

Cloning of the Glutamyl-tRNA Synthetase (*gltX*) Gene from *Pseudomonas aeruginosa*

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The glutamyl-tRNA synthetase (*gltX*) gene from *Pseudomonas aeruginosa* was identified. A plasmid containing a 2.3-kb insert complemented the temperature-sensitive *gltX* mutation of *Escherichia coli* JP1449, and GltX activity was demonstrated. The inferred amino acid sequence of this gene showed 50.6% identity with GltX from *Rhizobium meliloti*.

The nature of the genetic code is defined by the combined action of the 20 aminoacyl-tRNA synthetases of the cell. The specific interaction of these enzymes with their cognate tRNA and amino acid substrates is essential for proper translation of the nucleic acid sequence to proteins. Currently, several hundred tRNA synthetase sequences have been reported from archaeobacteria, eubacteria, mitochondria, chloroplasts, and eukaryotic cells. Initial sequence alignments revealed that these enzymes may be divided into two distinct structural classes (8), each containing 10 enzymes. The class I enzymes are distinguishable by the presence of the amino-terminal amino acid motifs HIGH and KMSKS (23). The crystal structures of the aminoacyl-tRNA synthetases for class I synthetases, such as glutamine from *Escherichia coli* (17) and glutamate from *Thermus thermophilus* (15), revealed that these sequences correlate with the presence of the classic α/β nucleotide binding domain termed the Rossmann fold. In contrast, the catalytic domain of class II enzymes, as exemplified by the crystal structure for the seryl-tRNA synthetase from *E. coli* (4), is composed of an antiparallel β pleated sheet. These enzymes may also be classified mechanistically; class I enzymes aminoacylate their cognate tRNAs at the 3' hydroxyl, whereas class II enzymes typically aminoacylate the tRNA at the 2' hydroxyl (8).

The *Pseudomonas aeruginosa* *gltX* gene, encoding glutamyl-tRNA synthetase, was recognized during sequence analysis of the cloned DNA in plasmid pLPS3, which contains genes for the synthesis of the lipopolysaccharide O antigen from the serogroup O11 *P. aeruginosa* strain PA103 (9). The *gltX* gene was found approximately 7.8 kb downstream of and transcribed in the same direction as genes of the lipopolysaccharide locus. Plasmid pLPS3 was able to complement the temperature-sensitive *gltX351* mutation in *E. coli* JP1449 (18). Further subcloning (Fig. 1A), by standard, previously described techniques (3), localized the *gltX* gene to a 2.3-kb *SalI* fragment on the recombinant plasmid pSal6F. In this construct, the *gltX* gene was in the same orientation as the plasmid-encoded *lac* promoter. A similar construct with the plasmid-borne promoter in the opposite orientation was also able to complement JP1449, suggesting that the insert DNA has its own promoter that is recognized in *E. coli*.

Nucleotide sequence determination of the 3,892-bp insert from pGltX was performed by standard techniques, as previously described (5), and compared to the *P. aeruginosa* codon usage table (2). Analysis of this region revealed a 1,482-bp open reading frame (ORF) potentially encoding a 494-amino acid protein with a predicted molecular mass of 56,777 daltons. This protein has 50.6% identity with GltX from *Rhizobium meliloti* and contains all of the class I motifs in the catalytic domain (Fig. 2). The overall G+C content of the insert, 64.3%, is typical for genes from this organism. The codon usage of *gltX* conforms to that of other well-expressed proteins from *P. aeruginosa*. A putative ORF was also found in the same position on the opposite strand from GltX. This predicted protein did not show significant similarity to any entries in GenBank, and codon usage was not consistent with that of other *P. aeruginosa* genes.

Upstream of *P. aeruginosa* *gltX* is a potential gene product with sequence similarity to putative transcriptional regulators (Fig. 1B). However, the presence of this ORF was not required for complementation of the *gltX351* mutation. Between the stop codon of this activator and the potential start codon of GltX is a potential ribosome binding site consistent with the orientation-independent complementation of *E. coli* JP1449.

It is interesting that downstream of the *P. aeruginosa* *gltX* gene and its rho-independent terminator are two tandemly organized tRNA genes, encoding tRNA^{Ala} and tRNA^{Glu} (Fig. 1B). The alanyl-tRNA is 94% identical to that of *E. coli* and contains the invariant G-U base pair at positions 3 and 70 that discriminates tRNA^{Ala} (1). The glutamyl-tRNA is 96% identical to that of *E. coli*. A large stem-loop structure, reminiscent of RNase P substrates, is predicted between these tRNAs. The frequencies of the use of codons specified by the two tRNAs by *P. aeruginosa* are 56% (Ala-GCC) and 40% (Glu-GAA) (2).

tRNAs are not always found adjacent to their respective tRNA synthetases. Of 29 bacterial *gltX* genes inspected, only the *Mycobacterium leprae* and *Mycobacterium tuberculosis* *gltX* genes have an adjacent tRNA^{Glu} (Fig. 3). In *E. coli* and *Haemophilus influenzae*, GltX is expressed divergently from an operon containing tRNA genes, none of which encode tRNA^{Glu}. In the case of the *P. aeruginosa* gene, it is clear that complementation of the *gltX* mutation in *E. coli* JP1449 is not simply due to pseudoreversion (i.e., increased levels of the cognate tRNA), since the pSalF construct does not contain the tRNA^{Glu} locus.

Glutamyl-tRNA synthetase activity was determined in *E. coli* JP1449 containing recombinant plasmids by a standard amino-

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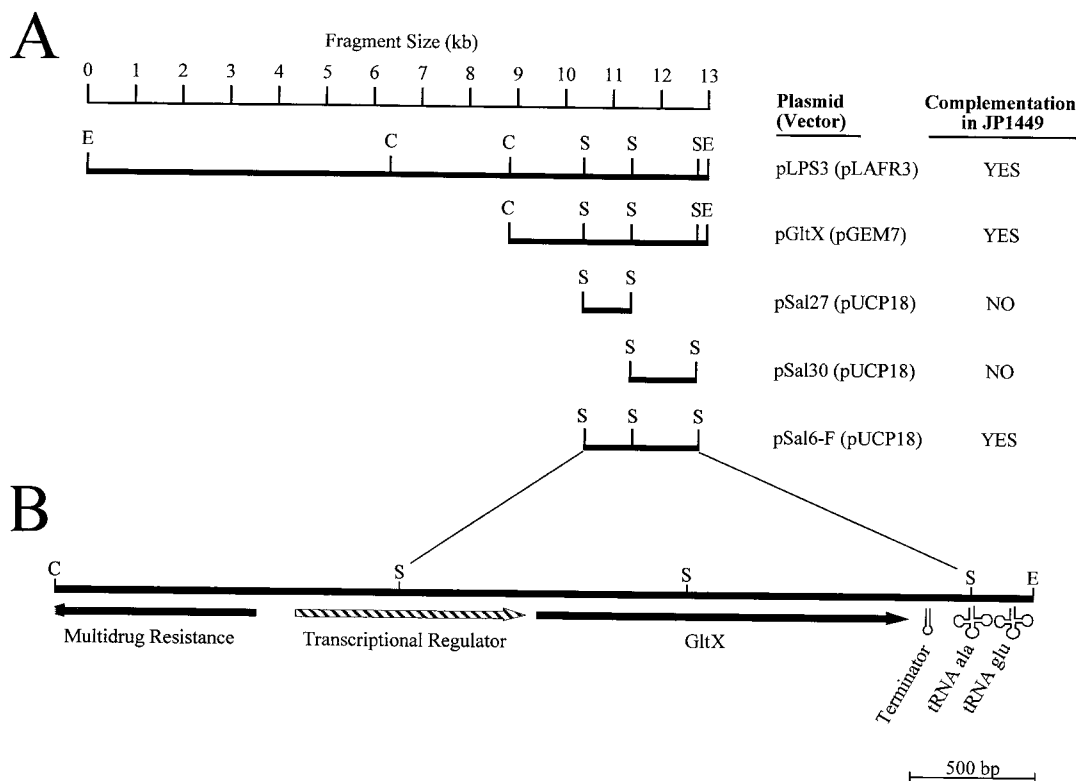


FIG. 1. (A) Complementation of the temperature-sensitive *gltX* mutation in *E. coli* JP1449 with various subclones of pLPS3 (9). Vectors used in cloning were pLAFR3 (22), pGEM7 (Promega Corp., Madison, Wis.), and pUCP18 (20). Complementation was detected as the ability of *E. coli* JP1449 containing recombinant plasmids to grow at 42°C. The smallest subclone capable of complementing this gene was a 2.3-kb *SalI* fragment in pUCP18. (B) Genetic organization of the 3.9-kb DNA insert from pGltX. Two complete ORFs, coding for a hypothetical transcriptional regulator and the glutamyl-tRNA synthetase (GltX), were detected. Upstream of these genes is a divergently transcribed partial ORF with similarity to multidrug resistance gene products. Directly downstream of the *gltX* gene is a rho-independent terminator; distal to this terminator is a short transcript encoding two tRNA molecules, alanyl-tRNA (GGC) and glutamyl-tRNA (TTC), indicated as cloverleaf structures. Restriction endonuclease recognition sites are abbreviated as follows: C, *Cl*I, E, *Eco*RI; S, *Sal*I.

acyl transferase assay (12) (Table 1). In brief, 1 liter of *E. coli* was grown in Luria-Bertani medium, with ampicillin (100 µg/ml) when needed, for 16 h at 30°C. Cells were pelleted and resuspended in 10 ml of 50 mM HEPES (pH 7.2) and then broken by one pass through a French pressure cell (10,000 psi). Cell debris was removed by low-speed centrifugation (4,000 × g, 4°C, 10 min), and cell membranes were pelleted by ultracentrifugation (100,000 × g, 4°C, 60 min).

The cell extract was then decanted and placed on ice for immediate use. The assay for GltX function contained the following components in a final volume of 100 µl: 50 mM HEPES (pH 7.2), 10% (vol/vol) glycerol, 25 mM MgCl₂, 0.5 mM dithiothreitol, 1 mM ATP, 3 mg of crude *E. coli* tRNA (Sigma Chemical Co., St. Louis, Mo.) per ml, and 0.1 mM [¹⁴C]glutamic acid (10 mCi/mmol) (ICN Inc., Costa Mesa, Calif.). Reactions were initiated by the addition of cell extracts, were allowed to proceed at either 30 or 42°C, and were terminated after 5 min by the addition of 20 µl of 100% (wt/vol) trichloroacetic acid (TCA). Incorporation of [¹⁴C]glutamic acid into the TCA-precipitable pool was determined by filtering onto glass filters, followed by two 5-ml washes of 5% (wt/vol) TCA and 95% ethanol. Dried filters were placed in 10 ml of Scintosafe EconoF LSC fluid and counted for 2 min on a Wallac 1409 liquid scintillation counter. Protein concentrations were determined with the Bradford dye-binding assay from Bio-Rad Laboratories (Hercules, Calif.) with bovine serum albumin as a standard.

E. coli JP1449 extracts contained a low, but detectable, level

of GltX activity at 30°C. This activity was abolished upon incubation of the reaction mixture at the nonpermissive temperature (42°C). Cells containing plasmid pSal6F expressed about sixfold more activity than JP1449 alone; this elevated activity was retained at the nonpermissive temperature.

The presence of tightly associated zinc has been reported for some tRNA synthetases from both class I and class II enzymes (13). Glutamyl-tRNA synthetases from *E. coli* and *Bacillus subtilis*, but not *T. thermophilus*, have been shown to contain one zinc atom per molecule. Removal of this ion with the metal chelator *o*-phenanthroline resulted in a conformational change and a concomitant loss of enzymatic activity (14). Sequence alignments revealed that the zinc-containing enzymes possessed two cysteine- and histidine-rich motifs, CXC and CRH SHEHH, in the tRNA acceptor domain. Extended X-ray absorption fine structure analysis of the *E. coli* glutamyl-tRNA synthetase demonstrated that the zinc atom was coordinated by three cysteine and one histidine residues. Site-directed mutagenesis of these motifs resulted in four variants—C98S, C100S, C125S, and H127Q—which no longer complemented the *gltX351* defect of *E. coli* JP1449 (13). Inspection of the multiple-sequence alignment (Fig. 2) revealed that *P. aeruginosa* GltX contains cysteine and histidine residues corresponding to those involved in zinc coordination. As expected, preincubation of *P. aeruginosa* cell extracts with either 1 or 10 mM *o*-phenanthroline diminished enzymatic activity at 42°C (Table 2).

Given both the degree of sequence similarity and the con-

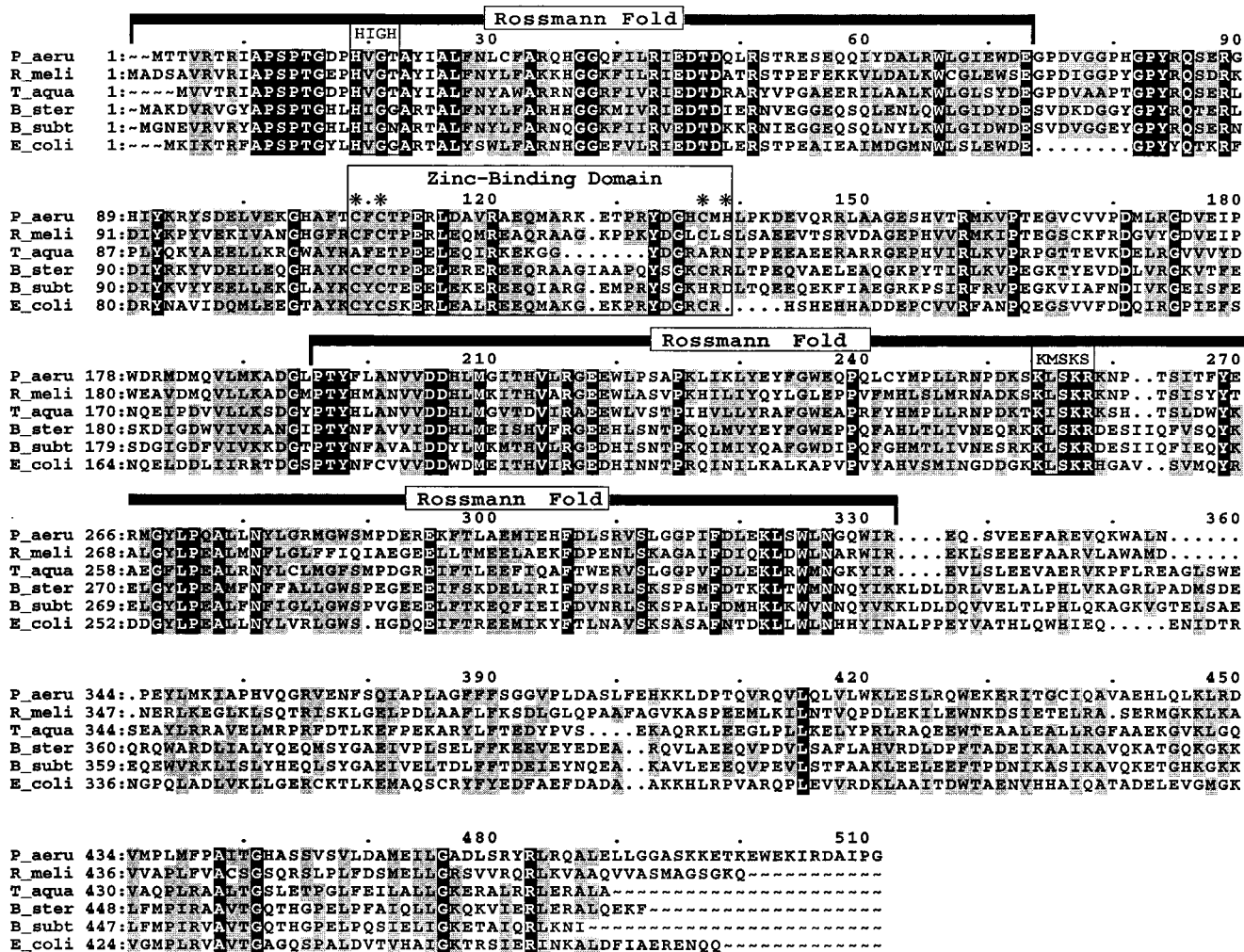


FIG. 2. Multiple-sequence alignment of GltX. The predicted protein product from *P. aeruginosa* *gltX* was compared to five other known glutamyl-tRNA synthetases by using the Pileup program from the Genetics Computer Group (Madison, Wis.) package (Wisconsin Package, version, 9.1). Comparisons were made with the blosum 62 matrix, a gap weight of 12, and a gap length weight of 4. The GltX sequences used were as follows: P_aeru, *P. aeruginosa* PA103 (this study); R_meli, *R. meliloti* (GenBank accession no. P15189); T_aqua, *Thermus aquaticus* (GenBank accession no. P27000); B_ster, *Bacillus stearothermophilus* (GenBank accession no. P43818); B_subt, *B. subtilis* (GenBank accession no. P22249); E_coli, *E. coli* (GenBank accession no. P04805). Residues which were identical in all six sequences are printed in white on black, while those conserved in at least four of the six sequences are shaded. The positions of the Rossmann fold, including the HIGH and KMSKS motifs, and the zinc-binding domain are indicated. The locations of the four residues implicated in coordinating zinc in the *E. coli* glutamyl-tRNA synthetase are marked with asterisks.

servation of functional domains between the glutamyl-tRNA synthetases from *E. coli* and *P. aeruginosa*, it is not surprising that the *Pseudomonas* gene can complement the *gltX351* mutation of *E. coli* JP1449. Interestingly, similar attempts to complement *E. coli* with *gltX* from *B. subtilis* were unsuccessful (16); recombinant plasmids containing an intact *B. subtilis* *gltX* gene were found to be lethal for *E. coli*. It has been postulated that this toxic effect is due to the misacylation of tRNA^{Gln} with glutamate. *B. subtilis*, like most other organisms, uses one tRNA synthetase, GltX, to charge both tRNA^{Glu} and tRNA^{Gln} with glutamate; the latter is subsequently converted to glutamine by a specific amidotransferase (11). Given the lack of lethality of *P. aeruginosa* *gltX*, we reasoned that *Pseudomonas*, like *E. coli*, may possess separate glutamyl- and glutaminyl-tRNA synthetases. A search of the incomplete *P. aeruginosa* genomic sequencing project (16a) revealed the presence of a glutaminyl-tRNA synthetase homolog that showed 60 and 59% identity with those of *E. coli* and *H. influenzae*, respectively.

Because of the crucial role that aminoacyl-tRNA synthetases

play in protein biosynthesis, and their high degree of sequence conservation, these enzymes are of interest from several perspectives. They are enticing targets for novel antimicrobials. Indeed, pseudomonic acid, a natural product of *Pseudomonas*

TABLE 1. Quantitation of GltX activity in *E. coli* JP1449

Plasmid ^a	Temp (°C) ^b	GltX sp act (nmol/min/mg of protein) ^c	Relative activity (%) ^d
None	30	1.15 ± 0.28	100
None	42	0.18 ± 0.06	15.6
pUCP18	30	0.99 ± 0.13	86.1
pUCP18	42	0.17 ± 0.05	14.8
pSal6F	30	7.57 ± 0.01	673.9
pSal6F	42	7.77 ± 0.37	675.6

^a Cell extracts were made from *E. coli* JP1449 containing the indicated plasmids.
^b Reaction incubation temperature.
^c Results are averages of five experiments ± standard deviations.
^d Activity compared to *E. coli* JP1449 cell extracts assayed at 30°C.

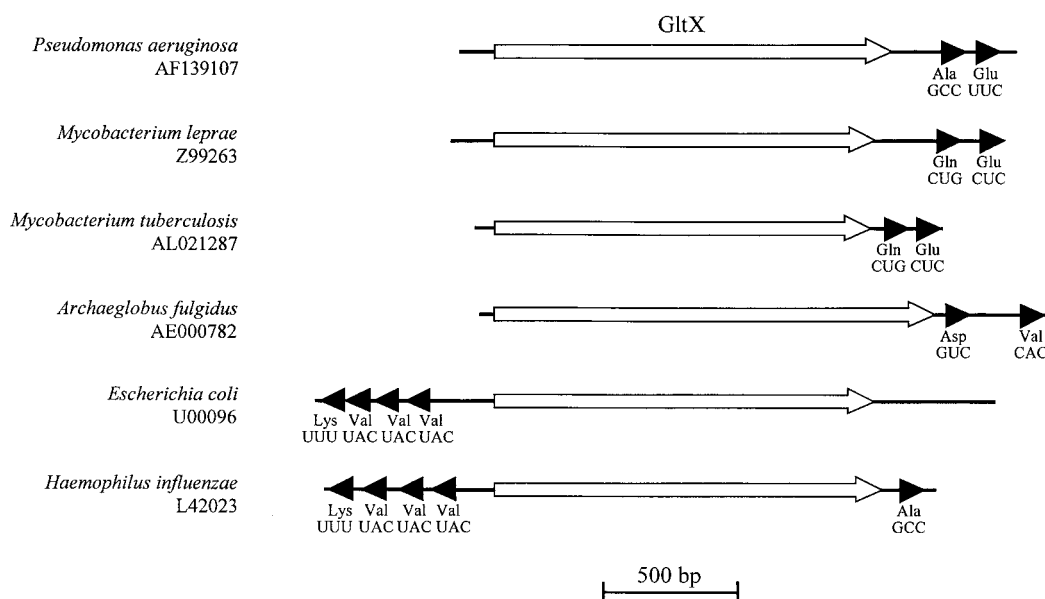


FIG. 3. Proximity of tRNA genes to glutamyl-tRNA synthetases. The *gltX* loci reported for 29 bacterial species were examined for the presence of adjacent tRNA genes; accession numbers are indicated. Species containing tRNAs adjacent to *gltX* are shown. Open arrows represent *gltX*, and solid arrows represent tRNA with amino acids and anticodons, as indicated. Species lacking tRNAs adjacent to *gltX* included the following, with accession numbers: *Aquifex aeolicus*, AE000657; *Azospirillum brasilense*, X99587; *Bacillus stearotherophilus*, M55072; *B. subtilis*, AL009126; *Borrelia burgdorferi*, AE00783; *Chlamydia psittaci*, U41758; *Chlamydia trachomatis*, AE001273; *Helicobacter pylori*, AE001439; *Methanobacterium thermoautotrophicum*, AE000666; *Methanococcus jannaschii*, L77117; *Mycoplasma genitalium*, L43967; *Mycoplasma pneumoniae*, U00089; *Mycoplasma pulmonis*, L25415; *Neisseria gonorrhoeae*, U76418; *Pyrococcus horikoshii*, pyro_h; *R. meliloti*, M27221; *Rickettsia prowazekii*, AJ235269; *Staphylococcus xylosum*, Y07614; *Streptococcus coelicolor*, AL031124; *Synechocystis* sp., AB001339; *T. thermophilus*, X64557; *Treponema pallidum*, AE000520; *Vibrio cholerae*, AF030977.

fluorescens, acts by blocking the action of isoleucyl-tRNA synthetases (10). Recently, several aminoacyl-tRNA synthetases (10). Recently, several aminoacyl-tRNA synthetases, some of which have been found to be potent antimicrobials (6, 19). Other researchers have used sequence comparisons of tRNA synthetases to investigate the phylogeny of prokaryotic cells (7, 21). In many cases, the phylogenetic relationships generated by these analyses differ from those obtained by analysis of rRNA genes. In such cases, horizontal gene transfer between organisms and/or gene duplications have been invoked. Although extensive, the database of tRNA synthetase genes is not yet sufficient to provide a single satisfactory model for the dissemination of these enzymes from a common ancestral gene. Thus, future discovery of aminoacyl-tRNA synthetases during genomic sequencing of organisms not only will lead to increased understanding of protein biosynthesis but also will provide possible leads to new antibiotics and clues to the origins of the diversity of life.

Nucleotide sequence accession number. The nucleotide sequence of *gltX* has been deposited in GenBank and assigned accession no. AF139107.

TABLE 2. Effects of *o*-phenanthroline on GltX activity

Concn (mM) ^a	GltX sp act (nmol/min/mg of protein) ^b	Relative activity (%) ^c
0	8.58 ± 0.68	100.0
1	0.79 ± 0.49	9.3
10	0.10 ± 0.11	1.2

^a GltX reactions were performed at 42°C with 20 μg of *E. coli* JP1449(pSal6F) extracts after preincubation of cell proteins with the indicated concentrations of *o*-phenanthroline for 10 min.

^b Results are averages of five experiments ± standard deviations.

^c Activity compared to assays with no added *o*-phenanthroline.

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