Increased Mutation Frequencies in Escherichia coli Isolates Harboring Extended-Spectrum β-Lactamases

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Since the early 1980s, several extended-spectrum beta-lactamases (ESBLs) have emerged in Escherichia coli strains, probably as a consequence of the use of broad-spectrum antibiotics (24). Classic ESBLs have evolved from plasmid genes encoding the previous broad-spectrum beta-lactamases (6), such as TEM-1, TEM-2, or SHV-1, by mutation in one to six positions in their sequences (http://www.lahey.org/studies). More recently, CTX-M-type ESBL genes have emerged in enterobacterial organisms by capture of chromosomal sequences of environmental bacteria and have undergone further genetic divergence by mutational and possibly recombinational events (5, 21). The sequential acquisition of mutations in ESBL genes is probably due to a stepwise process involving successive rounds of selection (19). Bacterial mutation rates might be variable (16). The modal frequency of mutation in E. coli strains originating either from clinical specimens or from fecal samples of healthy volunteers is, as expected (10), a constant value (1 × 10−8), but a consistent number of isolates present increased frequencies of mutation (f) (2). Most of these isolates are considered weak mutators, with mutation frequencies ranging from 4 × 10−8 to 4 × 10−7. Since mutator phenotypes increase both the rate of mutation and the rate of homologous recombination (4, 7, 26), it is conceivable that the emergence and dissemination of ESBLs could be facilitated in E. coli strains with increased frequencies of mutation. In fact, mutator strains have been used to predict the emergence of ESBLs under laboratory conditions (11, 22).

In order to examine this hypothesis, we studied a highly diverse collection of 89 E. coli strains (only 1 isolate per patient; 77 different pulsed-field gel electrophoresis clones) harboring 12 different ESBL types. The ESBL distribution was as follows: 18 TEM-4 strains, 2 TEM-12 strains, 2 TEM-24 strains, 1 SHV-1 strain, 5 SHV-2 strains, 1 SHV-5 strain, 7 SHV-12 strains, 2 SHV-13 strains, 5 CTX-M-1 strains, 31 CTX-M-9 strains, 9 CTX-M-10 strains, and 6 CTX-M-14 strains. The strains were isolated at our hospital from 1988 to 2002. Pulsed-field gel electrophoresis was carried out and analyzed as previously described (8, 25). ESBLs were characterized by isoelectric focusing, amplification of bla genes by PCR using primers and conditions previously described, and further sequencing of PCR products (8). The mutation frequencies of these strains were obtained by determining the proportion of rifampin-resistant colonies per total viable cell count (2). Results are means from at least three independent experiments for each strain. The technique was previously validated using classic Luria-Delbrück determinations (2).

The distribution of rifampin resistance mutation frequencies of the 89 E. coli strains harboring ESBLs is shown in Fig. 1. According to the categories previously described by our group (2), 2% of the strains were hypromutable (f ≤ 8 × 10−9), 55% were normomutable (8 × 10−9 < f < 4 × 10−8), and 43% were hypermutable (weak mutators; 4 × 10−8 ≤ f < 4 × 10−7). We could not find any strong mutators (f ≥ 4 × 10−7) in our ESBL-producing E. coli collection. The overall proportion of ESBL-producing strains with increased mutation frequencies was found to be the highest ever described after studying many different E. coli strains of different geographical and clinical origins (2, 14, 15). Indeed, the proportion of hypermutable strains was the highest (43%) compared with different collections (100 strains each) of E. coli from blood cultures (39%), urine cultures (26%), or feces from human volunteers (12%) studied in the same hospital (Fig. 1). Most of our ESBL-producing isolates (56%) were recovered from urinary tract infections. Uropathogenic E. coli strains had been shown to have a high frequency of mutators compared with isolates from commensal bacteria (2, 9, 18). We compared the observed mutation frequencies in the subset of ESBL-producing urinary isolates with those of the non-ESBL collection of strains from positive urine cultures recovered in our hospital (Fig. 2). It is noteworthy that 40% of urinary ESBL-positive E. coli strains had increased mutation rates, whereas the percentage of mutators among ESBL-negative isolates was just 26% (P = 0.03).

The mutation frequencies found for the different ESBL groups in our series are presented in Fig. 3. The highest percentage of hypermutation was found for strains carrying TEM derivatives.
whereas enzymes of the CTX-M-9 group (CTX-M-9 and CTX-M-14) were harbored by a group of strains with a lower proportion of hypermutators (38%). Probably due to low numbers, these differences did not reach statistical significance.

To investigate the possibility that hypermutable strains could eventually be better recipients of ESBL-encoding plasmids, we analyzed the frequency of transconjugants in otherwise isogenic hypermutable and wild-type normomutable strains. We introduced by conjugation four ESBL-encoding plasmids—two from _Escherichia coli_, pEC36 (TEM-4) and pEC16 (CTX-M-10), and two from _Klebsiella pneumoniae_, pRYCE11 (TEM-4) and pRYCE11.1 (TEM-4) (8)—into the nalidixic acid-resistant _E. coli_ K-12 BM21 recipient strain (1). The resulting _E. coli_ BM21 transconjugant strains harboring the ESBL plasmids listed above were then used as donors in further conjugation experiments. The recipients of these mating experiments were rifampin-resistant mutants of the normomutable strain _E. coli_ MI1443 (frdABCD::Tn10 Smr) and its isogenic hypermutable derivative GB20 (MI1443 mutS215::Tn10) (11). Transconjugants were selected onto LB plates containing rifampin (100 μg/ml) and cefotaxime (1 μg/ml). Three replicate experiments were run in parallel. Our results did not support the possibility that mutator strains could be better recipients of ESBL-encoding plasmids, since the frequency of conjugation per donor strain was not significantly different when the normomutable or the hypermutable strain was used as the recipient strain (data not shown). Another possibility to explain the high prevalence of hypermutable ESBL-producing strains could be a putative mutagenic effect on the recipient cell derived from the ESBL plasmid acquisition. In fact, this explanation has been suggested previously for _E. coli_ and _K. pneumoniae_ (3, 13). Again, such a hypothesis was not supported by our results. The mutation frequencies of _E. coli_ K-12 BM21 strains obtained before and after mating experiments with all ESBL-encoding plasmids tested were found not to be significantly different (data not shown).

A possible scenario is that the stepwise emergence of mutations in the ESBL ancestor genes (such as those encoding TEM-1 or SHV-1, ancestors of TEM-4 and SHV-12, respectively) might have preferentially, but not necessarily, occurred in plasmids harbored by hypermutable strains, followed by plasmid transfer, eventually to other, nonhypermutable strains. However, bacterial stress associated with epidemic spread of ESBL clones in the hospital might have favored hypermutable variants. Additionally, local horizontal transfer to neighbor hospital strains (where hypermutation is more frequent) may enrich the frequency of hypermutators with ESBLs. Since hypermutable strains are more resistant to antibiotics (20), the association of ESBL production with hypermutation may have
been favored by the local intensity of antibiotic selection. Indeed, hypermutable ESBL-producing strains could have been enriched by the use of fluoroquinolones, because of the easier emergence of fluoroquinolone resistance mutations (12, 23). In these hypermutable strains, and under appropriate selection, the emergence of mutations and recombinations leading to ESBLs could have been favored.

The results of our study suggest that in our hospital the presence of ESBLs is associated with a hypermutable phenotype in E. coli. Thus, the isolates harboring these enzymes might have an enhanced capacity for further adaptations to exposure to novel antibiotics or for acquisition of novel traits, allowing them to develop more-efficient mechanisms eventually increasing their epidemicity and virulence (17).

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