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Response of *Escherichia coli* hypermutators to selection pressure with antimicrobial agents from different classes

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The responses of hypermutable *Escherichia coli* strains to selection with antibiotics having different endogenous resistance potentials were determined. Selections with rifampicin or ciprofloxacin at 4 × MIC, i.e. conditions where they act as single target agents against RpoB and GyrA, respectively, demonstrated that some hypermutators generated resistant mutants with frequencies up to 1000-fold higher than normal strains. Furthermore, individual mutants recovered from hypermutable hosts often exhibited higher levels of resistance to the drugs than mutants arising in normal hosts. Exposure to ciprofloxacin at 16 × MIC, i.e. conditions where it has low endogenous resistance potential, failed to select resistant mutants in hypermutable or normal hosts (mutation frequency $<10^{-11}$). Consistent with these findings, the highest estimated mutation frequency for selection at 16 × MIC in a hypermutable host would be 4.4×10^{-15} (*mutT*), calculated by determining the individual mutation frequencies for first-step ciprofloxacin resistance and second-step resistance arising in hosts already harbouring single first-step mutations in *gyrA* at codons 83 or 87. The frequency with which second-step ciprofloxacin resistance mutations arose was suppressed in hypermutators and demonstrated at most a 10-fold increase in mutation rate compared with non-hypermutator hosts. Second-step mutants may contain mutations in *mar*, since a survey of 170 second-step ciprofloxacin-resistant mutants derived from both hypermutator and non-hypermutator parents demonstrated that they all possessed increased resistance to chloramphenicol, a phenotype associated with *mar* mutations. Exposure to 4 × MIC of D-cycloserine, cefotaxime or polymyxin B (agents with multiple targets or membrane activity) failed to select resistant mutants in normal or hypermutator hosts (mutation frequency $<10^{-11}$); however, continuous culture in the presence of sub-lethal concentrations of D-cycloserine (0.25 × MIC) selected resistant mutants in hypermutators after *c.* 33 generations, compared with *c.* 44 generations in normal hosts. Since hypermutable bacteria occur naturally, our data emphasize that successful new drugs will need to possess low endogenous resistance potentials.

Introduction

The emergence of antimicrobial drug resistance in pathogenic bacteria is a serious public health issue because it limits the therapeutic options for treatment of infection.[1] Antibiotic resistance can arise from a number of mechanisms involving mutation of chromosomal genes.[2–5] Such mechanisms, usually involving mutations in genes encoding drug targets or systems that affect drug accumulation, are defined as endogenous resistance mechanisms, to distinguish them from the exogenous resistance mechanisms that are typically mediated by the acquisition of plasmids and transposons.[2]

Although endogenous resistance to most antibiotic classes has been reported in clinical isolates,[3] current concepts on the ability of bacteria to adapt and survive in the presence of antibiotics are based primarily on the assumption that resistance arises in bacteria that show normal mutation frequencies. However, naturally occurring strains that exhibit elevated mutation frequencies have recently been reported amongst populations of pathogenic *Escherichia coli*, *Salmonella*

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enterica, *Pseudomonas aeruginosa* and *Neisseria meningitidis*.[6–8] In some cases hypermutators exhibit frequencies of mutation to endogenous antibiotic resistance that are 1000-fold higher than normal strains and such hypermutators can be found at frequencies of up to 20% of clinical isolates.[8] The naturally occurring *E. coli* and *S. enterica* hypermutators identified by LeClerc *et al.*[6] contained defects in methyl-directed mismatch repair (MMR), a post-replicative repair system that corrects errors on newly synthesized DNA strands to ensure the fidelity of chromosome replication. Amongst the hypermutators described by LeClerc *et al.*[6] variants defective in the *mutS* allele were the most common, followed by strains with deficiencies in *mutH* and *mutU (uvrD*).

Elevated mutation rates displayed by hypermutators are likely to benefit the organisms by increasing the frequency with which antibiotic resistance arises and enhancing the opportunity for compensatory mutations to reduce fitness costs sometimes associated with the acquisition of endogenous antibiotic resistance.[9] Nevertheless, despite the potential importance of hypermutable strains with respect to the emergence of endogenous resistance, their response to antimicrobial selection pressure has not been examined in detail. A recent report by Tanabe *et al*.[10] describes the *in vitro* development of resistance to ampicillin and ofloxacin by an *E. coli dnaQ* hypermutator strain. However, the significance of these observations for the emergence of resistance in the clinical setting is uncertain since variants with *dnaQ* deficiencies have not been reported amongst natural *E. coli* isolates.[6] We therefore examined the response of several *E.coli* hypermutators, including those with MMR defects found in naturally occurring strains, to a variety of antibiotics with different endogenous resistance potentials.[2] This included selection with polymyxin B, cefotaxime and D-cycloserine (low endogenous resistance potentials), rifampicin (high resistance potential) and ciprofloxacin under conditions of both high and low resistance potential.

Materials and methods

Organisms

Several *E. coli* strains displaying mutator phenotypes were used (Table 1). The wild-type parent of the isogenic series 1412–1419 is strain 1411 and the parent of the isogenic series CSH114–117 is strain CSH109.

Antibiotics and growth media

Antibiotics were from Sigma-Aldrich, Poole, UK, with the exception of ciprofloxacin, which was a gift from Bayer AG, Leverkusen, Germany. Mueller–Hinton broth (MHB) and agar (MHA) were from Fisher, Loughborough, UK.

Determination of susceptibility to antibiotics

MICs were determined by either two-fold serial antibiotic dilutions or narrower increments in MHA with an inoculum in MHB of 10^4 cfu/spot.[11] Plates were incubated aerobically for 18 h at 37°C and the MIC was defined as the lowest concentration that produced no visible growth. At least three, and up to five, replicate MIC determinations were carried out for all starting strains and mutants. The quoted MICs were reproducible on all occasions.

Table 1. *E. coli* strains and their susceptibility to various antibiotics

Strain	Source	Genotype	MIC (mg/L)				
			RIF	CIP	DCS	CTX	PMB
1411	R. Lloyd, University of Nottingham, UK	*lacI*3, *lacZ*118, *proB*, *trp*, *nalA*, *rpsL*	8	0.125	16	0.032	0.5
1412		*lacI*3, *lacZ*118, *proB*, *trp*, *nalA*, *rpsL*, *uvrD*210	8	0.125	16	0.063	0.5
1413		*lacI*3, *lacZ*118, *proB*, *trp*, *nalA*, *rpsL*, *mutS*3	8	0.25	16	0.063	0.5
1417		*lacI*3, *lacZ*118, *proB*, *trp*, *nalA*, *rpsL*, *mutH*34	8	0.25	16	0.063	0.5
1419		*lacI*3, *lacZ*118, *proB*, *trp*, *nalA*, *rpsL*, *uvrD*156	8	0.125	16	0.063	0.5
1499[48]		*argG*, *his*, *leu*, *metB*, *malA*, *gal*, *xyl*, *mtl*, *lac*, *mutL*13	8	0.063	16	0.125	0.5
CSH109	Cold Spring Harbor[49]	*pro*, *ara*Δ(*gpt-lac)*5, *rpsL*	8	0.016	16	0.063	1
CSH114		*pro*, *ara*Δ(*gpt-lac)*5, *rpsL*, *mutT*	8	0.016	16	0.125	0.5
CSH114A		*pro*, *ara*Δ(*gpt-lac)*5, *rpsL*, *nalA*, *mutT*	8	0.032	16	0.125	0.5
CSH115		*pro*, *ara*Δ(*gpt-lac)*5, *rpsL*,*mutS*:mini-Tn*10*	8	0.016	16	0.125	1
CSH116		*pro*, *ara*Δ(*gpt-lac*)5, *rpsL*, *mutD5 zae-502*:Tn*10*	8	0.016	16	0.032	0.5
CSH117		*pro*, *ara*Δ(*gpt-lac*)5, *rpsL*, *mutY*:mini-Tn*10*	8	0.016	8	0.032	0.5

RIF, rifampicin; CIP, ciprofloxacin; DCS, D-cycloserine; PMB, polymyxin B; CTX, cefotaxime.

Determination of mutation frequencies for resistance to antibiotics

This was performed essentially as described by O'Neill *et al.*[12] using MH growth media. Both standard and concentrated cell techniques were used, whereby mutation frequencies as low as 1 in 10^{11} can be detected.[12] In each case, cultures were started from single colonies of the test strain that were shown to be sensitive to the antibiotic under investigation. In most cases, antibiotic-resistant mutants were identified on medium containing the selective antibiotic at a concentration that was four-fold higher than the respective MIC for an individual strain. Frequencies of second-step mutations to ciprofloxacin resistance were determined following selections with ciprofloxacin at levels four-fold higher than the respective MICs for the primary mutants. Mutation frequencies were expressed as the number of resistant mutants recovered as a fraction of total viable bacteria.[13]

Determination of bacterial growth rates

The growth rates (doubling times) of bacteria cultured in MHB were determined from absorbance readings at 600 nm according to the method of Koch.[14] Absorbance readings were taken automatically in a Molecular Devices Spectra Max Plus 384 microplate reader (Sunnyvale, CA, USA). Microplates were shaken and incubated at 37°C within the instrument.

Selection of mutants by continuous subculture in the presence of sub-inhibitory concentrations of antibiotic

These experiments were only performed with cefotaxime, D-cycloserine and polymyxin B. Bacteria were grown overnight at 37°C in MHB in the absence of antibiotics and 5 μL aliquots of these cultures were used to inoculate 9 mL of fresh broth containing the appropriate antibiotic at one-quarter of the respective MIC for the strain. These cultures were incubated overnight (18 h). Control experiments, involving determination of viable cell numbers, established that such cultures progressed through 13 generations. After overnight incubation a 5 μL aliquot of the culture was used to repeat the passage through further cycles, continuously in the presence of 0.25× MIC of the antibiotic. Each day, samples of undiluted culture were plated on to MHA containing the selective antibiotic at 4× the original MIC, and plates were incubated at 37°C to identify the point at which resistant mutants emerged during the sequential passage.

Sequencing of the quinolone resistance-determining regions in gyrA and parC

Chromosomal DNA was prepared from bacteria according to Sambrook & Russell.[15] Primers described by Weigel *et al.*[16] were used to amplify the QRDRs (quinolone resistance-determining regions) of *gyrA* and *parC*. The PCR products were purified using Microcon PCR Centrifugal Filter Devices (Millipore, Bedford, MA, USA) according to the manufacturer's instructions. DNA sequencing was performed using an Applied Biosystems 377 DNA sequencer.

Results and discussion

Mutation frequencies for resistance to drugs with different endogenous resistance potentials

Endogenous resistance to antibiotics usually results from mutations that affect drug uptake into the cell or alter target structure, thereby decreasing drug binding at the molecular site of action. The mutational response of hypermutators to selection with antibiotics with a range of endogenous resistance potentials has not been determined previously. We carried out such studies with rifampicin, ciprofloxacin, D-cycloserine, cefotaxime and polymyxin B.

Rifampicin has a single molecular target, the RpoB subunit of RNA polymerase, and no specific mechanism of entry into bacteria.[17] Consequently, rifampicin has high endogenous resistance potential, and single-step point mutations in only one gene, *rpoB*, can confer high-level resistance in *E. coli*.[18]

In *E. coli*, fluoroquinolones, including ciprofloxacin, primarily inhibit DNA gyrase (GyrA) with topoisomerase IV (ParC) as a secondary target.[19] Single-site mutations at codon 83 or 87 of the *gyrA* QRDR are sufficient to confer low-level ciprofloxacin resistance, whereas higher levels of resistance either require double (codons 83 and 87) mutations in *gyrA*, or *gyrA* mutations in combination with *parC* or *marA* mutations, the latter enhancing drug efflux via the AcrAB–TolC system and reducing influx by suppressing expression of the outer membrane protein OmpF.[20,21] Thus, at low concentrations ciprofloxacin has a high endogenous resistance potential, but at higher selective concentrations there is a requirement to generate simultaneous mutations in two or more loci[4] and the agent consequently displays low endogenous resistance potential.

D-Cycloserine and cefotaxime are antibiotics that have multiple lethal targets, i.e. D-alanyl-D-alanine ligases A and B (DdlA, B) and alanine racemase (Alr) for D-cycloserine,[22] and PBPs 1a (MrcA), 1b (MrcB) and 3 (FtsI) for cefotaxime.[23] These antibiotics are predicted to have low endogenous resistance potentials, since at the level of target site interaction it is expected that independent mutations in more than one gene will be required to express a resistance phenotype.[4] However, cefotaxime uses the OmpF porin pathway for uptake across the *E. coli* outer membrane[24] and D-cycloserine is transported into the cell by the D-alanine permease system (Cyc).[25] In the case of cefotaxime, point mutations conferring resistance could also potentially occur in the *ampC*-encoded β-lactamase,[26] or *marA*, the latter both enhancing AcrAB-mediated β-lactam efflux and decreasing drug uptake through OmpF

porin channels.[20,27] Therefore, single-site mutations conferring resistance to these antibiotics could in principle arise in genes other than those encoding the drug targets, thereby making the antibiotics prone to endogenous resistance development.

Membrane-active agents, such as polymyxin B, have low endogenous resistance potentials because they have no specific routes of entry and it is difficult to alter fundamental bacterial membrane composition by mutation.[28,29] Polymyxin-resistant mutants of *E. coli* have been isolated.[30] However, multiple outer membrane protein and lipopolysaccharide alterations were detected, indicating that several mutations are required to express a resistant phenotype.

The susceptibilities of hypermutators and their parent strains to each of the antibiotics discussed above were determined (Table 1). The *nalA (gyrA)* genotypes of strains 1411, 1412, 1413, 1417, 1419 and CSH114A were consistent with the low-level ciprofloxacin-resistant phenotypes exhibited by these strains (Table 1). Sequence analysis of *gyrA* and *parC* from strains 1411–1419 indicated that they contain a single point mutation conferring a Ser-83→Leu substitution in the QRDR of GyrA. This is a common single-step mutation that confers low-level resistance to fluoroquinolones, including ciprofloxacin, in *E. coli*.[21]

Attempts to select mutants resistant to rifampicin, ciprofloxacin, D-cycloserine, cefotaxime and polymyxin B from the hypermutator strains and their parent strains were carried out by plating bacteria on to MHA containing antibiotics at 4× the respective MICs listed in Table 1. No resistant mutants were recovered, even for hypermutators, for selections at 4× MIC with D-cycloserine, cefotaxime or polymyxin B (i.e. frequencies were $<10^{-11}$). These data are consistent with low endogenous resistance potentials for these antibiotics.

For rifampicin, resistant mutants were recovered from all strains (Table 2) and hypermutators demonstrated elevated mutation frequencies compared with that of the parent strain. Mutation frequencies varied from *c*. 10^{-8} for non-hypermutator strains to as high as 10^{-5} for strain CSH114 (*mutT*). These results demonstrate the ease with which resistance can arise in hypermutators to agents that have a single molecular target and no specific uptake mechanism, i.e. exhibiting high endogenous resistance potential.

This generalization was confirmed by using ciprofloxacin under conditions where it behaves as an agent with high endogenous resistance potential. Preliminary experiments revealed that selection of first-step ciprofloxacin-resistant mutants in strains CSH115, CSH116 and CSH117 led to loss of hypermutator status (data not shown), apparently due to the elimination of the mini-Tn*10* elements integrated in the *mut* genes. Therefore, in subsequent studies on the selection of ciprofloxacin mutants from CSH115 to CSH117 the strains were cultured in the presence of 4 mg/L tetracycline to maintain mini-Tn*10* and hypermutator status. Control experiments established that growth in the presence of tetracycline did not influence the hypermutable properties of these strains, in that elevated mutation frequencies for rifampicin resistance were demonstrated when strains were cultured in the presence of 4 mg/L tetracycline (data not shown).

Table 2. First-step mutation frequencies for resistance to rifampicin and ciprofloxacin

Strain	Mutation frequency[a]	
	RIF	CIP
1411	$4.00 \pm 0.60 \times 10^{-8}$	NR
1412	$2.33 \pm 0.35 \times 10^{-6}$	NR
1413	$2.18 \pm 0.27 \times 10^{-6}$	NR
1417	$4.56 \pm 0.55 \times 10^{-6}$	NR
1419	$5.02 \pm 1.90 \times 10^{-6}$	NR
1499	$3.18 \pm 0.27 \times 10^{-6}$	NR
CSH109	$1.70 \pm 0.18 \times 10^{-8}$	$1.00 \pm 0.62 \times 10^{-9}$
CSH114	$1.70 \pm 0.90 \times 10^{-5}$	$1.50 \pm 0.31 \times 10^{-6}$
CSH114A	$5.40 \pm 0.57 \times 10^{-6}$	NR
CSH115	$6.65 \pm 0.59 \times 10^{-6}$	$1.67 \pm 0.52 \times 10^{-7}$
CSH116	$5.70 \pm 1.15 \times 10^{-6}$	$8.47 \pm 1.30 \times 10^{-8}$
CSH117	$3.82 \pm 0.85 \times 10^{-6}$	$1.31 \pm 0.10 \times 10^{-7}$

[a]± S.D.
Mutants were recovered from selection at 4 × MIC of each drug (see Table 1).
NR, not relevant: already contains a first-step *gyrA* mutation (see text and Table 3); RIF, rifampicin; CIP, ciprofloxacin.

The QRDRs of *gyrA* and *parC* in strains CSH109, CSH114 and CSH115–CSH117 were sequenced following PCR amplification of these regions. No mutations were detected. Using a low ciprofloxacin selection concentration, first-step ciprofloxacin-resistant mutants arose with a frequency of 10^{-9} with the parental strain (CSH109) and at elevated frequencies, up to 1000-fold higher, with the hypermutators (Table 2). Randomly selected, first-step ciprofloxacin-resistant mutants (strains KM1, KM2 and KM6–KM11) (Table 3) derived from strains CSH109, CSH114 and CSH115–CSH117 were chosen and their QRDRs of *gyrA* and *parC* were sequenced. KM1 and KM2 each demonstrated a single Ser-83→Leu mutation in GyrA, whereas the other mutants had Asp-87→Gly mutations in GyrA. Since involvement of these mutations in the expression of low-level fluoroquinolone resistance is well established,[21,31,32] it can be concluded that each of the first-step ciprofloxacin-resistant mutants KM1, KM2 and KM6–KM11 contains a single-step point mutation at codon 83 or 87 in *gyrA*. The sequence data confirm that the ciprofloxacin-resistant mutants arose readily from the CSH hosts (Table 2) under conditions in which ciprofloxacin displays high endogenous resistance potential.

Mutants KM1, KM2 and KM6–KM11 were examined for their potential to give rise to second-step ciprofloxacin-

Table 3. Susceptibility of first-step mutants and strains with pre-existing *gyrA* mutations to ciprofloxacin, and mutation frequencies for selection of second-step ciprofloxacin-resistant mutants

Strain (first-step mutants)	Mutation in *gyrA*	Parent	MIC (mg/L) (ciprofloxacin)	Second-step resistance mutation frequency	Theoretical simultaneous two-step resistance mutation frequency
KM1	Ser-83→Leu	CSH109	0.064	$5.41 \pm 1.80 \times 10^{-9}$	5.41×10^{-18}
KM2	Ser-83→Leu	CSH114	0.064	$2.95 \pm 1.96 \times 10^{-9}$	4.43×10^{-15}
KM6	Asp-87→Gly	CSH115	0.25	$1.54 \pm 0.31 \times 10^{-9}$	2.57×10^{-16}
KM7	Asp-87→Gly	CSH115	0.125	$1.30 \pm 0.58 \times 10^{-9}$	2.57×10^{-16}
KM8	Asp-87→Gly	CSH116	0.125	$2.36 \pm 0.12 \times 10^{-9}$	2.00×10^{-16}
KM9	Asp-87→Gly	CSH116	0.125	$2.88 \pm 0.42 \times 10^{-9}$	2.44×10^{-16}
KM10	Asp-87→Gly	CSH117	0.125	$3.44 \pm 0.99 \times 10^{-8}$	4.51×10^{-15}
KM11	Asp-87→Gly	CSH117	0.125	$5.87 \pm 0.92 \times 10^{-8}$	7.69×10^{-15}
1411	Ser-83→Leu	–	0.125	$8.33 \pm 3.81 \times 10^{-9}$	data unavailable
1412	Ser-83→Leu	–	0.125	$4.42 \pm 0.55 \times 10^{-8}$	data unavailable
1413	Ser-83→Leu	–	0.25	$5.74 \pm 0.85 \times 10^{-9}$	data unavailable
1417	Ser-83→Leu	–	0.25	$1.76 \pm 0.18 \times 10^{-9}$	data unavailable
1419	Ser-83→Leu	–	0.125	$2.69 \pm 1.00 \times 10^{-9}$	data unavailable

Ciprofloxacin selection concentrations for the second-step mutants were 4× respective MIC values of the first-step mutants.

resistant mutants. Selections were performed with ciprofloxacin at 4 × the respective MIC for strains KM1 and KM2 and for KM6–KM11 in the presence of 4 mg/L tetracycline (Table 3). As discussed above, the mutation frequencies recorded under these conditions are likely to reflect selection of second-site mutations either in *gyrA* (codon 83 or 87), *parC* (codon 80 or 84) or *mar*. In contrast to the generation of first-step ciprofloxacin-resistant mutants in the mutator CSH hosts (Table 2), the frequency with which second-step ciprofloxacin-resistant mutants arose in strains KM2 and KM6–KM11 was at most only 10-fold greater than the non-hypermutator strain KM1 (Table 3). Indeed, in some cases second-step mutation frequencies, e.g. for KM2 and KM6–KM9, were not significantly different from those obtained with KM1 (Table 3). This was not due to loss of mutator status in strains KM2 and KM6–KM11, since elevated mutation frequencies to rifampicin resistance, comparable to those exhibited by CSH114–CSH117, were found for KM2 and KM6–KM11 (data not shown). Further experiments to address the question of why second-step mutation frequencies to ciprofloxacin resistance were suppressed, or partially suppressed, in hypermutator hosts are considered in a subsequent section (see Further observations on second-step ciprofloxacin-resistant mutants).

By combining the individual mutation frequencies for ciprofloxacin resistance (Tables 2 and 3), it is possible to derive theoretical resistance mutation frequencies (Table 3) under conditions where ciprofloxacin has low endogenous resistance potential, i.e. 16 × MIC selective concentration. These values are extremely low for CSH109 and its *mutT* (CSH114), *mutS* (CSH115), *mutD* (CSH116) and *mutY* (CSH117) derivatives. Indeed, consistent with these theoretical values, it was not possible to select resistant mutants of strains CSH109–CSH117 directly when the ciprofloxacin selective concentration was 16 × MIC, i.e. the experimental mutation frequency was $<10^{-11}$ (data not shown). This confirms the low endogenous resistance potential of ciprofloxacin when there is a requirement to acquire simultaneous mutations in two or more genes to express moderate- or high-level resistance.

Selection of resistant mutants by continuous subculture in the presence of sub-inhibitory antibiotic concentrations

Experiments were also conducted with D-cycloserine, cefotaxime and polymyxin B to investigate the potential for the emergence of resistance in hypermutators during prolonged subculture. This involved attempts to select resistant mutants by continuous subculture in the presence of sub-inhibitory (0.25 × MIC) antibiotic concentrations. Separate experiments established that the antibiotics under these conditions imposed selection pressures. For example, the doubling time of strain CSH109 was increased 1.8-, 3.5- and 1.5-fold in the presence of 0.25 × MIC polymyxin B, cefotaxime and D-cycloserine, respectively. Repeated passage in the presence of D-cycloserine at 0.25 × MIC eventually led to the emergence of D-cycloserine-resistant mutants in both hypermutators and their parent strains (Figure 1). However, resistant mutants arose more frequently from hypermutators than their parental strains and resistance also emerged earlier from hypermutators (the majority between 26 and 39 generations) compared with the parents (39–52 generations). Each of the 18 D-cycloserine-resistant mutants recovered (Figure 1) exhibited a four-fold increase in resistance to the antibiotic

compared with the parent strain. The hypermutator status of D-cycloserine-resistant mutants arising in hosts CSH114A–CSH117 was confirmed by demonstrating that these strains retained resistance to tetracycline (MIC > 128 mg/L), indicating mini-Tn*10* insertions in the respective *mut* genes. Furthermore, the D-cycloserine-resistant mutants derived from strains 1412–1499 and CSH114–CSH117 each retained elevated mutation frequencies for rifampicin resistance, consistent with retention of hypermutator status. We have not explored the mechanism of D-cycloserine resistance in these mutants. However, in mycobacteria, resistance to D-cycloserine can arise by mutations that either increase the expression of alanine racemase (*alr*),[33] or alter the D-cycloserine uptake system (*cyc*).[34] Since D-cycloserine-resistant mutants could not be selected by direct plating of *E. coli* (i.e. mutation frequency $<10^{-11}$) (see previous section) it is possible that both types of mutation are required simultaneously to express resistance in *E. coli*. The frequency with which double mutations arise is predicted to be low and such mutants might only be selected by sequential passage in the presence of D-cycloserine.

Sequential passage for up to 65 generations failed to recover mutants resistant to either cefotaxime or polymyxin B. This indicates that these antibiotics have low endogenous resistance potential.

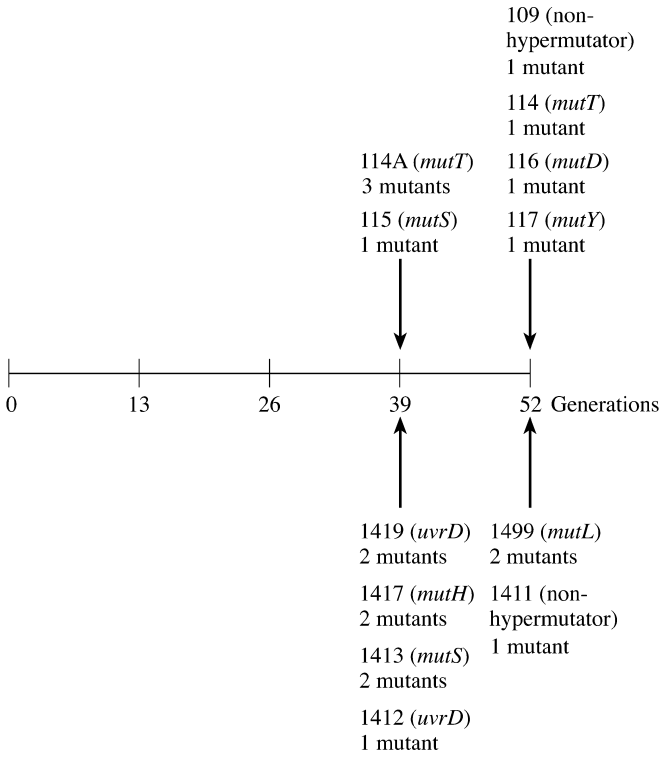

Figure 1. Timeline for emergence of mutants resistant to D-cycloserine during continuous subculture in the presence of antibiotic (0.25 × MIC). The diagram records the appearance of mutants recovered from one continuous growth experiment with each of the listed strains.

Population analysis of antibiotic-resistant mutants

Population analyses were performed to determine whether there was a bias towards the generation of mutants with higher, or lower, levels of antibiotic resistance from hypermutator strains compared with non-hypermutators. For rifampicin, 20 resistant mutants derived from each of the 12 strains listed in Table 1 were picked at random from colonies appearing on selection plates containing rifampicin at 4 × MIC levels. The individual MICs of rifampicin were determined for these 240 strains. Rifampicin MICs for mutants were in the range 32–256× that of the starting strain. For the majority (18 isolates, 90%) of rifampicin-resistant mutants from strain CSH109 (the parent of series CSH114–CSH117) the MIC was 1024 mg/L, i.e. 128 × MIC of rifampicin for strain CSH109 itself (Figure 2). The two further mutants derived from strain CSH109 displayed rifampicin MICs of 512 mg/L, i.e. 64 × MIC (one strain, 5%) and 2048 mg/L, i.e. 256 × MIC (one strain, 5%) (Figure 2). Similar determina-

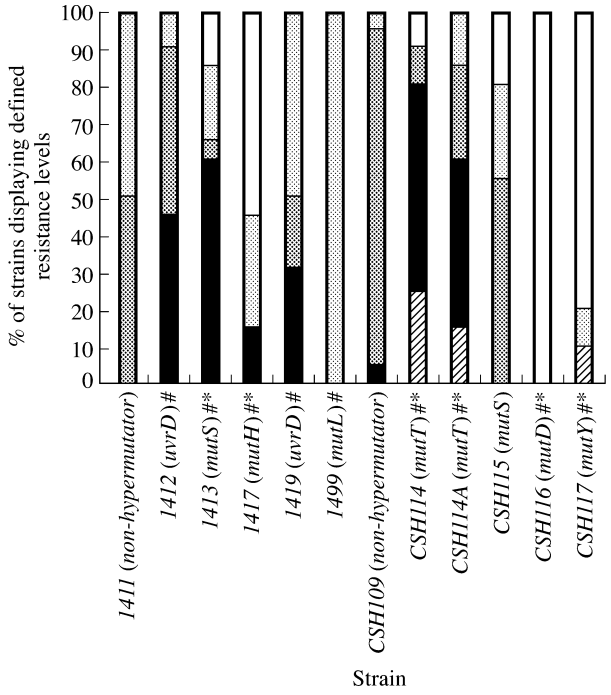

Figure 2. Population analysis of single-step rifampicin-resistant mutants derived from host strains CSH109–CSH117 and 1411–1499. Resistant mutants were selected from each of the starting strains by plating on agar containing rifampicin at 4 × MIC. Twenty mutants arising from each strain (i.e. 240 mutants in total) were picked at random from the primary selection plates and their susceptibilities (MIC) to rifampicin were determined. The numbers (%) of resistant mutants derived from each strain falling into the following resistance bands were recorded: hatching, 32 × MIC for starting strain; solid, 64 × MIC; dark shading, 128 × MIC; light shading, 256 × MIC; no shading, >256 × MIC. All starting strains have rifampicin MICs of 8 mg/L. #, Significant difference at 95% confidence limits; *, significant difference at 99% confidence limits; all others show no significant difference compared with the non-hypermutator strains, CSH109 and 1411.

tions were carried out for the other strains (Figure 2). Statistical analysis of the data by an $R \times C$ test of independence[35] indicated significant differences in the MIC distribution of rifampicin-resistant mutants arising from the majority of the hypermutators when compared with those of the respective parent strains (Figure 2). A greater proportion of high-level rifampicin-resistant mutants was recovered from the *mutD*-, *mutH*-, *mutL*- and *mutY*-deficient strains compared with the non-hypermutator strains. Furthermore, the highest level of resistance displayed by mutants arising from hypermutators (>256 × MIC for the starting strain) was greater than that of any mutant recovered from non-hypermutator strains, which never exceeded 256 × MIC (Figure 2). The range of rifampicin resistance levels exhibited by the 240 mutants studied here presumably reflects variations in the nature and position of point mutations occurring within *rpoB*.[18]

Similar experiments were carried out for first-step ciprofloxacin-resistant mutants derived from strains CSH109 and CSH114–CSH117. In the case of strains CSH115–CSH117, ciprofloxacin-resistant mutants were selected in the presence of 4 mg/L tetracycline to maintain mini-Tn*10* insertions in the *mut* alleles. Each of the 20 ciprofloxacin-resistant mutants of CSH109 (first-step) displayed four-fold increases in resistance to the drug (Figure 3). Compared with CSH109, significant differences in ciprofloxacin MIC distribution, confirmed by χ^2 independence testing,[35] were obtained for ciprofloxacin-resistant mutants of CSH114 (*mutT*), CSH115 (*mutS*), CSH116 (*mutD*) and CSH117 (*mutY*) (Figure 3). In each case, mutants were recovered from the mutator strains that exhibited higher ciprofloxacin resistance levels than mutants derived from the non-hypermutator strain CSH109. Thus, whereas ciprofloxacin-resistant mutants arising from the non-hypermutator strain CSH109 only demonstrated a four-fold increase in resistance to the drug, ciprofloxacin-resistant mutants with at least an eight-fold increase in resistance were recovered from all mutator hosts, and two mutants derived from CSH115 (*mutS*) demonstrated resistance levels up to 16-fold greater than the parent strain (Figure 3).

The first-step ciprofloxacin-resistant mutants described here (Figure 3) are all presumptive *gyrA* mutants since single-site mutations in this gene are sufficient to confer low levels of ciprofloxacin resistance.[21,31,36] We therefore sought evidence that first-step mutants contained *gyrA* mutations. One, and in some cases two, of the most resistant first-step ciprofloxacin-resistant mutants arising from each of the starting strains CSH109–CSH117 were randomly selected for sequence analysis of *gyrA* and *parC*. Alterations in these first-step mutants (KM1, KM2, KM6–KM11) were only located in *gyrA*, either at codon 83 or 87 (Table 3). In KM1 and KM2 the Ser-83→Leu mutation was associated with a four-fold increase in resistance to ciprofloxacin compared with the parent strains CSH109 and CSH114. Mutants KM7–KM11 possessed Asp-87→Gly mutations in the QRDR of *gyrA* and

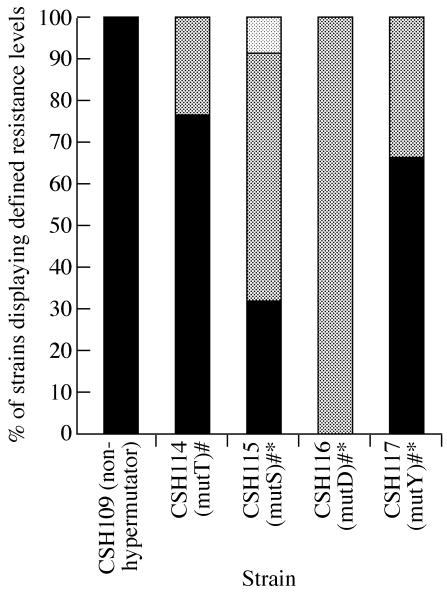

Figure 3. Population analysis of single-step, low-level ciprofloxacin resistant mutants derived from starting strains CSH109–CSH117. Resistant mutants were selected from each of the starting strains by plating on agar containing ciprofloxacin at 4 × MIC. Twenty mutants from each starting strain (i.e. 100 mutants in total) were picked at random from the primary selection plates and their susceptibilities (MICs) to ciprofloxacin determined. The numbers (%) of resistant mutants derived from each starting strain falling into the following resistance bands were recorded: solid, 4× MIC; dark shading, 8 × MIC, light shading, 16 × MIC. All starting strains have ciprofloxacin MICs of 0.016 mg/L. #, Significant difference at 95% confidence limits; *, significant difference at 99% confidence limits; all others show no significant difference in the profile compared with the non-hypermutator parent strain CSH109.

exhibited an eight-fold increase in resistance to ciprofloxacin. However, one mutant, KM6, although demonstrating an Asp-87→Gly mutation in the QRDR of *gyrA* consistently demonstrated a 16-fold increase in resistance to ciprofloxacin compared with its parent CSH115 (Tables 1 and 3). It is possible that two mutations have arisen simultaneously in KM6, one in *gyrA* and the second at another locus. These mutations would act synergically in KM6 to raise the level of resistance 16-fold compared with the parent strain CSH115. A candidate for the second locus might be *marA*, since such mutations have been identified as secondary to *gyrA* mutations in the course of *in vitro* selections for ciprofloxacin resistance.[21] However, KM6 did not exhibit increased resistance to chloramphenicol (data not shown), a phenotype associated with mutations in *mar*.[20,21] Therefore, the putative nature of the second-site mutation in KM6 is currently unknown, but it may reside in *crp*, *cya*, *icd*, *purB* or *ctr*, since mutations in any of these genes can confer low-level resistance to ciprofloxacin.[37] It is also interesting to note that although KM6 exhibits a

16-fold increase in resistance to ciprofloxacin compared with its parent CSH115, it was not possible to select ciprofloxacin-resistant mutants of CSH115 by direct selection on agar containing ciprofloxacin at 16 × MIC. This suggests that a second, unidentified mutation arose in KM6, probably spontaneously during subculture of the mutant before the final ciprofloxacin susceptibility testing that established the ciprofloxacin MIC of 0.25 mg/L for this strain. A similar explanation may account for the occurrence of a second mutant derived from CSH115, for which ciprofloxacin also exhibited an MIC of 0.25 mg/L (Figure 3).

To our knowledge this situation has not previously been reported. Furthermore, our data indicate that the occurrence of these spontaneous mutations in the absence of ciprofloxacin selection pressure may be enhanced in hypermutable hosts.

χ^2 Population analysis[35] was carried out on the second-step ciprofloxacin-resistant mutants (160 mutants) derived from strains KM1, KM2 and KM6–KM11 that had themselves been selected from the host strains CSH109 and CSH114–CSH117. Compared with KM1, derived from non-hypermutator strain CSH109, no significant differences in second-step ciprofloxacin MIC distribution were obtained for *mutD*-, *mutS*- and *mutY*-deficient strains (Figure 4). However, second-step mutants derived from KM2 (*mutT*) demonstrated a trend towards generation of mutants with higher levels of ciprofloxacin resistance than those derived from KM1 (Figure 4).

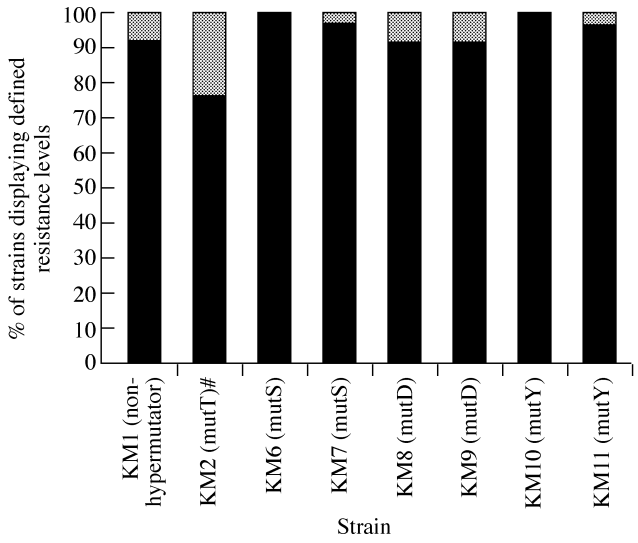

Figure 4. Population analysis of second-step, high-level ciprofloxacin-resistant mutants derived from starting strains KM1, KM2 and KM6–KM11. Resistant mutants were selected from each of the starting strains by plating on agar containing ciprofloxacin at 4 × MIC. Twenty mutants from each starting strain (i.e. 160 mutants in total) were picked at random from the primary selection plates and their susceptibilities (MICs) to ciprofloxacin determined. The numbers (%) of resistant mutants derived from each starting strain falling into the following resistance bands were recorded: solid, 4 × MIC; shading, 8 × MIC. Starting strains KM1 and KM2 have ciprofloxacin MICs of 0.64 mg/L; all other starting strains have MICs of 0.125 or 0.25 mg/L. #, Significant difference at 95% confidence limits; all others show no significant difference compared with non-hypermutator strain KM1.

Further observations on second-step ciprofloxacin-resistant mutants

As noted in previous sections, elevated mutation frequencies for selection of second-step ciprofloxacin-resistant mutants in strains KM2 (GyrA, Ser-83→Leu) and KM6–KM11 (GyrA; Asp-87→Gly) were not observed (Table 3), even though these strains retained hypermutator status for the generation of rifampicin-resistant mutants. Second-step ciprofloxacin-resistant mutants were also selected from strains 1411–1419 (GyrA; Ser-83→Leu). Mutation frequencies for second-step ciprofloxacin resistance were again suppressed in the mutator hosts (Table 3), even though these strains exhibited elevated frequencies for selection of rifampicin-resistant mutants. In addition, it was noted that the MIC distribution of second-step ciprofloxacin-resistant mutants arising from strains KM6–KM11 (Figure 4) and 1412–1419 (data not shown) did not differ significantly from that of second-step mutants arising from parent strains KM1 and 1411. These observations suggest that the nature of the second-step ciprofloxacin-resistant mutations arising from these strains is identical and that the locus responsible is not subject to elevated mutation frequencies despite the presence of defective MMR pathways in strains KM6–KM11 and 1412–1419.

The possibility that second-step mutations to ciprofloxacin resistance in hypermutator and parent strains arise in the *mar* regulatory locus was suggested by the observation that out of 170 second-step mutants analysed, each displayed four-fold increases in resistance to chloramphenicol (MIC 16 mg/L) compared with their immediate parents (chloramphenicol MIC 4 mg/L) and the parents of the first-step ciprofloxacin-resistant mutants (chloramphenicol MIC 4 mg/L). As already noted, the chloramphenicol-resistant phenotype is characteristic of *mar* mutations.[20,21]

Conclusions

In recent years progress has been made in the discovery and development of new agents to address problems caused by bacterial resistance to existing antibiotics.[38–41] Clearly, one of the critical elements in the selection of lead compounds for development rests upon choosing new compounds with low endogenous resistance potentials.[2,3,42] Since bacterial loads as high as 10^9 cells/mL can occur within infected tissues,[43] new agents should ideally possess endogenous mutation rates of $<10^{-9}$ at concentrations where they exhibit effective antibacterial action.

Recently, we[42] and others[4,44] advocated the use of bacterial hypermutators, i.e. strains with elevated mutation rates, as valuable tools for assessing the potential for the development of resistance to new antibiotics. Since hypermutators exist in the clinical setting,[6,7,44] their use in the laboratory can indicate worst case scenarios for resistance development.[42]

Despite the potential value of hypermutators for antibiotic discovery research, their response to selection pressure with antibiotics has only partially been characterized.[6,10] This paper demonstrates that the presence of hypermutators in the clinical setting presents an enhanced risk for the emergence of endogenous resistance to agents where mutations in single gene targets confer resistance phenotypes. Hypermutators could also present problems in the case of agents with multiple targets but single uptake routes, since resistance development may only need mutation in the uptake system. Although we did not characterize D-cycloserine-resistant mutants, we noted that they arose more readily from hypermutable hosts than from non-hypermutators, perhaps reflecting the enhanced ability of hypermutators to generate double mutations in both the transport system *cyc* and the alanine racemase (*alr*) promoter. In some cases hypermutators gave rise to mutants whose antibiotic resistance levels were greater than those recovered from non-hypermutator hosts. These results demonstrate that hypermutators possess superior genetic backgrounds for the selection of some antibiotic-resistant mutations and further emphasize the need to develop new drugs with minimal resistance potential.

We noted different relative mutation frequencies with some of the hypermutators for the selection of single-step rifampicin- and ciprofloxacin-resistant mutants. For instance, CSH116 (*mutD*) was relatively more proficient in generating rifampicin-resistant mutants than ciprofloxacin-resistant mutants under conditions where both agents were acting as single target drugs (Table 2). These observations probably reflect the outcome of several variables, including the preference for the introduction of transitions or transversions by the various hypermutators.[45]

Finally, we noted that the MICs for single-step rifampicin-resistant mutants, and in particular those arising in hypermutators, varied considerably. In contrast the MICs for single-step ciprofloxacin mutants fell into a more restricted MIC range that was not greatly influenced by hypermutator status. These differences probably reflect the fact that rifampicin resistance can be acquired by mutation at many sites in *rpoB*,[3,46] whereas mutations conferring ciprofloxacin resistance are confined to a more limited number of sites in *gyrA* or *parC*.[16,21,31,36,47]

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