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Stress-Induced β-Lactam Antibiotic Resistance Mutation and Sequences of Stationary-Phase Mutations in the *Escherichia coli* Chromosome▽

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In some enterobacterial pathogens, but not in *Escherichia coli*, loss-of-function mutations are a common route to clinically relevant β-lactam antibiotic resistance. We previously constructed an assay system for studying enterobacterial β-lactam resistance mutations using the well-developed genetics of *E. coli* by integrating enterobacterial *ampRC* genes into the *E. coli* chromosome. Like the cells of other enterobacteria, *E. coli* cells acquire β-lactam resistance by *ampD* mutation. Here we show that starvation and stress responses provoke *ampD* β-lactam resistance mutagenesis. When starved on lactose medium, Lac⁻ strains used in mutagenesis studies accumulate *ampD* β-lactam resistance mutations independent of Lac reversion. DNA double-strand break repair (DSBR) proteins and the SOS and RpoS stress responses are required for this mutagenesis, in agreement with the results obtained for *lac* reversion in these cells. Surprisingly, the stress-induced *ampD* mutations require DinB (DNA polymerase IV) and partially require error-prone DNA polymerase V, unlike *lac* mutagenesis, which requires only DinB. This assay demonstrates that real-world stressors, such as starvation, can induce clinically relevant resistance mutations. Finally, we used the *ampD* system to observe the true forward-mutation sequence spectrum of DSBR-associated stress-induced mutagenesis, for which previously only frameshift reversions were studied. We found that base substitutions outnumber frameshift mutations, as seen in other experimental systems showing stress-induced mutagenesis. The important evolutionary implication is that not only loss-of-function mutations but also change-of-function mutations can be generated by this mechanism.

Mutation is a primary cause of bacterial resistance to antibiotics. Some mutations promote resistance directly, such as *Escherichia coli gyrA and gyrB* mutations, which confer quinolone resistance (28). Other mutations ameliorate the otherwise deleterious effects on cell growth conferred by some antibiotic resistance mutations (34). Yet other mutations increase the mutation rate, thereby increasing the likelihood of acquiring a resistance mutation (71). Although antibiotic resistance is a major problem in modern medicine (75) and has been studied intensively, the mechanisms that generate resistance mutations are poorly understood.

Of particular interest in the clinical setting is whether environmental conditions encountered during bacterial infections might promote resistance mutagenesis. For example, variables such as antibiotic exposure can stimulate resistance mutagenesis (1, 10, 60, 64), and various natural environments have been shown to select cells with permanently increased general mutation rates due to mutator mutations (42, 50, 59). Presumably, these mutator mutations are selected because they promote rapid adjustment to changing environments, even though most mutations generated are likely to be deleterious. It is noteworthy that in the studies in which a "high" incidence of mutator mutants was found in commensal and pathogenic bacteria (42, 50, 59), although mutagenesis promotes adaptation, most of the colonizing bacteria were not mutator mutants, indicating that most cells adapted without permanent increases in the mutation rate (66). Environmental stresses have been demonstrated to induce transient, generally mutagenic pathways (for a review, see reference 21). For these reasons, common environmental stressors, such as starvation, have been postulated to stimulate mutagenesis leading to antibiotic resistance (34, 49). In this work we tested this idea directly and showed that starvation stress-induced mutagenesis can indeed induce β-lactam antibiotic resistance.

We studied β-lactam antibiotic resistance using an *E. coli* model system. β-Lactamases are enzymes that cleave and inactivate β-lactam antibiotics, promoting resistance. Chromosomally encoded AmpC β-lactamases confer β-lactam resistance in many pathogenic and opportunistic bacteria and are ubiquitous in all enterobacteria except the salmonellae, klebsiellae, and some others (33). *ampC* expression is inducible in all enterobacteria but *E. coli* and the shigellae (46). In inducible strains, *ampC* transcription is activated by the AmpR transcriptional activator (3) upon AmpR binding to its allosteric activator molecule, 1-6-anhydromuropeptide (30). AmpD converts the activator molecule to the blocker UDP-*N*-acetylmuramic acid-pentapeptide, which then binds AmpR and blocks *ampC* transcription (30). Thus, loss-of-function mutations in *ampD* lead to constitutive AmpC β-lactamase production and β-lactam resistance (31, 32, 43). *ampD* missense

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and nonsense mutations are common in AmpC-mediated β-lactam-resistant clinical isolates (40, 67).

Normally, *E. coli* lacks *ampR* and the *ampC* promoter that it controls (23) and so cannot become resistant via *ampD* mutation. However, *E. coli* cells carrying the *ampRC* genes of other enterobacteria in plasmids (44, 57) or in the chromosome (61) become resistant due to *ampD* loss-of-function mutations. We constructed a chromosomal model of *ampRC*-mediated β-lactam resistance to facilitate the use of *E. coli* genetics to study mutagenesis. This model has the following advantages: (i) the single-copy *ampRC* locus more closely resembles the situation in clinical isolates and (ii) the chromosomal *ampRC* system is not affected by mutations that increase the plasmid copy number so that (iii) it is possible to examine effects of mutations in genes affecting DNA repair-recombination and mutation, some of which destabilize plasmid replication (61). In this model, most or all β-lactam resistance mutations are *ampD* mutations, similar to those seen clinically (61).

We wanted to use the *ampRC* system as a forward mutational assay in the context of the well-characterized stress-induced mutagenesis in the Lac assay of *E. coli* (for a review, see reference 21). In this way, the experimental conditions could be replicated, and the results obtained could be directly integrated into the vast amount of data already available for the stress-induced mutagenesis pathway in this strain. Here, we used this *E. coli* model of enterobacterial β-lactam resistance mutation to address two problems. First, we demonstrated that starvation stress-induced mutagenesis mechanisms can induce β-lactam resistance in this clinically relevant model. Because starvation stress is thought to be a major feature of natural environments encountered by pathogens (14), this indicates that our extensive knowledge of stress-induced mutagenesis mechanisms and environmental conditions that induce them might be relevant to the generation of resistance mutations in nature.

Second, we show that the stress-induced mutagenesis mechanism that can generate a β-lactam resistance mutagenesis is a double-strand break (DSB) repair (DSBR)-associated mutagenesis mechanism, which is a mechanism that has been studied in detail using the *E. coli* Lac system (for a review, see reference 21), and we used the *ampD* assay to reveal the true sequence spectrum for DSBR-associated mutagenesis. Stress-induced mutagenesis in the Lac system requires DSBR proteins, the SOS DNA damage response, DinB error-prone DNA polymerase V (Pol IV), and the RpoS stationary-phase and general stress response transcriptional activator, and it results from a switch from high-fidelity DSBR to error-prone DSBR during stress (62). The requirement for an error-prone DNA polymerase and the requirement for stress responses are two features of mutagenesis in the Lac assay which are also found in many other stress-inducible mutagenesis mechanisms in *E. coli* and other species. DinB DNA polymerase is required for ciprofloxacin-induced resistance mutations in *E. coli* (10) and stationary-phase mutagenesis in starved cells of both *Pseudomonas putida* (38, 74) and *Bacillus subtilis* (70). The SOS response is required for ciprofloxacin-induced resistance mutations (10), as well as mutagenesis in aging colonies (72), in *E. coli*. RpoS is required for starvation-induced mutagenesis in *P. putida* (29), for mutagenesis in aging *E. coli* cells (5), and for stress-induced transposition-mediated deletions in *E. coli* (22). Another stress response, the competence regulon controlled by the *comA* and *comK* gene products, regulates mutagenesis in starved *B. subtilis* cells (69). Therefore, although several different stress-induced mutagenesis mechanisms seem to occur in nature, the Lac system shares many important features with many of these mechanisms and so is a reasonable general model.

Previously, although base substitution mutations were observed to be the products of other stress-induced mutagenesis mechanisms (10, 72), the mutations that were shown to be generated by the starvation stress-induced DSBR-associated mutagenic mechanism were only frameshift (−1 deletion) mutations. Only mutations that reverted frameshift alleles have been studied previously (7, 15, 16, 65, 76), and the DinB DNA polymerase required makes mostly −1 bp deletions when it is overproduced in vivo (36, 77). The *ampD* assay selects any loss-of-function mutation, and with this assay we found that stress-induced base substitutions outnumber frameshift mutations. The important evolutionary implication is that not only loss-of-function mutations but also change-of-function mutations can be generated by this stress-induced mutagenesis mechanism, as seen previously for quinolone-induced resistance mutations (10) and mutations in aging colonies (72).

MATERIALS AND METHODS

Strains and plasmids. The *E. coli* K-12 strains and plasmids used in this study are shown in Table 1. Phage P1-mediated transduction was performed using standard techniques (54).

Assay of stress-induced *ampD* mutations during starvation on lactose with *lac* strains. We used a modification of the procedure of Bull et al., who assayed chromosomal reversions of a *tet* frameshift allele during Lac assay experiments (7). For each strain tested, six independent 5-ml cultures were grown to saturation in minimal M9-glycerol medium (1×10^9 to 2×10^9 cells per ml), washed, and aliquoted in individual plating tubes, and a 20-fold excess of scavenger cells (cells of *lac* deletion strains that scavenge contaminating carbon sources [FC29 and SMR5522 when *ruvC* strains were tested]) was added to the "tester" cells. The cells were plated onto plates containing M9 medium with 10 μg/ml vitamin B_1 and 0.1% lactose in 2.5 ml of M9 top agar containing vitamin B_1 and lactose, and then they were overlaid with a second 2.5-ml layer of M9 top agar containing vitamin B_1 and lactose. At least 12 plates were generated for each culture (6 plates with 1× "tester" [*ampRC* mutation assay] cells and 6 plates with 2× "tester" cells) and then incubated at 37°C (or 30°C for experiments with poorly growing *ruv recG* strains). Every day or every other day, starting with the first day that cells were plated (day 0), one pair of plates per culture per strain was overlaid with 5 ml of M9 top agar containing 0.12 ml 50% glycerol, 20 μl of 100 mg/ml ampicillin (final concentration, 50 μg/ml), and 10 μl of 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal) (final concentration, 5 μg/ml). The ampicillin-containing plates were then incubated for an additional 3 days to allow growth of all Amp^r cells into colonies. After 3 days, white (Lac^-) colonies were counted. These colonies represented the number of cells that had mutated to Amp^r at any point in time between inoculation of the original culture and addition of the final ampicillin-glycerol overlay. In early experiments, ampicillin resistance was confirmed by patching colonies onto plates containing Luria-Bertani-Herskowitz medium (1% tryptone, 0.5% NaCl, 0.5% yeast extract, 2 μg/ml thymine, solidified with 1.5% agar) with ampicillin. Ampicillin resistance was attributed to mutation in the *ampD* gene by complementation with plasmid pJP19 (*ampD*$^+$) (61). Blue (Lac^+) Amp^r colonies were avoided, because the resistance mutation could have occurred during growth of the colony once the cell mutated to Lac^+. For all stress-induced Amp^r mutation experiments, a Lac^+ reversion assay (25) was performed in parallel to confirm that the strains tested reverted at expected rates (18, 24, 25, 53). The net cell viability (assayed as described by Harris et al. [25]) monitored during the experiments varied less than 2-fold for all experiments reported.

Sequencing *ampD* mutations. On day 5 or later Amp^r mutants were obtained as described above and purified on Luria-Bertani-Herskowitz medium containing 100 μg/ml ampicillin. The *ampD* gene was amplified using primers

TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or phenotype	Reference or source
Strains		
CS85	*ruvC53 eda-51*::Tn*10*	68
DM49	*lexA3*(Ind$^-$) *malB*::Tn*9*	45
FC29	Δ(*lac-proAB*)$_{XIII}$ *thi ara* [F′ *proAB*$^+$ Δ(*lacI-lacZ*)]	8
FC526	Δ*recG263*::Kan	18
JW2711	Δ*rpoS*::FRTKanFRT	2
N2731	*recG258*::Tn*10*mini-Kan	47
RW120	Δ*umuDC595*::*cat*	79
SMR580	FC40 *recB21 argA*::Tn*10*	24
SMR593	FC40 *recB21*	24
SMR4562	Δ(*lac-pro*)$_{XIII}$ *thi ara* Rif^r [F′ α45 *lacI*^{q} *lacI33*Ω*lacZ*] (genotype identical to that of FC40, independent construction)	52
SMR5201	Δ*att*λ::*ampRC*	61
SMR5222	SMR4562 Δ*att*λ::*ampRC*	61
SMR5225	SMR5222 Δ(*srlR-recA*)*306*::Tn*10*	61
SMR5228	SMR5222 *recB21 argA*::Tn*10*	SMR5222 × P1(SMR580)
SMR5522	FC29 Δ*ruvC64*::Kan	7
SMR5578	SMR5222 Δ*recG263*::Kan	61
SMR5652	SMR5222 Δ*recG263*::Kan Δ(*srlR-recA*)*306*::Tn*10*	61
SMR6064	SMR4562 *dinB10* (λ*xis1 cIts857*) [F′ *dinB10*]	53
SMR6371	SMR5222 *recB21*	SMR5228 × P1(SMR593)
SMR6373	SMR4562 *dinB10* Δ*att*λ::*ampRC* [F′ *dinB10*]	SMR6064 × P1(SMR5201)
SMR6485	SMR5222 *ruvC53 eda-51*::Tn*10*	SMR5222 × P1(CS85)
SMR6487	SMR5222 *recB21 recG258*::Tn*10*mini-Kan	SMR6371 × P1(N2731)
SMR6927	SMR5222 Δ*recG263*::Kan *ruvC53 eda-51*::Tn*10*	SMR5578 × P1(CS85)
SMR7055	SMR5222 Δ*recG263*::Kan *lexA*3(Ind$^-$) *malB*::Tn*9*	SMR5578 × P1(DM49)
SMR10316	SMR5222 Δ*umuDC595*::*cat*	SMR5222 × P1(RW120)
SMR10317	SMR5222 Δ*rpoS*::FRTKanFRT	SMR5222 × P1(JW2711)
Plasmids		
pJP2	pTGV-Light *ampRC*$^+$	61
pJP19	pACYC184 *ampD*$^+$	61
pKD46	*ori101 repA101*(Ts) pBAD-*gam-bet-exo* Amp^r *araC*$^+$ on plasmid	13

AmpD no. 1 (5′-GGGTTTTCATGAGAGGCGGCATGTTAAAACTCCAG-3′) and AmpD no. 2 (5′-GGGTTTAAGCTTTCATGTTGT-3′). The PCR products were sequenced (Lone Star Labs, Houston, TX) using primers AmpD no. 3 (5′GCGCGTCTCCGCTCACTGTTT-3′) and AmpD no. 4 (5′-GCATGC CATGCACGTTTATCG-3′).

RESULTS

Accumulation of *ampD* β-lactam resistance mutations during carbon starvation. We moved the chromosomal *ampRC*$^+$ system (61) into *E. coli* strains used to assay starvation stress-induced reversion of a *lac* +1 frameshift allele in an F′ episome during starvation (8). When spread on lactose minimal medium, the cells accumulate frameshift reversions in the F′-borne *lac* gene (16, 65) or a *tet* gene located either in F′ (15) or in the chromosome (7). This starvation-induced process requires the RpoS stationary-phase starvation and general stress response transcriptional activator (41, 48) and operates by a mechanism distinct from *lac* reversion in rapidly growing cells (see Introduction and below).

We found that prolonged starvation of the Lac$^-$ cells on lactose medium leads to accumulation of mutants resistant to the β-lactam antibiotic ampicillin (Amp^r mutants, *rec*$^+$) (Fig. 1A, C, and E). The mutations are recessive, forward mutations in the chromosomal *ampD* gene, as shown by complementation with an *ampD*$^+$ plasmid (see Materials and Methods) and sequencing (see below). The Amp^r mutants accumulate over several days of starvation on lactose (Fig. 1A to F). The increase is similar to that observed for reversion of a chromosomal *tet* frameshift (7), but the frequency of Amp^r mutants is about 10-fold higher, perhaps because any loss-of-function mutation in the ~580-bp *ampD* gene confers Amp^r, whereas only frameshift reversions in a 5-bp window restore the *tet*$^+$ function. The frequency of Amp^r mutants is ~2-fold lower than that of the F′ *lac* frameshift revertants in this system, which arise from frameshift mutations in a ~125-bp region (16, 65).

DSBR proteins in stationary-phase Amp^r mutation. We found that, like stress-induced *lac* reversion in the F′ (18, 24, 25) and Tet reversion in the chromosome (7), stationary-phase accumulation of Amp^r mutants requires homologous recombination (HR) and DSBR proteins RecA, RecBC, and RuvC and is elevated in cells lacking the RecG protein (Fig. 1A to F). *recG* cells show 10- to 25-fold more Lac$^+$ colonies (18, 25) and 4- to 10-fold more Amp^r mutants by day 4 (Fig. 1). The requirement for RecB, which is specific for double-strand ends (DSEs), is the first evidence implicating DSEs in stress-induced chromosomal mutagenesis.

Previously (18, 25) and in this study, we and other workers found that loss of RecG increases mutagenesis via a mechanism that is similar to or the same as the mutagenesis mechanism that operates in Rec$^+$ cells, a mechanism that requires the RecA, RecBC, and Ruv proteins (Fig. 1A to F). The current model for DSBR-associated mutagenesis indicates that invading 3′ ends are the priming site for new DNA synthesis that leads to mutations (for a review, see reference 21). The inhibitory effect of RecG in DSBR-associated mutagenesis is probably due to the unwinding and dissociation of this inter-

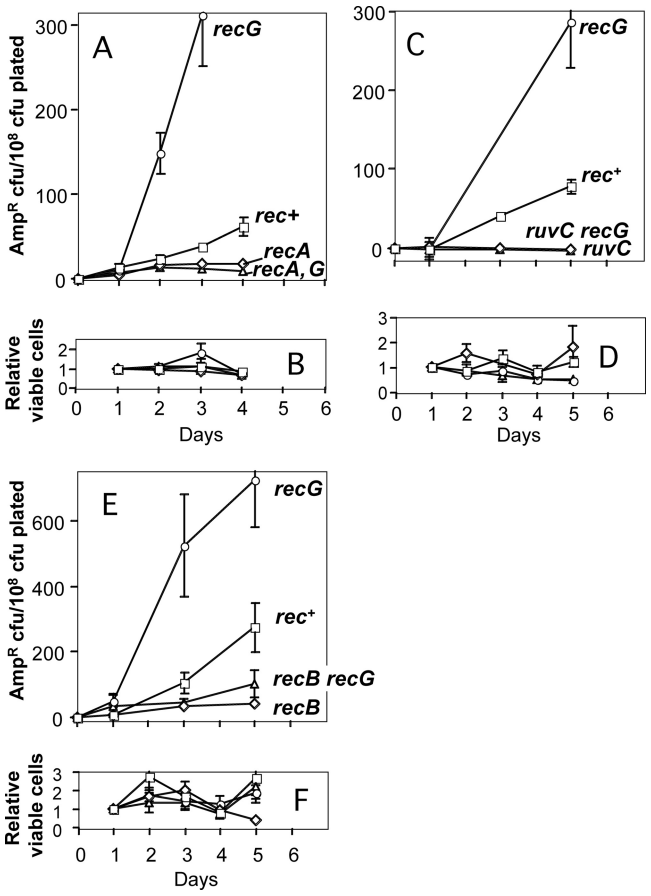

FIG. 1. DSBR protein-dependent stationary-phase mutation to ampicillin resistance (Amp^r) during prolonged starvation. (A, C, and E) Lac assay strains were starved on lactose medium for several days, and replicate plates were removed each day, overlaid with agar containing another carbon source and ampicillin, and reincubated to allow growth of Amp^r mutant colonies. The data are the means ± standard errors of the means of six independent cultures per strain assayed in parallel. Each graph is representative of at least three repetitions. The days indicated are the days on which the ampicillin overlay was performed, and the data show the number of Amp^r cells that were present on that day. The counts for day 0 (for the generation-dependent mutants present when the cultures were plated) were subtracted from the counts for stress-induced *ampD* mutants to more clearly show the increases in the numbers of mutants (when present) over time. Negative values were obtained when the average number of mutants from one set of replicate plates overlaid on a day was lower than the number of mutants for the set of plates overlaid on day 0. In all experiments, the *rec*$^+$ strain (open squares) was SMR5222, and the *recG* strain (open circles) was SMR5578. (A) Amp^r mutants accumulate during prolonged starvation on lactose. RecA is required for, and RecG inhibits, formation of these mutants. Open diamonds, *recA* strain SMR5225; open triangles, *recA recG* strain SMR5652. (C) Amp^r stationary-phase mutation requires *ruvC*. Open diamonds, *ruvC* strain SMR6485; open triangles, *ruvC recG* strain SMR6927. (E) Amp^r stationary-phase mutation requires *recB*. Open diamonds, *recB* strain SMR5228; open triangles, *recB recG* strain SMR6487. (B, D, and F) Daily levels of Lac$^-$ viable cells on the plates, normalized to the day 1 count, showing little net growth or death. For each panel the symbols are the same as those for the panel above it.

mediate that primes DNA synthesis (18, 25), an hypothesis which is further supported by the ability of this protein to perform similar reactions in vitro (51). Because the mutagenesis mechanism operating in *recG* and wild-type cells is similar, *recG* cells provided a sensitive way to compare the stress-induced mutagenesis phenotypes of other alleles in these experiments and some of the other experiments described below. *recG* cells have been used similarly as a sensitizing background in screens for stress-induced mutagenesis-defective mutants (41).

Stress responses in stationary-phase Amp^r mutation. Two stress responses have been implicated in stress-induced mutagenesis in *E. coli*: the SOS DNA damage response (8, 52) and the RpoS general stress response (41, 48). The SOS regulon is induced when regions of single-stranded DNA that are bound by the RecA protein accumulate. RecA then becomes activated and induces autocleavage of the repressor LexA, leading to an increase in the expression of roughly 40 genes in *E. coli* (11). *lexA* mutants unable to undergo autocleavage (Ind$^-$) are therefore impaired in induction of the SOS response (56). RpoS is an alternative sigma factor which accumulates in response to several different stresses, including entry into stationary phase and starvation (27), leading to increased transcription of hundreds of genes.

Figure 2A to D show that both stress responses are required for *ampD* mutagenesis, as observed previously for stress-induced *lac* mutagenesis (see the introduction). Additional data presented below confirm by independent means the requirement for the SOS response in *ampD* mutagenesis in starving cells. These data imply that chromosomal mutagenesis and F′ mutagenesis are triggered by similar environmental signals that stimulate the mutagenic response via RpoS and SOS induction. Because stress responses are required for formation of the *ampD* mutations, we call these mutations in starving cells "stress-induced" *ampD* mutations.

DinB and Pol V in Amp^r mutation. We found that, as observed for Lac and Tet stress-induced mutagenesis (7, 53), DinB is required for stress-induced *ampD* mutagenesis (Fig. 2E and F). Despite the requirement for DinB, we found that the data for the sequences of the stress-induced *ampD* mutations showed that there were more base substitutions than frameshift mutations (Fig. 3; see below). Because the base substitutions observed are not hallmarks of DinB action (see below), we hypothesized that another DNA polymerase(s) might also contribute to chromosomal mutagenesis under stress conditions. The most obvious candidate is Pol V (73), encoded by the *umuDC* genes. These genes are responsible not only for virtually all SOS-dependent mutagenesis that follows treatment of cells with DNA-damaging agents (35) but also for so-called "SOS untargeted mutagenesis," which occurs at undamaged sites or sites where there is endogenous DNA damage in SOS-induced cells (78). The *umuDC* genes are tightly regulated and are virtually not expressed in the absence of an SOS response (58).

Figure 2G and H show that a Δ*umuDC* strain is impaired in stress-induced mutagenesis in the chromosomal *ampD* locus, showing ~3-fold less mutagenesis upon starvation than the isogenic *umuDC*$^+$ strain. This result suggests that there is concerted action of both Pol V and DinB Y family DNA polymerases in stress-induced *ampD* mutagenesis in *E. coli*. The results also provide independent confirmation that the SOS response is required for stress-induced *ampD* mutagenesis, because the *umuDC* genes are expressed only during an SOS response (for a review, see reference 58).

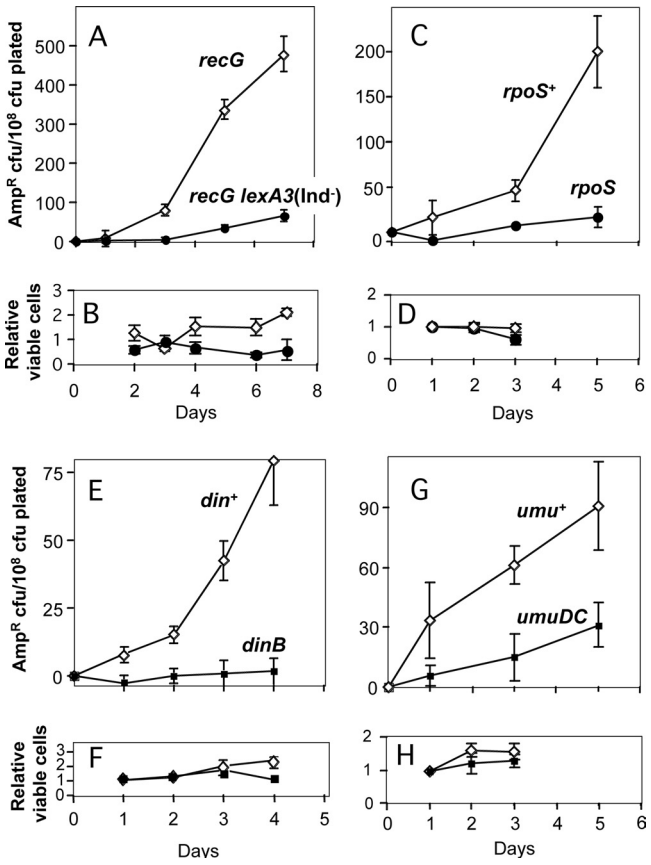

FIG. 2. Chromosomal Amp^r stationary-phase mutation requires stress responses and error-prone DNA polymerases. For an explanation of the experiments and data see the legend to Fig. 1. (A) Induction of the SOS response is required for *ampD* stress-induced mutagenesis. Open diamonds, *recG* strain SMR5578; filled circles, *recG lexA3* (Ind$^-$) strain SMR7055. The *lexA3*(Ind$^-$) allele encodes an uncleavable LexA repressor protein that prevents derepression of LexA-controlled SOS genes during an SOS response (45). (C) Amp^r stationary-phase mutation requires the general stress response controlled by RpoS. Open diamonds, *rpoS*$^+$ strain SMR5222; filled circles, *rpoS* strain SMR10317. (E) DinB is required for Amp^r stationary-phase mutagenesis. Open diamonds, *din*$^+$ strain SMR5222; filled circles, *dinB* strain SMR6373. (G) The *umuDC* genes are required for Amp^r stationary-phase mutagenesis. Open diamonds, *umu*$^+$ strain SMR5222; filled squares, Δ*umuDC* strain SMR10316. (B, D, F, and H) Daily levels of Lac$^-$ viable cells on the plates, normalized to the day 1 counts, showing little net growth or death. For each panel the symbols are the same as those for the panel above it.

Base substitutions prevalent in *ampD* stress-induced mutations. We sequenced chromosomal *ampD* stationary-phase mutations in mutants. One potential problem in obtaining stationary-phase *ampD* mutants is that resistant colonies on plates from later days may also include some early generation-dependent mutants. Plates contain colonies formed by mutant cells that arose at any time between inoculation of the culture and the time when the cells were rescued from starvation and Amp^r was selected by overlay with medium containing another carbon source and ampicillin on the lactose plates. To minimize the number of generation-dependent mutation sequences in the data, mutant colonies were chosen from experiments in which there were ≥5- to 7-fold more day 5 or day 7 mutants than day 0 (generation-dependent) mutants. Also, in some experiments preparations were plated using high dilutions so that few, if any, day 0 colonies would appear and the later colonies would be only stationary-phase mutant colonies. Because there were ≥5- to 7-fold more late (day 5) RpoS- and SOS-dependent *ampD* mutants than early stress response-independent mutants, we estimated that a maximum of ~20% of our stress-induced *ampD* mutations could be generation-dependent mutations.

We found 22 mutations in 20 generation-dependent Amp^r mutants sequenced and 40 mutations in 38 Amp^r mutants obtained at day 5 or later (some mutants had more than one mutation). The *ampD* generation-dependent mutations included small and large insertions (including insertions of mobile elements), small deletions, and base substitutions (61). Contrary to the expectation that the stress-induced mutations would be mostly −1 deletions in mononucleotide repeats (16, 65), substitutions predominated among the *ampD* stress-induced mutations (Fig. 3 and Table 2). The proportion of base substitutions resembled that seen in *ampD* generation-dependent mutations (Table 2) (61), although the kinds of substitutions differed (Table 3 and Fig. 3). Thirty-four of the 40 stress-induced mutations and 17 of the 22 generation-dependent mutations were substitutions (Fig. 3 and Table 2). However, ~30% (5 of 17) of the generation-dependent substitutions occurred at hot spots (sites showing the same substitution more than once), whereas only 5% (2 of 40) of the stress-induced mutations occurred at hot spots (Fig. 3 and Table 2). The stress-induced mutations also differed from the generation-dependent mutations in containing fewer mobile element insertions and more frameshift mutations in repeated sequences. The two types of mutations included similar numbers of transitions and transversions, but the difference in the types of substitutions observed is striking. In the stress-induced mutations G · C-to-T · A transversions were much more prevalent, comprising more than one-half of the substitutions detected, and more A · T-to-T · A transversions were also observed in these mutations than in the generation-dependent mutations. On the other hand, A · T-to-C · G transversions were observed only for generation-dependent mutations (Fig. 3 and Table 3).

DISCUSSION

Stress-induced β-lactam resistance mutagenesis. The data presented here show that starvation stress-induced mutagenesis can promote β-lactam resistance mutation in a clinically relevant model (Fig. 1 and 2). This is important because most antibiotic resistance acquisition and mutation have been modeled using mutation rates determined in nonstress environments, even though many natural environments of pathogens are likely to be stress-inducing environments (14, 49). For example, the stress-inducible mutation process shown here to promote *ampD* mutagenesis requires induction of the general stress response controlled by RpoS. RpoS is induced during *Pseudomonas* lung infections and may be a general feature of infection (14). Therefore, the stress-induced mutagenesis mechanisms that occur under RpoS-inducing conditions are likely to provide better models for generation of resistance mutations in situ. Considering that β-lactam antibiotics kill

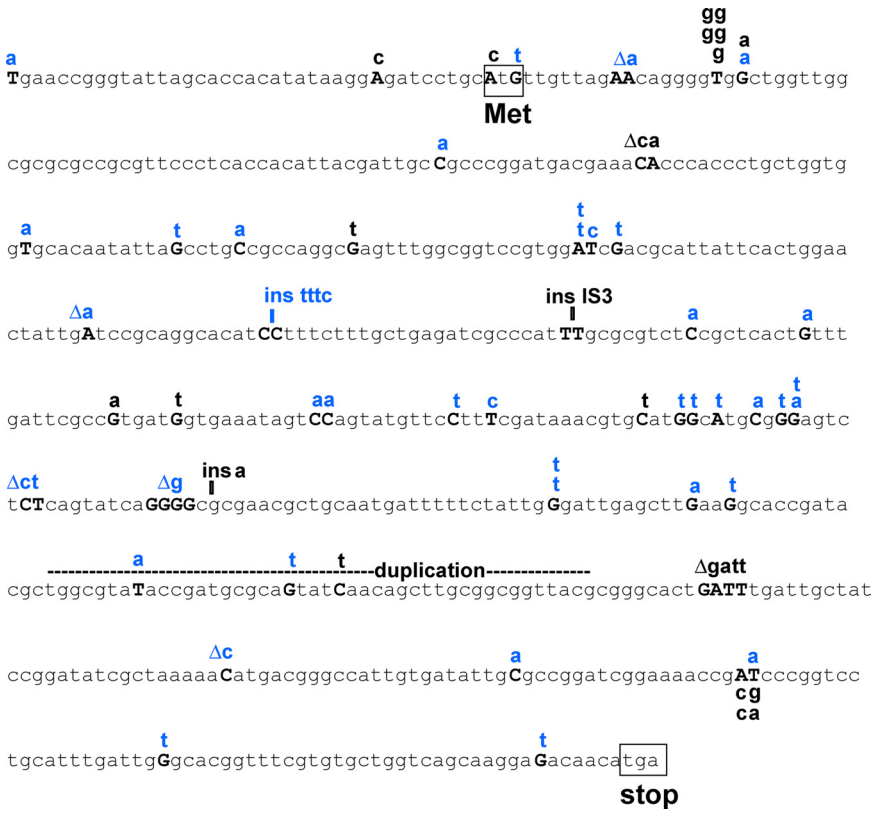

FIG. 3. Sequences of *ampD* generation-dependent and stress-induced mutations. The *ampD* genes of Amp^r mutants were sequenced as described previously (61). Above the *ampD* sequence, generation-dependent mutations determined in a previous study (61) are indicated in black type and stress-induced mutations (selected from overlay plates on day 5 or later) are indicated in blue type. Δ, deletion; ins, insertion.

dividing cells, it seems reasonable to conclude that a stationary-phase mutagenesis mechanism may lead to clinically relevant *ampD* mutations upon antibiotic treatment. For example, in enterobacteria and *Pseudomonas aeruginosa*, *ampD* null mutations are found in β-lactam-resistant clinical isolates, although in *Pseudomonas* other mutations also seem to be required for the β-lactam resistance (40, 67).

Possible error-prone DSBR mechanism of stress-induced *ampD* mutagenesis. Like stress-induced mutagenesis at the F′ *lac* locus (18, 24, 25, 41, 48, 52, 53), accumulation of chromosomal stress-induced *ampD* mutants during starvation on lactose medium required DNA DSBR and HR proteins RecA, RecB, and RuvC, the ability to induce the SOS DNA damage response, the RpoS stress response activator, and the SOS- and RpoS-inducible error-prone DNA polymerase DinB, and it was stimulated in the absence of RecG (Fig. 1 and 2). This is the first demonstration of a requirement for the DSE-specific RecBC enzyme in chromosomal mutagenesis during starvation stress, and the results imply that DSEs are molecular intermediates in the stress-induced *ampD* mutagenesis pathway as well. These genetic requirements are reminiscent of two other stress-induced mutagenesis mechanisms: *E. coli* ciprofloxacin-induced chromosomal ciprofloxacin resistance mutagenesis (10) and *Salmonella* bile-induced resistance mu-

TABLE 2. Profiles of generation-dependent and stress-induced *ampD* mutations in sequenced *ampD* mutants

Type of mutation	Generation dependent[a,b]	Stress induced[a]
Insertions of insertion element	1/22	0/40
−1 to −4 deletions	2/22 (0)[c]	6/40 (2)[c]
Transitions	2/22	7/40
Transversions	15/22	27/40
Duplications	1/22	0/40
1-base insertions	1/22	0/40
Substitutions at hot spots/total no. of substitution mutations	5/17	2/34

[a] Number of mutations observed/total number of mutations sequenced, unless indicated otherwise.
[b] Data from reference 61.
[c] The numbers in parentheses are the numbers of deletions in repeats.

TABLE 3. Different base substitutions in generation-dependent and stress-induced *ampD* mutants

Type of substitution	No. of substitutions observed/total no. of substitutions	
	Generation dependent[a]	Stress induced
G · C to A · T	4/17	5/34
G · C to T · A	2/17	20/34
A · T to T · A	1/17	7/34
A · T to C · G	10/17	0/34
A · T to G · C	0/17	2/34

[a] Data from reference 61.

tagenesis (63). In contrast to stress-induced *lac* reversion but like ciprofloxacin-induced resistance mutagenesis (10), a significant proportion of the mutagenesis requires the other *E. coli* error-prone DNA polymerase, Pol V. Because both *dinB* and *umuDC* strains show a severe defect in *ampD* stress-induced mutagenesis, it is likely that these polymerases cooperate in stress-induced mutagenesis in chromosomal genes.

For *lac* frameshift reversion, the hypothesis that there is an error-prone DSBR mutation mechanism is supported by evidence that DSBs generated by expression of a restriction enzyme in vivo increase mutagenesis nearby >1,000-fold (62). This occurs only in stationary phase or if RpoS is expressed inappropriately in log phase (62). The data indicate that there is a switch from high-fidelity DSBR to error-prone DSBR under stress conditions, mediated by RpoS (62). A similar mechanism of stress-induced switching to error-prone DSBR might also generate the *ampD* mutations studied here. This mechanism does not produce *ampD* mutations in rapidly growing cultures of the Lac assay strains, which are RecA and SOS independent and not stimulated by *recG* mutation (61). The DSEs whose error-prone repair provokes stress-induced mutagenesis might be spontaneous breaks and DSEs generated, for example, by replication mishaps.

Evolutionary significance of stress-induced chromosomal mutation sequences. The sequences of chromosomal, stress-induced *ampD* mutations provide the first look at the true sequences of mutations generated by the HR-DSBR-associated stress-induced mutagenesis mechanism, because any loss-of-function mutation in *ampD* can confer β-lactam resistance. The results are surprising because they show that there were more base substitutions than frameshift mutations (Fig. 3 and Table 2).

Previously, HR-DSBR-associated stress-induced mutagenesis was studied by examining reversion of frameshift alleles, so that only frameshift mutations could be recovered. Stress-induced *lac* reversions were nearly all −1 bp deletions in small mononucleotide repeats, unlike generation-dependent reversions, which were more heterogeneous, included larger insertions and deletions, and were not confined to mononucleotide repeats (16, 65). Also, the stress-induced mutation sequences in *lac* (16, 65) and in *tet* (7) resemble the errors made by DinB error-prone polymerase (36, 77); they are mostly −1 deletions in repeated sequences. Thus, one might have imagined that frameshift mutations would dominate the HR-DSBR-associated stress-induced-mutagenesis mechanism, with substitutions being a minor component. However, the role of Pol V in the type of mutagenesis demonstrated here provides an explanation for the abundance of base substitutions observed in *ampD* mutants (see below).

The evolutionary consequence of a frameshift-heavy mutation mechanism would be that genes would more often be inactivated and less often have their functions modified. Gene inactivation and reactivation by frameshift mutation in repeated sequences is an important strategy of pathogens that vary expression of surface protein antigens in this way (4), and stress-induced mutagenesis might promote this strategy. However, the results presented here (Fig. 3) imply that at least for chromosomal (and perhaps all) loci mutated during stress, substitutions are the predominant mutations. Therefore, modifications of gene functions, in addition to inactivation and activation, are predicted outcomes of this pathway, as observed in other instances of stress-induced mutagenesis (10, 72). This HR-DSBR stress-induced mutagenesis pathway could therefore contribute far more broadly to bacterial evolution under stress conditions.

Differences between F′ *lac* reversion and chromosomal *ampD* mutagenesis during stress. The involvement of the SOS-inducible Pol V in stress-induced *ampD* mutagenesis reveals a fundamental difference between the F′ *lac* gene and *ampD*. For stress-induced mutagenesis in *lac*, Pol I (26), Pol II (17), and Pol V (8, 52) are not required. Pol III is difficult to test, because it is essential, but some data suggest that it might compete with Pol IV, decreasing mutagenesis; we suggested that an antimutator Pol III lowered DinB-dependent stress-induced mutagenesis by increased processivity, leading to exclusion of Pol IV from the replisome (53). Thus, there is no compelling evidence that in *lac* more than one DNA polymerase produces most stress-induced mutagenesis. Additionally, we found that *dinB* is the only SOS gene required at induced levels for stress-induced mutagenesis in the *lac* gene (20).

The differences between mutagenesis in *lac* and mutagenesis in *ampD* that might account for the different uses of Pol V in mutagenesis are as follows. First, because *ampD* is a forward mutational target, analysis of *ampD* allows detection of base substitutions, which are not detectable in the *lac* reversion assay. Second, *ampD* is a chromosomal gene, and the DNA polymerases operating in the chromosome might differ somewhat. Third, whereas most of the mutational events in the F′ that occur during stress occur in acts of error-prone DSBR in *cis* to a DSB (as shown by Ponder et al. [62]), perhaps many chromosomal mutations occur in *trans* to a DSB (that is, not directly associated with a DSBR event). Different DNA polymerases might be responsible for the mutations generated by the two different mechanisms. This might explain the seemingly different sequence spectra observed for mutations at the two loci.

It is interesting that the following two other biological processes use more than one SOS DNA polymerase in the same mutagenesis pathway: survival in long-term liquid culture, which might include mutagenesis (80), and ciprofloxacin-induced mutation to ciprofloxacin resistance (10), as discussed below.

Mechanistic significance of stress-induced mutation sequences. Twenty of the 40 *ampD* stress-induced mutations were G · C-to-T · A transversions. The relatively high number of G · C-to-T · A mutations and the absence of −1 bp deletions in mononucleotide repeats seen here lead us to question whether DinB is the polymerase that is actually responsible for the Amp^r mutations, even though DinB is required for the vast majority of the stress-induced mutagenesis in both *lac* (53) and *ampD* (Fig. 2E).

First, the error spectrum of purified DinB DNA polymerase includes about 65% −1 bp deletions, mostly deletions in mononucleotide repeats, and there is only a very minor fraction of G-to-T (or C-to-A) changes (37). Second, by measuring mutations in the phage λ *c*II gene in vivo, Wagner and Nohmi (77) found that although overexpression of *dinB* from a plasmid increased the numbers of both frameshift and substitution mutations, the number of G-to-T mutations increased less than all the other substitution mutations examined. On the other hand, using the same *dinB* overexpression plasmid, Kim et al.

(36) found that G · C-to-T · A transversions were predominant base substitutions in the *lac* reversion assay of Cupples and Miller (12), even though −1 bp deletions were still by far the most common type of mutations found. Thus, it is not clear whether G · C-to-T · A transversions could be a major consequence of DinB activity in vivo.

A predominance of G · C-to-T · A and, to a minor extent, A · T-to-T · A transversions is a hallmark of "SOS untargeted mutagenesis," (i.e., mutagenesis occurring in cells not exposed to DNA-damaging agents but constitutively expressing the SOS response) (55). This is a heavily *umuDC*-dependent process (6, 9), and the observation that the same type of base substitution predominates in the *ampD* mutants supports the hypothesis that *umuDC* has a major role in this stress-induced mutagenesis mechanism. Furthermore, the *umuDC*-dependent SOS mutator phenotype giving rise to base substitutions is enhanced by DinB (39), particularly for mutagenesis occurring in the lagging strand. Similarly, here we observed base substitutions occurring in a *dinB*- and *umuDC*-dependent manner in stressed cells. The sequences of stress-induced Amp^r mutants in the *umuDC* background would, in principle, clarify whether the G · C-to-T · A mutations are really a result of the action of Pol V. However, an experiment to determine these sequences cannot be done, because the generation-dependent mutants outnumber the stress-induced mutants that remain for the *umuDC* strain.

Our results support a model in which the DNA synthesis that produces the mutations during DSBR-associated stress-induced mutagenesis involves both DinB and Pol V. Previously, Ponder et al. showed that DinB is not required for DSBR but becomes licensed to participate in DSBR and produces DSBR-associated mutations when RpoS is expressed (62). The predominance of Pol V-like mutation sequences in *ampD* despite a strong DinB dependence of the mutations could be explained by models suggested by others (19), in which one DNA polymerase makes the error and another DNA polymerase makes the initial extension from the mispaired primer terminus. This could account for the base substitutions, whereas the frameshift mutations might be made solely by DinB. This would explain the lack of a requirement for Pol V in stress-induced Lac frameshift reversion (20, 52) and the partial Pol V requirement and sequence signature for stress-induced *ampD* mutagenesis, which results in mostly base substitutions. This explanation was suggested for DinB-, Pol V-, and Pol II-dependent stress-induced ciprofloxacin resistance mutagenesis induced by ciprofloxacin in *E. coli*, which produces only base substitutions and is presumed to occur via error-prone DSBR, like Lac stress-induced mutagenesis (10). Apart from the use of Pol V and Pol II, Cirz et al. found requirements identical to those of DSBR-associated stress-induced Lac reversion. Thus, as those authors suggested (10), DSBR-associated stress-induced mutagenesis might involve Pol V and DinB and require both of these enzymes for substitution mutagenesis but only DinB for frameshift mutagenesis.

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