Bacterial hypermutation: clinical implications

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Heritable hypermutation in bacteria is mainly due to alterations in the methyl-directed mismatch repair (MMR) system. MMR-deficient strains have been described from several bacterial species, and all of the strains exhibit increased mutation frequency and recombination, which are important mechanisms for acquired drug resistance in bacteria. Antibiotics select for drug-resistant strains and refine resistance determinants on plasmids, thus stimulating DNA recombination via the MMR system. Antibiotics can also act as indirect promoters of antibiotic resistance by inducing the SOS system and certain error-prone DNA polymerases. These alterations have clinical consequences in that efficacious treatment of bacterial infections requires high doses of antibiotics and/or a combination of different classes of antimicrobial agents. There are currently few new drugs with low endogenous resistance potential, and the development of such drugs merits further research.

Introduction

Eradication of infectious diseases is constantly challenged by micro-organisms that develop new survival strategies. Previous studies suggest that mutational events play a predominant role in bacterial adaptation and confer a selective advantage (Chou et al., 2009; Cooper, 2007). Early experiments aimed at detecting mutators used mutagenized laboratory strains of bacteria, sometimes coupled with different selection strategies. LeClerc et al. (1996) reported high mutation frequency among Escherichia coli and Salmonella pathogens, challenging the theory that mutators were rare among bacterial populations. Taken together, these findings demonstrated that natural populations could respond to environmental selection in two ways, i.e. by enhanced mutation frequencies and by recombination. Transient mutator status, which involves reversion or recombination within the mutator alleles or depletion of the methyl-directed mismatch repair (MMR) system proteins, allows the organism to temporarily benefit from the elevated mutation frequency for adaptation while reducing the risk of accumulating deleterious mutations. Using a mathematical model, Rosche & Foster (1999) showed that transient hypermutators play a role in adaptive mutation in E. coli.

Molecular mechanisms of hypermutation

Proteins involved in the DNA mismatch repair pathway help replace nucleotides introduced erroneously into the replicated DNA and also hinder recombination between non-identical DNA sequences. Deficiencies in any of the DNA mismatch repair pathway mechanisms can lead to a hypermutator phenotype.

DNA repair

Siegel & Bryson (1967) discovered the mutS gene in an azaserine-resistant derivative of E. coli that had a mutator phenotype and carried a deletion in the mutS gene. The majority of naturally occurring strong mutators have defects in the MMR system; the mutations are mainly in mutS (Oliver et al., 2002), but deletions in genes encoding beta-clamp proteins (Chopra et al., 2003) and in mutH, mutL and mutU (uvrD) have also been described (Fig. 1). Inactivation of basal excision repair genes, e.g. mutY, mutM, mutT, can also cause a major increase in mutation rate. MutY, MutM and MutT reduce the level of 8-oxo-dG, and MutD supplies proofreading activity. In Bacillus

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anthracis, the mutY and mutM single knockouts are weak mutators by themselves, but the mutY mutM combination results in very high mutation rates due to G:C→T:A transversions (Zeilberg et al., 2007). Bacterial strains that are unable to perform any of these activities are unable to repair mismatches and, consequently, their mutation

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**Fig. 1.** MMR system DNA repair mechanism. Adapted by permission from Macmillan Publishers Ltd: *EMBO J* (Jiricny, 1998), copyright 1998; and by permission from Annual Reviews (Schofield & Hsieh, 2003). The initiation of mismatch repair requires MutS, MutL and MutH; the latter generates a nick in the unmethylated strand of a nearby hemimethylated d(GATC) sequence. Both MutS and MutL are dimeric ATPases: MutS recognizes and binds the mismatch and recruits MutL, which binds the MutS–mismatch complex. The endonuclease MutH is stimulated to catalyse endonucleolytic cleavage at the unmethylated d(GATC) site in the presence of MutL and MutS. DNA unwinding is then initiated at the nick by DNA helicase II (UvrD) and the repair itself is catalysed by the DNA polymerase III holoenzyme.
frequency increases. Hypermutators make up about 0.1% of the natural E. coli population (LeClerc et al., 1996), but this incidence is sometimes higher in clinical isolates (Baquero et al., 2004, 2005; Denamur et al., 2002). The mutation frequency is increased up to 13-fold in weak hypermutators and up to 200-fold in strong hypermutators (Matic et al., 1997).

Mismatch repair systems are highly conserved in bacterial populations and evolved from common ancestors, i.e. the Hex system in Gram-positive Staphylococcus pneumoniae and the Mut system in Gram-negative E. coli and Salmonella enterica serovar Typhimurium. There is homology between HexA and MutS and between HexB and MutL in Streptococcus pneumoniae (Haber et al., 1988; Prudhomme et al., 1991) and Saccharomyces cerevisiae (Reenan & Kolodner, 1992).

Disruptions of genes other than mut can also result in a hypermutator phenotype. For example, DNA adenine methylation by DNA adenine methyltransferase (Dam) plays an important role in DNA replication, gene expression regulation, and DNA methylation for strand discrimination during mismatch repair (Marinus, 2010). Strains deficient in Dam are hypersensitive to DNA-damaging agents or reactive oxygen species (Zaleski & Piekarowicz, 2004). Insertional inactivation of dam and/or drg (dam-replacing genes) results in hypermutator phenotypes (Chen et al., 2003).

Other mutants with inactivated DNA repair genes have also been reported. These include isolates with inactivation of the alkylating damage repair gene ada/alkA in several Mycobacterium tuberculosis isolates and in one Mycobacterium bovis strain (Nouvel et al., 2007) and E. coli isolates with an inactivated dnaQ gene, which encodes the epsilon subunit of DNA polymerase III (Itakura et al., 2008). Recently, Rodriguez-Rojas & Blázquez (2009) looked for additional genes involved in hypermutability by screening a Pseudomonas aeruginosa library with random insertions. Mutational inactivation of pfpl, which putatively encodes a member of the Djl-1/ThiJ/Pfpl superfamily, was found to confer a hypermutator phenotype.

Recombination

Recombination requires the genetic recombination genes recB, recC, recD and recA, which are part of the bacterial RecBCD recombination system. These proteins generally require near-perfect homology between the two complementary DNA strands, but this requirement is greatly relaxed in MMR-deficient mutants. Recombination-deficient recA and recB null mutant strains are deficient in adaptive reversion. A hyper-recombinogenic recD strain is hypermutable, and its hypermutation depends on functional recA and recB genes (Harris et al., 1994).

Worth et al. (1994) showed that MutS abolishes recA-catalysed strand transfer between the DNA of the fd and M13 bacteriophages, which vary by 3% at the nucleotide level, but has no effect on M13–M13 or fd–fd exchange. Although MutL has no effect on M13–fd heteroduplex formation, the protein dramatically enhances inhibition of MutS-mediated strand transfer. Analysis of strand-transfer intermediates that accumulate in the presence of MutS and MutL indicates that the proteins block branch migration, presumably in response to mispairs within the newly formed heteroduplex. Transduction between Salmonella Typhimurium and Salmonella Typhi is blocked by the activity of the recipient’s mismatch repair system, which senses sequence divergence and disrupts heteroduplexes in favour of recipient sequences (Zahrt et al., 1994).

Detection of hypermutable strains in laboratories

In vitro detection of hypermutable strains by disc diffusion and Etest methods

Using standard susceptibility testing methods, such as disc diffusion and the Etest method, mutant antibiotic-resistant subpopulations of P. aeruginosa were identified within the inhibition zones as hypermutable strains [CA-SFM recommendations (Comité de l’Antibiogramme de la Société Française de Microbiologie, 2009); Clinical and Laboratory Standards Institute guidelines (formerly the National Committee for Clinical Laboratory Standards) (NCCLS, 2003)] (Macià et al., 2004).

Determination of mutation frequencies

Hypermutable strains are recognized by their higher mutation frequency. The mutation rates are determined by plating dilutions of the tested bacterial strain grown in Luria–Bertani (LB) broth, on LB agar plates, and on LB agar plates containing one of several antibiotics: rifampicin (100 µg ml⁻¹) (Baquero et al., 2004; Galán et al., 2004), nalidixic acid, spectinomycin (LeClerc et al., 1996), novobiocin (Zaleski & Piekarowicz, 2004), fusidic acid (O’Neill et al., 2001) or fosfomycin (Denamur et al., 2002). Colony counts are performed after 24 h (LB plates) or 48 h (LB plus antibiotic), and mutation frequencies are often reported as the proportion of the number of antibiotic-resistant colonies compared to the total colony count on LB plates, for at least three experiments. Other alternative strategies for determining mutation rates have also been described (Eisenstadt et al., 1994). In E. coli, phenotypic categories were established by Baquero et al. (2004), who defined four categories of bacterial strains according to their mutation frequencies (f) for 696 E. coli clinical isolates: hypomorphic (f≤8×10⁻⁸), normomorphic (8×10⁻⁸ <f<4×10⁻⁷), weak mutator (4×10⁻⁸ ≤f<4×10⁻⁷) and strong mutator (f≥4×10⁻⁷). Denamur et al. (2002) proposed that a strain should be considered a mutator strain when the frequencies of mutations that conferred resistance to rifampicin were 10-fold higher than the median value of mutagenesis observed for all the studied strains (these were termed ‘10-fold mutators’). Strains that displayed a >50-fold increase in mutagenesis were considered strong mutators (50-fold mutators).
Phenotypic characterization

Some researchers found no differences in the phenotypic characteristics of hypermutable strains versus normomutator strains. However, a recent study using the API 32GN system (bioMérieux) showed some differences in the identification profiles of a hypermutable strain of Salmonella Heidelberg versus a wild-type strain. In contrast to the wild-type strain, Salmonella Heidelberg was not able to grow in the presence of L-alanine, L-serine, lactic acid and propionic acid (Le Gall et al., 2009).

Hypermutable phenotype incidence

Urinary tract infections (UTIs)

Denamur et al. (2002) studied a population of 603 E. coli strains, some commensal (i.e. faecal samples collected from healthy unrelated human subjects) and some pathogenic (from patients with bacteraemia, from patients with UTIs, enteroinvasive or enterohemorrhagic pathogen, or from newborn meningitis). They found no significant difference in the frequency of mutators between the two groups. However, mutator strains occurred significantly more frequently among UTI strains. These results were confirmed by Baquero et al. (2004), who found a higher frequency of weak mutators in urinary tract isolates (25 %) and in blood isolates (38 %). Studying 696 E. coli strains from patients with UTIs in Sweden, Denmark and Spain, Baquero et al. (2004) found that 23 % were weakly hypermutable and 0.7 % were strongly hypermutable. The proportion of weak mutators among the Danish isolates was significantly lower than in the Spanish blood isolates, indicating geographical differences in the mutation frequency distribution profiles.

Cystic fibrosis (CF)

CF airway mucus can contain opportunistic micro-organisms, notably P. aeruginosa and Staphylococcus aureus, and recently the frequency of isolation of Stenotrophomonas maltophilia and Burkholderia cepacia has increased (Turrientes et al., 2010). Of a small collection of 49 Staphylococcus aureus isolates from CF patients, only one showed a weak elevation in mutation frequency (three- to fourfold increase) compared with the laboratory reference strain (O’Neill & Chopra, 2002, 2003). There was a higher proportion of hypermutator strains in 89 staphyloccocal CF isolates (14.6 %) compared with a group of 74 non-CF controls (1.3 %) (Prunier et al., 2003).

P. aeruginosa is the most relevant pathogen in terms of development of chronic lung infections in patients with chronic underlying diseases such as CF, bronchiectasis and chronic obstructive pulmonary disease. Hypermutable P. aeruginosa strains have been found at high frequencies in the lungs of CF patients (Henrichfreise et al., 2007; Oliver, 2010), but their role in other chronic diseases is still unknown. Oliver et al. (2000) showed that 36 % of CF patients in their study were colonized by a hypermutable strain of P. aeruginosa, and in a second study Macià et al. (2005) detected a higher percentage (57 %). Hypermutable P. aeruginosa strains are thus extremely prevalent in chronic infections (CF infections) in contrast to what has been described in acute infections. This suggests a role for hypermutation in bacterial adaptation for long-term persistence (Macià et al., 2005; Mena et al., 2008) as well as a potential role in pulmonary exacerbations. These results are similar to those obtained for another opportunistic pathogen, Stenotrophomonas maltophilia: the proportion of hypomutators was significantly higher in environmental strains (58.3 %), with strong mutator strains found only in clinical isolates from CF patients (Turrientes et al., 2010).

The pathogenic role of Streptococcus pneumoniae and Haemophilus influenzae in CF remains controversial, although some think that these organisms predispose patients to acute and chronic airway infections by other commonly encountered CF organisms (Konstan & Berger, 1993). del Campo et al. (2005) studied 48 Streptococcus pneumoniae isolates recovered from the sputum samples of 26 CF patients. The percentage of Streptococcus pneumoniae strains with increased mutation frequencies leading to rifampicin resistance was significantly higher (P=0.02) in CF isolates (60 %) than in non-CF isolates (37 %). These results confirmed earlier results from a study of non-CF patients at the same institution (Morosini et al., 2003). Finally, 500 H. influenzae isolates were screened for increased mutation frequency by measuring resistance to rifampicin, nalidixic acid and spectinomycin. Interestingly, 12 of the 14 identified hypermutable strains were isolated from CF sputum (Watson et al., 2004).

It thus appears that chronic bacterial infections might select for hypermutator strains that promote the organism’s survival and persistence. Notably, such organisms must survive in an environment in which there is a strong inflammatory response that is dominated by polymorphonuclear cells and involves generation of oxidative stress, leading to further inflammation and tissue damage.

Food-related diseases

LeClerc et al. (1996) reported a higher incidence of mutators among pathogenic E. coli (1.2 %) and Salmonella enterica (4 %) isolates than in laboratory-attenuated strains. Le Gall et al. (2009) determined the incidence of hypermutable strains in a collection of 209 Salmonella isolates from humans and animals, finding that 3.3 % of the human clinical isolates were weakly hypermutable compared to 15.6 % (P=0.011) of the animal strains. Only 1 of 209 (0.7 %) bovine strains of Salmonella Heidelberg was strongly hypermutable. There was considerable variation in the mutation frequencies of 603 commensal and pathogenic E. coli and Shigella isolates (Denamur et al., 2002): enterohemorrhagic and enteroinvasive strains had higher frequencies of hypermutators (around 5 % and 4 %, respectively) than did commensal strains (1–3 %).
Meningitis

Richardson et al. (2002) found a high level of hypermutability (57%) in a collection of 95 serogroup A Neisseria meningitidis isolates, including hypermutators (mutation frequencies >100-fold) (11.5%).

Clinical implications of hypermutator strains

Role of environment and link to fitness

Fitness disadvantages are associated with having a permanent mutator phenotype, since mutations affecting metabolic pathways, mobility or environmental resistance are potentially lethal for strains. Modifications of environmental conditions in vitro have been associated with increased mutation rates, suggesting a possible role in bacterial fitness in vivo.

The antimutator effect of MutT has been demonstrated to be dependent on the growth phase of Pseudomonas putida and to cause a stronger mutator phenotype under carbon starvation conditions compared with normal growth conditions (Saumaa et al., 2007). It has also been observed that the strong mutator phenotypes of cells defective in both the mutM and mutY genes or in the mutT gene are completely suppressed under anaerobic conditions (Sakai et al., 2007).

Hypermutation has also been implicated in the phase variation of surface molecules, which is a major adaptive strategy for both pathogenic and commensal bacteria. One example of this is the coordination of switching rates in the phase-variable P-pili of uropathogenic bacteria (Bayliss et al., 2008). It was recently demonstrated in P. aeruginosa that the pfpI gene plays a protective role against DNA damage caused by oxidative stress (Rodríguez-Rojas & Blázquez, 2009). The mutation rates of pfpI mutants are dramatically increased in the presence of hydrogen peroxide, and inactivation of the pfpI gene affects biofilm formation. Under physiological conditions, the greater adaptability conferred by hypermutation may compensate for diminished stress protection. Ciofu et al. (2005) showed that the accumulation of polymorphonuclear leukocytes in inflammation processes is associated with a high occurrence of hypermutable bacteria. The release of reactive oxygen species promotes oxidative stress in lung disease, providing an opportunity for bacteria to adapt and establish a persistent infection.

Chronicity is presumed to have a role in emerging hypermutation. For example, thymidine-dependent small-colony variants of Staphylococcus aureus, which are frequently isolated in CF patients and are implicated in persistent and recurrent infections, are hypermutators (Besier et al., 2008). Inactivation of the MMR system favours long-term persistence of oropharyngeal colonization by P. aeruginosa in CF mice (Mena et al., 2007). Competition studies were performed in rats with a mutator Staphylococcus aureus strain and the parental strain: the deficient mutL strain was clearly at a disadvantage for long-term survival in experimental osteomyelitis (Daurel et al., 2007). In some infections, there are many latent bacterial pathogens (i.e. non-dividing or slow-growing bacteria). These ‘persisters’ may be the source of transient mutators because they have elevated mutation frequencies (Alonso et al., 1999; Wright, 1996). The molecular basis of high mutation frequencies in slow-growing bacteria is poorly understood, but one might imagine that the activation of global regulators could decrease the level of Mut proteins (Tsui et al., 1997). Labat et al. (2005) reported that a mutS mutator of a uropathogenic strain had an advantage in pathogenesis during the last stages of infection in a mouse model. Competition experiments showed that the proportion of mutators increased in the late stages of infection, suggesting an important role for hypermutation in bacterial persistence.

Possible links to virulence

A direct link between the mutator phenotype and virulence was investigated by studying the extraintestinal virulence of 88 commensal and extraintestinal pathogenic E. coli isolates in a mouse model. The virulence of MMR-deficient and MMR-proficient strains, which were otherwise isogenic, was compared. The results provided no support for the hypothesis that the mutator phenotype has a direct role in virulence or is associated with increased virulence (Picard et al., 2001). Mérimo et al. (2002) showed that the ΔmutSL deletion mutation in Listeria monocytogenes moderately affected bacterial virulence, with a 1-log increase in the lethal dose 50% (LD50) in the mouse model, but competition assays between wild-type and mutant strains showed a reduced capacity of the mutant intracellular pathogen to survive and multiply. This suggests that the hypermutator phenotype is more deleterious than advantageous in terms of virulence.

Mena et al. (2007) showed that hypermutation attenuated virulence in P. aeruginosa strains in CF mice and decreased initial oropharyngeal colonization potential. These results were confirmed by Hogardt et al. (2007), who showed that mutator P. aeruginosa strains isolated over a 3–6-year period had lost their destructive virulence factors. Another study of P. aeruginosa strains in CF patients showed that hypermutation was associated with a considerable biological cost and with reduced potential for colonization of new environments and strain transmissibility (Montanari et al., 2007). N. meningitidis adapts to environmental changes by high-frequency switches in the expression of specific phenotypes, such as phase variation. Bayliss and others (Bayliss, 2009; Bayliss et al., 2008) selected a hypermutator N. meningitidis isolate by subjecting the N. meningitidis strain 8047 to multiple cycles of growth in the presence of human serum and mAb B5 (bactericidal for N. meningitidis strain 8047 when phosphoethanolamine is present in the inner core LPS and IgG is switched ‘off’). In an in vivo infant rat model of bacteremia, they showed that the hypermutable variant could escape the bactericidal
activity of the serum and was more virulent than strain 8047, avoiding the passive protection of mAb B5. Mathematical epidemiology models and within-host infection dynamics previously predicted this result (Meyers et al., 2003).

**Links to antibiotic resistance**

Antibiotic resistance genes may be acquired by conjugation, transformation and/or transduction, and they are sometimes incorporated into the recipient strain by recombination events. Antibiotic-resistant phenotypes can also be caused by mutations in the promoter or coding regions of structural or regulatory genes. These mutations result in various levels of antibiotic resistance due to the following: reduced uptake of antibiotics; increased expression of efflux systems that confer resistance to multiple antibiotics; target modification; overproduction of the target; expression of extended-spectrum β-lactamases (ESBLs); overproduction of β-lactamases; sequestering of the antibiotic by protein binding; or binding of a specific immunity protein to the antibiotic. The probability of acquiring new functions by mutation and recombination is thus greatly increased in MMR-deficient strains (Fig. 2).

The connection between hypermutation and antibiotic resistance remains controversial. Denamur et al. (2002) demonstrated that uropathogenic *E. coli* mutator strains are not more antibiotic resistant than non-mutators, and they found no mutator strains that showed resistance to multiple antibiotics. These results were confirmed in a study of intensive care unit patients: there was no link between hypermutation and antibiotic resistance development in *P. aeruginosa* isolates (Gutiérrez et al., 2004). Linezolid-resistant enterococci contain mutations in the 23S rRNA genes, but mutations in the *mutS* and *mutL* genes of oxazolidinone-resistant *Enterococcus faecium* were not linked to a hypermutator phenotype (Willems et al., 2003). Studying the evolution of ESBLs in a *mutS*-deficient *P. aeruginosa* hypermutator suggested that hypermutable *P. aeruginosa* may not be the source of the ESBLs found in clinical isolates of this organism (Driffield et al., 2006).

![Fig. 2. Pathways of hypermutation and their relationship to antimicrobial agents, mobile genetic elements and the SOS system.](image-url)

1. MMR system (MutS, MutL, MutH, UvrD), proofreading (MutD), prevention of incorporation of 8-oxo-dG (MutT, MutM, MutY).
2. DNA Pol II, DNA Pol IV (DinB), DNA Pol V (UmuCD).
3. Pathogenicity island-encoded virulence factors, mobilization of integrative conjugative elements.
4. LexA is a transcriptional repressor protein and RecA* is a coprotease aiding the autocatalytic self-cleavage of LexA.
5. Inhibition of DNA gyrase and topoisomerase IV.
6. The most frequent mechanisms of resistance are mutations in *gyrA* (which encodes a subunit of a DNA gyrase), MexXY-OprM overproduction, OprD loss and AmpC overproduction.
Using insertional inactivation of the mutS gene in *Staphylococcus aureus*, O’Neill & Chopra (2003) showed that there were no mutators in a collection of 493 *Staphylococcus aureus* clinical isolates despite the potential existence of a mutator phenotype.

Conflicting results have been reported by other authors. Specifically, Baquero et al. (2005) reported that 40% of urinary ESBL-positive *E. coli* strains had increased mutation frequencies versus 26% in non-ESBL isolates. Increased mutation frequency correlated with fluoroquinolone resistance in clinical isolates of *E. coli* in another series (Komp Lindgren et al., 2003) as well as with fosfomycin resistance (Ellington et al., 2006). These results were confirmed in various other genera and species. Maciá et al. (2004, 2005) found multiple antimicrobial resistances in 42% of hypermutable *P. aeruginosa* strains isolated from patients with a lung disease. In contrast, none of the non-hypermutable strains showed antimicrobial resistance. These authors did not confirm their results by studying susceptibility to colistin: the increased colistin susceptibility observed with the hypermutable strains was not likely a direct consequence of the inactivation of the MMR system since the MICs for reference strain PAO1 and its mutS-deficient derivative PAOΔmutS were found to be essentially identical (2 μg ml⁻¹) (Maciá et al., 2007). A high rate of macrolide resistance (i.e. the presence of mutations in the target site of erythromycin) in *Staphylococcus aureus* strains from patients with CF also revealed a high proportion of hypermutable strains (Prunier et al., 2003). Nevertheless, a high prevalence of hypermutable strains has been described, essentially in chronically infected patients (such as CF patients). This could be a consequence of the adaptive mutations required for long-term persistence in infected patients. It may also represent coselection of a hypermutable variant that is also resistant to antibiotics. A higher prevalence of multiple antibiotic resistance among weak mutators has been predicted by computer modelling (Taddei et al., 1997) and confirmed by clinical studies (Baquero et al., 2004). This might be explained by a stronger selection of weak mutators and then a much longer persistence time than for strong mutators. Low migration rates in chronic infections could further lead to increased in situ selection of strong mutator alleles. The function of this hypermutable state could be to generate variability under stressful conditions rather than to enhance survival per se (Bridges, 2001).

**Do successive antibiotic treatments and/or prolonged antibiotic treatment increase acquisition of antibiotic-resistant bacteria?**

The presence of mutator strains in clinical samples may indicate an increased risk of acquiring antibiotic resistance through mutational and recombinational events (see below). The mutator phenotype is a risk factor during the treatment of bacterial infection, as it appears to enhance the selection of mutants with high- and low-level antibiotic resistance and can refine existing plasmid-located resistance determinants (Chopra et al., 2003).

The fluoroquinolone antibiotic ciprofloxacin, an inhibitor of type II DNA topoisomerases, stimulates intrachromosomal and conjugal recombination of DNA sequences, including resistance genes, in *E. coli* (López et al., 2007). This stimulation also occurs in a hyper-recombinogenic mismatch repair *mutS* mutant. *E. coli* populations can recover during treatment when recombination-stimulating concentrations of the antibiotic are present. Supporting this finding, a double topoisomerase mutant was selected *in vivo* after 10 days of ofloxacin treatment (Cattoir et al., 2006).

Single antibiotic selection or selection with successive antibiotics can increase the proportion of strong mutators in bacterial populations. Antibiotics have a direct effect in terms of selecting resistance by themselves; however, they can also indirectly select for strong mutators with an increased capacity for acquiring resistance to other antimicrobial agents (Giraud et al., 2002). Schaaff et al. (2002) subjected a *Staphylococcus aureus* strain and the isogenic mutator strain to stepwise vancomycin treatment and concluded that high mutation frequency leads to rapid development of vancomycin resistance followed by the emergence of resistant strains. However, other published results do not support a role for mutators in the emergence of vancomycin-intermediate *Staphylococcus aureus* (O’Neill & Chopra, 2002).

**Role of the SOS system?**

Environmentally stressful conditions, including antibiotics, can transiently increase the mutation frequency in a bacterial population (Kang et al., 2006). It is conceivable that some antibiotics act indirectly as promoters of antibiotic resistance by inducing the SOS system (Fig. 2). Fluoroquinolones (Ysern et al., 1990), which are DNA-damaging agents, or β-lactams, trimethoprim (Lewin & Amyes, 1991) or metronidazole (Quillardet & Hofnung, 1993) induce the SOS system and error-prone DNA polymerases (Fig. 2). Resistance to ciprofloxacin may require induction of a chromosomal mutation that is mediated by the cleavage of the SOS repressor LexA and the associated derepression of DNA polymerases II, IV and V (Cirz & Romesberg, 2006). LexA levels fluctuate with pH variation as well as in ageing colonies. LexA inactivation during starvation or under oxidative stress induces polymerase Pol IV, which is expected to increase the error rate of DNA synthesis (Foster, 2005) and to result in multiple mutations during the transient hypermutation state (Tomkins et al., 2003) (Fig. 2). In addition, fluoroquinolones act as damaging agents that can induce the SOS response, resulting in bacterial persistence and survival after treatment (Dörr et al., 2009). Dörr et al. (2010) showed that the *tisAB/istR* locus is implicated in *TisB* toxin production, but also persistor formation, after exposure to the DNA-damaging ciprofloxacin. This
LexA-controlled toxin TisB is able to decrease proton motive force and ATP levels, consistent with its role in forming dormant cells.

Role of prophages and mobile genetic elements?
Excision of mobile genetic elements in *Staphylococcus aureus*, as well as replication, can result from SOS induction due to fluoroquinolones (Ubeda et al., 2005) (Fig. 2). β-Lactams, on the other hand, can trigger prophage induction, promoting the spread of virulence factors (Maïques et al., 2006). *Streptococcus pyogenes* has been demonstrated to control MMR via dynamic prophage excision/reintegration in response to growth phase (Scott et al., 2008). Some antibiotics might serve as modulating agents of the hypermutation process, inducing an increase in mutation rates during the non-exponential growth phases (phage integration in *mutS*) that are often found in chronic infection. *Helicobacter pylori* exposed to reactive oxygen species has elevated spontaneous point mutation rates, deletions between direct repeats and intergenomic recombination (Kang et al., 2006). In addition, MMR-deficient strains have an increased chance of acquiring new antibiotic resistance genes as a result of the elevated recombination frequency (Blázquez, 2003).

And in clinical practice?
Selection pressure experiments reveal the pressing need for new drugs with low endogenous resistance potential, unlike rifampicin or fluoroquinolones, especially when used at sublethal doses (Miller et al., 2002). Lower antibiotic concentrations can promote an increase in mutators, as when a mixed culture of a hypermutable *hexA Streptococcus pneumoniae* mutant strain and its isogenic ancestor were challenged with low concentrations of cefotaxime. These conditions, i.e. low cefotaxime concentrations, selected for *hexA* mutants (Negri et al., 2002). Oliver et al. (2004) argued that in infections caused by hypermutable *P. aeruginosa*, the problem of acquired resistance to treatment and further selection by antibiotic pressure could be overcome by therapy with multiple antibiotics acting synergistically. Maciá et al. (2006) reached the same conclusion by investigating the therapeutic efficacy and potential for resistance selection of ciprofloxacin and tobramycin, alone or in combination, in a mouse model of lung infection that used a *P. aeruginosa* strain and its Δ*mutS* derivative. The authors concluded that it is possible to suppress resistance selection in infections caused by hypermutable *P. aeruginosa* strains, even in deficient MMR strains, by using appropriate synergistic combinations of antibiotics rather than monotherapy.

In clinical practice, the synergistic response of bacteria to counteract fluoroquinolone activity is a major obstacle to fluoroquinolone use in monotherapy. Low doses of antibiotics might also be discouraged, especially for chronic infections (Negri et al., 2002). When bacteria are stressed by adverse environmental conditions, including antibiotic treatment, the SOS repair system is induced to counter DNA damage and to facilitate recombination between bacterial DNA sequences. Some antibiotics used in clinical practice, such as fluoroquinolones, are good inducers of the SOS system. Thus, such antibiotics can also increase genetic evolution by stimulating recombination mechanisms of the treated bacteria (via an SOS-independent mechanism). As a consequence, this may favour the acquisition, evolution and spread of antibiotic resistance determinants. When the DNA-damaging agent is removed, the organism goes back to the original mutation rate. A transient hypermutable state may be acquired through MMR depletion in the stationary phase (Li et al., 2003), and it is tempting to speculate that antibiotics might have the same effect. If increased mutagenesis is associated with an important biological cost, it reduces potential for colonization of new environments and therefore strain transmissibility, but encourages bacterial persistence during chronic infections. We should therefore promote a good choice of initial antibiotic regimens, able to prevent secondary selection of hypermutators. We should also be vigilant in case of co-infection with a virus that may be involved in the emergence of mutators (Pal et al., 2007). It would also be useful to detect these strains in chronic infections and test their susceptibility to antibiotics under biofilm conditions, and maybe question evaluation standards for antimicrobial susceptibility testing, to reflect the singularity of these strains. Indeed, they could appear more resistant in vivo due to their ability to withstand adverse conditions (as persisters) and thus escape the treatment, especially if the antibiotic has a low tissue distribution. However, further work is still necessary to evaluate the role of hypermutators in disease progression.

Conclusions
The combination of hypermutation and hyper-recombination phenotypes rapidly generates genetic diversity and provides a way to restore lower mutation frequencies following adaptation, thus modulating the speed of bacterial evolution. Nevertheless, most of the generated mutations are neutral or deleterious for bacteria, which means that they must use a ‘transient mutator system’ that allows them to produce mutations only when needed and when it is less costly from an energetic standpoint. Antibiotics might act not only as selectors but also as promoters of antibiotic resistance. To suppress resistance selection in infections by hypermutable strains, the use of appropriate doses of combined synergistic regimens should always be encouraged.

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