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β-Puromycin Selection of Modified Ribosomes for *in Vitro* Incorporation of β-Amino Acids

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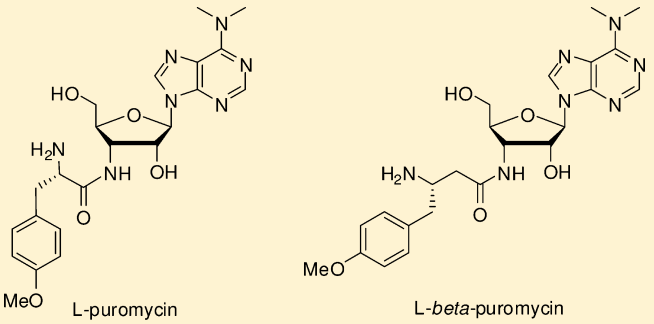
ABSTRACT: Ribosomally mediated protein biosynthesis is limited to α-L-amino acids. A strong bias against β-L-amino acids precludes their incorporation into proteins *in vivo* and also *in vitro* in the presence of misacylated β-aminoacyl-tRNAs. Nonetheless, earlier studies provide some evidence that analogues of aminoacyl-tRNAs bearing β-amino acids can be accommodated in the ribosomal A-site. Both functional and X-ray crystallographic data make it clear that the exclusion of β-L-amino acids as participants in protein synthesis is a consequence of the architecture of the ribosomal peptidyltransferase center (PTC). To enable the reorganization of ribosomal PTC architecture through mutagenesis of 23S rRNA, a library of modified ribosomes having modifications in two regions of the 23S rRNA (2057−2063 and 2496−2507 or 2582−2588) was prepared. A dual selection procedure was used to obtain a set of modified ribosomes able to carry out protein synthesis in the presence β-L-amino acids and to provide evidence for the utilization of such amino acids, in addition to α-L-amino acids. β-Puromycin, a putative mimetic for β-aminoacyl-tRNAs, was used to select modified ribosome variants having altered PTC architectures, thus potentially enabling incorporation of β-L-amino acids. Eight types of modified ribosomes altered within the PTC have been selected by monitoring improved sensitivity to β-puromycin *in vivo*. Two of the modified ribosomes, having 2057AGCGUGA2063 and 2502UGGCAG2507 or 2502AGCCAG2507, were able to suppress UAG codons in *E. coli* dihydrofolate reductase (DHFR) and scorpion *Opisthorcanthus madagascariensis* peptide IsCT mRNAs in the presence of β-alanyl-$tRNA_{CUA}$.



The successful incorporation of noncanonical amino acids (α-L-analogues of natural amino acids) into proteins demonstrates the flexibility of the ribosome peptidyltransferase center (PTC) toward a variety of side chains of α-L-amino acids. However, while numerous noncanonical amino acids have been incorporated into different proteins, not all of them have involved a high efficiency of incorporation during translation. The chirality of the amino acid substrates is also critical for effective incorporation of unnatural amino acids into proteins. Thus, naturally occurring D-amino acid-containing peptides and proteins are produced by nonribosomal synthesis or post-translational modification.[1,2] Clearly, the PTC discriminates between individual amino acids and can prevent the incorporation of L-α-amino acids having side chains of large or inappropriate geometry as well as α-D-amino acids.

Some efforts have been made to understand the scope of ribosomal selectivity. Thus, Sisido and co-workers used *E. coli* ribosomes programmed with streptavidin mRNA to identify structural features that enable the incorporation of amino acid analogues having large aromatic groups.[3,4] They suggested that the incorporation efficiency of a given noncanonical amino acid was directly correlated to its adaptability to the architecture of the A-site of the ribosome.

Our previous efforts to change the architecture of the PTC, and thereby facilitate the incorporation of D-amino acids, has shown that re-engineering the region 2447−2450 of the 23S rRNA, which is responsible for orienting N3 of the adenine nucleobase and 2′-OH of the ribose moiety of A2451 within hydrogen-bonding distance of the α-amino group of aminoacyl-tRNA,[5] can lessen the ribosomal exclusion of D-amino acids.[6,7] Two mutants, having 2447UUGU2450 and 2447UGGC2450 sequences instead of the wild-type 2447GAUA2450 sequence, displayed improved incorporation of D-methionine and D-phenylalanine into proteins. Both of these mutants shared G2447U and U2449G substitutions. These data are consistent with the results of Dahlberg and co-workers, who showed that the U2449G substitution does not affect ribosome function significantly but that the G2447U single mutation did not maintain cell viability.[8,9] In our experiments the double mutant G2447U/U2449G demonstrated good cell growth *in vivo* and protein synthesis ability *in vitro*.[6,7] Thus, multiple

Received: October 21, 2011
Revised: November 18, 2011
Published: December 6, 2011

mutations can dramatically change the architecture of the PTC and recover normal function by compensating for what would otherwise be deleterious changes to PTC architecture. The increased UAG codon suppression in the presence of D-aminoacyl-tRNAs verified that the new ribosome had altered PTC architecture more conducive to the utilization of D-aminoacyl-tRNAs.

β-Amino acids are found in cells but not in proteins or peptides.[10] During *in vitro* experiments, Roberts and co-workers[11] demonstrated that analogues of β-aminoacyl-tRNAs, L-β-(4-Me)-Phe-PANS and L-β-Ala-PANS, bound to the PTC and inhibited normal translation. Thus, β-amino acids can be accommodated in the A-site but apparently do not function in peptide bond formation. During our experiments with modified ribosomes, we were unable to identify any mutant in regions close to catalytic nucleotide A2451 which could significantly improve the *in vitro* incorporation of amino acids such as β-alanine or β-phenylalanine (unpublished data). This implies that the architecture of the ribosomal PTC may have a stronger bias against β-amino acids than D-amino acids. This may reflect a strict distance requirement between the nucleophilic amino group of aminoacyl-tRNA in the A-site and the activated carbonyl carbon atom of the bound peptidyl-tRNA.

To facilitate the incorporation of β-amino acids into proteins, the architecture of the substrate binding site would seem to require more significant changes. The nucleophilic amine of the A-site β-aminoacyl tRNA should be in close proximity to the carbonyl carbon atom of the peptidyl-tRNA to enable nucleophilic attack by the β-amino acid. However, the modified ribosomes must still be able to use α-amino acids for normal protein synthesis.

Accordingly, we constructed a new library of modified ribosomes having modifications in two regions of the PTC. β-Puromycin (Figure 1), a putative mimetic for β-aminoacyl-tRNAs, was used to select modified ribosome variants with altered PTC architectures. The *E. coli* containing selected variants with β-puromycin sensitive ribosomes were used to study the incorporation of β-amino acids *in vitro*. It was found that at least two modified ribosomes could direct the synthesis of wild-type and modified *E. coli* dihydrofolate reductase (DHFR) and scorpion *Opisthorcanthus madagascariensis* peptide IsCT. Suppression of UAG codons introduced into specific positions of the respective mRNAs resulted in enhanced full length protein synthesis in the presence of β-alanyl-$tRNA_{CUA}$. The wild-type DHFR was shown to retain good enzymatic activity, consistent with maintenance of the fidelity of protein synthesis involving α-amino acids.

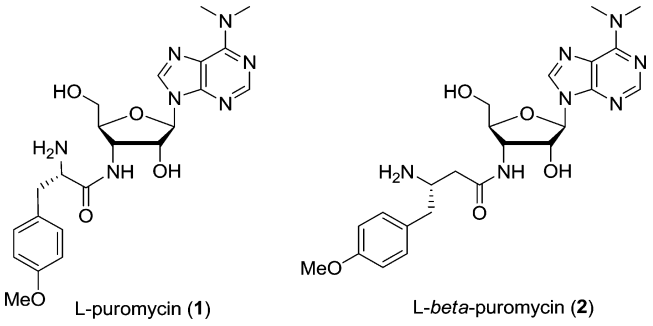


Figure 1. Structures of puromycin (**1**) and β-homopuromycin ("β-puromycin") (**2**).

MATERIALS AND METHODS

General Methods and Materials. Reagents and solvents for chemical synthesis were purchased from Aldrich Chemical Co. or Sigma Chemical Co. and were used without further purification. All reactions involving air- or moisture-sensitive reagents or intermediates were performed under argon. Flash chromatography was performed using Silicycle silica gel (40−60 mesh). Analytical TLC was performed using EM silica gel 60 F_{254} plates (0.25 mm) and was visualized by UV irradiation (254 nm). ^{1}H and ^{13}C NMR spectra were obtained using a 400 MHz Varian NMR instrument. Chemical shifts are reported in parts per million (ppm, δ) referenced to the residual ^{1}H resonance of the solvent ($CDCl_3$, δ 7.26; CD_3OD, δ 3.31; DMSO-d_6, δ 2.50). ^{13}C NMR spectra were referenced to the residual ^{13}C resonance of the solvent ($CDCl_3$, δ 77.16; CD_3OD, δ 49.00; DMSO-d_6, δ 39.52). Splitting patterns are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet; br, broad. High-resolution mass spectra were obtained at the Michigan State University High Resolution Mass Spectrometry Laboratory.

Tris, acrylamide, bis-acrylamide, urea, ammonium persulfate, *N,N,N′,N′*-tetramethylenediamine (TEMED), dihydrofolic acid, glycerol, ampicillin, pyruvate kinase, lysozyme, erythromycin, isopropyl-β-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), dithiothreitol (DTT), and 2-mercaptoethanol were purchased from Sigma Chemicals (St. Louis, MO). ^{35}S-Methionine (10 μCi/μL) was obtained from Amersham (Pitscataway, NJ). BL-21 (DE-3) competent cells, T4 RNA ligase, T4 polynucleotide kinase, and the luciferase assay system were from Promega (Madison, WI). The XAC-1 strain was obtained from Dr. Jeffrey H. Miller.[12] Taq DNA ligase, endonucleases *Eco*RI from *E. coli* RY13, and *Dpn*I from *Diplococcus pneumonia* were obtained from New England Biolabs (Beverly, MA). Pfu DNA polymerase was purchased from Agilent Technologies (Wilmington, DE). Synthetic oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA).

Plasmid MaxiKit (Life Science Products, Inc., Frederick, CO) and GenEluteHP plasmid miniprep kit (Sigma) were used for plasmid purification. The sequencing of rDNA samples was carried out in the ASU DNA Sequencing Laboratory.

Phosphorimager analysis was performed using a Molecular Dynamics 400E PhosphoImager equipped with ImageQuant version 3.2 software. Luminescence spectral measurements were made using a Hitachi F2000 fluorescence spectrophotometer. Ultraviolet and visible spectral measurements were made using a Perkin-Elmer lambda 20 spectrophotometer.

Synthesis of β-Puromycin and β-Alanyl-pdCpA Derivatives. *N-Boc-(S)-3-amino-4-(4-methoxyphenyl)butyric Acid Methyl Ester (**4**).*[13,14] To a cooled (0 °C) solution containing 161 mg (0.68 mmol) of β-L-homotyrosine hydrochloride in 2.6 mL of 1:1 dioxane−water was added 0.24 mL (1.7 mmol) of triethylamine, and the reaction mixture was stirred at 0 °C for 5 min. Di-*tert*-butyl dicarbonate (237 mg, 1.09 mmol) was added, and the reaction mixture was stirred at 0 °C for 30 min and then at 25 °C for 20 h. The reaction mixture was concentrated under diminished pressure, and the residue was diluted with 20 mL of water and washed with two 8 mL portions of Et_2O. The cooled aqueous phase (ice bath) was acidified to pH ∼3 with 5% aqueous $NaHSO_4$ and extracted with three 20 mL portions of ethyl acetate. The combined organic layer was washed with 10 mL of brine, dried over

dx.doi.org/10.1021/bi2016124 | *Biochemistry* 2012, 51, 401−415

anhydrous Na_2SO_4, filtered, and concentrated under diminished pressure to afford *N*-Boc-(*S*)-3-amino-4-(4-methoxyphenyl)butyric acid as a light yellow oil: crude yield 228 mg; silica gel TLC R_f 0.20 (9:1 chloroform−methanol). This compound was used directly in the next step without further purification.

To a suspension containing 228 mg of the crude carboxylic acid and 470 mg (3.41 mmol) of oven-dried K_2CO_3 in 4 mL of anhydrous acetone was added dropwise 0.20 mL (2.0 mmol) of dimethyl sulfate, and the reaction mixture was stirred at reflux for 36 h. The cooled reaction mixture was filtered and washed with ethyl acetate, and the combined filtrate was concentrated under diminished pressure. The light yellow residue was purified on a silica gel column (12 × 3 cm), which was eluted with 4:1 hexanes−ethyl acetate. Compound **4** was obtained as a colorless oil: yield 179 mg (81% over 2 steps); silica gel TLC R_f 0.30 (2:1 hexanes−ethyl acetate). ^{1}H NMR ($CDCl_3$) δ: 1.38 (s, 9H), 2.46 (dq, 2H, J = 15.8 and 5.7 Hz), 2.80 (m, 2H), 3.64 (s, 3H), 3.74 (s, 3H), 4.10 (m, 1H), 5.01 (br, 1H), 6.80 (d, 2H, J = 8.4 Hz), and 7.06 (d, 2H, J = 8.4 Hz). ^{13}C NMR ($CDCl_3$) δ: 28.4, 37.5, 39.5, 49.0, 51.6, 55.2, 79.3, 113.9, 129.7, 130.3, 155.1, 158.4, and 172.1. Mass spectrum (ESI), m/z 324.1800 $(M + H)^+$ ($C_{17}H_{26}NO_5$ requires m/z 324.1811).

*N-Boc-(S)-3-amino-4-(4-methoxyphenyl)butyric Acid (**5**).*[15] To a solution containing 76 mg (0.27 mmol) of **4** in 2.5 mL of THF was added a solution of 27 mg (1.1 mmol) of LiOH in 1.25 mL of water, and the reaction mixture was stirred at 25 °C for 20 h. The reaction mixture was diluted with 10 mL of Et_2O and 25 mL of water, and the phases were separated. The cooled aqueous phase (ice bath) was acidified to pH ∼ 3 with 5% aqueous $NaHSO_4$ and extracted with three 20 mL portions of ethyl acetate. The combined organic layer was washed with 10 mL of brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under diminished pressure to afford **5** as a colorless solid: yield 65 mg (91%); silica gel TLC R_f 0.17 (1:1 hexanes−ethyl acetate). ^{1}H NMR (CD_3OD) δ: 1.37 (s, 9H), 2.40, (m, 2H), 2.72 (m, 2H), 3.75 (s, 3H), 4.07 (m, 1H), 6.82 (d, 2H, J = 8.4 Hz), and 7.12 (d, 2H, J = 8.4 Hz). ^{13}C NMR ($CDCl_3$) δ: 28.7, 39.5, 40.8, 50.7, 55.6, 79.9, 114.8, 131.3, 131.5, 157.5, 159.7, and 175.0.

*N-Fmoc-(S)-3-amino-4-(4-methoxyphenyl)butyric Acid (**6**).* A solution containing 220 mg (0.71 mmol) of **5** in 4.2 mL of 4:1 CH_2Cl_2−CF_3COOH was stirred at 25 °C for 40 min. The reaction mixture was concentrated under diminished pressure, and the residual CF_3COOH was removed by coevaporation with five 4 mL portions of toluene. The solid residue was dissolved in 4.2 mL of 10% aqueous Na_2CO_3 and a solution containing 290 mg (0.86 mmol) of Fmoc-OSu in 4.2 mL of dioxane was added dropwise. The resulting mixture was stirred at 25 °C for 24 h. Water (25 mL) was added, and the aqueous layer was washed with two 10 mL portions of ether. The cooled aqueous phase (ice bath) was acidified to pH ∼ 3 with 5% aqueous $NaHSO_4$ and extracted with three 30 mL portions of ethyl acetate. The combined organic layer was washed with 15 mL of brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under diminished pressure. Compound **6** was purified by precipitation from hexanes−ethyl acetate and was obtained as a colorless solid: yield 202 mg (66%); silica gel TLC R_f 0.12 (1:1 hexanes−ethyl acetate); mp 178 °C. ^{1}H NMR (DMSO-d_6) δ: 2.37 (dd, 2H, J = 6.8 and 2.2 Hz), 2.66 (d, 2H, J = 6.8 Hz), 3.67 (s, 3H), 3.95 (dd, 1H, J = 14.9 and 7.0 Hz), 4.16 (dd, 1H, J = 12.9 and 6.0 Hz), 4.22 (dd, 2H, J = 7.0 and 2.9 Hz), 6.80 (d, 2H, J = 8.6 Hz), 7.08 (d, 2H, J = 8.6 Hz), 7.32 (q, 2H, J = 6.3 Hz), 7.41 (t, 2H, J = 7.4 Hz), 7.65 (d, 2H, J = 7.5 Hz), and 7.88 (d, 2H, J = 7.5 Hz). ^{13}C NMR (DMSO-d_6) δ: 46.7, 49.8, 54.9, 65.2, 113.6, 120.1, 125.2, 127.0, 127.6, 130.2, 130.4, 140.7, 143.8, 143.9, 155.3, 157.7, and 172.5. Mass spectrum (ESI), m/z 432.1820 $(M + H)^+$ ($C_{26}H_{26}NO_5$ requires m/z 432.1811).

*N-Fmoc-(S)-3-amino-4-(4-methoxyphenyl)butyric Acid Succinimidyl Ester (**7**).* To a suspension containing 46 mg (0.11 mmol) of **6** and 19 mg (0.16 mmol) of *N*-hydroxysuccinimide in 3 mL of anhydrous CH_2Cl_2 was added 33 mg (0.16 mmol) of *N*,*N*′-dicyclohexylcarbodiimide, and the reaction mixture was stirred at 25 °C for 24 h. The insoluble precipitate was filtered, and the filtrate was concentrated under diminished pressure. The residue was suspended in 3 mL of ethyl acetate and filtered, and the filtrate was concentrated under diminished pressure. The crude product was purified on a silica gel column (12 × 2 cm); elution was with 2:1 hexanes−ethyl acetate. Compound **7** was obtained as a colorless solid: yield 38 mg (68%); mp 172 °C; silica gel TLC R_f 0.75 (9:1 chloroform−methanol). ^{1}H NMR ($CDCl_3$) δ: 2.70−3.01 (m, 8H), 3.78 (s, 3H), 4.28 (dt, 4H, J = 14.1 and 6.7 Hz), 5.35 (d, 1H, J = 8.8 Hz), 6.85 (d, 2H, J = 8.3 Hz), 7.15 (d, 2H, J = 8.0 Hz), 7.30 (t, 2H, J = 7.4 Hz), 7.40 (t, 2H, J = 7.4 Hz), 7.56 (dd, 2H, J = 7.4 and 3.7 Hz), and 7.76 (d, 2H, J = 7.5 Hz). ^{13}C NMR ($CDCl_3$) δ: 25.7, 34.7, 38.7, 47.3, 49.6, 55.4, 66.9, 114.3, 120.0, 125.3, 127.1, 127.8, 129.0, 130.5, 141.4, 143.98, 144.02, 155.7, 158.6, 166.7, and 169.1. Mass spectrum (ESI), m/z 529.1956 $(M + H)^+$ ($C_{30}H_{29}N_2O_7$ requires m/z 529.1975).

*9-[3′-Deoxy-3′-(N-Fmoc-(S)-3-amino-4-(4-methoxyphenyl)butyramido)-β-D-ribofuranosyl]-6-(N,N′-dimethylamino)purine (**8**).* To a solution containing 14 mg (0.03 mmol) of **7** and 3.0 μL (0.02 mmol) of Et_3N in 0.6 mL of anhydrous DMF was added 5.0 mg (0.02 mmol) of puromycin aminonucleoside. The reaction mixture was stirred at 25 °C for 3.5 h (at which time silica gel TLC analysis showed complete consumption of **7**). The solvent was concentrated under diminished pressure, and the residue was purified on a silica gel column (14 × 2 cm); elution was with 25:1 chloroform−methanol. Compound **8** was obtained as a colorless solid: yield 12 mg (the product was slightly impure and was used directly in the next step); silica gel TLC R_f 0.52 (9:1 chloroform−methanol). ^{1}H NMR (DMSO-d_6) δ: 2.31 (d, 2H, J = 6.7 Hz), 2.55−2.82 (m, 2H), 3.50 (s, 6H), 3.68 (m, 5H), 3.97 (s, 2H), 4.09−4.32 (m, 3H), 4.45 (dd, 2H, J = 16.1 and 8.5 Hz), 5.17 (s, 1H), 5.97 (s, 2H), 6.80 (d, 2H, J = 8.1 Hz), 7.06 (d, 2H, J = 8.0 Hz), 7.18 (d, 1H, J = 8.3 Hz), 7.25−7.47 (m, 4H), 7.63 (t, 2H, J = 7.1 Hz), 7.87 (d, 2H, J = 8.0 Hz), 7.91 (d, 1H, J = 8.0 Hz), 8.20 (s, 1H), and 8.44 (s, 1H). ^{13}C NMR (DMSO-d_6) δ: 24.9, 33.8, 47.1, 50.5, 50.6, 55.3, 60.9, 65.6, 73.6, 83.3, 89.7, 113.9, 120.0, 120.5, 125.6, 127.4, 128.0, 130.7, 138.1, 141.1, 144.2, 144.4, 150.0, 152.2, 154.7, 155.8, 158.0, and 170.8; mass spectrum (MALDI) m/z 708.2 $(M + H)^+$ (theoretical m/z 708.3) and 730.1 $(M + Na)^+$ (theoretical m/z 730.3). Mass spectrum (ESI), m/z 708.3163 $(M + H)^+$ ($C_{38}H_{42}N_7O_7$ requires m/z 708.3146).

*9-[3′-Deoxy-3′-((S)-3-amino-4-(4-methoxyphenyl)-butyramido)-β-D-ribofuranosyl]-6-(N,N′-dimethylamino)-purine (β-Puromycin) (**2**).* A solution containing 12 mg (17.0 μmol) of **8** in 3 mL of 4:1 DMF−piperidine was stirred at 25 °C for 40 min. The solvent was concentrated under diminished pressure, and the residue was purified by chromatography on a silica gel column (5 × 2 cm). Elution with 9:1

dx.doi.org/10.1021/bi2016124 | *Biochemistry* 2012, 51, 401−415

chloroform−methanol afforded **2** as a colorless solid: yield 5 mg (61% over two steps); silica gel TLC R_f 0.12 (9:1 dichloromethane−methanol). ^{1}H NMR (CD_3OD) δ: 2.42 (dd, 1H, J = 15.9 and 8.4 Hz), 2.60 (dd, 1H, J = 15.9 and 4.2 Hz), 2.73−2.91 (m, 2H), 3.51 (s, 6H), 3.59 (m, 1H), 3.73 (dd, 1H, J = 12.6 and 2.9 Hz), 3.77 (s, 3H), 3.94 (dd, 1H, J = 12.5 and 2.0 Hz), 4.17 (d, 1H, J = 7.3 Hz), 4.53−4.71 (m, 2H), 6.01 (d, 1H, J = 3.0 Hz), 6.90 (d, 2H, J = 8.5 Hz), 7.17 (d, 2H, J = 8.5 Hz), 8.21 (s, 1H), and 8.35 (s, 1H). ^{13}C NMR (CD_3OD) δ: 38.8, 39.0, 40.5, 51.5, 52.0, 55.7, 62.2, 75.0, 84.8, 91.9, 115.3, 121.6, 129.6, 131.4, 139.1, 150.6, 153.0, 156.2, 160.4, and 173.3. Mass spectrum (MALDI) m/z 486.2 $(M + H)^+$ (theoretical 486.2) and 508.2 $(M + Na)^+$ (theoretical 508.2). Mass spectrum (ESI), m/z 486.2482 $(M + H)^+$ ($C_{23}H_{32}N_7O_5$ requires m/z 486.2465).

*N-(4-Pentenoyl)-β-alanine Cyanomethyl Ester (**9**).* To a solution containing 125 mg (1.40 mmol) of β-alanine hydrochloride in 12 mL of 1:1 water−dioxane was added 297 mg (1.68 mmol) of Na_2CO_3 followed by 332 mg (1.68 mmol) of 4-pentenoic acid succinimidyl ester. The reaction mixture was stirred at 25 °C for 24 h and then diluted with 10 mL of chloroform. The organic layer was discarded, and the aqueous layer was acidified with 6 mL of 1 N aqueous $NaHSO_4$ to pH ∼ 2−3. The aqueous layer was back-extracted with two 10 mL portions of EtOAc. The combined organic layer was washed with 5 mL of water, dried ($MgSO_4$), and concentrated under diminished pressure to give the crude *N*-4-pentenoyl-β-alanine as a colorless syrup. The crude product was dissolved in 12 mL of anhydrous CH_3CN and treated with 0.97 mL (7.0 mmol) of Et_3N followed by 0.88 mL (14.0 mmol) of chloroacetonitrile. The reaction mixture was stirred at 25 °C under argon for 22 h. EtOAc (15 mL) was added, and the mixture was washed with 10 mL of 1 N aqueous $NaHSO_4$ and 10 mL of brine. The organic layer was dried ($MgSO_4$) and concentrated under diminished pressure. The crude residue was purified on a silica gel column; elution was with 1:1 hexanes−ethyl acetate and then with ethyl acetate. *N*-(4-Pentenoyl)-β-alanine cyanomethyl ester (**9**) was obtained as a colorless syrup: yield 185 mg (62%) over two steps; silica gel TLC R_f 0.13 (1:1 hexanes−ethyl acetate). ^{1}H NMR ($CDCl_3$) δ: 2.24 (t, 2H, J = 7.2 Hz), 2.34 (m, 2H), 2.63 (t, 2H, J = 5.8 Hz), 3.51 (m, 2H), 4.72 (s, 2H), 5.01 (m, 2H), 5.77 (m, 1H), and 6.13 (br s, 1H). ^{13}C NMR ($CDCl_3$) δ: 29.5, 33.6, 34.7, 35.7, 48.5, 114.3, 115.7, 136.9, 171.2, and 172.6. Mass spectrum (ESI), m/z 211.1086 $(M + H)^+$ ($C_{10}H_{15}N_2O_3$ requires m/z 211.1083).

*N-(4-Pentenoyl)-β-alanine pdCpA Ester (**10**) and Bis[N-(4-pentenoyl)-β-alanine] pdCpA Ester (**11**).* To a conical vial containing 8.0 mg (38 μmol) of *N*-(4-pentenoyl)-β-alanine cyanomethyl ester (**9**) was added a solution of 7.0 mg (5.15 μmol) of the tris(tetrabutylammonium) salt of pdCpA[16] in 50 μL of DMF followed by 7.0 μL of Et_3N. The reaction mixture was stirred at room temperature. After 18 h the reaction mixture was diluted with 2:1 CH_3CN−50 mM NH_4OAc, pH 4.5, to a total volume of 600 μL and purified using a semipreparative C_{18} reversed phase column (250 × 10 mm). The column was washed with 1 → 63% CH_3CN in 50 mM NH_4OAc, pH 4.5, over a period of 45 min at a flow rate of 3.5 mL/min (monitoring at 260 nm). After lyophilization of the appropriate fractions two compounds were obtained as colorless solids: *N*-(4-pentenoyl)-β-alanine pdCpA ester (**10**) (retention times 15.3 and 15.7 min, for the two positional (2′,3′) isomers): yield 1.5 mg (37%). Mass spectrum (MALDI), m/z 811.97 $(M + Na)^+$ (theoretical 812.17). Mass spectrum (ESI), m/z 790.1974 $(M + H)^+$ ($C_{27}H_{38}N_9O_{15}P_2$ requires m/z 790.1963). Bis[*N*-(4-pentenoyl)-β-alanine] pdCpA ester (**11**) (retention time 20.1 min): yield 1.9 mg (39%). Mass spectrum (MALDI), m/z 965.1 $(M + Na)^+$ (theoretical 965.3). Mass spectrum (ESI), m/z 943.2774 $(M + H)^+$ ($C_{35}H_{49}N_{10}O_{17}P_2$ requires m/z 943.2752).

5′-Phosphorylation of Synthetic DNA Oligonucleotide Primers. Reaction mixtures (20 μL total volume) containing 100 pmol of primer, 1 mM ATP, 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, and 1 unit of T4 polynucleotide kinase were incubated at 37 °C for 1 h and then chilled on ice. Then 40 μL of deionized water, 240 μL of 0.5 M NH_4OAc, and 750 μL of cold ethanol were added. The combined solution was mixed and incubated at −20 °C for 20 min and then centrifuged at 12000*g* for 20 min. The pellet was washed with 70% ethanol, air-dried, and dissolved in 50 μL of RNase-free water.

Site-Directed Mutagenesis of the rrnB Operon. Mutagenesis of the rrnB operon at different positions of the 23S rRNA gene was carried out by using a modified QuikChangeTN site-directed mutagenesis protocol.[17] Two versions of mutagenesis were carried out.

Plasmid pUCrrnB, constructed previously,[6,7] and the randomized primer 5′-GTACCCGCGGCAAGACGHBBBHBDCCCGTGAACCTTTACTATAG-3′ were used for mutagenesis in region 2057−2063 of the 23S rRNA. Plasmids (01−08) from selected clones after the first mutagenesis and the randomized primers 5′-GTGGTACGCGAGCTGHHVVVBHAACGTCAGACAGTTC-3′ (01), 5′-GACGGCGGTGTTTGGDBDDVDGATGTCGGCTCATCAC-3′ (02), and 5′-CGGTGTTTGGCACCTCHBVHVDGGCTCATCACATCCTG-3′ (03) were used for positions 2582−2588, 2496−2501, and 2502−2507, respectively (B = C + G + T; H = C + A + T; V = A + G + C; D = A + G + T).

The polymerase chain reaction (PCR) was carried out in a 50 μL reaction mixture containing 300 ng of template, 14 pmol of primer, 10 nmol of dNTPs, 2.5 units of Pfu polymerase, and 20 units of Taq DNA ligase in 35 mM Tris-HCl, pH 8.0, containing 12 mM KOAc, 5 mM DTT, 0.05% Triton X-100, and 0.05 mM EDTA. The thermal cycler was programmed as follows: preincubation at 95 °C for 2 min, 18 cycles at 95 °C for 1 min, 50 °C for 1 min, and 65 °C for 24 min. The samples were then incubated at 72 °C for 7 min and then cooled to room temperature. Restriction endonuclease *Dpn*I (1 μL) was added, and the reaction mixture was incubated at 37 °C for 1 h. Then the samples were subjected to denaturation at 95 °C for 1 min, followed by 2 cycles at 95 °C for 1 min, 50 °C for 1 min, and 70 °C for 24 min. The samples were precipitated by the addition of NaOAc, pH 5.2, to a concentration of 0.1 M followed by 3 vol of cold ethanol. After incubation at −20 °C for 20 min, the samples were centrifuged and the pellets were washed with 70% ethanol, air-dried, and dissolved in 10 μL of deionized water. JM-109 cells were transformed (electroporation) by 5 μL of PCR products, and transformants were plated on the LB agar, prepared with 100 μg/mL ampicillin and 0.5 mM IPTG, and incubated at 37 °C for 18−24 h.

Selection of Erythromycin-Resistant Clones. For the selection of erythromycin-resistant clones (first mutagenesis reaction) the colonies were transferred to fresh LB agar plates containing 100 μg/mL ampicillin, 1 mM IPTG, and 3.5 μg/mL erythromycin (nitrocellulose filter replicates). Plates were

incubated at 37 °C for 48 h. The erythromycin-resistant colonies so obtained were transferred to 0.5 mL of LB medium containing 100 μg/mL ampicillin and were grown at 37 °C until OD_{600} 0.2−0.5 was reached (about 3−5 h). The erythromycin sensitivity (MIC) of prepared cultures was estimated in comparison with a culture containing the pUC-rrnB plasmid. Plasmids from erythromycin-resistant clones were isolated and sequenced in the region of mutagenesis.

Preparation of Libraries of Cells Having Modified Ribosomes. Each single colony from the corresponding agar plates was transferred into 0.5 mL of LB medium supplemented with ampicillin, grown at 37 °C for 5 h, and then mixed with 0.5 mL of 30% glycerol. A library containing "master plates" was organized in 96-well format (200 μL in each well; one clone per well). A culture having pUC-18 plasmid (no rrnB operon) was placed in a well of each plate as a control.

High Throughput Dual Selection with β-Puromycin and Erythromycin. Three new plates were prepared from each "master plate" by transferring 2 μL of cultures from each well of the master plate to the corresponding well of the new plates. Each culture was mixed with 98 μL of assay solution. LB medium, pH 8.25, containing 100 μg/mL ampicillin, 1 mM IPTG, 100 μg/mL of β-puromycin, or 3.5 μg/mL of erythromycin was employed in the assay solution. A solution without any β-puromycin or erythromycin was used for control plates. All plates were incubated at 37 °C for 16−18 h in a thermostated shaker, and the extent of cell growth was estimated by measuring the optical density at 600 nm. Inhibition of cell growth by antibiotics was estimated for each well as $[100 - (A_{600}\text{exp}/A_{600}\text{cont}) \times 100]$. Selection cutoff values were 50% and 70% for β-puromycin and erythromycin assays, respectively. The absence of inhibition (<1%) in the β-puromycin assay and full inhibition (>99%) in the erythromycin assay was observed for the control culture. Representative clones having sensitivity to β-puromycin and some resistance to erythromycin were chosen for the next selection step (verification). Plasmids from these clones were isolated and retransformed into fresh cells, and the sensitivity to both antibiotics was determined. Three colonies for each plasmid were grown in 0.5 mL of LB medium, supplemented with 100 μg/mL ampicillin and 1 mM IPTG until OD_{600} 0.15−0.3 was reached, and then diluted with the same medium about 15−30 times (final $OD_{600} \sim 0.01$). Each diluted culture was placed in 8 wells of a 96-well plate, having six different dilutions of antibiotics (200−6.25 μg/mL for β-puromycin and 25−0.78 μg/mL for erythromycin) and two wells without antibiotics. Plates were incubated at 37 °C for 18−24 h in a thermostated shaker, and the extent of cell growth was estimated by optical density (OD_{600}).

β-Galactosidase Assay. Plasmids, having the rrnB operon with wild-type and mutant sequences in different regions of 23S rRNA and selected by sensitivity to β-puromycin, were transformed into XAC-1 competent cells. Fresh single colony cultures were prepared in LB medium, supplemented with 100 μg/mL ampicillin and 0.5 mM IPTG ($OD_{600} \sim 0.3$−0.5), and then diluted to 0.01 OD_{600}/mL with LB medium containing 100 μg/mL ampicillin, 1 mM IPTG, and 100 μg/mL X-gal. Diluted cultures (100 μL) were placed in individual wells of 96-well plates (four wells for each culture) and incubated at 37 °C for 16−18 h. The optical densities at 654 and 490 nm were then determined. XAC-1 cells, either without plasmid or carrying a plasmid encoding $tRNA_{CUA}^{Gly}$, were used as negative and positive controls. β-Galactosidase activity (relative units, ru) was estimated in each sample using the formula $[(A_{654}/A_{490})\text{sample} - (A_{654}/A_{490})C^{-}] \times 1000$. Each culture was assayed at least three times, and the average data and standard deviation were determined.

S-30 Preparation. *E. coli* BL-21(DE-3) cells, harboring plasmids with a wild-type or modified rrnB gene, from liquid stocks (5−10 μL) were placed on LB agar supplemented with 100 μg/mL ampicillin and grown at 37 °C for 16−18 h. One colony was picked from each agar plate and transferred into 3 mL of LB medium supplemented with 100 μg/mL ampicillin and 0.5 mM IPTG. The cultures were grown at 37 °C for 3−6 h in a thermostated shaker until $OD_{600} \sim 0.15$−0.3 was reached (about 3−5 h), diluted with the same medium (having 3 μg/mL erythromycin in the case of cultures with modified ribosomes) until OD_{600} 0.01 was reached, and then grown at 37 °C for 12−18 h. The optimal concentration of the final cultures was OD_{600} 0.5−1.0. Cells were harvested by centrifugation (5000*g*, 4 °C, 10 min), washed three times with S-30 buffer (1 mM Tris-OAc, pH 8.2, containing 1.4 mM $Mg(OAc)_2$, 6 mM KOAc, and 0.1 mM DTT) supplemented with β-mercaptoethanol (0.5 mL/L), and washed once with S-30 buffer having 0.05 mL/L β-mercaptoethanol. The weight of the wet pellet was estimated, and 1.27 mL of S-30 buffer was added to resuspend 1 g of cells. The volume of the suspension was measured and used for estimating the amount of other components. Preincubation mixture (0.3 mL) (0.29 M Tris, pH 8.2, containing 9 mM $Mg(OAc)_2$, 13 mM ATP, 84 mM phosphoenol pyruvate, 4.4 mM DTT and 5 μM amino acids mixture), 15 units of pyruvate kinase, and 10 μg of lyzozyme were added per 1 mL of cell suspension, and the resulting mixture was incubated at 37 °C for 30 min. The incubation mixture was then frozen at −80 °C (∼30 min), melted (25 °C, 30 min), and again frozen and melted at room temperature (∼30 min). Ethylene glycol tetraacetic acid (EGTA) was then added to 2.5 mM final concentration, and the cells were incubated at 37 °C for 30 min and again frozen (− 80 °C, 30 min). The frozen mixture was centrifuged (15000*g*, 4 °C, 1 h), and the supernatant was stored in aliquots at −80 °C.

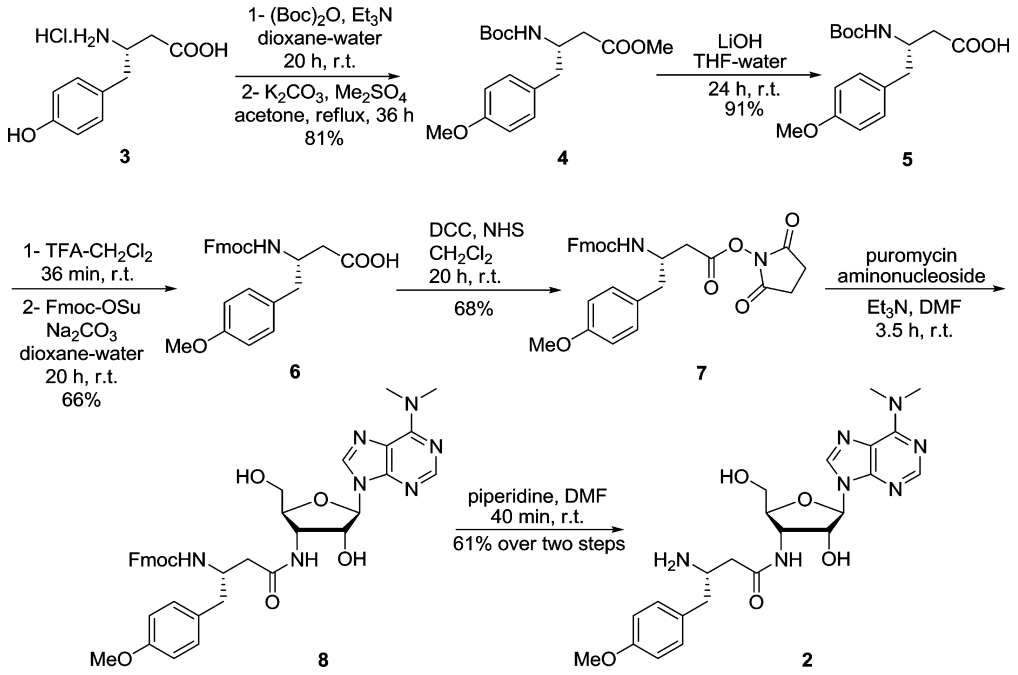
Aminoacylation of Suppressor $tRNA_{CUA}$. Aminoacylation of suppressor $tRNA_{CUA}$s was carried out in 100 μL of reaction mixtures (total volume) of 100 mM Na Hepes, pH 7.5, containing 1.0 mM ATP, 15 mM $MgCl_2$, 50 μg of suppressor $tRNA_{CUA}$-C_{OH}, 2.0 A_{260} units of pentenoyl-protected aminoacyl-pdCpA, 15% DMSO, and 100 units of T4 RNA ligase. After incubation at 37 °C for 1 h, the reaction was quenched by the addition of 0.1 vol of 3 M NaOAc, pH 5.2, and the aminoacylated tRNA was precipitated with 3 vol of cold EtOH. The efficiency of ligation was estimated by 8% polyarylamide−7 M urea gel electrophoresis (pH 5.0).

Pentenoyl-protected aminoacyl-$tRNA_{CUA}$s were deprotected by treatment with 5 mM aqueous I_2 (15 min, 25 °C). Following deprotection, the solution was centrifuged, and the supernatant was adjusted to 0.3 M NaOAc and treated with 3 vol of cold EtOH to precipitate the aminoacylated tRNA. The tRNA pellet was washed with 70% aqueous EtOH, air-dried, and dissolved in 50 μL of RNase free water.

In Vitro Protein Translation Reaction. Translation reactions were carried out in 20−200 μL of incubation mixture containing 0.2−0.4 μL/μL of S-30 system, 100 ng/μL of plasmid, 35 mM Tris acetate, pH 7.4, 190 mM potassium glutamate, 30 mM ammonium acetate, 2 mM DTT, 0.2 mg/mL total *E. coli* tRNA, 3.5% PEG 6000, 20 μg/mL folinic acid, 20 mM ATP and GTP, 5 mM CTP and UTP, 100 μM amino

dx.doi.org/10.1021/bi2016124 | *Biochemistry* 2012, 51, 401−415

Scheme 1. Synthesis of β-Homopuromycin from β-Homotyrosine and Puromycin Aminonucleoside



acids mixture, 0.5 μCi/μL of ^{35}S-methionine, and 1 μg/mL rifampicin. In the case of plasmids having a gene with a TAG codon, aminoacylated suppressor $tRNA_{CUA}$ was added to a concentration of 0.3 μg/μL. Reactions were carried out at 37 °C for 1 h and terminated by chilling on ice. Aliquots from *in vitro* translation mixtures were analyzed by SDS-PAGE followed by quantification of the radioactive bands by phosphorimager analysis.

Quantification of Enzyme Activity. DHFR activity was determined by the method of Baccanari et al.[18] Reaction mixtures (1 mL total volume) containing 50 mM morpholinoethanesulfonate (MES), 25 mM Tris, pH 7.0, 25 mM ethanolamine, 100 mM NaCl, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 100 μM dihydrofolic acid and 100 μM NADPH were incubated at 37 °C for 1−2 min in a thermostated cuvette (1 cm light path). Aliquots (5−10 μL) of DHFR samples were added, and the optical density at 340 nm was measured over period of 10 min. One unit of DHFR was defined as the amount of enzyme required to reduce 1 μM dihydrofolic acid/min at 37 °C and pH 7.0.

Luciferase activity was determined using a commercial luciferase assay system. Luciferase samples (5 μL) were added to 50 μL of luciferase assay reagent, and light emission was measured immediately. Enzyme activity was expressed as the level of light emission (Em) at the maximum wavelength (λ_{max}).

RESULTS

Synthesis of β-Puromycin. The synthesis of *N*-Fmoc-(*S*)-3-amino-4-(4-methoxyphenyl)butyric acid succinimidyl ester (**7**) commenced with Boc-protection of β-L-homotyrosine using di-*tert*-butyldicarbonate in the presence of triethylamine (Scheme 1). The crude *N*-Boc-β-L-homotyrosine[13] was subjected to exhaustive methylation using dimethyl sulfate and potassium carbonate in acetone to give the methyl ester **4**[14] in 81% yield. Saponification of the methyl ester with LiOH in aqueous THF afforded the acid **5**[15] in 91% yield. The Boc group of **5** was removed using CF_3COOH in dichloromethane, and the resulting amine was reprotected with a Fmoc group to give compound **6** in 66% yield. Finally, succinimidyl ester **7** was prepared by the use of *N*-hydroxysuccinimide and DCC in dichloromethane and was obtained in 68% yield. Puromycin aminonucleoside was then condensed with the succinimidyl ester **7** in the presence of triethylamine to give the Fmoc-protected nucleoside **8**. This slightly impure intermediate was treated with piperidine in DMF to give β-puromycin (**2**) in 61% yield (from **7**) after chromatography on silica gel.

Selection of Clones Having the Erythromycin Resistance Mutations in 23S rRNA. It is known, based on biochemical[19] and X-ray crystallographic[20] studies, that the antibiotic erythromycin binds ribosomes at a site close to the peptidyltransferase center. Accordingly, it seemed logical to assume that the erythromycin sensitivity of modified ribosomes would change as the architecture of the PTC was altered through systematic mutations. This would, in turn, facilitate the choice of ribosomal mutants for more detailed characterization. The initial selection of altered 23S rRNAs that conferred moderate erythromycin resistance was also intended to facilitate the identification of unwanted mutations that caused extensive alterations of PTC architecture. Finally, since all of the cells to be studied would contain ribosomes with both wild-type (chromosomally encoded) and modified (plasmid encoded) 23S rRNAs, the difference in erythromycin sensitivity of the wild-type and modified ribosomes to erythromycin would permit the latter species to be characterized in the absence of effects from wild-type ribosomes. It was also anticipated that cell growth in the presence of moderate concentrations of erythromycin would maximize the levels of the modified ribosomes.

On the basis of the literature data concerning the location of the erythromycin binding pocket, a library of *E. coli* having modifications of the 23S rRNA gene in nucleotides 2057−2063 was prepared by the use of specific random primers which lacked the wild-type nucleotide at each of the foregoing seven positions to avoid the wild-type phenotype among the derived mutants. The theoretical diversity of the library was 2187.

dx.doi.org/10.1021/bi2016124 | *Biochemistry* 2012, 51, 401−415

Before the evaluation of erythromycin-resistant variants from this library could be carried out, an efficient selection protocol was required. It is well-known that Gram-negative bacteria are not very sensitive to erythromycin. Therefore, the pH dependence of the erythromycin inhibition of cell growth for different *E. coli* strains was studied (Figure 2). As is clear from

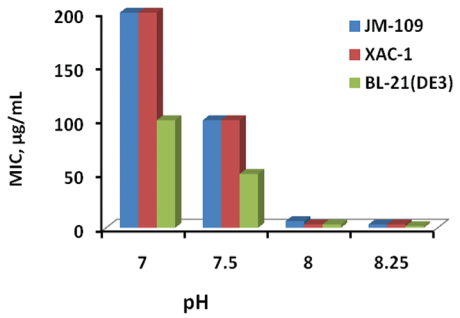


Figure 2. Erythromycin sensitivity of different *E. coli* strains having growth medium at different pH values.

Figure 2, pH is a critical parameter for inhibition of *E. coli* cell growth by erythromycin. It was found that the minimum inhibitory concentration (MIC) of this antibiotic could be decreased from 200 to 1.56 μg/mL as the pH of the growth medium was increased from 7.0 to 8.25. Therefore, LB medium having pH 8.25 was used in the selection experiments. The optimal erythromycin concentration, which allowed the greatest number of mutants to be obtained, was also investigated. Only a few colonies were found on LB agar plates supplemented with 3.5 μg/mL erythromycin vs >1000 colonies on a control plate lacking erythromycin for *E. coli* cells having only wild-type ribosomes. Under the same conditions, 27 clones were obtained from a library of cells containing modified ribosomes. The erythromycin sensitivity of these clones was verified in an MIC assay (Table 1); it was found that 15 clones had

Table 1. Characterization of Erythromycin-Resistant Clones Harboring Plasmids with Modified rrnB Operons

		inhibition by	
name/no. of clones	sequence in the 2057−2063 region of 23SrRNA gene	erythromycin (MIC, μg/mL)	β-puromycin (%)
01/5	TGCGTGG	12.5−6.25	26 ± 10
02/2	TTGGTCG	6.25−3.12	15 ± 6
03/1	ATGGTTG	6.25−3.12	20 ± 7
04/2	AGCGTGA	6.25−3.12	28 ± 13
05/2	TCGTCCA	12.5−6.25	16 ± 7
06/1	AGGGACA	12.5−6.25	27 ± 11
07/1	ATTCCGG	6.25−3.12	16 ± 7
08/1	AGTGAGA	25−12.5	23 ± 9
wt	GAAAGAC	1.56−0.78	<1

erythromycin MIC values 4−8 times greater than control cells. Plasmids from these cultures were isolated and sequenced (Table 1). Eight sequences with a high level of homology were found among these 15 clones. Eight of the 15 clones had A2058G and A2059C(or G) substitutions. Five of them had the sequence 2057U**GC**GUGG2063, and two had the sequence 2057U**C**GUCCA2063. Two other clones had 2057A**GG**GACA2063 and 2057A**G**UGAGA2063 mutations. The results obtained are in good agreement with earlier literature reports[21−25] which demonstrated that A2058G(C) and A2059G(C) substitutions could confer resistance to macrolide antibiotics.

All of the selected clones also had C2063G(A) substitutions. This nucleotide has been found to be involved in two types of hydrogen bonds, namely with the 3′-end of tRNA and with A2450. These interactions are important for the correct positioning of the 3′-end of aminoacyl-tRNAs. A2450−C2063 wobble pairing was shown to be important for conformational changes in the PTC.[26] The results of our previous *in vitro* studies have shown that both mutants in the 2447−2450 region had A2450C (or U) substitutions and were able to facilitate the improved incorporation of D-amino acids.[6,7] Therefore, we decided to check the β-puromycin sensitivity of clones having mutations in the 2057−2063 region. Accordingly, plasmids having different sequences in the region 2057−2063 were transformed into freshly grown *E. coli* cells, and their sensitivity to β-puromycin (100 μg/mL) was determined. As is clear from Table 1, all cells having mutations in 23S rRNA that conferred erythromycin resistance also showed some enhanced inhibition of cell growth by 100 μg/mL β-puromycin in comparison with the wild-type control.

Design and Construction of a 23S rRNA Library Having Two Modified Regions. The purpose of this study was to find combinations of nucleotides in the ribosomal PTC which permit the participation of both α- and β-amino acids in protein translation; it assumed the need for multiple rounds of 23S rRNA mutagenesis and selection. The first round of selection afforded eight 23S rRNA variants differing in the erythromycin binding pocket. This facilitated the maximal production of modified ribosomes in the bacterial cultures and provided a means of blocking wild-type ribosome function during the *in vitro* experiments. The next round of mutagenesis was carried out using these eight selected rrnB operon variants as templates for preparation of the second library. Thus, the new library contained mutations in two regions of the PTC: the first region 2057−2063 (all clones) and a second region, either 2582−2588 or 2496−2507. Initial mutagenesis reactions in these regions revealed that the region 2496−2507 was too large for effective PCR mutagenesis as only two clones were isolated. To increase the yield and variety of the library, two sets of random primers (2496DBDDVD2501 and 2502HBVHVD2507) (B = C + G + T; H = C + A + T; V = A + G + C; D = A + G + T) were used instead of one for this region. Finally, 24 PCR reactions (i.e., involving randomization of three new regions for each of the eight initially modified 23S rRNAs) were carried out and more than 6×10^3 clones were obtained (Table 2). The theoretical maximum number of sequences in the library was 29 160.

Analysis of the library afforded some interesting observations. As seen in Table 2, while some clones were obtained from mutagenesis in virtually all new regions, only a limited number of clones (<200) were obtained having modifications in the region 2582−2588 (denoted 0101−0801 mutations). Very limited numbers of clones (<100) were also observed in the case of mutations 0201−0203, 0501−0503, 0601−0603, and 0701−0703. Some specific combinations of mutations (0201, 0203, 0501, 0601, and 0701) afforded only a few colonies, and one (0503) gave none. The likely interpretation of these data are that some combinations of mutations in two regions represent lethal phenotypes of the modified ribosome and their high level expression results in low growth of cells. A small library (1270 different clones) was developed by amplification of the clones

dx.doi.org/10.1021/bi2016124 | *Biochemistry* 2012, 51, 401−415

Table 2. Variety of Library of Clones with Modified Ribosomes

2057−2063 mutants (template)	regions for second mutagenesis reaction (primers) 2582−2588 (01)	2496−2501 (02)	2502−2507 (03)
01	81	>1000	>1000
02	3	62	4
03	129	>1000	44
04	28	>1000	>1000
05	3	15	0
06	3	73	39
07	3	66	91
08	39	>1000	71
total clones	289	>4000	>2000
theoretical variety	17496	5832	5832

obtained in Table 2 (100 clones from PCR reactions affording >1000 clones), and these were arranged in the wells of 96-well plates to afford "master plates" for high-throughput selection.

Selection of β-Puromycin-Sensitive and Erythromycin-Resistant Clones. A double selection (for erythromycin resistance and β-puromycin sensitivity) was carried out. Erythromycin assay at 3.5 μg/mL permitted the removal of clones with putatively altered rrnB operon structures, while β-puromycin assay at 100 μg/mL identified clones having increased sensitivity (inhibition >50%) to this antibiotic. It was assumed that sensitivity to β-puromycin would be predictive of clones whose ribosomes would be more likely to incorporate β-amino acids into proteins. Thirty-four erythromycin-resistant and β-puromycin-sensitive clones were finally selected for more detailed evaluation.

Plasmids from these clones were isolated, transformed into the freshly grown *E. coli* cells, and tested for sensitivity to erythromycin and β-puromycin. Plasmids from clones affording the desired responses to erythromycin and β-puromycin were sequenced. The detailed characterization of 17 selected clones is shown in Table 3. As shown in Figure 3, the β-puromycin concentration dependence of growth inhibition of the cells harboring the plasmids with modified rrnB operons differed significantly between individual clones.

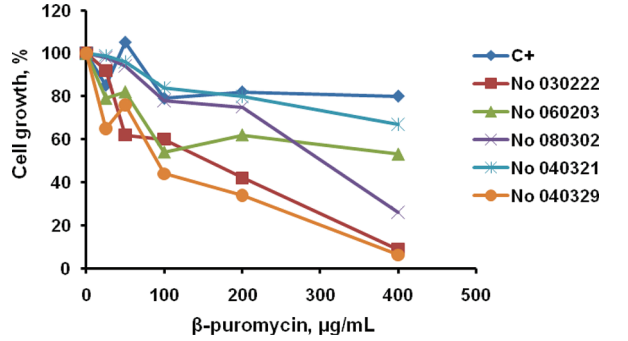


Figure 3. β-Puromycin sensitivity of cells harboring plasmids with modified rrnB genes. C+: cells harboring plasmid pUC-19.

All of the selected clones had mutations in the two anticipated regions of the PTC, namely in regions 2057−2063 (all of them), 2582−2588 (6 clones), 2496−2501 (6 clones), and 2502−2507 (5 clones). Some sequence homology was observed between the clones with mutations in the same second region. Thus, all selected clones with mutations in the region 2582−2588 had purine nucleotides in position 2585 (T2585G(A)) and pyrimidine nucleotides in position 2583 (G2583C(T)). All clones with a modified 2502−2507 region had U2506A and C2507G(A) substitutions. U2506, based on X-ray crystallographic structure data, is located in close proximity to A-76 of the A-site aminoacyl tRNA and may participate in positioning of the nucleophilic amino group of the activated amino acid.[27,28]

Nonspecific Readthrough Activity of Selected Clones. While ribosomes represent a potentially powerful tool for studying ribosome function and enhancing the repertoire of

Table 3. Characterization of Selected β-Puromycin-Sensitive and Erythromycin-Resistant Clones

name of plasmid	sequence in regions of mutagenesis: first region	second region	sensitivity to: β-puromycin (IC_{50}, μg/mL)	erythromycin (MIC, μg/mL)
wt	2057GAAAGAC2063	2496CACCTC2501 2502GATGTC2507 2582GGTTTAG2588	>1000	3.12−1.56
010102	2057TGCGTGG2063	2582TTCAAGA2588	390 ± 32	6.25−3.12
030116	2057ATGGTTG2063	2582TCAACTC2588	320 ± 90	6.25−3.12
030130	2057ATGGTTG2063	2582TCAGGGC2588	314 ± 20	6.25−3.12
030185	2057ATGGTTG2063	2582CCCGATT2588	320 ± 90	12.5−6.25
080110	2057AGTGCGG2063	2582TCAGATC2588	250 ± 50	12.5−6.25
080118	2057AGTGCGG2063	2582ATGGGCT2588	450 ± 90	12.5−6.25
020250	2057TTGGTCG2063	2496TCAGCG2501	260 ± 80	6.25−3.12
020252	2057TTGGTCG2063	2496TCGAGA2501	230 ± 40	6.25−3.12
030201	2057ATGGTTG2063	2496AGGTCT2501	290 ± 100	6.25−3.12
040217	2057AGCGTGA2063	2496ATAGAA2501	250 ± 30	12.5−6.25
060203	2057AGGGACA2063	2496ATAAAT2501	170 ± 45	6.25−3.12
060233	2057AGGGACA2063	2496ACAAAT2501	220 ± 97	12.5−6.25
020322	2057TTGGTCG2063	2502ACGAAG2507	145 ± 70	6.25−3.12
020328	2057TTGGTCC2063	2502ACGAAG2507	130 ± 90	6.25−3.12
040321	2057AGCGTGA2063	2502AGATAA2507	430 ± 85	12.5−6.25
040329	2057AGCGTGA2063	2502TGGCAG2507	320 ± 98	12.5−6.25
080302	2057AGTGCGG2063	2502TACAAG2507	210 ± 60	12.5−6.25

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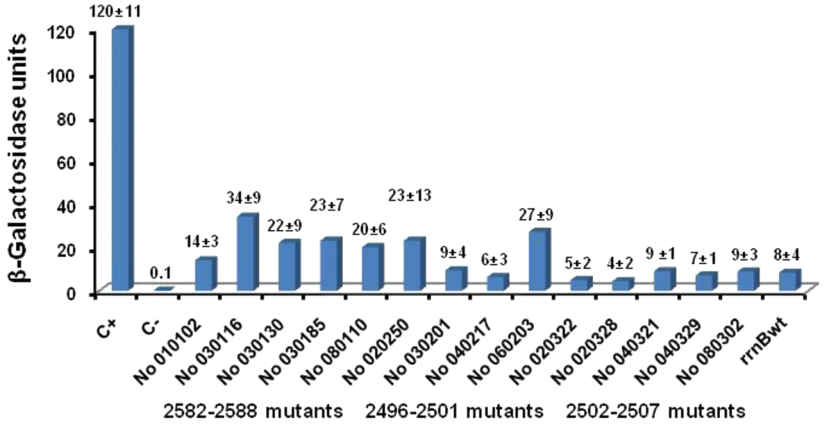


Figure 4. Nonspecific readthrough activity in selected clones having modified ribosomes.

amino acids of utility for ribosomal protein synthesis, the latter application can be realized only if the modified ribosomes maintain their fidelity of translation. As reflected in the data from published studies, some ribosome mutations significantly decrease the fidelity of protein synthesis.[9,29−31]

Therefore, the nonspecific mRNA readthrough activity of selected clones has been tested by the use of a β-galactosidase assay. *E. coli* strain XAC-1 (having a nonsense mutation in codon 17 of the β-galactosidase gene)[12] was chosen for this purpose. *E. coli* XAC-1 cells were transformed by plasmids having modified rrnB operons, and the activity of the expressed β-galactosidase was determined (Figure 4). XAC-1 cells without plasmids and transformed by a plasmid containing the $tRNA_{CUA}^{Gly}$ gene were assayed simultaneously as negative and positive controls.

As seen in Figure 4, the β-galactosidase activity in cultures having different mutations in the PTC varied from 4 to 40 relative units (vs 120 units for the positive control), demonstrating that some modified ribosomes had a greater propensity for nonspecific readthrough of the TAG codon in the β-galactosidase gene. It is interesting that all of selected clones having mutations in the region 2582−2588 region exhibited more readthrough activity than those with mutations in the other two (2496−2501 or 2502−2507) regions. Accordingly, only those ribosomes derived from mutations in the latter two regions were studied further.

Stabilization of Modified Ribosome Level in Culture Cells by the Use of Erythromycin Pressure. Although all of the selected clones having modified ribosomes demonstrated sensitivity to β-puromycin (Table 3), large deviations between individual experiments suggested that the levels of modified ribosomes in the cultures varied substantially. As all of the selected mutants exhibited some resistance to erythromycin, this antibiotic was used to exert selection pressure to increase the proportion of modified ribosomes in the cells. The working assumption was that in the presence of this antibiotic those cells containing higher levels of plasmid-borne ribosome would survive preferentially. To check this assumption, cells having different modifications in plasmid-borne ribosome fraction were grown in the presence of erythromycin and their rate of growth (doubling time) and β-puromycin sensitivity was compared (Table 4 and Figure 5). It was found that all of the clones could survive in the presence of erythromycin at concentrations which blocked the growth of control cells. However, some of the clones demonstrated significantly decreased doubling time in comparison with control cell growth in the absence of this antibiotic.

Table 4. Characterization of Rate of Growth and β-Puromycin Sensitivity of Cultures Having Modified Ribosomes in the Presence of Erythromycin

cultures	doubling time, min	β-puromycin inhibition (IC_{50}, μg/mL)
020322	137 ± 32	66 ± 20
020328	270 ± 25	41 ± 6
030201	540 ± 120	63 ± 10
040217	160 ± 80	46 ± 2
040321	220 ± 90	208 ± 130
040329	140 ± 25	53 ± 14
0403x4[a]	120 ± 40	76 ± 15
060203	102 ± 7	58 ± 4
080302	88 ± 20	53 ± 6
wt	no growth	
wt (no Ery)	38 ± 4	>1000

[a]Derived in analogy with 040329, but in a parallel set of experiments.

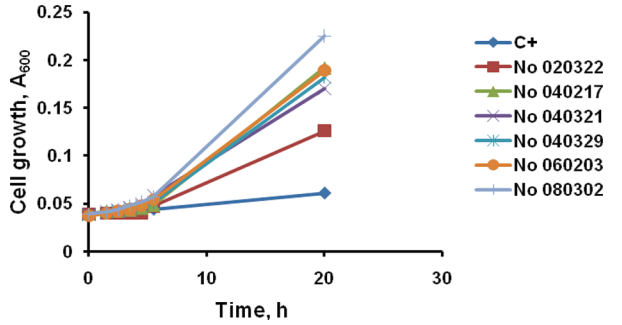


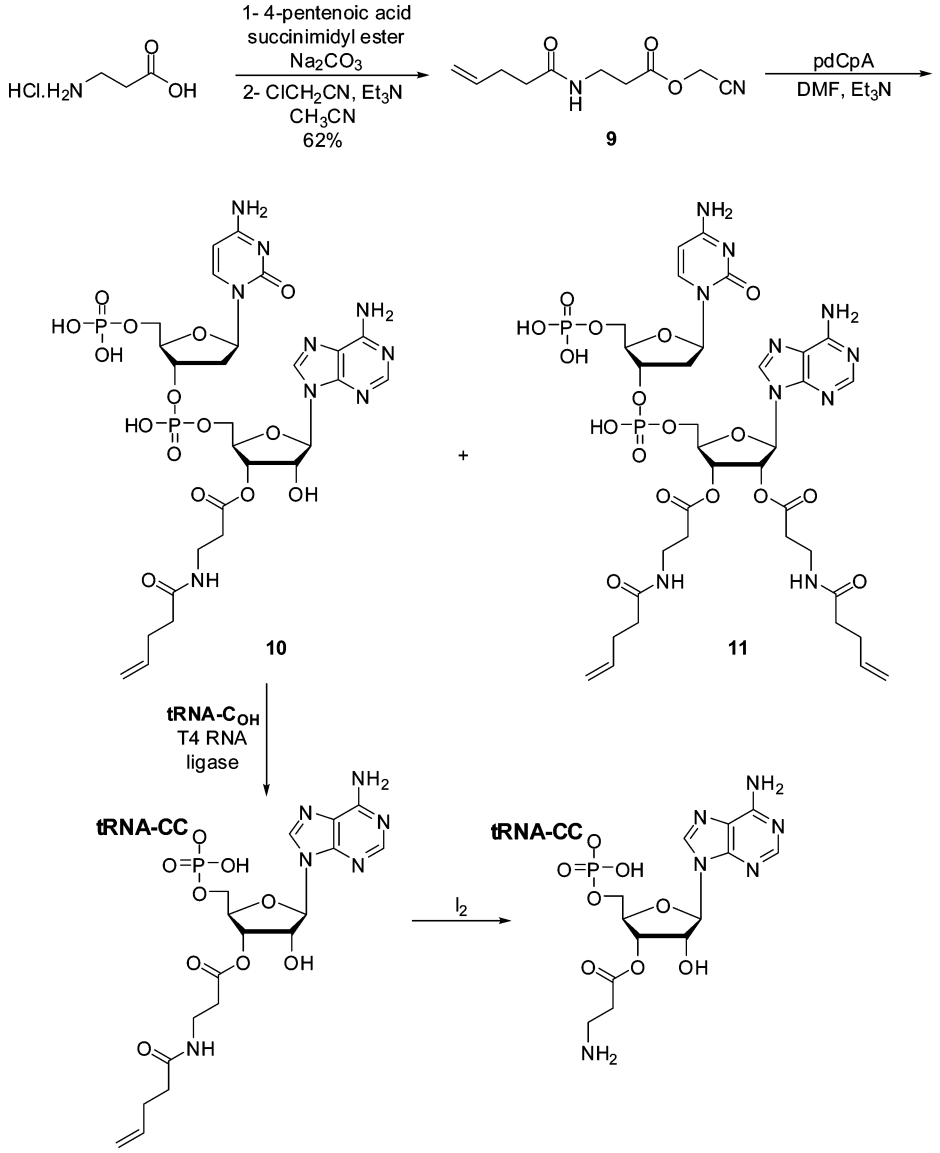
Figure 5. Time growth curves of cells having modified ribosomes in the presence of 3.5 μg/mL erythromycin. C+: culture having only wild-type ribosomes.

All clones grown in the presence of erythromycin exhibited increased β-puromycin sensitivity in comparison with the same culture growth in the absence of this antibiotic (cf. Tables 3 and 4). This provided good evidence that presence of erythromycin in the growth medium does result in an increase in the level of modified ribosomes.

Preparation of β-Alanyl-$tRNA_{CUA}$. *N*-(4-Pentenoyl)-β-alanine pdCpA ester (**10**) was prepared in three steps from β-alanine hydrochloride (Scheme 2). β-Alanine was N-protected by the use of 4-pentenoic acid succinimidyl ester in the presence of Na_2CO_3. Esterification of the carboxyl group was then effected by treatment with chloroacetonitrile in acetonitrile in the

dx.doi.org/10.1021/bi2016124 | *Biochemistry* 2012, 51, 401−415

Scheme 2. Preparation of β-Alanyl-tRNA$_{CUA}$



presence of triethylamine. *N*-(4-Pentenoyl)-β-alanine cyanomethyl ester (**9**) was obtained in 62% yield over two steps. Compound **9** was then treated with the tetrabutylammonium salt of pdCpA[16] to give the 2′(3′)-*O*-monoacylated pdCpA (**10**) and the 2′,3′-bis-*O*-acylated pdCpA (**11**) in 37 and 39% yields, respectively. The two products were easily separated by reversed phase HPLC.

2′(3′)-*O*-β-Alanyl-tRNA$_{CUA}$ was then prepared by the T4 RNA ligase-mediated condensation of aminoacylated dinucleotide **10** and an abbreviated suppressor tRNA$_{CUA}$-C$_{OH}$,[32] as described for other misacylated tRNAs.[33]

***In Vitro* Translation of Proteins and Peptides by the Modified Ribosomes.** Five S-30 systems were prepared from β-puromycin selected cultures and used for *in vitro* translation of proteins (*E. coli* DHFR and *Photinus pyralis* firefly luciferase) and a peptide (*Opisthacanthus madagascariensis* scorpion IsCT peptide). Initially, the synthesis of the proteins from wild-type ribosomes was carried out and the activity relative to wild type was determined (Figure 6). It was found that relative activities of proteins produced from different S-30 preparations of proteins produced by the modified ribosomes varied from 49 to 88% for luciferase and 64 to 94% for DHFR, indicating significant differences in the fidelity of protein synthesis by different clones. The best results were obtained using the S-30 preparations made from clones 0403x4 and 040329, which have the same modified sequence in first region (2057AGCGUGA2063) and a similar sequence in second region (2502AGCCAG2507 and 2502UGGCAG2507, respectively). Interestingly, the addition of erythromycin in amounts sufficient to suppress the activity of wild-type ribosomes did not have a major impact on the fidelity of DHFR synthesis, nor was there a strong correlation with the percent erythromycin-resistant ribosomes in the S-30 preparations studied.

Four S-30 systems were compared for β-puromycin sensitivity (Figure 7). It was found that only three of them (020322, 040217, and 040329) demonstrated a significant decrease in DHFR synthesis in the presence of β-puromycin. The S-30 preparation from the fourth clone (060203) lacked β-puromycin sensitivity in spite of the β-puromycin sensitivity of the clone from which it was derived (cf. Tables 3 and 4).

Finally, the ability of the S-30 preparations to suppress UAG codons in mRNAs from DHFR (Table 5 and Figure 8) or IsCT genes (Figure 9) in the presence of β-alanyl-tRNA$_{CUA}$ was measured. The level of full length DHFR synthesis from a

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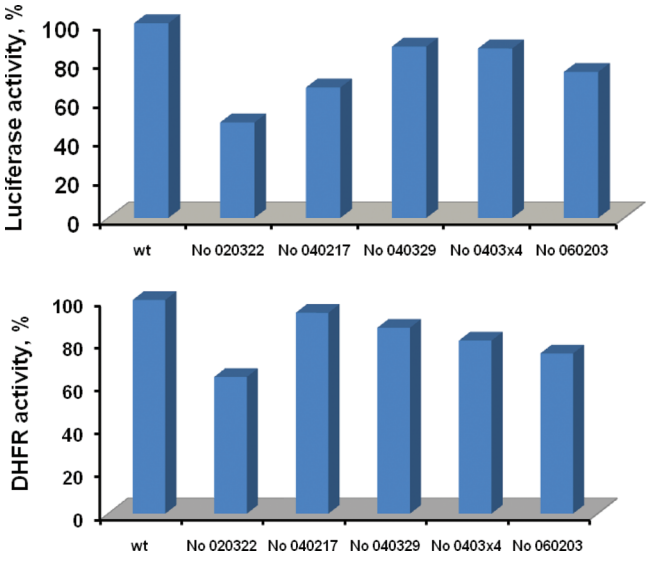


Figure 6. Comparison of the fidelity of translation for different S-30 preparations having modified ribosomes. *Photinus pyralis* luciferase synthesis (top panel) and *E. coli* DHFR synthesis (bottom panel) were carried out *in vitro*, and the activities of the resulting proteins were determined relative to wild type.

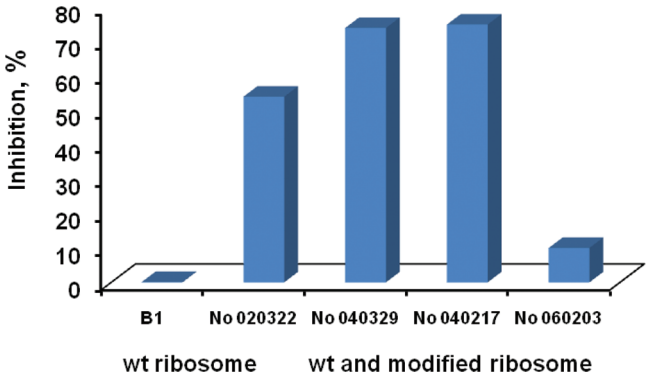


Figure 7. β-Puromycin sensitivity of *in vitro* translation of DHFR.

modified DHFR gene (having a TAG codon at position 10) in the presence of β-alanyl-tRNA$_{CUA}$ using S-30 preparations from clone 060203 was comparable with a control S-30 preparation containing wild-type ribosomes, but an S-30 preparation from clone 040329 resulted in a 3−4-fold enhancement of full length DHFR synthesis in the presence of β-alanyl-tRNA$_{CUA}$ (Table 5 and Figure 8). For the production of IsCT peptide by suppression of the sixth (UAG) codon of the mRNA, the efficiency was significantly greater. Thus, the S-30 preparation from clone

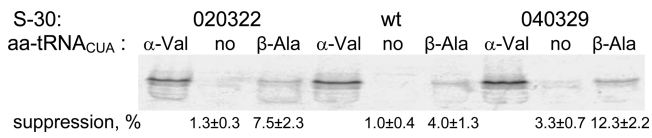


Figure 8. Translation of DHFR from a modified gene having a TAG codon in the position corresponding to Val10 by the use of S-30 preparations containing modified ribosomes. Suppression efficiencies using β-alanyl-tRNA$_{CUA}$ were determined relative to the suppression obtained using L-α-valyl-tRNA$_{CUA}$ (which was defined as 100%). The suppression efficiencies beneath the gels represent the results from replicate experiments, and the errors are expressed as SD. The efficiency of full length DHFR synthesis using this valyl-tRNA was 82.6 ± 14.6% of the amount of DHFR produced under the same experimental conditions using wild-type DHFR mRNA. Abbreviations: aa-tRNA, aminoacyl-tRNA; α-Val, L-α-valyl-tRNA$_{CUA}$; β-Ala, β-alanyl-tRNA$_{CUA}$; no, tRNA$_{CUA}$ lacking any amino acid.

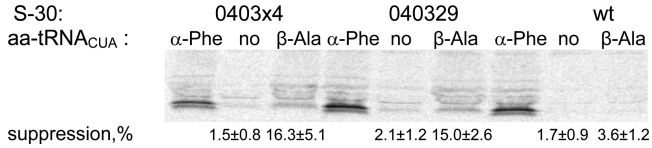


Figure 9. Translation of IsCT peptide from a modified gene having a TAG codon in the position corresponding to Trp6 of the wild-type peptide by the use of S-30 preparations containing modified ribosomes. The upper (darker) of the two bands in the "doublet" on the gel was the desired IsCT peptide. Suppression efficiencies using β-alanyl-tRNA$_{CUA}$ were determined relative to the suppression obtained using L-α-phenylalanyl-tRNA$_{CUA}$ (which was defined as 100%). The suppression efficiencies beneath the gels represent the results from replicate experiments, and the errors are expressed as SD. The efficiency of full length IsCT peptide synthesis using this phenylalanyl-tRNA was 77.1 ± 10.2% of the amount of IsCT peptide produced under the same experimental conditions using wild-type IsCT mRNA. Abbreviations: aa-tRNA, aminoacyl-tRNA; α-Phe, L-α-phenylalanyl-tRNA$_{CUA}$; β-Ala, β-alanyl-tRNA$_{CUA}$; no, tRNA$_{CUA}$ lacking any amino acid.

Table 5. Characterization of DHFR Synthesis Using S-30 Systems Prepared from Cultures with Modified Ribosomes[a]

	modified ribosome		suppression efficiency (%)	
S-30 name	sequence	abundance (%)	tRNA$_{CUA}$	β-alanyl-tRNA$_{CUA}$
B1 (wt)		no	1.0 ± 0.4	4.0 ± 1.3
020322	2057UUGGUCG2063 2502ACGAAG2507	22	1.3 ± 0.3	7.5 ± 2.3
040329	2507AGCGUGA2063 2502UGGCAG2507	66	3.3 ± 0.7	12.3 ± 2.2
0403x4	2507AGCGUGA2063 2502AGCCAG2507	58	3.2 ± 0.2	9.7 ± 3.6
040217	2507AGCGUGA2063 2496AUAGAA2501	50	0.9 ± 0.4	4.8 ± 2.0
060203	2507AGCGUGA2063 2496AUAAAU2501	53	0.8 ± 0.5	3.8 ± 1.5

[a]The mRNA utilized for translation had a UAG codon corresponding to position 10 of DHFR. The suppression efficiency reported is relative to that obtained using L-α-valyl-tRNA$_{CUA}$.

dx.doi.org/10.1021/bi2016124 | *Biochemistry* 2012, 51, 401−415

0403x4 afforded 16% full-length peptide in replicate experiments in comparison with the S-30 prepared from wild type, while 15% full length peptide resulted from use of the S-30 preparation made from clone 040329 (Figure 9).

Thus, our experimental results demonstrate that the use of β-puromycin for selecting clones (by inhibition of cell growth *in vivo* (Table 3) and inhibition of protein synthesis *in vitro* (Figure 7)) can identify clones having modified ribosomes capable of supporting the enhanced suppression of UAG codon in the presence of β-alanyl-tRNA$_{CUA}$, most logically consistent with the enhanced incorporation of β-amino acids, in a cell-free protein synthesizing system.

DISCUSSION

The use of misacylated tRNAs containing noncanonical amino acids has enabled the synthesis of proteins and peptides having these species at predetermined single and multiple positions.[33] This has enabled mechanistic studies that would otherwise have been problematic and has led to the genetic engineering of new cognate tRNA−amino acid−aminoacyl-tRNA synthetase sets that should permit larger quantities of the modified proteins to be prepared.[34]

In reflection of the normal function of the ribosome involving the synthesis of polypeptides from α-L-amino acids, most of the unnatural amino acids introduced into proteins from misacylated tRNAs have also involved α-L-amino acids. Studies involving α-hydroxy acids,[35,36] α-hydrazino amino acids,[37] and α-aminooxy acids[38] have been carried out, but the incorporation yields have been quite low, as might have been expected since the ribosome was not designed for the incorporation of such species. For the same reason, the use of tRNAs activated with β-amino acids[39−41] and D-amino acids[39−44] have afforded minimal amounts of proteins containing such amino acids. It is interesting, however, that *N*-acetyl-β-L-phenylalanyl-tRNA^{Phe} was found to be an efficient donor of *N*-acetyl-β-phenylalanine to L-phenylalanyl-tRNA^{Phe} bound to the A-site of *E. coli* ribosomes programmed with poly(U).[45,46]

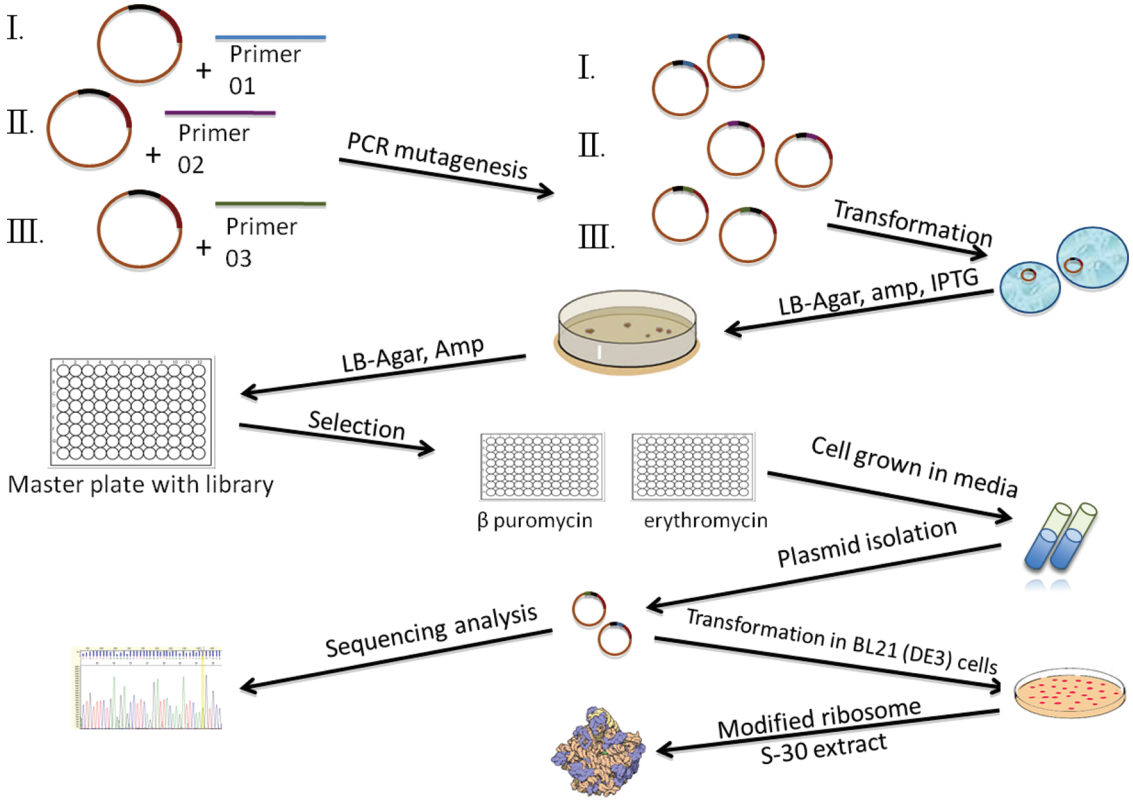
In an effort to obtain better incorporation of non-α-L-amino acids into proteins, Dedkova et al.[6,7] altered regions of 23S rRNA known[19,29,30] to be involved in maintaining the fidelity of protein synthesis. This was done by systematic alteration of regions 2447−2451 and 2457−2462 of the 23S rRNA gene and introduction of the modified gene into bacteria. Those colonies of bacteria exhibiting altered growth characteristics in the presence of the ribosomal ligand chloramphenicol, which is known[47,48] to bind in proximity to the PTC, were surveyed for their ability to mediate enhanced incorporation of D-phenylalanine and D-methionine into proteins, after each was used for the preparation of an S-30 extract. In spite of the laborious, low throughput nature of the strategy employed, a number of clones were obtained that exhibited enhanced incorporation of D-phenylalanine and D-methionine into firefly luciferase and dihydrofolate reductase. As might have been anticipated, the derived proteins exhibited position-dependent alteration of catalytic functions. Critically, the modified ribosomes employed for the incorporation of the D-amino acids afforded luciferase and DHFR that retained essentially full catalytic function when the unmodified mRNAs were used *in vitro* for the elaboration of the wild-type enzymes.

In parallel with this work, several laboratories have investigated the mechanism of peptide bond function at the level of the positioning of the activated amino acid attached to aminoacyl-tRNAs. To study the mechanism by which D-amino acids are excluded from incorporation into proteins, Yonath and co-workers[27] examined the high-resolution structure of the PTC in its native form and in complex with a tRNA acceptor stem mimic that included D- and L-phenylalanine. They found that D-phenylalanine can be bound in the A-site but results in steric interaction between the C$_\beta$ atom of the D-isomer and O4′ of U2506. Rather than assuming an orientation suitable for nucleophilic attack, the amine of the D-isomer is located within H-bonding distance from U2585; if formed, this H-bond could hinder rotation to the conformation required for peptide bond formation. The involvement of some regions of the PTC in the positioning of the amino acyl moiety of aminoacyl-tRNA has been well established in other reports.[28,49,50] Thus, Mankin and co-workers[49] found that U2506, U2584, and U2585 are strongly protected by biotinylated *N*-acetyltyrosyl-tRNA in footprinting experiments. X-ray crystallographic studies have verified that the O^4 atom of U2585 is within hydrogen-bonding distance of the 2′-OH group of the A-site substrate in the *H. marismortui* 50S subunit.[28] Additionally, it was found that the presence of an A-site substrate analogue induced the conformational shift of several nucleotides including U2506, G2583, U2584, and U2585.[50] Thus, both functional and X-ray crystallographic data make it clear that the architecture of the ribosome precludes the efficient use of activated tRNAs whose aminoacyl moieties contain species other than α-L-amino acids. Only by changing the architecture of the ribosomal A-site can non-α-L-amino acids be utilized efficiently.

In order to provide greater flexibility in altering the architecture of the ribosomal A-site, an iterative process was envisioned, as was the use of a dual selection procedure (Scheme 3). Puromycin, an "aminoacylated" adenosine analogue that mimics the 3′-end of aminoacyl-tRNAs, binds to the A-site of ribosomes and terminates protein synthesis by accepting peptides from peptidyl-tRNAs in the ribosomal P-site. It is capable of doing so at the level of intact cells, which results in cell death.[51] It seemed likely that puromycin analogues of non-α-L-amino acids[43] would act in the same way on modified ribosomes whose architecture permitted their use in incorporating non-α-L-amino acids into protein. Accordingly, we posited the utility of such species in identifying the emergence of promising A-site architectures. Also proposed for use in selection were alterations in resistance to erythromycin. Selection for altered erythromycin resistance monitored alteration of geometry at a site close to the PTC. The erythromycin resistance of the modified ribosomes also allowed them to be characterized distinct from (chromosomally encoded) wild-type ribosomes and allowed the induction of production of modified ribosomes in cells at the expense of the (erythromycin-sensitive) wild-type ribosomes.

For the selection of ribosomes able to incorporate both α- and β-amino acids, the rrnB operon was initially altered in the region 2057−2063 to confer some resistance to erythromycin. Eight clones having different sequences in this 23S rRNA region were identified (Table 1) and used for additional mutagenesis experiments (Scheme 3). The puromycin analogue employed was β-homopuromycin, which differs from puromycin only by the presence of a CH_2 group between the carbonyl moiety and C atom bearing the nucleophilic N atom. Its synthesis is outlined in Scheme 1. The validity of the envisioned dual selection scheme was supported by the finding that the eight clones selected for erythromycin resistance all exhibited increased sensitivity to β-puromycin.

Scheme 3. Selection of β-Puromycin-Sensitive Mutants



The plasmids encoding the rrnB operon from each of the eight erythromycin-resistant clones were further mutagenized in one of three other regions of the 23S rRNA gene. Several combinations of two mutagenized regions afforded large numbers of clones (Table 2), and these were screened for β-puromycin sensitivity and erythromycin resistance. As shown in Table 3 and Figure 3, 17 promising clones were identified, and these displayed a variety of behaviors in the two assays. Following the growth of these cells on 3.5 μg/mL erythromycin, a number of the cultures displayed increased sensitivity to β-puromycin, undoubtedly due to increased expression of the (erythromycin-resistant) modified ribosomes in the cells (Table 4).

Characterization of the properties of the modified ribosomes was initiated by assessing their propensity to support nonspecific readthrough of a nonsense codon. For this assay, the modified plasmids were transformed into *E. coli* XAC-1 cells, which have a chromosomal β-galactosidase gene with a TAG codon at position 17. Interestingly, clones having mutations in the region 2582−2588 all displayed relatively high levels of nonspecific readthrough (Figure 4), while only two of four clones having mutations in the region 2496−2501 exhibited high levels of nonspecific readthrough, and none of the clones modified in the region 2502−2507 had this property. Accordingly, only clones modified in the latter two regions were studied further.

The fidelity of protein translation was studied in more detail by using S-30 preparations from specific promising colonies to prepare *P. pyralis* luciferase and *E. coli* DHFR *in vivo* using the wild-type mRNAs. As shown in Figure 6, five different colonies were used to prepare both proteins. For luciferase, the specific activities were 49−88% of the same protein elaborated using an S-30 preparation having wild-type ribosomes. Clone 020322 produced protein having the lowest specific activity, while clone 040329 afforded the most favorable result. The range of specific activities for the five DHFR samples ranged from 64 to 94%. Again, clone 020322 afforded DHFR having the lowest specific activity. However, while clone 040329 again afforded a DHFR with a favorable specific activity (86% relative to the DHFR produced from wild-type ribosomes), the S-30 preparation from clone 040217 gave the best results, affording a DHFR having 94% specific activity relative to wild type. The generally more favorable results for DHFR noted here (as compared with luciferase) parallel the results reported previously for modified ribosomes selected for incorporation of D-amino acids.[6,7]

Although all of the colonies characterized in detail had exhibited significant sensitivity to β-puromycin, suggesting alteration of ribosomal architecture in proximity to the A-site, it seemed prudent to verify that β-puromycin sensitivity at the level of S-30 preparations. As shown in Figure 7, this was done for wild-type ribosomes and modified ribosomes from four different clones. Surprisingly, one of the clones (060203) afforded an S-30 preparation which did not display significant sensitivity to β-puromycin. The molecular basis for sensitivity of the clone to β-puromycin is not presently known.

As shown in Table 5 and Figure 8, S-30 preparations from the same four clones could be used to suppress a UAG nonsense codon at the mRNA position corresponding to amino acid 10 (valine) in DHFR in the presence of β-alanyl-tRNA$_{CUA}$, logically reflecting the incorporation of β-alanine into DHFR. The S-30 preparation from clone 060203 was no better than wild-type ribosomes in mediating the incorporation of β-alanine, but the preparations from all of the other clones did afford enhanced levels of full length product in the presence of β-alanyl-tRNA$_{CUA}$.

Additionally, two clones (0403x4 and 040392) which had functioned well in DHFR synthesis were used to suppress a

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UAG codon (peptide position 6) in the mRNA for IsCT peptide. As shown in Figure 9, the efficiency of incorporation, relative to the incorporation of phenylalanine, was 16% in replicate experiments using the S-30 preparation from clone 0403x4 and 15% using the S-30 preparation from clone 040329.

It is anticipated that additional rounds of mutagenesis will result in further improvements in the extent of incorporation of β-amino acids and that this can be achieved without unduly compromising the fidelity of incorporation of the appropriate α-amino acids that constitute the majority of the protein structure.

CONCLUDING PERSPECTIVE

The present study establishes a novel strategy for modifying the architecture of the ribosomal A-site to enable the additional recognition of β-amino acids. Key features of the strategy include the use of a puromycin analogue to identify emerging ribosomal architectures that may recognize the modified amino acids of interest. Also employed effectively was a dual selection strategy for erythromycin resistance and β-puromycin sensitivity that helped to guide the choice of colonies for detailed characterization. Iterative mutagenesis in additional regions of the 23S rRNA should permit further improvement of the selected clones. Further, the scheme outlined here may well prove applicable to many additional types of non-α-L-amino acids.

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ABBREVIATIONS

PTC, peptidyltransferase center; DHFR, dihydrofolate reductase; TEMED, *N,N,N′,N′*-tetramethylenediamine; IPTG, isopropyl-β-D-thiogalactopyranoside; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; DTT, dithiothreitol; PCR, polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; MES, morpholinoethanesulfonate; DMSO, dimethyl sulfoxide; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonate.

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