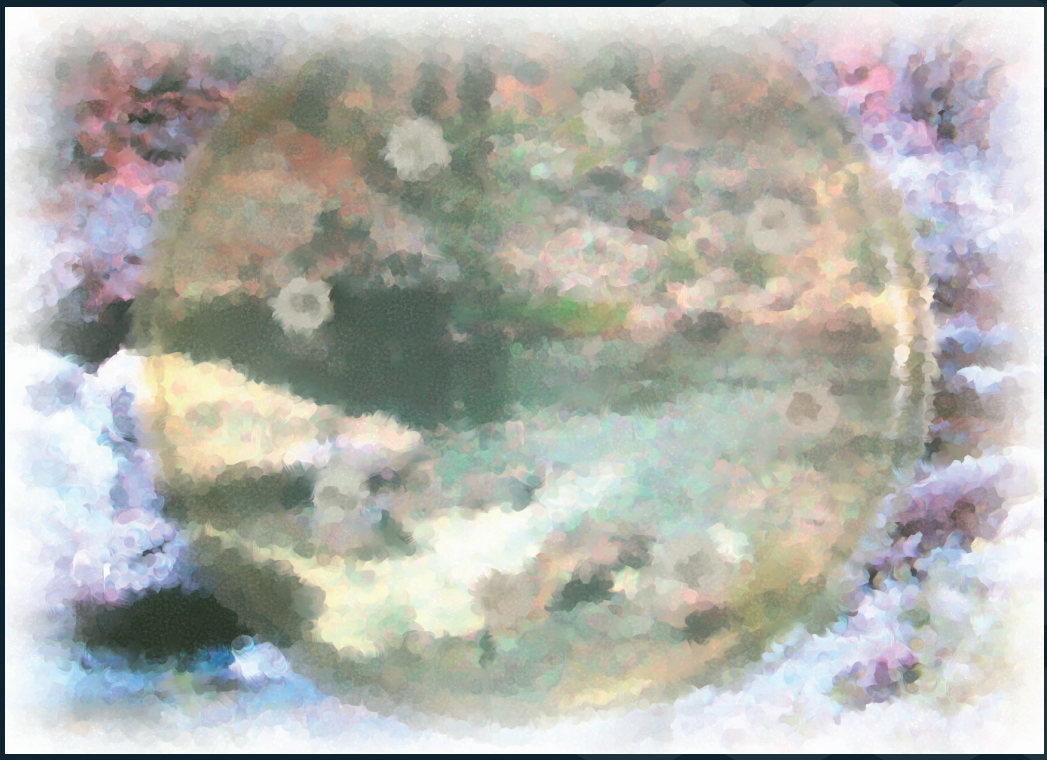
Antibiotics and Antibiotic Resistance in the Environment



CARLOS F. AMÁBILE-CUEVAS

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Preface

Antibiotic resistance is trendy once again. During the last few years, the World Health Organization, and the US and European Centers for Disease Control and Prevention, have all issued reviews on the subject, and stern calls to do something about it. The Infectious Diseases Society of America launched the 10 × '20 initiative, calling for a global commitment to have ten new antibiotics by the year 2020; even the US White House issued a *National Action Plan for Combating Antibiotic-Resistant Bacteria*. The finding of a new antibiotic produced by soil bacteria, something that would have been published in an obscure journal, if at all, had it happened during the last century, ended up as a full paper in *Nature*, and most news outlets worldwide afterwards. Is bacterial resistance to antibiotics something new? Was its emergence unexpected? Is it out of control as never before? Curiously enough, the answer to all three questions is 'no'.

Penicillin, the very first antibiotic, had its major clinical debut in 1942; it was used on the victims of the infamous fire at the "Cocoanut Grove" nightclub in Boston. That very year, Rammelkamp and Maxon reported the development of resistance in *Staphylococcus aureus* after long-term culturing in increasing concentrations of penicillin; and found the same phenotype in four clinical isolates obtained during the course of penicillin treatments (*Exp Biol Med* 51: 386–389). Three years later, Alexander Fleming himself, in his Nobel Lecture, warned that it was "not difficult to make microbes resistant to penicillin in the laboratory", and that "ignorant men" underdosing themselves, would make microbes resistant to the drug. By 1981, antibiotic abuse was so rampant, and antibiotic resistance so common, that Stuart Levy founded the Alliance for the Prudent Use of Antibiotics. Antibiotic resistance made the cover of *Newsweek* in March, 1994 ("Antibiotics – the end of the miracle drugs?") and, half a year later, of *Time* ("Revenge of the killer microbes – are we losing the war against infectious diseases?"). However, nothing spectacular did happen afterwards; mortality rates due to increased bacterial resistance kept rising, at a continuous but not dramatic pace. It wasn't until the end of 2007, when a "18,650" figure at the bottom of a busy table in a *JAMA* paper, created a new boom for resistance. That was the estimated number of yearly deaths caused by methicillin-resistant *S. aureus*, MRSA; that number, it turns out, was larger than the yearly deaths caused by AIDS. It was shocking: an obscure pathogen, unknown to most people, was killing more than the well-known HIV. During 2008, most US newspapers carried some information on antibiotic resistance at least once a week; *Dr. House* had a patient infected by MRSA, and a crucial witness in *Law & Order* died because of an MRSA infection before testifying in court. But the

MRSA crisis more or less subsided, only to be replaced by CRE, carbapenem-resistant enterobacteria. Antibiotic resistance is again trendy partially thanks to CRE.

Antibiotics are drugs and, as such, most people believe that they are mostly used to treat ill people and other animals. Exposure of bacteria to such compounds should, following this logic, occur only in clinical settings. Resistance hinders the ability of antibiotics to effectively cure infections; other than that, in most people's thoughts, resistance has little or no impact. Therefore, resistance is defined not based on the biological change enabling bacteria to survive and even thrive in antibiotic concentrations previously lethal to them; but only in terms of whether or not it can be related to therapeutic failure when using such antibiotic. All the notions above are plagued with misconceptions that had seriously limited research on antibiotic resistance outside the clinical settings. Why would sub-clinical concentrations of antibiotics, and low-level resistance, be of any relevance? Why look for antibiotic resistance in the environment? Who cares if an obscure bacteria from a lake, or from the gut of a wild animal, is resistant to an antibiotic? It is the purpose of this book to answer those questions and dispel those misconceptions. The field of antibiotic resistance in the environment is, however, hardly new: a paper dated more than 40 years ago, warned about the dangers of having resistant coliforms in water supplies, followed by descriptions of all sorts of environmental bacteria resistant to the drugs of that time (*e.g.*, chloramphenicol, tetracycline, ampicillin, nalidixic acid). Looking at more recent papers, the only things that have changed are the resistance figures (always higher) and the names of antibiotics (always stranger). It feels like André Gide was right: "Everything has been said before. But since nobody listens we have to keep going back and begin all over again". However, there has been an interesting twist recently. The arrival of powerful molecular technologies enabled us to look for resistance genes without culturing bacteria (which is important, as less than 1% of soil bacteria have been cultured), and to detect minute quantities of such genes. Using these technologies, a swarm of papers reported resistance genes of all kinds in samples from soil, water, feces … even very old permafrost. These papers gave the right notion that resistance is everywhere and is ancient; but this notion became distorted as to signify that it is therefore not worrisome to find resistance to, for instance, a new, synthetic drug, in an enteric bacteria from wildlife or a lake sediment. Something very akin to stating that climate has always changed – therefore human influence is irrelevant, an argument so popular amongst those that do not understand the difference between climate and weather. Interestingly, many of those papers emerged when people started to worry about resistance, alarmed also by the lack of new antibiotics – and by the lack of interest from pharmaceutical companies to develop them; only to give room for the grim reports mentioned at the beginning of this preface – including a forecast for a dramatic rise in yearly mortality due to resistance, from 700,000 to 10 million by 2050, along with a 100-trillion US dollars GDP loss, now that everybody seems convinced of the need for financial "incentives" for pharma companies to engage in antibiotic R&D. Curious timing.

Anyway, this book will ride the new wave of interest on antibiotic resistance. A (disproportionately long) introductory first chapter will establish working definitions of antibiotics, resistance and environment, as well as briefly describe the known mechanisms underlying resistance and its spread, and the methods used to investigate the presence of antibiotics and antibiotic resistance in the environment. From there, it will review available evidence of the causes and magnitude of the problem; and why it is a

very serious problem indeed. I would try to do so in a way that is accessible for most readers, requiring only basic notions of each of the involved topics. As the subject of antibiotics and environment has repercussions on human and animal medicine, microbiology, ecology, public health, environmental protection, pharmaceutical discovery and policy making, to mention a few, this book will try to provide a basic background for everybody to understand its content. Obviously, to achieve this goal, some sections will be boring to some readers, and some oversimplifications would have to be done. However, I hope that, at the end, all readers, regardless of their background, would get the "big picture" behind the issue of antibiotics and antibiotic resistance in the environment. This is, of course, a big task; and people do not undertake big tasks if not affected by a bit of arrogance (*i.e.*, "an exaggerated sense of one's own importance or abilities"). So, I confess: I am writing this book believing that a training in pharmacology, microbiology and molecular biology, a long time dealing with the clinical side of bacterial resistance, and some recent incursions in the field of resistance in the environment; along with a lifetime in a – so-called – "developing" country, do provide me with enough perspective and insight to convey an integrated view of this problem. Now, let's see if I can deliver.

Mexico City, September 2015

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Chapter 1

Definitions and basic concepts

When thinking of "antibiotics in the environment", perhaps a first image that comes to mind is a clandestine dumping of antibiotics from a drug factory into a river in a non-developed country; and when thinking of "antibiotic resistance in the environment", the natural consequence would be to think of an aquatic bacteria under the selection of the dumped antibiotic becoming resistant to the drug, and then causing an outbreak in a neighboring town. The actual scenario is far more complicated and complex: complicated, as there are much more elements at play; complex, as the interactions of those elements are many and multi-directional. Even from the semantic point of view, there could be confusion as to what we call "antibiotic", "resistance" or "environment", so it is important to begin with some working definitions. Although some definitions are not unanimously agreed upon, it is crucial to frame the content of this book within those definitions, in order to avoid misinterpretation.

In addition to formal definitions, this first section will deal with some concepts that are relevant and necessary to understand the reach and limitations of our current knowledge of the topic at hand. What is the "role" of natural antibiotics in microbial ecology? How is the definition of resistance limiting the perspective view of its emergence and evolution? What are the advantages and disadvantages of using molecular-based or culture-based techniques for assessing resistance in the environment? Many of these issues are clearly controversial, and the author's bias will become clear; but by actually stating a position, it is hoped that the readers will be able to reach their own conclusions much more easily.

1.1 ANTIBIOTICS: ORIGINS AND ACTIVITY

"Antibiotic", according to the Merriam-Webster dictionary, is "a substance produced by or a semisynthetic substance derived from a microorganism and able in dilute solution to inhibit or kill another microorganism". This definition would encompass things like natural penicillin (a product of a mold) and ampicillin (a semisynthetic derivative of penicillin); exclude entirely synthetic agents such as sulfonamides and quinolones; and leave in a limbo drugs like chloramphenicol which, although initially discovered as a product of soil bacteria, it is now produced entirely by chemical synthesis. A wider definition from Wikipedia states that "antibiotics [. . .] are a type of antimicrobial used in the treatment or prevention of bacterial infection", whereas "antimicrobial" is simply "an agent that kills microorganisms or inhibits their growth", which would also

include antiseptics and disinfectants. Then there are "antibacterials", "anti-infective chemotherapy", and so on. For the purposes of this book, as there is no evident advantage in discriminating at every sentence between natural and synthetic compounds, an antibiotic would be a chemical agent with a selective toxicity profile, capable of killing or inhibiting the growth of bacteria but mostly incapable of exerting toxicity upon eukaryotic cells at the same concentration (the "magic bullet" imagined by Paul Ehrlich), that is commonly used to treat or prevent bacterial infections. This definition would therefore include all drugs, of natural or synthetic origin, used against bacteria; and would exclude compounds used against viruses, fungi, protozoans or other microorganisms, as well as non-selective biocides, such as disinfectants and antiseptics.

1.1.1 Origin and mechanism of action of main antibiotic classes

Although it is not within the purview of this book to enlist and review the origin and mechanism of action of each class of antibiotics, having an overview included could be helpful for the reader not well versed into this mainly pharmacological area. It may be important to point out that a sort of unifying mechanism of action of bactericidal antibiotics, through a common pathway of generating reactive oxygen species, recently proposed (Kohanski et al., 2007), was first shown to be inconsistent with physiological evidence (Mahoney and Silhavy, 2013), and then most likely to be based on a laboratory artifact (Renggli et al., 2013). It is also important to emphasize that these are the mechanisms of bacteriostatic or bactericidal effects of high, clinically-attainable concentrations of antibiotics; as will be discussed below, this could very well be a human-made situation, with natural antibiotics actually exerting other physiological roles at much lower concentrations. The following paragraphs enlist some relevant information about each antibiotic class, with those that include mostly natural products first. For additional information on the chemistry, pharmacology and clinical uses of each drug, two comprehensive texts can be useful: Bryskier A. (ed.) *Antimicrobial agents, antibacterials and antifungals*; ASM Press, Washington DC, 2005; and Grayson M.L. *et al.* (eds.) *Kucers' The use of antibiotics*, 6th ed; Hodder Arnold, London, 2010.

– *Beta-lactams*. This class includes natural and semi-synthetic penicillins, natural and semi-synthetic cephalosporins and cephamycins (occasionally subgrouped as cephems), carbapenems, monobactams, and beta-lactamase inhibitors. Many members of this class are derivatives of natural products of fungi, *Penicillium* spp. and *Acremonium* (formerly *Cephalosporium*) spp.; while others (cephems, carbapenems, monobactams, beta-lactamase inhibitors) derive from soil bacteria from the genus *Streptomyces* and *Chromobacterium*. However, there is evidence that the genes necessary for the production of beta-lactams by fungi, actually originated from bacteria, making in the end all of these drugs of bacterial origin. With the partial exception of beta-lactamase inhibitors, beta-lactams inhibit the action of peptidoglycan transpeptidases, collectively known as penicillin-binding proteins, or PBPs. As a result, the synthesis of the main component of the bacterial cell wall is halted, while its hydrolysis during bacterial replication, and cellular growth, are not; the osmotic uptake of water occurs without the volume restriction imposed by the cell wall, leading to cytolysis. Apart, beta-lactamase inhibitors are used in conjunction with a penicillin or cephalosporin, so that they protect the

actual bactericidal agent from the action of bacterial enzymes responsible for resistance. However, one of these inhibitors, sulbactam, exerts by itself the inhibition of wall synthesis upon the pathogen *Acinetobacter*.

- *Aminoglycosides*. This class of antibiotics includes the natural and semi-synthetic products of soil bacterial of the genus *Streptomyces* and *Micromonospora*. The group's first member was streptomycin, which did open the door for looking into soil bacteria for new antibiotics. This further search led to natural aminoglycosides tobramycin, kanamycin, neomycin, sisomicin, and gentamicin; and semi-synthetic ones, such as amikacin, netilmicin and isepamicin (the "-mycin" suffix indicates a *Streptomyces* product, while the "-micin" one is used for *Micromonospora*-derived compounds). Aminocyclitol antibiotic spectinomycin, a natural product of *S. spectabilis*, is often included in the same group, although its chemical structure is different, as are some details of its mechanism of action. Aminoglycosides bind, sometimes irreversibly, to the 30S ribosomal subunit, leading to inaccurate translation (misreading), impaired proof-reading and/or premature termination of protein synthesis. Aminoglycosides are actively uptaken by components of the bacterial respiratory chain, hence they do not reach inhibitory concentrations intracellularly in anaerobes, or in facultative anaerobes growing under anaerobic conditions.
- *Macrolides*. They include erythromycin, a natural product of the actinomycete *Saccharopolyspora erythraea*; and semi-synthetic derivatives, sometimes classified, for marketing purposes, under individual "classes", such as the "azalide" azithromycin, or the "ketolide" telithromycin. Macrolides reversibly bind the 50S subunit of the bacterial ribosome, specifically the nascent peptide tunnel in the vicinity of the peptidyl transferase center, stalling the ribosome, hence blocking translation. Although chemically very different, lincosamides and streptogramins bind to the same ribosomal site.
- *Lincosamides*. A small class that includes lincomycin, a product of *Streptomyces lincolnensis*; and clindamycin, a semi-synthetic derivative of lincomycin; the main difference – and advantage of clindamycin, is its activity upon anaerobic bacteria. Their mechanism of action is similar to the one of macrolides.
- *Streptogramins*. There are two main subclasses of streptogramins, A and B. Both are products of *Streptomyces* bacteria and, although chemically different, they act in the same way and often synergistically. The combination of quinupristin (streptogramin B) and dalfopristin (streptogramin A) was used in human medicine, while virginiamycin is used in the industrial production of fuel ethanol, and as a "growth promoter" food additive for livestock. Their mechanism of action is similar to the one of macrolides.
- *Amphenicols*. Chloramphenicol, a product of *Streptomyces venezuelae*, is the main representative of this group; synthetic derivatives (chloramphenicol used today is chemically synthesized itself) include florfenicol, used only for veterinary purposes; and thiamphenicol, used for humans in some countries, and for animals in others. Amphenicols bind to the 23S rRNA of the 50S ribosomal subunit, inhibiting the peptidyl transferase activity of the bacterial ribosome.
- *Tetracyclines*. Natural (chlortetracycline, from *Streptomyces aureofaciens*; oxytetracycline, from *S. rimosus*) and semi-synthetic (minocycline, tigecycline, the later considered a "glycylcycline") are members of this group. Tetracyclines inhibit bacterial synthesis of proteins by binding to the small ribosomal subunit, blocking the

attachment of aminoacyl-tRNA to the A site; tetracyclines bind to both, the 30S bacterial subunit, and the 40S eukaryotic one, but bacteria uptake tetracyclines actively, leading to much higher intracellular concentrations.

- *Glycopeptides*. Vancomycin, a natural product of soil bacterium *Amycolatopsis orientalis*, was the first member of this group, followed by other natural (teicoplanin, ramoplanin, from *Actinoplanes* spp.; avoparcin, from *Streptomyces candidus*) and semi-synthetic (*e.g.*, telavancin, oritavancin), products. Glycopeptides inhibit the synthesis of the cell wall of gram-positive bacteria; gram-negatives are usually non susceptible due to the inability of glycopeptides to cross the outer membrane. These antibiotics bind to the D-alanyl-D-alanine moieties at the end of the short peptide hanging from acetylmuramic acid, before the cross-linking of peptidoglycan; the attached antibiotic prevent the cross-linking itself.
- *Lipopeptides*. Polymyxins (B, and E, known as colistin), products of *Paenibacillus polymyxa*; and daptomycin, obtained from *Streptomyces roseosporus*, are included in this group. Lipopeptides seem to alter the architecture of the phospholipid bilayer of the cell membrane; while polymyxins first attach to the lipopolysaccharide in the outer membrane of a few gram-negatives, and then gain access to the cell membrane; daptomycin binds to the cell membrane of gram-positives in a calcium-dependent manner. Lipopeptides are often regarded as "last-option" antibiotics: polymyxins are used only against multi-resistant bacteria (*i.e.*, carbapenem-resistant Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter* spp.), and daptomycin against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE).
- *Fosfomycin*. Fosfomycin is a small molecule isolated from *Streptomyces fradiae*; it inhibits the synthesis of bacterial cell wall, through the inhibition of MurA (UDP-acetylglucosamine-3-enolpyruvyltransferase), acting as a phosphoenolpyruvate analog. Fosfomycin is not widely used, despite a wide spectrum, low toxicity and low resistance rates. In countries where it is a preferred option (such as Spain, as the antibiotic was discovered there), it was mostly used against urinary tract infections; however, fosfomycin has shown relevant activity against multi-resistant organisms that are common in hospital settings, and is now regaining attention as an option in the management of infections caused by such bacteria.
- *Rifamycins*. Rifampicin (or rifampin), rifabutin, rifapentine, and orally-unabsorbable rifaximin, are all derivatives of rifamycin, a natural product of *Amycolatopsis rifamycinica* (formerly *A. mediterranei*, formerly *Nocardia mediterranei*, formerly *Streptomyces mediterranei*). They selectively inhibit bacterial RNA-polymerase. Rifamycins have mostly been used against tuberculosis, but have also been used against multi-resistant staphylococci and pneumococci. Rifaximin is used against intestinal bacteria; it should be used only for intestinal "sterilization" prior to gut surgery, and the management of hepatic encephalopathy, although it is also abused as an anti-diarrheic agent.
- *Sulfonamides (DHPS inhibitors)*. Prontosil, a prodrug of sulfanilamide, was the first synthetic antibiotic, patented in 1932. While "sulfonamide" refers to a chemical functional group, common to a very wide variety of molecules, many of clinical relevance (*e.g.*, diuretics, sulfonylureas, antiretrovirals, anti-inflammatory drugs), the term sulfonamide is often used to refer only to those of antimicrobial properties. Sulfonamides are structural analogs of *p*-aminobenzoic acid (PABA),

hence acting as competitive inhibitors of dihydropteroate synthase (DHPS), an enzyme that forms dihydropteroate (a precursor of folic acid) from dihydropteridinmethyl phosphate and PABA. There are dozens of sulfonamides, varying mostly in pharmacokinetic properties; today, sulfamethoxazole is the most widely used (in association with trimethoprim; see below); silver sulfadiazine is also used topically. DHPS is not present in mammals, as we need to have dietary folates.

- *Trimethoprim (DHFR inhibitors)*. At the end of the pathway that joins PABA and dihydropteroate diphosphate to form dihydropteroic acid, enzyme dihydrofolate reductase (DHFR) sequentially converts folic acid into dihydrofolate and then tetrahydrofolate, the actual cofactor for the synthesis of purines, thymidylic acid, and several amino acids. DHFR is inhibited by trimethoprim, the most widely used drug of this class, that also includes much less used brodimoprim, and failed drug iclaprim. Trimethoprim is often used in combination with the sulfonamide sulfamethoxazole; the combination, known as co-trimoxazole, was supposed to be synergic and less prone to select for resistance, which end up to be false expectations. While eukaryotes have also DHFR, trimethoprim and related drugs inhibit prokaryotic DHFR at concentrations several orders of magnitude lower.
- *Nitrofurans*. This group of synthetic molecules is mostly represented by nitrofurantoin, a drug only used against lower urinary tract infections; interest in nitrofurantoin has increased recently, as resistance among uropathogenic *E. coli* remains very low (<10%), despite decades of clinical use. The mechanism of action of nitrofurans seem to involve the formation of a reduced, reactive intermediate, that in turn disrupts DNA, ribosomes and respiratory chain. The formation of this intermediate depends on bacterial reductases, hence it is mainly produced inside bacterial cells. Other drugs in this group are nitrofurazone, used topically; and furazolidone, used against enteric pathogens.
- *Quinolones*. The quinolone antibiotic class is one of the latest ones to be introduced into clinical use; paradoxically, the first one of the family, nalidixic acid, is not quite a quinolone, but a naphthyridone. Second-generation quinolones are also the first fluoroquinolones, with an added fluoride atom: norfloxacin, ciprofloxacin, ofloxacin (and its homochiral formulation levofloxacin) and enrofloxacin, the latter used only on animals. These agents act as inhibitors of class 2 topoisomerases, gyrase and topoisomerase IV, although their bactericidal action is mostly due to gyrase inhibition. It is important to understand how class 2 topoisomerases work: they bind to DNA, cleave both strands (hence "class 2"), make other, intact double-stranded DNA pass across the cleavage, and bound the cleaved DNA back. While in the presence of quinolones, topoisomerases do cleave DNA, but are unable to rebound it; quinolones induce double-strand breaks in DNA, that are very difficult to repair. Many fluoroquinolones were introduced into clinical use, only to be withdrawn some few months later due to adverse effects. Nalidixic acid and second-generation quinolones are particularly active against gram-negative bacteria, although levofloxacin has been inadequately used against gram-positives. A third generation of fluoroquinolones (moxifloxacin, gemifloxacin) are better inhibitors of the gyrase of gram-positives, and are mostly used against respiratory tract diseases.
- *Oxazolidinones*. The first drug of this group, linezolid, was approved for clinical use in 2000, making oxazolidinones the latest antibiotic class to be introduced

(with a nearly 40-year gap between them and the first quinolone). Along with tedizolid, the only other oxazolidinone approved up to this date, these molecules block the initiation of protein synthesis, by binding to the 23S portion of the 50S ribosomal subunit, close to the binding site of amphenicols. Oxazolidinones are active only against gram-positive bacteria. Other drugs in this group that are likely to reach the market are posizolid and radezolid.

– *Nitroimidazoles*. Although mostly used against protozoans, metronidazole is also active against anaerobic bacteria (and facultative anaerobes under anaerobic conditions). A reduced derivative, only produced in the absence of free oxygen, interacts with DNA resulting in cell death. An exception occurs in *Helicobacter pylori*, against which metronidazole is also effective, but that thrives in environments still too rich in oxygen; in this species, an oxygen-insensitive nitroreductase converts metronidazole into its reduced toxic derivative. Related drug tinidazole supposedly acts in the very same way, although is mainly used against protozoal infections.

Aside from the antibacterial properties of antibiotics, briefly described above, some of these drugs have been used for an entirely different purpose: by reasons that are not fully understood, sub-therapeutic doses of some antibiotics can promote the growth of farm animals. Possible ways antibiotics can achieve this include: (a) reducing microbial use of nutrients, (b) diminishing the thickness of the intestinal wall, thus enhancing uptake of nutrients, (c) preventing some sub-clinical infections, and (d) reducing microbial metabolites that can depress animal growth (Laxminarayan et al., 2015). Although the actual mechanism of animal growth promotion is not clear, this represents by far the main use of antibiotics, as will be analyzed in Chapter 3.

1.1.2 Antibiotics: chemical warfare, intercellular signaling, prebiotic remains?

The term "antibiotic", introduced by streptomycin "discoverer" Waksman, could be understood as derivative of *antibiosis*: "antagonistic association between organisms to the detriment of one of them or between one organism and a metabolic product of another". This suggests, as it is a common belief, that natural antibiotics are a sort of chemical weapon used by producing bacteria, to colonize new environmental niches and/or to keep invaders at bay from an already colonized place. In addition to its simplicity, this explanation has the appeal of assuming that chemical warfare is something natural and not an abomination. Furthermore, in such war-like scenario, resistance seems like a normal, ubiquitous trait to emerge wherever an antibiotic-producing, aggressive bacterial strain is around. However, natural antibiotics are much more complicated than that.

Julian Davies was the first to suggest that antibiotics are unlikely weapons for chemical warfare. Based on the complexity of their synthetic pathways, the fact that they are mostly produced when bacteria are in stationary phase, and the effector activity many of them have upon gene expression and transfer, Davies proposed that antibiotics could be remnants of prebiotic molecules that interacted with early nucleic acids, even before the emergence of ribosomes, and are now adopted as secondary regulators (Davies, 1990). Antibiotics act as bacteriostatic or bactericidal agents only

at concentrations much higher than those found in nature. At sub-inhibitory concentrations, antibiotics are known to act as signaling molecules, having very subtle effects on gene expression at different levels. Among the many aspects of bacterial physiology that are affected by low concentrations of antibiotics, are those related to quorum, biofilm formation, and others involved in the coexistence of different bacterial species in the same ecological niche (Sengupta et al., 2013). At the clinical perspective, subinhibitory concentrations of antibiotics act upon the expression of virulence determinants (Linares et al., 2006). As the notion of coexistence and non-competitiveness clash with the anthropocentric warfare view, antibiotics are still mainly regarded as microbial chemical weapons, despite the evidence in contrary. To be fair, there are some few known examples where antibiotics – none used clinically – seem to be produced to suppress the growth of competitor microorganisms (Sengupta et al., 2013).

While the controversy around the actual role of natural antibiotics may seem purely academic, it holds the key to understand the emergence of resistance and its role in open environments. Again, it was Julian Davies who first demonstrated the biochemical similarity between some of the resistance mechanisms found in antibiotic-producing bacteria and in clinically-relevant ones (Benveniste and Davies, 1973). There is now some genetic evidence of the presence of antibiotic-resistance genes in antibiotic-producing bacteria; protection from their own products, as well as having some role in the very biosynthesis of the antibiotics themselves, are among the proposed roles of these genes (Sengupta et al., 2013). However, many resistance genes have been reported in non-producing, environmental bacteria, the so-called "resistome". If natural antibiotics occur only at very low concentrations, it is unlikely that they exert a selective pressure for a full-resistant phenotype. Are antibiotics actually present at high concentrations in nature? Are there other, non-antibiotic agents that select for antibiotic-resistance genes? Or could it simply be that we are using the word "resistance" in a very loose way? All this will be discussed in the next chapter.

1.2 RESISTANCE: WHAT IT IS AND HOW WE MEASURE IT

As the main – recognized-role of human-made antibiotics is to cure infections, resistance has always been considered from a clinical, instead of a biological point of view. This is to say that it is not merely a matter of comparing the ability of one strain against another, to withstand an antibiotic at a given concentration; but to try to correlate such ability to the likelihood of the antibiotic to fail if used therapeutically against the "resistant" strain. This notion has resulted in dangerous generalizations and oversimplifications. For instance, a set of concentration breakpoints have been established: if a strain grows *in vitro* in the presence of antibiotic concentrations above such breakpoint, it is deemed resistant. Such breakpoints purportedly consider the antibiotic concentrations reached clinically when the drug is administered at standard doses; also, the cure rate when using the drug, related to the inhibitory concentrations for each isolate in a number of patients. However, for these breakpoints to be adequate, they should consider the wide variation of tissular concentrations achieved by an antibiotic within a single patient; and the even wider variation between different patients. Also, the pharmacokinetics and pharmacodynamics of each drug should be considered, along with the dosing schemes. If all these issues were to be included, the

breakpoint list would be endless and completely unpractical; therefore, all we have are single breakpoints for each antibiotic against each major bacterial group of clinical relevance. For the subject of this book, that is, bacterial resistance in the environment, this poses great difficulties, as for many environmental, innocuous bacteria, there are simply no established breakpoints, in addition of the whole concept of resistance, from the clinical point of view, would be mostly irrelevant. Moreover, the limitations do not end there.

The activity of antibiotics *in vitro* is generally assessed in two ways: by determining the minimum inhibitory concentration (MIC) of the antibiotic, usually in liquid media; or by measuring an inhibitory halo produced by the antibiotic diffusing from a small paper disk into a solid culture media. Media used for these assays have been selected to optimize bacterial growth, and to minimize their interference with antibiotic activity (*e.g.*, devoid of para-aminobenzoic acid, as it antagonizes the effect of sulfonamides). Pure cultures, at standardized innocula, are tested; bacteria are therefore growing in artificial conditions entirely different than they do in clinical or environmental settings. When assessing the MIC, bacteria are suddenly exposed to high antibiotic concentrations, reducing the ability of adaptive responses to be activated; and in a way that is very different from the gradual exposure that typically occurs in nature (the disk diffusion technique allows for a gradual exposure, as the antibiotic diffuses slowly from the disk into the agar medium). The effect of the antibiotic is measured in the very short term —18 to 24 hours, but as short as 4 hours if using an automated system. This would hinder the ability to detect slow-growing varieties, as well as hetero-resistance (see below). Bacterial growth is measured just by the turbidity they produce in liquid media, by eye or using a nephelometer; or by trying to assess the diameter of an often irregular, diffuse halo surrounding a paper disk. Finally, MIC determinations rely on series of double-fold dilutions, that analyze with detail the effect of low concentrations (*e.g.*, 0.01, 0.02 and 0.04 μg/mL), but that leave great gaps at high concentrations (*e.g.*, 32, 64, 128 μg/mL). In any case, we end up with a MIC value, or a halo's diameter. These values are then to be compared to breakpoints' tables that enlist antibiotics and bacterial groups (*e.g.*, enterobacteria, non-fermentative bacteria, staphylococci), so that we can interpret whether a 12 μg/mL MIC, or a 17-mm halo, is indicative of susceptibility of resistance of that particular species and for that particular antibiotic. These tables change from time to time, resulting in the curious paradox of having a phenotype classified as resistance one year, and as susceptible the following year (this has had a very negative impact in assessing the evolution of resistance along time, especially for rapidly changing breakpoints, such as penicillin susceptibility in pneumococci). And, to make it all worst, each geopolitical region has its own set of breakpoints: the US and, by extension, most of the American countries (America, by the way, is the name of a continent, not of a country), follow the ones set by the Clinical Laboratory Standards Institute (CLSI); the EU have their owns (EUCAST); as do the UK (BSAC); and they do not always match. As a result, a bacterial strain can be considered resistant in Europe, but susceptible in America.

For an adequate assessment of resistance in the environment, a biological rather than clinical definition should be used. A useful approach is to consider the natural variation of antibiotic activity upon a large number of isolates of a given bacterial species. This would enable the separation of susceptible and resistant bacteria within each species or other relevant taxa, independently of the clinical nuances of achievable

plasma concentrations and relatedness to treatment outcomes. An ECOFF, or epidemiological cut-off value for resistance breakpoint has been proposed (Martínez et al., 2015), aimed at a biological rather than clinical description of resistance. However, as this data is mostly missing, resistance would have to be defined through this book, based on the clinical breakpoints, when available. When referring to diminished susceptibility not reaching said breakpoints (*e.g.*, the one conferred by plasmid-borne *qnr* quinolone-resistance genes); or to data obtained using selecting concentrations of antibiotics different from said breakpoints (*e.g.*, using agar plates containing ampicillin concentrations of 50–100 μg/mL, while the breakpoint for resistance in enterobacteria is 32 μg/mL); or to species and/or antibiotics for which breakpoints are not available (*e.g.*, streptomycin for *E. coli*), an aclaratory note would be made, along with the reference to "resistance", within quotation marks.

1.2.1 Resistance mechanisms, horizontal gene transfer, and adaptive responses

Bacteria can withstand the effect of antibiotics in many different ways. This section will first review the main biochemical and physiological mechanisms through which bacteria can survive and even thrive in the presence of antibiotics; then, it will review the genetic phenomena that allow such biochemical and physiological mechanisms to arise and spread. Although of considerable interest, this section will not further discuss other related phenotypes, such as tolerance (an increase in the concentration needed to kill bacteria, while the inhibitory concentration remains unchanged); subsistence (the ability to use antibiotics as a carbon or nitrogen source); and dependence (a rare phenomenon where affected bacteria can grow *only* in the presence of an antibiotic). Variations of these phenomena, and even of the ones that will be described in following paragraphs, can be considered as "noninherited" resistance mechanisms (Levin, 2004), which are of great interest but that are still far from being adequately understood.

1.2.1.1 Three main kinds of resistance: intrinsic, acquired, and adaptive

There are many ways in which resistance mechanisms can be classified. For the purposes of this book, resistance mechanisms will be categorized as intrinsic, acquired, or adaptive. It is important, however, to state that the boundaries for each of these three categories can be diffuse and some times confusing. This confusion is particularly relevant to the subject of this book, as it seems to pervade the research in the area, as will be further discussed.

1.2.1.1.1 Intrinsic resistance

Intrinsic resistance can be defined in different ways. It can be thought as an inherent characteristic of a given bacterial species, to be unaffected by an antibiotic at concentrations achieved clinically. Many cases of intrinsic resistance are related to permeability issues: the outer membrane of *Pseudomonas aeruginosa*, for instance, is mostly impermeable to aminopenicillins (ampicillin or amoxicillin), as is the outer membrane of many gram-negative bacteria to macrolides and glycopeptides. But there are some other mechanisms underlying intrinsic resistance: the inability of anaerobic bacteria to uptake aminoglycosides render them intrinsically resistant to such compounds; a

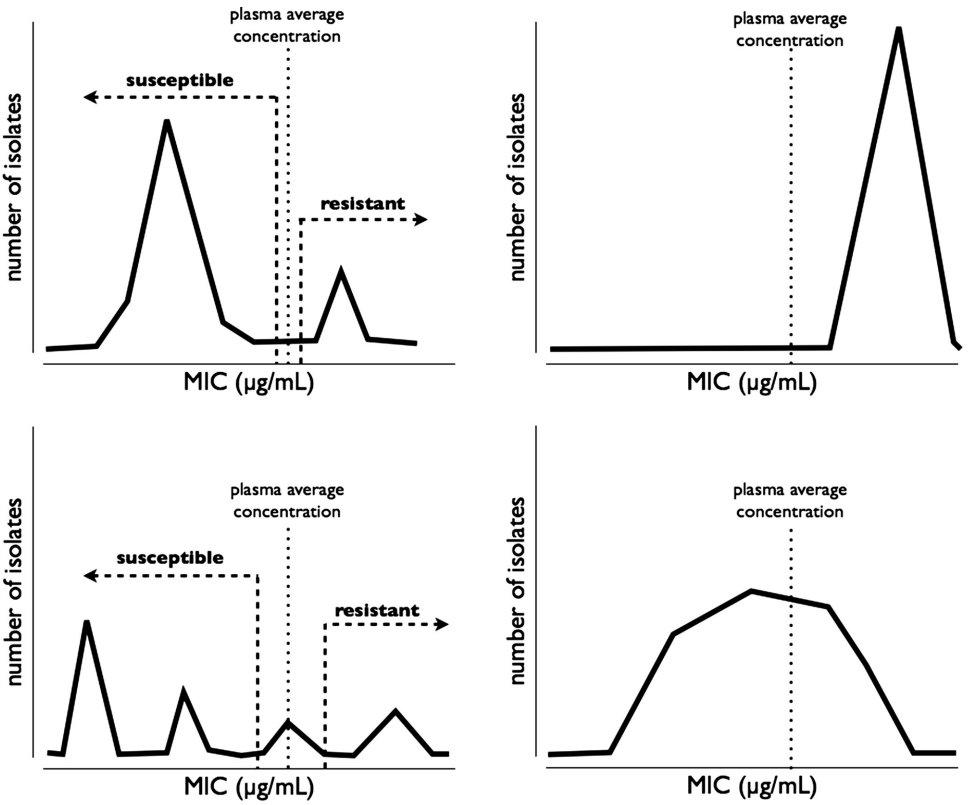

Figure 1.1 **Definitions of resistance; a graphic summary.** Top left, the easiest scenario, where members of a bacterial species have either, a very low or a very high MIC, and plasma (or tissue) concentrations are always enough to inhibit the low-MIC varieties. A breakpoint for resistance follows both, the biological variation, and the clinically-achievable concentrations. Top right, a clear example of intrinsic resistance, where all members of a bacterial species are inhibited by an antibiotic, but at concentrations too high to be reached clinically. Bottom left, the most usual scenario with clinical isolates of a bacterial species, having a variety of phenotypes, some under, some above, and some even overlapping clinical concentrations. This variety some times creates the messy definition of "intermediate susceptibility", a MIC range between resistant and susceptible, that physicians seldom know how to use. From the biological point of view, all three peaks at the right have gained resistance; but from the clinical point of view, only the one at the far right is truly resistant. Bottom right, a very wide distribution of MICs that makes the determination of resistance and susceptibility a very hard – and often useless task.

complex and distinctly different set of enzymes involved in peptidoglycan synthesis in enterococci render this genus intrinsically resistant to cephalosporins (Vesić and Kristich, 2012). When considering a timeframe, intrinsic resistance is a defining characteristic of both, a bacterial species and an antibiotic, a characteristic that has not changed in time, especially within the "antibiotic era". Intrinsic resistance defines the original spectrum of activity of each antibiotic; there is no known antibiotic capable of inhibiting all bacterial species at clinically relevant concentrations. For the purposes

of this book, intrinsic resistance is considered to be irrelevant, as it is an unchanged, inherent feature of each bacteria/antibiotic combination. The mechanisms mediating intrinsic resistance are part of the defining characteristics of each bacterial species, residing in housekeeping genes, extremely unlikely to be transferred from an intrinsically resistant bacteria to a susceptible one. Intrinsic resistance cannot be considered a public health threat, as it is an inherent bacterial characteristic – or an inherent antibiotic limitation.

While the examples provided in the last paragraph are straightforward enough, some other bacterial abilities may qualify as "intrinsic" resistance, but will be considered separately in this section. Three examples are singled out below: (1) increased unspecific efflux, elicited by the presence of an antibiotic; (2) the persistence of biofilms; and (3) the almost universal presence of chromosomal beta-lactamases in some enteric bacteria. The first two will be discussed under the adaptive resistance category; the third one within the acquired mechanisms.

1.2.1.1.2 Acquired resistance

The accidental discovery of penicillin occurred because an agar plate with *Staphylococcus aureus* growing all across, became contaminated with a *Penicillium* mold growing in an edge of the plate. At that time, most *S. aureus* isolates had a penicillin MIC $\ll 1\,\mu g/mL$. Strains reported as resistant to penicillin, in 1942 and from that date forward, have penicillin MIC $>100\,\mu g/mL$. These strains have acquired a resistance phenotype, some times even during the course of an antibiotic treatment, within a single patient. This is of course named acquired resistance. In addition of being a recent acquisition, these traits are from that moment on, more or less stably inherited to daughter cells; and usually qualify as full-resistance (*i.e.*, well above clinical resistance breakpoints). Genetically, these traits can be acquired through two main mechanisms that will be further discussed: mutations and horizontal gene transfer; this section will deal with the biochemical mechanisms that enable bacteria to resist the effects of antibiotics.

While several hundreds of resistance genes have been characterized up to this date (and probably a few more while this book is being prepared), all of them can be mechanistically categorized in four groups: (1) enzymatic inactivation of the antibiotic, mostly of naturally-occurring antibiotics; (2) protection or modification of the target of antibiotic action; (3) diminished accumulation of the antibiotic achieved by active efflux and/or diminished permeability; and (4) acquisition of a by-pass route for an antibiotic-blocked pathway, or overproduction of enzymes within such pathway. There are possibly hundreds of reviews on this matter, from one of the earliest but still useful (Foster, 1983); to a recent extraordinarily comprehensive listing of acquired resistance genes (van Hoek et al., 2011). The following is just a brief overview on the subject, with most information coming from the van Hoek paper, except when specifically stated.

– *Resistance to beta-lactams*. Resistant bacteria can (1) enzymatically inactivate beta-lactams, using hydrolases known as beta-lactamases; or (2) produce a modified PBP (paradoxically, a "penicillin-binding protein" that does not bind to penicillin) that enables the synthesis of peptidoglycan even in the presence of beta-lactams. Additionally, diminished accumulation can yield a low-level "resistance" or, if coexisting with a beta-lactamase gene, enhance the protective spectrum of the enzyme. There are around 1,000 known beta-lactamase genes, that encode,

from very narrow spectrum enzymes, capable of hydrolysing only penicillins (also known as penicillinases); to extended-spectrum beta-lactamases (ESBL), capable of inactivating third-generation cephalosporins; to carbapenemases, that inactivate all beta-lactams, including carbapenems. Some of these enzymes have a zinc ion within their active site (metallo-beta-lactamases); some are resistant to beta-lactamase inhibitor clavulanate. Most are plasmid-borne, but *ampC* genes are chromosomal in many enterobacteria and in *Pseudomonas aeruginosa* (although also present in plasmids and somehow linked to other mobile genetic elements (Jacoby, 2009)); AmpC enzymes can inactivate several cephalosporins, and are often inducible, even by clavulanate, resulting in a wide-spectrum, clavulanate-resistance phenotype. Mutations in the regulatory genes result in the constitutive overproduction of AmpC, along with increased resistance. As to altered PBPs, these are commonly found in two clinically relevant bacterial groups: (a) streptococci, particularly *S. pneumoniae*, which altered PBP genes were mobilized by transformation, and a mosaicism phenomena resulting from homologous recombination; also, low-affinity PBPs have been found to be plasmid-encoded in enterococci (Raze et al., 1998); and (b) *S. aureus*, mediating the phenotype known as methicillin-resistance (an archaism, as methicillin is no longer in clinical use; "methicillin-resistance" therefore refers to resistance to anti-staphylococcal penicillins, such as oxacillin, and actually includes most beta-lactams), the well-known MRSA.

- *Resistance to aminoglycosides*. Most acquired resistance to aminoglycosides depend on the enzymatic inactivation of the antibiotic, through acetyl-, phosphoryl- or nucleotidyl-transferases; altogether, there are more than 150 different known genes encoding such enzymes. A few methyltransferases, encoded by *rmt* genes, have been more recently described in gram-negatives; and a bifunctional acetyl- and phosphoryl-transferase, that inactivates most aminoglycosides in clinical use, is common in gram-positive cocci.
- *M and MLS*$_B$ *resistance*. In the MLS$_B$ (macrolide, lincosamide, streptogramin B) antibiotic group, macrolides are the most diverse and commonly used ones. Macrolide resistance is mediated by two main mechanisms: specific efflux, mediated by *mef* genes, whose products expel only macrolides (M phenotype); and ribosomal protection via methylation of the 23S rRNA by methylases encoded by *erm* genes, which result in cross-resistance towards lincosamides and streptogramins (MLS$_B$ phenotype), as these antibiotics bind to the same ribosomal region. From the clinical point of view, *mef*-mediated resistance is of low level and, perhaps, of little relevance, as increased MIC are still below plasmatic and tissular concentrations (Anzueto and Norris, 2004). However, as many *erm* genes are inducible, resistance breakpoints in the clinical lab have been set low enough to include fully-resistant, *erm*-bearing bacteria along with *mef*-bearing "resistant" organisms. Aside, a short list of macrolide-inactivating enzymes have been recently described, although many of them in Enterobacteriaceae and other gram-negatives considered to be intrinsically resistant to these antibiotics (except for *vat* transferases, found in gram-positive cocci).
- *Resistance to amphenicols*. The most common mechanism of resistance to chloramphenicol and thiamphenicol is enzymatic inactivation through acetyltransferases, CAT; florfenicol, only used in animals, is not affected by these enzymes. A few other genes encode for efflux systems: *cmlA*, also ineffective

against florfenicol; and *floR*, which confers resistance to chloramphenicol and florfenicol alike. A ribosomal methyltransferase encoded by *cfr* genes, whose action prevents the binding of florfenicol to the ribosome, is important as it also mediates resistance to other clinically-relevant antibiotics, such as linezolid.

- *Tetracycline resistance*. There are more than 40 *tet* genes mediating tetracycline resistance. Most of them encode specific efflux pumps, while about 10 mediate a ribosomal protection mechanism, and 5 or so an inactivating enzyme found only in gram-negatives. Although *tet* genes have not been implicated in tigecycline resistance, the extensive use of tetracyclines for agricultural purposes, along with the linking of *tet(M)* and *erm(B)* genes in mobile genetic elements (Moritz and Hergenrother, 2007), have fostered the interest in tetracycline resistance determinants.
- *Resistance to glycopeptides*. Being antibiotics that uniquely bind to a substrate rather than an enzyme or ribosome component, resistance mechanisms are also peculiar. The binding of glycopeptides to the D-Ala-D-Ala terminus of a peptidoglycan precursor is prevented by changing this terminus to D-Ala-D-lactate or, less commonly, to D-Ala-D-Ser. The changing of D-Ala-D-Ala to D-Ala-D-Lac involves at least a D-D dipeptidase to remove the last D-Ala, a lactate dehydrogenase that synthesize the D-lactate, and a ligase that binds the D-lactate. *vanA* and *vanB* are actual operons; although some of the genes included into these came probably from soil bacteria (*Paenibacillus popilliae*), the codon usage of the genes differs, suggesting a different origin for each of them. The expression of these operons is induced by the glycopeptides themselves: *vanA* is induced both by vancomycin and teicoplanin, while *vanB* is only induced by vancomycin (Fraimow, 2003).
- *Resistance to lipopeptides*. Acquired resistance to polymyxins are often mediated by changes in outer-membrane lipopolysaccharides, or even the complete loss of them, which are necessary for the initial binding of the drug to the bacterial envelope (Olaitan et al., 2014). Although such modifications can be accompanied by diminished virulence and resistance to other antibiotics, they still pose a significant health threat. Resistance to daptomycin is still rare; resistant enterococci isolates have mutations in enzymes involved in phospholipid metabolism, as well as in a putative membrane protein (Arias et al., 2011); no inactivation was detected in a previous report, although *Actinoplanes utahensis* is capable of deacylating daptomycin to an inactive derivative (Montero et al., 2008). In *S. aureus*, resistance is also linked to membrane and wall changes: genes responsible for the synthesis of lysyl-phosphotidylglycerol and for the D-alanylation of teichoic acids are among those mutated in resistant isolates (Bayer et al., 2013).
- *Resistance to DHPS inhibitors*. Mutations in the chromosomal dihydropteroate synthase gene, *dhps*, have been identified as causing sulfonamide resistance; these can go from point mutations in *E. coli*, to a 10% difference in nucleotide sequence found in *Neisseria meningitidis*, more likely to have arisen by transformation-recombination. However, in most pathogens is much more common to find genes *sulI* and *sulII*, both closely related but with significant sequence divergence from chromosomal *dhps* genes. These *sul* genes encode drug-insensitive enzymes that by-pass the effect of sulfonamides; they are often found in plasmids and, more precisely, in the conserved regions of integrons (Huovinen et al., 1995).
- *Resistance to DHFR inhibitors*. A number of *dfr* genes, encoding a non-allelic, drug-insensitive dihydrofolate reductase, can by-pass the blockage exerted by

trimethoprim and related agents upon the synthesis of tetrahydrofolate. Most *dfr* genes are grouped into families A and B, and are mostly found in gram-negatives; while a few other individual genes (*dfrC*, *dfrD*, *dfrG* and *dfrK*) coming from gram-positives.

– *Resistance to nitrofurans*. Being nitrofurans pro-drugs that must be "activated" by bacterial reductases, the loss of such enzymes (nitroreductases encoded by *nfsA* and *nfsB*) is an obvious and easy way to acquire resistance. However, such a loss has a high fitness cost (Sandegren et al., 2008) that makes resistant bacteria very weak, hence their low prevalence. A similar resistance mechanism has been reported for related drug furazolidone (Martínez-Puchol et al., 2015). It is likely that intrinsically resistant bacteria lack this kind of reductases. A 30-years old report (Breeze and Obaseiki-Ebor, 1983) of plasmid-mediated nitrofuran resistance did not advance much on the possible biochemical mechanism; however, plasmids were more recently reported to be linked to nitrofurantoin resistance in clinical isolates of uropathogenic *E. coli* (Arredondo-García and Amábile-Cuevas, 2008).
– *Resistance to quinolones*. Most clinically relevant, quinolone resistant bacteria, bear mutations on target genes, *i.e.*, those encoding affected topoisomerases (*gyr* and *par* genes in enteric bacteria). While single mutations are often enough to confer resistance to nalidixic acid, two or more are necessary to confer resistance to fluoroquinolones. Many combinations of mutations are known to enable the survival of bacteria in previously inhibitory concentrations of the drugs (Fuchs et al., 1996). During the early years of quinolone usage, the lack of plasmid-mediated resistance (Courvalin, 1990) was highlighted as an interesting feature of this class of antibiotics, perhaps predicting a slower spread of resistance among clinically relevant bacteria. The recessive nature of the trait supported the notion that horizontal transfer was not likely. This was shown to be wrong, as the acquisition of mutated topoisomerase genes through transformation was demonstrated among streptococci (Balsalobre et al., 2003, Ferrándiz et al., 2000) (homologous recombination after transformation enabled the replacement of the wild-type gene). Also, a number of plasmid-borne, horizontally transferable *qnr* genes have been reported, starting from the 1990's. These genes encode pentapeptide repeat-containing proteins that possibly prevent the binding of topoisomerases to DNA, preventing DNA cleavage, but without actually inhibiting the enzymes' activity somehow. *qnr* genes do not confer full fluoroquinolone resistance, and only increase the MIC, from $\leq$0.01 μg/mL, to 0.12–0.5 μg/mL (resistance breakpoints are usually 4 μg/mL or higher). However, they seem to play an important role in fostering the ability of bacteria to survive in higher concentrations of quinolones, increasing the likelihood of gaining full resistance by other means. A quinolone-modifying enzyme, encoded by *aac(6′)-Ib-cr*, derived from an aminoglycoside acetyltransferase gene, has also been found in plasmids, mediating a low-level resistance phenotype as well (Strahilevitz et al., 2009).
– *Resistance to oxazolidinones*. Resistance to linezolid is still rare. A number of mutations in the domain V of the 23S rRNA gene, and in L3 and L4 ribosomal proteins, have been found in clinical resistant isolates (Campanile et al., 2013). Most interesting for the purposes of this book, is the ribosomal methyltransferase encoded by the *cfr* gene: this gene is plasmid-borne, and confers resistance to antibiotics used in veterinary medicine, such as florfenicol, lincosamides and

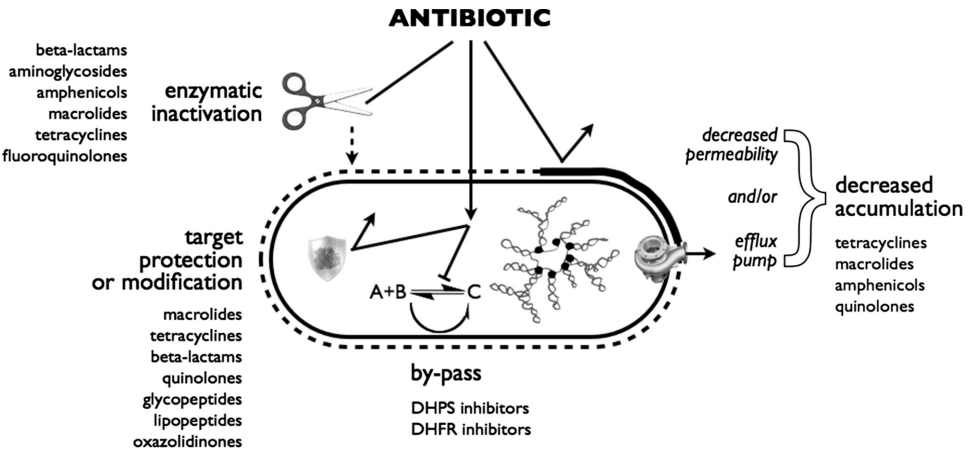

Figure 1.2 **Mechanisms of acquired, specific resistance; a graphic summary.** Four general mechanisms of resistance to individual antibiotics (or individual classes of antibiotics): enzymatic inactivation, decreased accumulation (in turn derived from decreased permeability and/or efflux pumps), target protection or modification, and pathway by-pass. This figure does not include a fifth mechanism, *i.e.*, the loss of target (as in lipopeptide-resistant bacteria that do not produce LPS) or of activating enzyme (as in nitrofuran-resistant bacteria that do not produce the nitroreductases needed for activation of the prodrug). Also, non-specific mechanisms are not included, such as unspecific efflux systems that provide a multi-resistance phenotype, as discussed below.

pleuromutilins. Staphylococcal strains of animal origin may be acting as reservoirs of these genes, that are now found in linezolid-resistant clinical isolates (Tewhey et al., 2014). The activity of tedizolid, a newer oxazolidinone, is not affected by *cfr* genes, and minimally affected by ribosomal mutations. A recently reported *otprA* gene, found in enterococci both, from food-animals and humans, confer resistance to both, linezolid and tedizolid, as well as to amphenicols (Wang et al., 2015).

– *Resistance to metronidazole*. In anaerobic *Bacteroides*, *nim* genes have been identified as able to confer resistance to metronidazole. Apparently, *nim* genes encode a reductase that reduces the 5-nitroimidazole to a 5-amino inactive derivative (Soares et al., 2012).

1.2.1.1.3 Adaptive resistance: stress responses

Bacteria often change suddenly from one environment to another, each change involving conditions that vary widely. To survive these variations, many bacterial species are equipped with complex, overlapping, but unspecific defense systems; some of them can confer "resistance" to several antibiotics, usually by diminishing the permeability of the outer membrane, and/or by overexpressing efflux pumps. These "resistance" phenotypes are unspecific, providing protection towards a variety of antibiotics and other xenobiotics; are transient, their effects lasting only the duration of the exposure to the inducing stimuli; and increases in antibiotics' MICs are only mild and rarely above full-resistance clinical breakpoints. From the clinical diagnostic point of

view, these mechanisms do represent a challenge, as inducing conditions that can be found *in vivo*, are seldom present *in vitro*, being therefore undetectable by the typical susceptibility assays. Mutations in the regulatory genes that result in the constitutive expression of these systems can be construed as "acquired resistance"; however, due to the unspecificity of their protective capabilities, there could be a vast variety of selective and maintenance pressures favoring such mutants, other than antibiotics themselves. Hence, the presence of such mutations can hardly be related to an specific antibiotic, or even to antibiotics as a group. Finally, a wide variety of unrelated mutations can enhance the ability of bacteria to tolerate slightly inhibitorial concentrations of antibiotics; affected genes can be called "susceptibility genes", instead of "resistance" ones, but the end result is the same if mutated: low-level resistance, but allowing for an additive nature that can result in full-resistance (Girgis et al., 2009).

Two well-known regulons of *E. coli* and related enteric bacteria that are involved in antibiotic resistance phenotypes, are the *soxRS* regulon, governing the response to superoxide stress; and the *marRAB* regulon, that regulates a response to a number of chemical insults (Demple and Amábile-Cuevas, 2003). These two regulons overlap extensively. The genes for the efflux system AcrAB-TolC, and *micF*, a gene that encodes an antisense RNA that post-transcriptionally represses the expression of OmpF, are included in both regulons. Overexpression of AcrAB-TolC and repression of OmpF result in decreased accumulation of several antibiotics and, in turn, diminished susceptibility. "Resistance" achieved through these mechanisms is rarely enough to be of clinical relevance alone (although *marR* was formerly called *cfxB*, a quinolone resistance gene from a clinical isolate). However, several features of the *soxRS* and *marRAB* regulons are of particular relevance to the general issue of antibiotic resistance, and to the particular situation in the environment: (1) gained "resistance" can add up to a full-resistance phenotype, if coexisting with other low-level resistance determinants, *e.g.*, a *gyr* single-mutation (Heinemann et al., 2000); (2) the activity of different, structurally-unrelated antibiotics is affected by the overexpression of these regulons (*mar* is, after all, an acronym for *m*ultiple *a*ntibiotic *r*esistance); (3) a wide variety of compounds can induce the expression of these regulons, including oxygen and nitrogen reactive species released by activated macrophages (Nunoshiba et al., 1993); several antibiotic (*e.g.*, chloramphenicol, tetracycline (Davin-Regli and Pagès, 2007)) and non-antibiotic drugs (*e.g.*, aspirin (Demple and Amábile-Cuevas, 2003), phenazopyridine (Amábile Cuevas and Arredondo García, 2013)); the lack of iron (Fuentes et al., 2001); and environmental pollutants such as mercury (Fuentes and Amábile Cuevas, 1997) and herbicides (Kurenbach et al., 2015), to mention a few; and (4) mutations in the regulatory genes can result in the constitutive expression of the "resistance" phenotype; such mutations can then be selected by the wide variety of compounds these regulons protect against, including those that act as inducers, and/or other agents against which protection is elicited (*e.g.*, ozone (Jiménez-Arribas et al., 2001) or triclosan (Levy, 2002)). Although protection against oxidative stress can be considered mostly a chromosomal trait, a recent report of a mobile genetic element in *Legionella pneumophila* that confers resistance to hydrogen peroxide and bleach, along with beta-lactam antibiotics (Flynn and Swanson, 2014), opens the possibility of such oxidative stress protective genes to be horizontally acquired.

There is a number of other regulatory proteins that control the expression of efflux pumps in Enterobacteriaceae (*e.g.*, Rob, RamA, PqrA, AarP, AcrR, EmrR), each with

its own set of chemical or physical effectors; and a number of efflux pumps other than AcrAB-TolC –37 putative genes in *E. coli* alone (Davin-Regli and Pagès, 2007). The description of the role of each one in adaptive resistance to antibiotics is far from completion. In *P. aeruginosa* the scenario is also complex: the outer membrane porin OprD, and several efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY and MexJK, all of the resistance-nodulation-division, RND family) are involved in multiple antibiotic resistance (Lister et al., 2009). Again, the overexpression of Mex efflux pumps, or the repression of OprD porin, alone, are not enough to confer full-resistance; but when coexisting with other low-level resistance mechanisms, they can add up to a complete protection against clinically-achievable antibiotic concentrations. For instance, diminished expression of OprD, and overexpression of chromosomal beta-lactamase AmpC, can result in carbapenem resistance; and overexpression of MexXY along with aminoglycoside-modifying enzymes can allow *P. aeruginosa* to withstand extremely high concentrations of amikacin. As discussed before, mutations that result either in diminished expression of the porin, and/or increased expression of efflux pumps, lead to a stable multi-resistance phenotype. A number of regulated efflux systems have also been implicated in antibiotic multi-resistance in the opportunistic pathogen *Acinetobacter calcoaceticus-baumannii* (Coyne et al., 2011).

Back to *E. coli*, it is worth to mention that plasmid-mediated efflux pumps known to mediate antibiotic resistance have been described: a conjugative plasmid bearing genes *oqxAB* encode a TolC-dependent efflux system that confers resistance to olaquindox, a bacterial DNA-synthesis inhibitor used as growth promoter in pigs, and also to chloramphenicol (Hansen et al., 2004). By "jumping" to mobile elements, genes that have so far been considered to mediate adaptive or perhaps even intrinsic resistance, could be transferred to other, clinically-relevant bacterial species.

In gram-positives, of course, outer membrane porins are absent; but unspecific efflux systems have been identified, such as the PmrA pump in *S. pneumoniae*, and the Bmr and Blt pumps in *Bacillus subtilis*, whose overexpression reduce the susceptibility to quinolones and other, unrelated compounds (Brenwald et al., 2002). An overview of bacterial antibiotic efflux pumps can be found in Van Bambeke et al. (2003).

1.2.1.1.4 Adaptive resistance? Biofilms

An entirely different set of conditions that enhance the bacterial ability to survive antibiotic exposure result from biofilm formation. Unlike the inducible mechanisms described above, in this case the resistance phenotype is not gained by individual cells, but by the whole biofilm community, by a variety of mechanisms that are yet to be fully understood, and that vary from one bacterial species to the other (Gilbert et al., 2007). Rather than resistance, the word "persistence" seems more adequate: when a biofilm is exposed to antibiotics, many bacterial cells are killed, mainly at the outer layers of the biofilm; but a number of "persisters" are not, making the whole biofilm to survive the exposure and to thrive again afterwards. From the clinical perspective, this means that once the antibiotic treatment is completed, persisting biofilm is still there to grow again, restarting the infectious process; as most infectious episodes are caused by biofilms, and as this phenotype cannot be detected by the usual antibiotic susceptibility assays, it is likely that biofilms are behind therapeutic failure of antibiotic treatments against bacteria deemed susceptible by the clinical lab.

The diminished activity of antibiotics against biofilms is listed here as a form of adaptive resistance, as biofilm formation is often regarded as an inducible, chromosome-mediated ability that allows bacteria to colonize surfaces, hence resistance is only gained when bacteria are growing within such microbial communities. However, biofilm formation itself can be an acquired trait, as conjugative plasmids can mediate biofilm development (Ghigo, 2001). This imply that acquired biofilm-formation traits could also be considered as acquired resistance mechanisms.

In addition to the persistence phenomenon, biofilms and antibiotics interplay at multiple levels: (a) aminoglycoside antibiotics can induce the formation of biofilms (Hoffman et al., 2005); (b) macrolide antibiotics can inhibit the formation of some biofilms (Wozniak and Keyser, 2004), while promoting the formation of others (Wang et al., 2010); (c) biofilm-forming *P. aeruginosa* strains carry more resistance traits than non biofilm-formers (Delissalde and Amábile-Cuevas, 2004); (d) a biofilm can be considered as a significant playground for all sorts of horizontal gene transfer (Amábile-Cuevas, 2013, Amábile-Cuevas and Chicurel, 1996), hence promoting the spread of antibiotic resistance genes, although plasmids seem to be less frequently found in biofilm-forming Vibrionaceae (Xue et al., 2015) and *P. aeruginosa* (Delissalde and Amábile-Cuevas, 2004); and (e) species interactions within biofilms can favor mutations that enable symbiotic, specialized associations (Hansen et al., 2007) that, although not been demonstrated in the context of antibiotic exposure, remain as an intriguing possibility.

1.2.1.2 Co-selection: the plot thickens

Antibiotic usage leds to antibiotic resistance; this we know for sure. But it is not quite as simple: there are non-antibiotic agents that can select for antibiotic resistance; and antibiotics can select traits different from antibiotic resistance. Furthermore, some antibiotics can select for resistance to other, unrelated antibiotics. Most of these interactions are based on co-selection, and have been reviewed before (Amábile-Cuevas, 2013); cross-resistance (*i.e.*, a single resistance mechanism providing protection against several drugs on the same family, or even against chemically unrelated compounds, such as the MLS_B phenotype) that can also account for some of these phenomena, is rather obvious. Important to keep in mind while discussing resistance in the environment are the following:

– Genetic linkage of antibiotic resistance determinants and some other traits, can explain why non-antibiotic agents, or unrelated antibiotics, select for antibiotic resistance genes. For this to happen, resistance genes must reside on the same genetic element: many antibiotic resistance genes have been found along with heavy-metal (*e.g.*, mercury, cadmium) and/or disinfectant (*e.g.*, quaternary ammonium compounds) resistance genes in the same plasmids or other mobile elements. Hence, the presence of such compounds select for the entire genetic element that carries antibiotic resistance determinants, in the absence of antibiotics. It is perhaps relevant to state that the same, or even worst confusion over the definition of "resistance" prevails when referring to disinfectants (Gilbert and McBain, 2003). However, while the role of genes that confer only protection against slightly higher disinfectant concentrations could be negligible in houses or hospitals, it may be

particularly relevant in environmental settings where such biocides are diluted. About the same can be said about antibiotics: integrons and transposons (see below) often carry a sulfonamide-resistance gene along with genes conferring resistance to other, unrelated antibiotics. Sulfonamides are among the very few antimicrobial compounds that can be detected at relatively high concentrations in wastewater; therefore, it can select for such multi-resistance genetic elements, in the absence of other, more labile compounds.

– Typical examples of cross-resistance, such as *gyr/par* mutations that protect against almost all quinolones, or *erm* genes that protect against macrolides, lincosamides and streptogramins, are very obvious: the presence of ciprofloxacin would select for ofloxacin or norfloxacin resistance, and the presence of streptogramins would select for clarithromycin resistance. Despite the very simple nature of this assertion, the use of some antibiotics as "growth promoters" circumvented a restriction for using clinical antibiotics by neglecting known cross-resistance: enrofloxacin, while not used in humans, select for resistance to other fluoroquinolones; avoparcin select for resistance to other glycopeptides, such as vancomycin; virginiamycin select for resistance to other streptogramins, such as quinupristin/dalfopristin. Furthermore, other mechanisms of low-level multi-resistance, such as those resulting from unspecific efflux, can be induced by a variety of non-antibiotic agents, and mutants constitutively expressing such mechanisms can be selected by the same kind of compounds.
– By the same token, antibiotics can select for a variety of traits different from antibiotic resistance. Virulence genes have been found linked to resistance ones on the same genetic element, for instance; antibiotics can therefore be selecting for resistant, virulent bacteria. It is even possible that antibiotics can be increasing the prevalence of mobile elements in bacterial populations, either by selecting bacteria that carry resistance plasmids, transposons or integrons; and/or by selecting bacteria that are more permissive of such kinds of extrachromosomal DNA molecules.

1.2.1.3 Inter-molecular gene mobilization: the gene "cut & paste" bacterial kit

Perhaps the most striking feature of the antibiotic resistance crisis is the very high frequency with which multi-resistant (*i.e.*, resistant to three different classes of antibiotics) organisms are isolated in clinical settings. These multi-resistance phenotypes, that are also often transferable in single HGT events, indicate that the accumulation or gathering of resistance determinants in single genetic elements is common. Although the accumulation of resistances in single organisms, due to successive exposure to individual drugs, is merely the consequence of such successive exposure, along with a low rate of spontaneous loss of resistance determinants; the accumulation of resistance genes in single genetic elements, mainly plasmids, is the result of additional phenomena. These involve a number of genetic elements capable of mobilizing between DNA molecules: insertion sequences (IS), transposons, integrons, and gene cassettes, are among the best characterized of them. Although it is not within the purpose of this book to make a detailed review of the nature of each of these elements, there are some important features to highlight.

An old classification of the gene rearrangements mediated by transposons – and extensive to integrons and gene cassettes, put them under the "illegitimate recombination" label; this is to mean that no extensive sequence homology is needed for the insertion of such elements into target DNA molecules, other than short regions (hotspots, attachment sites) that serve as substrate for transposase or integrase enzymes. Through these recombinatorial events, gene cassettes can be inserted, excised and shuffled within integrons; integrons can become linked to transposons; and transposons can "jump" between plasmids, and between plasmids and chromosomes. The result is a sort of Matrioshka doll of nested mobile genetic elements (Amábile-Cuevas and Chicurel, 1992). This picture describe more or less accurately elements such as plasmid R100: a 94.5-kb conjugative plasmid that contains transposons Tn*10* and Tn*2670* (the later formed by the insertion of Tn*21* into Tn*9*), which in turn contains a class-1 integron, with a single gene cassette inserted; as such, this plasmid, isolated from a *Shigella flexneri* strain in the 1950's, encode resistance to tetracycline, chloramphenicol, sulfonamides, streptomycin, spectinomycin, quaternary ammonium disinfectants, and mercury (Bushman, 2002). Complex arrays of resistance genes can be found in a single integron, as is the case of In53, carrying genes for two beta-lactamases, four aminoglycoside-modifying enzymes, a chloramphenicol-modifying enzyme, along with a *sul1* sulfonamide-resistance gene, and two *qac* quaternary-ammonium compounds' resistance genes (Naas et al., 2001). Some particular details of each of these mobile elements, that are important to understand their role in the spread of antibiotic resistance genes, are:

- ISs apparently play a minimal role on antibiotic resistance in gram-negatives (*e.g.*, increasing mutagenesis, or inserting promoters upstream silent resistance genes (Amábile-Cuevas, 1993)); but IS*257* seems pivotal in the mobilization of resistance genes in gram-positives (Firth, 2003).
- Transposons seem to mobilize preferentially to plasmids than to chromosomes. This was recognized since the early characterization of these elements, as plasmids were described as "collections" of transposons 40 years ago (Cohen, 1976). About one half of plasmids in a genome database carry at least one IS, with an average density of one copy every 19 kb (contrasting with only 8% of phages), an observation later extended to transposons (Leclercq et al., 2012). Furthermore, some transposons seem to prefer conjugating plasmids as targets for transposition (Wolkow et al., 1996).
- There are several classes of integrons, but class-1 and -2 are the most commonly linked to antibiotic resistance, and are considered to be mobile, because they are mostly found in plasmids and other mobile elements. Integrase I seems to derive from XerC/D recombinases, having Vibrionales as a sort of bridge towards clinically-relevant enteric bacteria and Pseudomonadales (Díaz-Mejía et al., 2008). While integrons are mostly chromosomal in aquatic bacteria, such as *Vibrio* and *Shewanella*, they are more commonly found in plasmids when in *Pseudomonas* and enterics. Integrons are also frequently found in gram-positives, especially those with similar codon usage, such as *Corynebacterium* (Díaz-Mejía et al., 2008).
- For gene cassettes to be integrated into integrons, two integrase-specific recombination sites are necessary: an *attI* site at the integron, and an *attC* site at the cassette. Most curiously, gene cassettes are composed of a single gene and the *attC*

site, suggesting that reverse transcription of mRNA molecules are at the origin of such elements. Group IIC introns have been proposed as responsible for the formation of gene cassettes, providing the *att*C site to unrelated genes, and the retrotranscription machinery to form the cassette (Léon and Roy, 2009). A class 1 integron bearing a group II intron was found in an *E. coli* from a wild Norway reindeer (Sunde, 2005), indicating that this conjunction likely existed before and without antibiotic intervention.

– Transposition and integration, in several instances, are increased when the host cell has activated its SOS response. From the early reports of the transposase of IS*50* and derived composite transposons (*e.g.*, Tn*5*) being repressed by LexA (Kuan and Tessman, 1991); to the more recent discovery of induction of integrons' integrases by an activated SOS response, which fosters the acquisition and rearrangement of gene cassettes (Guerin et al., 2009). Antibiotics themselves play a role in inducing the mobilization of transposons and integron gene cassettes (Courvalin, 2008).

Through transposition and integration, resistance genes can be mobilized between DNA molecules residing within a single bacterial cell. Such rearrangements can allow a better expression profile of gene cassettes, "adequating" it to the environmental conditions; can be fostered by the exposure to environmental stress; and allow the assembly of complex, nested, multi-resistance mobile elements. But the substrate genes for such formidable arrangements come from very different cell lineages, as has been demonstrated by sequence homology and codon usage. Therefore, these mechanisms alone may have been meaningless without the ability to exchange genetic information between cells: the horizontal gene transfer.

1.2.1.4 Horizontal gene transfer: the main means for resistance spread

Horizontal gene transfer (HGT) seems to be a peculiar feature of prokaryotes: 30–50% of Bacteria have at least one protein domain acquired through HGT, while less than 10% of Eukarya do (Choi and Kim, 2007). The extent of this transfer establishes a "network of genomes" (Dagan and Martin, 2009) among bacteria, of a magnitude and consequences difficult to grasp. If an old calculation of foreign DNA in the genome of *E. coli*, of about 12.8% (Ochman et al., 2000), still holds, and if it is somehow representative of similar bacteria, it is at least easy to realize that HGT is quite common. There are three main mechanisms of HGT (transformation, transduction, and conjugation), each with a number of theme variations; but, before briefly reviewing these mechanisms, lets enlist some traits that are known to have been mobilized in these ways.

Of obvious relevance for the purpose of this book, hundreds of genes directly mediating antibiotic resistance are known to have been mobilized horizontally. But resistance genes have arguably been caught in this gene flux only recently, as the human use of antibiotics mounted a sudden and dramatic selective pressure. Aside from resistance genes, it is generally accepted that not all kinds of genes are equally transferable, establishing an initial bias as to the kind of genetic information that can be exchanged. For instance, genes that are part of complex systems, such as transcription or translation (named "informational genes") are less frequently transferred than those that act relatively on their own mediating housekeeping traits ("operational genes") (Jain et al., 1999). Within these housekeeping genes, those coding for secreted

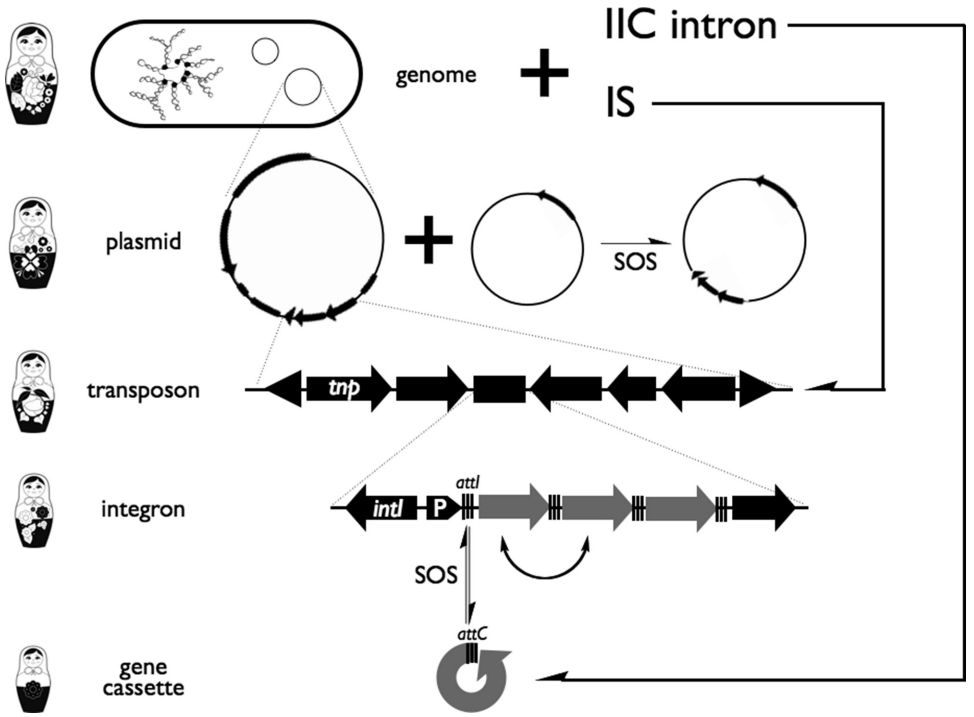

Figure 1.3 **Intra-cellular gene mobilization; a graphic summary.** A series of genetic elements abound within bacterial cells, many in a nested fashion resembling a Matrioshka doll. The whole genome of a bacterial cell is formed by one (or two) chromosomes, and one or several plasmids. Plasmids, usually ranging from 1 to 10% of the size of the chromosome, can be seen as "accessory" gene elements, or as sub-cellular forms of life. They can act as collections of transposons, that can go from the simple insertion sequence (IS, containing only genes for transposase and resolvase, and substrate sequences for transposase), to very complex sets of genes, including antibiotic resistance ones. Some transposons include other kind of element, the integron, that is essentially formed by an integrase (*intI*) gene, a promoter sequence, and an attachment site (*attI*); the integrase allows the recombination between *attI* site of the integron, and the *attC* site of a gene cassette, a non-replicative DNA circle containing a single gene and an *attC* sequence. SOS responses can induce transposition and integration, allowing the mobilization of transposons between coresiding DNA molecules, and/or the acquisition, excision, or rearrangement of gene cassettes. Composite transposons can result from the insertion of ISs flanking a gene or set of genes; and gene cassettes from group CII introns and their associated retrotranscription activities.

proteins and for outer membrane proteins seem to be more prone to mobilization than those that encode periplasmic, cell membrane, or cytoplasmic proteins (Nogueira et al., 2009). Furthermore, most analysis of mobilized genes are centered on protein-coding sequences; but a recent paper suggests that there is a sort of regulatory "switching" that can explain the expression divergence between strains; and that such switching occur through HTG of regulatory regions (Oren et al., 2014). With regulatory genes also included in the gene pool formed by HGT, and adaptive resistance included in