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The emergence of antibiotic resistance by mutation

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ABSTRACT

The emergence of mutations in nucleic acids is one of the major factors underlying evolution, providing the working material for natural selection. Most bacteria are haploid for the vast majority of their genes and, coupled with typically short generation times, this allows mutations to emerge and accumulate rapidly, and to effect significant phenotypic changes in what is perceived to be real-time. Not least among these phenotypic changes are those associated with antibiotic resistance. Mechanisms of horizontal gene spread among bacterial strains or species are often considered to be the main mediators of antibiotic resistance. However, mutational resistance has been invaluable in studies of bacterial genetics, and also has primary clinical importance in certain bacterial species, such as *Mycobacterium tuberculosis* and *Helicobacter pylori*, or when considering resistance to particular antibiotics, especially to synthetic agents such as fluoroquinolones and oxazolidinones. In addition, mutation is essential for the continued evolution of acquired resistance genes and has, e.g., given rise to over 100 variants of the TEM family of β-lactamases. Hypermutator strains of bacteria, which have mutations in genes affecting DNA repair and replication fidelity, have elevated mutation rates. Mutational resistance emerges *de novo* more readily in these hypermutable strains, and they also provide a suitable host background for the evolution of acquired resistance genes *in vitro*. In the clinical setting, hypermutator strains of *Pseudomonas aeruginosa* have been isolated from the lungs of cystic fibrosis patients, but a more general role for hypermutators in the emergence of clinically relevant antibiotic resistance in a wider variety of bacterial pathogens has not yet been proven.

Keywords Antibiotic resistance, evolution, hypermutator, mutation, phenotypic changes, review

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INTRODUCTION

Darwin recognised that variation is the raw material for 'natural selection', and that the frequency at which evolutionary change occurs can fluctuate [1]. It is now clear that the accumulation of genetic mutations underlies much of this variation. Bacteria typically have short generation times and are able to evolve in what is perceived to be real-time. This ability to adapt rapidly to changing conditions is illustrated well by the development of antibiotic resistance. Each new antibacterial agent presents bacteria with a new challenge (i.e., adverse growth conditions on exposure), and they have consistently risen to, and overcome, the challenges set.

It is possible to generate spontaneous mutants *in vitro* that confer resistance to virtually any antibiotic, although frequencies vary dramatically according to the bacterial species and the agent tested (with most frequencies typically $\leq 10^{-6}$). Bacteria carrying resistance mutations may be 'less fit' initially than wild-type organisms, but this is often a temporary phenomenon, and compensatory mutations can arise that limit the negative effects of the resistance mutations [2–4]. In some cases, resistant mutants with compensatory mutations may be more fit than susceptible revertants possessing the same compensatory mutations [5], which might explain why some mutational resistance can be maintained stably in the absence of selective pressure, i.e., antibiotic use. This review discusses mutational resistance

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using specific examples. As such, it cannot be comprehensive, but rather, seeks to illustrate the importance of mutation and the principles by which mutation contributes to the emergence and diversification of resistance mechanisms.

MUTATION AS THE PRIMARY EFFECTOR OF RESISTANCE: ANTIBIOTIC CLASSES

Mutational resistance to rifampicin, fusidic acid and streptomycin arises readily and may pose clinical problems in some settings, limiting the use of these agents as monotherapy. Mutation is also a common mechanism of resistance to many synthetic antibacterial agents, such as the fluoroquinolones and oxazolidinones, although resistance to these classes arises less readily.

Increasing dependence on the fluoroquinolone class began in earnest with the launch of ciprofloxacin in the mid-1980s, and has continued to the present day. Despite the undoubted clinical value of the fluoroquinolones, their development has been beset by many hurdles, and several compounds have either been withdrawn in late development or have been withdrawn or strictly limited post-licensing [6]. Current incarnations include levofloxacin and moxifloxacin, but further development is ongoing, with the chief goal being to extend and improve activity, especially against Gram-positive cocci and bacteria resistant to existing members of the class [7].

Fluoroquinolones are bactericidal and target two homologous enzymes, namely DNA topoisomerases II (also known as DNA gyrase) and IV [8], which are essential for the supercoiling of bacterial DNA. Both enzymes are composed of subunits, encoded by *gyrA* and *gyrB* (for DNA gyrase) or *parC* and *parE* (for topoisomerase IV). Development of resistance to fluoroquinolones is a stepwise process, resulting from the accumulation of amino-acid substitutions in these subunits, and with increasing numbers of mutations generally correlating with increasing MICs [8,9]. Most, but not all, of the mutations map into defined regions of the subunits, designated the quinolone resistance-determining regions [10].

In Gram-negative bacteria, DNA gyrase tends to be the primary target for fluoroquinolones. Mutations in *gyrA* are found in isolates with low-level resistance, whereas higher MICs are associated with additional mutations, particularly in *parC*, but also in *gyrB* and, less commonly, in *parE* [10]. Resistance mediated by these mutations is enhanced by other mutations affecting the expression of efflux pumps [11–16]. In Gram-positive species the primary target is usually *parC* [17–19], but this varies among quinolones. For example, ciprofloxacin targets *parC*, while sparfloxacin targets *gyrA* [20]. This target preference can be engineered by substitutions at the C-7 position of the quinolone pharmacophore [21]. Other quinolones, including the des-fluoroquinolone garenoxacin [7], target both DNA gyrase and topoisomerase IV equally [22], and may be less prone to select resistance as this would require simultaneous mutations affecting both proteins.

Linezolid is the first licensed member of the oxazolidinone class, and has a novel mechanism of action which prevents protein synthesis by inhibiting formation of the 70S ribosomal initiation complex. Prevalent mechanisms of resistance to other antibiotics inhibiting protein synthesis do not confer cross-resistance to oxazolidinones [23–25]. As the oxazolidinones are synthetic agents, bacteria had no pre-existing reservoir of resistance genes, but linezolid-resistant enterococci were isolated during pre-licensing clinical trials [26], and resistance has emerged post-licensing, albeit infrequently, in enterococci [27–30], *Staphylococcus aureus* [31–33], coagulase-negative staphylococci [34] and streptococci [35,36]. This resistance is mediated typically by mutations in the genes that encode 23S rRNA. Several different mutations have been reported in laboratory-generated mutants, but a G2576T mutation is particularly common among resistant clinical isolates; mutations in the ribosomal protein L4 have also been described [36].

Unlike, e.g., resistance to rifampicin, linezolid resistance emerges extremely rarely in the laboratory, and accurate quantification of mutation frequencies is often not possible. The low mutation frequency occurs, in part, because the target 23S rDNA genes are present in multiple copies in most relevant bacterial species. A single mutational event in one gene copy is generally insufficient to confer phenotypic linezolid resistance. Rather, subsequent intra-chromosomal recombination (also known as gene conversion [37]) is needed to distribute the mutation(s) to multiple rDNA alleles. As a result of this, the number of rDNA alleles carrying known linezolid resistance mutations correlates with the linezolid MIC

[38–41]. Also, linezolid-resistant mutants were selected at lower frequencies from a recombination-deficient strain of *Enterococcus faecalis* [42], and emerged more slowly in a recombination-deficient strain of *Staph. aureus* [43]. In the latter study, the *Staph. aureus* mutants carried distinct mutations in different rDNA gene copies, consistent with independent mutational events (rather than gene conversion); this route to resistance is statistically improbable in the clinical setting, and most natural isolates (arising from one mutation and subsequent recombination) have gene copies with a single mutation type.

MUTATION AS THE PRIMARY MECHANISM OF RESISTANCE: BACTERIA

Although mutational resistance can be selected *in vitro*, it may not represent the most prevalent resistance mechanism in clinical isolates of a particular species. For example, chloramphenicol-resistant mutants of *Staph. aureus* have 23S rDNA mutations and may be cross-resistant to linezolid [44], but most clinically significant chloramphenicol resistance in this species is mediated by acetyltransferase enzymes [45]. However, in certain species, mutation is the main, or sole, cause of clinical resistance problems. One of the best examples is *Mycobacterium tuberculosis*. Resistance to all therapeutic agents in this species is mediated by mutations: i.e., rifampicin resistance in *rpoB*; isoniazid resistance in *katC*, *inhA*, *oxyR*, *ahpC* and *furA*; streptomycin resistance in *rrs* and *rpsL*; pyrazinamide resistance in *pncA*; ethambutol resistance in *embB*; and fluroquinolone resistance in *gyrA* and *gyrB* [46]. The treatment of tuberculosis requires combination therapy to tackle a difficult pathogen that causes prolonged disease [46,47]; monotherapy is not an option because of the very real risk of resistance emerging. Combination therapy decreases, but does not eliminate, this risk, which is influenced by the frequencies of mutation to resistance for each individual agent, and by the number of bacteria at a focus of infection [46].

Multidrug therapy is also recommended for *Helicobacter pylori* infections, typically comprising triple combination regimens, including two of clarithromycin, metronidazole, amoxycillin or tetracycline, plus a proton pump inhibitor [48]. Chromosomal mutations are responsible for resistance of *H. pylori* to clarithromycin (in 23S rRNA) [49–52], amoxycillin (changes in penicillin-binding protein 1) [53], metronidazole (in *rdxA* and other genes) [48], tetracycline (in 16S rRNA and other undefined genes) [54,55] and, *in vitro*, streptomycin (in *rpsL*) [56].

RESISTANCE PHENOTYPES CAN BE ALTERED BY MUTATION

Intrinsic antibiotic resistance is defined as resistance that is characteristic of all members of a given bacterial species or genus. In many cases, such resistance results from an inability of the antibiotic to reach its target (e.g., glycopeptides are too large to penetrate the outer-membrane of Gram-negative bacteria), a lack of affinity for the target (e.g., the penicillin-binding proteins of Gram-positive bacteria have low affinity for the monobactam aztreonam), the presence of efflux pumps (e.g., the multi-substrate pumps of *Pseudomonas aeruginosa* [57] and the *lsa*-encoded pump of *Ent. faecalis*, which confers resistance to the streptogramin combination quinupristin–dalfopristin [58]), or possession of other chromosomal resistance mechanisms (e.g., *Enterococcus gallinarum* and *Enterococcus casseliflavus/flavescens* possess D-alanine-D-serine ligases, encoded by *vanC* genes, which confer low-level vancomycin resistance [59]). Mutation can affect how such resistances are expressed. For example, nonsense mutations, resulting in the production of a truncated Lsa pump, have been identified in occasional quinupristin–dalfopristin-susceptible isolates of *Ent. faecalis* [60], while *env* mutations, affecting the cell envelope, can render *Neisseria gonorrhoeae* and *Eschericha coli* susceptible to glycopeptides [61,62], and mutations that result in over-expression of the numerous intrinsic efflux pumps of *P. aeruginosa* make a significant contribution, complemented by resistance gene acquisition, towards the multiresistance that is shown regularly by this species [57,63,64].

AmpC enzymes provide a further example of an intrinsic resistance mechanism that may be affected markedly by mutation. These chromosomally-encoded cephalosporinases are found in many members of the Enterobacteriaceae [65,66]. In some species, such as *Esch. coli*, AmpC is usually expressed only at low levels and rarely has clinical significance. However, AmpC enzymes have therapeutic significance in many

Enterobacter spp. and *Citrobacter* spp., which typically express the enzymes inducibly, with the induction process intimately entwined with cell wall recycling by AmpD, a process that is affected by β-lactams. Isolates with an inducible AmpC are not resistant phenotypically to, e.g., cefotaxime and ceftazidime because these agents are poor inducers of β-lactamase synthesis. However, if these agents are used to treat infections caused by AmpC-inducible species, there is a significant risk that mutants derepressed for AmpC production will be selected. For *Enterobacter* spp., phenotypic resistance to cefotaxime and ceftazidime emerges in *c.* 20% of bacteraemic patients treated with third-generation cephalosporins [67], and can lead to therapeutic failure [68]. Cephalosporin therapy selects for *ampD*-null mutants, in which *ampC* transcription, no longer regulated negatively by AmpD, is constitutive [69–71].

The introduction of new antibacterial agents into clinical use provides new pressures, sets new challenges for bacteria, and can result in the selection of new resistance types. For example, AmpC enzymes typically have little activity against fourth-generation cephalosporins, such as cefepime and cefpirome, but mutant AmpC enzymes with activity spectra expanded to include cefepime and cefpirome have been reported, both *in vitro* [72,73] and, more recently, in rare clinical isolates with chromosomal [74–76] or plasmid-borne AmpC enzymes [77].

VanA and VanB glycopeptide resistance in enterococci is mediated by complex clusters of resistance genes. Both types are usually inducible and are tightly regulated; membrane-bound VanS and $VanS_B$ peptides sense environmental glycopeptides and activate VanR and $VanR_B$, which then initiate transcription of resistance genes [78–80]. The VanS sensor of a VanA strain recognises both vancomycin and teicoplanin. However, the $VanS_B$ sensor of a VanB strain does not recognise teicoplanin, with the result that teicoplanin fails to induce resistance and VanB isolates appear susceptible to teicoplanin *in vitro* [59]. In the absence of glycopeptides, the VanS/$VanS_B$ peptides also act as negative regulators; they have a phosphatase domain which deactivates VanR/$VanR_B$ and switches off resistance gene expression [81]. Mutation can affect the VanS and $VanS_B$ sensors, resulting in changes to the type of glycopeptide resistance phenotype expressed; changes in the N-terminal sensor domain of VanS (L50V, E54Q and Q69H) can prevent it from recognising teicoplanin, leading to a 'VanB' phenotype in a genotypic *vanA* strain [82,83], while a six-residue deletion in the phosphatase domain of $VanS_B$ results in constitutive glycopeptide resistance, including to teicoplanin, i.e., a VanA phenotype in a genotypic *vanB* strain [84]. Mutation can also cause VanA and VanB enterococci to acquire moderate levels of resistance (MICs ≤ 16 mg/L) to the second-generation glycopeptide oritavancin [7,85].

Pleiotropic effects, including increases in antibiotic resistance, can be mediated by mutations at global regulatory loci. The transcriptional activator MarA, encoded by the *marRAB* operon [86,87], regulates transcription of over 60 genes in *Esch. coli*, with 76% of these being up-regulated [88]. Homologues of this regulation system are found in several enteric Gram-negative genera [88]. Mutations in *marR*, encoding a negative regulator, or *marO*, the operator to which MarR binds, affect MarA expression. The Mar phenotype is associated with induction of MarA, or with its constitutive expression following mutational inactivation of *marR*. This phenotype includes non-specific, low-level resistance to fluoroquinolones, some β-lactams, chloramphenicol, rifampicin and tetracycline, and also to disinfectants and organic solvents, as the proteins regulated by MarA include the AcrAB–TolC efflux pump (up-regulated) and various outer-membrane proteins (down-regulated) [87]. Other mutations in MarR can act as super-repressors of MarA expression [89]. Resistance associated with MarA expression can complement other resistance mechanisms in clinical bacterial isolates [87].

Global regulatory loci occur in many other bacteria. For example, the virulence regulatory locus *agr* [90] may have a role in the development of intermediate-level resistance to glycopeptides (GISA phenotype; vancomycin MICs 8–16 mg/L) in *Staph. aureus* [91–94]. The precise molecular basis of this resistance has not been defined, but the phenotypic characteristics include thickened cell walls and over-production of D-alanyl-D-alanine-containing peptidoglycan precursors [93,95–97]. Glycopeptide-intermediate *Staph. aureus* (GISA) isolates tend to have a disrupted *agr* function, and an *agr*-null mutant gained heteroresistance to vancomycin and was tolerant of its bactericidal effects [98]. Mutations that inactivate *tcaA*, which encodes a putative trans-membrane protein, have also been associated with the GISA

phenotype, but the precise mechanism of resistance has not been defined [99].

THE ROLE OF MUTATION IN THE EVOLUTION OF RESISTANCE GENES

Many clinically relevant antibiotic resistance mechanisms are acquired traits. The resistance genes encoding them, which may be incorporated into plasmids, transposons or integrons, or may exist either as gene cassettes or as partial gene fragments released from dead bacterial cells, are acquired by new host strains via horizontal transfer, mediated by conjugation, transformation or transduction. These processes are the primary means of dissemination of acquired resistance genes, but mutation is essential for the evolution and diversification of these acquired genes.

In addition to resistance to most penicillins, extended-spectrum β-lactamases (ESBLs) confer resistance to second and subsequent generations of cephalosporins, such as cefuroxime, cefotaxime and ceftazidime, and to monobactams, but not to cephamycins or carbapenems [100,101]. Most plasmid-mediated ESBLs fall into one of two major families, the TEM- or SHV-type enzymes [102], found primarily in members of the Enterobacteriaceae. The prototypes of these enzyme families are TEM-1 and TEM-2, which differ from one another by a glutamine (TEM-1) to lysine (TEM-2) substitution at residue 39, and SHV-1. Each is primarily a penicillinase, with no significant activity against extended-spectrum cephalosporins, and is susceptible to inhibitors such as clavulanic acid and tazobactam. However, many variants of these enzymes have additional mutations which render the enzymes capable of hydrolysing, e.g., cefotaxime and ceftazidime, or able to resist the action of β-lactamase inhibitors [103–105].

At the time of writing, there are 150 TEM variants and almost 90 SHV variants (http://www.lahey.org/studies). Among the numerous variants of the TEM family, likely derivatives of the TEM-1 or TEM-2 progenitors can be distinguished by the presence or absence of residue Lys39. The evolution of progenitor enzymes by accumulation of multiple mutations has been mimicked *in vitro* [106–108]. Structural studies have helped to define those mutations that are critical for expanding the substrate profile of the TEM enzyme, and others that stabilise enzyme structure [103,107]. Further mutant variants of TEM (previously called inhibitor-resistant TEM or IRT enzymes) and SHV variants confer resistance to β-lactamase inhibitors. These mutations are distinct from those that confer ESBL activity. However, continued selection pressure caused by wide use of β-lactams has resulted in the emergence of complex mutant TEM enzymes [109–111] that possess combinations of mutations conferring ESBL activity with other mutations that provide small degrees of inhibitor resistance.

Another multi-allelic family of plasmid-mediated ESBLs, the CTX-M enzymes, is currently causing significant public health concern [112,113]. In contrast with the TEM and SHV families, all CTX-M enzymes are ESBLs, with no known progenitor possessing penicillinase activity only. Many, possibly all, members of this family bear witness to the 'escape' to plasmids of chromosomal β-lactamases from *Kluyvera* spp. [114–116]. More than 50 CTX-M variants have been defined, clustering in five phylogenetically distinct subgroups [112,113] (http://www.lahey.org/studies). Thus, the appearance of CTX-M enzymes belonging to separate subgroups in members of the Enterobacteriaceae represents a series of independent escape events. However, intra-subgroup diversification of CTX-M enzymes again owes much to the accumulation of mutations that alter enzyme activity.

CTX-M enzymes are primarily cefotaximases, with far greater activity against cefotaxime than against ceftazidime. However, representatives of several subgroups (including CTX-M-15, -16, -25, -27, -28, -29 and -32) have an Asp240Gly substitution, associated with increased catalytic activity against ceftazidime [113,117–120]. A Pro167Ser mutation similarly enhances the ceftazidimase activity of CTX-M-19 [121]. A Ser130Gly mutation decreased the activity of CTX-M-9 against cefotaxime, but increased its resistance to β-lactamase inhibitors significantly [122].

Besides ESBLs, diverse carbapenemases belonging to β-lactamase molecular classes A, B and D are beginning to emerge in non-fermentative bacteria, especially *Pseudomonas* spp. and *Acinetobacter* spp., and also in members of the Enterobacteriaceae [123–128]. As with CTX-M ESBLs, molecular comparisons reveal that these enzymes represent multiple escape events, and also that mutation has led to the diversification of alleles within particular subtypes, most dramat-

ically with the class D OXA enzymes of *Acinetobacter* spp. [129,130], and among the IMP (22 variants) and VIM (12 variants) class B metalloenzymes (http://www.lahey.org/studies).

THE ROLE OF THE HYPERMUTABLE HOST IN MUTATIONAL RESISTANCE

Genetic change, through the incorporation of mutations in bacterial DNA, can arise via various mechanisms, including oxidative [131] and alkylation [132] damage, and via errors introduced during DNA replication. Replication errors can result from failure of three separate processes, namely base selection, proof-reading and DNA mismatch repair (MMR), which act sequentially to ensure the fidelity of replication [133,134]. The first two processes allow DNA replication to proceed with a fidelity of 10^{-7} per bp replicated. The final step, MMR, recognises DNA base mispairs and initiates a DNA repair cascade, contributing to genomic fidelity and yielding a final error rate of 10^{-10} per bp [135–138]. In *Esch. coli*, the MMR pathway is composed of four proteins: MutS (HexA), MutL (HexB), MutH and UvrD [136,139].

The MMR pathway is crucial in avoiding mutations and maintaining replicative fidelity, thus modulating genomic fidelity within and between generations. In any bacterial population, the majority of isolates has low mutation rates, which suggests that longer-term genetic stability is favoured among wild-populations [140]. Bacteria with defects in their MMR and other repair pathways have reduced ability to repair DNA damage, and are more likely to develop and accumulate mutations. Such bacteria are said to be 'hypermutable' and to express a 'mutator' phenotype. As MMR also imposes a barrier to recombination between divergent sequences, MMR-negative strains are also hyper-recombinogenic. Such mutator phenotypes are not selectable directly but, as an increased mutation rate can facilitate rapid adaptation to changing environments, they can be co-selected by association with favourable mutations, such as antibiotic resistance [141,142]. Mutator strains of *Esch. coli* can become predominant in a mixed *Esch. coli* population under relatively stable growth conditions [143], and occur at frequencies of 1–7.5% among pathogenic [144] and commensal isolates [145]. This indicates the importance of mutator strains in conferring a short-term selective advantage under stressful or changing growth conditions in the wild; moreover, the fact that the majority of isolates are non-mutators suggests that mutators are not advantaged under 'normal' or more prevalent growth conditions.

Hypermutators and the cystic fibrosis lung

P. aeruginosa and *Staph. aureus* are isolated from the sputum of cystic fibrosis (CF) patients at frequencies of *c.* 80% and 33–60%, respectively [146]. The long-term antibiotic prophylaxis for CF patients offers good conditions for high rates of mutation; moreover, an often low rate of bacterial killing maintains exogenous stress, potentially elevating further the cellular mutation rate [142,147] through induction of general stress systems, such as the SOS response [148,149]. Furthermore, as a highly stressful and fluctuating environment [150], the CF lung can impose significant physiological stress on the bacterial cell in the presence of high concentrations of antibiotics for protracted periods.

Among CF patients colonised with *P. aeruginosa*, 29–36% harbour multiply-resistant, hypermutable isolates [151,152]. Most natural isolates of hypermutable *P. aeruginosa* harbour lesions in MMR genes or *mut*Y [150,151] at frequencies similar to those observed in *Esch. coli* [145]. While the frequency of resistance to several antibiotics was about two-fold greater among the mutator strains [151], a dearth of molecular investigation of the resistance mechanisms means that the importance of hypermutability in the emergence of multiple resistance in these isolates is unknown.

During a study of macrolide resistance in *Staph. aureus* isolates from CF patients, Prunier *et al*. [153] found a higher proportion of hypermutator strains among CF isolates than among non-CF control isolates. Lesions in *mutS* were found in five of 11 hypermutable strains and three non-hypermutable strains [153]. It was argued that the higher proportion of hypermutable strains in CF patients could explain the numerous ribosomal mutations observed and the associated macrolide resistance.

Hypermutators and mutators in other environments

Of almost 30 mutator genes identified in *Esch. coli*, only a small proportion have been associated with

mutator phenotypes among natural isolates of *Esch. coli* and salmonellae; these include the *mutS*, *mutL*, *mutH* and *mutU* (or *uvrD*) genes [144]. In natural populations of *Esch. coli*, mutators may occur at frequencies between 0.1% and several per cent [144,154,155]. Indeed, up to 7.5% of isolates in uropathogenic populations of *Esch. coli* and *Shigella* spp. have mutation rates elevated ≥50-fold above average [156]. Among isolates from cases of bacteraemia, *c.* 1% were found to be mutators, with a ten-fold elevation in mutation frequency [156]. Recently, <1% of geographically diverse isolates from blood and urine cultures were found to be hypermutators, with mutation frequencies elevated by >100-fold. Moreover, among the study isolates, 23% were found to be weak mutators, with mutation frequencies elevated by <100-fold compared with normal [157]. A proposal that a more mutable phenotype could be related to the pathogenicity of the organism [144] has been disputed and, to date, remains unproven [145,156]. Denamur *et al*. [156] found no correlation between increased resistance, multiple resistance, ciprofloxacin resistance or an overexpressed cephalosporinase and a mutator phenotype. In contrast, Gustafsson *et al*. [158] detected a significant increase in the rate of mutation to rifampicin resistance among isolates from patients with high antibiotic usage, suggesting that antibiotic usage could enrich for bacterial populations with elevated mutation rates.

In *Acinetobacter baumannii*, mutations in *mutS* increase the rate of mutation to rifampicin resistance by *c.* 50-fold [159]. Recently, Davies *et al*. [160] reported a clinical isolate with an elevated rate of mutation to ciprofloxacin resistance (via *gyrA* mutations). Part of its MutS sequence was identical to those of ciprofloxacin-resistant clinical isolates, which suggests that selection of ciprofloxacin-resistant *A. baumannii* could also select for strains with elevated mutation frequencies. Among the epidemic serogroup A of *Neisseria meningitidis*, 11% of the isolates tested were mutators, correlating with defects in *mutS* or *mutL* [161]. Hypermutable strains have also been found among clinical isolates of *H. pylori*, but the role of *mutS* seems doubtful, raising the possibility that other mutator loci could be important for a hypermutable phenotype in this organism [162].

Studies of Gram-positive pathogens outside of the CF lung have found mixed evidence for the existence of mutators. A laboratory study has shown that in mixed cultures of hypermutable (*hexA*) and wild-type *Staphylococcus pneumoniae*, exposure to low concentrations of cefotaxime enriched the hypermutable isogen [163], thus increasing the risk of further acquisition of antibiotic resistance. Among 200 clinical isolates of *Streptococus pneumoniae*, 8.5% were found to be hypermutable on the basis of their frequencies of mutation to rifampicin resistance. However, mutations in *hexA* (*mutS*) and *hexB* (*mutL*) could not be associated unequivocably with hypermutability, raising the possibility that additional mutator loci could be involved in the observed mutator phenotypes [164].

As discussed above, resistance to linezolid is rare, and is a compound function of mutation and recombination events. The emergence of linezolid resistance during therapy has provided pairs of pre- and post-therapy isolates from the same patient, allowing examination of the relationship between the emergence of resistance, the cellular mutation rate and the genes encoding the MMR system pre- and post-resistance development. As many as seven amino-acid substitutions were found in the MutSL proteins among 13 clinical isolates of *Enterococcus faecium*, which included two pairs of resistant/susceptible isolates and two epidemiologically unrelated susceptible isolates [165]. The relevance of some of these substitutions was questioned, but the multiresistant nature of the isolates did not allow determination of mutation frequencies for resistance to other antibiotics [165].

During testing of a further oxazolidinone, AZD2563, a low rate of emergence of mutational resistance was observed *in vitro* [166], as for linezolid. The isolation of a resistant mutant of a clinical isolate of *Ent. faecalis* was possible at a frequency of *c.* 10^{-8}, and the mutant was heterozygous for a G2576T mutation in the 23S rRNA. This strain was not thought to show a hypermutable phenotype [166], but re-evaluation indicated that it possessed an elevated rate of mutation to fosfomycin resistance at 4 × MIC (M. J. Ellington, unpublished observation). To date, no study has determined the mutation rates among a series of clinical isolates in which linezolid resistance has emerged. Studies to examine mutation frequencies and correlate them with MMR defects and polymorphisms should be a priority for the future.

A survey of rates of mutation to rifampicin and fusidic acid resistance among 493 clinical isolates

of *Staph. aureus* from non-CF patients did not reveal any mutators, suggesting that they are rare outside of the CF lung [167]. Mutations are almost certainly responsible for the low levels of vancomycin resistance (MIC 8 mg/L) that are typical of GISA. The precise molecular mechanism has not been defined, but thickening of the cell wall is a consistent phenotypic consequence [95,168–170]. Theoretically, this phenotype could arise more readily in a hypermutable background, and a frameshift causing a premature truncation of the MutS protein was reported in the original GISA strain, Mu50 [171,172]. Two *mutS*-disrupted constructs of *Staph. aureus* RN4220 showed elevated frequencies of mutation to antibiotic resistance, including resistance to vancomycin [167,172]. However, O'Neill and Chopra [173] found no evidence for a mutator phenotype in Mu50 or six other GISA clinical isolates [173], and demonstrated that the *mutS* gene of strain Mu50 is intact [167]. Further studies have revealed that GISA phenotypes tend to develop more readily in methicillin-resistant *Staph. aureus* strains than in related methicillin-susceptible *Staph. aureus* strains [174], and that some isolates have amino-acid changes in MutS [175]. However, the absence of mutation frequency data for these strains means that the phenotypic relevance of these changes is unknown. Overall, there is no convincing evidence supporting a positive role for mutators in the emergence of GISA strains.

In summary, there is significant evidence for the presence of mutator strains among natural populations of some bacterial pathogens. However, the frequency of mutators varies dramatically from study to study, and often does not correlate well with antibiotic-resistant strains known to have developed mutational resistance. The diversity of findings perhaps reflects the complexity and variability of the environments in which pathogenic bacteria exist. Moreover, it also seems possible that some mutator phenotypes may easily revert genetically or be inducible. Clearly, hypermutation has a potential role in the development of antibiotic resistance [152,176–178], but work is required to gain a comprehensive understanding of its impact in the clinical setting. Potential areas of significance for further investigations include patients receiving long-term nitrofurantoin treatment, as this antibiotic appears to require many mutations for resistance to emerge, and animals reared using antibiotics as growth promoters.

CONCLUSIONS

Mutation as a cause of antibiotic resistance has the greatest clinical impact on particular antibiotic classes or in particular bacterial pathogens. However, it can also alter the way in which resistance genes are expressed and, in the longer term, can play a significant role in the evolution and diversification of acquired resistance determinants. If chromosomal mutation remains the main resistance mechanism for a 'bug–drug' combination, then there should be concern regarding the potential for spread of the resistant bacterial strains, rather than of resistance genes. There is clear potential for mutational resistance to emerge, including resistance to agents not yet licensed for clinical use, and this aspect should be investigated during the development process of new compounds. Such studies may help to inform the choice of suitable dosing regimens for agents awaiting a licence, in order to better prevent the emergence of resistance.

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