Structure–function analysis of tRNA^{Gln} in an *Escherichia coli* knockout strain

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ABSTRACT

The diverse and highly specific interaction between RNAs and proteins plays an essential role in many important biological processes. In the glutamine aminoacylation system, crystal structures of the free and ligated macromolecules have provided a description of the tRNA–protein interactions at the molecular level. This data lays the foundation for genetic, biochemical, and structural analyses to delineate the set of key interactions that governs the structure–function relationships of the two macromolecules. To this end the chromosomal tRNA^{Gln} genes were disrupted in *Escherichia coli* to produce a tRNA^{Gln} knockout strain that depends upon expression of a functional tRNA^{Gln} from a plasmid for cell viability. Mutants of an inactive tester tRNA derived from tRNA^{Ala} were generated by hydroxylamine mutagenesis, and the active derivatives were selected by their ability to support knockout cell growth. Two of the mutants contained substitutions in the first base pair of the acceptor stem that likely facilitate the formation of a hairpin loop that places A76 in the active site. The third mutation was located at position 13 in the D loop region of the tRNA, and suggests that an interaction with residue 13 contributes to a specific conformational change in unliganded GlnRS, which helps configure the enzyme active site in its catalytically proficient form. This work demonstrates the efficacy of an integrated approach that combines genetic selections and biochemical analyses with the physical data from crystal structures to reveal molecular steps that control the specificity of RNA–protein interactions.

Keywords: Aminoacylation; in vivo RNA function; mutation; plasmid; RNA structure–function; X-ray structure

INTRODUCTION

RNA–protein recognition is an essential element of many fundamental cellular processes, yet the molecular mechanisms that control the specificity of these interactions are not well understood. Although in many cases crystal structures of RNA–protein complexes have provided atomic descriptions of the interactions, these structures do not differentiate between the key interactions that govern the structure–function relationships of the two macromolecules and the many interactions that do not. These functionally relevant interactions must be defined experimentally using genetic, biochemical, and structural analyses.

In the *Escherichia coli* glutamine aminoacylation system, 3D structures of glutamyl–tRNA synthetase (GlnRS) and the tRNA^{Gln}–GlnRS complex (Rould et al. 1989; Rath et al. 1998; Sherlin and Perona 2003) have provided an atomic description of the interacting surfaces, thereby laying the

foundation for further delineation of the structure–function relationship of the two macromolecules. It is known that GlnRS is a class I synthetase that functions as a monomer (Rould et al. 1989), and that tRNA^{Gln} is required for the formation of the aminoacyl-adenylate (Kern and Lapointe 1980). Comparison of the crystal structure of free *E. coli* GlnRS with the tRNA-bound complex suggests an induced fit mechanism in which substrate binding is required for the formation of the active site. In addition, the binding of tRNA results in structural changes in the enzyme, which properly position active site peptides that are crucial for the binding of glutamine and ATP (Sherlin and Perona 2003). Like many tRNAs, the acceptor stem of tRNA^{Gln} is important for specificity. The protein directly recognizes the bases at positions 2 and 3 at the end of the acceptor stem (Rould et al. 1991), while the 1–72 base pair of enzyme-bound tRNA^{Gln} is disrupted by residue Leu136 of GlnRS, which allows the 3' end of the acceptor arm to form a hairpin that positions the accepting A76 residue in proper proximity to the aminoacyl adenylate within the active site of GlnRS for aminoacylation.

We are investigating the nature of tRNA–protein interactions in the glutamine system using an integrated ap-

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proach that combines biochemical and genetic analyses with the physical data from crystal structures. Thus we have constructed an *E. coli* strain in which all the chromosomal tRNA^{Gln} genes have been inactivated. Cell viability of the resulting knockout strain depends upon an active tRNA^{Gln} expressed from a plasmid, which enabled us to readily analyze synthetic tRNA genes with precise mutations for functional activity in vivo. These studies cannot be carried out in the nonsense suppression system because the tRNA^{Gln} anticodon is important for accurate aminoacylation (Ibba et al. 1996) and suppression efficiency is an indirect assay of tRNA function (McClain 1993). Using the new knockout approach we have uncovered a critical contact that contributes to an induced fit between tRNA^{Gln} and GlnRS. Information from continued analyses of this kind, in combination with information from the crystal structures of tRNA^{Gln} and the tRNA^{Gln}–GlnRS complex, will generate a more comprehensive description of the aminoacylation process.

RESULTS

Construction and characterization of the *E. coli* tRNA^{Gln} knockout strain

Because tRNA^{Gln} is essential for cell viability, a plasmid that expresses a functional tRNA^{Gln} is required for construction and maintenance of the knockout strain. We constructed maintenance plasmids containing the genes for tRNA^{Gln}–UUG (gln1) and/or tRNA^{Gln}–CUG (gln2) isoacceptors (Fig. 1). Synthetic gln1 and gln2 genes were cloned separately into the medium copy, chloramphenicol resistant vector

pSAD (Gabriel and McClain 2001) to give pSADgln1 and pSADgln2. After sequence confirmation, the two tRNA genes were subcloned together to give pSADgln1,2. The tRNA^{Gln} genes in these maintenance plasmids are under the control of an *araCpBAD* promoter, which is induced by arabinose and repressed by glucose. The amount of tRNA^{Gln} produced from these plasmids in *E. coli* wild-type MG1655 when grown under inducing or repressing conditions was determined by Northern analysis (data not shown). When the pSAD tRNA genes were repressed, the amount of tRNA^{Gln} produced was similar to that of MG1655 cells, indicating that the tRNA^{Gln} was being produced only from the chromosomal genes. Under inducing conditions, however, the amount of tRNA^{Gln} more than doubled, reflecting the expression of tRNA^{Gln} from the pSAD plasmid. Northern probes specific to gln1 or gln2 indicated that both isoacceptors were produced from pSADgln1,2. Strain MG1655/pSADgln1,2 was subsequently used to construct the tRNA^{Gln} knockout strain.

The tRNA^{Gln} knockout strain was constructed by replacement of the chromosomal tRNA^{Gln} genes with a DNA fragment in which the tRNA^{Gln} genes were deleted. The four *E. coli* tRNA^{Gln} genes (*glnX*, *glnV*, *glnW*, and *glnU*; Fig. 2) are located in a gene cluster that also contains the tRNA genes *metU*, *leuW* and *metT* (Komine et al. 1990). To avoid disrupting the latter three tRNA genes, we used PCR to construct a replacement DNA fragment in which an *NcoI* restriction site replaced the *glnX* and *glnV* genes, a *XhoI* site replaced the *glnW* and *glnU* genes, and the *metU*, *leuW*, and *metT* genes were retained (Fig. 2). This mutated DNA fragment was ligated into the ampicillin resistant, temperature sensitive plasmid pBAT (Gabriel and McClain 1999) to give the gene replacement vector pBATglnΔXVWU.

FIGURE 1. Cloverleaf representations of *E. coli* tRNA^{Gln}–UUG (gln1), tRNA^{Gln}–CUG (gln2), phage T4 tRNA^{Gln}, phage T5 tRNA^{Gln}, and the tester tRNA^{Ala}. Arrows indicate base changes incorporated into the phage tRNAs (see text) and mutations obtained in the tester tRNA^{Ala} gene that confer a gain of growth phenotype on glnΔ114 cells. The ' symbol indicates an alignment gap. Northern probes for tRNAs were complementary to base positions 29–43.

FIGURE 2. Diagram of wild-type MG1655 chromosomal tRNA genes (top), logic for deleting tRNA^{Gln} genes (middle) and plasmid construct pBATglnΔXVWU (bottom). Genetic loci are not to scale.

Because pBAT replicates poorly at 42°C, transformed cells in which the plasmid is integrated into the chromosome possess a growth advantage when grown at high temperatures with ampicillin selection. We therefore transformed pBATglnΔXVWU into MG1655/pSADgln1,2 and selected colonies that grew at 42°C in the presence of am-

picillin. The diploid colonies were selected on minimal medium plates containing arabinose so that wild-type tRNA^{Gln}-UUG and tRNA^{Gln}-CUG would be induced from maintenance plasmid pSADgln1,2. The ampicillin resistant colonies were subjected to several rounds of growth at 30°C to allow resolution of the diploid and then were grown at 42°C for a few hours to remove any remaining cytoplasmic pBAT plasmid. Approximately half of the resolved, ampicillin sensitive colonies were unable to grow on minimal medium with glucose as the carbon source, indicating that cell viability depended on tRNA^{Gln} from pSADgln1,2.

The deletion of the tRNA^{Gln} genes was confirmed by PCR analysis of the parental strain (MG1655/pSADgln1,2), the diploid (glnΔ1/pSADgln1,2), and the knockout strain (glnΔ114/pSADgln1,2). Primers *prm1* and *prm4* (see Fig. 2) generated a 1878-kb fragment from the wild-type parental strain, while a 1477-kb fragment was generated from the glnΔ114 knockout strain, reflecting the deletion of the four chromosomal tRNA^{Gln} genes and their intervening sequences. The diploid strain generated two PCR fragments: one from the wild-type portion of the chromosome, and the other from the inserted pBATglnΔXVWU replacement plasmid. Restriction analysis of the PCR products demonstrated that the NcoI and XhoI sites, which replaced the *gln* genes, are present in the diploid and knockout strain but not in the wild-type parent (Fig. 3). The 1878-bp fragment is present in lower amounts (most notably in the diploid strain) because Taq I polymerase used in the PCR reaction preferentially amplifies smaller fragments.

Streak plates of the parental strain, the diploid derivative, and two representative knockout strains (glnΔ114/pSADgln1,2 and glnΔ141/pSADgln1,2; Fig. 4) show that all four strains grow well under the permissive condition where arabinose induces tRNA^{Gln} from the maintenance plasmid pSADgln1,2. However, the two knockout strains did not grow on rich LB medium or minimal medium with glucose where expression of tRNA from the maintenance plasmid is repressed (McClain and Gabriel 2001). The growth dependence on

FIGURE 3. PCR amplification using primers prm1 and prm4 (see Fig. 2, top) of glnΔ114/pSADgln1,2 and glnΔ1/pSADgln1,2 and MG1655/pSADgln1,2. The PCR products were digested with EcoRI and BamHI (EB), NcoI (N), or XhoI (X), and run on a 1.5% agarose gel.

plasmid-produced tRNA^{Gln} means that no functional tRNA^{Gln} gene capable of sustaining knockout cell growth remains on the modified MG1655 chromosome.

Analysis of tRNA^{Gln} genes for ability to support glnΔ114 knockout cells

We analyzed various tRNA^{Gln} genes for their ability to support growth of the glnΔ114 knockout strain. The genes for gln1 and gln2 were cloned separately between the constitutive lipoprotein promoter and *rrnC* terminator of the high copy, ampicillin-resistant plasmid pGFIB to give pGFIBgln1 and pGFIBgln2. The gln1 gene was also cloned into pGAD2 to give pGAD2gln1. The pGAD2 plasmid (Gabriel and McClain 2001) is a high-copy, ampicillin-resistant vector containing the same *araCpBAD* promoter and *rrnC* terminator as pSAD. In addition, synthetic genes for bacteriophage T4 tRNA^{Gln} and T5 tRNA^{Gln} (Fig. 1) were cloned into pGFIB to give pGFIBT4glnU38 and pGFIBT5glnU32U38A72. Mutations were introduced into the T4 and T5 tRNA^{Gln} genes at positions 32, 38, and 72, because the crystal structure of the *E. coli* tRNA^{Gln}–GlnRS complex (Rould et al. 1991) indicates that the bases at these positions may play a role in tRNA interactions with the synthetase. Therefore, to improve the possibility that T4 and T5 tRNA^{Gln} would support knockout cell growth, we substituted the *E. coli* wild-type tRNA^{Gln} bases at those positions, although we have not determined if mutations at these positions affect functional activity in knockout cells.

Plasmid bearing the tRNA^{Gln} gene to be tested was in-

roduced into glnΔ114/pSADgln1,2 by electroporation, and the electroporated cells were grown on medium selective for the test plasmid. All cultures grew except those receiving pGFIBgln2 or pGFIBT5glnU32U38A72 indicating that the gln2 and T5glnU32U38A72 tRNA are unable to support knockout strain growth. Although the basis of the functional impairment of T5 tRNA^{Gln} is not known, the impairment of *E. coli* tRNA^{Gln}–CUG (gln2) may result from the unfavorable CxA mispair between the C of the anticodon with the 3' A of CAA glutamine codons (see Fig. 1). Cultures containing a functionally active tRNA^{Gln} gene were streaked on agar plates containing the appropriate medium and grown overnight. Plasmid switches of the test plasmid for the maintenance plasmid were carried out by growth in liquid medium that is selective for the test plasmid. In some cases, several rounds of growth in selective medium were needed to remove the initial maintenance plasmid. Plasmid switches were confirmed by sequencing the tRNA gene and its promoter using a primer located about 40 nucleotides to the 3' side of the tRNA gene terminator.

Streak plates (Fig. 5) show the growth phenotypes of strain glnΔ114 with plasmid pGFIBglnT4U38, pGFIBgln1, pGAD2gln1, pSADgln1, or pSADgln1,2, as well as the parental strain MG1655/pSADgln1,2. All the knockout cells grew on minimal medium plates with arabinose and casamino acids except those containing pGFIBglnT4U38. Growth inability of the latter construct may result from

FIGURE 4. Streak plates of the parent strain (MG1655/pSADgln1,2), the diploid derivative (glnΔ1/pSADgln1,2), and two representative knockout strains (glnΔ114/pSADgln1,2 and glnΔ141/pSADgln1,2) on the indicated media. The cultures were incubated overnight at 37°C. The key at the bottom right identifies the strains.

FIGURE 5. Streak plates of the parent strain (MG1655/pSADgln1,2) and the *glnΔ114* knockout strain containing plasmid pGFIBglnT4U38, pGFIBgln1, pGAD2gln1, pSADgln1, or pSADgln1,2. The cultures were incubated overnight at 37°C, and antibiotics were omitted due to the variable sensitivities of the strains. The key at the bottom right identifies the strains.

reduced function of tRNA^{Gln}-T4U38 and/or the poorer growth of strains containing the plasmid pGFIB in minimal media (McClain and Gabriel 2001). As expected, all knockout strains containing pSAD or pGAD2 maintenance vectors were not viable on minimal media with glucose or rich media, confirming the absence of functional chromosomal tRNA^{Gln}. The parental MG1655 grew on both media.

A Northern analysis (Fig. 6; Table 1) was performed to examine tRNA^{Gln} levels in the parent strain MG1655/pSADgln1,2 and the *glnΔ114* knockout strain containing plasmid pSADgln1,2 or plasmid pGFIBgln1 or plasmid pGFIBglnT4U38. The samples were hybridized separately to probes for tRNA^{Gln}-CUG, tRNA^{Gln}-T4U38, and tRNA^{Gln}-UUG. A probe for 5S RNA was included in all hybridization reactions to allow relative quantitation of the tRNA^{Gln} species. MG1655 samples and *glnΔ114*/pSADgln1,2 samples hybridized to tRNA^{Gln}-UUG and tRNA^{Gln}-CUG probes but not to the tRNA^{Gln}-T4U38 probe. *GlnΔ114*/pGFIBgln1 samples hybridized only to the tRNA^{Gln}-UUG probe, while *glnΔ114*/pGFIBglnT4U38 samples hybridized only to the tRNA^{Gln}-T4U38 probe. The results with *glnΔ114*/pGFIBglnT4U38 demonstrate that no expressed *E. coli* tRNA^{Gln} gene remains on the modified MG1655 chromosome (Fig. 6; Table 1, lanes 5,15). Moreover, the tRNA^{Gln} originating from the plasmid is in excess relative to chromosomal tRNA^{Gln}. For example, MG1655/pSADgln1,2 grown in minimal arabinose medium contained about four

times the amount of tRNA^{Gln}-CUG (Table 1, lanes 1,2) and seven times the amount of tRNA^{Gln}-UUG (Table 1, lanes 11,12) compared to the strain grown with glucose.

Selection of active tRNA^{Gln} mutants in *glnΔ114* knockout cells

The *E. coli* tRNA^{Gln} knockout strain was used to select derivatives of an inactive tRNA having compensating mutations that permit aminoacylation by GlnRS. The inactive tester tRNA was derived from alanine tRNA by modification of two regions: G3-U70 in the acceptor stem was mutated to G3-C70 to remove the alanyl-tRNA synthetase recognition site (Hou and Schimmel 1988; McClain and Foss 1988), and the anticodon was changed to UUG of tRNA^{Gln}. The tester tRNA^{Ala} gene was constructed by PCR and ligated into plasmid pGFIB. *GlnΔ114* knockout cell containing this plasmid were unable to grow after 72 h of incubation in LB medium, indicating that the tRNA is inactive in knockout cells. In addition, the tester tRNA^{Ala} gene was cloned with the *glnS* gene in plasmid pGFIB to give pGTRQ. Expression of wild-type tRNA^{Gln} and *glnS* genes from plasmid pGTRQ was tested through Northern and Western gel analyses, respectively. The tRNA^{Gln} gene showed a fivefold overproduction over chromosomal expression of isoacceptor tRNA^{Gln1} (data not shown). Likewise, GlnRS showed an increase in expression of 5.6-fold over chromosomal expression of *glnS* (Fig. 7). The DNA sequence of tRNA^{Gln} and GlnRS genes was confirmed in pGTRQ.

Hydroxylamine mutagenesis was performed directly on the pGTRQ plasmid with hydroxylamine (Tessman 1968). The mutated plasmid was then transformed into *glnΔ114* knockout cells and plated onto LB plus ampicillin plates. Three active tRNA mutants were obtained from this mutagenesis method: two changed the first base pair of the acceptor stem of the tester tRNA from G1-C72 to A1-C72 (HAX7) or G1-U72 (HAX3); the third mutation changed the base pair next to the D loop from C13-G22 to U13-G22 (HAX15; Fig. 1). The three mutants give medium size colonies after 20 h of growth (Fig. 8). No mutations were found in the *glnS* gene that was coexpressed on the pGTRQ plas-

FIGURE 6. Northern analysis of tRNA^{Gln} in the parent strain MG1655/pSADgln1,2 and the *glnΔ114* knockout strain containing plasmid pSADgln1,2, plasmid pGFIBgln1 or plasmid pGFIBglnT4U38. Membranes were hybridized with probes for the indicated tRNA^{Gln} species and 5S RNA as an internal standard. Lanes 1–15 of Table 1 report the strain/plasmid combinations, growth media, and quantitative analysis of tRNA^{Gln}.

TABLE 1. Levels of tRNA^{Gln} isoacceptors calculated from Figure 5

Lane	Strain	Plasmid	Medium	Probed tRNA ^{Gln} /5S RNA
Probe used: <i>CUGprb</i>				
1	MG1655	pSADgln1,2	MinGG+aa+cam	(1.00)
2	MG1655	pSADgln1,2	MinGA+aa+cam	3.96
3	<i>glnΔ114</i>	pSADgln1,2	MinGA+aa+cam	5.50
4	<i>glnΔ114</i>	pGFIBgln1	LB+amp	0.05
5	<i>glnΔ114</i>	pGFIBT4U38	LB+amp	0.02
Probe used: <i>T4U38prb</i>				
6	MG1655	pSADgln1,2	MinGG+aa+cam	0.02
7	MG1655	pSADgln1,2	MinGA+aa+cam	0.03
8	<i>glnΔ114</i>	pSADgln1,2	MinGA+aa+cam	0.03
9	<i>glnΔ114</i>	pGFIBgln1	LB+amp	0.03
10	<i>glnΔ114</i>	pGFIBT4U38	LB+amp	(1.00)
Probe used: <i>UUGprb</i>				
11	MG1655	pSADgln1,2	MinGG+aa+cam	(1.00)
12	MG1655	pSADgln1,2	MinGA+aa+cam	7.30
13	<i>glnΔ114</i>	pSADgln1,2	MinGA+aa+cam	10.94
14	<i>glnΔ114</i>	pGFIBgln1	LB+amp	12.73
15	<i>glnΔ114</i>	pGFIBT4U38	LB+amp	0.20

The ratio on the right shows the amount of tRNA^{Gln} standardized to 5S RNA in each sample relative to the indicated reference in lane 1 [sample tRNA^{Gln}/sample 5S RNA]/(reference tRNA^{Gln}/reference 5S RNA)]. No background correction was performed.

mids. The mutant tRNA genes were subcloned into pGFIB to determine if the growth phenotype conferred by the mutations is dependent on overexpression of the *glnS* gene. No change in the growth phenotype was observed indicating that the overexpression of *glnS* from the pGTRQ plasmid had no effect. The cellular steady-state level of glutaminyl-tRNA in the mutants was determined by acid gel analysis, which showed that the mutant tRNAs were charged to similar extents as wild-type tRNA^{Gln} (Fig. 9). This analysis also revealed that the amount of the HAX7 tRNA is reduced relative to the others, indicating that mutant tRNA amounts exceed that required for cell growth. The glutamine charging specificity must approach 100% for each mutant because the tRNA inserts its amino acid into glutamine codons in global proteins.

DISCUSSION

Using the *glnΔ114* knockout strain, we have begun to probe the glutamine aminoacylation system with an inactive tester

tRNA derived from alanine tRNA. Two of the active derivatives of the tester tRNA (HAX3 and HAX7) contained compensating mutations in the first base pair of the acceptor stem. Analogous mutations in other tRNAs have been described previously as facilitating 3' hairpin loop forma-

FIGURE 8. Streak plates of the *glnΔ114* knockout strain expressing wild-type or mutant tRNA from plasmid pGTRQ (A) or plasmid pGFIB (B). The cultures were grown on LB plus ampicillin medium for 20 h (A) or 25 h (B) at 37°C.

FIGURE 7. Western blot analysis of GlnRS present in the indicated dilutions of protein samples prepared from XAC/A16 cells expressing the plasmids pGFIB tRNA^{Gln} or pGTRQ tRNA^{Gln}. The position of purified GlnRS is noted.

FIGURE 9. Steady-state aminoacylation levels of wild-type and mutant tRNAs expressed from plasmid pGTRQ in the *glnΔ114* knockout strain.

tion, which places A76 in the active site (Rould et al. 1989). The mutations in HAX3 and HAX 7 break or somehow modulate the strong terminal G-C base pair of the inactive tester tRNA^{Ala}, presumably allowing GlnRS to bend the acceptor arm, at a lower free energy cost, into a position suitable for attachment of glutamine to the terminal adenosine of the tRNA. In wild-type *E. coli* tRNA^{Gln}, the weaker U-A base pair occurs at the terminal position of the acceptor stem. The concurrence of these results with previous crystallographic work validates our new genetic system.

The third mutation at residue 13 gives new structure–function information. Specifically, it reveals that a tRNA backbone interaction with residue 13 (the base is not contacted) is associated with a specific conformational change in GlnRS that helps configure the active site in its catalytically proficient form. Structural coupling between the complex core of the tRNA including residue 13 and the enzyme active site has been proposed (Nissan and Perona 2000). The backbone of residue 13 of tRNA^{Gln} interacts with Thr316 (2.7 Å), and also within 3.5 Å of Ile313 and Gln318, which are all part of the turn-helix motif that acts as a connector between anticodon binding and active site domains of GlnRS. The loop formed by residues 313 to 323 is disordered in the crystal structure of uncomplexed GlnRS, but is packed against the active site of GlnRS in the crystal structure of the tRNA^{Gln}–GlnRS complex (Fig. 10; Sherlin and Perona 2003). In fact, the active site region that the loop packs against contains Glu257, which—along with Asp219—is thought to order a water molecule (Wat1050) involved in recognizing one of the amide hydrogens of glutamine (Rath et al. 1998). It is less obvious, however, how a change from one pyrimidine (cytosine) to another (uridine) could cause the tRNA to be aminoacylated, because the wild-type base of tRNA^{Gln} at position 13 is an adenosine

(Fig. 1), and the tRNA–protein interaction at this position is between the tRNA backbone and GlnRS.

The inactive tester tRNA has a C-G Watson-Crick pair at position 13–22, while the wild-type sequence of tRNA^{Gln} has an AxA mispair, which reduces the number of Watson-Crick base pairs on the D stem and extends the D loop. The U13-G22 mutant may gain activity in two nonexclusive ways. Perhaps the U-G mispair at position 13–22 in HAX15 extends the D-loop to include residue 13 (and 22) of the tRNA so that it is more like the D-loop of wild-type tRNA^{Gln}. Also, due to the special geometry of the U-G pair (Varani and McClain 2000), U13 acquires the orientation close to that of A13 in wild-type tRNA^{Gln}, which would facilitate the standard interaction of its backbone with the nearby region of the protein. In interacting with the synthetase, the modified D region pushes the flexible loop formed by GlnRS residues 313–323 against the active site of GlnRS, thus structuring the active site pocket for formation of the aminoacyl-adenylate. The activation of the inactive tester tRNA in this way suggests that recognition of this region is accomplished through an indirect readout. The indirect readout refers not only to recognition of the backbone of residue 13 of the tRNA, but also to the consequential formation of a catalytically proficient site in the enzyme for specifically binding glutamine. An alternative interpretation for the increased activity resulting from the mutation at residue 13 is that the tRNA binds GlnRS more tightly. Determining the basis of increased

FIGURE 10. Comparison of uncomplexed GlnRS (A) and tRNA^{Gln}–GlnRS complex (B) structures (Rath et al. 1998; Sherlin and Perona 2003). The loop formed by residues 313–323 (colored green) of GlnRS packs against the active site (colored red) of the tRNA^{Gln}–GlnRS complex, but is disordered in the uncomplexed GlnRS structure. The aminoacyl-adenylate (colored purple) is absent in the uncomplexed structure because it was not present during the crystallization. The tRNA of the tRNA^{Gln}–GlnRS complex is shown as an orange backbone with the sphere designating the backbone phosphate of residue 13, while the aminoacyl-adenylate is shown in wire frame. The packing of the 313–323 residue loop against the active site in the tRNA^{Gln}–GlnRS complex is thought to facilitate assembly of the GlnRS active site.

mutant tRNA activity is an interesting area for future research.

The number of active mutants obtained by the hydroxylamine mutagenesis in the initial screen and the fact that no repeats were found suggest that the set of mutations in the inactive tester tRNA^{Ala} that can confer a growth phenotype upon *gln*Δ114 knockout cells has not been saturated. Because the pGTRQ plasmid contains both the tester tRNA gene and the *glnS* gene, mutagenesis performed on this plasmid allows selection of compensating mutation in the tRNA or the synthetase. In these initial experiments, no mutations were found in the *glnS* gene, which may suggest that either one or a few specific mutations or multiple mutations are needed in GlnRS to compensate for the inactive tester tRNA. Also, the one-dimensional nature of hydroxylamine, which only changes G-C pairs to A-T, may have contributed to the limited number of mutants obtained. Future experiments will focus on finding more compensating mutations in both the tester tRNA and GlnRS to further dissect the structure–function relationship in the glutamine system.

MATERIALS AND METHODS

Strains and plasmids

The genome of wild-type *E. coli* strain MG1655 has been sequenced (Blattner et al. 1997). Plasmids pSAD, pGAD2 (Gabriel and McClain 2001), and pGFIB (Masson and Miller 1986) have been described. Synthetic tRNA genes were inserted into the vectors and/or moved between vectors using 5' EcoRI and 3' PstI restriction sites located between promoter and terminator sequences, respectively. Plasmid pBAT–AESH is a derivative of pBATS (Gabriel and McClain 1999) and was constructed by inserting a linker containing restriction sites for AatII–EcoRI–SmaI–HindIII between resident AatII and HindIII sites. Liquid minimal medium MinA and MinA plates were supplemented with MgSO₄ (1 mM), B1 (4 μg mL^{−1}) and glycerol (0.2% (v/v) and designated MinG. When added, casamino acids (aa), arabinose (A), and glucose (G) concentrations were each 0.2% (w/v). LB broth and plates were used as rich medium. Ampicillin (amp) and chloramphenicol (cam) concentrations were 100 μg mL^{−1} and 20 μg mL^{−1}, respectively. The SOC broth used for electroporation contained added MgCl₂ (100 mM), MgSO₄ (100 mM) and KCl (25 mM).

Replacement vector pBATglnΔXVWU was constructed as follows (see Fig. 2). Using Taq I polymerase, two PCR fragments were synthesized from *E. coli* strain MG1655: one 5' of *glnX* (using prm1: 5'-TATAGAATTCTGGGCATCGCTGGTGTGG-3' and prm2: 5'-AGCCATGGTAAAAAGCTCGCTTC-3') and the other 3' of *glnV* (using prm3: 5'-CCATGGATATTCGATACATTTCAG AATTTG-3' and prm4: 5'-TATTGGATCCTTCCGTCGCTTGT TCTTC-3'). The PCR fragment from prm1 and prm2 contained a 5' EcoRI site and a 3' NcoI site, while that from prm3 and prm4 contained 5' NcoI and 3' BamHI sites. The fragments were ligated together and inserted into EcoRI–BamHI digested pBAT–AESH plasmid to make pBATglnΔXV. Next, two PCR fragments, one 5' of *glnW* (from prm1 and prm5: 5'-GTACCTCGAGCATCGAAG

AAACAATCT-3') and the other 3' of *glnU* (from prm6: 5'-TACTCGAGATCCGTACAACGCTTTCT-3' and prm4), were synthesized from plasmid pBATglnΔXV. The PCR fragment from prm1 and prm5 contained a 5' EcoRI site and a 3' XhoI site, while the fragment from prm6 and prm4 contained 5' XhoI and 3' BamHI sites. The fragments were ligated into EcoRI–BamHI digested pBAT–AESH to construct pBATglnΔXVWU. We used sequence primer glnXVWUprm (5'-ATTGAATGAACGCAGAA-3') to confirm that the four tRNA^{Gln} genes had been deleted.

The pGTRQ plasmid is a derivative of pGFIB and was constructed as follows. The *glnS* gene from *E. coli* strain MG1655 was synthesized by PCR using the primers 5'-TACGTATCTAGAAC GCTTTGAGGAATCCAC-3' and 5'-GTAAACGCATGCTCCGGC ATAGCGAAACTT-3'. The PCR product was cloned as an SphI–XbaI DNA fragment into the same sites located 3' of the tRNA^{Gln}–UUG gene in plasmid pSAD to give pSTRQ (W.H. McClain and D. Lee, unpubl.). The pGTRQ plasmid was constructed by subcloning both the tRNA^{Gln}–UUG gene and the *glnS* gene as an EcoRI–HindIII DNA fragment from plasmid pSTRQ into the same sites of the pGFIB vector.

Knockout strain construction

pBATglnΔXVWU was transformed into strain MG1655/pSADgln1,2 and incubated at 37°C overnight on LB plates with both ampicillin and chloramphenicol. Five transformed colonies were picked into MinGAaa minimal medium (MinG + 0.2% arabinose + 0.2% casamino acids) + amp + cam and grown overnight at 37°C. The cultures were then either streaked or spread on MinGAaa + amp + cam plates and grown for 24 h at 42°C to obtain diploid cells that had integrated the pBATglnΔXVWU plasmid into the chromosome. Several medium-large colonies were restreaked on MinGAaa + amp + cam plates and incubated overnight at 37°C. A single colony from each of five independent cultures was again grown overnight at 37°C in the MinGAaa + amp + cam liquid medium, and stocks were made of these diploid cells. To resolve the diploid cells, an aliquot of culture was added to MinGAaa plus chloramphenicol liquid medium, and the culture was grown at 30°C to saturation. This process was repeated four times. The cultures were diluted again and grown for 5 h at 37°C to remove by nonreplication the resolved, temperature-sensitive pBAT plasmid. The cultures were diluted and plated on MinGAaa + cam overnight at 37°C. Ampicillin-sensitive colonies were identified either by picking and stabbing or by replica plating onto MinGAaa plates with ampicillin. Ampicillin-sensitive colonies were picked and stabbed onto MinGAaa + cam, MinGGaa + cam (0.2% glucose to repress tRNA from pSADgln1,2) and MinGAaa + amp + cam. Four of the five cultures contained resolved colonies, approximately half of which had undergone a gene replacement as indicated by lack of growth on minimal medium glucose plates where tRNA^{Gln} from the pSAD maintenance plasmid was repressed.

Plasmid switches

To prepare cells for a plasmid switch, an overnight culture was pelleted, washed three times in an equal volume of ice-cold sterile H₂O, and resuspended in 1/20 volume H₂O. Because electroporated cells lose their resident plasmids, 20 μL of the cells were

electroporated without added plasmid at 2.5 kV (GIBCO BRL E. coli Pulser) and allowed to recover for 20 min in 1 mL SOC broth at 37°C. The cells were pelleted and made electrocompetent again. A replacement plasmid was then electroporated into the cells under the same conditions described above. When the replacement plasmid was pGFIB, the cells were grown in SOC broth for 1 h at 37°C then subcultured into LB plus the appropriate antibiotic. When the replacement plasmid was pSAD or pGAD, the cells were grown in MinGA for 1 h then subcultured in the same media with the appropriate antibiotic. Cells were grown overnight at 37°C and plated on the same media. Colonies that lost the original plasmid were found by picking and stabbing, or by replica plating, onto plates with different antibiotics or carbon sources. Ampicillin sensitivity and/or inability to grow on LB plates indicated loss of pGFIB, while chloramphenicol sensitivity indicated loss of pSAD. Presence of the new plasmid and the loss of the original plasmid was indicated by antibiotic resistance and growth properties, and was confirmed by sequencing using a primer effective for both plasmids and located about 40 nt 3' of the tRNA terminator. Loss or gain of a tRNA^{Gln} isoacceptor was confirmed by Northern analysis.

Northern blot analysis

Knockout cells (glnΔ114) were grown to A₅₅₀ = 0.5 in LB + amp when containing pGFIB, or MinGAaa + cam when containing pSAD plasmid. MG1655 cells also were grown in minimal medium containing glucose as the carbon source. Cells (1.5 mL) were pelleted, resuspended in 0.3 M sodium acetate (pH 5) and extracted with 100 μL of acid phenol (GIBCO BRL). The aqueous phase was precipitated with ethanol, washed in 70% ethanol, dried, and resuspended in 15 μL TE. Three aliquots (2 μL) from each sample were mixed with 1 μL formamide, heated to 65°C for 5 min, and run on a 10% polyacrylamide, 7 M urea, TBE minigel at room temperature, and the samples were electroblotted at 20 V for 30 min in 1 × TAE onto a Nytran SuperCharge membrane. The membrane was divided into three sections, and each section was hybridized separately to probes for tRNA^{Gln}-CUG or tRNA^{Gln}-T4U38 or tRNA^{Gln}-UUG. A probe for 5S RNA was included in all hybridization reactions to allow relative quantitation of the tRNA^{Gln} species. Membranes were hybridized overnight in Sigma PerfectHybTM Plus buffer to 30 pmole of (5'-³²P)-labeled 5S RNA probe (5'-TTCTGAGTTCGGCATGGGGT-3') and similarly labeled gln1 probe (5'-CCGGTATCAAAAACC-3') or gln2 probe (5'-CCGGAATCAGAATCC-3') or glnT4U38 probe (5'-TAGCAATCAAAATGC-3'). Quantitations were determined using a Molecular Dynamics Storm 860 instrument and ImageQuant 4.2a software. A background correction was not made.

Western blot analysis

Isolation of whole-cell protein was performed as described (McClain et al. 1999). Various dilutions of the protein samples were run on a 9% polyacrylamide SDS gel, and electroblotted onto Hybond-P membrane (Amersham Biosciences). The membrane was blocked with 3% BSA (FractionV), PBS, and Tween20. After incubation with a 1:100 dilution of rabbit polyclonal antibody to the glutaminyl-tRNA synthetase (obtained from the Research Animal Resources Center of the University of Wisconsin Medical

School), the membrane was then incubated with antirabbit antibody, and an AP conjugate (Amersham Biosciences). The membrane was developed through Electro-chemiluminescence by exposing the AP conjugate to ECL substrate. The membrane was quantified using a Molecular Dynamics Storm 860 instrument and ImageQuant 4.2a software.

Hydroxylamine mutagenesis

Plasmid DNA (10 ng) containing the mutagenesis target genes was combined with 300 μL phosphate buffer (0.2 M NaH₂PO₄·H₂O, 0.2 M Na₂HPO₄·3H₂O, pH6, 1 mM EDTA), and 200 μL of 1 M hydroxylamine. The solution was incubated at 50°C for 2 h. The plasmid DNA was then isolated using a microcon30 microconcentrator kit (Micon) and was resuspended in 40 μL of TE. The mutated plasmid was then electroporated into glnΔ114 knockout cells. The electroporated cells were plated on LB plus ampicillin and were incubated at 37°C for 48 h.

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REFERENCES

- Blattner, F.R., Plunkett III, G., Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., et al. 1997. The complete genome sequence of *Escherichia coli* K-12. *Science* **277**: 1453–1462.
- Gabriel, K. and McClain, W.H. 1999. A set of plasmids constitutively producing different RNA levels in *Escherichia coli*. *J. Mol. Biol.* **290**: 385–390.
- . 2001. Plasmid systems to study RNA function in *Escherichia coli*. *J. Mol. Biol.* **310**: 543–548.
- Hou, Y.-M. and Schimmel, P. 1988. A simple structural feature is a major determinant of the identity of a transfer RNA. *Nature* **333**: 140–145.
- Ibba, M., Hong, K.-W., Sherman, J.M., and Söll, D. 1996. Interactions between tRNA identity nucleotides and their recognition sites in glutaminyl-tRNA synthetase determine the cognate amino acid affinity of the enzyme. *Proc. Nat. Acad. Sci.* **93**: 6953–6958.
- Kern, D. and Lapointe, J. 1980. Catalytic mechanism of glutamyl-tRNA synthetase from *Escherichia coli*. Reaction pathway in the aminoacylation of tRNA^{Glu}. *Biochemistry* **19**: 3060–3068.
- Komine, Y., Adachi, T., Inokuchi, H., and Ozeki, H. 1990. Genomic organization and physical mapping of the transfer RNA genes in *Escherichia coli* K12. *J. Mol. Biol.* **212**: 579–598.
- Masson, J.-M. and Miller, J.H. 1986. Expression of synthetic suppressor tRNA genes under the control of a synthetic promoter. *Gene* **47**: 179–183.
- McClain, W.H. 1993. Rules that govern tRNA identity in protein synthesis. *J. Mol. Biol.* **234**: 257–280.

- McClain, W.H. and Foss, K. 1988. Changing the identity of a tRNA by introducing a G-U wobble pair near the 3' acceptor end. *Science* **240**: 793–796.
- McClain, W.H. and Gabriel, K. 2001. Construction of an *Escherichia coli* knockout strain for functional analysis of tRNA^{Asp}. *J. Mol. Biol.* **310**: 537–542.
- McClain, W.H., Gabriel, K., Bhattacharya, S., Jou, Y.-Y., and Schneider, J. 1999. Functional compensation by particular nucleotide substitutions of a critical G-U wobble base-pair during aminoacylation of a transfer RNA. *J. Mol. Biol.* **286**: 1025–1032.
- Nissan, T.A. and Perona, J.J. 2000. Alternative designs for construction of the class II transfer RNA tertiary core. *RNA* **6**: 1585–1596.
- Rath, V.L., Silvanm L.F., Beijer, B., Sproat, B.S., and Steitz, T.A. 1998. How glutaminyl-tRNA synthetase selects glutamine. *Structure* **6**: 439–449.
- Rould, M.A., Perona, J.J., Söll, D. and Steitz, T.A. 1989. Structure of *E. coli* glutaminyl-tRNA synthetase complexed with tRNA^{Gln} and ATP at 2.8Å resolution. *Science* **246**: 1135–1142.
- Rould, M.A., Perona, J.J., and Steitz, T.A. 1991. Structural basis of anticodon loop recognition by glutaminyl-tRNA synthetase. *Nature* **352**: 213–218.
- Sherlin, L.D. and Perona, J.J. 2003. tRNA-dependent active site assembly in a class I aminoacyl-tRNA synthetase. *Structure* **11**: 591–603.
- Tessman, I. 1968. Mutagenic treatment of double- and single-stranded DNA phages T4 and S13 with hydroxylamine. *Virology* **35**: 330–333.
- Varani, G., and McClain, W.H. 2000. The G-U Wobble base pair: A fundamental building block of RNA structure crucial to RNA function in diverse biological systems. *EMBO Rep.* **1**: 18–23.

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