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The forgotten Gram-negative bacilli: What genetic determinants are telling us about the spread of antibiotic resistance

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ABSTRACT

Gram-negative bacilli have become increasingly resistant to antibiotics over the past 2 decades due to selective pressure from the extensive use of antibiotics in the hospital and community. In addition, these bacteria have made optimum use of their innate genetic capabilities to extensively mutate structural and regulatory genes of antibiotic resistance factors, broadening their ability to modify or otherwise inactivate antibiotics in the cell. The great genetic plasticity of bacteria have permitted the transfer of resistance genes on plasmids and integrons between bacterial species allowing an unprecedented dissemination of genes leading to broad-spectrum resistance. As a result, many Gram-negative bacilli possess a complicated set of genes encoding efflux pumps, alterations in outer membrane lipopolysaccharides, regulation of porins and drug inactivating enzymes such as beta-lactamases, that diminish the clinical utility of today's antibiotics. The cross-species mobility of these resistance genes indicates that multidrug resistance will only increase in the future, impacting the efficacy of existing antimicrobials. This trend toward greater resistance comes at a time when very few new antibiotics have been identified capable of controlling such multi-antibiotic resistant pathogens. The continued dissemination of these resistance genes underscores the need for new classes of antibiotics that do not possess the liability of cross-resistance to existing classes of drugs and thereby having diminished potency against Gram-negative bacilli.

1. Introduction

In the past 2 decades significant activity has been expended to discover new antibiotics with improved potency against Gram-positive organisms. This focus has been due to concerns raised over the continued spread of multidrug resistant (MDR) staphylococci and vancomycin-resistant enterococci [1,2]. However, the pharmaceutical industry has not pursued with equal urgency discovery of new agents in response to the more recent increase in MDR Gram-negative bacteria. Little emphasis has been given to such agents, in part, due to the perception that well established drug classes such as the aminoglycosides, beta-lactams and fluoroquinolones have little to offer by way of derivatives with substantially improved potency against Gram-negative pathogens. Furthermore, most of the recent structurally novel classes of antimicrobials such as the oxazolidinones and peptide deformylase inhibitors demonstrate poor activity against Gram-negatives due to poor permeability [3,4].

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Unfortunately, Gram-negatives have not remained idle under the incredible selective pressure placed upon them by heavy antibiotic use. The MDR strains of Gram-negative bacilli found in hospitals today demonstrate the extensive plasticity of their genomes, characterized by the ability to acquire exogenous genetic elements and groups of resistance genes that confer broad-spectrum antibiotic resistance [5–7]. There are also genetic barriers to resistance, as evidenced by resistance genes from non-fermentative species such as *Pseudomonas aeruginosa* that are poorly transferred or expressed in the *Enterobacteriaceae* [8]. Despite this, it is evident that some species are working diligently to overcome even these genetic limitations, allowing acquisition and expression of some of the most potent resistance genes that scientists have ever observed. This review will first focus broadly on the many classes of resistance determinants that confer antibiotic resistance in Gram-negative bacilli. In addition, specific examples of elegant genetic systems that are at work to escalate the resistance war in certain of these pathogens will be covered in some detail.

2. Recent resistance trends in Gram-negative bacilli

The focus on clinical resistance in Gram-positive cocci is due to the decrease in susceptibility observed over the past decade in staphylococci, enterococci and MDR *S. pneumoniae*, where acquisition of resistance genes markedly decreases the clinical utility of entire classes of antibiotics. For instance, in hospitals today it is not unusual for up to 60% of *S. aureus* isolates to have acquired the *mecA* gene that encodes the low affinity penicillin binding protein 2a that confers clinical resistance to most beta-lactam antibiotics [9]. In a similar fashion, acquisition of a complex cassette of genes that encode resistance to vancomycin in enterococci imparts dramatically high-level resistance to vancomycin [10]. Such strains change from highly antibiotic susceptible to highly resistant after a single genetic event. In contrast, many Gram-negative bacilli contain several intrinsic resistance factors (e.g. low outer membrane permeability, chromosomally encoded efflux pump genes) that confer moderate decreases in susceptibility to many chemically unrelated antimicrobial agents [11,12]. These intrinsic factors serve to establish a baseline level of resistance characteristic for each class of antibiotic. While this baseline may establish the MIC to specific agents in the susceptible or intermediate resistance range, subsequent acquisition of mobile resistance determinants can lead to strains that are highly resistant to these antibiotics.

While some strains of every Gram-negative species are multidrug resistant, the highest incidence of resistance is limited to a rather small number of species. Surveillance studies funded by government and pharmaceutical sponsors now provide contemporary information concerning the most problematic species. Fig. 1 illustrates data from the 2004 National Nosocomial Infection Surveillance Report [13] regarding antibiotic resistance trends in isolates from intensive care units from a selected set of hospitals in the United States. While the highest rates of resistance are found in Gram-positive cocci, certain Gram-negatives are also of significant concern. By comparing the study periods 1998–2002 with 2003, resistance to imipenem, quinolones and third-generation cephalosporins (ceftriaxone, cefotaxime and ceftazidime) is clearly on the rise in *P. aeruginosa*. As described in subsequent sections of this review, this shift in resistance represents a complicated mixture of the effects of intrinsic resistance determinants and acquisition of mobile resistance genes that can act synergistically to limit the potency of multiple classes of antibiotics. Other problems of resistance are seen consistently in some members of the *Enterobacteriaceae* such as *Klebsiella pneumoniae* and *Enterobacter* spp. This review will focus on some of the most important resistance determinants found in these key species.

3. Mechanisms of resistance to antibiotics in Gram-negative bacilli

The most common mechanisms of antibiotic resistance in Gram-negative bacilli are listed in Table 1. These mechanisms fall into four categories: (1) inactivation of antibiotic by endogenous enzymes, (2) mutation in the structural or regulatory genes of the target protein (3) alterations in the outer membrane that result in decreased drug permeability and (4) extrusion of antibiotic from the Gram-negative cell by active efflux.

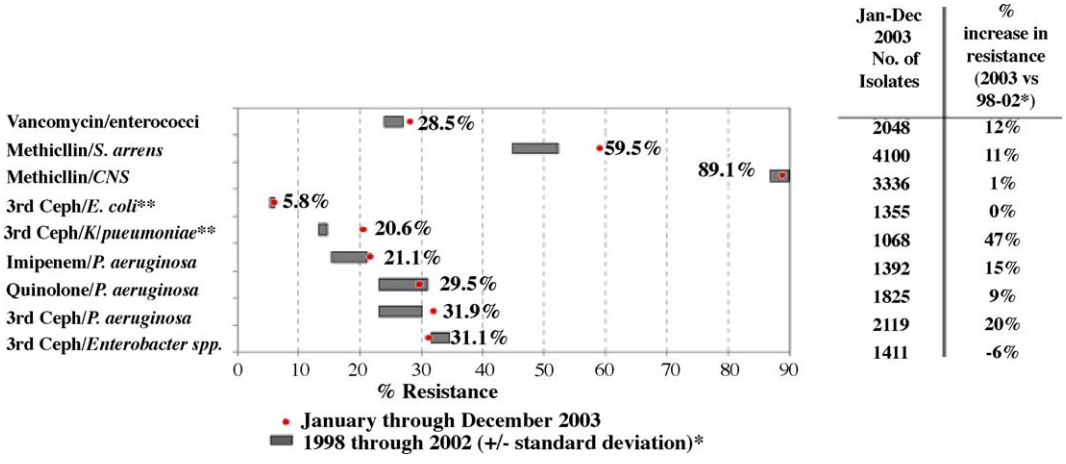

Fig. 1 – 2004 NNIS report [13].

Table 1 – Resistance mechanisms to some antibiotics

Antibiotic class	Resistance mechanism	Determinants	Mobile genes
Aminoglycosides	Compound modification	Adenyltransferases, acetyltransferases phosphotransferases	Plasmids, trransposons integrons
Streptomycin	Target modification	Mutation in small ribosomal proteins	No
Cephalosporins	PBP changes	Rare in Gram-negatives	No
	Beta-lacatamases	Ambler classes A–D[a]	Plasmids, transposons, integrons
	Efflux systems/Omp mutations	RND–MFP–OMF[b]/ΔOmpF, OmpC	No, usually chromosome
Carbapenems	PBP changes	Rare	No
	Beta-lactamases	Ambler classes A, B, D[a]	Integrons
	Efflux/Omp changes	RND–MFP–OMF[b]/ΔOprD	Chromosome
Fluoroquinolones	Target mutation	Gyrase primary Topoisomerase IV secondary	No
	Target rescue	qnr	Plasmid
	Efflux/Omp mutations	RND–MFP–OMF[b]/ΔOmpF and OmpC	Chromosome

[a] Ambler functional classification.
[b] Definitions in text.

In MDR strains of today, it is the norm to have all four of these mechanisms playing a role in resistance with strains having multiple determinants affecting susceptibility to each class of antimicrobial. Indeed, while it was once hypothesized that resistant strains carrying determinants on multiple plasmids or mobile genetic elements were less pathogenic than their susceptible counterparts, it is now common to find virulent clinical strains equipped with such multiple resistance weaponry. Genetic studies with MDR strains tell us that resistance genes for cephalosporinases and aminoglycoside inactivating enzymes are encoded on the same element. This also means that a wide variety of structurally unrelated antibiotics can provide selective pressure to maintain these genes in pathogenic bacteria. All of the determinants listed in Table 1 have significantly reduced the activity of antibiotics currently used in treating infections, with some of the determinants proving to be particularly problematic in their ability to limit our antibiotic armamentarium. In particular, determinants for efflux alone or combined with enzymatic inactivation of drug tend to confer the highest levels of resistance in many problematic species. Non-fermentative species such as *P. aeruginosa*, *Acinetobacter baumanii* and *Stenotrophomonas maltophilia* demonstrate the highest frequency of antibiotic resistance due to their intrinsic mechanisms of efflux and low outer membrane permeability. While it has been known for some time that these species contain multiple drug modifying and inactivating enzymes for aminoglycosides and beta-lactams [14–16], recent studies have clearly established that chromosomal genes encoding active efflux pumps play an equally important role in resistance [17–19]. A lesson from the genetic studies with non-fermenters is that they continue to represent the most problematic group of Gram-negatives from a therapeutic viewpoint since they possess such a comprehensive collection of resistance determinants.

3.1. *Intrinsic antibiotic resistance in Gram-negative bacilli: role of efflux pumps*

Many studies have established that Gram-negative bacilli contain chromosomally encoded genes for multiple efflux pumps, some that are expressed constitutively and others that are induced only under certain environmental stimuli [11,12,19]. Efflux pumps, reviewed extensively elsewhere [11,12,19] are classified into five families based on structure and energy source: the MFS or major facilitator superfamily, the ABC group, which all have ATP binding cassettes, the RND or resistance nodulation-division family, multidrug and toxic compound extrusion family (MATE) and the small multidrug resistance (SMR) family [12]. Of these, the RND efflux pumps play the most important role in conferring antibiotic resistance in Gram-negatives. Structurally, RND pumps are composed of a periplasmic membrane fusion protein (MFP) functionally linked to an outer-membrane protein termed the outer-membrane fusion protein (OMF). The RND–MFP–OMF efflux pump is represented by the common AcrRAB-TolC tripartite pump extensively characterized in *E. coli* and many other members of the *Enterobacteriaceae* [19]. Recent genetic data involving detection of efflux genes in Gram-negative bacilli has helped us to understand the importance of this mechanism in conferring resistance to a wide array of antibiotics.

The genetic dynamic regarding efflux pumps is not that the genes encoding them are extensively transferred between Gram-negative species, but rather that most strains encode multiple, native efflux pumps that help to establish some level of intrinsic resistance to a broad array of antibiotics since the pumps primarily serve to extrude harmful metabolites out of the cell [11,12,19]. Due to the broad substrate range of these efflux pumps, the selective pressure conferred by one class of antibiotics cannot by itself account for the maintenance of efflux pump genes in Gram-negatives. In addition, most of the genes encoding these pumps are located on the chromosome and are not frequently transferred between bacterial species. However, there are dramatic differences in the number of individual effux pump genes encoded on the chromosome of Gram-negative organisms, with *E. coli* having 37 putative or proven efflux genes encoded on the chromosome as indicated in a recent review [11]. Other studies have identified up to 52 putative efflux pump genes on the chromosome of *P. aeruginosa* [20]. In order to establish this level of resistance, efflux pumps work

synergistically with the outer cell membrane found in all Gram-negatives that provides a permeability barrier limiting antibiotic access into the cell. Deletion mutants of even one pump such as the AcrRAB-TolC in *E. coli* and *Salmonella enterica* serovar Typhimurium Type DT204 strains markedly lowers the minimal inhibitory concentrations (MICs) for fluoroquinolones and beta-lactams [19,21,22]. For instance, in the *E. coli* 4*-AG100 DNA gyrase double mutant (Gly87Asn and Leu83-Ser), deletion of the functional AcrAB pump lowered the MICs to ofloxacin and ciprofloxacin from 4 and 2 μg/ml, respectively, to 0.125 μg/ml for both compounds [23]. This example illustrates the synergy between mutations occurring in antibiotic target proteins and intrinsic efflux pumps in determining MICs. Other examples provide evidence that efflux alone may confer high levels of resistance; the constitutively expressed MexAB-OprM efflux pump in *P. aeruginosa* confers intrinsic resistance to the potent class of penem antimicrobials [24]. All isolates of this species show poor susceptibility to the penem class but when the *mexAB-oprM* genes are disrupted, the cells become susceptible to penems [24]. Even the new agent tigecycline possesses modest activity (4 μg/ml) against isolates of *Proteus mirabilis* due to expression of the AcrRAB efflux system [25]. Transposon-mediated mutagenesis showed that deletion of the *acrB* gene was accompanied by a 16-fold drop in the tigecycline MIC. This species specificity is particularly interesting since tigecycline is relatively potent against most other species of *Enterobacteriaceae* that have an orthologue of this pump [26].

The degree of intrinsic resistance mediated by efflux pumps is influenced by regulation of the pump structural genes. Point mutations in the *acrR* regulatory gene in the *acrRAB* efflux pump result in over-expression of the pump, elevating MICs to amoxicillin (8–16 μg/ml) and macrolides (4–8 μg/ml) in beta-lactamase-negative, amoxicillin-resistant isolates of *Haemophilus influenzae* [27]. This study demonstrates how facile Gram-negatives are in accumulating mutations leading to resistance in multiple genes (in this case *pbp3* and *acrR*) that can be selected and maintained by pressure from diverse classes of antibiotics such as the beta-lactams and macrolides. Several studies have described regulation of the efflux pumps in *P. aeruginosa* such as those in *mexR* that affect expression of the *mexRAB-oprM* operon [11,28–31]. While most of the changes influencing expression of efflux pumps presumably occur at chromosomal loci, it is likely that a wide variety of antimicrobials can select for these regulatory mutations, perpetuating their existence in the clinical environment. Given the chromosomal locus of efflux pump genes and the chemical diversity of compounds recognized by these pumps, we can conclude that efflux will continue to be a significant problem in limiting antimicrobial activity for a long time to come.

3.2. *Lipopolysaccharide and porin alterations*

Mutations in genes encoding the outer membrane lipopolysaccharide of Gram-negatives may also contribute to intrinsic resistance to antimicrobials. Several studies have identified changes in the O-specific side chains of the polysaccharide molecule that change the structure and charge of the outer polysaccharide with concomitant increases in resistance to certain antibiotics [12,32,33]. Such changes are generally in response to antibiotic selective pressure, characterized by mutation in key genes responsible for synthesis and assembly of outer membrane polysaccharide [34]. These types of changes in polysaccharide are thought to decrease binding of some cationic antibiotics, leading to development of resistance to polymyxin B, an old antibiotic that often remains active against current MDR strains of *P. aeruginosa* and other non-fermentative species [35].

Outer membrane porins (Omps) are water-filled protein channels through which many hydrophilic antibiotics pass in order to reach the cell membrane and interior [12,36]. Size, shape and charge all affect a drug's ability to pass through the typical porins OmpF, OmpC and OmpE of *E. coli* and the porin orthologues found in other species [12,36]. These chromosomally encoded proteins are intrinsic to each species, and loss of one or more key porins is a common contributing factor in establishing resistance to a wide variety of antibiotics, usually in combination with the presence of a drug modifying or inactivating enzyme [37–42]. Loss of porins resulting in increased MICs to hydrophilic antibiotics has been observed in many clinical pathogens including *E. coli*, *K. pneumoniae*, *Serratia marcescens*, *Enterobacter* spp. and *P. aeruginosa* [37–42]. As an example, loss of the outer membrane porin OprD in clinical isolates of *P. aeruginosa*, is sufficient to confer resistance to imipenem and meropenem [43]. Mutation in the *mexR* gene causing over-expression of MexAB-OprM efflux, further increases carbapenem resistance in the background of OprD deficiency [43].

In *E. coli*, the OmpF porin is the major channel through which the diffusion of many different antibiotics occurs and loss of this channel can lead to multidrug resistance [12,44]. While *ompF* regulation normally occurs only in a low osmolarity environment [12], this physiological regulation can be overridden by the selective pressure of antibiotics, resulting in decreased OmpF in the outer membrane [12,44]. The loss of porins can occur by several mechanisms including post-transcriptional regulation by *micF* anti-sense that prevents translation of *ompF* mRNA in *E. coli* [45,46]. The *micF* gene encodes a non-translated 93 nt long antisense RNA that in *E. coli* binds to the upstream regulatory region of *ompF* mRNA, preventing translation. Many growth conditions affect *micF* expression and several transcriptional regulators such as Rob control expression by binding to the *micF* gene [46]. *MicF* orthologues exist in many Gram-negative bacilli, including *K. pneumoniae*, *Citrobacter freundii*, *Enterobacter* spp., *S. enterica* serovar Typhimurium, *S. marcescens* and *P. aeruginosa* [45,46]. This complex system of gene regulation offers numerous ways that mutation in any one gene could affect porin expression and antibiotic susceptibility.

The multiple antibiotic resistance phenotype (Mar), first identified in *E. coli* [47], involves a complex expression of resistance genes including up-regulation of the efflux genes AcrAB as well as a loss of porin expression. Mutation in the *marR* repressor gene allows an increased expression of the *marA* activator [48]. MarA expression imparts pleiotropic effects in terms of antibiotic resistance, since MarA influences the expression (increases or decreases) of at least 60 chromosomal genes in *E. coli* [48]. Rob and SoxA are additional regulatory proteins found in *E. coli* and *E. cloacae* that also

decrease *ompF* mRNA translation via posttranscriptional regulation of the *micF* gene [46]. RobA, which is highly homologous in these two species, apparently functions similarly to MarA in that it stimulates expression of *micF* antisense [49]. Most of the genetic work with these regulatory genes has been done with *E. coli* and it will be important to demonstrate their presence in other *Enterobacteriaceae* as well as to prove their relevance in conferring antibiotic resistance.

In addition to alterations in the regulatory genes controlling porin expression, mutations leading to substitutions of amino acids facing the interior of the OmpF channel can decrease the permeability of certain hydrophilic beta-lactams across the outer membrane [50]. In *E. coli*, substitutions of G119D and G119E resulting in insertion of an acidic side chain protruding into the OmpF pore, decreases the diffusion of cefepime through the pore [50]. There are also examples of complete loss of porin expression in *K. pneumoniae* resulting from insertional inactivation of *omp* structural genes by insertion sequences residing in the genome of *K. pneumoniae* [51]. This genetic mechanism for inactivation of porin genes in *Klebsiella* is a key factor in development of resistance to carbapenems in strains containing certain Ambler class C (bla_{AmpC}) beta-lactamases [41]. The genetic capability of strains to inactivate structural genes by insertion suggests a powerful mechanism by which such strains can achieve multidrug resistance under antibiotic pressure. Acquisition of plasmids encoding AmpC beta-lactamases together with elegant mechanisms for the control of porin and efflux genes in such strains of *K. pneumoniae* are now known to represent highly complicated mechanisms of multidrug resistance.

3.3. *Acquired resistance genes: dissemination of beta-lactamase variants*

There is no better example of genetic diversification leading to antibiotic resistance than the extensive evolution that has occurred in beta-lactamases over the past 20 years. While mechanisms of beta-lactam resistance related to changes in penicillin-binding proteins, porin loss and efflux are documented in Gram-negative bacilli, beta-lactamases play the most prominent role in conferring resistance to these agents. Extensive reviews describing the various classes of beta-lactamases illustrate the tremendous effect of point mutations in *bla* structural genes that significantly alter substrate utilization [52–56]. Point mutations in the structural genes of some major families of beta-lactamases have led to classification of over 300 of these enzymes based on structure in the active site and substrate utilization [55]. This degree of genetic diversity reminds us that the overuse of beta-lactam antibiotics comes at a price in terms of the propensity of beta-lactamase genes to mutate at the active site. This promotes an evolution of beta-lactamases with decreased affinity at the active site for the older beta-lactam molecules. Using the Ambler classification scheme of beta-lactamases (four classes A–D), the greatest number of variants resulting from mutations within the structural genes occur in class A and D, which include the TEM, SHV and OXA enzymes [53,56]. Over 139 TEM, 63 SHV and 15 OXA variants exist possessing an extended-spectrum (ESBLs) capable of hydrolyzing most cephalosporins and penicillins [57]. More recently in Europe, the CTX-M group has become the most frequently isolated ESBLs, demonstrating the constantly shifting gene pool in Gram-negatives [58].

Since many of the genes encoding these variants are on plasmids or integrons on the chromosome, ESBLs have disseminated widely in most species of the *Enterobacteriaceae* [59] and more recently in *P. aeruginosa* [60], although this has proven to be a clinical problem predominantly in *E. coli* and *K. pneumoniae* ([61] and Fig. 1). In these species, the ESBL phenotype is characterized by elevated MICs to first- and second-generation cephalosporins as well as to the third generation, oxyimino cephalosporins and aztreonam [53,55]. A presumptive positive ESBL test involves addition of clavulanic acid to the MIC or disk test. A $\geq$3-fold decrease in cephalosporin MIC in the presence of the beta-lactamase inhibitor signifies the presence of an ESBL. There is also a major geographic difference in the frequency of isolation of ESBL-positive *K. pneumoniae*, with rates of 45% in Latin America, 22% in the Western Pacific compared with around 10% in the U.S. in one study [61]. Within the ESBL group, some enzymes (such as certain CTX-M variants) confer only a modest increase in the MIC to ceftriaxone and cefotaxime [58]. The Clinical and Laboratory Standards Institute (formerly NCCLS) has therefore suggested lowering the breakpoints to all third-generation cephalosporins from 8 to 2 μg/ml in order to more easily identify all ESBL-containing isolates [62]. This may be an example of genetic studies being ahead of medicine, since little data exist documenting clinical failure with cephalosporins against ESBL-positive strains with intermediate susceptibility to cephalosporins. Nevertheless, CLSI guidance indicates that all *E. coli* and *Klebsiella* in which an ESBL is detected should be considered as resistant to all third-generation cephalosporins [62].

While the ESBLs probably represent the best example of genetic diversity within a class of antibiotic resistance determinants, other potent beta-lactamases previously encoded only on the chromosome of Gram-negative bacteria, have migrated to mobile plasmids and integrons, promoting their extensive dissemination across species. For instance, it is now common to find genes for some class C beta-lactamases such as variants of CMY, MOX, FOX, ACT-1, DHA-2 and CFE-1 encoded on self-transmissible plasmids in MDR strains of *K. pneumoniae*, *E. cloacae*, *Proteus mirabilis* and *C. freundii* [54,63–67]. These beta-lactamases are highly effective against third-generation cephalosporins and cefoxitin [63,66]. Since the genes for these beta-lactamases originally resided only on the chromosome they have often been referred to as the class C, species-specific enzymes. The movement of the structural genes for many of these beta-lactamases to mobile plasmids and integrons is a major genetic event that negatively impacts the clinical utility of third-generation cephalosporins and cefoxitin against several genera of *Enterobacteriaceae* [58,59]. These beta-lactamases can also decrease the potency of the fourth-generation cephalosporin cefepime, particularly a higher inocula of organism [55]. In addition to *bla* genes, these mobile genetic elements can also encode determinants for resistance to aminoglycosides, tetracyclines and chloramphenicol. Therefore, it is often important to study the genetic context in which a *bla* gene is found in order to understand the evolution of co-resistance. This genetic organization of mobile plasmids and integrons promotes

the dissemination of resistance to multiple classes of antibiotics. Likewise, the clinical use of many unrelated classes of antibiotics provides common selective pressure that forces the maintenance of these genes in clinical pathogens. This is a case where the genetic complexity of bacteria has evolved ahead of clinical medicine. Merely switching from third-generation cephalosporin use to aminoglycosides for instance, will not remove the selective pressure placed on these genetic determinants resulting from the use of other classes of antibiotics. Thus, the genetics of bacterial resistance have evolved to a point that demands discovery of structurally novel classes of antibiotics that will not be part of the existing MDR profiles. This has proven to be a very difficult task, since no chemically novel classes of antibiotics possessing potent activity against Gram-negative bacilli have been discovered since the fluoroquinolones. Glycylcyclines such as tigecycline are derivatives of an existing antibiotic class (tetracyclines) and their place in antimicrobial therapy of Gram-negatives is still being established.

While the ESBLs and class C enzymes present a problem in terms of conferring resistance to third-generation cephalosporins, representatives of the Ambler class A (NMC-A, SME-1 to -3, IMI-1, KPC-1 to -3, GES-2 and SHV-38), class B (IMP-1 to -13, VIM-1 to -7 and SPM-1) and class D (OXA-23 to -27, -40, -48, -54) beta-lacatamases broaden this resistance profile to include carbapenems [54–56,58,59,68]. There has been endemic spread of imipenem-resistant *Klebsiella* spp. containing the class A, KPC beta-lactamases in New York City hospitals for some time [69–71]. In a recent report [69] the bla_{KPC-2} enzyme was present in the same strain as the bla_{TEM-30} variant that encodes resistance to the beta-lactamase inhibitor clavulanic acid. Since the carbapenems imipenem, meropenem and ertapenem are often the last antibiotics active against MDR Gram-negatives, the spread of beta-lactamases such as the bla_{KPC} variants that can directly hydrolyze carbapenems is of great concern.

Recent publications describing the NYC outbreaks of *K. pneumoniae* containing KPC beta-lactamases are troubling, given their extent of resistance to many other antimicrobials [72,73]. One surveillance study involving 96 strains isolated from ten Brooklyn hospitals [72] indicated that all were resistant to carbapenems (MICs $>$ 32 μg/ml) and most contained at least one ESBL, ensuring cephalosporin resistance. Most of the strains were also resistant to fluoroquinolones. Eighty percent of the isolates were clonal and encoded a KPC beta-lactamase. Antimicrobials with the greatest activity against these isolates included polymyxin B (90% were susceptible) and the new agent tigecycline (MIC_{90} of 1 μg/ml). Interestingly, the combination of polymyxin B at 0.5× MIC plus rifampicin (1 μg/ml) was synergistic against 15/16 isolates tested. However, these in vitro results are tempered by the fact that 89% of these *Klebsiella* were resistant to rifampicin by the MIC test [72].

The Ambler class B metallo-beta-lactamases (MBLs) are carbapenemases previously limited to *P. aeruginosa* and other non-fermenters from Southeast Asia and Europe [68,74]. The first *P. aeruginosa* encoding a metallo-beta-lactamase (bla_{VIM-7}) in the U.S. was found in Texas in 2004 [75]. This enzyme was of particular interest since the gene encoding it was located on a 24-kb plasmid that could be transferred into *Enterobacteriaceae* and other pseudomonads. The MBLs are a unique group in that they are zinc-dependent beta-lactamases whose activity is inhibited by EDTA but not by the traditional inhibitors that target beta-lactamases with serine in their active site [68,74]. MBLs are divided into two major subclasses (IMP and VIM), each with multiple allelic variants. These carbapenemases have only recently been found in the *Enterobacteriaceae*, generating major concern in countries that have been free from these resistance genes. Further spread of MBLs from *P. aeruginosa* to other Gram-negative species may be anticipated since the genes encoding them are often on mobile plasmids or integron-borne gene cassettes [68,76–79]. Molecular studies with the first MBL-containing strain isolated in the U.S. provides a good deal of important information regarding the global spread of these genes. As mentioned, the bla_{VIM-7} gene isolated from this *P. aeruginosa* was located on a plasmid that could be electroporated into *Enterobacteriaceae* [75]. Comparison of the sequence of this gene with other MBL genes indicated that it was highly divergent, showing 77% identity at the amino acid level with bla_{VIM-1}, but having poor identity with other bla_{VIM} or bla_{IMP} genes. An analysis of the genetic context in which the gene was located revealed sequences of the *attI*1 attachment sites and the 59-bp elements characteristic of class 1 integrons, yet other characteristic sequences of these integrons were absent. These genetic data taken together confirm that the *bla* VIM-7 gene from the Texas isolate is not highly related to the known VIM genes isolated from other parts of the world, suggesting that the origin of the first MBL detected in the U.S. is probably not a clone directly disseminated from Asia or Europe [75]. This variant of VIM may have been present in the North American population for some time, or was transferred here from a geographic location where the most common VIM variants are not prevalent.

A major question relevant to the future of antimicrobial therapy is how easily will the genes encoding the VIM and IMP carbapenemases transfer to and be expressed in the *Enterobacteriaceae*. Recent literature clearly shows that the genes encoding MBLs are on mobile cassettes of class 1 integrons that can move into enteric organisms [74,77,80–82]. The levels of MBL expression and magnitude of the resultant imipenem/meropenem MICs appear to be strain dependent. In one study, a strain of *E. coli* acquired the bla_{VIM-1} gene on a plasmid-encoded, class 1 integron where expression of the MBL gene was under a strong P1 promoter [80]. This *E. coli* had MICs to imipenem and meropenem of 8 and 2 μg/ml, respectively. Another study showed that 17 different strains of *K. pneumoniae* from hospitals in Athens contained the bla_{VIM-1} gene and displayed varying imipenem MICs from 4 to 32 μg/ml [77]. A study that examined a large number of enteric isolates from Taiwan, found IMP-8 in *E. cloacae* and VIM-2 in *C. freundii* isolates, all of which remained susceptible to carbapenems [78]. In another recent study [83] 12 enteric isolates were tested as recipients in transformation studies with MBL-encoding plasmids derived from *P. aeruginosa* ranging in size from 24 to 400 kb. Using ceftazidime or imipenem as the selecting agents, only one *C. freudii* recipient was transformable with a *Pseudomonas* plasmid encoding bla_{VIM-7} carbapenemase gene. There were elevated MICs to imipenem from 8 to $>$32 μg/ml in the transformants obtained. These results illustrate that transfer, maintenance and expression of plasmid-encoded

genes from *P. aeruginosa* to members of the *Enterobacteriaceae* is strain dependent. This may be a positive result, indicating that the dissemination of MBLs into the *Enterobacteriaceae* may not rapidly occur in the clinic. Few studies of this type have tested for the presence of other factors such as outer membrane porin loss or efflux that may act synergistically with the MBL to confer high levels of carbapenem resistance. The genetic elements enabling the transfer and expression of class B *bla* genes from *P. aeruginosa* to the *Enterobacteriaceae* need to be studied in greater detail in order to gain further insight into the factors that control expression of metallo-beta-lactamases in this family of bacteria.

Currently there is an overwhelming amount of information documenting the evolving diversity of beta-lactamases that exist in Gram-negative bacilli. Further dissemination of ESBLs and KPC plasmid-encoded bla_{AmpC} enzymes, as well as metallo-beta-lactamases, all threaten the future clinical utility of cephalosporins and carbapenems. The genetic data tell us that it is almost a certainty that these important *bla* genes will become more widespread in the hospital environment over the next decade. Understanding those factors that influence their spread across species should be helpful in managing patients infected with these MDR organisms. We can predict that without the development of new classes of antibiotics, this management will include costly patient isolation procedures and a demand for more advanced diagnostic tests that could enable us to identify problem pathogens directly from patient samples.

3.4. *Development of resistance to fluoroquinolones*

The fluoroquinolones were developed in part due to their excellent potency against Gram-negative bacilli. While fluoroquinolones still remain the ''workhorse'' class of antimicrobials in many institutions, their potency has been substantially limited against several species due to resistance development. As illustrated in Fig. 1, flouroquinolone resistance is on the rise in *P. aeruginosa* from ICU isolates in the U.S. and global surveillance studies indicate that this organism and several species of the *Enterobacteriaceae* are experiencing higher rates of resistance to these agents as well [84–87]. In one recent surveillance study involving 3049 hospital isolates from 17 countries, the percent of strains susceptible to levofloxacin were: *Acinetobacter* spp. 56.8%, *E. coli* (ESBL producers) 50%, *E. coli* (ESBL non-producers) 81.4%, *K. pneumoniae* (ESBL producers) 78.1%, *K. pneumonie* (ESBL non-producers) 93.4% and *P. aeruginosa* 74.8% [84]. In a recent SENTRY study involving 65,746 isolates from 48 North American Medical Centers the observed rates of susceptibility to ciprofloxacin were: *Acinetobacter* spp. 60.6%, *E. coli* (ESBL producers) 68.7%, *E. coli* (ESBL non-producers) 93.1%, *Klebsiella* spp. (ESBL producers) 60.2%, *Klebsiella* spp. (ESBL non-producers) 94.0%, indole-positive *Proteus* spp. 74.1% and *P. aeruginosa* 75.2% [85]. In a European surveillance study [86] comparisons of percent susceptible to ciprofloxacin in isolates obtained in 1997 with those from 2002, revealed a change in the percent susceptible in: *E. coli* (90.2–82.1%), *K. pneumoniae* (94.7–88.6%), *Proteus* spp. (94.9–86.1%) and *Serratia* spp. (89.7–71.8%). These studies show a trend toward decreased activity of fluoroquinolones in some Gram-negative bacilli around the world. A highly consistent finding globally is that fluoroquinolone resistance is markedly higher in strains containing ESBLs. There appears to be no predisposed genetic linkage between ESBL genes and those encoding resistance to fluoroquinolones, yet the loss of activity of the cephalosporins and fluoroquinolones in these strains severely limits therapeutic options that frequently leave carbapenems as the only choice of therapy.

There are many good reviews explaining resistance mechanisms to flouroquinolones in Gram-negative bacilli and a recent one covers many aspects of this problem [88]. The major determinants of resistance to these agents in this group of bacteria involve the selection in a step-wise fashion of multiple mutations in the structural gene for the A-subunit of DNA gyrase (*gyrA*). While both DNA gyrase A and topoisomerase IV subunit C (*parC*) appear to be more or less equal targets in Gram-positive isolates to the newer fluoroquinolones, topoisomerase IV appears to remain a minor target in Gram-negatives [88]. The topoisomerase genes are encoded on the chromosome of bacteria and have not been found on mobile genetic elements. One recently recognized determinant *qnr*, encodes a protective protein of DNA gyrase and this determinant has been found on plasmids in strains of *K. pneumoniae* conferring only a modest increase in fluoroquinolone MICs [89]. Efflux-mediated resistance and changes in porins as well as outer membrane lipopolysaccharide, all can play an additive role in conferring resistance to fluoroquinolones [88].

It is clear that in some species of Gram-negative bacilli, flouroquinolone resistance is a real problem clinically, and it can be expected that this problem will only worsen over the next decade in the face of the continued extensive use of these antimicrobials.

4. ''Superbugs'' that contain numerous resistance genes: the wave of the future

Resistance development among the *Enterobacteriaceae* to beta-lactams and other antibiotics has been widely studied over the past several years. One of the species from this group that is often multidrug resistant is *K. pneumoniae*. While *K. pneumoniae* has long been a serious respiratory tract pathogen in hospitalized patients, it has recently surfaced as one of the most antibiotic resistant organisms in localized outbreaks [70,77]. Isolates of this species have been reported to be resistant to almost all classes of antibiotics through progressive mutations in chromosomally encoded genes and through acquisition of genes encoded on mobile plasmids and integrons. As previously discussed, acquisition of transmissible carbapenemase genes such as bla_{KPC}, bla_{IMP} or bla_{VIM}, can confer carbapenem resistance through a single genetic event. However, resistance to carbapenems and combination agents such as piperacillin-tazobactam can occur in the absence of carbapenemase acquisition. Such resistance usually results from the interplay of ESBLs or bla_{AmpC} enzymes and some other determinant such as loss of outer membrane porins. *K. pneumoniae* contains three known porins in the outer membrane OmpK35, 36 and 37 [38]. While OmpK37 is a small porin related to OmpN of *E. coli* and is not normally expressed, porins

OmpK35 and OmpK36 play an important role in the penetration of antibiotics. Their loss can confer resistance to cephalosporins and carbapenems particularly in strains containing certain Ambler class A, B, C or D beta-lactamases [90]. OmpK35 plays a major role in antibiotic resistance as illustrated in the *K. pneumoniae* CSUB10R deletion mutant (Δ*ompk35* and Δ*ompk36*) under complementation by transformation with a plasmid-encoded *ompK35* gene [90]. The transformant had ≥128-fold lower MICs to cephalosporins and meropenem and ≥8-fold lower MICs to imipenem, ciprofloxacin and chloramphenicol than did the deletion mutant, illustrating the importance of a functional OmpK35 in antibiotic permeation [90]. Since *K. pneumoniae* normally lacks a chromosomally encoded, class C beta-lactamase, acquisition of a plasmid-encoded bla_{AmpC} or another broad-spectrum beta-lactamase can confer high-level carbapenem resistance in porin deficient strains [41,42,44,91–93]. In an example of this ''synergistic'' resistance, a *K. pneumoniae* clinical isolate encoding the bla_{SHV-2} along with reduced expression of OmpK36, had MICs to cefoxitin, ceftazidime-clavulanate and piperacillin-tazobactam of >256 μg/ml [42]. This strain also showed elevated MICs (8–16 μg/ml) for meropenem and imipenem. The important lesson taken from these strains is that enteric organisms need not acquire a KPC or metallo-beta-lactamase to become resistant to carbapenems.

A useful set of *K. pneumoniae* strains has been constructed involving the combination of reduced permeability and beta-lactamase expression for assessing the activity of carbapenems and cephalosporins [41]. An ertapenem-resistant *K. pneumoniae* strain was cured of a resistance plasmid, lowering the ertapenem MIC from 16 to 1 μg/ml. This strain was shown to be defective for OmpK35 and OmpK36 production, accounting for the elevated MIC to ertapenem. This porin-deficient strain was used as recipient for an extensive plasmid collection encoding most of the common Ambler class A–C beta-lactamases. Results indicate that MICs for cefepime and ertapenem were ≥8 μg/ml when the transformant contained a plasmid-encoded ESBL (TEM/SHV, OXA variant) or class C beta-lactamase. The combination of the porin loss and beta-lactamase activity led to elevated MICs to imipenem and meropenem indicating the importance of both decreased permeability and beta-lactamase expression in limiting the activity of carbapenems in this species. This observation further underscores that carbapenem and cephalosporin resistance will occur in *Klebsiella* spp. even in the absence of the potent class B metallo-carbapenemases. These observations are important, since in North America mutations in multiple genes leading to step-wise resistance development to carbapenems appears to be more prevalent than acquisition of metallo-beta-lactamases.

These observations highlight the importance of understanding the mechanisms in *Klebsiella* spp. and other enterics that can confer MDR particularly when carbapenem resistance is observed. One recent study extensively evaluated the genotypes of two highly related MDR *K. pneumoniae* strains isolated from the same patient sample taken in a NYC hospital in 1998 [94]. Both isolates had identical mutations in *gyrA* leading to elevated MICs to quinolones and both contained the class 1 integron 9 with gene cassettes encoding resistance to aminoglycosides and chloramphenicol. Both strains were highly resistant to third-generation cephalosporins, cefoxitin and piperacillin-tazobactam (MICs > 64 μg/ml) but only one of the isolates was highly resistant to imipenem and meropenem (16–32 μg/ml versus 0.5 μg/ml). While both strains contained a plasmid-encoded, class C bla_{ACT-1}, no difference was found between them with respect to DNA sequence of upstream regulatory regions or the bla_{ACT-1} structural gene. As is typical for many *Klebsiella* isolates, both had insertion elements within the *ompk35* and *ompk36* structural genes, resulting in their inactivation. Further characterization revealed that the carbapenem-susceptible strain had a constitutively expressed PhoE porin, while this porin was absent in the membranes isolated from the carbapenem-resistant strain. Lack of expression of the *phoE* gene in the resistant strain was confirmed by comparison of mRNA products using RT-PCR [94]. A backcross experiment conducted with a plasmid-encoded *phoE* construct under control of a heterologous *lac* promoter in the resistant isolate completely reversed the carbapenem resistance phenotype. PhoE is normally repressed in *E. coli* and is only expressed under minimal phosphate growth conditions [95]. Furthermore, PhoE porin expression has not previously been implicated in antibiotic resistance in *K. pneumoniae* or *E. coli*. One explanation for the involvement of PhoE in carbapenem resistance is that in this background, upregulation of PhoE expression was likely selected for in the carbapenem-susceptible variant in order to compensate for the loss of OmpK35 and OmpK36. This compensation may have been over-ridden under the selective pressure of carbapenems or other beta-lactams, leading to selection of the carbapenem-resistant variant in which *phoE* was repressed. These results suggest that in the background of bla_{ACT-1} and *ompk35/36* deletion, regulation of PhoE can affect resistance to carbapenems, contributing to a very complicated MDR genotype. This recent study highlights the genetic plasticity of the genomes of Gram-negative bacilli such as *K. pneumoniae*. While investigators closely monitor for the acquisition of MBLs and class A carbapenemases in this species, it is clear that clinical isolates are capable of becoming carbapenem-resistant through mutation in intrinsic genes present in this species.

5. Summary

This review proposes to ask what the current information regarding the genetics of antibiotic resistance in Gram-negative bacilli is telling us about the future utility of antibiotics. Global surveillance data indicate that *P. aeruginosa* remains one of the most problematic species in terms of resistance, with fluoroquinolones, cephalosporins and carbapenems losing activity. The presence of multiple efflux pumps, outer membrane changes and acquisition of metallo-beta-lactamases, remain significant resistance problems in this species as well as in other non-fermenters. While MBLs in non-fermenters largely remained a regional problem in Europe and Asia, the first MBL-containing *P. aeruginosa* was recently isolated in the United States. Molecular studies describing the genetic context of the bla_{VIM-7} gene in this strain indicates that it is not highly related to other VIM or IMP

genes. This suggests that the bla_{VIM-7} gene in this U.S. isolate was not recently transferred from Europe or Asia, but rather may have existed in the U.S. bacterial population for some time. These genetic data may tell us that there are more point sources for these genes that could enhance the rate of their global dissemination.

Recent data from surveillance studies and molecular analyses of MDR strains of *Enterobacteriaceae* are identifying resistant isolates with the potential of being as refractory to antibiotic therapy as the most resistant strains of *P. aeruginosa*. Studies that have characterized the genetic mechanisms responsible for such resistance have involved only a limited number of species such as *K. pneumoniae*, but the results obtained are nonetheless predictive of future events that will likely occur in other enteric species. Over the past decade the genetic data tell us that *K. pneumoniae* has acquired genes encoded on plasmids or integrons that confer resistance to older beta-lactams, aminoglycosides and chloramphenicol. Genes for potent carbapenemases such as the *bla* KPC group are now often identified in MDR outbreak strains of *K. pneumoniae* in NYC. Genetic studies reveal that most of these outbreak strains are clonal, probably disseminating through different hospitals on patients and staff. In many cases, these strains are only susceptible to polymyxin B and the new agent tigecycline.

A limited amount of genetic data tells us that some enteric isolates can acquire and express genes encoding MBLs from *P. aeruginosa* further threatening the future utility of carbapenems. In addition, studies have also demonstrated the tremendous genetic plasticity of the genomes of some enteric species including *K. pneumoniae*. Here resistance occurs through collecting multiple point mutations or insertions in target genes, as well as regulating multiple genetic determinants to achieve resistance to important classes of antibiotics including the fluoroquinolones, third-generation cephalosporins and carbapenems. Characterizing all of these genetic changes in clinical isolates is very labor intensive, but the data significantly adds to our knowledge of how these organisms achieve the necessary changes in so many different resistance determinants. Many of the genetic studies described in this review focus only on a small number of species. Is it likely that the complex genetic events described in *P. aeruginosa* and MDR *K. pneumoniae* will occur in more commonly isolated enteric species? Unfortunately, there is little genetic evidence to prove that it will not.

These and other examples given in this review highlight the complexity of resistance development and illustrate that these processes are still evolving in Gram-negative bacilli. We must continue to investigate these mechanisms if we hope to design new antibiotic classes that will be effective against such multiresistant pathogens. More importantly, the genetic data are telling us that we must look to developing novel structural classes of antibiotics that are not recognized by the plethora of determinants disseminating within the clinical environment. This accomplishment has proven to be very difficult, as few novel agents have emerged with significant spectrum to cover Gram-negative bacilli. The medical and pharmaceutical communities must recognize the magnitude of this problem and underscore the urgency to discover such new drugs before the problem worsens.

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