Cloning and Characterization of the 23S RNA Pseudouridine 2633 Synthase from *Bacillus subtilis*†

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ABSTRACT: A *Bacillus subtilis* ORF, *ypuI*, 41% homologous to *rsuA*, the gene for the synthase which forms pseudouridine 516 in *Escherichia coli* 16S rRNA, was cloned and the protein expressed and affinity-purified by the His tag procedure. Reactions with *E. coli* 16S and 23S rRNA transcripts were performed in vitro. The protein did not form pseudouridine 516 as expected but did produce pseudouridine 552 in 16S rRNA and pseudouridines 1199, 2605, and 2833 in 23S rRNA. Of these, only pseudouridine 2605 is found naturally in either *E. coli* or *B. subtilis* rRNA. Kinetic experiments confirmed that pseudouridine 2605 was the primary target. Comparison of the four pseudouridine sites yielded a consensus recognition sequence for the synthase. This consensus sequence was not present at any other site in either *E. coli* or *B. subtilis* 16S or 23S RNA. We propose that YpuL is the *B. subtilis* pseudouridine 2633 (2605 in *E. coli*) synthase. Since the closest gene sequence homologue in *E. coli* is *yciL*, we suggest that its gene product is the corresponding *E. coli* pseudouridine 2605 synthase.

Pseudouridine (5-ribosyl uracil or Ψ) is one of the enigmas of the RNA world. Discovered almost 40 years ago (*1*), it is found in tRNA (*2*), rRNA (*3*), and sn- and snoRNA (*4*, *5*) but not so far in mRNA or viral RNA. Ψ is formed by isomerization of selected uridines after the RNA chain has been made. The mechanism of N-glycosyl bond breakage and C-glycosyl bond formation is not known, although an intriguing hypothesis involving catalysis by an aspartic acid carboxyl group at the active site has recently been proposed (*6*).

Ψ is found in the rRNA of all organisms so far examined (*7*). It is the predominant modified nucleoside in the *Escherichia coli* ribosome (*8*), and in eukaryotes is present in amounts about equal to the sum of the amounts of all four 2′-O-methylated major nucleosides (*3*). In higher eukaryotic rRNA, Ψ can be as much as 8−9% of the total uridines (*3*, *7*). In ribosomal small subunits, the number of Ψ residues varies widely from species to species, and their distribution does not coincide with known functionally important sites (*9*). By contrast, in ribosomal large subunits, although the number of Ψ also varies widely from species to species, their distribution is, in all examples known, confined to three distinct areas, all of which are at or near the peptidyl transferase center in the three-dimensional structure (*7*).

Nothing is known about the functional role of Ψ in rRNA. To address this issue, we have initiated a program for identifying the genes for the synthases which make up the 10 Ψ residues found in *E. coli* rRNA. Our intent is to inactivate these genes, one at a time, to block the formation of specific Ψ residues. Unlike the situation in eukaryotes, where guide RNAs specify the uridines to be converted (*10*, *11*) possibly using only a single synthase (*12*), in *E. coli* there appear to be specific synthases for specific sites (*13*−*16*). So far, the genes for five synthases, which together are responsible for synthesis of nine of the 10 Ψ residues, have been identified. *rsuA* is the gene for the synthase which makes the sole Ψ in the 30S subunit, Ψ516 (*13*). *rluA* is the corresponding gene for forming Ψ746 in the 50S subunit (*14*). *rluC* makes the synthase for formation of Ψ955, Ψ2504, and Ψ2580 in the 50S subunit (*15*). *rluD* is the gene for the enzyme catalyzing the synthesis of Ψ1911, Ψ1915 [this is further modified to $m^3\Psi$ (*17*) in the cell], and Ψ1917 in the 50S subunit (*16*). *rluE* is the gene for the Ψ2457 synthase (J. Conrad, C. Alabiad, and J. Ofengand, unpublished results). The synthase gene for Ψ2605 has not yet been identified. Except for *rluE*, these synthase genes have all been disrupted. Gene disruption of *rsuA*, *rluA*, and *rluC* had little or no effect on cell growth (*15*; S. Raychaudhuri, J. Conrad, L. Niu, and J. Ofengand, unpublished results), whereas disruption of *rluD* caused a severe growth defect (*16*).

In this work, we describe the cloning and characterization of another Ψ synthase. The protein, overexpressed from the *ypuL* gene of *Bacillus subtilis*, makes primarily Ψ2605 when *E. coli* 23S RNA is used as a substrate in vitro, and thus may be the *B. subtilis* counterpart to the missing *E. coli* enzyme.

EXPERIMENTAL PROCEDURES

Materials. 5-[^{3}H]UTP and α-[^{32}P]dATP were from Amersham. RNasin was from Promega. Restriction enzymes were from New England Biolabs. T7 RNA polymerase was from Ambion. Plasmid pET-15b, the BL21/DE3 and Nov-

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ablue strains of *E. coli*, His-Bind resin, and protein molecular mass standards were obtained from Novagen. T4 DNA ligase was from U.S. Biochemicals. *B. subtilis* DNA (catalog no. D-4041), yeast inorganic pyrophosphatase, and Norit A washed with HCl were obtained from Sigma. Nuclease-free bovine serum albumin was from BRL. Deoxyoligonucleotide primers were prepared as described previously (*8*). The PCR[1] reagent kit was from Perkin-Elmer (part no. N801-0043).

Buffers. Buffer A is 20 mM Hepes (pH 7.5), 20 mM NH_4-Cl, 0.1 mM EDTA, 5 mM mercaptoethanol, and 10% glycerol. Buffer B is 20 mM Hepes (pH 7.5), 100 mM NH_4-Cl, 5 mM mercaptoethanol, 0.1 mM EDTA, 10% glycerol, and 6 M urea. Buffer LB is 50 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% SDS, 10% glycerol, and 0.1% bromphenol blue. Binding buffer is 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, and 5 mM imidazole. Elution buffer is binding buffer with the imidazole concentration raised to 1.0 M.

RNA Transcripts and RNP Particles. The rRNA transcript of full-length *E. coli* 23S RNA (residues 1−2904) was prepared as described previously (*14*). Transcripts of *E. coli* 16S rRNA and its fragment (residues 1−678), assembly of the 16S RNA fragment with 30S proteins into the RNP (1−678) particle, and in vitro reconstitution of 30S ribosomes with full-length *E. coli* 16S RNA transcripts were as described previously (*13*). $tRNA^{Val}$ and $tRNA^{Phe}$ transcripts were prepared as described previously (*18*). $tRNA^{Leu}$ transcripts were prepared similarly from plasmid pUC19-$tRNA^{Leu4}$, a gift of J. Sampson (California Institute of Technology, Pasadena, CA).

Cloning and Overexpression of the B. subtilis ypuL Gene. The candidate ORF was amplified and prepared for insertion into pET-15b by PCR. The N-terminal primer extended from residue −17 to +23, where the A of the initiating codon ATG is residue +1, with changes at positions 0 and −2 to create an *Nde*I site. The C-terminal primer extended from residue +695 to +734, where the T of the termination codon TAA is residue +688, with a mismatch at position +715 to generate a *Bam*HI site. Primers were removed by membrane filtration (Amicon Microcon 100), and the amplified product was digested by *Nde*I and *Bam*HI. After digestion, salts were removed by membrane filtration (Amicon Microcon 100) and the solution was heated at 65 °C for 20 min to inactivate the restriction enzymes and then used directly for ligation. The pET vector was digested with *Nde*I and *Bam*HI, purified by gel electrophoresis, and incubated with the gene insert in a ligation mixture containing 50 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 25 μg/mL nuclease-free bovine serum albumin, 10 mM DTT, 1 mM ATP, 1000 units/mL T4 DNA ligase, 30 μg/mL vector, and 25 μg/mL insert for 20 h at 16 °C. Transformation of Novablue cells was achieved by standard methods and yielded four clones with the correct insert in the pET vector out of 24 tested. The plasmid from one clone carrying the correct insert was used to transform BL21/DE3 cells.

For overexpression, the transformed BL21/DE3 cells were grown in Luria-Bertani medium (*19*) at 37 °C to an A_{600} of 0.5. IPTG (1 mM) was added, and cells were grown at 37 °C for 4 h. Cells were recovered and quick-frozen on dry ice in aliquots. For analysis of the whole cell contents, one aliquot was thawed in $^1/_{10}$ of the original culture volume of LB buffer, heated to 100 °C for 5 min, and then chilled. Other aliquots were disrupted by sonication in $^1/_{40}$ of the original culture volume of binding buffer and centrifuged at 15000*g* to obtain the S15 supernatant fraction. The pellet was suspended in $^1/_{25}$ of the original volume of binding buffer with 6 M urea, incubated at 0 °C for 1 h, and then centrifuged at 39000*g* for 20 min to obtain the solubilized pellet fraction.

Affinity Purification of the Synthase. The S15 supernatant and the solubilized pellet fraction were applied to 2.5 mL columns of His-bind resin under the native and denatured chromatography conditions, respectively, as described in the *pET System Manual* (4th ed., Novagen, Inc.). Upon addition of elution buffer, the tagged protein was released. The eluted S15 fraction was dialyzed against buffer A for 6 h. The pellet fraction, eluted with 6 M urea, was dialyzed against buffer B with decreasing concentrations of urea from 6 to 3 M at 1.0 M intervals and then from 3 to 0 M at 0.5 M intervals for 1 h each. Both protein solutions were adjusted to contain 50% glycerol and stored at −20 °C.

Quantitation of Ψ *Sequencing Gels*. Quantitative analysis of the Ψ sequencing gels was carried out on a Molecular Dynamics PhosphorImager model 445 SI system. The value of each band in the plus CMC plus enzyme lane was corrected for background by subtraction of the value for the equivalent band in the plus CMC minus enzyme lane. Since analysis of each site required the use of a different primer with a different efficiency of reverse transcription, the sites were normalized to each other by comparison to the sum of the 10 bands immediately downstream of the band of interest (see Figure 5).

Other Methods. Ψ sequencing was according to Bakin and Ofengand (*8*, *20*). The assay of pseudouridine formation by the ^3H release assay was as described previously (*13*). SDS gels were 12% and contained 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS. The 5% stacking gel contained 0.127 M Tris-HCl (pH 6.8) and 0.1% SDS. Samples were heated at 95 °C for 5 min in buffer LB and then quenched on ice before loading. Gels were stained with Coomassie Blue. The protein content was assayed by a modified Bradford procedure (Bio-Rad Protein Assay catalog no. 500-0006) using bovine serum albumin as a standard. Equivalent positions in *E. coli* and *B. subtilis* 16S and 23S RNAs were deduced by comparison of their secondary structures as described in ref *7*.

RESULTS

Cloning and Overexpression of the ypuL Gene. A comparison of the amino acid sequence of *rsuA*, the gene for the synthase responsible for formation of the single Ψ516 in *E. coli* 16S RNA, with the database available at the time (November 1994) identified the *B. subtilis* gene *ypuL* (SWISSPROT P35159) as the closest homologue with 30% identity and 76% similarity over 225 residues (*13*). Because of this homology and the fact that *B. subtilis* has a Ψ in its 16S RNA at position 523 (*13*), corresponding to Ψ516 in *E. coli*, we proposed that *ypuL* was the gene for the synthase responsible for Ψ523 formation (*13*). To confirm this proposal, the gene was cloned and overexpressed in *E. coli*, and characterized in vitro.

As shown in Figure 1A, a highly overexpressed band at ca. 30 kDa was found (lane 3) only when the *ypuL* insert

[1] Abbreviations: PCR, polymerase chain reaction; RNP, ribonucleoprotein; ORF, open reading frame; IPTG, isopropyl β-D-thiogalactopyranoside.

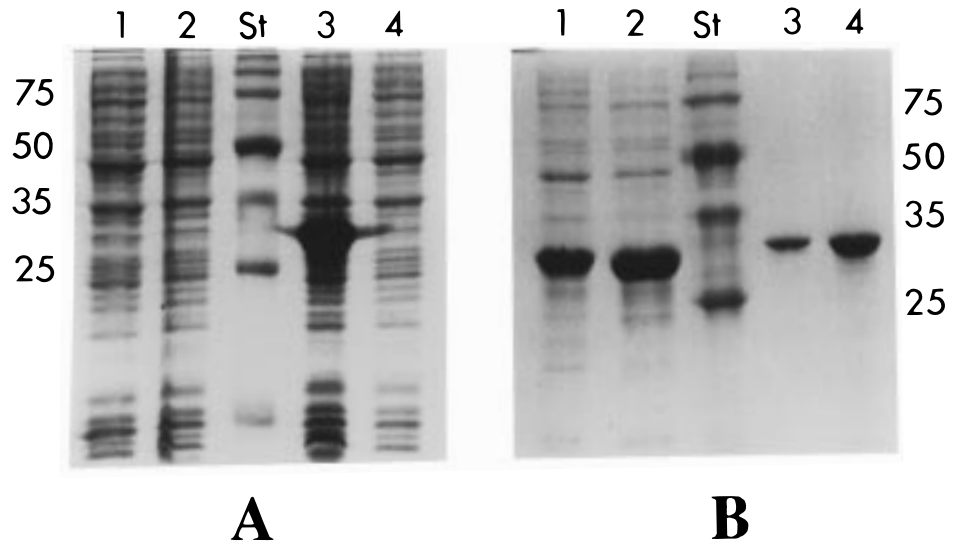

FIGURE 1: Overexpression (A) and affinity purification (B) of the *ypuL* gene product. (A) Cells were transformed with the indicated plasmids, harvested either before or after induction with IPTG, and lysed as described in Experimental Procedures: lanes 1 and 2, pET vector lacking the gene insert before (1) and after (2) induction; and lanes 3 and 4, pET vector carrying the gene insert after (3) and before (4) induction. (B) Cells disrupted by sonication were fractionated into an S15 supernatant and a pellet: lane 1, S15 supernatant; lane 2, solubilized pellet; lane 3, 1.0 μg of the affinity-purified S15 supernatant fraction; and lane 4, 3.2 μg of the affinity-purified pellet fraction after removal of urea. In each lane, aliquots from approximately equivalent amounts of cells were loaded except for lanes 3 and 4 in panel B, where the indicated amounts were used. St lanes contained molecular mass standards with the indicated values.

was present in the vector and only after IPTG induction. The yield of the protein, determined after affinity purification, was 45 mg/L of culture in one experiment and 49 mg/L in a second preparation. Figure 1B (lanes 1 and 2) shows that the protein was found both in the cell sap and in inclusion bodies. The percent in the soluble fraction varied from 6 to 12% in the two preparations, measured after affinity purification on a Ni^{2+}-containing column by virtue of the N-terminal His tag sequence (lanes 3 and 4). The mass of the purified protein was 31 kDa, in good agreement with that calculated from the gene (26.0 kDa) and 2.2 kDa of the N-terminal leader. The renaturation protocol described in Experimental Procedures successfully restored the activity of the enzyme. Specific activities of the soluble and renatured preparations were within 2% of each other (data not shown). Nevertheless, the soluble enzyme was used for all subsequent work since we were concerned that renaturation might have altered the specificity properties of the enzyme. When stored as described, the preparation was stable for at least 2 months.

The YpuL Synthase Is Not a Homologue of RsuA. The *E. coli* Ψ516 synthase, RsuA, specifically recognizes an RNP particle consisting of a transcript of residues 1−678 of 16S RNA complexed with those *E. coli* 30S ribosomal proteins which stably bound to it in an in vitro reconstitution reaction (*13*). 30S subunits reconstituted from full-length 16S RNA transcripts (*21*, *22*) were very poor substrates, and neither full-length 16S RNA nor its fragment (residues 1−678) was active. Moreover, an RNP particle consisting of residues 1−526 of 16S RNA associated with 30S subunit proteins S4, S16, S17, and S20 (*23*) was also inactive, as was the RNA fragment (residues 1−526) alone (*13*). We anticipated a similar specificity for the YpuL synthase because of its sequence similarity with RsuA. However, when the enzyme was tested with *E. coli* RNAs and RNPs, a very different specificity was found (Figure 2). The RNA fragment (residues 1−678) was most active, followed by the full-length 16S RNA. RNP (1−678), which was the only strongly active

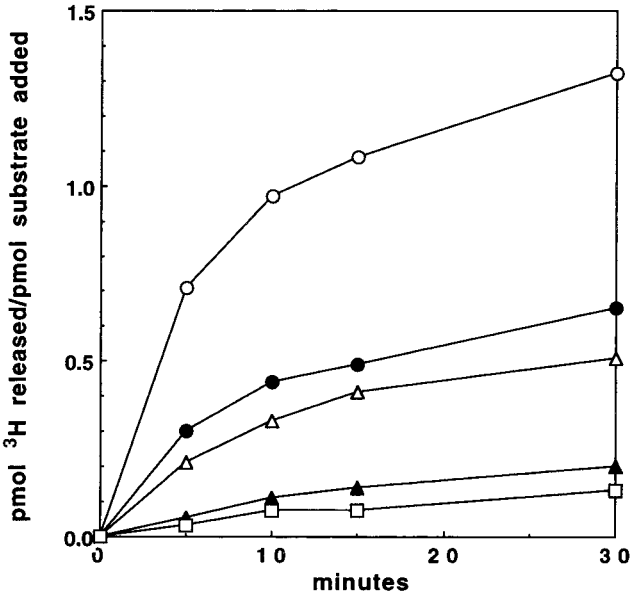

FIGURE 2: Specificity of the recombinant *B. subtilis* YpuL synthase. [5-^{3}H]Uracil-containing *E. coli* 16S RNA (●), *E. coli* 16S RNA (residues 1−678) (○), *E. coli* 16S RNA (residues 1−526) (□), reconstituted *E. coli* 30S (▲), and RNP (1−678) (△) were prepared and the extents of pseudouridine formation measured at 3 mM Mg^{2+} by the release of ^{3}H as described in Experimental Procedures. The substrate concentrations were 76 nM.

substrate with RsuA, was even less active than full-length 16S RNA. These results were obtained at 3 mM Mg^{2+}. At 10 mM Mg^{2+}, similar results were obtained except that all values were decreased by $^2/_3$ (data not shown). These same substrate preparations, when tested with the RsuA enzyme, showed the same behavior as before (*13*). Thus, at 3 mM Mg^{2+} and a single time of reaction, a mole:mole ratio of 0.58 was obtained with RNP (1−678), whereas RNA (1−678) and full-length 16S RNA gave values of 0.02 and <0.01, respectively (data not shown).

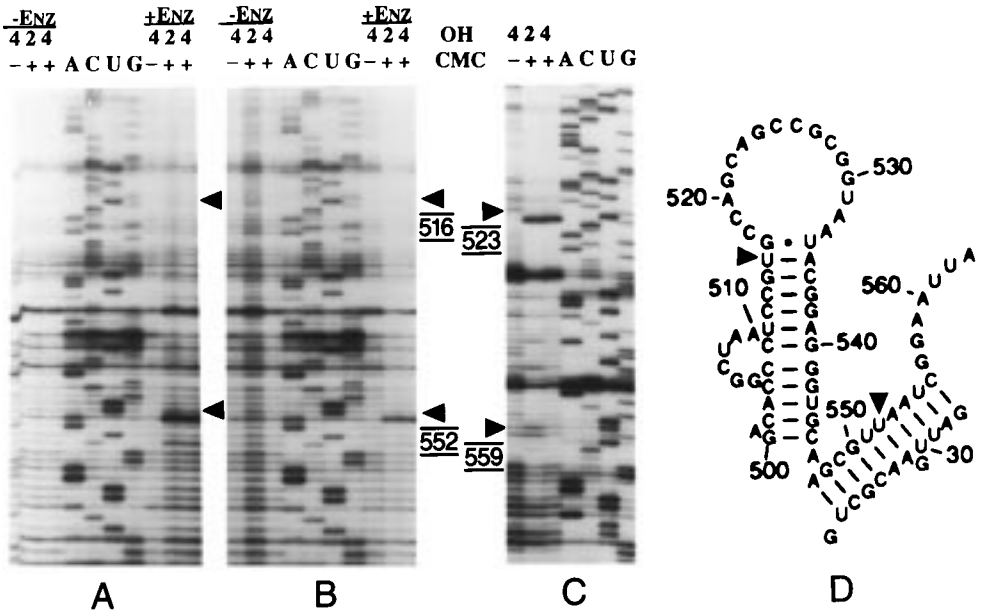

FIGURE 3: Site of Ψ formation in *E. coli* 16S RNA transcripts by the recombinant *B. subtilis* YpuL synthase. (A) The transcript was incubated with (+) or without (−) enzyme and then treated with (+) or without (−) CMC followed by incubation for 2 or 4 h at pH 10.4 (OH). The methodology was that of Bakin and Ofengand (*8*, *20*). A, C, U, and G were sequencing lanes in which the in vitro transcript was used. The RNA was modified in 3 mM Mg^{2+} to a level of 0.72 mol of ^3H released per mole of RNA. (B) The same as panel A except that modification was in 10 mM Mg^{2+} to a level of 0.44 mol of ^3H released per mole of RNA. (C) Mapping of Ψ residues in *B. subtilis* wild type 16S RNA. Total *B. subtilis* RNA was treated with (+) or without (−) CMC followed by incubation for 2 or 4 h at pH 10.4 (OH). A, C, U, and G were sequencing lanes in which total *B. subtilis* RNA without CMC treatment was used. (D) Secondary structure of the segment of *E. coli* 16S RNA examined in panels A and B. In all panels, arrows show the positions of actual or potential Ψ residues as described in the text.

Sequence analysis provided conclusive evidence that this synthase did not form Ψ516 (Figure 3). Panels A and B show sequencing ladders for *E. coli* 16S RNA reacted with the YpuL synthase at 3 and 10 mM Mg^{2+}, respectively. There was no Ψ at position 516. Therefore, it is clear that YpuL is not the homologue of RsuA. On the other hand, there was a strong band corresponding to Ψ552 which was dependent on both reaction with enzyme and CMC. Recall that in this sequencing method the band occurs one residue 3′ to the CMC-Ψ (*8*, *20*). The same results were obtained when the fragment (residues 1−678) reacted with YpuL at 10 mM Mg^{2+} was sequenced (data not shown). Ψ552 does not occur naturally in *E. coli* 16S RNA (*24*), nor is its equivalent in *B. subtilis*, Ψ559, found in that 16S RNA (panel C). Ψ523, the equivalent of Ψ516 in *E. coli*, is present, however. No other Ψ residue was found over the region scanned, residues 440−570 (*E. coli*) and 360−590 (*B. subtilis*). The locations of these two sites, 516 and 552, are shown in panel D on a secondary structure diagram of this part of the molecule. Since there is no Ψ552 in *E. coli* or its equivalent in *B. subtilis*, we conclude that the formation of this Ψ occurs only in vitro.

YpuL Appears To Be the 23S RNA Ψ2633 (2605 in E. coli) Synthase. The true specificity of the YpuL synthase became clear when the relative kinetics of Ψ formation on 23S RNA and 16S RNA were compared (Figure 4). 23S RNA was at least a 2-fold better substrate at 3 mM Mg^{2+}, and more than 3-fold better at 10 mM Mg^{2+}. tRNA transcripts, on the other hand, were largely inactive. Although a low level of activity was seen with $tRNA^{Leu4}$, $tRNA^{Phe}$ and $tRNA^{Val}$ were not recognized. It appears that the true substrate of YpuL is 23S RNA or some RNP-containing 23S RNA. Our inability to reconstitute active 50S particles

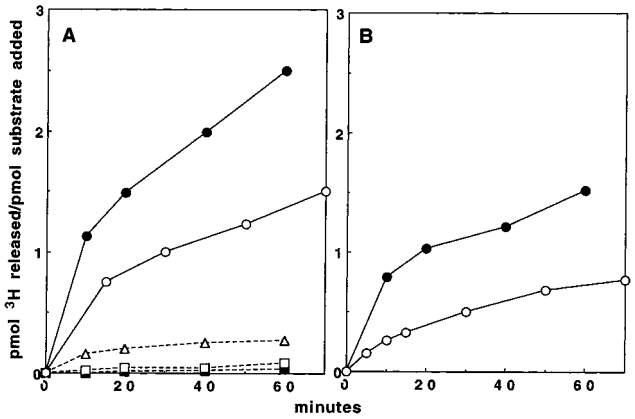

FIGURE 4: Kinetics of Ψ formation on 23S RNA, 16S RNA, and tRNA transcripts by the YpuL synthase. Assays were carried out with [5-^3H]uracil-containing *E. coli* RNA transcripts by the ^3H release method. (A) Reaction at 3 mM Mg^{2+} and (B) reaction at 10 mM Mg^{2+}: 23S RNA (●), 16S RNA (○), $tRNA^{Leu}$ (△), $tRNA^{Phe}$ (■), and $tRNA^{Val}$ (□).

containing 23S RNA transcripts precluded the testing of such RNPs.

Sequencing analysis established which U residues had been converted to Ψ (Figure 5). There was a major band corresponding to Ψ2605 (Ψ2633 in *B. subtilis*) and two minor bands corresponding to Ψ1199 (Ψ1238 in *B. subtilis*) and Ψ2833 (Ψ2857 in *B. subtilis*). There was no detectable reaction at U2818 (see Discussion). Additional primers were used to screen residues 110−994, 1120−1369, and 1447−2854 for a total of 2543 residues or 88% of the *E. coli* 23S RNA sequence. No other sites of Ψ formation were detected. In particular, all nine of the known Ψ sites in *E. coli* 23S RNA were included in this screen, as well as the five known Ψ in *B. subtilis* 23S RNA (*7*). Of the three sites which

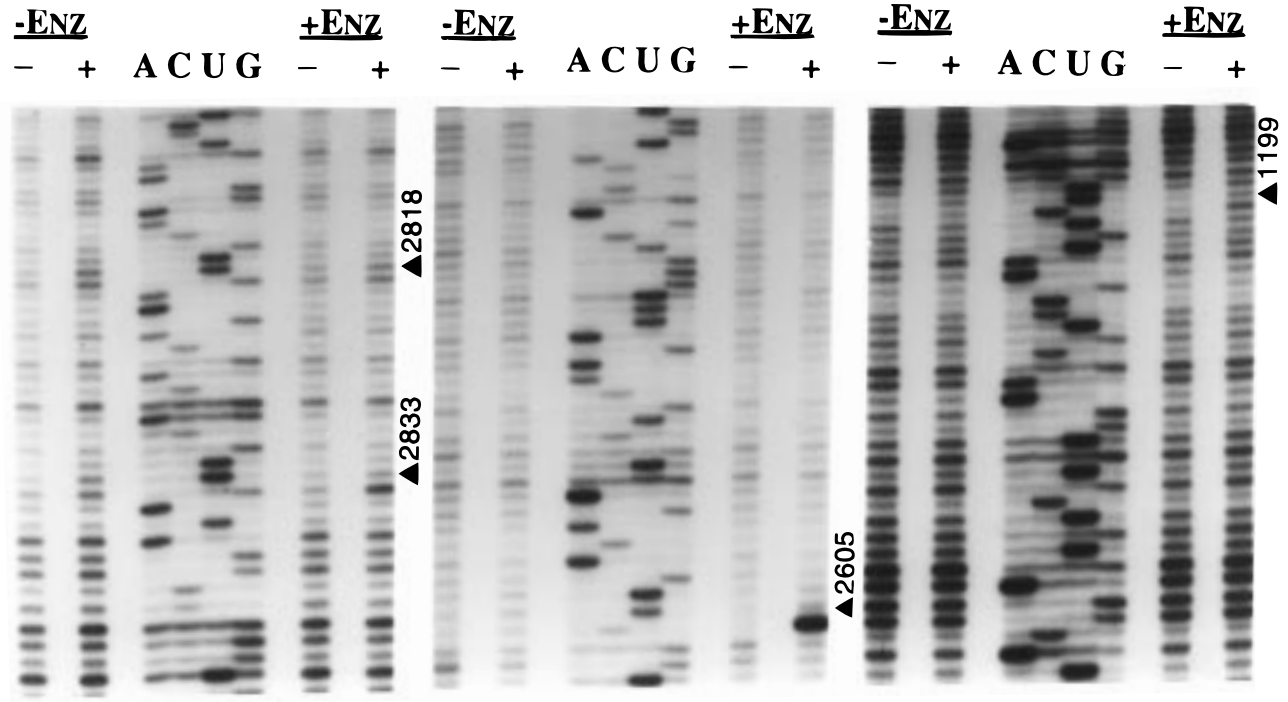

FIGURE 5: Sites of Ψ formation in *E. coli* 23S RNA transcripts by the recombinant *B. subtilis* YpuL synthase. Transcripts were incubated with (+) or without (−) enzyme and then treated with (+) or without (−) CMC followed by incubation for 4 h at pH 10.4 according to the methods of Bakin and Ofengand (*8*, *20*). A, C, U, and G were sequencing lanes in which the in vitro transcript was used. The RNA was modified in 3 mM Mg^{2+} to a level of 0.89 mol of ^3H released per mole of RNA. Each panel shows reverse transcription with different primers. Numbered arrows show the positions of Ψ residues.

Table 1: Sites of Pseudouridine Formation on *E. coli* 23S RNA by the *B. subtilis* YpuL Synthase[a]

	moles of pseudouridine formed per mole of RNA	
site	0.89 mol of Ψ[b]	1.48 mol of Ψ[b]
1199	0.09 ± 0.02	0.34 ± 0.04
2605	0.72 ± 0.02	0.71 ± 0.04
2833	0.08 ± 0.00	0.43 ± 0.00

[a] Sequencing gels were quantitated as described in Experimental Procedures. Two independent reverse transcription sequencing reactions were performed on a single RNA sample incubated with the YpuL synthase and subsequently treated with CMC in preparation for Ψ sequencing. The values shown are the average of two determinations. The average deviation is indicated. [b] Determined by the ^3H release pseudouridine assay described in Experimental Procedures.

reacted in vitro, only Ψ2605 and its equivalent in *B. subtilis* are found in native 23S RNA of either *E. coli* or *B. subtilis* (*7*). Previous work (*7*) had eliminated U2857 as a naturally occurring Ψ site in *B. subtilis*, and in this work, additional sequencing of residues 1165−1260 established that there was no Ψ at position 1238 (data not shown). Thus, the major site of reaction at U2605 corresponds to a naturally occurring site, whereas the minor reactions at U1199 and U2833 occur only in vitro.

A quantitative analysis of the Ψ bands in Figure 5 was performed to determine the degree of preference of YpuL for U2605 over the other reactive U residues. The results are shown in Table 1 along with data from a second reaction in which modification had proceeded further, to 1.48 mol of Ψ formed per mole of RNA. These results show clearly that Ψ2605 was formed more rapidly than the other Ψ. Its absolute amount remained constant when the total amount of Ψ increased by 1.7-fold, whereas the amounts of Ψ1199 and Ψ2833 which were low initially increased 4−5-fold. At 0.89 mol of Ψ formed per mole of RNA, 81% was found as Ψ2605. We conclude that YpuL is probably the *B. subtilis* 23S RNA Ψ2633 synthase.

DISCUSSION

Specificity of the YpuL Ψ Synthase. On the basis of amino acid sequence homology, we initially had predicted that YpuL would be a homologue of the *E. coli* RsuA Ψ synthase which makes Ψ516 in *E. coli* 16S RNA. Instead, we have found that the preferred substrate is 23S RNA and the preferred site is U2605 in *E. coli* 23S RNA. Two lines of evidence suggest that U2605 is the intended site of Ψ formation. First, the rate of reaction was faster with 23S RNA than with 16S RNA or tRNA, and within 23S RNA, U2605 reacted more readily than either U1199 or U2833. Second, of the four sites shown to react with YpuL, only U2605 is naturally Ψ in both *E. coli* and *B. subtilis*. This site corresponds to Ψ2633 in *B. subtilis* which is found in the same secondary structural context as Ψ2605 of *E. coli*. Therefore, we propose that YpuL is the Ψ2633 synthase in this organism and suggest that the gene be re-named *rluB-(Bs)* for (r)ibosomal (l)arge subunit pseudo(u)ridine synthase B in *B. subtilis*. In *E. coli*, RluA (*14*), RluC (*15*), RluD (*16*), and RluE (J. Conrad, C. Alabiad, and J. Ofengand, unpublished results) have previously been identified as large ribosomal subunit pseudouridine synthases. Pertinent details of these and all the other currently known eubacterial synthases are summarized in Table 2.

Reactions were performed at both 3 and 10 mM Mg^{2+} with the expectation that conformational changes in the two ribosomal RNAs in solution might occur that would influence the amount and/or the location of Ψ formation. There was no effect on the site of Ψ formation in 16S RNA (Figure 3), but there was a considerable decrease (by $^2/_3$) in the extent

Table 2: Currently Known Eubacterial Pseudouridine Synthases

organism	name	previous gene name(s)	RNA substrate	modified position	SWISSPROT file name	ref
E. coli	RsuA	yejD	16S rRNA	516	P33918	*13*
E. coli	RluA	yabO	23S rRNA	746	P39219	*14*
			tRNA	32		
B. subtilis	RluB	ypuL	23S rRNA	2633[a]	P35159	this work
E. coli	RluC	yceC	23S rRNA	955	P23851	*15*
				2504		
				2580		
E. coli	RluD	yfiI or sfhB	23S rRNA	1911	P33643	*16*
				1915		
				1917		
E. coli	RluE	ymfC	23S rRNA	2457	P75966	*b*
E. coli	TruA	hisT	tRNA	38−40	P07649	*25*
E. coli	TruB	yhbA	tRNA	55	P09171	*18*

[a] Equivalent to position 2605 in *E. coli*. [b] Unpublished results of J. Conrad, C. Alabiad, and J. Ofengand.

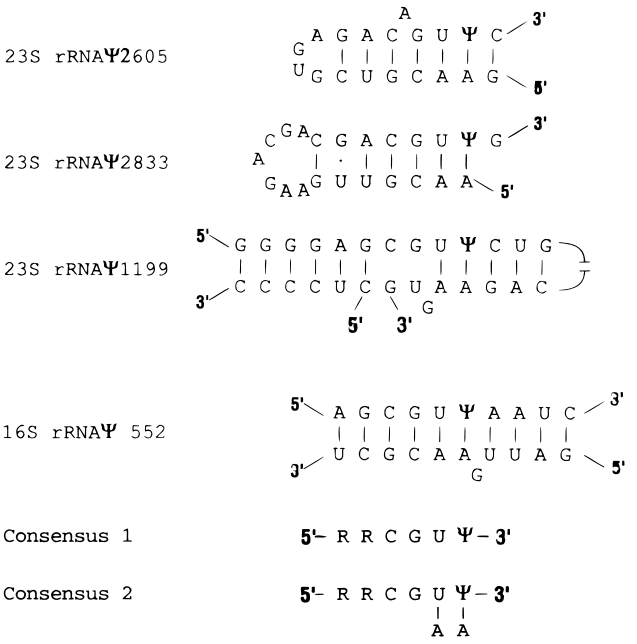

FIGURE 6: Secondary structure context of the YpuL Ψ formation sites. The four sites of Ψ formation are shown. Consensus 1 was derived from the structures shown. Consensus 2 was obtained by consideration of the other consensus 1 sites in *E. coli* rRNA which do not form Ψ (see the text).

of reaction, suggestive of a condensation of the RNA structure, at least in the vicinity of Ψ552. Similarly, the extent of reaction in 23S RNA was decreased when the Mg^{2+} concentration was raised (Figure 4).

We also observed reaction in vitro at three other sites in *E. coli* ribosomal RNA, positions 552 in 16S RNA and 1199 and 2833 in 23S RNA. Because these sites are not normally Ψ in *E. coli* or in their equivalent positions in *B. subtilis*, they appear to be artifacts of the in vitro system. Nevertheless, these sites can be used to our advantage to deduce a consensus recognition motif for YpuL. Figure 6 shows the four sites in *E. coli* rRNA in their accepted secondary structural context. A minimum consensus sequence derived from these structures is shown as consensus 1. Since only the Ψ2605 site has the bulged A residue, it has not been considered in construction of the consensus structure (but see below). Moreover, there are no other (bulged A)GUU sequences in either *E. coli* or *B. subtilis* 23S or 16S RNAs. In *B. subtilis*, the residue 2605 stem loop is identical, the residue 2833 stem loop does not have the consensus sequence, and the residue 1199 and 552 segments are the same except for base pair substitutions outside of the consensus region. There are no other consensus 1 sequences in *B. subtilis* 16S or 23 RNA, but five were found in *E. coli*. Four potential Ψ sites were found at positions 653, 1326, 2203, and 2818 in 23S RNA and one at position 486 in 16S RNA. None of these sites are Ψ in *E. coli* in vivo (*7*, *8*, *24*), and none were found to be Ψ in vitro in this work. Position 2818 is shown in Figure 5 and is clearly not Ψ. The other sites were checked in the screens shown in Figures 3 and 5 and described in the text.

The minimum distinguishing characteristic between the four reactive sites and the five that did not react is opposite strand base pairing of the two U residues, as shown in consensus 2. We propose, therefore, that the consensus 2 structure is the basic recognition element for YpuL (RsuB) and, therefore, that the bulged A residue is not *essential* for recognition even though it may markedly enhance the process, thus explaining the strong preference for U2605 in vitro. In *B. subtilis* in vivo, Ψ2633 (2605 in *E. coli* 23S RNA) is found but not Ψ1238 (1199 in *E. coli* 23S RNA) or Ψ559 (552 in *E. coli* 16S RNA) even though all three sites share the consensus 2 structure. One explanation may be that U1238 and U559 are structurally shielded in some way from reaction. Another possibility is that the bulged A residue, only present at the Ψ2633 site, may be a strong recognition element, even if not the only one.

Homology with Respect to Other E. coli ORFs. Although YpuL was first identified by its homology to RsuA, when YpuL was used as the query sequence in a BLAST 2.0.6 (*26*) search, the closest *E. coli* homologue was no longer RsuA, but YciL. This gene product was previously identified as a likely Ψ synthase (*27*, *28*), and we have shown that the gene product possesses Ψ synthase activity on 23S RNA (*29*; I. Pfeifer and J. Ofengand, unpublished results). However, the specificity of YciL has not yet been determined. On the basis of the above-described amino acid homology, we suggest that *yciL* may be the *E. coli rluB* gene. Experiments for testing this hypothesis are currently underway.

Even closer homologues to YpuL exist in other organisms. The same BLAST search detected a hypothetical protein in *Aquifex aeolicus* (Genbank file 2983856), *Mycobacterium tuberculosis* (Genbank file 2326754), and *Mycobacterium leprae* (Genbank file 2065214) with considerably more homology to YpuL than to YciL. There were also two others with homology only slightly greater than YciL in *Synechocystis* sp. (Genbank file 1001457) and in *Chlamydia trachomatis* (Genbank file 3329180). This last protein was predicted to be a Ψ synthase (*30*). These hypothetical proteins may all be Ψ synthases with a specificity like that of YpuL since the equivalents of U2605 or U2606 have been shown to be Ψ in a variety of prokaryotes and eukaryotes (*7*). The persistence of Ψ residues and their cognate synthases in widely divergent species certainly provide evidence for their importance in some aspect of cell function. Unfortunately, that function or functions have so far remained elusive, except in one instance (*16*).

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