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TITLE: Mechanisms of Mutation in Non-Dividing Cells

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designated by other documentation.
Understanding how mutations arise in non-growing cells will help illuminate mechanisms of oncogenesis, tumor progression, and resistance to chemotherapeutic drugs. To this end, I have been addressing how antibiotic resistance mutations occur in non-, or slowly-growing enterobacteria cells. Previously, our laboratory discovered that RecA (an hRAD51 homolog) and RecBCD recombination repair proteins are necessary for the acquisition of β-lactam drug-resistant mutations in the Escherichia coli chromosome during stationary-phase. The data suggest that either the SOS DNA damage-repair response, recombinational DNA repair, or both, are involved in the mutation pathway. I have improved the E. coli-based system to examine the genetic and biochemical processes involved in this mutational mechanism in detail. Initial results in this improved system suggest that β-lactam resistance mutations occur not only in a growth-dependent manner but also in response to stress induced by starvation. The starvation conditions may mimic the environment pathogens or tumor cells may encounter in inflicted patients under stress, chemotherapeutic treatment, or other anti-tumor drug regimen in which cells are in a state of slow-, or non-growth. In addition, I have engineered a reporter construct that will allow me to enrich for those cells undergoing stationary-phase mutation so I may study the genetic and biochemical intermediates involved in this mutation mechanism.
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Introduction

When cancers develop in tissues of non-dividing or slowly growing cells, the first cancerous cell must escape growth regulation and acquire mutations to become transformed. Mutational mechanisms specific to non-growing cells may facilitate this progression from quiescence to division. Similarly, when tumors develop resistance to chemotherapeutic drugs, such as mitotic inhibitors, they acquire mutations and other changes that allow growth in the presence of the drugs. Moreover, the growth stage when mutation is most active is not known. G₀, the analog of bacterial stationary phase, could be an important period for spontaneous mutation. To understand these processes, it is important to elucidate the mechanism(s) of mutation in non-dividing and slowly growing cells.

A mutation mechanism has been described in stationary-phase Escherichia coli that requires recombination proteins, and produces sequences different from growth-dependent mutations (7, 9, 10, 19, 20). Stationary-phase mutation is best understood in the E. coli Lac assay in which cells with a lac +1 frameshift allele on an F’ plasmid generate Lac⁺ mutants upon starvation on lactose medium (2). The stationary-phase mutations, but not growth-dependent Lac⁺ mutations, require recombination proteins (RecA, RecBCD, RuvABC) and the bacterial SOS DNA damage-repair response (5, 9, 10, 14). Furthermore, DNA mismatch-repair protein MutL is transiently limited during stationary-phase mutation (8, 13). Recently, our lab determined that error-prone DNA polymerase IV (encoded by dinB, the ortholog of DINB1 in humans) is the SOS component required for Lac⁺ stationary-phase mutation (15).

The E. coli recombination and DNA repair proteins important for stationary-phase mutation have human homologs implicated in breast and other types of cancer. For example, Rad51, a homolog of E. coli RecA, interacts with breast cancer tumor suppressor proteins BRCA1, and BRCA2 as well as the p53 tumor suppressor protein (16, 21, 22). Mutations in these proteins are associated with predisposition to breast cancer. In addition, defects in human homologs of the E. coli mismatch repair proteins MutS and MutL underlie hereditary nonpolyposis colorectal cancer (HNPCC).

Mutation mechanisms that occur in non-dividing or stationary-phase cells may generate β-lactam antibiotic resistance mutations. β-lactam antibiotics kill actively dividing bacteria, and so those cells that are not dividing have an opportunity to acquire resistance mutations via stationary-phase mutation. Chromosome-encoded AmpC β-lactamases inactivate specific β-lactam antibiotics and are widespread in enterobacteria. E. coli carries all of the genes required for ampC production except for ampR, the transcriptional activator of ampC. Loss-of-function mutations in ampD, an indirect regulator of ampR, are found in β-lactam resistant pathogens and cause resistance in E. coli when the Enterobacter ampRC genes are expressed from a plasmid (12). The focus of this project is on the mechanisms of chromosomal ampD mutation in stationary-phase E. coli to improve the understanding of how antibiotic resistance mutations can arise in quiescent cells.

Recently, our lab demonstrated stationary-phase mutation occurring directly on the E. coli chromosome (1). However, some targets on the E. coli chromosome are less prone than others to stationary-phase mutation (18). The reasons for this observation are not clear and probably relate to the fundamental mechanism underlying stationary-phase
mutation. Many aspects of the mutation processes involved may be relevant to how oncogenic mutations occur in non-dividing cells in humans, and how tumors treated with drugs that kill dividing cells may still acquire mutations that enable the drugs to be evaded.

**Body**

**Year 1 Key Research Accomplishments (items 1-5 published in *Antimicrob. Agents Chemother.* 46:1535-9: Appendix 1):**

- The *ampRC* genes were integrated into the *E. coli* chromosome.
- Mutant alleles of the *recA, recB, recG, lexA3, sulA, sulA lexA51*, and *dinB* recombination and SOS genes were incorporated into *ampRC*-containing cells.
- Growth-dependent and stationary-phase mutation were quantified using this system.
- Stationary-phase, but not growth-dependent, mutation requires *recA* and increases in *recG*-deficient cells.
- 20 out of 20 growth-dependent Amp⁰ mutations in rec⁺ and *recA* cells occurred in *ampD* as determined by plasmid complementation and sequence analysis.
- A *sulA* promoter-∗ fusion was constructed and integrated into the *E.coli* chromosome to isolate SOS-induced cells.
- The *sulA* promoter-∗ fusion was demonstrated to be LexA-regulated as intended.

**Response to first year summary technical issues:**

The reviewer of the year one summary made several interesting and insightful comments that are addressed here.

**Technical issues:**

a) The reviewer commented on an apparent contradiction in mutation results reported in the *recA* background.—Fluctuation tests were done in RecA⁺ cells to show that recombination functions are not required for growth-dependent, spontaneous mutations in *ampD*. Spontaneous mutations are traditionally related to strand-slippage events and polymerase-errors and are not perceived to require recombination functions, such as those encoded by *recA*. These fluctuation tests were performed partly as proof-in-principle experiments for the chromosomal *ampRC* system, as well as to confirm our presumption that little or no recombination-dependent spontaneous mutation is occurring in these cells.

The other mutation studies demonstrating *recA* dependence relate solely to mutation happening in stationary-phase cells after they are starved in the presence of lactose. In studies performed to date by our lab and others, mutation in stationary-phase cells is strikingly different to mutation in growing cells. One of the fundamental mechanistic differences is the requirement for recombination functions in stationary-phase, but not spontaneous (i.e. growth-dependent), mutation.

b) The reviewer questioned how roles for the RecA and RecG proteins in stationary-phase mutation are assigned, as well as how will we determine which function(s) of the multi-purpose RecA protein is important for stationary-phase mutation.—Various genetic
requirements for stationary-phase mutation were determined in experiments where strains isogenic to the parent <i>ampRC</i> E. coli were deleted for genes of interest (e.g. <i>recA</i> and <i>recG</i>). It was found that <i>recA</i> is absolutely required for <i>ampD</i> stationary-phase mutation, and deletion of the <i>recG</i> gene leads to a greater frequency of <i>ampD</i> mutation. As a result, <i>recA</i> is believed to be a primary component of the stationary-phase mutation mechanism, while the role of <i>recG</i> is not clear.

As the reviewer points out, RecA is also a critical component of the bacterial SOS DNA damage-repair response. To discern which functions of RecA are required for stationary-phase mutation, genes specific to RecA-mediated DNA recombination/repair and genes belonging to the SOS response were deleted in isogenic <i>ampRC</i> strains and tested for their ability to undergo stationary-phase <i>ampD</i> mutation. It was found that both recombination and the SOS-induced <i>dinB</i> gene are required for mutation in this system (described below in Year 2 results). (DinB, or DNA polymerase IV, is an error-prone DNA polymerase which has homologs in all three domains of life [archaea, bacteria, and eukaryotes—including humans].) Therefore, it appears both the SOS inducing- and recombination functions of RecA are required for stationary-phase <i>ampD</i> mutation.

c) The reviewer brought up complications with working with a starvation model for studying stationary-phase mutation, particularly the presence of free radicals, which may promote stress. The role of damaging radicals and DNA base-analogs which may be present in higher quantities during starvation and/or stationary phase is of great interest to our laboratory in both the context of the original Lac system as well as our <i>ampRC</i> system and, of course, in eukaryotic cells. Preliminary Lac stationary-phase mutation studies have been initiated (by others in our lab) with strains deleted for genes involved in the repair of oxidative damage in the cell. These studies have generated potentially interesting results that need to be pursued in both the Lac and <i>ampRC</i> systems. The relevance to breast/eukaryotic cancer systems will be explored as well.

Year 2

The genetic requirements for β-lactam resistance mutation was studied further in stationary phase cells starving on lactose. Among other interesting results, I found that DNA PolIV is required for stationary-phase ampicillin resistance mutation as it was found to be for Lac stationary-phase mutation. When the <i>dinB</i> gene is interrupted by a kanamycin-resistance gene cassette, mutation to ampicillin resistance drops to background levels in cells starving on lactose. Recent papers suggest the possible involvement of three of the four DinB (PolIV)/UmuDC superfamily polymerases present in vertebrates in somatic hypermutation of the immunoglobulin genes (3, 17, 23).

I have also found that stationary-phase β-lactam resistance mutation requires the DNA recombination protein <i>recB</i> which, when mutated, eliminates the RecBCD double-stranded DNA (dsDNA) helicase/exonuclease. RecBCD normally prepares DNA for recombination by unwinding dsDNA and loading RecA protein onto the newly created ssDNA to prepare it for recombination (4). The requirement of RecBCD suggests that DNA double-strand ends (DSEs) are an intermediate in the stationary-phase mutation mechanism. How these DSEs are generated is of great interest and is currently under
investigation. Damage from free radicals, as mentioned by the reviewer from my year one report, is one hypothesis that we are testing.

In year one, we analyzed growth-dependent $ampD$ mutant sequences to ascertain the sequence spectrum of growth-dependent mutation in our system (Appendix 1). In year two, I began sequencing $ampD$ mutants that were isolated from our stationary-phase mutation experiments to compare the sequence spectra of growth-dependent and stationary-phase mutation in our system. So far, I have collected 20 $ampD$ stationary-phase mutant sequences and have found that the sequence spectrum appears significantly different in stationary-phase, compared to that of growth-dependent, mutants. Hotspots (where mutations seem to cluster) in the growth-dependent mutants, were non-existent in the stationary-phase mutants. Also, there is a high proportion of substitution mutations in the stationary-phase mutants compared to the growth-dependent mutants. Among the substitution mutations, I found a high-proportion of G to T transversions. Interestingly, G-C to T-A transversions were found to be increased nearly 100-fold when PolIV is overexpressed in $E. coli$ (11). Also, G to T transversions are found in cells unable to repair 8-oxyguanine oxidative damage. This sequence information therefore points to/supports two potential pathways leading to mutation: one through PolIV (already shown through genetic requirement testing), and one possibly through oxidative damage. The role of oxidative damage in the stationary-phase mutation pathway can be tested by deleting genes required for the repair of 8-oxyguanine damage (e.g. $mutY$—such experiments are underway in our laboratory), however the results of such experiments have to be carefully interpreted and followed up, because as the reviewer from year 1 mentioned, primary and secondary causes and effects of this mutation process will be detected in our system.

We are currently conducting control experiments that will be necessary to present for publication of our findings. One major set of controls is reconstruction experiments to show that each of the ampicillin resistant mutants that arises is capable of growing in our test conditions in each of the genetic backgrounds we have examined. For example, it's possible that mutations in $ampD$, in a recA background, prohibit growth in our assay conditions and is what causes the decreased mutation phenotype we observe. To eliminate this possibility, we subject approximately 100 recA$ampD$ cells (or other relevant background) to the experimental conditions (i.e. starve on a lactose plate and overlay with ampicillin and glycerol after 4 days) and observe whether all, or only a fraction of, the cells plated are able to grow into colonies. This control is currently being repeated for each strain background I have examined.

Another control I need to perform is one to eliminate the possibility that the ampicillin resistant mutants that arise are not mutators (capable of mutation at higher frequencies than the rest of the bacterial population—often the result of a defect in mismatch repair). It has been shown that, in a given bacterial population, there always may be a small percentage of cells that are mutator (6). Simple mutation experiments with $\beta$-lactam resistant mutants compared to $\beta$-lactam sensitive cells will easily discern whether or not mutator populations are skewing our results. (Preliminary data suggest that they are not.)

Last year, I reported on a $sulA$ promoter/gfp construct that will enable us to enrich for starving, stationary-phase cells undergoing an SOS response from cells which are not at different times during experimental trials. It is expected that the sorted cells that are
green will demonstrate hypermutability to β-lactam resistance and to Lac⁺ compared to cells sorted against. The DNA and proteins in this subpopulation can be purified to further examine the changes and signals occurring in these cells during stationary-phase mutation. Over the past year, I performed several follow-up experiments with these cells to help me ascertain whether future sorting experiments will be successful. In addition to the original sulA promoter/gfp construct, I have made recombination protein-deficient derivatives as controls and additional strains to test in our sorting experiments. These cells were examined by fluorescence microscopy to determine their level of SOS induction at the cellular level. It was found that certain recombination-deficient backgrounds have a higher fraction of cells induced for the SOS response compared to the recombination-proficient parent strain. However, never were all cells chronically SOS-induced. These observations suggest that our construct will enable us to sort subpopulations of SOS-induced cells from those not experiencing an SOS response.

**Key Research Accomplishments:**
- Additional alleles have been introduced into the ampRC⁺ tester strain including recB and ruvC
- β-lactam resistance mutation was shown to be PolIV, RuvC (Holliday Junction resolvase), and RecB dependent
- Sequence spectra of stationary-phase mutants shows a different mutation pattern compared to growth-dependent mutants (supports PolIV requirement and suggests possible role of oxidative damage in stationary-phase mutation)
- gfp- fusion studies show discrete levels of SOS induction within bacterial populations
- Control experiments leading to the publication of our stationary-phase mutation work are underway
- Paper describing the ampRC⁺ system has been published (*Antimicrob. Agents Chemother.* 46:1535-9: Appendix 1)

**Reportable Outcomes:**

**Abstract submissions:**


Conclusions

The genetic requirements observed for stationary-phase \( \beta \)-lactam resistance mutation so far suggest that a similar mechanism underlies both the \( \text{ampRC}^+ \) and the Lac systems. However, \( \text{ampD} \) mutation takes place on the bacterial chromosome and not on the F' and differences between mutation in these two regions is currently being investigated. The \( \text{ampRC}^+ \) system provides a first look at the full sequence spectra of recombination/PoIV-dependent stationary-phase mutation. The F' plasmid-based Lac and chromosomal Tet\(^R\) systems specifically select for -1 frameshift mutations and therefore display a strong bias when looking at their respective sequence patterns. Data thus far suggest that \( \text{ampD} \) stationary-phase mutations are significantly different from \( \text{ampD} \) growth-dependent, spontaneous mutations. The data supports the role of PoIV as the polymerase generating the mutations, and also hints at the role of oxidative damage as a potential component of the mutational mechanism as well.

Preliminary experiments leading to flow-cytometry sorting of the hypermutable cell subpopulation show that DNA damage is constantly occurring in a fraction of a given cell population and that this fraction is different depending on the genetic background of the cells being observed. Actual sorting of the cells is scheduled for the upcoming year.

References:


Chromosomal System for Studying AmpC-Mediated β-Lactam Resistance Mutation in Escherichia coli

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In some enterobacterial pathogens, but not in Escherichia coli, loss-of-function mutations in the ampD gene are a common route to β-lactam antibiotic resistance. We constructed an assay system for studying mechanism(s) of enterobacterial ampD mutation using the well-developed genetics of E. coli. We integrated the Enterobacter ampRC genes into the E. coli chromosome. These cells acquire spontaneous recombination- and SOS response-independent β-lactam resistance mutations in ampD. This chromosomal system is useful for studying mutation mechanisms that promote antibiotic resistance.

Mutation is a primary cause of bacterial resistance to antibiotics. Some mutations promote resistance directly (e.g., quinolone resistance mutations in genes encoding its Escherichia coli targets, gyrA and gyrF [16]). Other chromosomal mutations can cause mutator phenotypes that increase the likelihood of acquiring a resistance mutation (42). Mutations also ameliorate the otherwise deleterious effects on cell growth and physiology of some antibiotic resistance-conferring mutations (22). Although antibiotic resistance has been studied intensively, the mechanisms that generate resistance mutations are poorly understood.

In addition to spontaneous mutation in exponentially growing cells (growth-dependent mutation), other mutation mechanisms exist that may pertain to antibiotic resistance (34, 36, 37). For example, factors such as antibiotic concentration (23), environmental conditions (12), or other stress-inducing phenomena (1, 34, 36) may enhance or repress mutational machinery that leads to resistance mutations (for a review, see reference 31). Some mutation mechanisms or factors may be more important when the organism is under suboptimal growth conditions, as is probably the case during certain stages of an infection. In this study, we utilize a relatively well-described β-lactam resistance pathway as a model system to begin dissecting the mechanism(s) of antibiotic resistance mutation using the tools of E. coli genetics.

Chromosomally encoded AmpC β-lactamases confer β-lactam antibiotic resistance in many pathogenic and opportunistic bacteria and are ubiquitous in enterobacteria, except for the salmonellae, klebsiellae, Proteus mirabilis, Shigella flexneri, and Shigella dysenteriae (30, 32). Their expression is inducible in all but E. coli and the shigellae (30). In inducible strains, ampC transcription is controlled by the transcriptional activator AmpR (2). AmpR activity is regulated allosterically by two cell wall components, 1,6-anhydromuropeptide and UDP-N-acetylglucuronic acid-pentapeptide (UDP-MurNAc-pentapeptide). The first allows, and the second blocks, AmpR transcriptional activator activity at ampC (19). AmpD converts (activator-promoting) 1,6-anhydromuropeptide to (activator-blocking) UDP-MurNAc-pentapeptide, which then binds AmpR and blocks ampC transcription. Thus, loss-of-function mutations in ampD cause 1,6-anhydromuropeptide accumulation and constitutively induced AmpC β-lactamase production (7, 20, 21, 27). ampD missense and nonsense mutations are common in AmpC-mediated, β-lactam-resistant clinical isolates (25, 38). Also, some β-lactam antibiotics can induce expression of ampC by causing an increase in the cytoplasmic concentration of 1,6-anhydromuropeptide (7, 30).

E. coli lacks ampR, and low-level ampC expression results from a promoter embedded in the overlapping fumarate reductase (frdABCD) operon (13). High-level β-lactam resistance, mediated by ampD loss-of-function mutation, can be reconstituted in E. coli when the ampR and ampC genes of other enterobacteria are expressed from a plasmid (28, 35). We have integrated the ampRC genes from Enterobacter cloacae into the E. coli chromosome to assay ampD mutation, as selected by its β-lactam resistance phenotype. Background resistance imparted by the native ampC gene does not interfere with assays involving the reconstituted system. Integrating the ampRC genes into the chromosome improves upon previous plasmid-based ampRC expression systems by allowing genetic analyses not possible previously, first, because many mutant alleles used to study DNA repair, recombination, and mutation cause plasmid instability (e.g., reference 6). Second, the single-copy ampRC locus more closely models the situation in clini-
Strains

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SMR821

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Plasmids

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| * Full genotype is Δ(lec-pro)[XII] *attX* and RII* [F* aux5, lacZ* lacI33[delCZ]]. 

| * Plasmid pJF19, carrying the E. coli amprD* gene and promoter, was created by amplifying amprD* from E. coli wild-type strain MG1655 (3) chromosomal DNA using primers AmpD no. 1, 5'-GGGTTTTTCATAGGGCCCATGT TAAAACCGAC-3'; and AmpD no. 2, 5'-GGGTTTAAAGCTTCAATGCAGCGCCATG TCTTATCTGATCAG-3'. The primers incorporate terminal BgII and HindIII restriction sites at the 5' and 3' ends (respectively) of the amplified fragment. Amplified amprD* DNA and pACYC184 DNA (5) were digested with BgII and HindIII, and the amprD* fragment was ligated into pACYC184. pJF19-mediated amprD expression was confirmed by complementation to β-lactam sensitivity of four independent amprD β-lactam-resistant mutants.

| Strains used were SMR5222, recA* (recombination and SOS response proficient); SMR5225, recA* (recombination and SOS response proficient [40]; SMR5578, recG (recombination proficient, elevated for stationary-phase-mutation [11, 15]; SMR5652, recG recA (recombination, SOS, and stationary-phase-mutation deficient [11, 15]; SMR5715, lexA3 (recombination proficient, SOS gene induction defective [40]); SMR5749, sul4 (allows viability in the presence of a lacA-null mutation [40]); and SMR5725, sul4 lexA3(Def) (SOS-induced genes expressed constitutively [40]).

| Strains used were SMR5222, recA* (recombination and SOS response proficient); SMR5225, recA* (recombination and SOS response proficient [40]; SMR5578, recG (recombination proficient, elevated for stationary-phase-mutation [11, 15]; SMR5652, recG recA (recombination, SOS, and stationary-phase-mutation deficient [11, 15]; SMR5715, lexA3 (recombination proficient, SOS gene induction defective [40]); SMR5749, sul4 (allows viability in the presence of a lacA-null mutation [40]); and SMR5725, sul4 lexA3(Def) (SOS-induced genes expressed constitutively [40]).

An amprC expression cassette in the E. coli chromosome was constructed as follows. The strains and plasmids used are shown in Table 1. SMR5222, an E. coli strain carrying the E. cloaca amprC* genes in the E. coli chromosome, was constructed using the TGV (transgenic vector) system for integrating linear DNA cassettes into the E. coli genome (14). E. cloaca MHN1 amprC* genes were isolated from plasmid pEcl1 (35) by digestion with BamHI and SalI and were ligated into BglII- and XhoI-digested pTGV-light (14) plasmid DNA, creating pJF2. pJF2 was digested with NdeI to generate an amprC* fragment flanked on both sides by homology to the E. coli attachment site for phage λ (attX) for linear trans-
played only small differences in growth-dependent mutation recA, recG, lexA3(lnd~), sulA, — are not sulA lexA(Def) and tation (Table 2).

important for most growth-dependent ß-lactam resistance mutation rates, indicating that recombination and SOS genes are not growth-limiting conditions of carbon starvation (for a review, (rec +) cells is about 1.4 in recombination- and SOS-proficient (rec +) resistance mutation rates, indicating we wished to test. The ß-lactam (40) whose possible involvement in resistance mutation we wished to test. The ß-lactam SOS response controls several mutation-promoting proteins (37; see also references 41 and 43). Moreover, the required for a mechanism of mutation observed under method of the median (26).

The recombination and SOS genes examined—recA, recG, recA recG, lexA3(Ind~), sulA, and sulA lexA(Def)—are not required for most growth-dependent mutation; however, they are required for a mechanism of mutation observed under growth-limiting conditions of carbon starvation (for a review, see reference 37; see also references 41 and 43). Moreover, the SOS response controls several mutation-promoting proteins (40) whose possible involvement in ß-lactam resistance mutation we wished to test. The ß-lactam resistance mutation rate in recombination- and SOS-proficient (rec +) cells is about 1.4 \times 10^{-7} \text{cell}^{-1} \text{generation}^{-1} (Table 2). The strains tested displayed only small differences in growth-dependent mutation rates, indicating that recombination and SOS genes are not important for most growth-dependent ß-lactam resistance mutation (Table 2).

The following experiments demonstrated that the ß-lactam resistance mutations are in ampD. Based on prior observations in an E. coli model and in clinical isolates of enterobacterial pathogens (7, 27), we expected most of the ß-lactam resistance mutations to be in ampD. To test this, 20 independent ampicillin resistant mutants (each from a separate independent culture) from the rec + and recA fluctuation test experiments were examined further. ß-Lactam sensitivity was restored to each of the 40 mutants by transforming each with pH19 (Table 1) carrying the ampD + gene. This complementation test shows that the ampicillin resistance mutations of the 40 independent mutants are recessive, loss-of-function mutations in ampD.

We determined the ampD sequences for each of the 40 mutants. Little difference was found between the rec + and recA backgrounds (Fig. 1), suggesting similar mutation mechanism(s) in each. Many different mutations and insertions in ampD conferred AmpC-mediated ß-lactam resistance.

Among the eight substitution mutations identified here, two were identified previously from ß-lactam resistant isolates. A Trp7Gly substitution in AmpD occurred in both the rec + and recA strains (Fig. 2) and previously in E. coli ampicillin-resistant mutants of cells expressing the E. cloacae ampRC genes from a plasmid (18). Also, we found Asp164Glu and Asp164Ala in both rec + and recA. Asp164Glu was found previously in Citrobacter freundii (39). Both previous ampD mutations were shown to cause full derepression of ampC, as ampD-null mutations do (28, 39).

Other ampD substitution mutations found include
Fig. 2. Amino acid substitutions, insertions, and deletions identified in the rec + and recA mutants aligned with the ampD genes from nine enterobacteria. Also included are previously identified ampD mutant proteins from other laboratories (10, 18, 24, 28). rec + mutations are in black, recA mutations are in red, and previously identified mutations are in blue. Insertions are indicated by a plus sign (+), deletions are indicated by a minus sign (−), and nonsense mutations are indicated by an asterisk (*). Mutations isolated multiple times show the number of times that each was encountered for each strain (rec + or recA). Abbreviations: S. typh., Salmonella enterica serovar Typhimurium; C. freu., C. freundii; E. cloa., E. cloacae; V. chol., Vibrio cholerae; P. aeru., Pseudomonas aeruginosa; A. vine., Azotobacter vinelandii; A. acti., Actinobacillus actinomycetemcomitans; and R. sola., Ralstonia solanacearum. Alignment was performed using ClustalW (17) and formatted using BOXSHADE.

Ser37Arg, Arg80His, Gly82Cys, Ala94Val, and Leu117Arg (Fig. 2). Although these might or might not inactivate ampD fully, substitutions that alter or abolish AmpD function reveal amino acids that are important for AmpD structure and/or function. Conservative substitutions, such as Ala94Val or Asp164Glu, highlight the specific steric and/or chemical requirements of the wild-type amino acids. For example, the intolerance for Ala94Val suggests that the smaller size of alanine is important here, because both amino acids are similarly hydrophobic. The Asp164Glu substitution involves similar charges, suggesting that this amino acid position makes important catalytic or structural contacts disrupted by the larger glutamic acid side chain. Asp164 is probably not simply a surface amino acid, because it has a seemingly stringent size requirement and because alanine at this position is also not tolerated.

Alignments show that ampD is highly conserved among various bacteria (e.g., Fig. 2). Asp164, Ala94, and the other substituted amino acids from our mutation studies are highly conserved among the aligned ampD sequences (Fig. 2) and further highlight their potential structural and/or functional importance.

The variety of loss-of-function mutations observed in this system suggests its utility for studying many kinds of mutation...
mechanisms. This system may be useful additionally for studying the forward mutation spectra caused by potential damaging agents and environmental factors, because mutations in the small ampD gene are easily selected and sequenced.

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