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Protein Synthesis in *Escherichia coli* with Mischarged tRNA

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Two types of aspartyl-tRNA synthetase exist: the discriminating enzyme (D-AspRS) forms only Asp-tRNA^{Asp}, while the nondiscriminating one (ND-AspRS) also synthesizes Asp-tRNA^{Asn}, a required intermediate in protein synthesis in many organisms (but not in *Escherichia coli*). On the basis of the *E. coli trpA34* missense mutant transformed with heterologous ND-*aspS* genes, we developed a system with which to measure the in vivo formation of Asp-tRNA^{Asn} and its acceptance by elongation factor EF-Tu. While large amounts of Asp-tRNA^{Asn} are detrimental to *E. coli*, smaller amounts support protein synthesis and allow the formation of up to 38% of the wild-type level of missense-suppressed tryptophan synthetase.

Aspartyl-tRNA synthetase (AspRS) exists in two different forms with respect to tRNA recognition (7). The discriminating enzyme (D-AspRS) recognizes only tRNA^{Asp}, while the nondiscriminating one (ND-AspRS) also recognizes tRNA^{Asn} and therefore forms both Asp-tRNA^{Asn} and Asp-tRNA^{Asp}. Most bacteria and archaea lack asparaginyl-tRNA synthetase and are unable to synthesize Asn-tRNA^{Asn} by direct acylation of tRNA. These organisms rely on the ND-AspRS to produce the misacylated Asp-tRNA^{Asn}, which is then converted by a tRNA-dependent amidotransferase to the correctly acylated Asn-tRNA^{Asn} (1, 4, 5, 19). Thus, the ND-AspRS is essential in organisms that form Asn-tRNA by transamidation.

The primary sequence distinguishes two general groups of AspRS. There is a predominantly bacterial type of AspRS that is about 580 amino acids, in addition to a shorter archaeal-eukaryotic type of about 430 amino acids. In vitro data have made clear that discriminating and nondiscriminating enzymes exist in both groups (16, 20). The determinants in the protein sequence responsible for tRNA discrimination are not known.

The two AspRS types are usually separated in nature. Genome analyses of bacteria and archaea have revealed that the presence of the ND-AspRS is always accompanied by the occurrence of the heterotrimeric GatCAB amidotransferase, an enzyme capable of converting the misacylated Asp-tRNA^{Asn} to Asn-tRNA^{Asn} (2, 5, 19). Presumably, this is to avoid introducing the misacylated Asp-tRNA^{Asn} into an organism's translational apparatus and potentially endangering protein synthesis. This reasoning is supported by the fact that the heterologous expression of ND-AspRS or ND-GluRS in *Escherichia coli*, which lacks GatCAB, is highly toxic to the cell, especially when the synthetase genes are overexpressed (15). However, some organisms (e.g., *Deinococcus radiodurans* and *Thermus thermophilus*) contain a D-AspRS in addition to an ND-AspRS and a GatCAB amidotransferase (1, 3, 5, 9).

We wanted to observe how *E. coli* copes with in vivo mischarging effected by the ND-AspRS, as this organism is unable to eliminate the toxic Asp-tRNA^{Asn}. Therefore, we developed an approach that would, in fact, require *E. coli* to be dependent on the presence of mischarged Asp-tRNA^{Asn} for growth. To this aim, we used missense suppression of a specific mutation in the *trpA* gene brought about by transformation of *E. coli* with the genes of several different ND-AspRS enzymes.

MATERIALS AND METHODS

Plasmids and strains. AspRS genes were cloned into pCR2.1-TOPO (Invitrogen), while *aspS* complementation studies were carried out with pCBS1 (6) and pBAD-TOPO (Invitrogen). Expression of the desired gene in the latter vector is induced by arabinose. *E. coli* DH5α was used for most of the cloning experiments. *E. coli trpA34* strains (17) carrying a D60N mutation in *trpA* were used in missense suppression tests. *E. coli* strain A2/A2 (10) was used for synthesis of indole-3-glycerol phosphate (IGP), the substrate for the tryptophan synthetase assay.

AspRS enzymes used. The standard bacterial-type D-AspRS in our experiments was the *E. coli* enzyme (11). *D. radiodurans* provided both a larger D-AspRS1 and a small ND-AspRS2 (9). The *Chlamydia trachomatis* ND-AspRS resembling the standard bacterial enzyme (16) was used, as well as the *Halobacterium salinarum* archaeal-type ND-AspRS (accession no. BAA20527).

Plasmids carrying AspRS and tRNA^{Asn} genes. With genomic DNA, the *aspS* genes (from the start codon to the stop codon) were PCR amplified and cloned into the pCR2.1-TOPO or pBAD-TOPO vector. After sequence confirmation, they were recloned into the pCBS1 vector behind the *trpS* promoter for low-level constitutive expression. The *H. salinarum* tRNA^{Asn} gene was constructed from two oligonucleotides (91 and 94 nt) inserted between the *lpp* promoter and the *rrn* terminator of the chloramphenicol resistance-encoding pTECH vector, derived from pGFIB (13) and pACYC184 by Tong Li and Makoto Kitabatake (Yale University).

Suppression of the *E. coli trpA34* strain. The *trpA34* strain was transformed with each of the pCBS1 (for low-level expression) and pBAD-TOPO (for high-level expression) plasmids containing *aspS* genes from the sources mentioned previously. Ampicillin-resistant colonies were streaked onto M9 minimal agar plates supplemented with 19 amino acids (20 μg/ml) in the presence or absence of tryptophan (20 μg/ml), incubated at 37°C for 5 days, and scored daily.

Tryptophan synthetase assay. Freshly grown seed cultures in Vogel-Bonner minimal medium with or without Trp (20 μg/ml) were inoculated into 500 ml of the same medium. The cultures were grown at 37°C to late log phase, harvested by centrifugation, washed twice with ice-cold NaCl (0.9%) solution, and resuspended in buffer A (0.05 M KPO_4 [pH 7.0], 0.1 mg of pyridoxal-5-phosphate per ml, 10 mM 2-mercaptoethanol). Cell extracts were prepared (18), dialyzed against buffer A containing 50% glycerol, and stored at −20°C. IGP was freshly prepared as described by Mosteller (10). Tryptophan synthetase was assayed in the IGP→Trp conversion with [^{3}H]Ser (28.0 Ci/mmol) and [^{14}C]Trp (58.1 mCi/mmol) (18).

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TABLE 1. Growth of *trpA34* strains containing *aspS* genes from *D. radiodurans*, *C. trachomatis*, or *H. salinarum* in the absence of tryptophan[a]

aspS gene[b]	pCBS1	pBAD-TOPO + Glc + Ara	pBAD-TOPO + Glc	pBAD-TOPO, pTECH-HStRNA^{Asn} + Glc
—	−	−	−	−
DR1	−	−	−	ND
DR2	+	ND	ND	ND
CT	−	−	+	ND
HS	−	−	−	+

[a] The strains were grown in minimal medium containing 19 amino acids without tryptophan. Glc; 0.2% glucose, Ara; 0.2% arabinose; ND, not determined.
[b] —, empty pCBS1 vector; DR1, *D. radiodurans aspS1*; DR2, *D. radiodurans aspS2*; CT, *C. trachomatis aspS*; HS, *H. salinarum aspS*.

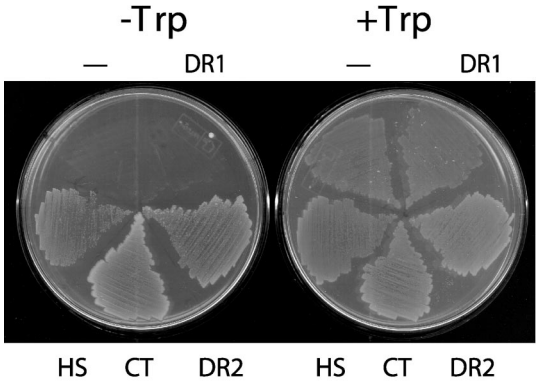

FIG. 1. Growth of *E. coli trpA34* mutant strains transformed with *aspS* genes from *D. radiodurans*, *C. trachomatis*, and *H. salinarum* on minimal agar plates in the absence (−Trp) or presence (+Trp) of tryptophan. —, empty pCBS1 vector; DR1, *D. radiodurans aspS1* in pCBS1; DR2, *D. radiodurans aspS2* in pCBS1; CT, *C. trachomatis aspS* in pBAD-TOPO; HS, *H. salinarum aspS* in pBAD-TOPO plus tRNA^{Asn} in pTECH. The picture was taken after 5 days of incubation at 37°C.

RESULTS AND DISCUSSION

Missense suppression of *trpA34*. The *E. coli trpA34* mutation is a GAT→AAT change in codon 60 of the *trpA* gene (17); the resulting D→N alteration causes loss of the catalytically essential D60 residue in the α subunit of tryptophan synthetase and leads to enzyme inactivation. As a consequence, the *E. coli trpA34* mutant strain is a Trp auxotroph (17). However, the presence in *E. coli* of mischarged Asp-tRNA^{Asn} should lead to reinsertion of D at the AAU codon (specifying N) and enable synthesis of wild-type tryptophan synthetase and restoration of prototrophic growth. This should provide a sensitive test for the presence of Asp-tRNA^{Asn} and allow in vivo examination of the tRNA recognition properties of AspRS enzymes.

Asp-tRNA^{Asn} formation in vivo. The ability of the ND-AspRS enzymes from *C. trachomatis*, *H. salinarum*, and *D. radiodurans* to form the missense suppressor Asp-tRNA^{Asn} in vivo in *E. coli* was tested by transforming the *trpA34* mutant strain with the relevant cloned *aspS* genes. The results summarized in Table 1 show that the *E. coli trpA34* mutant strain transformed with the empty vector or with *D. radiodurans aspS1* did not grow on minimal medium lacking Trp. However, the *D. radiodurans aspS2* gene (cloned in pCBS1) supported growth in minimal medium (Table 1) but *C. trachomatis* and *H. salinarum aspS* did not. While increased expression of *C. trachomatis aspS* (the pBAD-TOPO transformant in the absence of arabinose) allowed growth on minimal medium, *H. salinarum aspS* suppressed *trpA34* only when the *H. salinarum* tRNA^{Asn} gene was also expressed in the *E. coli* strain (Fig. 1 and Table 1). This indicates that the *H. salinarum* AspRS does not recognize *E. coli* tRNA^{Asn} but charges the RNA product of the homologous *H. salinarum* tRNA^{Asn} gene expressed in *E. coli*. Under the conditions described above, the *C. trachomatis aspS* transformant grew best on minimal medium while the strains transformed with *D. radiodurans aspS2* and *H. salinarum aspS* grew two and three times slower, respectively.

We then proceeded to measure tryptophan synthetase activity in the cell extracts of the transformed strains (Table 2). As expected, *E. coli aspS* (the empty-vector control) and *D. radiodurans aspS1* did not confer any tryptophan synthetase activity. However, the three ND-*aspS* genes all gave rise to sizable tryptophan synthetase activities, i.e., up to 38% of the amount measured in the wild-type *E. coli* W3110 strain. This suggests that if the observed levels of tryptophan synthetase (Table 2) are a consequence of the amount of Asp-tRNA^{Asn} formed in *E. coli* by the heterologous ND-AspRS enzymes, then the higher levels of the mischarged tRNA may be correspondingly more toxic to the cell because of a certain level of general misincorporation of aspartate specified by asparagine codons. Therefore, it is reasonable that the *trpA34* mutant strain transformed with the *H. salinarum* AspRS and tRNA^{Asn} displayed the slowest growth.

These results raise a number of questions. What levels of mischarged tRNA can a cell tolerate? The phenomenon of missense suppression (12, 14) mandates that a cell can cope with a small level of mischarging. However, this has never been investigated in detail. Furthermore, it is assumed that misacylated tRNA is discriminated against by elongation factor EF-Tu (1). While this is supported by elegant biochemical studies (8), the levels of discrimination in vivo have not been established. It may also be possible that the properties with EF-Tu in this regard may vary depending on the whether or not the organism synthesizes amide aminoacyl-tRNAs by the transamidation route. Additionally, the concentrations of correctly acylated versus misacylated tRNA may affect the dis-

TABLE 2. Tryptophan synthetase activities in W3110 and *trpA34* strains containing AspRSs from *D. radiodurans*, *C. trachomatis*, and *H. salinarum* grown in the absence of tryptophan

aspS gene[a]	Avg activity: (U/mg)[b] ± SD	Relative activity (%)
W3110	15.74 ± 1.43	100
—	0	0
DR1	0	0
DR2	3.59 ± 0.49	23
CT	2.54 ± 0.21	16
HS	5.98 ± 0.84	38

[a] —, empty pCBS1 vector; DR1, *D. radiodurans aspS1* in pCBS1; DR2, *D. radiodurans aspS2* in pCBS1; CT, *C. trachomatis aspS* in pBAD-TOPO; HS, *H. salinarum aspS* in pBAD-TOPO plus tRNA^{Asn} in pTECH.
[b] One activity unit is the amount of enzyme producing 0.1 μmol of Trp in 20 min at 37°C per mg of protein (18).

crimination process. Future genetic experiments based on the *trpA34* system should further our knowledge of specificity in the process of protein biosynthesis.

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