Chapter 6
Mutations as a Basis of Antimicrobial Resistance

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1 Prefatory Remarks

The past two decades have witnessed a disturbing increase in antimicrobial resistance. Bacterial isolates that are resistant to all currently available antimicrobial agents are emerging. Bacteria with this phenotype are designated multidrug-resistant (MDR) or pan-drug resistant (PDR) strains. What is the genetic basis of this remarkable survival skill? Are advantageous changes in the genome always random? Is antibiotic pressure the cause of growing resistance rates, or does it merely serve as a trigger that selects the archived defense armamentarium within bacteria? In this chapter, we will explore these concepts and discuss: (1) genetic diversity and mutations as its basis; and (2) hypermutators and the mechanisms responsible for high mutation rates. Our chapter will conclude with examples of specific point mutations in bacterial enzymes that confer resistance to certain antibiotic classes.

2 Genetic Diversity and Mutator Strains

Nearly six decades ago, Luria and Delbrück developed the field of modern bacterial genetics. Until then, it was believed that mutations (as defined by antibiotic resistance) emerged by an unknown process in which the antibiotic "trained" the bacteria. In a classical set of experiments called "fluctuation analysis," Luria and Delbrück demonstrated the role of chance and selection in the recovery of a novel phenotype (1). An example of fluctuation analysis as it relates to antimicrobial resistance follows. One inoculates streptomycin susceptible cells of *Escherichia coli* in a flask containing 100 mL of broth, and also in 100 tubes each containing 1 mL of broth. After reaching full growth, 1 mL samples of both groups are plated on a streptomycin-containing medium and incubated overnight. If resistant mutations arise spontaneously, before exposure to antibiotics, parallel cultures in a liquid medium should have their first mutation at different times, resulting in a wide variation in the colony count of resistant bacteria. If, however, resistance does not arise until "directed" by the antibiotic, the samples from different tubes should all be equivalent, just like the aliquots from a single flask. The numbers of streptomycin-resistant colonies on the 100 plates from the flask are all similar. On the other hand, the number of colonies "fluctuated" significantly on the plates originating from the 100 different tubes. This experiment showed that the resistant mutants appeared before antibiotic exposure and were only selected, not directed, by the agent (2). Statistically, these random outcomes follow a Poisson distribution. This experiment is based upon earlier studies examining the susceptibility of *E. coli* bacteria to bacteriophage lysis.

We recognize now that genetic diversity is based on mutations. DNA polymerases, the enzymes that replicate bacterial genomes, are of limited fidelity. If a polymerase introduces the incorrect nucleotide, repair enzymes generally correct the "mistake" (3, 4). If the incorrect nucleotide is introduced without "correction," a point mutation occurs. Nucleotide sequences in a codon are permanently changed as a result of substitutions, deletions, or additions. Point mutations can be "silent" if the new codon encodes the same amino acid. They can be "nonsense mutations" if the new codon is one of the chain-terminating ones, or they can be "missense mutations" that encode a different amino acid in the peptide chain. Missense mutations are point mutations that can sometimes confer resistance to an antibiotic, because point mutations can affect the key amino acid residues that are important in protein function. Deletions or insertions usually cause "frame shifting" mutations that are deleterious to the tertiary structure of a protein, and may also result in premature chain termination. In general, mutations that are harmful or deleterious to a particular bacterial phenotype do not get passed to the next generation (5).

In times of normal growth, a perfectly adapted clonal population has a mutation rate close to zero. The absence of mutations, however, may prevent adaptation to the environment, should something suddenly change. A high mutation

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rate is desirable in times of stress or drastic changes in the environment (e.g., antibiotic selection pressure), where advantageous mutations (drug resistance) are selected and rapidly propagated to ensure survival. Conversely, a very high mutation rate can introduce lethal changes. This "fine-tuning" of the global mutation rate is postulated to be a function of hypermutators. It has been estimated that hypermutators represent approximately 0.0001–0.001% of some bacterial populations. Under selective pressure, this percentage can increase up to 0.5%. Many natural isolates of *E. coli* and *Salmonella* spp. were found to have even higher numbers of mutators, 1–5% (6). A high proportion of bacteria with increased mutation frequencies has recently been described in *Pseudomonas aeruginosa* isolates from sputum of cystic fibrosis patients (7). Two distinct types of hypermutators have been described: constitutive or permanent hypermutators and transient hypermutators (8). In the next section, we discuss the differences between constitutive and transient hypermutators and their evolutionary significance.

3 Mismatch Repair-Deficient Permanent Hypermutators

During evolution, bacteria have developed safety mechanisms that recognize mismatched bases and remove them. In that way, the genetic information is kept intact and passed on to daughter cells unchanged. One of the best-described DNA repair mechanisms in bacteria is the methyl-dependent mismatch repair system (MMR) in *E. coli*. The MMR system consists of three proteins; MutS, MutL, and MutH. Once MutS recognizes a distorted double helix caused by a mismatched base (e.g., an insertion or a deletion), it undergoes an ATP-dependent conformational change and binds to MutL. This MutS-MutL complex activates MutH, which functions as an endonuclease and nicks the unmethylated nascent DNA strand upstream from the mismatch. Helicase II then unwinds the DNA toward the mismatch, and a specific exonuclease excises the nascent strand. This is followed by re-synthesis and ligation. Bacteria that have an inactive MMR system have an increased mutation rate, because they do not repair mismatches efficiently. These MMR-deficient strains are permanent hypermutators, and they exhibit up to a 10,000-fold increase in mutation rates compared to wild-type bacteria (6). According to recent evidence, permanent hypermutators are responsible for pre-exposure mutations that are present in the population prior to selective antibiotic pressure. In terms of survival value, MMR inefficiency may come at too high a price for the random protection it offers against noxious agents (7, 9).

Hypermutators are utilized in evaluating the frequency at which resistant genotypes arise in vitro while assessing a novel antimicrobial agent. The recovered mutants can provide insight into likely mechanisms of resistance. Hypermutators can potentially be utilized for selecting rare, interesting mutations with modified metabolic capabilities of biotechnological relevance (10). For example, taking a culture of fully grown *E. coli* with a density of 10^{10} CFU/mL and resuspending this culture in 1/10 the volume, followed by incorporation of 1-mL aliquots on ten agar plates, will detect mutants that arise at a frequency of about 10^{-12}. If hypermutators of *E. coli* exhibiting a 1,000-fold higher mutation rate are used, mutants that arise at frequencies as low as 10^{-15} can potentially be identified. This approach has been used to detect rare *ampC* promoter mutations in *E. coli* that confer increased ampicillin resistance (10).

4 Transient Hypermutators and the *SOS* System

Transient hypermutators have an inducible, genetically programmed *SOS* system that allows them to mutate at a higher frequency only under times of stress. The SOS system is composed of a number of polymerases – "mutases" – that introduce errors at high rates. One of the best known DNA mutase groups is the SOS system in *E. coli* (3). In response to DNA damage, for example damage brought about by exposure to fluoroquinolones, a protein designated RecA activates and wraps around the single-stranded DNA (ssDNA), forming a nucleoprotein filament (11, 12). This nucleoprotein filament is a poor substrate for the chromosomal replicase. However, this nucleoprotein filament triggers the specific proteolytic cleavage of a suppressor protein called LexA. Under conditions of normal bacterial growth, LexA suppresses a group of nearly 40 genes involved in the "SOS response." In the absence of LexA, the SOS system is activated.

SOS consists of three major polymerases – Pol II, Pol IV, and Pol V – that actively generate mutations in the genome (13, 14). All three polymerases collaborate in generating nucleotide substitutions, the so-called "translesions," by dNTP mis-insertions followed by mis-pair extension (15) (see Fig. 1).

As a result of exposure to DNA-damaging antibiotics, SOS^+ bacteria actively increase the number of mutations. Therefore, transient hypermutators are responsible for postexposure mutations, arising under selective antibiotic pressure

DNA damage→RecA activation→LexA proteolysis→Derepression of SOS genes→Pol II+PolIV+PolV→Translesions

Fig. 1 Derepression of SOS

and offering a better evolutionary tool for diversity, incurring an overall lower cost (9). The SOS system renders itself a suitable target for new antimicrobial agent development, as the inhibition of mutation could serve as a novel strategy in combating the evolution of antibiotic resistance (9).

5 Antimicrobial Resistance Determinants

Mutations that confer antimicrobial resistance can occur in different parts of the genome, and are spread among the population by diverse mechanisms. Based on the origin of the mutated gene, antimicrobial resistance determinants can be classified into three distinct groups (5):

1. Acquisition of foreign DNA
2. Mutations of preexisting genetic determinants
3. Mutations in acquired genes.

Acquisition of foreign DNA in bacteria can occur by transduction, transformation, and conjugation. Briefly, transformation refers to the uptake of naked DNA; conjugation is plasmid-mediated mating between cells in contact; and transduction involves infection of the bacteria by a nonlethal bacteriophage carrying bacterial genes (2). These topics are covered elsewhere in this book. In this section of the chapter, we will focus primarily on point mutations in both preexisting and acquired genes.

Mutations of preexisting genetic determinants can affect either *structural* or *regulatory* genes. Select examples of antimicrobial resistance acquired through a one-step mutation in a *structural* gene are effective resistance mechanisms for β-lactams, fluoroquinolones, streptomycin, and rifampin. Mutations involving *regulatory* genes in a number of different species are known to confer resistance to various classes of antimicrobials, including fluoroquinolones, tetracyclines, and β-lactams.

6 β-Lactam Resistance Mediated by Low-Affinity Penicillin Binding Proteins

In most Gram-negative bacteria, resistance to β-lactam antibiotics generally involves inactivation of β-lactam antibiotics by β-lactamases. The majority of clinically important Gram-positive bacteria, along with a handful of Gram-negative organisms, demonstrate low-affinity penicillin binding proteins (PBPs) that confer resistance to β-lactam agents. PBPs are cell wall synthesizing enzymes. Based on size, PBPs are divided into high molecular weight and low molecular weight enzymes. The high molecular weight group is comprised of transpeptidases and transglycosidases, which are essential for cell wall synthesis. Low molecular weight enzymes are carboxypeptidases, which re-arrange and degrade the three-dimensional murein structure. Low molecular weight PBPs serve some regulatory functions, but are not essential (see Table 1). All cell wall containing organisms described to date have from four to eight PBPs. To illustrate, *Staphylococcus aureus* has five PBPs, whereas *E coli* has eight different PBPs.

All PBPs have a highly conserved serine residue in their active site that forms an ester with the carbonyl group of an "opened" β-lactam ring (16, 17). This serine ester is a structural analogue of the PBP's actual substrate, the C terminal D-Ala-D-Ala that is excised from the disaccharide-pentapeptide building block of the cell wall. Unlike the natural substrate, the β-lactam formed ester is hydrolyzed very slowly, rendering the PBP nonfunctional.

The bactericidal activity of β-lactams is based on their effective inhibition of high molecular weight essential PBPs. Some bacteria manage to escape this action by the presence of PBPs that do not readily bind to the β-lactam and are thus not inactivated by the drug. The origins of these "low affinity PBPs" are very diverse. Point mutations have been described only in the high molecular weight essential PBPs (18, 19). In transformable species like *Streptococcus pneumoniae*, "mosaic genes," acquired through homologous recombination and natural transformation from neighboring intrinsically resistant organisms, have given rise to highly resistant strains. PBP2b, 2x, and 1a are encoded by mosaic genes that can be transferred between *Streptococcus sanguis, S. oralis, S. mitis,* and *S. pneumoniae* (20). A succession of seven amino acid substitutions in PBP2b is responsible for penicillin resistance (21). An interesting point mutation also causes significant modification of PBP2b affinity codes for the substitution of Thr446 by an Ala. This mutation alone confers significant resistance to penicillin when found in wild-type *S. pneumoniae* strains. PBP2b production is associated with much slower cell wall hydrolysis at high β-lactam concentrations. While all other PBPs are inhibited, PBP2b continues active synthesis of the cell wall and thereby counters the action of cell wall autolytic enzymes, which are activated by a process unleashed by interference with cell wall synthesis. This effect is great enough to slow the hydrolysis down and prevent cell lysis. Resistant PBP2x variants differ from the wild-type by only 8–10 amino acids. Apart from the major

Table 1 Major PBP characteristics[a]

PBP	Size	Function
Essential	HMW	Transpeptidases
1, 2, 3, 4		Transglucosidases
Nonessential	LMW	Endopeptidases
5, 6, 7, 8		Carboxypeptidases

[a]*PBP* penicillin binding proteins; *HMW* high molecular weight; *LMW* low molecular weight

mutation involving a Thr to Ala substitution immediately following the active-site Ser337, the Thr550 to Ala change is noteworthy for conferring resistance to extended spectrum cephalosporins, and also for producing increased susceptibility to oxacillin.

Methicillin-resistant *S. aureus* (MRSA) possesses the *mecA* gene, which has probably evolved from a closely related gene by point mutations and codes for PBP2a, a novel additional PBP. This low-affinity PBP functions as a transpeptidase and mediates the cell wall synthesis in lieu of other PBPs, which are all inhibited by β-lactam concentrations that do not inhibit PBP2a (22).

Enterococci are intrinsically resistant to all cephalosporins. This resistance is based on the structure of enterococcal PBP5, which does not bind cephalosporins. The mechanism for resistance toward penicillins among enterococci is somewhat more complex. In certain enterococcal species, a point mutation in the regulatory gene (*psr*) causes hyperproduction of PBP5 that translates into high-level penicillin resistance (23). This does not appear to be the case in *Enterococcus faecium*, where highly ampicillin-resistant clinical isolates do not have increased level of PBP5 expression, but achieve higher MIC values to ampicillin by point mutation in the *pbp5* gene, thereby lowering the affinity of PBP5 for ampicillin binding (24).

7 Quinolone-Resistance Determining Region in Fluoroquinolone-Resistant Bacteria

Fluoroquinolones are inhibitors of DNA replication. Quinolones target prokaryotic topoisomerase enzymes whose major function is unwinding of DNA (25). In binding to the enzyme-DNA complex, they stabilize it. This inhibits the movement of proteins such as DNA and RNA polymerases along the DNA chains, thus arresting the replication fork. In Gram-negative bacteria, resistance to fluoroquinolones arises from alterations in the DNA gyrase (topoisomerase II), an enzyme responsible for the relaxation of supercoiled DNA. The DNA gyrase enzyme has two subunits: A and B. Amino acid substitutions resulting in quinolone resistance usually occur in Gyrase A. In *E. coli*, these mutations are clustered between amino acid positions 67 and 106 at the amino terminus of the polypeptide chain. This domain is called the quinolone-resistance determining region (QRDR). The most common mutations encountered in resistant strains involve Ser83 and Asp87. It appears that the above amino acid changes caused by point mutations in the QRDR region of Gyrase A alter the structure of the quinolone binding area at the interface of the enzyme-DNA complex, thereby reducing its affinity for the drug. Many other Gram-negative bacteria, *Mycobacteria,* and atypical pathogens with amino acid substitutions in positions equivalent to Ser83 and Asp87 display fluoroquinolone resistance. Amino acid substitutions in Gyrase B usually result in low-level resistance. In Gram-positive bacteria like *S. aureus*, resistance to fluoroquinolones usually involves point mutations in Topoisomerase IV, which separates intertwined DNA rings. Topoisomerase IV also has two subunits (ParC and ParE). High-grade resistance to fluoroquinolones is linked to amino acid substitutions in ParC (26).

8 Streptomycin Resistance and *Mycobacteria*

In *Mycobacteria*, point mutations in genes that encode ribosomal proteins confer resistance to streptomycin. Most resistant strains have one isolated nucleotide change from adenine to guanine in codon 43 of the *rpsL* gene. This changes the tertiary structure of the ribosomal protein S12, resulting in the inability of streptomycin to bind to the ribosome and inhibit protein synthesis (27). It is intriguing that *Mycobacteria* rely on generating resistance solely by de novo mutations and vertical transmission. Mycobacteria seem not to exchange genetic determinants horizontally (i.e., by conjugation or transformation).

9 Rifampin Resistance

In *E. coli*, rifampin resistance arises from point mutations in highly conserved regions of the *rpoB* gene, which encodes the β subunit of RNA polymerase. One amino acid change in the β subunit causes a large change in the binding-affinity of rifampin to the DNA-dependent RNA polymerase, thereby hindering rifampin's inhibition of mRNA transcription. Curiously, resistance to rifampin occurs at high frequency in many genera of bacteria. *M. tuberculosis, Neisseria meningitidis,* and *Mycobacterium leprae* develop rifampin resistance by accumulating point mutations in the same highly conserved regions of the *rpoB* gene (28). Thus, rifampin is never used as monotherapy, primarily because of the high frequency at which resistant mutants arise. Combining rifampin with a second agent significantly reduces the chances of rifampin resistance arising on therapy. This paradigm forms the basis of our therapy against *M. tuberculosis*. Point mutations that confer resistance to two antibiotics are separate events, and the chance of both mutations occurring

in one organism is the product of the frequencies of each of them occurring alone (29).

10 Fluoroquinolone Resistance Caused by Overexpression of Active Efflux Pumps

Multi-drug resistant (MDR) strains of *P. aeruginosa* display cross-resistance to a number of structurally unrelated antimicrobial agents. A major role for this type of resistance has recently been attributed to an active efflux pump system encoded by the *mexA-mexB-OprM* operon. The MexA-MexB-OprM efflux pump system has wide substrate specificity, including β-lactams, β-lactamase inhibitors, tetracyclines, quinolones, macrolides, chloramphenicol, trimethoprim, and novobiocin (30). Expression of the efflux operon is under control of the *mexR* regulator gene. A point mutation in *mexR* (substitution of Trp to Arg at position 69) alters the function of the MexR protein, causing overexpression of the MexA-MexB-OprM efflux system. This, in turn, leads to higher levels of resistance to a variety of antibiotics, as seen in the *nalB* multi-drug resistant mutant, OCR1 (31).

11 Constitutive Tetracycline Resistance due to a Mutated Repressor Gene

Tetracycline antibiotics are bacteriostatic agents that inhibit protein synthesis by blocking the attachment of amino-acyl-tRNA to the acceptor site on the 30S ribosomal subunit, as reviewed in (32). Resistance to tetracyclines is mediated by either ribosomal protection proteins or efflux pumps. The tetracycline efflux pumps belong to the Major Facilitator Superfamily (MFS). MFS efflux pumps are approximately 46-kDa membrane = bound proteins that expel tetracyclines against a concentration gradient. In Gram-negative organisms, the efflux system determinants are comprised of two genes: a gene coding for the efflux pump and another coding for a repressor molecule. Both are regulated by the presence of tetracyclines. In the absence of tetracycline, the repressor binds to the operator and blocks the transcription of the efflux pump (33). In certain strains of *H. influenzae* constitutive expression of the efflux protein has been attributed to a single omission of thymidine, causing a frame shift mutation The resultant truncated repressor molecule is half the usual size and nonfunctional. The constitutive expression can be reversed by addition of functional repressor molecules (34).

12 Constitutive and Inducible Glycopeptide Resistance Caused by Point Mutations in the Regulatory System

Glycopeptide antibiotics, both vancomycin and teicoplanin, act as inhibitors of cell wall synthesis by binding to the D-Ala-D-Ala terminus of the pentapeptide precursor of the peptidoglycan molecule (35). Two types of gene clusters, designated *vanA* and *vanB*, account for the majority of acquired resistance to glycopeptides (36). The gene clusters include three genes, v*anH, vanA*, and *vanX*, which encode enzymes involved in incorporating D-Ala-D-Lac instead of D-Ala-D-Ala into the peptidoglycan precursors, thereby reducing the binding affinity of glycopeptides by approximately a 1,000-fold. Though the number of genes in the *Van* cluster is variable, there are five "core genes" present, as illustrated in Fig. 2 and Table 2. The expression of the *vanA* and *vanB* gene clusters are regulated on the transcriptional level by a set of two other genes, *vanS* and *vanR*, whose products comprise the VanRS and VanRbSb regulatory system. VanS and VanSb are transmembrane kinases that autophosphorylate a histidine residue in the presence of glycopeptides, and thereupon transfer the phosphoryl group to an aspartate residue on the VanR regulator protein. The phosphorylated regulator protein activates transcription of both the resistance and the regulatory genes. VanS also functions as a phosphatase, switching off the VanR regulator protein in the absence of glycopeptides. Alterations in the functions of VanS and VanSb give rise to a variety of phenotypical expressions of vancomycin (or glycopeptide) resistance. The phenotypes fall into several major categories: (1) constitutive expression; (2) inducible expression by vancomycin and teicoplanin; (3) inducible expression by vancomycin alone; and (4) repressed under all conditions. Mutations in the transmembrane segments of VanSb affect

Fig. 2 Vancomycin resistance *VanA* operon

Table 2 *VanA* operon gene function table

Gene	Product
VanR	Response regulator
VanS	Histidine kinase
VanH	Dehydrogenase
VanA	Ligase
VanX	D-D dipeptidase
VanY	D-D carboxypeptidase
VanZ	Unknown

signal transduction and lead to inducible expression of resistance genes. Mutations in VanSb, causing substitutions at two specific positions located on either side of the His233, give rise to constitutive expression of VanB by conveying resistance to the dephosphorylation of VanR.

13 Unique Regulation of Inducible Macrolide Resistance by Translational Attenuation

Macrolide antibiotics inhibit protein synthesis by binding to the peptidyl-tRNA binding region of the larger ribosomal subunit, thereby preventing translocation of the peptidyl-tRNA molecule from the donor to the acceptor site on the ribosome. In Gram-positive organisms, there are two major mechanisms of resistance to macrolides: (1) methylation of the ribosome and (2) macrolide efflux pumps (*mef*). The ribosomal methylation is accomplished by erythromycin ribosomal methylases (*erm*), which are products of a variety of *erm* genes (37). Posttranscriptional methylation of a single adenine residue in 23S rRNA confers resistance to macrolides, the related lincosamides (clindamycin and lincomycin), and streptogramin B (MLSb resistance) (38). This type of resistance is inducible by erythromycin, but not by clindamycin, and it is regulated by a proposed unique mechanism of translational attenuation. This unusual regulatory mechanism does not involve repressor genes, but relies on conformational isomerization of the *ermC* message to a translationally active form. Mutations in the messenger RNA cause different conformational changes, which result in constitutive expression of MLS resistance (39). In a clinical microbiology laboratory setting, inducible resistance to clindamycin brought about by erythromycin is detected by the so-called "D-test" (40).The D-test is used to alert clinicians to avoid the use of clindamycin in treating staphylococcal and streptococcal infections.

14 β-Lactam Resistance Caused by AmpC β-Lactamase Hyperproduction

β-Lactam antibiotics are therapeutically important bactericidal agents. However, both Gram-negative as well as Gram-positive organisms have developed enzymes able to degrade the β-lactam ring, thereby rendering the β-lactam inactive. Gram-positive organisms produce extracellular β-lactam hydrolyzing enzymes only when needed (i.e., by induction upon exposure to the agent). The majority of Gram-negative beta-lactamases are expressed constitutively and are contained in the periplasmic space, where they inactivate incoming β-lactams. In addition to the constitutive β-lactamases in Gram-negative bacteria, AmpC is an inducible chromosomally encoded β-lactamase. The *ampC* gene is found in all Gram-negative bacteria. Its product, the AmpC β-lactamase, is primarily a cephalosporinase, but, when produced in large amounts in the presence of an efficient "inducer" like cefoxitin or imipenem, it can confer resistance to all cephalosporins, penicillins, β-lactam-β-lactamase inhibitor combinations, and the monobactam, aztreonam.

Table 3 AmpC regulatory system-specific protein functions

Protein	Function
AmpR	Repressor
AmpG	Permease
AmpE	Transmembrane protein
AmpD	Amidase
AmpC	Cephalosporinase

The regulatory system responsible for the induction mechanism is rather complex, and under strict control of several other genes: *ampR*, *ampD*, *ampE*, and *ampG*. The most widely accepted explanation of how AmpC production is regulated postulates that the gene product of AmpR has a dual function. It serves as a repressor of *ampC* transcription at baseline, but turns into an activator upon exposure to β-lactams. Current experimental evidence suggests that peptidoglycan breakdown products (i.e., muramyl peptides, and not the β-lactam molecule itself) serve as the activation trigger (41). The product of *ampG* is a transmembrane protein through which peptidoglycan breakdown products enter the cytoplasm. The *ampD* product linked to the transmembrane AmpE protein is a soluble cytosolic *N*-acetylmuramyl-L-alanine amidase that helps to recycle the breakdown products. In the presence of agents whose actions lead to cell wall destruction, like certain β-lactams, the recycling capacity of the amidase is exceeded and AmpR activates the production of AmpC. As a consequence of point mutations in AmpD that render it inactive, the regulatory system breaks down and AmpC production is permanently switched on, conferring resistance to all penicillins and cephalosporins. Strains that hyperproduce AmpC as a consequence of AmpD mutations are designated "derepressed mutants" (42, 43). The functions of individual proteins of the AmpC regulatory system are summarized in Table 3.

15 Point Mutations in Acquired Resistance Genes: The New-Generation β-Lactamases

Ampicillin was the first synthetic aminopenicillin active against *E. coli* and other Gram-negative bacteria. Before long, enzymes capable of hydrolyzing ampicillin and first-generation cephalosporins were discovered. The genes encoding

these β-lactamases were transferred onto plasmids, and propagated with astonishing rapidity among *E. coli* and other *Enterobacteriaceae*. These first "broad-spectrum" β-lactamases were the TEM- and SHV-type. Their mechanism of action is based on catalytically disrupting the amide bond in the β-lactam ring by forming an acyl–enzyme complex. With the help of a strategically positioned water molecule in the active site, the covalent ester link is disrupted, the free enzyme released, and the β-lactam transformed into inactive penicilloyl and cephalosporyl moieties.

The need for antibiotics resistant to hydrolysis by plasmid-borne β-lactamases of Gram-negative bacilli, namely TEM-1 and SHV-1, was the stimulus for the development of "extended-spectrum" cephalosporins. These newer-generation extended-spectrum cephalosporins managed to avoid hydrolysis by alterations in the β-lactam molecule that interfered with effective interaction with the β-lactamase, while still retaining their binding affinity to target PBPs. As the modifications in the β-lactam molecule were relatively minor, it was reasonable to predict that β-lactamases able to hydrolyze these new β-lactams would soon evolve. No one, however, expected they would do so as easily and rapidly as they did – threatening the utility of the entire class of extended-spectrum cephalosporins. These novel β-lactamases were called "extended-spectrum β-lactamases" or ESBLs.

Another strategy of battling the growing problem of β-lactamase-mediated resistance to penicillins was the development of effective inhibitors of the enzymes to protect the penicillins from inactivation. Clavulanic acid, sulbactam, and tazobactam are β-lactam compounds that occupy the active site of the β-lactamase and act as "suicide" substrates (or β-lactamase inhibitors), resulting in inactivation of the enzyme. When combined with a penicillin, these β-lactamase inhibitors protect the penicillin from inactivation by the β-lactamase. The success of β-lactamase inhibitors was compromised by subsequent mutational resistance. As early as 1992, an ampicillin-resistant clinical isolate of *E coli* resistant to the ampicillin-sulbactam inhibitor combination was discovered, possessing a β-lactamase with reduced affinity for sulbactam and clavulanic acid (44).

The bases for resistance to extended-spectrum cephalosporins by ESBLs and resistance to β-lactam β-lactamase inhibitor combinations are point mutations in the β-lactamase gene, which cause amino acid substitutions that alter the structure or dynamics of the enzyme. The majority of β-lactamases have more than one amino acid substitution compared to the wild-type enzyme. Interestingly, only a few point mutations at selected loci in the β-lactamase gene give rise to the above-mentioned phenotypes. The corresponding major amino acid positions at which substitutions conferring new resistance occur most frequently are summarized in Table 4.

Table 4 Sites for phenotype-altering amino acid substitutions in TEM and SHV β-lactamases[a]

	Position of amino acid (Ambler numbering) substitutions[45]	
Phenotype	TEM	SHV
ESBL	Gly 104	Gly 238
	Arg 164	Glu 240
	Gly 238	
	Glu 240	
IRT	Met 69	Met69
	Ser 130	Ser 130
	Arg244	
	Arg275	
	Asp276	
CMT	(Gly 238 or Glu 240) + (Met 69 or Ser 130 or Arg 275)	SHV-10

[a]*ESBL* extended-spectrum β-lactamases; *IRT* inhibitor-resistant TEMs; *CMT* complex mutants of TEM

16 The G238S ESBL Mutation

This is one of the most frequently encountered, and therefore most studied, mutations that codes for the G238S amino acid substitution. In nature, the substitutions of -Ser, -Ala, or -Asp for Gly at the Ambler position ABL 238 are mutations in SHV β-lactamase that confer resistance to extended-spectrum cephalosporins. There are currently 33 TEM and 25 SHV β-lactamase variants with the substitution Gly238Ser (www.lahey.org). Numerous hypotheses have been advanced to explain why the Gly238Ser substitution results in significant resistance to broad-spectrum cephalosporins. In 2003, the crystallographic structure of SHV-2 was elucidated and compared to the structure of SHV-1 from which it differs in only the one G238S substitution (46).

17 Inhibitor-Resistant TEMs

There are currently 23 inhibitor-resistant TEM and 2 inhibitor-resistant SHV mutants. In general, the inhibitor-resistant mutants are devoid of ESBL activity and are less active against narrow-spectrum cephalosporins than classical TEM (47). The number of Inhibitor-Resistant TEMs (IRTs) in TEM far exceeds the number in the SHV series, although the mutation sites are the same. The reason for this is a subject of ongoing studies.

SHV-10 was the first inhibitor-resistant SHV enzyme discovered in 1997, in a clinical isolate of *E. coli*. It is a derivative of SHV-5, an ESBL enzyme. As a result of a single point mutation in which adenine transitioned to guanine, a glycine is substituted for a serine at Ambler position 130. The enzyme partially retains its ability to hydrolyze penicillins, but loses significant activity against cephalosporins. Only recently, a

second inhibitor-resistant SHV was discovered, SHV-49. This novel β-lactamase was found to be a derivative of chromosomal SHV-1, and differs from the original gene only by the substitution of guanine by adenine at nucleotide position 195, leading to the amino acid substitution M69I. Experiments using site-directed mutagenesis have shown that this change in the SHV-1 conveys inhibitor resistance (48).

Inhibitor-resistant variants of TEM are more numerous (49, 50). The largest group involves changes at position 69, where Met is substituted with one of the hydrophobic, aliphatic amino acids Leu, Ile, or Val. Although distant from the cross-linking S130, the majority of IRT mutations cause a change in the local environment of S130. For example, in TEM-32 the M69I substitution distorts S70, causing S130 to adopt a new conformation, moving its O γ 2.3 Å away from where the inhibitor would bind. Similarly, in TEM-34 the M69V substitution leads to a conformational change in Ser-130, causing it to hydrogen bond with K73 and K234 and reducing its nucleophilicity for cross-linking (51).

18 Complex Mutants of TEM

Both ESBLs and IRTs arose from the common plasmid-mediated TEM and SHV-1 penicillinases by single point mutations. These substitutions either conferred resistance to inhibitors or resulted in the ability to hydrolyze oxyimino-β-lactams, but not both. Since the 1990s, a new subgroup of enzymes has emerged in different species of the *Enterobacteriaceae* family that combine mutations responsible for inhibitor resistance (i.e., Leu-69 and Asp-276) with those responsible for the extended-spectrum phenotype, (Lys-104 and Ser-238). These mutants were termed Complex Mutants of TEM (CMT) (52, 53).To date, there have been five CMTs described.

19 CTX-M

CTX-Ms are a growing group of plasmid-borne enzymes that belong to the same class as SHVs and TEMs (class A). They share only 40% sequence identity with TEM and SHV, and are thought to be derived from the chromosomal *ampC* gene of the *Kluvyera* spp. (54). In general, CTX-Ms confer resistance to most oxyimino-cephalosporins and cefepime, but do not efficiently hydrolyze ceftazidime. Therefore, when screening for ESBL production, in addition to checking for susceptibility to ceftazidime, cefotaxime should also be tested to reduce the risk of overlooking a CTX-M enzyme. Recently, new members of this group have evolved with a point mutation resulting in an Asp240Gly or a Pro167Ser substitution. These new mutants phenotypically display increased resistance to ceftazidime and higher susceptibility to cefepime, and must have evolved under ceftazidime selection pressure. Neither one of the substitutions has ever been found in naturally occurring TEM or SHV ESBLs, which may suggest that CTX-Ms have a distinct evolutionary potential (55). It is rather unusual that CTX-M β-lactamases only possess the ESBL phenotype.

20 Global Suppressors

In the mid-1980s, experimental work was carried out in an attempt to elucidate genes that would code for the tertiary structure of a protein. Using random gap misrepair mutagenesis, a number of missense mutations were introduced into the gene for staphylococcal nuclease, rendering the mutant strains nuclease negative (*nuc*$^{-}$). Most of the detrimental mutations, as expected, affected amino acids located in the active site of the enzyme or in close proximity to it. There were, however, several distinct mutations involving remote sites. Surprisingly, after subjecting these "remote-site mutants" to another round of mutagenesis, nuclease activity was restored. Introducing the remote site mutations into other *nuc*$^{-}$ mutants had the same protein restoring effect. The term "global suppressors" was applied to outlying mutations capable of suppressing the deleterious effects of active-site mutations.

At that time, it was hypothesized that, in some way, the peripherally located amino acid substitutions were involved in preserving the tertiary structure of the protein (56). One striking example is the unique mutation involving the substitution of Met with Thr at position 182. Residue 182 is located in the hinge region between two different domains of the protein. Amino acids in this area, around position 182 and leading to the catalytic site, generally do not tolerate substitutions well. They are believed to play an essential role in core packing and catalytic site orientation. M182T is found in several different TEM enzymes (TEM-32, TEM-43, TEM-52) (57). It is thought that M182T functions as a global suppressor by affecting protein folding and thereby stabilizing the enzyme. This ability of M182T to compensate for the deleterious effects of other mutations makes it a powerful tool in acquiring resistance. As a natural polymorphism in β-lactamases, it will permit sampling of a much greater number of positions that tolerate substitutions. On the other hand, small inhibitor molecules could be designed against the hinge region of a β-lactamase, hindering folding to the active conformation of the enzyme and opening a new avenue for antimicrobial development (58). Investigations are under way to find a global suppressor in other class A β-lactamases, such as SHV.

21 OXA

The OXA-type enzymes are classified as a group of ESBLs that are different from SHV, TEM, and CTX-M, but share a common substrate spectrum. They are not inhibited by clavulanic acid, and they hydrolyze oxacillin and cloxacillin very efficiently. OXAs are primarily found in *P. aeruginosa* and *Acinetobacter baumannii*. Although the group is genotypically diverse, the most recent additions show some degree of homology to the existing members (59). Most OXAs, including OXA-11, -14, -16, and -17, are derivatives of OXA-10. They differ from the parental enzyme by one to several amino acid substitutions. The two most important substitutions in OXA-10 derivatives are Ser73Asn and Gly157Asp. The latter appears to be necessary for high-level ceftazidime resistance. This substitution is lacking in OXA-17, which, in contrast to the rest of the group, hydrolyzes cefotaxime and ceftriaxone much better than ceftazidime. OXA-31 differs from OXA-1 in only three amino acid substitutions, and was found to confer a rather unusual susceptibility pattern. OXA-31 hydrolyzes cefepime, but not ceftazidime. It is therefore important to avoid reporting ceftazidime resistance solely on the basis of cefepime resistance, as is the routine in most clinical laboratories (60).

22 Concluding Remarks

To summarize, emerging antibiotic resistance is often a consequence of chance mutations. The vast majority of mutations are detrimental to the host bacterium and do not spread. The ones that offer a survival advantage are selected. From a Darwinian standpoint, antibiotics function as a "selection tool." By killing the susceptible bacteria, antibiotics provide a new niche for the resistant organisms. Yet, mutations come at a price. They usually confer decreased "fitness" upon the mutant compared to the wild parental strain. This is readily seen among β-lactamases. We are learning how to discover and screen for mutants, and are gaining knowledge of the structural and functional impact of mutations. In many instances, we have described the mechanism of resistance at the molecular level. We have even simulated natural evolution and predicted new resistance determinants years before they were isolated clinically. Yet, it seems that we are losing the battle against resistance. A single "mistake" at the codon level occurs at random. And evolution takes over.

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