The Promiscuous *sumA* Missense Suppressor from *Salmonella enterica* Has an Intriguing Mechanism of Action

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ABSTRACT While most missense suppressors have very narrow specificities and only suppress the allele against which they were isolated, the *sumA* missense suppressor from *Salmonella enterica* serovar Typhimurium is a promiscuous or broad-acting missense suppressor that suppresses numerous missense mutants. The *sumA* missense suppressor was identified as a *glyV* tRNA Gly3(GAU/C) missense suppressor that can recognize GAU or GAC aspartic acid codons and insert a glycine amino acid instead of aspartic acid. In addition to rescuing missense mutants caused by glycine to aspartic acid changes as expected, *sumA* could also rescue a number of other missense mutants as well by changing a neighboring (contacting) aspartic acid to glycine, which compensated for the other amino acid change. Thus the ability of *sumA* to rescue numerous missense mutants was due in part to the large number of glycine codons in genes that can be mutated to an aspartic acid codon and in part to the general tolerability and/or preference for glycine amino acids in proteins. Because the *glyV* tRNA Gly3(GAU/C) missense suppressor has also been extensively characterized in *Escherichia coli* as the *mutA* mutator, we demonstrated that all gain-of-function mutants isolated in a *glyV* tRNA Gly3(GAU/C) missense suppressor are transferable to a wild-type background and thus the increased mutation rates, which occur in *glyV* tRNA Gly3(GAU/C) missense suppressors, are not due to the suppression of these mutants.

KEYWORDS tRNA; suppression; missense suppressor

THE *sumA* (suppressor of missense) mutation has been used by researchers to identify missense mutants in *Salmonella enterica* serovar Typhimurium based on its ability to restore the enzymatic function of inactive proteins (Hughes *et al.* 1991; Galitski and Roth 1996). *sumA* or *su537*, like most missense suppressors, was originally isolated as an extragenic suppressor of the *hisC537* missense mutant (Whitfield *et al.* 1966). *sumA* suppressed 3 of 21, or 14.29%, of the *hisC* missense mutants that were characterized in this study but could not suppress *hisC* amber, ochre, or frameshift mutants. In a study involving a greater number of missense mutants, *sumA* was shown to suppress 11 of 72, or 15.28% of *nadC* missense mutants (Hughes *et al.* 1991). *sumA* has been mapped near the *purA* locus in *S. enterica* (Sanderson and Hartman 1978).

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Missense suppressors are mutated transfer RNAs (tRNAs) that recognize an aberrant codon instead of the usual codon the tRNA is supposed to recognize (for general reviews see Hill 1975 and Murgola 1985, 1995). For example, the *glyT* tRNA Gly2(AGA) missense suppressor in *Escherichia coli*, the first missense suppressor that was thoroughly characterized, contains a C-to-U mutation at the 3′ end of the wild-type *glyT* tRNA Gly2(GGA/G) anticodon and reads AGA codons instead of GGA codons, thus inserting a glycine amino acid instead of arginine, which is normally coded for by AGA codons (Brody and Yanofsky 1963; Roberts and Carbon 1975). Missense suppressors can be grouped into four classes based on the change that occurs in the tRNA; those that contain a nucleotide substitution in the anticodon, those that contain a nucleotide insertion in the anticodon loop outside of the anticodon, those that contain a mutation in the amino acid acceptor stem, and those that contain a nucleotide substitution in the base-paired region of the D arm. The vast majority of missense suppressors that have been characterized contain a nucleotide substitution in the anticodon of the tRNA.

In general, both the efficiency of missense suppressors, or their ability to restore the functionality of defective missense mutants, and the specificity of missense suppressors, or their ability to rescue different missense mutants, have been found to be quite low, because presumably a highly efficient missense suppressor with broad specificity would be incredibly detrimental to the cell and most likely lethal. In most of the efficiency studies that have been conducted, the efficiency of missense suppressors ranged from 1.10 to 3.60% as measured by the ability of a missense suppressor to restore the enzymatic activity of a mutant inactive gene compared to the enzymatic activity of the wild-type gene (Brody and Yanofsky 1963; Berger and Yanofsky 1967; Hill *et al.* 1970, 1974). In the specificity studies that have been conducted, most of the missense suppressors have been found to be allele specific and only suppress one or two alleles (Brody and Yanofsky 1963; Eggertsson and Adelberg 1965; Eggertsson 1968). There are two notable exceptions. The *glyU* tRNA Gly1(GAG) missense suppressor rescued 5 of the 12 or 35.71% of the mutants that were tested (Eggertsson and Adelberg 1965; Hill *et al.* 1974), and the *sumA* missense suppressor as discussed above rescued 15.05% of the mutants that were tested.

In this study, we have characterized the *sumA* missense suppressor. The mutation that causes *sumA* was mapped and sequenced and the efficiency and specificity of the *sumA* missense suppressor was determined using a collection of missense mutants in either the *lacZ* gene from *E. coli*, which codes for the β-galactosidase enzyme, or the *xylE* gene from the *Pseudomonas putida* pWW0 TOL plasmid, which codes for the catechol 2,3-dioxygenase enzyme.

Materials and Methods

Media, bacterial strains, and plasmids

Lysogeny broth (LB) (Bertani 1951) or M9 (Miller 1972) were used as rich or minimal defined media, respectively. The antibiotics chloramphenicol and tetracycline were used at a final concentration of 20 μg/ml in LB, while rifampicin was used at a final concentration of 50 μg/ml in LB. Ampicillin was used at 100 μg/ml in LB to provide selective pressure for Amp^R plasmids or at 30 μg/ml in LB to select for the *amiB*::*mudA* chromosomal insertion. The *S. enterica* strains used in this study are listed in Table 1 and the plasmids used in this study are listed in Table 2. To construct ALS2583, a P22 HT105/1 *int-201* lysate (Schmieger 1972) prepared from GT467 was used to transduce *amtB211*::*mudA* into TR4780. LB Amp^R transductants were selected that could not grow on minimal M9 glucose. To construct ALS234, a P22 HT105/1 *int-201* lysate prepared from TT16237 was used to transduce *zjf-3693*::Tn*10*dTet into TR3359. LB Tet^R transductants were selected that could not grow on minimal M9 glucose. To construct ALS2241 and ALS2242, a P22 HT105/1 *int-201* lysate prepared from TT20702 was used to transduce *btuB12*::Tn*10*dCam into TT16237 and ALS234, respectively. The construction of ALS1442, which contains the *proB*::*xylE*(*cat*) insertion is described later in the *Materials and Methods* section. Two *E. coli* strains were also used in this study, CS520, *glyV50 metB1 relA1 spoT1 trpA58 tyrT58*(AS), from the Carbon laboratory and MC1061, Δ(*araABOIC-leu*)*7679 araD139 hsr*$^-$ *hsm*$^+$ *galU galK* Δ(*lac*)*X74 rpsL*, Casadaban and Cohen (1980).

Determining the mutation responsible for sumA

Because the *zjf-U130*::Tn*10*dTet insertion was 99% linked to *sumA*, it was expected to be <500 bases from the mutation that caused *sumA*. To determine the mutation that caused *sumA*, the tetracycline resistance of the Tn*10*dTet insertion, which was 99% linked to *sumA*, was replaced with chloramphenicol resistance from pACYC184 (Chang and Cohen 1978) to facilitate cloning of the region surrounding the Tn*10*dTet insertion, using the lambda Red recombination system (Datsenko and Wanner 2000; Yu *et al.* 2000). Using the forward primer 5′ CTGATGAATCCCCTAATGATTTTGGTAAAAATCATTAAGTTAAGGTGGATTTGAGAAGCACACGGTCACA 3′ and the reverse primer 5′ CTGATGAATCCCCTAATGATTTTGGTAAAAATCATTAAGTTAAGGTGGATTACCTGTGACGGAAGATCAC 3′, a 1163-bp fragment was amplified using the polymerase chain reaction (PCR), *Pfu* polymerase, and pACYC184 as a template, which contained the last 50 bases of the inverted repeats from Tn*10*dTet (del16 del17 TetR, Way *et al.* 1984) and chloramphenicol resistance from pACYC184. The homology for the pACYC184 chloramphenicol resistance from 419 to 3601 bp is underlined. The 1163-bp fragment was gel isolated and electroporated into LT2 pKM201 cells that were prepared as described by Murphy and Campellone 2003 to express the lambda gam and red recombination genes. Chloramphenicol-resistant colonies were selected and checked for tetracycline sensitivity to ensure that tetracycline resistance had been replaced with chloramphenicol resistance in the resulting strain. Genomic DNA was prepared from the chloramphenicol-resistant strain, partially digested with *Sau*3AI to generate 5000-bp fragments and ligated into pTrc99A (Amann *et al.* 1988), which had been digested with *Bam*HI and dephosphorylated with calf intestinal alkaline phosphatse. MC1061 transformants that were both ampicillin and chloramphenicol resistant were selected. Plasmid DNA was prepared from pTrc99A-*sumA*, one of the clones that harbored a 5000-bp insert and sequenced using the following two primers; 5′ TGTGACCGTGTGCTTCTCAA 3′, a primer that sequences outwards from the beginning of the chloramphenicol resistance region from pACYC184 and 5′ TGATCTTCCGTCACAGGT 3′, a primer that sequences outwards from the end of the chloramphenicol resistance region from pACYC184. The site of the Tn*10*dTet insertion that was 99% linked to *sumA* was determined to be at bp 4,596,266 of the *S. enterica* chromosome (McClelland *et al.* 2001, GenBank accession number NC_003197).

Since the *glyV*, *glyX*, *glyY* tRNA locus, which could be mutated to generate a missense suppressor, was immediately downstream of the Tn*10*dTet insertion, genomic DNA was prepared from ALS233, which harbors the *sumA* missense suppressor, PCR amplified, and sequenced using the 5′ GCGAAAAATGCGTTCAGGG 3′ and 5′ GCCCTGTGGATAAGTCTGTT 3′

Table 1 S. *enterica* strains

Name	Genotype	Source
ALS234	*hisC537 zjf-3693*::Tn*10*dTet (40% linked to *sumA*)	This study
ALS1442	*proB*::*xylE*(*cat*)	This study
ALS2241	*btuB12*::Tn*10*dCam *hisC537 sumA10 zjf-3693*::Tn*10*dTet	This study
ALS2242	*btuB12*::Tn*10*dCam *hisC537 zjf-3693*::Tn*10*dTet	This study
ALS2583	*amiB211*::*mudA hisC537 purA155*	This study
GT2086	*zjf-U130*::Tn*10*dTet (99% linked to *sumA*)	Björk laboratory
GT467	*amtB211*::*mudA* (*amiB211*::*mudA*)	Björk laboratory
LT2	Wild-type	Roth laboratory
TR3359	*hisC537*	Roth laboratory
TR4780	*hisC537 purA155*	Roth laboratory
TT2337	*hisC527*(UAG) *leuA414*(UAG) *supF* (UAG, *tyr*) *zde-94*::Tn*10* (50% linked to *supF*)	Roth laboratory
TT2344	*hisC527*(UAG) *leuA414*(UAG) *supE* (UAG, *gln*) *zbf-604*::Tn*10* (51% linked to *supE*)	Roth laboratory
TT2839	*hisC527*(UAG) *leuA414*(UAG) *tyrU90* (*supM*) *zii-614*::Tn*10* (45% linked to *supM*)	Roth laboratory
TT4029	*hisO1242 hisB2135 supU1283*(UGA) *zhb-736*::Tn*10* (10% linked to *supU*)	
TT7610	*supD501* (UAG, *ser*) *zeb-609*::Tn*10* (60% linked to *supD*)	Roth laboratory
TT13029	*hisC527*(UAG) *leuA414*(UAG) *supC80*(UAA, UAG, *tyr*) *zde-605*::Tn*10* (54% linked to *supC*)	Roth laboratory
TT16237	*hisC537 sumA10 zjf-3693*::Tn*10*dTet (40% linked to *sumA*)	Roth laboratory
TT18519	ara-9 hisC10081::MudF(*lac*$^+$)	Roth laboratory
TT20702	*btuB12*::Tn*10*dCam (30% linked to *rpoB*)	Roth laboratory

primers designed to amplify the *glyV*, *glyX*, *glyY* region between bp 4,596,321 and 4,597,058 of the *S. enterica* chromosome. The mutation in *sumA* was determined to be a C-to-T change at bp 4,596,687 of the *S. enterica* chromosome.

A *glyV* tRNA Gly3(GAU/C) missense suppressor, which should be identical to the *sumA* missense suppressor from *S. enterica* has also been identified in *E. coli* (Guest and Yanofsky 1965; Fleck and Carbon 1975). Since the *glyV* tRNA Gly3(GAU/C) missense suppressor was never sequenced, we did so to confirm this fact. Genomic DNA was prepared from CS520, which harbors the *gly*V tRNA Gly3(GAU/C) missense suppressor and the *glyV*, *glyX*, *glyY* region between bp 4,391,858 and 4,392,795 of the *E. coli* chromosome was PCR amplified using the 5′ TGAACTGGCAACGCTCGAAT 3′ and 5′ CACCGTGCGAAGTTTCTTTG 3′ primers and then sequenced using the 5′ CGGCGTGATTTTGACGCTAA 3′ and 5′ CACCGTGCGAAGTTTCTTTG 3′ primers. The mutation in *glyV* tRNA Gly3(GAU/C) was determined to be a C-to-T change at bp 4,392,617 of the *E. coli* chromosome (Blattner *et al.* 1997, GenBank accession number U00096.3).

Construction of pACYC184-xylE

Initially the *xylE* gene was cloned into the pTrc99A expression vector. To construct pTrc99A-*xylE*, which maximized the expression of *xylE*, forward primer 5′ ATCAGACTGCAGGAGGTAACAGCTATGAACAAAGGTGTAATGCGACC3′ and reverse primer 5′ TAGCAGTGGCAGCTCTGAAAGCTTTGCACAATCTCTGCAATAAGTCG 3′ and *Pfu* polymerase were used to PCR amplify a 1006-bp fragment from the pXE60 plasmid (Delic *et al.* 1992), which contained the wild-type *P. putida xylE* gene isolated from the TOL pWW0 plasmid and a strong Shine–Dalgarno ribosome binding . The regions of homology to *xylE* are underlined. The resulting fragment was gel isolated, digested with *Pst*I and *Hin*dIII, and then ligated into the pTrc99A vector, which had been digested with the same two restriction enzymes. To construct pACYC184-*xylE*, forward primer 5′ ATATCCATAAGCTTCGCCGACATCATAACGGTTC 3′ and reverse primer 5′ TCATGACCGTGCTGACCTGATGATCATTGGATAT 3′ and *Pfu* polymerase were used to PCR amplify a 1090-bp fragment from pTrc99A-*xylE* that contained the *trc* promoter and the *xylE* gene. The resulting fragment was gel isolated, digested with *Hin*dIII and *Bcl*I, and then ligated into the pACYC184 vector, which had been digested with the same two restriction enzymes.

Construction of the proB::xylE(cat) insertion

Using pACYC184-*xylE* as template DNA, the forward primer 5′ ATGAGTGACAGCCAGACGCTGGTCGTAAAACTCGGCACCAGCGTGCTAACGCCGACATCATAACGGTTCT 3′ and the reverse primer 5′ TTATCGAGTAATCATGTCATCACGATGAACAGCGACCGGGCCATATTCATTACCTGTGACGGAAGATCAC 3′ and *Pfu* polymerase was used to PCR amplify a 2312-bp fragment that contained the start of the *proB* gene along with the following 47 bases, the *trc* promoter, and the *xylE* gene through the *cat* promoter region and the *cat* gene of pACYC184-*xylE*, and the stop of the *proB* gene along with the preceding 47 bases. The homology to the pACYC184-*xylE* plasmid is underlined. The resulting fragment was gel isolated, electroporated into LT2 pKM201 cells that were prepared as described by Murphy and Campellone (2003), and plated on LB chloramphenicol plates at 37° to kick the pKM201 plasmid. Recombinant colonies were streaked on LB chloramphenicol plates at 37° and patched on LB chloramphenicol plates, M9 glucose plates plus or minus proline, and LB ampicillin plates to verify that the desired *proB*::*xylE*(*cat*) insertion had been isolated. The *proB*::*xylE*(*cat*) insertion produced two forms

Table 2 Plasmids

Name	Relevant characteristics	Reference
pACYC184	Tet^R, Cam^R, p15A ori	Chang and Cohen (1978)
pACYC184-*xylE*	Tet^R, Cam^R, p15A ori, *trc* promoter/operator, *xylE* gene	This study
pKM201	Amp^R, pAMPts ori, *tac* promoter, lambda *gam* and *red* genes	Murphy and Campellone (2003)
pTrc99A	Amp^R, *trc* promoter/operator, $lacI^Q$, ColE1 ori	Amann *et al.* (1988)
pTrc99A-*sumA*	Amp^R, *trc* promoter/operator, $lacI^Q$, ColE1 ori, *sumA* gene	This study
pTrc99A-*xylE*	Amp^R, *trc* promoter/operator, $lacI^Q$, ColE1 ori, *xylE* gene	This study
pXE60	Amp^R, ColE1 ori, TOL pWWO *xylE* gene	Delic *et al.* (1992)

of the catechol 2,3-dioxygenase protein as verified using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The majority of the protein produced was from the wild-type *xylE* gene. A minority protein product was also produced that contained the wild-type *xylE* gene and a 20 amino acid amino terminal extension due to the in-frame ATG start from the pTrc99A expression vector.

Generating lacZ and xylE missense mutants

Strains TT18519 (*hisC*10081::MudF[lac^+]) or ALS1442 (*proB*::*xylE*[*cat*]) were mutagenized with ethylmethane sulfonate (EMS) as described by Miller (1972). The *lacZ* mutants were isolated using LB plates that contained 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and 40 μg/ml 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-gal). White or very light blue colonies were selected. The *xylE* mutants were isolated on LB plates that contained 1 mM IPTG and then the resulting colonies were sprayed with a light mist of a 100 mM potassium phosphate buffer, pH 7.5, that contained 100 mM catechol. White or very light yellow colonies were selected. Nonsense mutants due to amber, ochre, or opal mutations were identified using the *supD* (TT7610), *supE* (TT2344), and *supF* (TT2337) amber suppressors, the *supC* (TT13029) and *supM* (TT2839) ochre suppressors, and the *supU* (TT4029) opal suppressor. Missense mutants suppressible by *sumA* were identified using the *sumA* missense suppressor (TT16237).

The potential *lacZ* and *xylE* missense mutants were sequenced by PCR amplifying the *lacZ* or *xylE* genes using *Pfu* polymerase and then using overlapping primers to sequence both strands of either the *lacZ* or *xylE* genes. The GenBank accession no. for the sequence of the *xylE* gene is M64747.1 (Harayama *et al.* 1991). The sequence of the *lacZ* gene was taken from the genomic sequence of MG1655, the first *E. coli* strain to be sequenced (Blattner *et al.* 1997, GenBank accession no. U00096.3). During the sequencing of the *lacZ* missense mutants we noticed that the sequence of the *lacZ* gene in the *hisC*10081::MudF(lac^+) insertion differed from the sequence of the *lacZ* gene in MG1655 by one codon. The CAA glutamine codon at amino acid 703 in the *lacZ* gene in MG1655 was changed to a UUA leucine codon in the *lacZ* gene in the *hisC*10081::MudF(lac^+) insertion. The glutamine-to-leucine codon change is in the last amino acid of a β-sheet and by either Prevelige and Fasman (1989), Garnier *et al.* (1978), or Qian and Sejnowski (1988) protein secondary structure analysis, is predicted to be a neutral change.

Suppression tests

Suppression tests were conducted using amber, ocher, or opal nonsense suppressor or *sumA* missense suppressor strains, which also harbored a Tn*10* or Tn*10*dTet transposon insertion that was linked to the suppressor. All mutants to be tested were transduced with a P22 lysate prepared from the appropriate suppressor strain. Transduced *hisC* or *hisD* mutants were plated on LB tetracycline plates and suppression was determined by patching 50 transductant colonies onto M9 glucose plates and scoring whether the presence of the suppressor restored the ability of *hisC* or *hisD* mutants to grow on minimal M9 glucose. Transduced *lacZ* mutants were plated on LB tetracycline plates supplemented with both 1 mM IPTG and 40 μg/ml X-gal and suppression was determined by whether the presence of the suppressor restored blue color. Transduced *xylE* mutants were plated on LB tetracycline plates supplemented with 1 mM IPTG and suppression was scored by spraying the transductant colonies with 100 mM catechol and determining whether the presence of the suppressor restored yellow color.

β-Galactosidase and catechol 2,3-dioxygenase enzyme assays

β-Galactosidase assays were performed as described by Miller (1972). Because the β-galactosidase and catechol 2,3-dioxygenase enzyme assays both utilize a colorless substrate that is converted to a colored product, we optimized the catechol 2,3-dioxygenase assay described by Sala-Trepat and Evans (1971) to generate a more robust easy to use assay where catechol 2,3-dioxygenase activity could be measured in units similar to the units that were developed for the β-galactosidase assay (Miller 1972). The optimal buffer was determined to be Z buffer, which is used in β-galactosidase assays, the maximal adsorption of the yellow product, 2-hydroxymuconate semialdehyde, was determined to be 368 nm, and the optimal substrate concentration was determined to be 0.067 mM catechol as specified by Sala-Trepat and Evans (1971).

To conduct the catechol 2,3-dioxygenase assays, 100 μl of a bacterial overnight was added to 3.0 ml of Z buffer that contained 0.27% sodium dodecyl sulfate. A total of 50 μl of chloroform was added and the sample was vortexed thoroughly to lyse the cells and liberate any catechol 2,3-dioxygenase enzyme that was present. After 10 min of equilibration at room temperature, 100 μl of 100 mM potassium phosphate buffer, pH 7.5, that contained 2.15 mM catechol was added,

the samples were briefly vortexed, and the start time of the assay was recorded. After optimal yellow color development occurred due to the conversion of catechol to 2-hydroxymuconate semialdehyde, the reaction was stopped by the addition of 1.5 ml of methanol to inactivate the catechol 2,3-dioxygenase enzyme and the time of the reaction was recorded. The assay samples were centrifuged to remove cell debris and the 2-hydroxymuconate semialdehyde in the supernatant was measured at 368 nm (OD_{368}). XylE units were determined using the formula, 10,000 × (OD_{368} of the 2-hydroxymuconate semialdehyde)/(T × V × OD_{550} of the concentrated cells), where T was the reaction time in minutes and V was the volume of the concentrated cells that were used in the assay.

Inheritance test of rifampicin-resistant mutants isolated in a sumA missense suppressor

A total of 0.2 ml of a saturated LB overnight of the isogenic strains ALS2241 (*sumA*) and ALS2242 (wild type), both of which contained the *btuB12*::Tn*10*dCam transposon insertion were plated on LB plates that contained rifampicin. *btuB12*::Tn*10*dCam is 30% linked to the *rpoB* locus, which can be mutated to yield rifampicin resistance. A total of 0.1 ml of 10^{-6} dilutions of the saturated overnights were also plated on LB plates to determine the number of cells present. ALS2242 (wild type) yielded an average of four rifampicin-resistant mutants per 1.136×10^9 cells, while ALS2241 (*sumA*) yielded an average of 102 rifampicin-resistant mutants per 7.62×10^8 cells. P22 HT105/1 *int-201* lysates were prepared from 50 independent rifampicin-resistant mutants isolated in ALS2241 (*sumA*) and used to transduce the *btuB12*::Tn*10*dCam into LT2. Two hundred transductant colonies from each transduction were patched onto LB rifampicin plates to determine whether rifampicin resistance was transferable from ALS2241 (*sumA*) to LT2 (wild-type *S. enterica*). Transferable or inheritable mutations would be transduced 30% of the time, while nontransferable or noninheritable mutations would not be transduced. All of the rifampicin-resistant mutants were transduced at ~30%, which is consistent with the linkage of *btuB12*::Tn*10*dCam to *rpoB*.

Growth rate studies

LB overnights of the isogenic strains TT16237 (*sumA*) and ALS234 (wild type) were diluted 1:200 in fresh LB media and OD_{550} readings were taken every 15 min until the OD_{550} reached 0.75. The growth rates (min^{-1}) were determined by calculating the slope of a plot of the growth time in minutes *vs.* the natural logarithm of the OD_{550} reading, while the doubling times were calculated as ln2/slope.

Data availability

All of the data required for confirming the conclusions presented in the article are represented fully within the article. All of the bacterial strains and plasmids that were used in this study are available upon request.

Results

Determining the mutation responsible for the sumA missense suppressor

The *sumA* mutant has been mapped near the *purA* locus in *S. enterica* (Sanderson and Hartman 1978) and based on this information, we conducted a three-factor cross with the *amiB*, *purA*, and *sumA* genes to determine the exact location of *sumA*. A P22 HT105/1 *int-201* lysate prepared from a donor *amiB*$^+$, *purA*$^+$, *sumA* missense suppressor strain was used to transduce a recipient *amiB*::mudA, *purA*155, *sumA*$^+$ (wild-type) strain. Table 3 shows the results of this cross and a linkage map indicating the location of the *sumA*, *amiB*, and *purA* genes is shown in Figure 1. Because the *sumA* mutation was highly linked to the *amiB* locus, we screened a collection of Tn*10*dTet transposon insertions from the Björk laboratory that mapped to the *amiB* locus and identified a *zjf-U130*::Tn*10*dTet insertion that was 99% linked to *sumA* (data not shown). The location of the *zjf-U130*::Tn*10*dTet and the base pair change that is responsible for the *sumA* mutation was determined as described in the *Materials and Methods* section.

The mutation in the *sumA* missense suppressor was determined to be a C-to-T change at bp 4,596,687 of the *S. enterica* chromosome (McClelland *et al.* 2001, GenBank accession no. NC_003197), which changes the anticodon of *glyX*, the second of three duplicate copies of the Gly3 tRNA, from GCC to GUC. Thus the *sumA* missense suppressor should be classified as a *glyV* tRNA Gly3(GAU/C) missense suppressor and is expected to insert a glycine amino acid instead of aspartic acid at GAU or GAC codons. The *glyV* tRNA Gly3(GAU/C) missense suppressor has been previously identified in *E. coli* but not verified by DNA sequencing (Guest and Yanofsky 1965; Fleck and Carbon 1975). To show that the *glyV* tRNA Gly3(GAU/C) missense suppressor from *S. enterica* and *E. coli* were identical, we sequenced the *glyV* tRNA Gly3(GAU/C) missense suppressor from *E. coli* as described in the *Materials and Methods* section. The mutation in the *glyV* tRNA Gly3(GAU/C) missense suppressor from *E. coli* was determined to be a C-to-T change at bp 4,392,617 of the *E. coli* chromosome (Blattner *et al.* 1997, GenBank accession no. U00096.3), which changes the anticodon of *glyY*, the third of three duplicate copies of the Gly3 tRNA, from GCC to GUC.

Characterizing the efficiency and specificity of the sumA missense suppressor

Initially we tested the well-characterized *hisD* mutant collection that was isolated by Greeb *et al.* (1971) and found that 14 of 57, or 24.56%, of the missense mutants analyzed from this collection could be suppressed by *sumA*. To further characterize the efficiency and specificity of the *sumA* missense suprressor, we created a collection of missense mutants in both the *lacZ* and *xylE* genes. These genes were chosen because they both encode well-characterized enzymes, for which robust colorimetric assays are available.

Table 3 Three-factor cross using the *amiB*, *purA*, and *sumA* genes

Recombinant class	Total number out of 300
sumA amiB::*mudA*	8
sumA amiB$^+$ (wt)	112
sumA$^+$ (wt) *amiB*::*mudA*	63
sumA$^+$ (wt) *amiB*$^+$ (wt)	117

A P22 HT105/1 *int-201* lysate prepared from a donor *amiB*$^+$, *purA*$^+$, *sumA* missense suppressor strain was used to transduce a recipient *amiB*::*mudA*, *purA155*, *sumA*$^+$ (wild-type) strain, which also contained the *hisC537* mutation, known to be suppressible by *sumA*, to *purA*$^+$ on M9 glucose plates supplemented with histidine. The 300 transductants were analyzed and the number of *sumA amiB*::*mudA*, *sumA amiB*$^+$, *sumA* (wild-type) *amiB*::*mudA*, and *sumA* (wild-type) *amiB*$^+$ recombinants were scored on M9 glucose plates and LB Amp plates. Based on this information, the gene order was determined to be *sumA*, *amiB*, *purA*, due to the rare *sumA amiB*::*mudA* class. The linkage of *purA* to *sumA* was calculated to be 40.0%; the linkage of *purA* to *amiB* was calculated to be 76.3%; and based on these linkages, the linkage of *sumA* to *amiB* was predicted to be 63.7%.

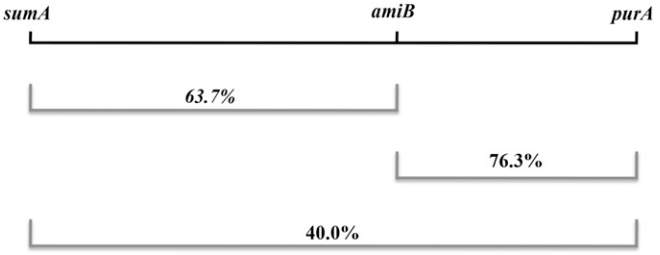

Figure 1 Linkage map of the *sumA*, *amiB*, and *purA* genes in *S. enterica*. The gene order of the *sumA*, *amiB*, and *purA* genes are shown and the linkages based on the data from Table 3 are included.

EMS mutagenesis was employed to isolate 100 *lacZ* and *xylE* missense mutants as described in the *Materials and Methods* section. The *sumA* missense suppressor was able to rescue 15 of the 100, or 15%, of the *lacZ* missense mutants and 10 of the 100, or 10%, of the *xylE* missense mutants. To better understand the efficiency and specificity of the *sumA* missense suppressor, 10 of the *lacZ* and *xylE* mutants were sequenced and the enzymatic activities of the rescued β-galactosidase and catechol 2,3-dioxygenase enzymes was determined. Table 4 and Table 5 list the *lacZ* and *xylE* missense mutants that were characterized, the results of the mutation on both the gene and protein, as well as the phenotypic and enzymatic activities of the parental missense mutant in the absence or presence of the *sumA* missense suppressor. The fold increase in enzymatic activity due to the *sumA* missense suppressor is also given along with the percentage of wild-type enzymatic activity that is restored by the *sumA* missense suppressor. The phenotypes listed in Table 4 and Table 5 regarding the ability of the *sumA* missense suppressor to rescue *lacZ* or *xylE* missense mutants are shown in Figure 2 and Figure 3. As in the missense suppressor studies conducted in *E. coli* with *trpA* missense mutants or in *S. typhimurium* with *hisC* or *nadC* missense mutants, the suppression of the *lacZ* missense mutants could also be analyzed on minimal media, since the *sumA* missense suppressor could restore the growth of *lacZ* missense mutants on M9 lactose plates. However, as seen in Figure 2, the use of LB X-gal media to analyze the suppression of the *lacZ* missense mutants was much more sensitive.

Most of the *lacZ* and *xylE* missense mutants contained glycine-to-aspartic acid codon changes as expected, given that the *sumA* missense suppressor is a glyV tRNA Gly3 (GAU/C) missense suppressor that inserts a glycine amino acid instead of aspartic acid. All of the glycine-to-aspartic acid mutated codons were GGC to GAC changes and no GGU to GAU changes were observed. Interestingly, a large number of the missense mutants, 6 of 20, or 30%, contained codon changes other than a glycine-to-aspartic acid. For most of the missense mutants, the *sumA* missense suppressor restored an average of 2.53% of the wild-type enzyme activity. There was one notable exception. The *sumA* missense suppressor restored 30.28% of the wild-type enzyme activity of the *lacZ2454* missense mutant. While the *sumA* missense suppressor caused a lower fold increase in the activities of the *xylE* mutants than the *lacZ* mutants, the ability of *sumA* to restore wild-type enzymatic activities for the two proteins was very similar.

Analyzing the missense mutants with codon changes other than glycine-to-aspartic acid that are suppressible by sumA

The simplest explanation for why the *sumA* missense suppressor could suppress missense mutants with codon changes other than a glycine-to-aspartic acid was that an adjoining (connecting) or neighboring (contacting) amino acid was an aspartic acid whose conversion to glycine rescued the defective codon change. Using PyMOL, we determined whether any of the missense mutants that contained amino acid changes other than a glycine-to-aspartic acid also contained neighboring amino acids that were aspartic acid. All six of the missense mutants in question contained aspartic acid neighboring amino acids. Table 6 lists the codon changes in these missense mutants and the position and distance in angstroms of the neighboring aspartic acid. Half of the neighboring aspartic acids were coded by the GAC aspartic acid codon and half of the neighboring aspartic acids were coded by the GAU aspartic acid codon, both of which can be suppressed by the *sumA* missense suppressor. The neighboring aspartic acids were an average of 8.85 Å from the affected codon. For comparison purposes, the distance between aspartic acids and adjoining aspartic acids, or asparagine or leucine amino acids, the two amino acids that are structurally very similar to aspartic acid, ranged from 4.65 to 5.88 Å, with an average of 5.30 Å, for the β-galactosidase and catechol 2,3-dioxygenase proteins.

Inheritance test of mutations generated by sumA

The *glyV* tRNA Gly3(GAU/C) missense suppressor has also been identified as the *mutA* mutator in *E. coli* (Michaels *et al.* 1990; Slupska *et al.* 1996). While it is clear that the mutation rate is significantly higher in the *glyV* tRNA Gly3(GAU/C) missense suppressor than in wild-type *E. coli* (Michaels *et al.* 1990;

Table 4 Characterization of *lacZ* missense mutants that are suppressible by *sumA*

					With the *sumA* missense suppressor			
Mutant[a]	Base change[b]	Amino acid change[b]	Phenotype[c]	Activity Miller units[d]	Phenotype[c]	Activity Miller units[d]	Fold increase	Percentage of wild-type activity[e]
lacZ2234	1694 (G to A)	565 (G to D)	W	3.74	LB	99.65	26.64	2.71
lacZ2343	2828 (G to A)	943 (R to H)	VLB	9.78	LB	146.96	15.03	3.87
lacZ2381	2291 (G to A)	764 (G to D)	VLB	6.87	LB	89.17	12.98	2.32
lacZ2382	1697 (G to A)	566 (G to D)	W	3.84	LB	109.67	28.56	2.99
lacZ2396	1643 (G to A)	548 (G to D)	VLB	4.31	LB	65.55	15.21	1.73
lacZ2454	1061 (G to A)	354 (G to D)	W	3.86	LB	122.09	31.63	3.34
lacZ2504	623 (G to A)	208 (G to D)	VLB	4.19	LB	1076.28	256.87	30.28

[a] Of the 10 *lacZ* missense mutants that were sequenced, *lacZ231* was identical to *lacZ2234*, *lacZ2352* was identical to *lacZ2382*, and *lacZ2232* was identical to *lacZ2454*, and are not included in the table.
[b] The *lacZ* gene is 3075 bp in length and codes for the 1024 amino acid β-galactosidase protein. The resulting amino acid changes are given based on the coded protein predicted by the base sequence and not the Protein Data Bank file.
[c] The plate phenotypes are depicted as W for white, VLB for very light blue, and LB for light blue.
[d] β-Galactosidase assays were repeated in triplicate and the SD was <10%.
[e] The percentage of wild-type enzyme activity restored by the *sumA* missense suppressor is calculated with respect to the enzyme activity of wild-type β-galactosidase protein in TT18519 (*his*C10081::MudF[*lac*$^+$]), which was 3540.86 Miller units, after subtracting the activity of the nonsuppressed mutant.

Slupska *et al.* 1996; Murphy and Humayun 1997; Al Mamun *et al.* 1999, 2002; Dorazi *et al.* 2002; Balashov and Humayun 2004), no one has determined whether gain-of-function mutants generated in a *glyV* tRNA Gly3(GAU/C) missense suppressor are inheritable. Because it is quite possible that the increased mutation rate in gain-of-function mutants was due to the tolerance or potential benefit of aspartic acid-to-glycine amino acid changes induced by the *glyV* tRNA Gly3(GAU/C) missense suppressor, we decided to conduct an inheritance test. If some of the gain-of-function mutations occurred because of the aspartic acid-to-glycine amino changes generated by the *glyV* tRNA Gly3(GAU/C) missense suppressor then not all of the mutations isolated in a strain containing the *glyV* tRNA Gly3(GAU/C) missense suppressor would be functional in a wild-type strain that lacked the *glyV* tRNA Gly3(GAU/C) missense suppressor.

Initially we confirmed that the mutation rate of gain-of-function rifampicin-resistant mutants was significantly higher in a *S. enterica sumA* strain than in wild-type *S. enterica*. Rifampicin-resistant mutants were obtained and quantified as described in the *Materials and Methods* section and it was determined that rifampicin-resistant mutants occurred at a rate of 0.35 per 1×10^8 cells in wild-type *S. enterica* and 13.39 per 1×10^8 cells in *S. enterica sumA*. Thus the formation rate of rifampicin-resistant mutants increased by 38.26-fold in *S. enterica sumA* cells *vs.* wild type, a value that is consistent with previous studies. The inheritance test was conducted as described in the *Materials and Methods* section and we observed that the mutations that caused 50 independent rifampicin-resistant mutants in a *S. enterica sumA* strain could all be transduced back into the wild-type *S. enterica* strain and that rifampicin resistance was transferred. Thus

Table 5 Characterization of *xylE* missense mutants that are suppressible by *sumA*

					With the *sumA* missense suppressor			
Mutant[a]	Base change[b]	Amino acid change[b]	Phenotype[c]	Activity XylE units[d]	Phenotype[c]	Activity XylE units[d]	Fold Increase	Percentage of wild-type activity[e]
xylE1571	380 (G to A)	127 (G to E)	VLY	24.78	LY	34.69	1.40	2.64
xylE1579	26 (G to A)	9 (G to D)	VLY	24.15	LY	30.91	1.28	1.80
xylE1582	191 (G to A)	64 (G to D)	VLY	24.06	LY	30.32	1.26	1.67
xylE1583	473 (G to A)	158 (G to D)	VLY	24.12	LY	31.40	1.30	1.94
xylE1584	727 (C to T)	243 (P to S)	W	18.05	VLY	25.63	1.42	2.02
xylE1585	25 (G to A)	9 (G to S)	W	22.01	LY	31.03	1.41	2.40
xylE1587	532 (G to A)	178 (E to K)	W	18.71	LY	32.74	1.75	3.73
xylE1588	740 (G to A)	247 (G to D)	W	22.14	LY	33.65	1.52	3.06
xylE1589	845 (G to C)	282 (W to S)	W	23.50	LY	29.85	1.27	1.69

[a] Of the 10 *xylE* missense mutants that were sequenced, *xylE1570* was identical to *xylE1583* and is not included in the table.
[b] The *xylE* gene is 921 bp in length and codes for the 307 amino acid catechol 2,3-dioxygenase protein. The resulting amino acid changes are given based on the coded protein predicted by the base sequence and not the Protein Data Bank file.
[c] The plate phenotypes are depicted as W for white, VLY for very light yellow, and LY for light yellow.
[d] Catechol 2,3-dioxygenase assays were repeated in triplicate and the SD was <10%.
[e] The percentage of wild-type enzyme activity restored by the *sumA* missense suppressor is calculated with respect to the enzyme activity of wild-type catechol 2,3-dioxygenase protein in ALS1442 (*proB*::*xylE*[*cat*]), which was 375.87 XylE units, after subtracting the activity of the nonsuppressed mutant.

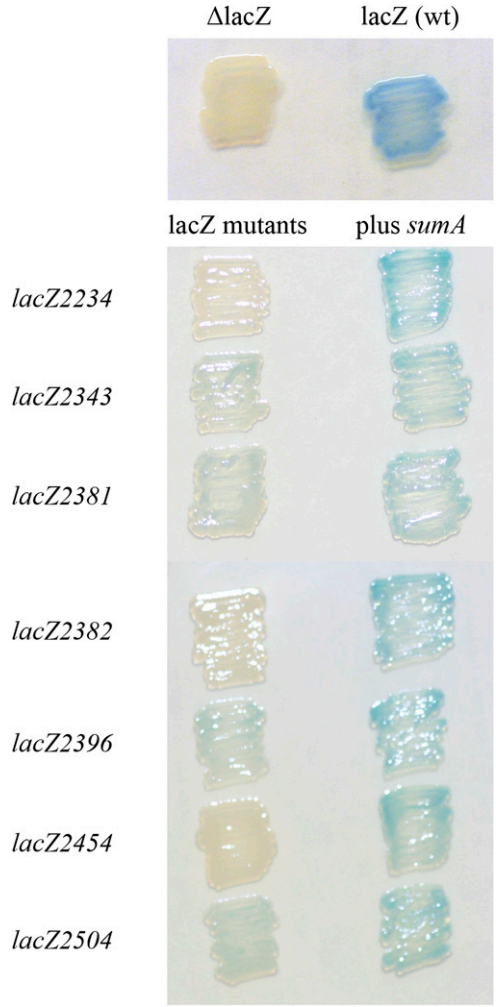

Figure 2 Suppression of *lacZ* missense mutants by the *sumA* missense suppressor. *lacZ* missense mutants with or without the *sumA* missense suppressor were patched onto LB, 1 mM IPTG, 40 μg/ml X-Gal plates, and incubated at 37° for 16 hr. Wild-type *S. enterica* (*lacZ*$^-$) and *S. enterica* with the *hisC10081*::MudF[*lac*] insertion are included as controls.

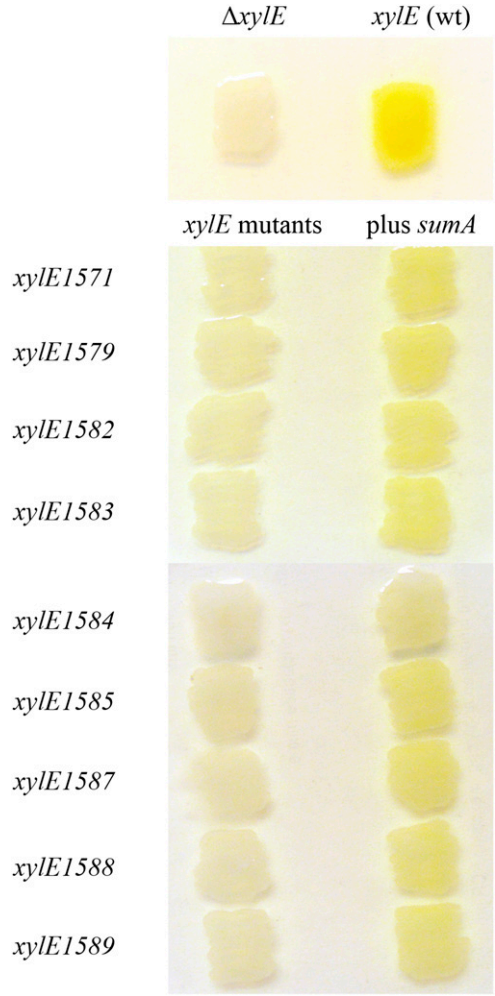

Figure 3 Suppression of *xylE* missense mutants by the *sumA* missense suppressor. *xylE* missense mutants with or without the *sumA* missense suppressor were patched onto LB, 1 mM IPTG plates, and incubated at 37° for 16 hr after which the plates were sprayed with a light mist of 100 mM catechol. Wild-type *S. enterica* (*xylE*$^-$) and *S. enterica* with the *proB*::*xylE*(*cat*) insertion are included as controls.

all of the rifampicin-resistant mutations isolated in the *S. enterica sumA* strain were inheritable by the *S. enterica* wild-type strain.

The sumA missense suppressor causes a significant reduction in the growth rate

We noticed that strains containing the *sumA* missense suppressor grew significantly slower than strains that lacked the *sumA* missense suppressor under both suppressing and nonsuppressing conditions. For this reason, we determined the growth rate of the isogenic strains TT16237 (*sumA*) and ALS234 (wild type) in LB media. The growth rate and doubling time of the strain containing the *sumA* missense mutation was $1.0486 \times 10^{-2} \cdot \text{min}^{-1}$ and 66.10 min, respectively, while the growth rate and doubling time of the wild-type strain was $1.5460 \times 10^{-2} \cdot \text{min}^{-1}$ and 44.83 min, respectively. Thus the presence of the *sumA* missense suppressor reduced the growth rate or doubling time of wild-type *S. enterica* by 47.43%.

Discussion

In this study, we have shown that the *S. enterica* serovar Typhimurium *sumA* missense suppressor is caused by a GCC-to-GUC change in the anticodon of one of the three copies of the Gly3 tRNA, which can cause a glycine amino acid to be inserted instead of an aspartic acid at GAU or GAC codons. Thus the *sumA* missense suppressor is more accurately designated as a *glyV* tRNA Gly3(GAU/C) missense suppressor. While the efficiency of the *sumA* missense suppressor was similar to the efficiency of other missense suppressors that have been characterized, it was verified to be promiscuous or broad acting and able to rescue a large number of missense mutants, a trait that has only been observed in one other missense suppressor, the *glyU* tRNA Gly1(GAG) missense suppressor, which can cause a glycine amino acid to be inserted instead of a glutamic acid. The *sumA* missense suppressor was verified to be identical to the *glyV* tRNA

Table 6 Position and distance of the neighboring (contacting) aspartic acids in missense mutants that contain codon changes other than a glycine to aspartic acid

Mutant	Amino acid change	Neighboring aspartic acid	Distance (Å)
lacZ2343	943 (R to H)	660	9.16
		955	5.93
xylE1571	127 (G to E)	51	6.72
		133	8.39
xylE1584	243 (P to S)	295	10.66
xylE1585	9 (G to S)	51	8.31
xylE1587	178 (E to K)	271	8.49
xylE1589	282 (W to S)	182	12.08
		285	9.93

PyMOL Molecular Graphics System (Schrödinger) was used to determine the distance of neighboring aspartic acids. The Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) was the source of the three-dimensional crystal structure data. The 1DP0 file was used for the β-galactosidase crystal structure data (Juers *et al.* 2000) and the 1MPY file was used for the catechol 2,3-dioxygenase crystal structure data (Kita *et al.* 1999).

Gly3(GAU/C) missense suppressor that was isolated in *E. coli* and like its *E. coli* counterpart, was shown to be capable of acting as a mutator. Just as the *glyV* tRNA Gly3(GAU/C) *sumA* missense suppressor has proven useful as a research tool in *S. enterica*, it could be used in *E. coli* as well to identify missense mutants.

The suppression efficiencies for the *sumA* missense suppressor ranged from 1.67 to 3.87% of the wild-type enzyme activity for the *lacZ* and *xylE* missense mutants that were tested with one exception. The *sumA* missense suppressor restored 30.28% of the wild-type enzyme activity for the *lacZ2504* missense mutant. In the suppression efficiency studies that have been conducted on other missense suppressors, the suppression efficiencies ranged from 1.10 to 3.60% with two notable exceptions (Brody and Yanofsky 1963; Berger and Yanofsky 1967; Hill *et al.* 1970, 1974). The *glyU* tRNA Gly1(AGA) missense suppressor restored 10.50% of the wild-type enzyme activity of the *trpA36* missense mutant and the *glyT* tRNA Gly2(AGA) missense suppressor restored 27.00% of the wild-type enzyme activity of the *trpA36* missense mutant (Brody and Yanofsky 1963; Hill *et al.* 1970). Thus the results of our efficiency studies with the *sumA* missense suppressor are in very close agreement with the efficiency studies for other missense suppressors. With only a couple of exceptions, the suppression efficiencies of missense suppressors range from 1.10 to 3.87%.

If one combines the data on the specificity of suppression of the *sumA* missense suppressor from the two previous studies (Whitfield *et al.* 1966; Hughes *et al.* 1991) and our study, *sumA* suppressed 53 of 320, or 16.56%, of the missense mutants that have been tested. Only one other broad-acting missense suppressor has been characterized, the *glyU* tRNA Gly1(GAG) missense suppressor. Table 7 compares the data on the narrow *vs.* broadly acting missense suppressors that have been definitively characterized. The data on the frequency of codon usage is from Maloy *et al.* (1996) and the data on the abundance of the tRNAs is from Dong *et al.* (1996). If one considers all the factors that determine whether a missense suppressor might be predicted to be broad acting, the data in Table 7 clearly indicates that the *sumA* missense suppressor is expected to be broad acting. Most important among these factors is codon usage. The GGC glycine codon, which is the codon most often mutated in the *sumA* suppressible missense mutants that were sequenced, is the sixth most abundant codon found in translated genes and occurs at a frequency of 30 codons per 1000 total codons. The GGU glycine codon, which can also be mutated to generate *sumA* suppressible missense mutants, is the eighth most abundant codon found in translated genes and occurs at a frequency of 28 codons per 1000 total codons. Only the CUG leucine codon, the GAA glutamic acid codon, the AAA lysine codon, the GAU aspartic acid codon, and the GCG alanine codon, which occur at frequencies of 52, 44, 38, 33, and 32 codons per 1000 total codons, respectively, are more widely utilized than the GGC glycine codon. A second factor to consider is how prevalent the mutated codon change is in translated genes. The GAU and GAC aspartic acid codons are both suppressible by the *sumA* missense suppressor and occur at very high frequencies in translated genes. The GAU aspartic acid codon is the fourth most abundant codon found in translated genes and the GAC aspartic acid codon ranks in the upper third of codons that are found most often in translated genes. A third factor to consider is tRNA abundance, since mutated tRNAs that are more abundant are more likely to be available for suppression. Among the tRNAs, only the Arg2, Glu2, and Leu1 tRNAs, which constitute 7.37, 7.32, and 6.95%, respectively, of the total tRNA population, are more abundant than the Gly3 tRNA, which constitutes 6.76% of the total tRNA population and is modified to form the *sumA* missense suppressor. Additionally, as detailed later in the *Discussion*, aspartic acid-to-glycine amino acid changes are very tolerable with respect to protein structure.

While not as strong an argument can be made for the *glyU* tRNA Gly1(GAG) missense suppressor being a broad-acting missense suppressor compared to the *sumA* missense suppressor based on the data in Table 7, a compelling case can be made in comparison to the *glyT* tRNA Gly2(AGA) and *serU* tRNA Ser2(UUG) missense suppressors, which have very narrow specificities. The abundance of the GGG glycine codon in translated genes is below average, but definitely not considered to be a rare codon, and the mutated GAG glutamic acid codon ranks in the upper third of codons that occur at very high frequencies in translated genes. Only eight other tRNAs are more abundant than the Gly2 tRNA. Additionally, glutamic acid-to-glycine changes should also be very tolerable with respect to protein structure. One must exercise caution, however, because the assessment of the broad-acting ability of the *glyU* tRNA Gly1(GAG) missense suppressor is based on a single study with a very limited number of missense mutants (Eggertsson and Adelberg 1965). It would be very interesting to determine whether

Table 7 Data on allele specific *vs.* broad acting missense suppressors

Original tRNA	Missense suppressor	Suppression	Original codon abundance[a]	Mutated codon abundance[a]	tRNA abundance[b] (%)
glyT tRNA Gly2(GGA/G)	*glyT* tRNA Gly2(AGA)[c]	Narrow	GGA (7/1000) GGG (9/1000)	AGA (2/1000)	3.31
glyU tRNA Gly1(GGG)	*glyU* tRNA Gly1(GAG)[d]	Broad	GGG (9/1000)	GAG (19/1000)	3.31
glyV tRNA Gly3(GGU/C)	*glyV* tRNA Gly3(GAU/C)[e]	Broad	GGU (28/1000) GGC (30/1000)	GAU (33/1000) GAC (23/1000)	6.76
serU tRNA Ser2(UCG)	*serU* tRNA Ser2(UUG)[f]	Narrow	UCG (8/1000)	UUG (11/1000)	0.53

[a] The frequency that the codon occurs per 1000 amino acids among all *E. coli* proteins. Data are taken from Maloy *et al.* (1996).
[b] The abundance of the tRNA per the total tRNA. Data are taken from Dong *et al.* (1996).
[c] Brody and Yanofsky (1963) and Roberts and Carbon (1975).
[d] Eggertsson and Adelberg (1965) and Hill *et al.* (1974).
[e] Whitfield *et al.* (1966) and this study.
[f] Eggertsson and Adelberg (1965) and Thorbjarnardóttir *et al.* (1985).

the *glyU* tRNA Gly1(GAG) missense suppressor was truly broad acting in a study involving a greater number of missense mutants.

Given that most mutants, which are isolated spontaneously or through the use of ultraviolet radiation or chemical mutagenesis, are either C-to-T or G-to-A transitions, if one considers the factors required to yield a broad-acting missense suppressor that were discussed above, one other missense suppressor should be isolatable that would be expected to rescue a large number of missense mutants similar to the *sumA* missense suppressor. A tRNA Glu2(AAA/G) missense suppressor that recognizes AAA or AAG lysine codons could be derived from tRNA Glu2(GAA/G), which normally recognizes GAA or GAG glutamic acid codons. The GAA glutamic acid codon is the second most abundant codon, the AAA lysine codon is the third most abundant codon, and the Glu2 tRNA is the second most abundant tRNA. Additionally, missense mutants that resulted from glutamic acid-to-lysine changes would be very disruptive to protein structure and function due to the acidic-to-basic change and thus be expected to be quite prevalent. The equivalent of the predicted tRNA Glu2(AAA/G) missense suppressor has been isolated in *Saccharomyces cerevisiae* (Su *et al.* 1990). The *S. cerevisiae SOE1* tRNA 3Glu(AAA/G) missense suppressor suppressed five of eight, or 62.5% of the potential missense mutants that were analyzed. The mutants that were analyzed in this study were not known definitively to be missense mutants and it would be interesting to isolate and characterize a tRNA Glu2(AAA/G) missense suppressor in *S. enterica*, where large sets of missense mutants are available for analysis.

Six of the 20, or 30%, of the missense mutants that were suppressible by the *sumA* missense suppressor contained a mutation other than glycine to aspartic acid. Interestingly, all six of these missense mutants contained neighboring (contacting) aspartic acids that were in close proximity to the mutated amino acid. These neighboring aspartic acids, which could be changed from an aspartic acid to a glycine by the *sumA* missense suppressor, were within an average of 8.85 Å from the mutated amino acid. To put these distances into perspective, the distances between aspartic acids and adjoining aspartic acids or adjoining asparagine or leucine amino acids, the amino acids most structurally similar to aspartic acid, ranged from 4.65 to 5.88 Å for the β-galactosidase and catechol 2,3-dioxygenase proteins. Thus in all cases, missense mutants that contained mutations other than a glycine-to-aspartic acid change also contained a very near neighboring aspartic acid that was in close contact and enabled suppression to occur. This observation reinforces the fact that aspartic acid-to-glycine changes are very well tolerated in proteins. Seven of the nine neighboring aspartic acids were in connecting loops or turns. Glycine is the most prevalent amino acid that is found in connecting loops or turns and the conversion of aspartic acid to glycine by the *sumA* missense suppressor would arguably give the connecting loop or turn greater flexibility. In a study of the amino acids found in turns between two α-helices, glycine was the most prevalent amino acid and occurred 39.39% of the time, followed by valine and serine, both of which occurred at a frequency of 18.18% (Shestopalov 1988). In a study of the amino acids found in the loops between two β-sheets, glycine was the most prevalent amino acid and occurred 14.42% of the time, followed by aspartic acid, serine, and asparagine, which occurred at a frequency of 9.67, 9.23, and 9.09%, respectively (Minuchehr and Goliaei 2005). In the β-galactosidase and catechol 2,3-dioxygenase proteins, glycine was the most prevalent amino acid found in connecting loops or turns and occurred 11.26% of the time, followed by proline and aspartic acid, which occurred at a frequency of 9.92 and 8.91%, respectively. While aspartic acid is a prevalent amino acid in connecting loops or turns, glycine is preferred and arguably an aspartic acid-to-glycine conversion gives the connecting loop or turn greater flexibility.

The *glyV* tRNA Gly3(GAU/C) *sumA* missense suppressor has also been identified and characterized as the *mutA* mutator (Michaels *et al.* 1990; Slupska *et al.* 1996) and numerous studies have confirmed that the presence of the *glyV* tRNA Gly3(GAU/C) missense suppressor, or *mutA*, increases the spontaneous generation rate of mutants (Michaels *et al.* 1990; Slupska *et al.* 1996; Murphy and Humayun 1997; Al Mamun *et al.* 1999, 2002; Dorazi

et al. 2002; Balashov and Humayun 2004). The glyV tRNA Gly3(GAU/C) missense suppressor appears to cause a change in DNA polymerase III, which acts as a mutator, although the nature of this change has not been determined (Al Mamun *et al.* 2002). In all of the studies that have been conducted on the ability of the *glyV* tRNA Gly3(GAU/C) missense suppressor to act as a mutator, not one has determined whether the increase in the mutational rate is due in part to an increase in mutants whose function would be restored by the presence of the *glyV* tRNA Gly3(GAU/C) missense suppressor, so we conducted a gain-of-function inheritance test to determine whether this was the case. Fifty independent rifampicin-resistant mutants were isolated in a *glyV* tRNA Gly3(GAU/C) missense suppressor and transduction tests were conducted to verify that all 50 of the rifampicin-resistant mutants were functional when transduced back into a wild-type background that lacked the *glyV* tRNA Gly3(GAU/C) missense suppressor, thus definitively proving that the *glyV* tRNA Gly3(GAU/C) *sumA* missense suppressor can act as a mutator due to the change in DNA polymerase III, and that the increase in the mutational frequency is not due to an increase in mutants, which can be rescued by the *glyV* tRNA Gly3(GAU/C) missense suppressor.

Just as the *glyV* tRNA Gly3(GAU/C) *sumA* missense suppressor can act as a mutator by altering DNA polymerase III, it is possible that the reason *sumA* acts broadly and suppresses missense mutants caused by amino acid changes other than a glycine to aspartic acid, is due to its ability to alter the translational process by the ribosome. Alterations in the ribosomal P, A, or E sites or ribosomal release or elongation factors could be expected to cause mistranslation. While this might be the case, we feel that it is unlikely, since all of the suppressible missense mutants that were not glycine-to-aspartic acid amino acid changes as expected on the basis of codon anticodon pairing contained neighboring aspartic acids that could be converted to glycine by the *sumA* missense suppressor.

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