and the middle G were mutated in both sequences, and the effects on growth, expression, ribosomal assembly, and functions were studied in this and the accompanying paper (28).

### MATERIALS AND METHODS

**Plasmids and Strains**—The plasmid pNO2680 contains the *E. coli* rrnB operon under the control of the λ leftward promoter (29). This plasmid with the single point mutation A1067U in the 23 S rRNA gene (30) is called pNOt. Plasmids ptac-1 and ptac-2 carry the rrnB operon or the 23 S rRNA gene, respectively, under the control of the tac-promoter (31). Plasmids ptac-1 and ptac-2 also contain the A1067U mutation that confers thiostrepton resistance. *E. coli* CJ236 (dut1, ung1, thi-1, relA1) was used for the preparation of uracil containing single-stranded DNA template for site-directed mutagenesis, and strain XL-1 Blue was used for propagating the M13 derivatives as well as the plasmids ptac-1 and ptac-2. XL-1 transformed with pC1857, which encodes the temperature-sensitive λ repressor and a neomycin resistance marker, was the host of the pNO2680 derivatives.

**Site-directed Mutagenesis**—The *EcoRI*-*Bam*HI fragment, containing the 3' part of the rrnB 23 S rRNA gene, was cloned into M13mp18 and used for oligo-directed construction (32) of the mutations U2249C, G2250A, C2254U, C2507U, Ψ2580C, G2581A, and G2250A/C2254U. The double mutation C2507U/G2581A was constructed taking advantage of an M13mp18 derivative already carrying the mutation G2581A as a template for the introduction of the second mutation, C2507U. The *EspI*-*PvuII* fragments containing the base changes G2581A and G2250A were cloned into ptac-1 by performing a partial restriction of

\* This work was supported by DFG Grant Ni174/7-1 and EC Grant ERBCIPA CT930136. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviation used is: PTF, peptidyltransferase.

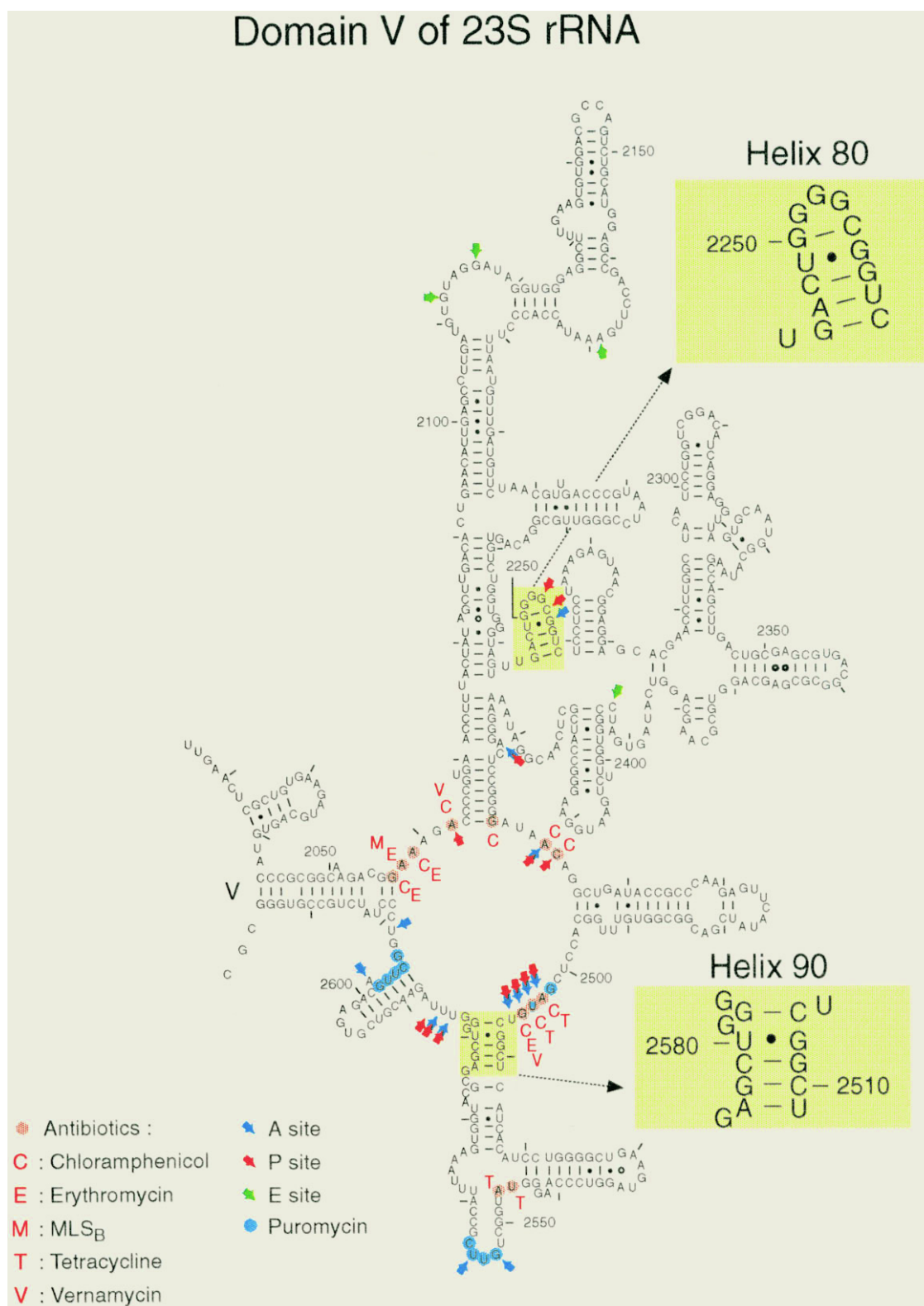


FIG. 1. Schematic representation of the secondary structure of the domain V of *E. coli* 23S rRNA according to Ref. 44. Helix 80 and helix 90 containing the two highly conserved UGG sequences are highlighted and enlarged. Site-specific protections by or cross-links to tRNAs are indicated by colored arrows, RNA regions cross-linked to puromycin are in blue, and nucleotides at or near the binding site of antibiotics identified by cross-links, protections, or resistance mutations are in red (for review see Refs. 8, 9, 14, 16, and 20)

the vector with *Pvu*II. The other mutants were cloned into ptae-2 using *Bam*HI and *Ecl*XI. The *Esp*I-*Xba*I fragment of the mutated ptae-1 or ptae-2 vectors was cloned into pNO2680, yielding a pNO2680 derivative carrying the A1067U mutation as well as the mutation in domain V of the 23S rRNA gene. All base changes were verified by DNA sequencing (33) of the M13 inserts and the reconstructed expression vectors. The DNA manipulations followed standard procedures (34, 35), and compe-

tent cells for plasmid transfection were prepared according to the SEM protocol (36).

**Polysome Preparation and Quantification of Plasmid-born 23S rRNA**—100 ml of  $2 \times$  YT (16 g/liter tryptone, 10 g/liter yeast extract, 5 g/liter NaCl) supplemented with 0.2 mg/ml ampicillin and 0.05 mg/ml neomycin was inoculated with XL-1/pCI857 transformed with the pNO derivatives. Cultures were grown until  $A_{560} = 0.1$  and then shifted to

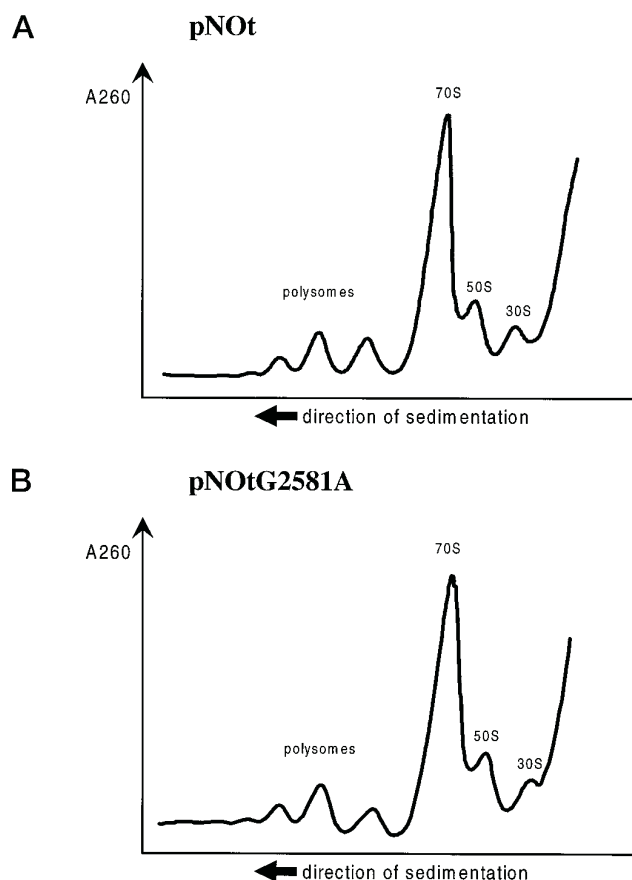


FIG. 2. Sucrose gradient patterns from S30 lysates of XL1-Blue/pCI857/pNot (A) and XL1-Blue/pCI857/pNotG2581A (B).

42 °C. After 90 min at 42 °C, the cultures were poured over crushed ice. The cells were pelleted and resuspended in 1 ml of buffer A (20 mM Tris-HCl (pH 8.0 at 0 °C), 6 mM MgCl<sub>2</sub>, 100 mM KCl) containing 16% (w/v) sucrose and lysozyme (0.5 mg/ml), and broken by freezing and thawing. Lysates were applied onto a 10–40% sucrose gradient in binding buffer (20 mM Hepes-KOH (pH 7.6), 6 mM MgCl<sub>2</sub>, 150 mM NH<sub>4</sub>Cl, 4 mM  $\beta$ -mercaptoethanol, 2 mM spermidine, and 0.05 mM spermine) and centrifuged in a Beckman SW-40 rotor for 7.5 h at 80,000  $\times g$ . Fractions of polysomes, 70 S ribosomes, and 50 S subunits were collected, and rRNA was isolated by phenol extraction. The relative amount of mutant 23 S rRNA was determined by the primer extension method (37) using the 1067U as a common marker for all constructs. The 5'-<sup>32</sup>P-labeled oligonucleotide used as primer was complementary to bases 1069–1087 of 23 S rRNA. Extension products were quantified by densitometry using a laser scanner (Epson GT-8000) and the WinCAM 2.0 software (Cybertech, Berlin).

**Preparation of Ribosomes**—*E. coli* XL-1/pCI857 transformed with the pNO plasmids were grown at 30 °C in 2  $\times$  YT (16 g/liter tryptone, 10 g/liter yeast extract, 5 g/liter NaCl) supplemented with 0.2 mg/ml ampicillin to A<sub>560</sub> = 0.2. Neomycin was omitted in the preparative culture because it interferes with the thiostrepton resistance of the isolated A1067U ribosomes,<sup>2</sup> but it was added to 0.05 mg/ml in all stages before. The culture was shifted to 42 °C to induce synthesis of the plasmid-born rRNA. After 2 h, cells were collected and lysed by lysozyme (0.5 mg/ml) in buffer A (20 mM Tris-HCl (pH 8.0), 6 mM MgCl<sub>2</sub>, 100 mM KCl) containing 16% (w/v) sucrose by three freeze-thaw cycles. S-30 lysate was prepared by centrifugation at 12,000  $\times g$  for 30 min in an SA600 rotor (Sorvall). The volume of the lysate was enlarged fourfold, and the final concentrations were 20 mM Hepes-KOH (pH 7.6), 6 mM MgCl<sub>2</sub>, 500 mM NH<sub>4</sub>Cl, and 4 mM  $\beta$ -mercaptoethanol. The sample was loaded onto a 5-ml sucrose cushion (20% sucrose in 20 mM Hepes-KOH (pH 7.6), 6 mM MgCl<sub>2</sub>, 500 mM NH<sub>4</sub>Cl, 4 mM  $\beta$ -mercaptoethanol) in a Beckman SW-40 rotor, and the ribosomal particles were pelleted by centrifugation at 55,000  $\times g$  for 20 h. The crude ribosomes were redissolved in standard buffer (20 mM Hepes-KOH (pH 7.6), 6 mM MgCl<sub>2</sub>, 150

mM NH<sub>4</sub>Cl, 4 mM  $\beta$ -mercaptoethanol), loaded onto a 10–30% (w/v) sucrose gradient in standard buffer, and centrifuged in a Beckman SW-27 rotor for 17 h at 40,000  $\times g$ . The fractions containing 70 S ribosomes were combined, and the ribosomes were pelleted in a Beckman SW-40 rotor by centrifugation at 55,000  $\times g$  for 20 h. Pellets were redissolved in standard buffer, shock-frozen in liquid nitrogen, and stored in aliquots at –80 °C.

**Cell-free Protein Synthesis**—The protocol essentially followed Bartetzko and Nierhaus (38) with the modifications of Rheinberger and Nierhaus (39). The ionic conditions in all steps were 20 mM Hepes-KOH (pH 7.6), 3 mM MgCl<sub>2</sub>, 150 mM NH<sub>4</sub>Cl, 4 mM  $\beta$ -mercaptoethanol, 2 mM spermidine, and 0.05 mM spermine. Long poly(U) chains used in this system were isolated over a Sephacryl S-400 gel filtration column. In a volume of 225  $\mu$ l, 45 pmol of 70 S ribosomes were incubated with 67.5 pmol of [<sup>14</sup>C]AcPhe-tRNA<sup>Phe</sup> (1,030 dpm/pmol) and 55  $\mu$ g of poly(U) for 30 min at 37 °C. Where indicated, thiostrepton was added to a final concentration of 1.6  $\mu$ M. Two aliquots of 25  $\mu$ l containing 5 pmol of 70 S were withdrawn and filtered over nitrocellulose to determine the amount of AcPhe-tRNA<sup>Phe</sup> bound to ribosomes. Poly(Phe) synthesis was started by mixing the rest of the binding mix (35 pmol of 70 S ribosomes) with 105  $\mu$ l of charging mix (preincubated for 2 min, 37 °C) containing 31.5  $\mu$ l of 100,000  $\times g$  supernatant enzymes freed from tRNA, 0.2 mM GTP, 2 mM ATP, 4 mM phosphoenolpyruvate, 10.5  $\mu$ g of pyruvate kinase, 350 pmol of tRNA<sup>Phe</sup>, and 70,000 pmol of [<sup>3</sup>H]Phe (22 dpm/pmol). Aliquots of 25  $\mu$ l were withdrawn at the indicated times, and the reaction was stopped by hot trichloroacetic acid precipitation.

## RESULTS

**Construction of Mutants, in Vivo Effects and Distribution of Mutant rRNA**—The two highly conserved UGG sequences in 23 S rRNA, UGG2582 and UGG2251, are shown in Fig. 1. The mutations C2507U,  $\Psi$ 2580C, G2581A, and C2507U/G2581A are located in helix 90, and U2249C, G2250A, C2254U, and C2250A/G2254U are in helix-loop 80. C2507 $\Delta$ /G2581A was obtained by chance and was included in the further analysis. The plasmids pNO2680 (29) and ptac-1 or ptac-2 (31) were used as expression vectors. ptac-1 contains the rrnB operon and, ptac-2 contains the 3' part of the rrnB operon, including the gene for 23 S rRNA. Both genes are under the control of the isopropyl-1-thio- $\beta$ -D-galactopyranoside-inducible tac promoter and contain the mutation A1067U of 23 S rRNA, which confers resistance to thiostrepton. pNO2680 contains the rrnB operon under the control of the  $\lambda$  leftward promoter  $\lambda_{PL}$ . The promoter was blocked by the heat-labile CI<sub>857</sub> repressor that was supplied by the plasmid pCI<sub>857</sub>. For the sake of clarity, we call the pNO2680 plasmid pNO(wt), where wt stands for wild type. The derivative pNot carries the A1067U mutation in the 23 S rRNA gene (pNot, where t stands for thiostrepton resistance) (30).

The effects of mutations under the control of the tac promoter (ptac derivatives) were tested by streaking the cells on plates in the absence or presence of isopropyl-1-thio- $\beta$ -D-galactopyranoside, whereas the plates with the mutations under the  $\lambda_{PL}$  promoter (pNot derivatives) were incubated at 30 or 42 °C. Cells containing the mutation C2507U, G2581A, C2507U/G2581A, and C2507 $\Delta$ /G2581A were not able to form colonies when expressed, *i.e.* the mutations were dominant lethal.  $\Psi$ 2580C showed a reduced size of the colonies when expressed from ptac-2 but grew well when expressed from pNot. All the mutations within helix-loop 80 grew normal. The type of induction had no effect on the expression with the exception of  $\Psi$ 2580C.

The constructs C2507U, G2581A, C2507U/G2581A, and C2507 $\Delta$ /G2581A in pNot also showed a reduced growth in liquid cultures. When expression was induced at a cell density of A<sub>560</sub> = 0.2 by shifting the temperature from 30 to 42 °C, the growth was severely reduced after one dilution of the culture, and the doubling time was enlarged by a factor of two to three as compared with that of the wild type or the 1067U control. However, after prolonged incubation at 42 °C, we observed that the cells resumed a normal growth rate, and after streaking

<sup>2</sup> C. M. T. Spahn and K. H. Nierhaus, unpublished result.



TABLE I

Growth on plates and distribution of mutant 23 S rRNA in polysomes, 70 S ribosomes, and 50 S subunits

For details see text. ND, not determined.

	Growth on plates		fraction of mutant rRNA		
	tac promoter	$\lambda_{PL}$ promoter	Polysomes	70 S	50 S
				%	
Wild type	+	+	ND	ND	ND
1067U	+	+	44	45	46
C2507U	—	—	46	39	49
$\Psi$ 2580C	$\pm$	+	49	46	51
G2581A	—	—	47	45	43
C2507U/G2581A	—	—	27	48	50
C2507 $\Delta$ /G2581A	—	—	33	47	48
U2249C	+	+	43	45	40
G2250A	+	+	49	40	45
C2254U	+	+	48	55	32
G2250A/C2254U	+	+	48	57	36

TABLE II

Binding of AcPhe-tRNA to mutant ribosomes given as AcPhe-tRNA bound per 70 S ribosome

AcPhe-tRNA was bound to the site in the presence of poly(U) at 3 and 6 mM  $Mg^{2+}$ , respectively. The  $NH_4^+$  concentration was 150 mM, spermine was 50  $\mu$ M, and spermidine and 2 mM in both systems (under "Materials and Methods"). When MF-mRNA was used, the P site was prefilled with deacylated tRNA<sup>Met</sup> directing the AcPhe-tRNA to the A site (–EF-G); the binding was also assessed in the presence of EF-G with and without thiostrepton (thio).

	Poly(U) 3 mM $Mg^{2+}$		Poly(U) <sub>2</sub> 6 mM $Mg^{2+}$	MF-mRNA		
	–thio	+thio		–EF-G –thio	+EF-G –thio	+EF-G +thio
Wild type	1.06	0.86	1.11	0.55	0.58	0.49
1067U	0.96	0.84	1.08	0.56	0.57	0.53
C2507U	0.92	0.75	1.09	0.54	0.58	0.50
$\Psi$ 2580C	0.95	0.76	1.03	0.51	0.54	0.52
G2581A	1.01	0.77	1.07	0.49	0.53	0.48
C2507U/G2581A	0.87	0.79	1.06	0.51	0.56	0.49
C2507 $\Delta$ /G2581A	0.70	0.67	1.10	0.50	0.51	0.46
U2249C	0.80	0.68	1.04	0.49	0.50	0.47
G2250A	0.88	0.74	1.08	0.50	0.48	0.47
C2254U	1.10	0.88	1.03	0.52	0.51	0.47

them onto plates, they were also able to form single colonies at 42 °C. We pursued this effect a little further with the mutant G2581A. A second site mutation in the plasmid was responsible for the observed effect.<sup>2</sup>

The severe growth effects seen with some of the mutants must not necessarily be due to ribosomes defective in a distinct essential function but rather due to assembly effects preventing the formation of active ribosomes. A sensitive measure for assembly defects are sucrose-density profiles of S30 extracts. Assembly defects will lead to accumulation of precursor particles and distort in a characteristic way the profile derived from wild-type cells (31). S30 extracts were prepared from all pNot constructs; the profiles of all the mutants were indistinguishable from that of the wild type. An example is shown in Fig. 2. None of the mutants, therefore, affected the assembly of 50 S ribosomal subunits.

50 S particles, 70 S ribosomes, and polysomes were isolated from sucrose gradients as indicated in Fig. 2. The 23 S rRNA was isolated from all fractions, and the relative amount of plasmid-born 23 S rRNA was determined using the A1067U mutation as a general marker (primer extension method, see Ref. 37); this mutation, as mentioned above, mediates thiostrepton resistance. Generally, the fraction of plasmid-born 23 S rRNA was around 45% in all three ribosomal populations. The double mutations C2507U/G2581A and C2507 $\Delta$ /G2581A were reduced in polysomes (27 and 33%, respectively). The fact that the same fraction of plasmid-born 23 S rRNA was found in the 50 S subunits and the 70 S ribosomes of all mutants indicate that the mutant 50 S subunits flow as easy into the pool of the 70 S ribosomes as the wild-type 50 S subunits and that they associate with 30 S subunits as well as wild-type 50

S subunits. This is another indication that the ribosomes containing mutant 23 S rRNA have achieved the same level of assembly maturation as the wild-type 50 S subunits. A reduced relative amount of the mutant 23 S rRNA in the polysomal fraction indicates that the functional turnover of mutant 50 S subunits in the ribosomal cycle is impaired. Possible reasons are a hampered capability to form initiation complexes, a reduced processivity, and/or an increased tendency toward frameshifts, which will cause a premature termination at out-of-frame stop codons.

A summary of the growth effects and the relative amounts of the various mutant 23 S rRNAs in the 50 S subunits, 70 S ribosomes, and the polysomes is given in Table I.

**Functional Studies**—One can anticipate that a mutation that exclusively affects the PTF center should not impair tRNA binding since only a small fraction of the A and P sites belong to the PTF center. Therefore, a normal tRNA binding to the A and P sites and a block in the PTF activity would be important indications for the specificity of the provoked ribosomal defect. Various functional tests were performed in this and the accompanying study (28) that require a tRNA at a specific site but differ in their ionic conditions. Therefore, 70 S ribosomes were isolated from all mutants and analyzed for binding of AcPhe-tRNA under the various conditions. Poly(U) and the heteropolymeric MF-mRNA, which is 46 nucleotides long and contains the codons AUG-UUC only once in the middle (40), were used as templates (Table II). It is clear that in all cases mutant and wild-type ribosomes bound the tRNA to the same extent and that, therefore, none of the mutations alters the basic interactions between the sites (A and P) and tRNAs.

The next step was to check whether the mutations influence

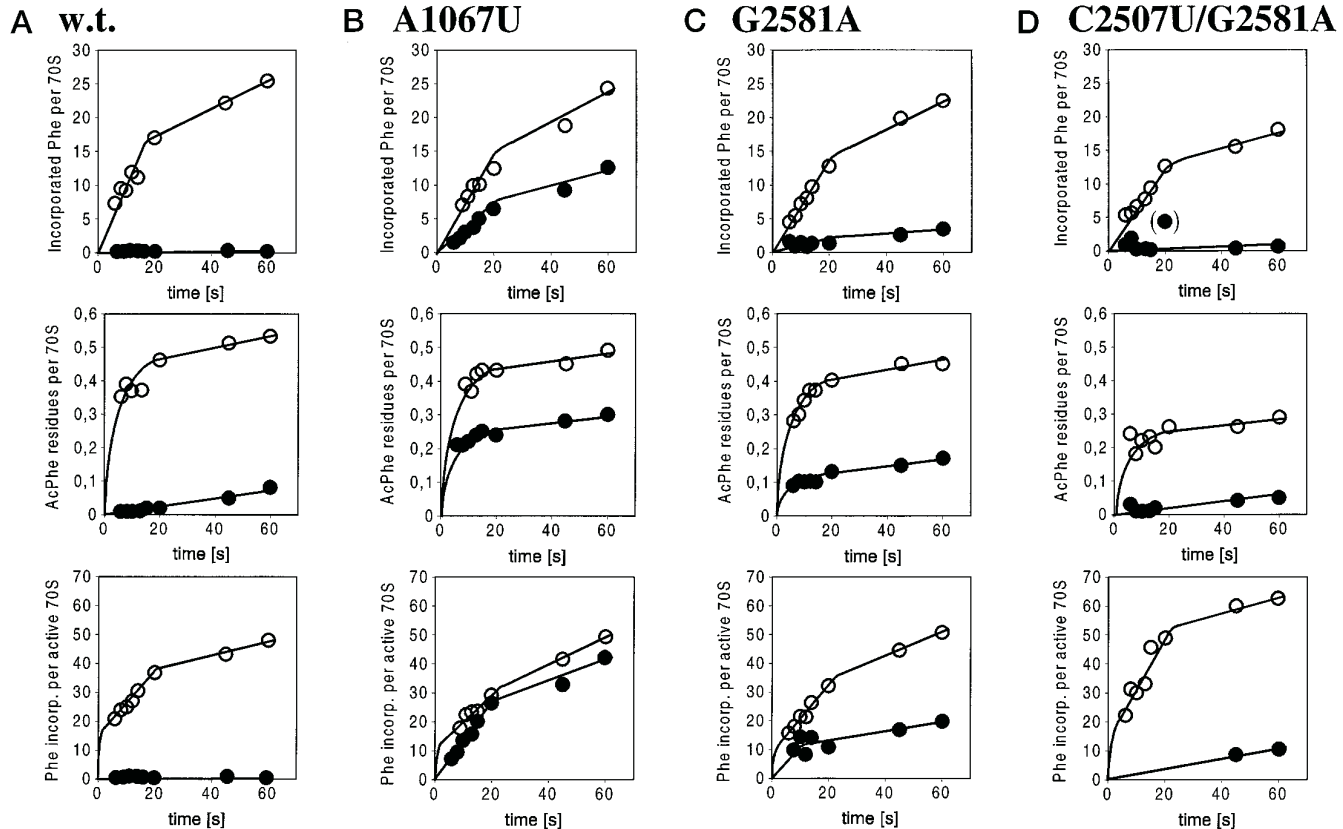


FIG. 3. **Kinetics of poly(U)-dependent poly(Phe) synthesis.** Ac[ $^{14}$ C]Phe-tRNA was prebound to the P sites of poly(U)-programmed 70 S ribosomes, and the incorporation of [ $^3$ H]Phe was measured. Kinetics were performed in the absence (open circles) and in the presence of 1  $\mu$ M thiostrepton (filled circles). The panel at the top shows the overall poly(Phe) synthesis given as Phe incorporation per ribosome. The panel in the middle indicates the hot TCA precipitable AcPhe residues per ribosome, which is the fraction of ribosomes participating in the poly(Phe) synthesis. The bottom panel presents the ratio Phe/AcPhe in the hot TCA precipitable material, which corresponds to the statistical chain length of the synthesized poly(Phe) chains.

the overall elongation cycle that can be tested with the poly(U)-dependent poly(Phe) synthesis. An *in vitro* system with near *in vivo* characteristics concerning speed and accuracy was used to look at the influence of the mutations (38, 39). [ $^{14}$ C]AcPhe-tRNA was bound to poly(U)-programmed 70 S ribosomes, and the incorporation of [ $^3$ H]Phe was followed kinetically. In order to distinguish between chromosomal- and plasmid-born 23 S rRNA, the assays were performed in the presence or absence of thiostrepton (41). The effect of the addition of thiostrepton can be studied by comparing thiostrepton-sensitive ribosomes (wild-type ribosomes derived from pNO(wt)) with thiostrepton-resistant ribosomes (A1067U, derived from pNOt). The overall incorporation of Phe per ribosome is comparable for both ribosome preparations (Fig. 3, A and B). Addition of thiostrepton abolishes completely the activity of wild-type ribosomes (first panel), whereas the A1067U mutant still shows half of the activity in good agreement with the 46% fraction of plasmid-born ribosomes (Table I). Similar results are obtained when the hot TCA precipitable AcPhe residues are considered (second panel). Under this condition, AcPhe precipitates only if it heads a chain of oligo(Phe) with a minimal length of three Phe residues (42). The number of precipitable AcPhe residues directly gives the number of ribosomes active in poly(Phe) synthesis since ribosomes do not recycle on poly(U) but translate as far as possible toward the 3'-end. Furthermore, under the conditions used, ribosomes with AcPhe-tRNA initiate poly(Phe) synthesis much faster than ribosomes without (38, 43). The statistical length of the poly(Phe) chains can thus be simply calculated from both the total Phe incorporation and the number of precipitable AcPhe residues (third panel), since practically all

poly(Phe) chains carry an AcPhe residue at their N-termini. Fig. 3 shows that in the presence of thiostrepton, the chain growth of poly(Phe) occurs nearly as fast as that of the total ribosome population in the absence of thiostrepton, *i.e.* the A1067U mutation has practically no consequence for the ribosomal elongation cycle in our test systems.

Results obtained with the mutants G2581A and C2507U/G2581A are demonstrated in Fig. 3, C and D. In the presence of thiostrepton, the total Phe incorporation per 70 S ribosome (first panel) is strongly reduced for the G2581A ribosomes compared with the control ribosomes A1067U. The overall Phe incorporation is the product of the active ribosomes (second panel) and the chain length (Phe/AcPhe; third panel). Both factors are significantly reduced. Furthermore, the average speed of elongation within the first 20 s is reduced by a factor of two for the mutant ribosomes (Fig. 4C). It follows that these mutant ribosomes are strongly affected in peptide synthesis and that the processivity is significantly impaired. The double mutant shows no significant activity in this assay.

Fig. 4 shows a summary of the relative Phe incorporation per 70 S ribosome in the presence of thiostrepton compared with the corresponding activity without the drug (Fig. 4A), the active fraction of ribosomes in the presence and absence of the drug (Fig. 4B), and the average velocity of the ribosomes with plasmid-encoded 23 S rRNA compared with that of the control ribosomes A1067U (Fig. 4C). Note that the thiostrepton-sensitive part of the active fraction of ribosomes (Fig. 4B) is hardly affected by all ribosomes derived from pNOt derivatives. These thiostrepton-sensitive signals correspond to the wild-type fraction within the mixed population of ribosomes and represent a

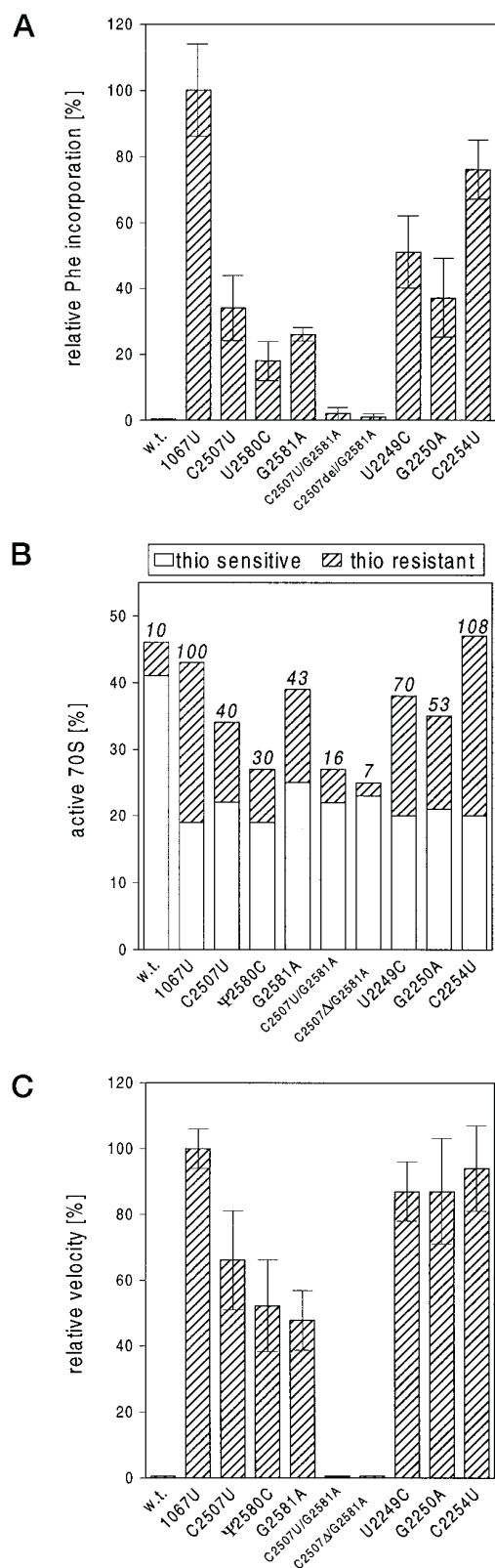


FIG. 4. Summary of the results of the poly(Phe) synthesis obtained with the various constructs. The poly(Phe) synthesis was described in detail for some ribosome preparations in Fig. 3. A, Phe incorporation in the presence of thiostrepton relative to the pNot derived ribosomes carrying only the A1067U mutation. The average value for all time points  $\geq 20$  s from at least two independent experiments is shown, and the standard deviation is given. B, the active fractions of ribosomes (AcPhe/70 S). All time points  $\geq 20$  s of the kinetics were used for the calculation. The total height of the columns represents the active fraction without drug. The standard deviation of the active fractions was around  $\pm 10\%$ . The thiostrepton-sensitive fraction (*thio sensitive*)

good internal control that only the mutated ribosomes are affected.

All mutations in the helix 90 severely affect poly(Phe) synthesis. The G2581A and Ψ2580C ribosomes show a three-fold reduced Phe incorporation (Fig. 4A) caused by a reduction of the active fraction (Fig. 4B), and the elongation rate was retarded by a factor of two (Fig. 4C). As already mentioned, the C2507U/G2581A and also the C2507Δ/G2581A double mutants are practically inactive in the poly(U) translation system. The effects seen with the mutations in the helix-loop 80 are less pronounced but still significant. All mutations show a slightly reduced speed of elongation (Fig. 4C). The relatively strongest effect is seen with the G2250A mutation concerning the overall Phe incorporation (Fig. 4A). This effect is mainly due to a reduction of the active fraction of ribosomes (Fig. 4B).

#### DISCUSSION

In this study and in the following paper (28), regions of helices 80 and 90 of the 23 S rRNA have been investigated for their possible involvement in the PTF reaction. Both regions contain a UGG sequence that is universally conserved among non-mitochondrial 23 S-type rRNA and has, therefore, the potential to bind the universal conserved CCA-3'-end of tRNAs via base pairing. Only a few mitochondrial 23 S-type rRNA from animals contain only two out of the three otherwise universally conserved UGG sequences (44); the corresponding ribosomes synthesize proteins with low efficiency and translate only a few mRNAs (45).

In agreement with the high evolutionary conservation of both sequences, all mutations tested show some defect in the *in vitro* poly(Phe) translation system. However, none of the mutations introduced in helix 80 (U2249C, G2250A, C2254U, and G2250A/C2254U) affects the growth of the bacteria. This is not necessarily a contradiction. For example, the G2661C mutation of 23 S rRNA has no effect on bacterial growth until expressed in a mutant S12 background (46), but the 23 S rRNA mutation alone significantly reduces the translational efficiency (47). As shown in the following paper (28), the mutations introduced in helix 80 only slightly reduce the puromycin reaction. Therefore, none of the changed bases is essential for peptide bond formation. The effects of these mutations on the poly(Phe) synthesis is not caused by a specific block of a single elongation step, but rather the kinetic efficiency of A site binding, the translocation reaction, or the processivity might be affected. Among the altered bases is C2254, a base protected by an A site-bound tRNA (18). Our data suggest that this protection is either an indirect effect or, alternatively, the interaction between tRNA and 23 S rRNA at this position is not essential. Furthermore, weakening or destruction of the base pair G2250-C2254 (*i. e.* the mutations C2254U and G2250A, respectively) have no disastrous effect on any of the ribosomal activities tested. It follows that this base pair does not seem to be required for functions of the ribosomal elongation cycle and might not even exist within the ribosome.

represented by the *lower columns* was obtained as the difference between the active fraction of ribosomes without thiostrepton and the active fraction in the presence of thiostrepton. The *numbers* over the columns indicate the ratio of thiostrepton-resistant active fraction *versus* the thiostrepton-sensitive fraction, relative to the control ribosomes carrying only the A1067U mutation. 56% of the active fraction of the control ribosomes were resistant against thiostrepton; 100% corresponds, therefore, to a ratio  $56/44 = 1.27$ . C, the relative average velocity of the growth of the chain length Phe/AcPhe within the first 20 s in the presence of thiostrepton derived from a regression line (see Fig. 3, *third panel*). The control ribosomes (relative velocity of 100) derived from pNot had an average velocity of 1.3 Phe/AcPhe/sec in the presence of thiostrepton.



In contrast, most of the mutations within helix 90 (C2507U, G2581A, C2507U/G2581A, and C2507Δ/G2581A) have a dominant lethal phenotype. At least the single mutations do not affect the assembly of the 50 S subunits and the flow of the assembled subunit into the polysome fraction. Poly(Phe) synthesis is severely impaired. In the following paper, we were able to demonstrate that the helix 90 mutations G2581A, C2507U/G2581A, and C2507Δ/G2581A completely block the puromycin reaction, and the mutation Ψ2580C drastically impairs this reaction (28). Also C2507U reduces the AcPhe-puromycin formation by approximately 50%.

Comparison of the poly(Phe) translation data with the results of the puromycin reactions (28) raises the following question. How is it possible that a ribosomal activity essential for protein synthesis such as peptide-bond formation is completely blocked in assay systems specifically testing this activity, whereas in systems testing the overall elongation such as poly(Phe) synthesis a significant although residual activity can be detected?

Let us consider the G2581A mutation. In contrast to the total inhibition of the puromycin reaction, there is a strongly decreased but still significant activity of this mutant 70 S in the poly(U) translation system (~30% compared with the 70 S derived from pN0t). Various factors have to be considered for an explanation of this apparent discrepancy. First of all, one has to consider that the PTF activity is not the rate-limiting step of elongation and is much faster than A site occupation or translocation. It follows that even a severe effect on the rate of the PTF activity is not necessarily detected in a complete translation system. Second, the puromycin reaction takes place at 0 °C and the poly(U) translation at 37 °C. The higher temperature might cause a higher flexibility of the PTF center, thus partially overcoming the negative effect of the mutation. And third, the reactants in the poly(U) translation system, peptidyl-tRNA and aminoacyl-tRNA, are bound to the ribosome mainly outside of the PTF center and are tightly fixed. If the mutation weakens the binding of the P site-bound CCA-3'-end but does not affect the stably fixed aminoacyl-tRNA at the A site, peptidyl transfer will occur when the loosely bound peptidyl residue has accidentally reached the correct position. In contrast, puromycin has only a low binding affinity to the A site ( $1.35 \times 10^3 \text{ M}^{-1}$ ) (48). Therefore, the sticking time is very short and most of the encounters of puromycin with the ribosome should be unproductive if the peptidyl residue is not tightly fixed at the PTF center. This feature of the puromycin reaction explains why a mutant can affect the puromycin reaction much more severely than an overall protein synthesis system such as poly(Phe) synthesis. Furthermore, a binding analysis of ligands for the A and P site regions of the PTF center revealed a positive cooperativity between these sites (49, 50). This cooperativity might be destroyed by a mutation that weakens the binding of the P site bound CCA-3'-end, therefore further reducing the low affinity of puromycin or a fragment that only binds to the A site region of the PTF center.

Effects of this kind were also observed with other mutants, i.e. a strong or even complete inhibition in an assay system testing a partial but essential reaction for protein synthesis in contrast to an almost normal growth or activity in a complete translation system. The G2583A, G2583C, and G2505A mutations are active in a poly(U) translation system but are inactive in AcPhe-puromycin formation.<sup>3</sup> The A2503G mutation did not affect growth unless erythromycin was added, but the derived ribosomes were inactive or nearly inactive in a fragment assay

(24). Similar observations also have been reported with peptidyl-tRNA mutations. A mutated CCA-3'-end at position 74 abolished the activity as a PTF donor in a fragment reaction (27), but the corresponding tRNAs are active *in vivo* (51). Mutations at position A-76 also affect the fragment reaction more (27) than an *in vitro* translation system (52).

It is therefore likely that the effects of the helix 90 mutations on poly(Phe) synthesis are caused by a severe distortion of the active center of the ribosomal PTF (28). The data clearly indicate that not only the single-stranded 23 S rRNA regions in the central loop of domain V are of high importance for ribosomal function but also the nucleotides at the top of helix 90.

**Acknowledgments**—We are grateful to Drs. C. O'Neal, N. Burkhardt, R. Jünemann, and G. Diedrich for help and discussion and to J.-U. Bittner for expert assistance.

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