An editing-defective aminoacyl-tRNA synthetase is mutagenic in aging bacteria via the SOS response

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Mistranslation in bacterial and mammalian cells leads to production of statistical proteins that are, in turn, associated with specific cell or animal pathologies, including death of bacterial cells, apoptosis of mammalian cells in culture, and neurodegeneration in the mouse. A major source of mistranslation comes from heritable defects in the editing activities of aminoacyl-tRNA synthetases. These activities clear errors of aminoacylation by deacylation of mischarged tRNAs. We hypothesized that, in addition to previously reported phenotypes in bacterial and mammalian systems, errors of aminoacylation could be mutagenic and lead to disease. As a first step in testing this hypothesis, the effect of an editing defect in a single tRNA synthetase on the accumulation of mutations in aging bacteria was investigated. A striking, statistically significant, enhancement of the mutation rate in aging bacteria was found. This enhancement comes from an increase in error-prone DNA repair through induction of the bacterial SOS response. Thus, mistranslation, as caused by an editing-defective tRNA synthetase, can lead to heritable genetic changes that could, in principle, be linked to disease.

aminoacylation errors | error-prone DNA polymerases | amino acid misincorporation | genetic code ambiguity

As organisms age, mutations accumulate over time owing to direct environmental insults that are incompletely or inaccurately repaired. Recent work directed our attention to the possibility that mistranslation could play a role in producing mutations in aging populations. In particular, mistranslation can arise from editing defects in aminoacyl-tRNA synthetases. These enzymes catalyze the first step of protein synthesis, where each amino acid is linked to its cognate tRNA bearing the anticodon triplet associated with the amino acid. Because of inherent physiochemical limitations of some enzymes to discriminate between structurally similar amino acids, mischarged tRNAs, such as Val-tRNA^{Ile} or Ser-tRNA^{Ala}, are produced. Normally, these mischarged tRNAs are cleared by hydrolytic editing, which occurs at a distinct active site within the synthetase. But even a small defect, caused by a mutation in the editing center, can lead to extreme cell pathologies and disease.

For example, mice that carry a mild mutation in the editing domain of alanyl-tRNA synthetase suffer from ataxia (1). The mutant alanyl-tRNA synthetase mischarges tRNA^{Ala} with serine and, as a consequence, mistranslation occurs that leads to induction of the unfolded protein response. That, in turn, leads to the degeneration of Purkinje cells in the cerebellum, beginning within 3 weeks of age. Similarly, in human cell culture, an inducible, editing-defective valyl-tRNA synthetase caused cellular degradation and apoptosis (2).

With these recent results in mind, we were motivated to investigate the possibility that mistranslation could also lead to heritable genetic changes. Previously, we generated a knock-in mutant allele of *ileS* that encodes no functional editing domain (3). This *ileS*_{Ala} allele has a critical section of the coding sequence of the editing domain replaced with 11 codons for alanine. Relative to the isogenic wild-type strain, this strain had a diminished growth rate, was highly sensitive to a structural

analogue of isoleucine, and had an increased sensitivity to antibiotics (4). Surprisingly, the editing-defective mutant was no more sensitive to mutagens and had no greater mutation frequency than the isogenic wild-type strain (4). Given the gradual cellular degeneration observed in Purkinje cells in response to an editing-defective alanyl-tRNA synthetase (1), we wondered whether aging might also lead to accumulated genetic degeneration in a bacterial model system. If so, then a connection of mistranslation might be made to the long-standing observations of the accumulation of genetic mutations in aging individuals that lead to disease.

Results

Testing for the Mutagenesis in Aging Colonies (MAC) Phenotype. As stated above, cellular degeneration has been observed *in vitro* and *in vivo* as a result of genetic code ambiguity (1, 2). In light of these observations, we hypothesized that mutation-causing systems might also be induced in editing-defective cells. Here the rationale was that mistranslation could in principle generate statistical variants of proteins associated with DNA replication and repair. Some fraction of these variants could cause DNA breaks and thereby induce the SOS response. To initiate investigations and tests of this hypothesis, we chose a bacterial system where the DNA replication machinery has been well studied and where aging organisms could be examined in some detail.

Following this line of reasoning, the susceptibility to mutation of wild-type and *ileS*_{Ala} (editing-defective) *Escherichia coli* was explored under conditions of aging. Such studies are referred to as MAC. These require plating bacteria on solid media for either 1 or 7 days, followed by determining the frequency of spontaneous rifampicin (Rif)-resistant (Rif^R) mutants in the population after each time period (5–7). This phenotype is known to at least partially depend on the SOS response. The latter response includes, among others, the LexA-dependent induction of error-prone DNA polymerase (pol) II (encoded by *polB*), pol IV (encoded by *dinB*), and pol V (encoded by *umuDC*). [DNA pol II is a high-fidelity DNA pol when restarting replication, but error-prone when correcting certain types of DNA damage (8).]

We determined the MAC phenotype for wild-type and *ileS*_{Ala} *E. coli*. Bacteria were allowed to grow as colonies for either 1 or 7 days. At the end of each period, all of the colonies on each assay plate were collectively resuspended in liquid media and titered for viable cells and the number of Rif^R cells in the population. Little difference in the spontaneous appearance of Rif^R colonies was observed between strains after 1 day (Mann–Whitney *U* test; *n* = 67 and 68 for wild-type and *ileS*_{Ala} strains, respectively; *P* = 0.8) (9). After 7 days incubation on LB agar, the frequency of

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Abbreviations: MAC, mutagenesis in aging colonies; Ci, ciprofloxacin; Ci^R, Ci-resistant; Rif, rifampicin; Rif^R, Rif-resistant; pol, polymerase; MIC, minimum inhibitory concentration; Kan, kanamycin; Kan^R, Kan-resistant.

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Fig. 1. MAC of *ileS_{Ala}* editing-defective and wild-type *E. coli*. Frequencies of spontaneous mutants that had acquired resistance to Rif were determined for >60 replicates of each genotype after 1 and 7 days of growth on LB. (A) Shown are the median *Rif^R* frequencies for day 7 relative to those of day 1 for wild-type and editing-defective strains. (B and C) Each frequency observation was converted to its log value, binned, and counted. Shown is the fraction of observations in each bin. (B) No differences were observed between wild-type and *ileS_{Ala}* strains after 1 day (Mann–Whitney *U* test; $n = 67$ and 68 for wild-type and editing-defective strains, respectively; $P = 0.8$). (C) After 7 days, the frequency of spontaneous *Rif^R* had increased in both wild-type and editing-defective strains. The increase in *Rif^R* frequency was significantly greater in the editing-defective strain (Mann–Whitney *U* test; $n = 69$ for each strain; $P < 0.05$). See Results for further discussion of the distribution of day-7 *Rif^R* mutants.

spontaneous mutants increased in both strains, but to a significantly greater extent in the editing-defective strain (Mann–Whitney *U* test; $n = 69$ for both strains; $P < 0.05$).

Wild-type *E. coli* (strain MG1655) is reported to have a 5.5-fold increased frequency of *Rif^R* clones after MAC (comparing median of day 7 to median of day 1) (5). Similarly, the wild-type strain used here, which is derived from strain MG1655, had an increased mutation frequency of ≈ 6 -fold (Fig. 1A). By comparison, the median value of *Rif^R* frequency increased ≈ 15 -fold in the editing-defective strain. The change in mutation frequency at day 7 is highly significant relative to day 1, for both editing-defective and wild-type strains (Mann–Whitney *U* test; $P \ll 0.0005$ for each).

Analysis of Frequency of *Rif^R* Spontaneous Mutants. From the total number of observations of the frequency of *Rif^R* spontaneous mutants, the observations that fell within given ranges of *Rif^R* frequencies were grouped into “bins” (Fig. 1B). The frequency distributions of binned frequencies of *Rif^R* mutants illustrate the variation inherent in genotype/time period combinations. In addition, the binned frequencies of spontaneous *Rif^R* mutants on day 7 revealed a bimodal distribution. This distribution may be indicative of a subpopulation that is experiencing an increased mutation rate.

The putative high- and low-mutation frequency subpopulations were separately analyzed for statistical significance. A Mann–Whitney *U* test revealed a significant difference between the highly mutated *ileS_{Ala}* and wild-type subpopulations ($P < 0.005$), whereas the *ileS_{Ala}* and wild-type subpopulations that experienced lower levels of mutations were not significantly different ($P = 0.14$). (The same test revealed that the subpopulations with lower mutation rates after 7 days still had a significantly greater mutation frequency than after 1 day; $P \ll 0.0005$ for both wild-type and *ileS_{Ala}* strains.) The bimodal distribution is indicative of a subpopulation that is experiencing a higher mutation rate, and this effect is greatly exacerbated in the editing-defective strain. Median mutation frequencies for the more frequently mutating subpopulations were much greater than for the population as a whole. The editing-defective strain had increased in mutation frequency 330-fold, whereas the wild-type strain had an increased mutation frequency of ≈ 95 -fold.

DNA Sequence Analysis of Mutated *rpoB*. The target of Rif is the β -subunit of RNA pol, so that mutations that confer *Rif^R* localize to the *rpoB* gene (10). In particular, *Rif^R* mutations typically fall in one of three clusters of *rpoB*. These regions have been extensively sequenced and characterized in earlier work (10–12). To examine the mutations in detail, 48 clonal DNA isolates were sequenced for each genotype (wild-type and *ileS_{Ala}*) after each time period (1 and 7 days). Of course, clones isolated from the same plate (LB plus Rif) could not be definitively characterized as independent mutations if the genetic change was identical. As a consequence of this consideration, we were limited to 39 and 29 unique mutants for *ileS_{Ala}* strains and 38 and 30 unique mutants for the wild-type strains on days 1 and 7, respectively.

The frequencies of each type of transition and transversion and the total frequency of insertions were determined (Fig. 2A). The GC $\rightarrow$ AT transition was most common and was identified in >50% of all mutations for both the wild-type and *ileS_{Ala}* strains. The frequency of GC $\rightarrow$ TA transversions increased by ≈ 2 -fold on day 7 relative to day 1. This distribution of base-pair changes is similar to previous *Rif^R* mutations that were caused by MAC in other tested strains (5). Thus, the enhanced mutation rate of the *ileS_{Ala}* strain yields the same distribution of specific transitions and transversions as seen with the wild-type organism (χ^2 test, $P = 0.82$ for day 1; χ^2 test, $P = 0.47$ for day 7).

Certain mechanisms of mutation are known to result in “signatures,” where only specific types of genetic changes occur (11, 12). The GC $\rightarrow$ AT mutation is a signature for DNA-repair-

Fig. 3. Ci -induced mutations in the editing-defective and wild-type strains. Editing-defective and wild-type strains were exposed to Ci to determine the extent of SOS response-induced mutagenesis in each strain. (A) The cumulative frequency of Ci^R mutants after incubation on minimal media plates plus Ci is shown for editing-defective and wild-type strains. (B) The MICs of clones resistant to Ci from day 2 (preexposure) and days 5–7 (postexposure) reveal a functional difference in Ci^R mutations acquired before and after exposure to Ci . Shown is the relative MIC of preexposure and postexposure mutants [i.e., $\log_2$ (mean preexposure MIC) – $\log_2$ (mean postexposure MIC)]. Error bars represent SEM. (C) The *ileS_{Ala}* editing-defective mutation was combined with *lexA*(wt)- Kan^R and *lexA*(S119A)- Kan^R . The latter mutation inhibits autocleavage of LexA, making the mutant protein a constitutive repressor and preventing induction of the SOS response. Shown is the accumulation of Ci^R mutants in editing-defective and editing-defective/SOS response-inhibited strains after incubation on minimal media plus Ci .

overnight cultures of both the wild-type and editing-deficient strain. This result is consistent with the SOS response being active even in culture conditions. The differences in mutation rate between the *ileS_{Ala}/lexA*(S119A) and the *ileS_{Ala}/lexA*⁺ strains was especially striking (Fig. 3C). In particular, the *lexA*-deficient, editing-defective strain had a far lower mutation rate. These results demonstrated that the inability to induce the SOS response resulted in a dramatically diminished accumulation of Ci^R mutants.

Discussion

Our work has associated translational errors to mutagenesis via the induction of the SOS response (Fig. 3). Although work by others (29–31) showed translational errors could increase mutation rates under certain conditions, the connection to the SOS response established in this work may be specific to the way that mistranslation was induced. Because of functional ablation of the editing domain of the isoleucyl-tRNA synthetase, all proteins are synthesized as statistical entities, meaning that isoleucine is replaced at a low frequency by valine (and other naturally occurring loose structural analogs of isoleucine). Some of these statistical proteins are involved in DNA replication and repair. Some may fail to function adequately because of their statistical nature.

There are several candidate enzymes that may induce the SOS response by increasing the frequency of breaks in DNA (25). These include topoisomerases and gyrases (the same class of enzymes that are the targets of Ci), which relax supercoiling. Similarly, recombinases such as RecG or RuvC promote strand migration to begin the process that ultimately relieves lesion blockage of pols. PriA and pol II are candidates as well because they reestablish strand polymerization after lesion repair. If any of these enzymes were to malfunction because of translational errors, free DNA ends may result. [In addition to the effect of mistranslation on SOS-induced enzymes, we considered more general effects of mistranslation that could induce the SOS response. For example, based on the types of *rpoB* mutations (especially GC → AT; Fig. 2A), one or both of oxidation and alkylation are occurring at elevated levels in aging bacteria (5, 13, 14). Lesions that cause DNA replication to stall or arrest may occur if the enzymes normally responsible for repair of DNA oxidation or alkylation are impaired because of mistranslation in the editing-defective strain (25). These lesions would result in

DNA breaks.] In either case, the free DNA ends would initiate the formation of RecA nucleoprotein filaments that can then induce LexA self-cleavage and induce the SOS response. Of course, the SOS response itself is likely to be mutagenic.

It was a formal possibility that LexA with translational errors have a diminished ability to regulate the SOS response, which results in SOS response induction. However, the SOS response remains inhibited in the *lexA*(S119A) mutant (Fig. 3C), showing that the regulatory function of LexA is retained.

Historically, the major assay for MAC has been the spontaneous accumulation of mutations that confer Rif resistance (5–7). We followed this precedent, recognizing that the detection of mutations caused by MAC can vary depending on the antibiotic resistance being tested (5). This variance is doubtless caused by intrinsic differences in the mechanism of action of each antibiotic, making some better than others for mutation detection. (We confirmed this kind of variance by testing for resistance to fosfomycin, which, although weaker with regard to the frequency of spontaneous mutants that result in resistance to Rif, has previously been shown to have a significant MAC phenotype. The results with the wild type and the editing-defective strain were inconclusive.)

In our work with Rif, the dramatic increase after 7 days in the frequency of *rpoB* mutations at R529 suggested a selective advantage conferred by this particular mutation. This particular mutation may result in similar effects to mutations known to affect the interaction between ppGpp, the alarmone signal of the stringent response, and RpoB. These mutations are known to limit RNA polymerization (26), resulting in fewer ribosomes to synthesize proteins (27). The mutants, therefore, suffer less from amino acid starvation, the cause of the stringent response (27). Following this line of reasoning, all combinations of *relA/relA*⁺ and *ileS_{Ala}/ileS*⁺ were constructed in *E. coli* and tested for the MAC phenotype. However, no differences were observed for *relA/relA*⁺ strains (data not shown).

We also considered the possibility that the enhanced mutation rate coming from mistranslation in aging bacteria may be caused, in particular, by the variant protein structures resulting from misincorporation of amino acid metabolites in the cell that are not among the canonical 20 amino acids. Examples include the naturally occurring metabolites α -aminobutyrate, homocysteine, and norvaline (28). [Owing to its large hydrophobic binding pocket that must accommodate the isopropyl side chain of

isoleucine, isoleucyl-tRNA synthetase weakly activates a broad spectrum of amino acids (28).] Thus, the inclusion in the media of an amino acid analog such as norvaline might increase the misfolded proteins within the cells, thereby increasing the SOS or stress response. Pursuing this possibility, sublethal levels of norvaline were tested for their effect on the SOS response in the editing-defective versus the wild-type strain. Each strain accumulated Ci^R mutants in the presence of norvaline at a comparable rate to when the analogue was absent (data not shown). Thus, the enhanced mutation rate caused by mistranslation in aging bacteria most likely occurs through mistranslation that inserts one or more of the standard, canonical amino acids and thereby sets up the system for induction of error-prone DNA pols.

Translational errors have been shown to result in an increased mutation frequency. In particular, *mutA* and *mutC* are alleles of *glyV* and *glyW*, respectively, which encode tRNAs specific for glycine (29). These mutants replace the tRNA anticodon for glycine with that for asparagine, resulting in statistical proteins that incur Asp → Gly substitutions. The source of the increased mutation rate is DnaQ, which provides an error-correcting exonuclease function to the replicative DNA pol III (30). This cause of increased mutation rate is known as translational stress-induced mutagenesis (TSM). The mechanism of TSM is not completely understood, however, as the phenotype appears to require several recombination-associated enzymes (31). By comparison, we have shown here the unambiguous involvement of a known pathway for mutagenesis, namely the SOS response. Other studies have shown that mutations that result from the SOS response are specifically caused by the activity of the error-prone DNA pols (21).

A number of human homologues of error-prone DNA pols have been identified (8). Several of these have been associated with various types of cancer. For example, DNA pol ι , an error-prone DNA pol, is expressed at elevated levels in breast cancer cells (32). Pol ι causes an increased level of mutagenesis, and immunodepleted nuclear extracts exhibited a diminished mutation frequency. Similarly, DNA pol κ , the human homolog of the *E. coli* DNA pol IV (*dinB*), is capable of translesion synthesis that can result in error-free, error-prone and -1 frameshifting, depending on the lesion (33–35). Pol κ can be up-regulated in cultured lung cancer cells and leads to loss of heterozygosity (LOH), the most common alteration found in tumors (36). LOH is likely induced by replication slippage, which is especially likely to be caused by the frameshifting effect of pol κ slippage, indicating that up-regulation of pol κ predisposes a cell to cancer. These specific cases are further supported by a more general study in which the expression of specialized DNA pols were examined in a number of tumor samples (37). In >45% of the samples, at least one specialized DNA pol was expressed at least 2-fold higher relative to the replicative DNA pols.

These examples serve to illustrate that the acquisition of a mutator phenotype may play a crucial role in susceptibility to cancer (38–40). Our bacterial system has shown that mutations in the editing site of a tRNA synthetase can stimulate induction of error-prone DNA pols. Thus, while recent previous work demonstrated that a heritable mutation in the editing domain of a tRNA synthetase leads to cellular degeneration of Purkinje cells because of misfolded proteins (1), this work showed that such mutations also cause heritable genetic degeneration from the induction of error-prone DNA pols.

Methods

Strains and Media. *E. coli* strains PS8078, PS8079, and PS8080 all carry the *ileS_{Ala}* chromosomal mutation, encoding an 11-codon substitution of Ala codons in the isoleucyl-tRNA synthetase editing domain. Strains PS8229, PS8231, and PS8233 are wild-type derivatives. All six strains have been described (4). Strains

RTC0184 and RTC0185 are *E. coli* *lexA*(wt)–kanamycin-resistant (Kan^R) and *lexA*(S119A)– Kan^R , respectively, and were the gift of Ryan Cirz and Floyd Romesberg (The Scripps Research Institute). These strains carry the *lexA* gene, either wild-type or an uninducible mutant, in tandem with the gene that confers Kan^R , and are otherwise isogenic. These mutations were transferred to the *ileS_{Ala}* strains by P1 transduction using standard methods (41), generating three clones each of which combine *ileS_{Ala}* with either *lexA*(wt)– Kan^R or *lexA*(S119A)– Kan^R . Rich media were LB, supplemented as necessary with Rif (170 μ g/ml), Kan (50 μ g/ml), or fosfomycin (30 μ g/ml). Minimal media (42) were supplemented with 0.2% glucose (MSglc) and Ci (8 ng/ml) or norvaline (4 μ M) as necessary. Plates were made with 15 g/liter agar and measured to be 25 ml.

MAC. An increased propensity for mutation caused by aging was assayed essentially as described (7). In brief, 100–1,000 colonies were allowed to grow on solid LB agar for either 1 or 7 days. After this period, bacteria were scraped off of the plate in 2 ml of LB, and the titer of viable colonies was determined by serial dilution and spotting a 5- μ l aliquot on LB plates. In addition, the titer of spontaneous Rif^R mutants was determined. Growth of Rif^R mutants was rapid, and colonies were counted after overnight growth to ensure that Rif (which is moderately unstable) had not degraded. In each of six separate experiments, either three or four separate plates were assayed at each time point for each strain, PS8078, PS8079, PS8080, PS8229, PS8231, and PS8233, thereby resulting in >60 separate data points (Rif^R frequency determinations) for each genotype after each period. The Mann–Whitney *U* test was used to determine the statistical significance of differences in distributions (9). One additional experiment testing the acquisition of fosfomycin resistance was conducted.

Sequencing of *rpoB* Mutations. Two clones from each plate of two independent MAC experiments were isolated and grown in microplates such that a total of 48 clones of each genotype were isolated after 1 and 7 days. Clones were stored in LB plus 15% glycerol at -80°C . Clones were revived in 250 μ l of LB and grown overnight at 37°C . A 2- μ l aliquot of these overnight cultures was diluted to 10 μ l with water, lysed by boiling, and used as a template for PCR of the region of the *rpoB* gene that is known to harbor the locus for the majority of Rif^R -conferring mutations. As described, primers 1525-up (5'-ggcgatctggataccctgatgc) and 2198-down (5'-cggagtcacacggcaacagcac) were used to PCR-amplify this region (5) by using Platinum Taq HiFi (Invitrogen, Carlsbad, CA) and the thermocycling program, 35 $\times$ (94 $^\circ\text{C}$, 30 s/56 $^\circ\text{C}$, 30 s/68 $^\circ\text{C}$, 2 min, 30 s), 68 $^\circ\text{C}$ 10 min, 4 $^\circ\text{C}$. PCR products were purified (Qiagen, Valencia, CA) and sequenced with the primer 1525-up.

Mutation Rate Determination. Overnight MSglc cultures of strains were diluted 1:10 in PBS, and a 50- μ l sample was plated on MSglc plus Ci or MSglc plus Ci plus norvaline for *lexA*⁺ and *lexA*(wt)– Kan^R strains, whereas a 50- μ l sample of undiluted cultures was plated for *lexA*(S119)– Kan^R strains. The starting titer of each culture was determined by serial dilution and subsequent spotting of 5 μ l on LB. The number of new colonies on minimal media plates was counted each day after the first, and the positions of colonies were marked (21). Six replicate plates were counted for each strain. However, to determine the mutation rate, the number of viable cells that had not formed colonies needed to be determined (21). Therefore, on days 2 and 5, all of the visible colonies were excised from one plate per strain (equating to three plates per genotype). The remaining agar was mixed with 10 ml of PBS to elute the surviving bacteria. The number of viable cells in solution was then determined by spotting serial dilutions (5 μ l) on LB. The mass of agar used for

elution of viable, Ci^S cells was compared with the total mass of agar (before excision of visible colonies) to calculate the total number of viable, Ci^S cells that had not formed colonies.

Ci^R clones were saved by storing overnight cultures in LB plus 15% glycerol. To determine whether there was a functional difference between preexposure and postexposure mutations, the MIC of Ci^R clones was determined. Clones isolated from growth on MSglc plus Ci were revived in LB from day 2 (representing preexposure Ci^R mutants) and days 5–7 (representing postexposure Ci^R mutants). LB cultures were diluted 1:100 in MSglc and again grown overnight. These cultures were diluted 1:500 in 2 ml of PBS and allowed to coat the surface of MSglc plates, and excess liquid was then removed. Plates were

dried, and then a Ci Etest strip (AB Biodisk, Piscataway, NJ) was placed on the plate. Etest strips allow for the precise determination of the MIC.

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