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Molecular Mechanisms of Antimicrobial Resistance in Bacteria of Public Health Important

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Abstract: Antibiotics underpin modern medicine; their use has reduced childhood mortality and increased life expectancy, and they are crucial for invasive surgery and treatments such as chemotherapy. However, the emergence of antimicrobial resistance has become a problem of public health concern both in developed and developing countries. There are different mechanisms by which bacteria are either intrinsically resistant or acquire resistance to available antimicrobials. These include prevention of access to drug targets, changes in the structure and protection of antibiotic targets, direct modification or inactivation of antibiotics, mutagenesis of key amino acids in the macromolecular targets of specific chemotherapeutics, or drug efflux from the cell, among others. All these mechanisms are encoded by several genes and other genetic elements, many of which can transfer between bacteria. Also, new resistance mechanisms are constantly being described, and new genes and vectors of transmission are identified on a regular basis. This article reviews the molecular mechanisms of drug resistance in bacteria of public health importance including *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Vibrio cholerae* and *Neisseria gonorrhea*.

Keywords: Antibiotic resistance; Bacteria; Molecular Mechanisms.

1. Introduction

In the early 1970s, physicians were finally forced to abandon their belief that, given the vast array of effective antimicrobial agents, virtually all bacterial infections were treatable. Their optimism was shaken by the emergence of resistance to multiple antibiotics among such pathogens as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*. The evolution of increasingly antimicrobial resistant bacterial species stems from a multitude of factors that includes the widespread and sometimes inappropriate use of antimicrobials, the extensive use of these agents as growth enhancers in animal feed, and, with the increase in regional and international travel, the relative ease with which antimicrobial-resistant bacteria cross geographic barriers [1-4].

Bacteria can be intrinsically resistant to certain antibiotics but can also acquire resistance to antibiotics via mutations in chromosomal genes and by horizontal gene transfer. The intrinsic resistance of a bacterial species to a particular antibiotic is the ability to resist the action of that antibiotic as a result of inherent structural or functional characteristics [5]. The simplest example of intrinsic resistance in an individual species results from the absence of a susceptible target of a specific antibiotic; for example, the biocide triclosan has broad efficacy against Gram-positive bacteria and many Gram-negative bacteria, but it is unable to inhibit growth of members of the Gram-negative genus *Pseudomonas*. Although this was initially thought to be due to active efflux, it has more recently been shown that it is instead due to the carriage of an insensitive allele of *fabI* that encodes an additional *enoyl-ACP reductase* enzyme — the target for triclosan in sensitive species [6]. Also, the intrinsic resistance of some Gram-negative bacteria to many compounds is due to an inability of these agents to cross the outer membrane: for example, the glycopeptide antibiotic vancomycin inhibits peptidoglycan crosslinking by binding to target d-Ala-d-Ala peptides but is only normally effective in Gram-positive bacteria as, in Gram-negative organisms, it cannot cross the outer membrane and access these peptides in the periplasm [7]. Understanding the genetic basis of intrinsic bacterial resistance, and hence the spectrum of activity of an antibiotic, can therefore guide the development of new combinations of agents with improved or expanded activity against target species.

In addition to intrinsic resistance, bacteria can acquire or develop resistance to antibiotics. This can be mediated by several mechanisms, which fall into three main groups: first, those that minimize the intracellular concentrations of the antibiotic as a result of poor penetration into the bacterium or of antibiotic efflux; second, those that modify the antibiotic target by genetic mutation or post-translational modification of the target; and third, those that inactivate the antibiotic by hydrolysis or modification. Many authors have reviewed the mechanisms of antimicrobial

resistance in different bacterial species [4, 5, 8-14], in the present paper, the author reviewed the molecular mechanisms of drug resistance in selected bacteria of medical importance and presented a framework towards solving the menace of antibiotic resistance. The word antibiotic has become synonymous with 'antimicrobial agents or antibacterial drug' therefore, in this article the terms are used interchangeably.

2. *Staphylococcus aureus*

S. aureus is perhaps the pathogen of greatest concern among the pyogenic cocci because of its intrinsic virulence, its ability to cause a diverse array of life threatening infections, and its capacity to adapt to different environmental conditions. The mortality of *S. aureus* bacteremia remains approximately 20–40% despite the availability of effective antimicrobials [9]. However, *S. aureus* has developed resistant to common antibiotics such as penicillin, methicillin, fluoroquinolones, and vancomycin. Staphylococcal resistance to penicillin is mediated by *blaZ*, the gene that encodes *β-lactamase*. This predominantly extracellular enzyme, synthesized when *staphylococci* are exposed to β-lactam antibiotics, hydrolyzes the β-lactam ring, rendering the β-lactam inactive. *blaZ* is under the control of two adjacent regulatory genes, the anti-repressor *blaR1*and the repressor *blaI* [15]. Research has demonstrated that the signaling pathway responsible for *β-lactamase* synthesis requires sequential cleavage of the regulatory proteins *BlaR1* and *BlaI*. Following exposure to β-lactams, *BlaR1*, a transmembrane sensor-transducer, cleaves itself. Then, the cleaved protein functions as a protease that cleaves the repressor *BlaI*, directly or indirectly and allows *blaZ* to synthesize enzyme [16].

Methicillin, introduced in 1961, was the first of the semisynthetic penicillinase resistant penicillins. Its introduction was rapidly followed by reports of methicillin-resistant isolates. The therapeutic outcome of infections that result from methicillin-resistant *S. aureus* (MRSA) is worse than the outcome of those that result from methicillin-sensitive strains. Methicillin resistance requires the presence of the chromosomally localized *mecA* gene [16]. *mecA* is responsible for synthesis of penicillin-binding protein 2a (PBP2a; also called PBP2′) a 78-kDa protein [17]. PBPs are membrane-bound enzymes that catalyze the transpeptidation reaction that is necessary for cross-linkage of peptidoglycan chains. PBP2a substitutes for the other PBPs and, because of its low affinity for all β-lactam antibiotics, enable *staphylococci* to survive exposure to high concentrations of these agents. Thus, resistance to methicillin confers resistance to all β-lactam agents, including cephalosporins. The *mecA* gene is part of a mobile genetic element found in all MRSA strains. Katayama, *et al.* [18] demonstrated that *mecA* is part of a genomic island designated staphylococcal cassette chromosome mec (SCCmec). These islands may also contain additional genes for antimicrobial resistance and insertion sequences, as well as genes of uncertain function.

Fluoroquinolone resistance develops as a result of spontaneous chromosomal mutations in the target of the antibiotic, topoisomerase IV or DNA gyrase, or by the induction of a multidrug efflux pump. The quinolones act on DNA gyrase, which relieves DNA supercoiling, and topoisomerase IV, which separates concatenated DNA strands. Amino acid changes in critical regions of the enzyme DNA complex (quinolone resistance–determining region [QRDR]) reduce quinolone affinity for both of its targets. The *ParC* subunit (*GrlA* in *S. aureus*) of topoisomerase IV and the *GyrA* subunit in gyrase are the most common sites of resistance mutations; topoisomerase IV mutations are the most critical, since they are the primary drug targets in *staphylococci* [19]. An additional mechanism of resistance in *S. aureus* is induction of the *NorA* multidrug resistance efflux pump. Increased expression of this pump in *S. aureus* can result in low-level quinolone resistance [19].

Staphylococcal resistance to vancomycin in a clinical isolate was first reported in a strain of *Staphylococcus haemolyticus* [20]. In 1997, the first report of vancomycin intermediate-resistant S. aureus (VISA) came from Japan, and additional cases were subsequently reported from other countries. Vancomycin resistance result from conjugal transfer of the *vanA* operon from a vancomycin-resistant *E. faecalis.* Showsh *et al.* (Showsh *et al.*, 2001) reported that the *enterococcal* plasmid containing *vanA* also encodes a sex pheromone that is synthesized by *S. aureus*, suggesting a potential facilitator of conjugal transfer. These VRSA isolates demonstrate complete vancomycin resistance; with MICs of ≥128 μg/ml. Resistance in these isolates is caused by alteration of the terminal peptide to D-Ala-D-Lac instead of D-Ala-D-Ala. Synthesis of D-Ala-D-Lac occurs only with exposure to low concentrations of vancomycin. As a result, the additional biosynthetic demands are limited and the VRSA strain is ecologically fit [21]. This ecological fitness, the possibility that this plasmid exchange will occur more frequently (due to the ever increasing likelihood of patients being colonized with both MRSA and vancomycin-resistant enterococci), and the resistance of these strains to both β-lactams and glycopeptides all increase the likelihood that VRSA strains will rapidly become more prevalent.

3. *Vibrio cholerae*

As the causative agent of cholera, the bacterium *Vibrio cholerae* represents an enormous public health burden, especially in developing countries around the world. The acute diarrhoeal disease cholera is responsible for approximately 120000 deaths every year and has a major impact on the health of young children between the ages of 1 and 5 years [22]. *V. cholerae* becomes drug resistant by exporting drugs through efflux pumps, chromosomal mutations or developing genetic resistance via the exchange of conjugative plasmids, conjugative transposons, integrons or self-transmissible chromosomally integrating SXT elements [10].

V. cholerae uses multidrug efflux pumps to export a broad range of antibiotics, detergents and dyes that are chemically and structurally unrelated [10]. The two major groups of *V. cholerae* efflux pumps are distinguished by

their energy sources: ATP hydrolysis, or the proton-motive force (PMF) of transmembrane H+ or Na+ gradients [23]. PMF pump families include MATE (multidrug and toxic compound extrusion), MFS (major facilitator superfamily), RND (resistance–nodulation–cell division) and SMR (small multidrug resistance) [10]. One of the few bacterial ATP-driven pumps is *VcaM*, a *V. cholerae* ABC (ATP-binding cassette) multidrug resistance efflux pump. *VcaM* confers resistance to structurally divergent drugs (e.g. tetracycline, norfloxacin, ciprofloxacin and doxorubicin).

V. cholerae uses an array of MATE-family efflux systems, namely *VcmB*, *VcmD*, *VcmH*, *VcmN*, *VcmA* and *VcrM* [10]. In addition, the *V. cholerae O1 El Tor N16961* genome carries a homologue of *NorM* in *Vibrio parahaemolyticus* that mediates resistance to hydrophilic fluoroquinolones, aminoglycosides and norfloxacin [24].

MFS transporters in *V. cholerae* include the *V. cholerae* efflux systems that confer resistance to bile (deoxycholate), antibiotics (e.g. chloramphenicol and nalidixic acid) and the proton gradient-uncoupling agent carbonyl cyanide m-chlorophenylhydrazone [10]. Research has shown that the classical O395 strain carries the MFS efflux protein *EmrD-3*, which confers resistance to linezolid, rifampicin, erythromycin and chloramphenicol when expressed in a drug-hypersensitive *Escherichia coli* strain [25].

The *V. cholerae* RND efflux systems are encoded by six operons *(vexRAB, vexCD, vexEF, vexGH, vexIJK* and *vexLM)* [26] and exhibit particularly broad substrate specificity. Interestingly, the *V. cholerae* RND systems play a role not only in the efflux of a variety of compounds (e.g. Triton X-100, SDS, polymyxin B, erythromycin, bile salts, penicillin), but also in colonization [26]. Collectively, these results indicate that efflux pumps are not employed exclusively for drug resistance, but also play a role in the expression of important virulence genes in *V. cholerae*.

Antibiotic-resistance in *V. cholerae* is also facilitated by horizontal gene transfer via self-transmissible mobile genetic elements, including SXT elements – mobile DNA elements belonging to the class of integrative conjugating elements (ICEs). Besides conferring antibiotic resistance, SXT elements have the capacity to mobilize conjugative plasmids and genomic islands *in trans*, thus, providing alternative mechanisms for antibiotic resistance gene transfer [10]. Dissemination of antibiotic resistance genes is also facilitated when *V. cholerae* cells share mobile integrons with other bacterial cells. All *V. cholerae* isolates harbour large chromosomal integrons, giving them the capacity to rapidly transfer gene cassettes containing antibiotic resistance genes. Class 1 integrons are by far the most frequent type and are closely associated with a *Tn402* transposon, whereas class 2 integrons are associated with a *Tn21* transposon. Both classes carry multiple gene cassettes encoding antibiotic resistance genes, such as *dfrA1* (trimethoprim resistance) [27].

4. *Enterobacteriaceae*

Resistance of the *Enterobacteriaceae* to antibiotics, especially of the β lactam type, is increasingly dominated by the mobilization of continuously expressed single genes that encode efficient drug modifying enzymes. Strong and ubiquitous selection pressure has seemingly been accompanied by a shift from "natural" resistance, such as inducible chromosomal enzymes, membrane impermeability, and drug efflux, to the modern paradigm of mobile gene pools that largely determine the epidemiology of modern antibiotic resistance. In this way, antibiotic resistance is more available than ever before to organisms such as *Escherichia coli* and *Klebsiella pneumoniae*; two medically important genera in the family of *Enterobacteriaceae* that are implicated in severe sepsis and septic shock [12].

Beta-lactamase production is the main mechanism of beta-lactam resistance in *Enterobacteriacea*e [14]. These highly diversified enzymes hydrolyze beta-lactams in the periplasmic space, thus preventing penicillin-binding protein inhibition. Inhibitor-susceptible *TEM* and *SHV* penicillinases emerged first in the 1960s, and spread rapidly afterwards. They spread successfully in healthcare-associated strains of *K. pneumoniae, Enterobacter sp*. and, in a lesser extent, *E. coli*, causing major hospital outbreaks in the 1990s [12]. Nowadays, *TEM*-type and *SHV*-type ESBL (extended spectrum beta-lactamases) are still endemic in many hospitals around the world; nevertheless, they tend to be outnumbered by another ESBL class, referred as *CTX-M, which was first*, described in the early 1990s [28]. A key epidemiological aspect of *CTX-M*-type ESBL is to be mostly found in *E. coli* colonizing subjects with no medical condition, antibiotic exposure, or previous contact with the healthcare setting [12, 29]. This community reservoir fuels a continuous influx of ESBL into the hospital system [12, 29]. Extended spectrum beta-lactamases producing *enterobacteriaceae* (ESBL-PE) are resistant to most beta-lactams except cefoxitin, carbapenems and, for a subset of strains, temocillin. Meanwhile, co-resistances to fluoroquinolones, cotrimoxazole and aminoglycosides are commonly observed in ESBL-PE [12], leaving few alternatives to carbapenems for the treatment of severe infections. However, *TEM, SHV* and *CTX-M* are all class A beta-lactamases, and many ESBL variants remain susceptible in vitro to beta-lactamase inhibitors.

In parallel to ESBL, plasmid-borne *cephalosporinases* have gained increasing prominence in *Enterobacteriaceae*, including in community-acquired strains [12]. These beta-lactamases are actually encoded by chromosomal *blaAmpC* genes of *Enterobacteriaceae* that have been captured on MGE. *CMY-2* from *Citrobacter freundii* is the most frequently encountered type [30]. Most of plasmid-borne *cephalosporinases* confer a similar pattern of resistance to that of derepressed *AmpC*. As the prevalence of ESBL and plasmid-borne *cephalosporinases* rose, so did the consumption of carbapenems, which promoted the emergence of carbapenems resistant *enterobacteriaceae* (CRE) through the diffusion of plasmid-borne *carbapenemases.* CRE are currently spreading worldwide through travelers and repatriated patients [31], and are now isolated in subjects with no previous stay in endemic areas [31]. It should be underlined that *carbapenemase* production is not the sole mechanism of carbapenem resistance in *Enterobacteriaceae*, since this phenotype may also emerge under therapy in ESBL-PE or

AmpC hyper producers with acquired impermeability to carbapenems due to mutation-derived loss of outer membrane porins [12, 32].

Aminoglycosides resistance in *Enterobacteriaceae* mainly relies on AMEs that hampers antibiotic activity by engrafting various radicals (*aminoglycoside phosphotransferase*, APH, *aminoglycoside nucleotidyltransferase*, ANT and *aminoglycoside acetyltransferase*, AAC). An intrinsic AME production is met in *Providencia stuartii* (*AAC (2'),* resistance to gentamicin and tobramycin) and *Serratia marcescens* (*AAC (6')-I,* low-level resistance to tobramycin and amikacin). Methylases of the 16S ribosomal subunit (i.e., the target of aminoglycosides) have been more described, notably in NDM-producing strains [32]: these enzymes, named *ArmA* and *Rmt*, confer resistance to all aminoglycosides except neomycin.

All *Enterobacteriaceae* are naturally susceptible to quinolones and fluoroquinolones. High-level resistance emerges after successive chromosomal mutations in the DNA gyrase- and topoisomerase IV-encoding genes (*gyrA* and *parC*, respectively), each mutation causing a rise in the MICs [33]. Chromosomal mutations may also lead to decreased permeability or overexpression of efflux pumps, resulting in reduced susceptibility. Besides mutations, plasmid-encoded resistance has emerged in the 2000s with *Qnr* (A, B, C, D and S subtypes), a small DNA-mimicking protein that confers low-level fluoroquinolone resistance, *AAC (6')-Ib-cr*, an AME for which two mutations extend the resistance spectrum to ciprofloxacin and norfloxacin, and the *QepA* efflux pump [12]. It is noteworthy that these plasmid-borne determinants of fluoroquinolone resistance are frequently associated with ESBL [33].

5. *Pseudomonas aeruginosa*

Similarly to AmpC-producing *Enterobacteriaceae*, *P. aeruginosa* harbors an inducible AmpC-type *cephalosporinase* that can be derepressed following mutations in the regulation system [12]. Wild-type strains of *P. aeruginosa* are resistant to amoxicillin (with or without clavulanate), 1GC, 2GC, cefotaxime, ceftriaxone and ertapenem, while they remain susceptible to ticarcillin, piperacillin, ceftazidime, cefepime, imipenem, meropenem and doripenem. Unlike tazobactam, clavulanate is a strong inducer of AmpC in *P. aeruginosa*, and experimental data suggest a risk of clinical failure with the ticarcillin–clavulanate association [34]. *P. aeruginosa* has several three-component efflux systems, some of which confer resistance to beta-lactams when strongly expressed after mutations in their promoter regions [34]. The most frequently involved system is *MexAB-OprM*, whose overexpression confers resistance to ticarcillin, aztreonam, cefepime and meropenem.

Efflux pumps are also major determinants of the multidrug resistance phenotypes that are increasingly observed in *P. aeruginosa*. A key feature is that different antimicrobial classes may be substrates of a single pump: exposure to a given class (e.g., beta-lactams) may thereby select mutants with resistance to other classes (e.g., beta-lactams plus fluoroquinolones or aminoglycosides) [35]. *P. aeruginosa* has the ability to develop resistance to all beta-lactams as the sole result of chromosomal mutations. Nonetheless, the species can acquire MGE encoded beta-lactamases, including ESBL and *carbapenemases* [12]. Resistance to tobramycin mostly occurs through the acquisition of AMEs, while resistance to amikacin mostly depends on the over-expression of efflux pumps [36]. MGE-borne 16S rRNA methylases such as *ArmA*, *RmtA* and *RmtD* are also reported as an emerging mechanism of aminoglycoside resistance in *P. aeruginosa* [37]. Fluoroquinolone resistance results from mutations in the topoisomerase-encoding genes and/or the hyper-expression of efflux systems [35].

6. *Neisseria gonorrhoeae*

The strict human pathogen *Neisseria gonorrhoeae* has caused gonorrhea for thousands of years, and currently gonorrhea is the second most prevalent bacterial sexually transmitted infection worldwide [38]. As with other heritable changes, resistance to antibiotics in gonococci develops due to spontaneous mutation and/or gene (whole or parts) acquisition, which are effectively selected due to antibiotic pressure in patients and, in general, in society. In general, resistance determinants seem to be stably maintained in gonococci even though the antibiotic has been removed from treatment regimen decades earlier. This maintenance of the resistance determinants may be due to the antibiotics used to treat other bacterial infections, inappropriate use of the antibiotic, or anti-gonococcal agents used topically to prevent STIs and HIV transmission or pregnancy (e.g., the spermicide nonoxynol-9), which could inadvertently maintain selective pressure in the community for resistant strains. However, the persistence may also be because the resistance determinants (i) do not affect the biological fitness (no benefits for the bacteria to get rid of them); (ii) do lower the biological fitness; however, this fitness cost is compensated by second-site mutation (not influencing the resistance); or (iii) the resistance determinants may even cause a higher biological fitness and, in fact, make these clones more successful with regard to transmission and virulence [38].

Transformation has played a key role in the evolution of antibiotic resistance in the gonococcus. Gonococci are highly competent for transformation (natural competence during the entire life cycle) by their own DNA and to a lesser extent, although still quite significant, that of other closely related bacteria, that is especially commensal *Neisseria species* and *N. meningitidis*. Accordingly, for example, pharyngeal gonorrhea, where gonococci frequently coincide with other neisserial species, may act as an asymptomatic reservoir for infection but also for initial emergence of antibiotic resistance, by transformation, in the gonococci [38, 39]. Donor DNA from these other species can create mosaic genes in recipient gonococci, such as *penA* mosaic alleles, that encode variants of

penicillin-binding protein 2 (PBP2) having reduced affinity for β-lactam antibiotics. These emerged mosaic genes (commonly resistance determinants) can subsequently effectively spread among gonococcal strains [39].

The development of penicillin resistance in gonococci is illustrative of how antibiotic pressure and selection can drive resistance. The development of the chromosomally determined resistance took nearly 40 years and was the result of changes (mutations and gene acquisition) in at least five single locus (or at least in some cases "cooperative loci"). Early work by Sparling and coworkers showed that sequential accumulation of polymorphisms in loci termed *penA, mtr*, and *penB* resulted in graded increases in penicillin resistance. The latter two resistance determinants also affect the susceptibility to several other antimicrobials, such as tetracycline, macrolides, and cephalosporins, which are also affected by polymorphisms in *pen A*. The *penA* gene encodes PBP2, which is the main lethal target for penicillin (and other β-lactam antibiotics) and responsible for peptidoglycan cross-linking at the septum during cell division [38]. *Mtr* (multiple transferable resistance) was first thought to decrease the outer membrane permeability of gonococci to antimicrobials, but is now known to be mainly due to mutations in a gene encoding a transcriptional repressor *(MtrR)* or its promoter [40]. *MtrR* binds to and represses an adjacent, but divergent, promoter used for transcription of an efflux pump operon *(mtrCDE),* which encodes a tripartite export system that expels antimicrobials from the bacterial periplasmic space [40] Research has shown that overexpression of the *mtrCDE*-encoded efflux pump is important in strains expressing high level penicillin resistance and determined that mutations that abrogate pump function can result in a mutant strain expressing hyper susceptibility to penicillin [40]. The *penB* resistance determinant is due to specific mutations in the gene encoding the major outer membrane porin protein termed *PorB1b* [38]. This porin, *PorB*, exists in two allelic forms termed *PorB1a* and *PorB1b*. *PorB1b*–producing gonococci are often slightly less susceptible than *PorB1a* strains to penicillin and *penB* mutations can further decrease such susceptibility. Specific amino acid replacements in loop 3 (G120K and A121D) of *PorB1b* have been linked to the *penB* resistance determinant (42). These mutations were thought to decrease entry of penicillin through the *PorB1b* porin, [38, 41] but a conflicting view has been presented. Interestingly, *penB* mutations are only phenotypically evident when the strain has a co-resident *mtrR* mutation, suggesting some interaction between *PorB1b* and the *MtrCDE* efflux pump [39, 41]. At least two additional mutations are needed for penicillin resistance (MIC of ≥2 μg/mL), but these are less well understood. Specific mutation in *ponA* (*ponA1*; results in the amino acid replacement L421P) causes a decreased affinity for penicillin to the encoded PBP1, and further decreased susceptibility to penicillin [42]. Finally, the *penC* (currently more commonly named *pilQ2*) mutation occurs in the *pilQ* gene, which encodes the secretin *PilQ* of the type IV pilin [42]. *pilQ*2 (encoding the amino acid replacement E666K) can decrease the stability of the *PilQ* doughnut-like multimeric structure in the outer membrane, which seems to decrease entry of penicillin [38, 42]. However, since *pilQ2* mutations influence proper piliation, which is important for gonococcal disease, it is hard to envision how *pilQ* mutations would afford a selective advantage in the community and accordingly be of importance for wide spread of clinical penicillin resistance.

The evolution of gonococcal resistance to third-generation cephalosporins also seems to be highly similar to the evolution of penicillin resistance, that is, the most common mechanism for decreased susceptibility is alteration of *penA*, including acquisition of a *penA* mosaic allele or alterations of amino acid A501 in *PBP2* [38, 43, 44]. The same mutations, as seen in penicillin resistance, in especially the promoter of *mtrR* further decrease the susceptibility [43, 44]. Moreover, alterations of amino acid G101 and A102 in *PorB1b* (*penB* resistance determinant) result in further decreased susceptibility. [43, 44]. However, on the relatively few studies and the currently circulating gonococcal strains *ponA1* or mutations in *pilQ* do not seem to substantially enhance the MICs. As in chromosomally mediated penicillin resistance, at least one unknown ("factor X"), non-transformable resistance determinant seems to exist [44]. Worryingly, the detailed characterization (including also transformation experiments verifying the resistance mechanisms) of the first gonococcus displaying high level clinical resistance to ceftriaxone showed that only a few additional amino acid replacements in a "traditional" mosaic *PBP2 (PBP2* mosaic X allele, which has been correlated with cefixime treatment failures in Japan) were needed, that is, together with the resistance determinants *mtrR*, *penB*, and "factor X," to develop the ceftriaxone MIC of 2–4 μg/mL (cefixime MIC of 8 μg/mL) [45]. This novel *PBP2* allele contained only 12 polymorphic *PBP2* amino acids compared to the *PBP2* mosaic X allele, and four of these alterations were unique compared with any neisserial *PBP2* sequence previously described. These four unique amino acid alterations consisted of *A311V, T316P, A328T, and T484S*. Although additional studies are needed, *A311V* and *T316S* are likely the alterations causing the high resistance to ceftriaxone (and all other extended-spectrum cephalosporins), that is, due to the proximity to the β-lactam active site in *PBP2* [45]. Based on all historical precedents, strains with decreased susceptibility and resistance to third-generation cephalosporins will become more common and analysis of their genetic profiles are crucial, in order to understand the mechanisms for emergence and spread (national and international) of this resistance. Accordingly, it also seems inevitable that strains with clinical resistance to ceftriaxone will emerge and spread internationally, and the only question is when, and not if, we will identify these strains spreading worldwide.

7. *Mycobacterium tuberculosis*

Tuberculosis (TB) remains one of the leading public health problems worldwide. Declared as a global emergency in 1993 by the WHO, its control is hampered by the emergence of multidrug resistance (MDR), defined as resistance to at least rifampicin and isoniazid, two key drugs in the treatment of the disease. More recently, severe forms of drug resistance such as extensively drug-resistant (XDR) TB have been described. Major advances in molecular biology and the availability of new information generated after sequencing the genome of *Mycobacterium*

tuberculosis increased our knowledge of the mechanisms of resistance to the main anti-TB drugs. Better knowledge of the mechanisms of drug resistance in TB and the molecular mechanisms involved will help us to improve current techniques for rapid detection and will also stimulate the exploration of new targets for drug activity and drug development.

Isoniazid is one of the main drugs for the treatment of TB. It has a simple structure containing a pyridine ring and a hydrazide group, with both components being essential for the high activity against *M. tuberculosis.* Resistance to isoniazid is a complex process, mutations in several genes, including *katG, ahpC, inhA, kasA* and *ndh,* have all been associated with isoniazid resistance. Isoniazid is a pro-drug requiring activation by the catalase/peroxidase enzyme encoded by *katG*. [13]. Activated isoniazid interferes with the synthesis of essential mycolic acids by inhibiting NADH dependent enoyl-ACP reductase, which is encoded by *inhA* [46]. Two molecular mechanisms have been shown to be the main cause for isoniazid resistance: mutations in *katG* and mutations in *inhA*, or more frequently in its promoter region [13, 46]. A decrease in or total losses of catalase/peroxidase activity as a result of katG mutations are the most common genetic alterations associated with isoniazid resistance [46]. So far, more than a hundred mutations in *katG* have been reported, with MICs ranging from 0.2 to 256 mg/L. Missense and nonsense mutations, insertions, deletions, truncation and, more rarely, full gene deletion have been observed. The most common mutation is *S315T*, which results in an isoniazid product that is highly deficient in forming the isoniazid-NAD adduct related to isoniazid antimicrobial activity. Furthermore, down-regulation of *katG* expression has also been shown to be associated with resistance to isoniazid [46]. Three novel mutations in the *furA-katG* intergenic region were identified in 4% of 108 isoniazid-resistant strains studied; none of these was present in 51 isoniazid-susceptible strains. Reconstructing these mutations in the *furA-katG* intergenic region of isogenic strains decreased the expression of katG and conferred resistance to isoniazid.

Mutations in *inhA* cause not only resistance to isoniazid, but also resistance to the structurally related second-line drug ethionamide [13]. The most common *inhA* mutation occurs in its promoter region *(1-15C-T)* and it has been found more frequently associated with mono-resistant strains [47]. Studies have shown that mutations in the intergenic region *oxyR-ahpC* can reduce the level of expression of *inhA* and have been associated with resistance to isoniazid. A study by Dalla Costa, *et al.* [47] found mutations in the intergenic region *oxyR-ahpC* in 8.9% of 224 isoniazid-resistant strains studied, confirming its less frequent involvement as a cause of resistance to isoniazid. The role of some of these genes in isoniazid resistance, however, has not been completely elucidated. `

Another first line drug for TB drug is rifampicin, a lipophylic ansamycin introduced in 1972. Due to its efficient antimicrobial action, it is considered, together with isoniazid, to be the basis of the short-course treatment regimen for TB. The target of rifampicin in *M. tuberculosis* is the b-subunit of RNA polymerase, where it binds and inhibits the elongation of messenger RNA. The great majority of M. tuberculosis clinical isolates resistant to rifampicin show mutations in the gene *rpoB* that encodes the b-subunit of RNA polymerase. This results in conformational changes that determine a low affinity for the drug and consequently the development of resistance [48]. Mutations in a 'hot-spot' region of 81 bp of *rpoB* have been found in about 96% of rifampicin-resistant *M. tuberculosis* isolates. This region, spanning codons 507–533, is also known as the rifampicin resistance-determining region (RRDR). Some studies have also reported mutations outside of the hot-spot region of rpoB in rifampicin-resistant *M. tuberculosis* isolates [13, 48]. An important finding related to resistance to rifampicin is that almost all rifampicin-resistant strains also show resistance to other drugs, particularly to isoniazid. For this reason, rifampicin resistance detection has been proposed as a surrogate molecular marker for MDR [13].

Pyrazinamide, another TB drug is a structural analogue of nicotinamide and is a pro-drug that needs to be converted into its active form, pyrazinoic acid, by the enzyme *pyrazinamidase/nicotinamidase (PZase)* [49]. *PZase* is encoded in *M. tuberculosis* by the gene *pncA*. It has been postulated that the mechanism of action of pyrazinamide is through pyrazinoic acid, its active moiety, by disrupting bacterial membrane energetics and inhibiting membrane transport. Mutations in *pncA* are the main mechanisms for pyrazinamide resistance in *M. tuberculosis*. Most alterations occur in a 561 bp region of the open reading frame or in an 82 bp region of its putative promoter [49].

M. tuberculosis resistance to streptomycin emerged quite rapidly after the introduction of the drug. The genetic basis of resistance to streptomycin is mostly due to mutations in *rrs* or *rpsL*, which has been identified in slightly more than 50% of the strains studied. [50]. The majority of point mutations resulting in streptomycin resistance occur in *rpsL*, with the most common mutation being *K43R*. Also it has been shown that mutations in *gidB,* which encodes a conserved 7-methylguanosine methyltransferase specific for the 16S rRNA, can confer a low level of streptomycin resistance [50].

Fluoroquinolones are bactericidal antibiotics currently in use as second-line drugs in the treatment of TB. Both ciprofloxacin and ofloxacin are synthetic derivatives of nalidixic acid, the parent compound discovered in 1965 as a by-product in the purification of the antimalarial drug chloroquine. In *M. tuberculosis*, only type II topoisomerase (DNA gyrase) is present and thus is the only target for fluoroquinolone activity. Initial studies performed in laboratory strains of *M. tuberculosis* and *M. smegmatis* showed that resistance to fluoroquinolones was the result of amino acid substitutions in the putative fluoroquinolone binding region in *gyrA* or *gyrB* [51]. This association of mutations in the so-called quinolone resistance-determining region (QRDR) of *gyrA* and *gyrB* and resistance to fluoroquinolones has been confirmed now in multiple studies. Fluoroquinolone-resistant strains of *M. tuberculosis* show mutations in a conserved region of *gyrA*, with Ala-90 and Asp-94 as the most frequently mutated positions; nevertheless, mutations at Ala-74, Gly-88 and Ser-91 have also been reported [51].

Resistance to other second line drugs has also been reported. Kanamycin and amikacin are aminoglycoside antibiotics, while capreomycin and viomycin are cyclic peptide antibiotics. All four are used as second-line drugs in the treatment of MDR-TB. Although belonging to two different antibiotic families, all exert their activity at the level of protein translation. Cross-resistance among kanamycin, capreomycin and viomycin has been reported. Several other studies have also reported cross-resistance between kanamycin and amikacin or between kanamycin and capreomycin or viomycin to variable degrees [52, 53]. The most common molecular mechanism of drug resistance has been associated with an A1401G mutation in the *rrs* gene coding for 16S rRNA. This mutation occurs more frequently in strains with high-level resistance to kanamycin and amikacin [53]. Mutations in the gene tlyA have also been implicated in resistance to capreomycin and viomycin. This gene codes an rRNA methyltransferase specific for 2′-O-methylation of ribose in rRNA. When mutated, it determines an absence of methylation activity [52].

8. Conclusion

As a result of the widespread use of antibiotics in human medicine — as well as in animal treatment, horticulture, beekeeping, anti-fouling paints (used in the marine and oil industries) and laboratories carrying out genetic manipulation — the evolutionary pressure for the emergence of antibiotic resistance is great. Antibiotic resistance is a natural phenomenon, and bacteria have been evolving to resist the action of natural antibacterial products for billions of years. Although the ability of bacteria to become resistant to antibiotics has long been appreciated, our knowledge of the remarkable diversity of mechanisms involved has increased greatly in recent years. Advances in genomics, systems biology and structural biology have dissected many of the precise events underpinning resistance and will continue to provide greater understanding. This information, if used properly, should aid the discovery and development of new agents that can circumvent or neutralize existing resistance mechanisms. Indeed, increased understanding of resistance has also provided new targets for discovery. Studies of resistance development and mechanisms of resistance must be a mandatory requirement at an early stage of drug development; such studies will enable academic institutions and industry to work together. We now have the ability to rapidly evaluate the potential for the emergence of resistance to novel drugs, identify where and when this might occur and determine the mechanisms responsible.

Furthermore, knowledge of the molecular basis of drug resistance will allow more rational development of new drugs; something that is urgently needed, when taking into account the increasing rates of MDR around the world. In this framework it is useful to have additional sources of information and new drugs should bypass the molecular mechanisms of resistance in currently available drugs and also offset intrinsic resistance, as, for example, that provided by efflux mechanisms.

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