A truncated aminoacyl–tRNA synthetase modifies RNA

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Aminoacyl–tRNA synthetases are modular enzymes composed of a central active site domain to which additional functional domains were appended in the course of evolution. Analysis of bacterial genome sequences revealed the presence of many shorter aminoacyl–tRNA synthetase paralogs. Here we report the characterization of a well conserved glutamyl–tRNA synthetase (GluRS) paralog (YadB in *Escherichia coli*) that is present in the genomes of >40 species of proteobacteria, cyanobacteria, and actinobacteria. The *E. coli yadB* gene encodes a truncated GluRS that lacks the C-terminal third of the protein and, consequently, the anticodon binding domain. Generation of a *yadB* disruption showed the gene to be dispensable for *E. coli* growth in rich and minimal media. Unlike GluRS, the YadB protein was able to activate glutamate in presence of ATP in a tRNA-independent fashion and to transfer glutamate onto tRNA^{Asp}. Neither tRNA^{Glu} nor tRNA^{Gln} were substrates. In contrast to canonical aminoacyl–tRNA, glutamate was not esterified to the 3′-terminal adenosine of tRNA^{Asp}. Instead, it was attached to the 2-amino-5-(4,5-dihydroxy-2-cyclopenten-1-yl) moiety of queuosine, the modified nucleoside occupying the first anticodon position of tRNA^{Asp}. Glutamyl–queuosine, like canonical Glu–tRNA, was hydrolyzed by mild alkaline treatment. Analysis of tRNA isolated under acidic conditions showed that this novel modification is present in normal *E. coli* tRNA; presumably it previously escaped detection as the standard conditions of tRNA isolation include an alkaline deacylation step that also causes hydrolysis of glutamyl–queuosine. Thus, this aminoacyl–tRNA synthetase fragment contributes to standard nucleotide modification of tRNA.

Faithful translation of the mRNA genetic information into proteins relies on the correct attachment of an amino acid to its corresponding tRNA(s) by enzymes collectively known as aminoacyl–tRNA synthetases (aaRSs). These essential enzymes are structurally conserved (1, 2) and easily detected in the analysis of genome sequences (e.g., ref. 3). Prokaryotes usually have one gene for each aaRS, although the presence of two functional paralogous genes for a synthetase (e.g., *lysS* and *lysU* for *Escherichia coli* lysyl–tRNA synthetase; ref. 4) is well known. Analysis of the ever larger number of bacterial genome sequences revealed the occurrence of many aaRS paralogs containing regional deletions (compared to the full-length genes also present in the genome). Given the modular nature of the aaRS proteins (5), the presence of synthetase gene pieces in a genome was not surprising, because they are likely remnants of a complex evolutionary history (2, 3, 6, 7). Although these truncated aaRSs were considered to be pseudogenes (8), more recent attention to particular aaRSs paralogs revealed their involvement in important biological processes. For instance, HisZ, a histidyl–tRNA synthetase-like protein lacking the anticodon domain, was shown to be essential for histidine biosynthesis in a number of bacteria (9). Likewise, an archaeal asparaginyl–tRNA synthetase paralog, lacking the anticodon-binding domain, is able to carry out tRNA-independent asparagine synthesis (10). The function of aaRS paralogs is not restricted to amino acid biosynthesis. Truncated alanyl–, prolyl–, and threonyl–tRNA synthetase-like proteins were shown to possess esterase function and be capable of specific hydrolysis of misacylated

tRNA in *trans* (11, 12), a mechanism that should increase the fidelity of the translation process.

Here we report the characterization of another widely distributed aaRS paralog, a glutamyl–tRNA synthetase (GluRS)-related protein encoded by *yadB* in *E. coli*.

Materials and Methods

Oligonucleotides, DNA Sequencing, Radiochemicals, and Bacterial Strains. Oligonucleotides synthesis and DNAs sequencing was performed by the Keck Foundation Biotechnology Resource Laboratory at Yale University. Uniformly labeled [^{14}C]Glu (254 mCi/mmol; 1 Ci = 37 GBq), sodium [^{32}P]pyrophosphate (1–60 Ci/mmol), and [γ-^{32}P]ATP (6,000 Ci/mmol) were from Amersham Biosciences. [^{14}C]Asp (208 mCi/mmol) was from American Radiolabeled Chemicals, St. Louis.

E. coli strains W3110, W3110Δ*yadB*, DH5α, and the queuosine-deficient JE7350 (13) were used for tRNA preparations.

Cloning of the *E. coli yadB* Gene. The *E. coli yadB* gene (GenBank accession no. AE000123) was obtained by PCR from *E. coli* W3110 genomic DNA using the Expand High Fidelity PCR System (Roche Molecular Biochemicals). Oligonucleotide primers introduced an *Nde*I site at the 5′ end and a *Bam*HI site at the 3′ end of the gene. The PCR product was cloned into the pCR2.1–TOPO vector (Invitrogen). After sequence confirmation, the DNA was digested with *Nde*I and *Bam*HI and cloned into the pET15b vector (Novagen) digested with the same enzymes to form plasmid pDJSyadB.

Inactivation of *yadB* in *E. coli*. The deletion of the *yadB* gene was carried out by using the Red disruption system (14). Confirmation of the deletion was done by PCR using oligonucleotides complementary to the upstream and downstream sequence of *yadB* and complementary to the kanamycin gene. The sizes of the PCR products expected are 850 bp for the PCR using primers upstream of *yadB* and internal to the kanamycin gene, 1,280 bp for the PCR using primers downstream of *yadB* and internal to the kanamycin gene, and 471 bp as the amplified fragment of the kanamycin gene. Fragments of these lengths were observed on gel analysis (data not shown).

Purification of YadB. The plasmid pDJSyadB was transformed into *E. coli* BL21(DE3) (Invitrogen). The expression of *yadB* was performed by incubating the cells at 37°C until an absorbance of 0.6–0.8 A$_{600}$ was reached, at which point 1 mM IPTG was added and incubated for 3 h at 25°C. Purification was performed with an Amersham Biosciences Äkta FPLC system using a TALON superflow Co^{2+}-column (BD Biosciences/Clontech), followed by gel filtration on Superdex 200.

Preparation of *E. coli* tRNAs. Unfractionated tRNA was prepared from freshly grown or frozen *E. coli* cells as described (15). *E. coli*

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tRNA was fractionated by reverse-phase HPLC (Varian Pro-Star, Palo Alto, CA): 5 mg of unfractionated *E. coli* tRNA in 0.1 ml of sterile water was injected on a ProSphere 300 C4 column (250 mm × 10 mm, Alltech). The column was washed with 5 volumes of buffer A [20 mM NH_4OAc/10 mM $Mg(OAc)_2$/400 mM NaCl] at a 5 ml/min flow rate. RNA was eluted in a 300 ml (0–55%) gradient of buffer B [20 mM NH_4OAc/10 mM $Mg(OAc)_2$/400 mM NaCl/60% EtOH]. Fractions were collected (1 ml) and ethanol precipitated in the presence of glycogen (20 μg/ml), washed with cold 70% ethanol, dried, and resuspended in sterile water.

Purification of tRNA^{Asp}. *E. coli* tRNA^{Asp} was purified from unfractionated tRNA by affinity chromatography on immobilized *Thermus thermophilus* EF-Tu (16) as follows. Unfractionated *E. coli* tRNA was aminoacylated with pure *E. coli* aspartyl–tRNA synthetase (AspRS) and the aminoacyl–tRNA was separated from the uncharged tRNA by the formation of a ternary complex with the EF-Tu-GTP immobilized on a nickel nitrilotriacetic–agarose column. The eluted Asp–tRNA^{Asp} was deacylated for 1 h at 37°C in 0.2 mM Tris-acetate, pH 9.0. This tRNA^{Asp} could be charged to 1,100 pmol/A_{260}.

Cloning and *in Vitro* Transcription of the *E. coli* tRNA^{Asp} Gene. The tRNA^{Asp} gene suited for *in vitro* T7 RNA polymerase transcription was cloned by annealing of two complementary DNA oligonucleotides and direct ligation into pUC18 plasmid by using *Bam*HI and *Hin*dIII restriction sites. The clone was transformed into *E. coli* DH5α, sequenced, and amplified. The 3′ end of the transcription template was digested overnight with *Bst*NI at 60°C. *In vitro* T7 RNA polymerase run-off transcription was conducted according to standard procedure (17). tRNA^{Asp} transcripts were purified on a denaturing 12% polyacrylamide gel and recovered by soaking into 1M NaOAc pH6.0, followed by desalting on a Nap-5 Column (Amersham Biosciences).

Aminoacylation Assay. *In vitro* acylations with *E. coli* YadB and AspRS enzymes were carried out at 37°C in a 70-μl reaction mixture containing 100 mM Hepes-KOH, pH 7.2, 30 mM KCl, 12 mM $MgCl_2$, 2 mM ATP, 2 mM DTT, 25 μM radioactive amino acid ([^{14}C]Glu or [^{14}C]Asp) plus 75 μM unlabeled amino acid, and 40–60 μg of unfractionated tRNA or 0.7–1.3 μg of pure tRNA. Enzyme concentration was 900 nM (YadB) or 225 nM (AspRS). Reactions were started by amino acid addition. Aliquots (15 μl) were taken at different times, spotted on Whatman 3MM filter paper discs, and washed twice with 5% TCA.

ATP-^{32}PP$_i$ Exchange. Reactions were conducted in 100 mM Hepes-KOH, pH 7.2, 30 mM KCl, 12 mM $MgCl_2$, 2 mM DTT, 2 mM KF, 2 mM sodium [^{32}PP$_i$] (2 cpm/pmol), varying concentrations of ATP (0–5 mM), varying concentrations of glutamate (0–50 mM), 150 nM YadB in a final volume of 0.2 ml at 37°C. To detect possible tRNA-dependent glutamate activation by YadB, unfractionated *E. coli* tRNA was added to the reaction mixture in a variety of concentrations (0.1–2 μM of estimated tRNA^{Asp}). [^{32}P]ATP formation was followed by specific absorption on acid-washed Norit [200 μl of a 1% suspension (wt/vol) of Norit in a solution of 0.4 M sodium pyrophosphate solution containing 15% (vol/vol) perchloric acid] and filtration on Whatman GF/C filters followed by washing with 15 ml of water and 5 ml of ethanol.

Analysis of Aminoacyl–tRNA. As an additional test to determine aminoacyl–tRNA formation with YadB, periodate oxidation followed by β elimination was used (18) with the following modifications. After aminoacylation with YadB or AspRS (positive control), half of the reaction was deacylated with 0.2 mM Tris-acetate, pH 9.0. After ethanol precipitation, the RNA of both samples was treated in the dark with 2.5 mM $NaIO_4$ in 50 mM sodium acetate, pH 5.0, for 1 h at 37°C. The reaction was stopped by adding 50 mM glucose and incubated at 0°C for 1.5 h. The tRNA was precipitated and further processed as described (18). tRNAs were detected by Northern blotting. For this purpose, the portion of the gel containing the tRNAs was electroblotted onto a Hybond-N+ membrane (Amersham Bioscience) by using a Hoefer Electroblot apparatus (Amersham Pharmacia) at 20 V for 1 h with 40 mM Tris-acetate, pH 4.2 and 2 mM EDTA as transfer buffer. Membranes were then baked at 72°C for 2 h, and tRNAs detected by hybridization with a 5′-[^{32}P]-oligo-deoxyribonucleotide probe complementary to nucleotides 32–51 of tRNA^{Asp}.

Digestion of tRNA to Nucleosides. tRNA was hydrolyzed to nucleosides by using nuclease P1 and Antarctic (acid) phosphatase (New England BioLabs); enzyme amounts are per 20 μg tRNA. Typically, 1/10 vol 10× Antarctic phosphatase buffer (pH 5) was added to the tRNA solution in water, followed by 2 units of nuclease P1 (2 units/μl; Boehringer). After incubation for 2 h at 37°C, 1 unit of acid phosphatase (1 unit/μl) was added, and the reaction was continued a further 2 h.

Liquid Chromatography/Electrospray Ionization-Mass Spectrometry of Digested tRNA. tRNA hydrolyzates were analyzed directly, without cleanup. Digests of bulk W3110 tRNA were analyzed on an HP1090 liquid chromatograph coupled directly to a Micromass Quattro II mass spectrometer. Nucleosides were separated on a Develosil C30 column by using a multilinear gradient essentially as described (19), except that the flow rate was 0.3 ml/min and the concentration of buffer A was 5 mM. The ion source and nebulizer temperatures were 140°C and 275°C, respectively. Data were collected by selected ion recording of m/z 410 (MH$^+$ for queuosine), and m/z 539 (expected MH$^+$ ion for glutamyl–queuosine). Purified tRNA^{Asp} was analyzed by using a Waters CapLC liquid chromatograph coupled without solvent stream splitting to a Micromass QTof2 mass spectrometer. Both instruments were controlled by using a Micromass MASSLYNX v. 3.3 data system. Nucleosides were separated on a Luna C18(2) column (150 × 0.5; Phenomenex) at a flow rate of 17 μl/min using a multilinear gradient essentially as described (19) with buffer A, 5 mM ammonium acetate (pH 5.3), and buffer B, 40% (aq.) CH_3CN. Ion source and nebulizer temperatures were 80°C and 135°C, respectively; the eluate was conducted directly into the mass spectrometer without splitting. Data were collected over a mass range of m/z 100–600.

Results

The GluRS Paralog YadB. Analysis of bacterial genome sequences revealed the presence of a widely occurring GluRS paralog (named *yadB* in *E. coli*). *YadB* genes are found in >40 organisms that belong to the proteobacterial, cyanobacterial, and actinobacterial groups. The *E. coli yadB* gene encodes a protein of 308 aa. The known YadB proteins constitute a well conserved group (>45% amino acid identity) related to bacterial GluRS. Sequence alignment of the bacterial YadB orthologs and comparison with bacterial GluRS revealed the presence of the conserved Rossmann fold class I defining signature sequences (HVGG and KLSK for *E. coli* GluRS) and a well conserved amino acid binding pocket. The crucial glutamate binding site residues Arg-5, Ser-9, Glu-41, Asn-191, and Arg-205 of *T. thermophilus* GluRS (20) were strictly conserved in the YadB proteins, indicating that glutamate probably binds in a similar fashion in both GluRS and YadB. However, YadB is only 65% as large as GluRS; it lacks the C-terminal portion (171 aa for *E. coli* GluRS) that serves as the anticodon binding domain of this protein.
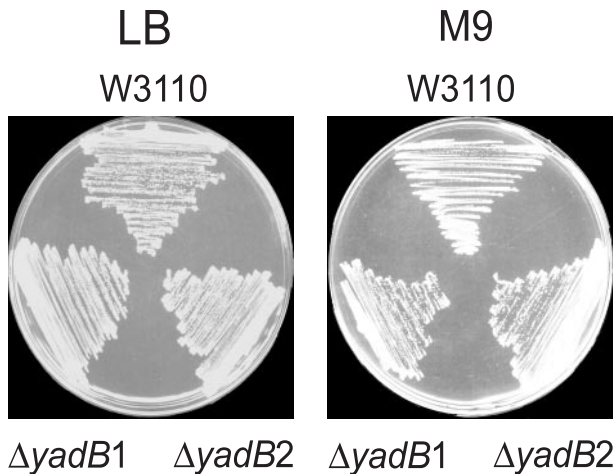
Fig. 1. Growth of the W3110Δ*yadB* strain. Growth at 37°C of *E. coli* strain W3110 and two independent isolates of W3110Δ*yadB* on LB and M9 plates.

YadB Is Dispensable in *E. coli*. To assess the essentiality of the *E. coli* YadB protein, we used deletion and insertion strategy (14) to construct a *yadB* deletion in *E. coli* strain W3110, which was confirmed by PCR analysis of the resultant DNA (see *Materials and Methods*). This deletion strain was viable (Fig. 1) and grew in both rich (LB) and minimal (M9 glucose) medium at 37°C (and also at 42°C, data not shown). Thus, under the conditions tested, *yadB* is not essential. During the course of this investigation, a similar result was obtained by a genomic deletion strategy (21).

YadB Activates Glutamate in a tRNA-Independent Manner. The enzymatic functions of the recombinant N-terminal His$_6$–YadB protein, purified to homogeneity by two-step column chromatography, was tested in amino acid activation. ATP-PP$_i$ exchange was performed in the presence and absence of unfractionated *E. coli* tRNA with either glutamate, aspartate, or with a mixture of 19 amino acids (except glutamate). YadB is capable of activating glutamate specifically (Fig. 2*A*); however, in contrast to GluRS, YadB can carry out this process in the absence of tRNA (a similar finding was published very recently, ref. 22). This observation is consistent with the fact that the amino acid-binding site is conserved in GluRS and YadB, but the lack of the anticodon

binding domain in YadB should prohibit the major conformational rearrangements observed in the active site of the *T. thermophilus* GluRS upon tRNA binding (19). Determination of the kinetic parameters in ATP-PP$_i$ exchange experiments revealed a much lower glutamate affinity of YadB than of *E. coli* GluRS (7.5 mM vs. 0.66 mM, ref. 23), whereas the K_M for ATP was similar for both enzymes (440 μM vs. 420 μM, ref. 23). The turnover rate for glutamate activation was also in the same range as the one observed for GluRS (11 s^{-1} vs. 1.77 s^{-1}, ref. 24).

YadB Transfers Glutamate to tRNA^{Asp}. Given that YadB lacks a large part of the tRNA binding domain (compared to GluRS), we wanted to see whether YadB was capable of transferring activated glutamate onto tRNA. Surprisingly, ≈3% of unfractionated *E. coli* tRNA could be glutamylated under standard conditions. Because some GluRS enzymes can charge tRNA^{Glu} and tRNA^{Gln} (e.g., refs. 25 and 26), we purified and tested these tRNA species; however, neither tRNA was a substrate for YadB (data not shown). To identify the tRNA substrate for YadB, *E. coli* tRNA was fractionated by HPLC reverse-phase chromatography (27). Assaying the column fractions (Fig. 2*B*) showed a coincidence of glutamylation by YadB with aspartylation by AspRS, suggesting that tRNA^{Asp} might also be a YadB substrate and be glutamylated. This was confirmed by charging of EF-Tu affinity chromatography (16) purified tRNA^{Asp}; this tRNA proved to be an equally good substrate for both pure YadB and pure *E. coli* AspRS (Fig. 2*C*). As expected for the labile ester linkage between glutamate and tRNA, Glu–tRNA^{Asp} could be deacylated at pH 9.0 (data not shown). An independent report of Glu–tRNA^{Asp} formation by YadB has recently appeared (28).

Glutamate Is Not Transferred to the tRNA's 3′-Terminal Adenosine. The nature of Glu–tRNA^{Asp} was further examined by electrophoretic mobility shifts on a sequencing gel (18). Periodate oxidation of the vicinal *cis* diols of the tRNA's terminal ribose, followed by mild base treatment, leads to elimination of the terminal adenosine (29). However, if the tRNA's terminal adenosine is aminoacylated, periodate oxidation is blocked. After subsequent deacylation, the originally charged tRNA retains its length, whereas the originally free tRNA species will be 1 nt shorter and can be separated by a sequencing gel (e.g., ref. 18). Comparison of the electrophoretic mobilities (Fig. 3*A*) of periodate-treated Glu–tRNA^{Asp}, Asp–tRNA^{Asp}, and deacylated tRNA showed that, as expected, Asp–tRNA^{Asp} was 1 nt longer than deacylated tRNA. However, the oxidation product
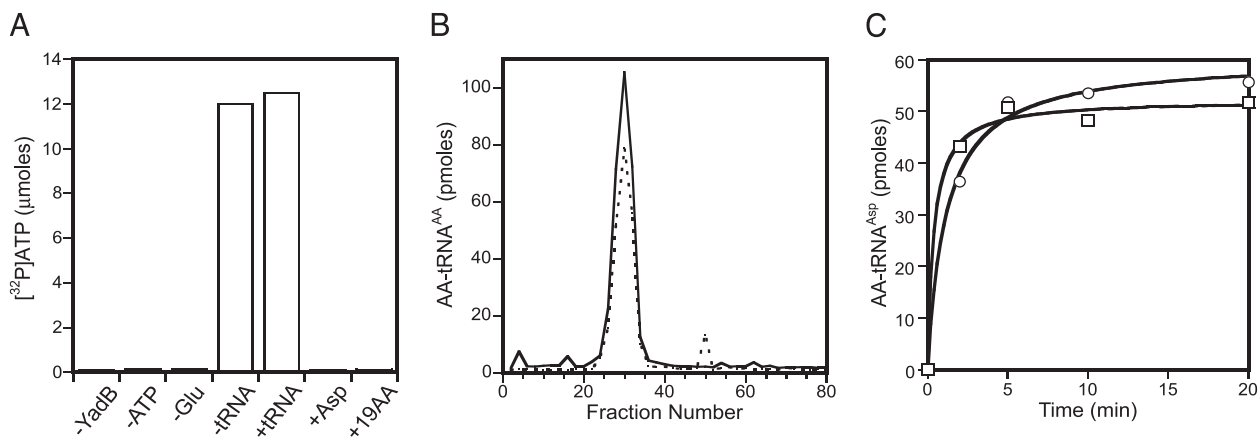

Fig. 2. Glutamate activation and transfer onto tRNA by YadB. (*A*) YadB-dependent amino acid activation measured by ATP-PP$_i$ exchange. The lanes are: -YadB; -ATP; -Glu; -tRNA, Glu in the absence of tRNA; +tRNA, Glu in the presence of tRNA; +Asp, Asp alone; +19 AA, all amino acids except Glu. (*B*) Charging of tRNA fractions from reverse-phase HPLC with aspartate using *E. coli* AspRS (dotted line) and with glutamate using YadB (solid line). (*C*) Charging of purified tRNA^{Asp} with aspartate using *E. coli* AspRS (□) and with glutamate using YadB (○).
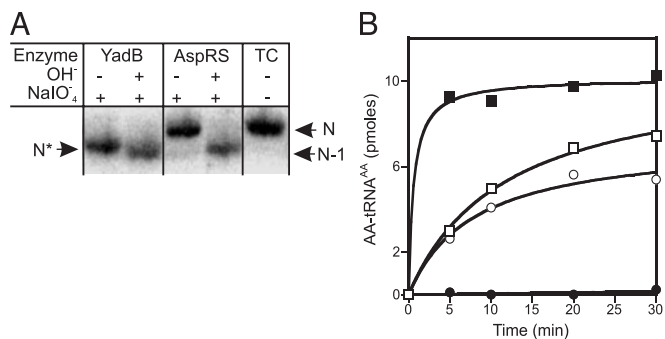
Fig. 3. Analysis of periodate-treated Glu–tRNA^{Asp} and Asp–tRNA^{Asp}. (*A*) Glu–tRNA^{Asp} and Asp–tRNA^{Asp} was treated with periodate and then lysine, deacylated, and subjected to gel electrophoresis for size determination. tRNA was visualized by hybridization with ^{32}P-labeled probes against tRNA^{Asp}. N, position of intact tRNA^{Asp}; N-1, tRNA^{Asp} without the terminal adenosine; N*, YadB-generated Glu–tRNA^{Asp} lacking the terminal adenosine; tRNA control. (*B*) Glu–tRNA^{Asp} was treated with periodate, deacylated and then recharged with either glutamate and YadB (○) or aspartate and *E. coli* AspRS (●). Untreated tRNA is aminoacylated by YadB (□) and AspRS (■).

of Glu–tRNA^{Asp}, revealed only a very small mobility difference compared to that of oxidized deacylated tRNA, indicating that glutamate is not attached to the tRNA's 3′ end. This result was confirmed by checking the acceptor activities of periodate-treated aminoacyl–tRNA. Glu–tRNA^{Asp} was subjected to periodate treatment, deacylated, and recharged either by *E. coli* AspRS with aspartate or by YadB with glutamate. Periodate-treated tRNA could be successfully reaminoacylated with glutamate by YadB, but could no longer be aspartylated by AspRS (Fig. 3*B*). Conversely, periodate-treated Asp–tRNA^{Asp} could only be recharged with aspartic acid (data not shown). The results of both experiments show that glutamate is not esterified to the 3′-terminal adenosine, but to some other nucleotide in tRNA^{Asp}.

Pure *E. coli* tRNA^{Asp} Can Be Acylated with Aspartate and Glutamate Simultaneously. The results above suggested that it might be possible to charge aspartate and glutamate simultaneously to tRNA^{Asp}. This was indeed the case; Fig. 4 shows that simultaneous acylation with [^{14}C]Asp and [^{14}C]Glu in the presence of the *E. coli* AspRS and YadB enzymes led to a tRNA molecule carrying both amino acids, aspartate attached at the tRNA's acceptor end and glutamate bound to an unknown position in tRNA^{Asp}.
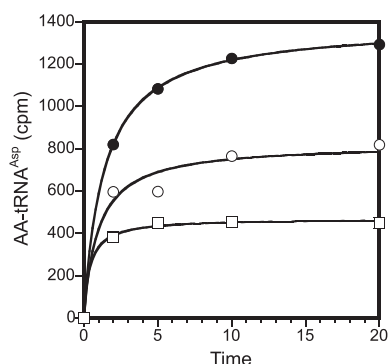

Fig. 4. Simultaneous acylation of purified *E. coli* tRNA^{Asp} with aspartate and glutamate. Charging with [^{14}C]Asp and AspRS (□), [^{14}C]Glu using YadB (○), and [^{14}C]Asp and AspRS in the presence of [^{14}C]Glu and YadB (●). Accounting for the different specific activities of Asp and Glu (see *Materials and Methods*), aminoacylation at the 20 min time point is >90%.
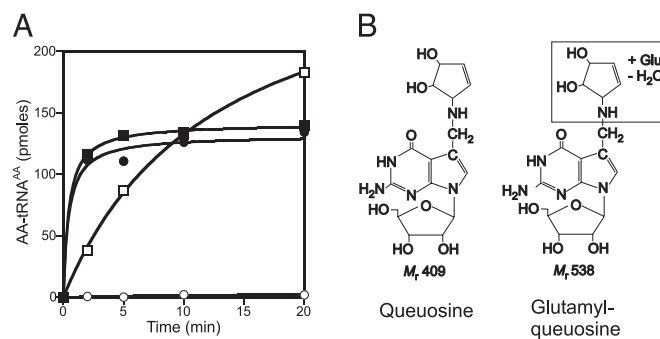

Fig. 5. Glutamylation and aspartylation of queuosine-deficient tRNA and structures of queuosine and glutamyl–queuosine. (*A*) tRNA isolated from the queuosine-deficient *E. coli* JE7350 strain and wild-type tRNA was acylated *in vitro*. (■) wild-type tRNA, [^{14}C]Asp, and AspRS; (●) Q-deficient tRNA, [^{14}C]Asp, and AspRS; (□) wild-type tRNA, [^{14}C]Glu, and YadB; (○) Q-deficient tRNA, [^{14}C]Glu, and YadB. (*B*) Queuosine and glutamyl–queuosine.

The Hypermodified Nucleoside Queuosine Is the Possible Site of Glutamate Attachment. Given the alkali lability of the glutamyl–tRNA linkage and the sensitivity of the glutamylation activity to periodate-treated tRNA, we surmised that the site of glutamate attachment may have chemical features similar to the ribose at the 3′ end of tRNA. Additionally, the *in vitro* transcript of tRNA^{Asp}, lacking all of the posttranscriptional modifications, could not be glutamylated by YadB, whereas it was a very good substrate for the AspRS (data not shown). This strongly suggested that glutamate might be acylated to a modified nucleoside present in tRNA^{Asp} that would have the appropriate properties is queuosine, the first anticodon nucleoside (position 34) in tRNA^{Asp} (30). Queuosine, 7-{5-[(4S,5R-dihydroxy-2-cyclopenten-1-yl)amino]methyl}-7-deazaguanosine (Fig. 5*B*) (31) presents both a possible functional amino group and a cyclopentene moiety bearing vicinal *cis* diols. These hydroxyl groups are reactive, as some mammalian tRNAs contain modified versions of queuosine in which the 5-hydroxyl is mannosylated or galactosylated (32). To determine whether queuosine is the "acceptor" for glutamate, we prepared tRNA from an *E. coli* mutant strain deficient in the first step of queuosine formation (13). This Q-deficient tRNA could not be glutamylated by YadB (Fig. 5*A*), strongly suggesting that queuosine provides the site for glutamate attachment.

In addition to tRNA^{Asp}, queuosine is also found in tRNA^{Asn}, tRNA^{His}, and tRNA^{Tyr}. We have not yet tested the ability of these tRNAs to be glutamylated by YadB.

YadB Glutamylates the Nucleoside Queuosine in tRNA^{Asp}. Therefore we decided to prepare pure Glu–tRNA^{Asp} from the W3110Δ*yadB* deletion strain *in vitro* and subject the RNA hydrolyzate to electrospray ionization liquid chromatography MS analysis. The calculated molecular mass of glutamyl–queuosine is 538.24 (MH$^+$ ion, m/z 539.24). As shown in Fig. 6*A*, the glutamylated tRNA^{Asp} contains a nucleoside eluting at 19.2 min whose measured mass is 539.2, consistent with an assignment as glutamyl–queuosine. The control tRNA lacks this species (Fig. 6*C*). In a separate tandem mass spectrometry analysis of this component (from unfractionated tRNA; data not shown), an m/z 163 ion, assigned as loss of the cyclopentenediolamine side chain from the 7-deazaguanine moiety of queuosine, is likewise produced from the glutamyl–queuosine nucleoside. These data establish that the 2-amino-5-(4,5-dihydroxy-2-cyclopenten-1-yl) moiety of queuosine is glutamylated (Fig. 5*B*). However, the nature of the functional group (OH- or NH-) and the position of glutamate attachment, as well as the nature of the glutamate carboxyl involved (α or ε), remains to be established.
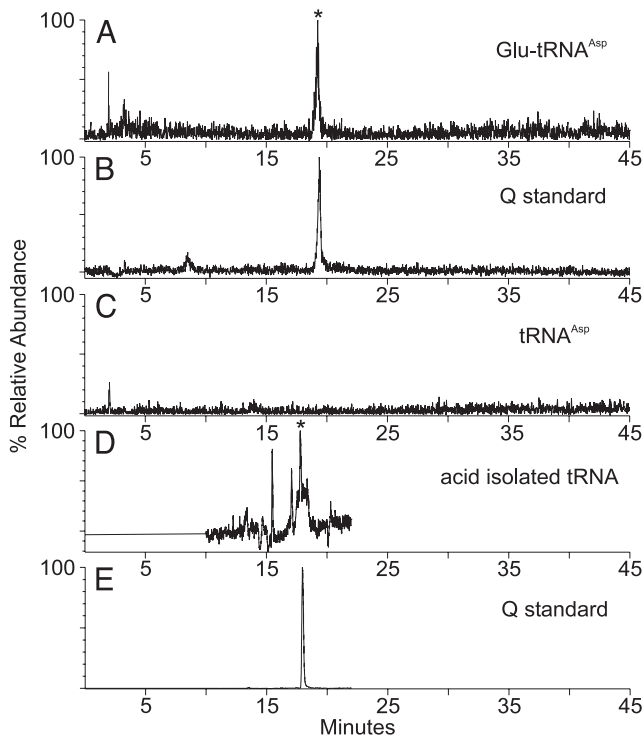
Fig. 6. Liquid chromatography/electrospray ionization-mass spectrometry analysis of nucleoside mixtures derived from purified W3110Δ*yadB* tRNA^{Asp} before and after YadB-catalyzed glutamylation (*A–C*) and from unfractionated W3110 tRNA isolated under acidic conditions (*D* and *E*). Reconstructed ion chromatograms for *m/z* 539.2, the MH$^+$ ion expected for glutamyl–queuosine (*A*); *m/z* 410.2, the MH$^+$ ion expected for queuosine (*B*); *m/z* 539.2 from tRNA^{Asp} (*C*). Selected ion recording for *m/z* 539.2, the MH$^+$ ion expected for glutamyl–queuosine (*D*) and *m/z* 410.2, the MH$^+$ ion expected for queuosine (*E*). The glutamyl–queuosine peak is marked with an asterisk (*A* and *D*). Because this figure contains two different experimental runs (*A–C*, and *D* and *E*), two queuosine standards (*B* and *E*) are included.

Glutamyl–Queuosine Is a Normal Constituent of *E. coli* tRNA. Like the glutamyl–tRNA linkage at the 3′ terminus of tRNA, glutamyl–queuosine could be hydrolyzed by mild alkaline treatment. This raised the question whether glutamyl–queuosine is present *in vivo* in normal tRNA, as this modification may have previously escaped detection because the standard conditions of tRNA isolation (in preparation for modified nucleoside analysis) include an alkaline deacylation step. We therefore used acidic conditions to isolate tRNA from the wild-type W3110 *E. coli* strain. Analysis of this unfractionated tRNA (Fig. 6*D*) revealed the presence of glutamyl–queuosine. Therefore we conclude that this modified nucleoside is present in normal *E. coli* tRNA,

and that YadB contributes to posttranscriptional nucleotide modification of tRNA.

Discussion

It should not be unexpected that an aaRS paralog acylates tRNA at a position different from the acceptor helix to which the amino acid is normally attached. YadB has lost most of the GluRS tRNA binding region, and as such may not have sufficient binding surface to recognize an entire tRNA. However, tRNA modifying enzymes usually recognize much less of the tRNA molecule. For instance, the minimal requirement of tRNA–guanine transglycosylase, the enzyme involved in queuosine base insertion into tRNA, is a UGU sequence in a 7-base loop (33, 34). Most importantly, the orientation in which the tRNA interacts with YadB is very different from that with GluRS, because the anticodon, not the acceptor helix, must be close to the enzyme's active site.

Several other modified nucleosides are known that contain "intact" amino acid building blocks. Lysidine in the first anticodon position of tRNA^{Ile} is important for both codon and aaRS recognition; this modified nucleoside is formed by direct attachment of lysine to cytidine in the presence of ATP (35). N^6-threonylcarbamoyladenosine and its derivatives are also amino acid containing nucleosides; however, their ATP-dependent biosynthesis (36, 37) requires introduction of an additional carbonyl group.

The biological function(s) of queuosine are relatively unknown; however, the presence of this modified nucleoside correlates with modulation of translational fidelity (e.g., ref. 38). What might be the role of glutamyl–queuosine? The fact that *yadB* is absent from many bacterial genomes indicates that this modified nucleoside does not have a central universal importance. On the other had, even queuosine is not essential under normal growth conditions in *E. coli*, as viable queuosine-deficient strains exist. However, such strains show a marked reduction of viability when the cells are kept under unsuitable conditions for growth (13). The high K_M for glutamate of YadB might indicate that this reaction only takes place when glutamate concentration rises in the cell. Interestingly, a *yadB* ortholog is present in *Shigella flexnerii*, where the intracellular concentration of the virulence-related transcriptional regulator VirF was shown to be dependent on the presence of queuosine (39). Thus, it would be interesting to determine the effect of the glutamate modification. In any case, differences in the modification states of mannosyl–queuosine and galactosyl–queuosine in mammalian tRNAs from normal and cancer cells (40) suggest that hypermodifications of queuosine are relevant.

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