An overview of multi-antibiotic resistance in pathogenic bacteria - from selected genetic and evolutionary aspects - A review

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ABSTRACT

The challenge posed by multi-drug resistance (MDR) of pathogenic organisms, spectacularly manifested in the 6 “ESKAPE” bacterium (two Gram-positive, four Gram-negative) species, should invoke new comprehensive strategies, and needs cooperation of scientists with medical, veterinary and natural science background. This review is aimed at informing newcomers, coming from the field of biology and genetics, about problems related to rapidly emerging, new multi-drug resistant, pathogenic bacteria. Unlike persistence, the antibiotic resistance is inherited. A functioning “resistance gene” makes a susceptible organism resistant to a given antibiotic, encoding for polypeptides capable of acting either as decomposing enzymes, or acting as trans-membrane pumps, or membrane structure components capable of modifying the permeability implementing a “by pass” mechanism enabling the antibiotic molecule to reach its cellular target(s). A functioning “sensitivity gene” encode for a polypeptide, capable (directly or indirectly) of transferring toxic molecules into target cells, or of metabolizing non-transferable to transferable, or non-toxic molecules to toxic derivatives. A gene of a normal function could act as a “sensitivity” gene in the presence of antibiotics of chemical structures similar to the natural substrate of the gene product, (enzyme or binding/trans-membrane protein). The Agrocin 84 story is a good example. Multi-drug resistance is a phenotypic consequence of the sequential accumulation of mutations, and/or up-take of plasmids or genomic islands carrying resistance genes from the environment via horizontal gene transfer, mediated by conjugative plasmid or bacteriophage carrying mobile genetic elements. Both multi-drug resistance and collateral sensitivity are evolutionary products. Some revealed evolutionary process and their Lamarckian and Darwinian interpretations are discussed. Toolkits of comparative full-genome sequencing, genomics, experimental evolution and population genetics may provide perspectives for overcoming the invincibility of multi-drug panresistance. The status of some recently emerging pathogenic bacterium species with zoonic features and of veterinary background is also discussed.
**KEYWORDS:** ESKAPE-bacteria; Persistence; Resistance; Intrinsic/Acquired/ Multidrug (MDR) and Pan – Resistance; Genetic background; Experimental Evolution; Collateral sensitivity; Agrocin.
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1. Introduction

Multi-drug resistance (MDR) of pathogenic bacteria is an extremely complex field of life sciences that needs the expertise of physicians, (docs, vets), microbiologist, biochemists, theoretical and preparative organic chemists, bioinformatics, geneticists, and evolutionary biologists. This review is prepared first of all for geneticists and biologists who are newcomers to life science background without clinical or veterinary experience. It is well known today, that although the antibiotics are extremely important therapeutic tools in human, veterinary and even plant medicine, their use has gradually become limited because of resistance problems. The phenomenon of antibiotic-resistance was first discovered as early as 1940 [1]. Whenever pathogenic microorganisms are exposed to the selective pressure of antimicrobials, either in the laboratory, medicine, or agriculture, it is favorable for the development, survival and spread of resistant clones [2]. Resistance means non-susceptibility to given antibiotics. When an isolate of a given pathogenic bacterium is resistant to more than one antibiotic, the options for antibiotic therapy of the disease caused by this pathogen is decreased. The emergence of antibiotic multi-resistance in pathogenic bacteria has become alarming in the recent decades. As an example, 1,481 patients died in Hungarian hospitals in 2016. In 174 of those deaths, infection was the cause of death, or was involved in it, according to the (Hungarian) National Epidemiological Center. Last year, 4,830 MDR infections were reported, compared with 4,187 in 2015 and 3,998 in 2014. Mostly urinary tract infections occurred, followed by infected wounds, blood vessel infections, and hospital-related pneumonia. A majority of the patients were above 60 [3].

Infections caused by multi-resistant bacteria have dramatically increased not only in Hungary, but all over the world, invoking an enormous public concern. There are not human clinical, [4-7] but zoonic [8] and veterinary [9-15], as well as plant health aspects [16-18] come forward alarmingly.

A spectacular plant example is the increasing number of streptomycin-resistant Erwinia amylovora isolates, (the pathogen of the “fire blight” of Rosaceae, including apple trees) causing serious difficulties in the treatment of severe plant infections both in the USA [19] and in Europe [20]. Although application technology has been improving revolutionarily [21], the trend is that the application of antibiotics for clinical use as plant medicines has been increasingly more restricted [22].

All this has been motivating research to introduce not only new antibiotics, but environmentally friendly plant medicines as well, with novel modes of action. A rational approach for elaborating effective therapies has been based on the better understanding of the different bacterial mechanisms of drug resistance, especially for Gram-negative pathogens, [4, 5, 7].

When reporting a radical and continuous decrease in the number of new antibiotics in the market, Canadian authors [23] asked in 2005:

- “Where are all the new antibiotics?”

Eleven-years later, a late answer appeared in ‘Nature’:

- “Antibiotics” (are) ”right under our nose”! [24].

It is good news and may be true. But unfortunately they still have not been in the market; at least not in the required numbers. Their number seems to be much less than needed for really effective control of multi-resistant pathogens [4]. In the period 2003-2007 only 5 new antibiotics appeared. In 2009 there were 16 new molecules listed as being in clinical trials phase, with only 2 in the pipeline; including 3 glycopeptides, 4 quinolones, 2 oxazolidinones, 2ß-lactams, 1ß-lactamase inhibitor, 1 trimethoprim, 1 macro-ketolide, 1 streptogramin, and 1 glycylic-cycline [25].

It is encouraging that well-qualified top scientists have been working on better understanding of the process, solving the newly appearing and spreading resistance problems, and working on new approaches all over the world [26]. The scientific approach based on a better understanding of the different bacterial, genetic and evolutionarily mechanisms of drug resistance [5], aimed at “reducing the bottle neck in the discovery of new antibiotics” [27]. This new approach is based on transcriptome analysis, and exploiting the options provided by using RNA sequencing (RNAseq) to identify promising novel antimicrobial compounds from microbial extracts.

The analysis of the reasons of the moderate interest of the pharmaceutical industry toward new antibiotics is not in the scope of this article.

This review intends to focus on a few selected aspects, such as:

1. A short overview of the list of the most significant bacterial pathogens which cause the most striking examples of MDR outbreaks;

2. Some genetic and evolutionary mechanisms, leading to increase or decrease in the frequencies of multi-drug

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resistant pathogen bacteria around us;

(3) Some evolutionary and coevolutionary mechanisms (co-existence, horizontal gene transfer) channelizing these
two-way movements, weighted by the genetic load of newly acquired antibiotic resistance.

2. The “Card Game” of antibiotics research scientists and antibiotic resistance. New antibiotic drugs
invoke new resistances; it is just question of time.

2.1. Multi-drug resistance: Definitions and nomenclature (Based on phenotype and origin of multi-drug
resistance)

The resistance to an antimicrobial compound means non-susceptibility to a given antibiotic molecule. From practical
aspects, one can distinguish between multi-resistant pathogens based on qualitative and quantitative profiles. A recent
classification defines (i) multi-drug resistant (MDR) strains and isolates, which are not susceptible to (at least) one
representative of each of three categories of antimicrobial compound families; (ii) extreme drug resistant, (XDR),
which are not susceptible to (at least) one representative of all but very few categories of antimicrobial compound
families; and (iii) pan-drug resistant (PDR) ones, which are not susceptible to any of the tested representatives of all
known antimicrobial compound families [28].

The resistance to an antimicrobial compound is an inherited character (phenotype) determined by the presence and
expression of a respective “resistance gene”. This gene can be localized in the bacterial chromosome, or in an
extrachromosomal element, which is most frequently a plasmid, and in a rarer, but worse case, an episome, capable
of being inserted into the chromosome permanently. The origin of the resistance could be a mutation, changing the gene
which had originally been present, resulting in structural and functional changes of the original gene product. When
this is the case, the literature calls it “intrinsic” resistance. If the resistance to an antimicrobial compound is a
phenotypic consequence of the activity of a resistance gene that has been harbored by a plasmid taken-up from the
environment, the literature calls it “acquired” resistance [29]. An antibiotic resistance gene is most frequently a coding
gene, (an open reading frame) located, organized and regulated in a so-called antibiotics resistance cassette, which is
most frequently harbored and transferred by some mobile genetic element. Considering that mobile genetic elements
are capable of separating from, and integrating into, any available bacterial DNA (chromosome, plasmid or even
phage), and vice-versa, that event, called horizontal gene transfer (HGT), is possible in more than one step between
bacteria, (including pathogens of rather different taxa), on condition that the plasmid is compatible with the new
“host”. The gastro-intestinal track of humans (and of course that of the animals), has a densely populated mixed
microbial community (“microbiota”), and therefore it is an optimal “market place” for such exchanges during horizontal
gene transfer In addition, hospitals are ideal meeting place for pathogens harboring different resistance genes [30].

2.2. The “ESKAPE Club” of omnipotent multi-drug resistant bacterium species

In 2006 The Antimicrobial Availability Task Force (AATF) of Infectious Diseases Society of America (IDSA)
prepared a review that highlighted frequently resistant pathogens to licensed antimicrobials, and for which only a few,
if any, potentially effective drugs are shown in late-stage drug development [31]. This “Six Bad Bugs” originally
comprised a notorious group of 5 pathogen bacterium species, and Aspergillus, characterized by an enormously high
rate of antibiotic resistance, and extremely versatile MDR phenotypes, that are responsible for the majority of
nosocomial infections [31].

The present list (without the fungus Aspergillus), includes 6 bacterium species called the ESKAPE Pathogen
Bacterium Species list. The name of the six letters involve the initials of genus names of these bacteria: Enterococcus
faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa, and
Enterobacter [32]. The explanation: these groups of bacteria may produce omniresistant (panresistant) pathogen
strains, against which there is “NO DRUG” (no protecting antibiotics), and therefore there is “NO ESKAPE” [32].

The “club” of the worst 6 “bad bugs” includes 4 extended spectrum β-lactamase (ESBL)-producing Gram-negatives -
A. baumannii, P. aeruginosa; Enterobacteriaceae species (such as E. coli), Klebsiella pneumoniae – and 2 Gram
positive - meticillin resistant S. aureus (MRSA) and vancomycin-resistant gastrointestinal Enterococci, (E.
faecalis, E. faecium) – “club-members”.

This internationally accepted list which has been refreshed yearly, and should be considered still authentic, but
probably will be expanding soon, and may not be considered as complete. This authentic list has been renewing
from time-to-time [4], and has been expected to be expanded. The Clostridium genus for instance, which provides
examples of MDR pathogens, (in C. difficile: see [33, 34]; in C. perfringens: see [35]), is not included, but is a potential
candidate. Similarly, the Salmonella genus has not been included in the ESKAPE list, despite alarming publications
related with signs of MDR pathogen evolution in this taxonomic group [36-40].
This subsection is restricted only to the species “officially” registered in the ESKAPE “club” and tries to draw the attention of the respected Reader to some new candidates.

### 2.2.1. Methicillin resistant *Staphylococcus aureus*, MRSA

The increased use of antibiotics in clinical practice has been followed by an increase of the frequencies of antibiotic multiresistant pathogen strains. Since the discovery and revelation of some details of the genetic background of high-level methicillin resistance in *S. aureus* [41], a rapid evolution of multi-drug resistance could be monitored in Gram-positive bacteria [42]. The best known classic example is the spread of methicillin-resistant *S. aureus* (MRSA). The history started at about 1960, right after the clinical applications of penicillin and tetracycline derivatives (second generation beta-lactam antibiotics) were introduced into clinical practice, and *S. aureus* rapidly acquired resistance to them [43-46].

MRSA causes many types of serious infections, especially in infants. Nosocomial infection caused by *S. aureus* substantially increases the hospital death toll rates. MRSA is a problematic multi-drug-resistant pathogen around the world nowadays. It rapidly develops complete resistance to most applied antibiotics [47]. Strains of community-associated MRSA are readily transmitted from person-to-person when crowding occurs [31].

There have been several approaches aiming at overcoming this problem. One of them is to synergistically apply active antibiotics, such as triple β-lactam, in combination with meropenen/ piperacillin/tazobactam [48]. A comprehensive, 15-year study, (completed in 2014) of the evolution of resistance of *Staphylococcus* species to different antimicrobials, and of corresponding mechanisms and their molecular backgrounds, confirmed the key role and useful indicator of the mecA gene [48].

The mecA gene, the predominant determinant of methicillin resistance in *S. aureus* is very probably not native to this species, but may have originated in the animal commensal species *S. sciuri*. At least all known *S. sciuri* strains carry a close homologue of mecA in the form of pbpD, the genetic determinant of penicillin binding protein 4 (PBP 4) of *S. sciuri*.

An experimental system has been elaborated and used confirming that the resistance determinant mecA of MRSA strains has evolved from *S. sciuri* pbpD [49].

It has generally been accepted that the resistance appeared right after the mecA gene, (encoding methicillin resistance carried on a SCCmec element) was horizontally transferred to an originally sensitive strain of *S. aureus*, and an international working group (called (IWG-SCC) has been working on the classification of “Staphylococcal Cassette Chromosome Elements” [50]. It was found that the intensity of antimicrobial treatments, as well as the risk of transferring them to humans or human isolates, was somehow correlated [48].

Many clonal lineages of MRSA, and methicillin-resistant *S. epidermidis*, were found circulating in hospitals, suggesting that companion animals could contribute to the dissemination of highly successful human clones [51].

The recently accomplished whole genome, sequencing a collection of the first MRSA isolates, followed by applied Bayesian phylogenetic reconstruction, provided an option for reconstructing the evolutionary history of the archetypal MRSA. It has been assumed that the approximate date at which the earliest MRSA lineage harboring the SCCmec appeared, was about the mid-1940s. This time was much prior to the application of methicillin [52]. Naturally, it is quite plausible to suppose that inducible enzymes play a role in the biochemical mechanisms. Consequently, the resistance to a new antibiotic could not have appeared before application of a lower (not immediately fatal) dose of the given compound.

From this important discovery, one concludes pessimistically that genomic sources of intrinsic antibiotics resistances in genus *Staphylococcus* are non-exhaustible. The reviewers hope that both the options provided by the possibly very high number of structure/activity combinations, as well as by the natural sources of antimicrobial peptides effective in MRSA, are also non-exhaustible.

### 2.2.2. Extended spectrum β-lactamase (ESBL) producing Enterobacteriaceae and *Klebsiella pneumoniae*

The phenotype of resistance to antibiotics can result by four mechanisms: (1) enzymatic detoxification; (2) efflux; (3) reduced cell wall permeability, that is a decreased affinity of the target for the antibiotic molecule; and (4) a bypass of the target. β-lactams detoxification of antibiotics by β-lactamases is widespread in nearly all bacterial phyla. In Gram-negative bacteria, β-lactamase production is frequently associated with reduced permeability of the outer membrane and efflux [53].

From an evolutionary point of view, probably the most important discovery is that the extended spectrum β-lactamase background exerts an unexplained, but demonstrated positive, kind of channelizing effect on the
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...evolutionary mechanisms, leading to the appearance of other antibiotic resistant mutants, at a significantly higher degree than in the normal population [54, 55].

The emergence of ESBL producing Enterobacteriaceae [56] is another classical example of the threat coming from multiple resistance pathogens. Klebsiella species and E. coli have most frequently caused diseases of the urinary tract, biliary tract, gastrointestinal tract, and wounds due to trauma in humans. Bacteremia, hospital-acquired pneumonia, postoperative meningitis, and nosocomial infections produce life-threatening diseases. The prevalence of ESBL production among E. coli and Klebsiella species varies depending on geography, nature of the institution and age of the population. In vitro resistance to ceftazidime, and/or aztreonam, can be used as a phenotypic marker of one of these new groups of enzymes, referred to as ESBLs [56].

The biochemical background is the increasing number of β-lactamase enzymes with enlarged substrate specificities. The substrate range includes cephalosporins (ceftaxime and ceftriaxone) [57-59], a monobactam (aztreonam) [60-62], the amino-penicillin combinations [63], ampicillin sulbactam (a lactamase inhibitor) [64-67], the urode-penicillins [68, 69], including piperacillin [70], tazobactam [71-73], temocillin and piperacillin/tazobactam [74], and ceftolozane-tazobactam [75]. Furthermore, the appearance of new enzymes is not associated with the loss of ability to hydrolyze the earlier-lactams, such as ampicillin. The prevalence of ESBL production among E. coli and Klebsiella species is variable [31, 4].

To overcome ESBL problems, carbapenem antibiotics were developed. They are relatively resistant to hydrolysis by most β-lactamases. In some cases they act either as “slow substrate” β-lactamases inhibitors, and are capable of binding to penicillin binding proteins. This “value-added feature” of inhibiting β-lactamases serves as a major rationale for expansion of this class of β-lactams [76]. Carbapenem antibiotics including imipenem (a Gram-negative cell-wall synthesis interrupting molecule which binds to penicillin-binding proteins); cilastatin (a human enzyme dehydropeptidase in the human kidney, inhibiting imipenem degradation); meropenem [77, 78] and ertapenem [79-82] have served as a putative last line of defense against multi-drug-resistant Gram-negative organisms.

Since 2006, however, the number of carbapenem-resistant Enterobacteriaceae (CRE) has also significantly emerged, providing a serious public health threat [83]. As for the molecular genetics, a novel, epidemic, serine class-A type enzyme (KPC) is behind that, encoded by the Bla (Oxa) gene family [84], exhibiting powerful activity against all types of -lactam agents [85].

As for K. pneumoniae, the primary mode of spread is clonal dissemination, while that for E. coli and other Enterobacteriaceae species is polyclonal dissemination. Several alleles, promoters and carriers localized on mobile genetic elements, have been identified [86]. Since the above mentioned review, in the carbapenem-resistant K. pneumoniae population, colistin resistance has also appeared [87].

As for future perspectives, an important question is what is the role of antibiotic resistance from the aspect of a given pathogenic species. Is it axiomatic that antibiotic resistance is a positive selection factor?

Recent population dynamics analysis, carried out by using comparative intra-species genomics to analyze a systematic decade-long survey of the most successful lineages within a broader E. coli population associated with disease in England, led to the unexpected conclusion that that multi-drug resistance was not the dominant reason for (evolutionary) prevalence of E. coli lineages in this population [88]. The most frequently identified lineage, (ST73), was susceptible to most antibiotics, while the virulent, globally disseminated, multi-drug-resistant, lineage (ST131) was much less successful! The conclusion was that E. coli lineages in invasive diseases have been driven by negative frequency-dependent selection occurring outside of the hospital, most probably in the commensal niche where the drug resistance is not necessarily a primary determinant of success [88].

The reviewers suppose that maybe an uptake of a mobile genetic element, or a plasmid, means a genetic load acting as a negative determinant of the success.

2.2.3. Pseudomonas aeruginosa, a pan-genomic hot bed of multi-drug resistance

Armed with a full arsenal of persistence (Biofilm Formation), and resistance (Genome Plasticity), P. aeruginosa is one of the two pangenomic Gram-negative pathogenic species acquiring an extraordinary large scale of MDR phenotypes. It has been considered as threatening sources of resistance coding genes transmittable via horizontal gene transfer [89].

2.2.3.1. Taxonomy

Pseudomonas aeruginosa (Class: Gammaproteo-bacteria; Family: Pseudomonadaceae; Phylum: Proteobacteria) is a common Gram-negative, rod-shaped, bacterium species that can cause disease in plants, animals, and humans. It is a ubiquitous, invasive, pangenomic, Gram-negative, opportunistic, pathogen, (Wikipedia).
2.2.3.2. A short list of intrinsic and acquired antibiotic resistances

The original intrinsic MDR-arsenal of this species includes production of beta-lactamases, loss of outer membrane proteins and up-regulation of efflux pumps. Most strains of *P. aeruginosa* which are resistant to third-generation cephalosporins, produce a chromosomally mediated molecular class C beta-lactamase, and the AmpC enzyme [89]. This species also acquired resistance to aminoglycosides and fluoroquinolones. One of these acquired enzymes was taken-up by *P. aeruginosa*, (PER, *Pseudomonas* extended resistance) [90] a class called an extended-spectrum beta-lactamase (ESBL) occurs less frequently, but still is of clinical importance [91]. It confers resistance to oxyimino beta-lactams, and also to carbapenases [92]. From burn wounds in a Hungarian patient, a victim of a terror attack in Egypt, several *P. aeruginosa* strains, including ESBL-producing *P. aeruginosa* (PA1), an imipenem-resistant *P. aeruginosa* (PA2), and an EMBL-producing *P. aeruginosa* (PA3), were observed [93]. Furthermore, ESBL-producing *K. pneumoniae* (ESBL-KP), methicillin-resistant *S. aureus* (MRSA) and *E. faecalis* (EF) isolates have also been found [93].

2.2.3.3. Incomplete list of diseases and pathomechanisms

*Pseudomonas aeruginosa* has a large genome [94, 95], flexible metabolic capabilities, biofilm formation [96], and a virulent factor biosynthesis [97]. Integrated whole-genome sequences revealed the genetic background of the exploitation potential for conquering so many different environmental niches. As an opportunistic pan-genomic pathogen, *P. aeruginosa* is considered an invasive pathogen that causes a wide range of diseases. In human clinical practice, *P. aeruginosa* strains are especially endangering immune-system-depressed or deficient patients. It causes serious, predominantly nosocomial, human infections of the lower respiratory tract, the urinary tract and wounds in children and elder patients in hospitals, causing different diseases through host-specific pathogenesis, and the pathogenicity is always host-specific [98].

It was found in the lower respiratory airways of children with cystic fibrosis, inciting inflammation that inexcogically destroys lung tissue, and ultimately leads to respiratory failure and death [31]. The fatal consequences are most apparent in cystic fibrosis patients, where a high degree of biofilm formation was detected [99, 100]. *Pseudomonas aeruginosa* has also been a causative agent of infections in burn wounds [101], chronic wounds, chronic obstructive pulmonary disorder, surface growth on implanted biomaterials, on hospital surfaces [102], and in water supplies [103], where it poses a host of threats to vulnerable patients.

In the pathogen mechanism, extracellular signals are capable of synchronizing group behaviors through a process called quorum sensing (QS) [104], making this bacterium capable of forming massive biofilms [105, 106, 100]. In the pan-genomic *P. aeruginosa* a complex QS system controls the expression of more than 300 genes [104], including many involved in host colonization and disease. The problems have been aggravated by multiple effects of multi-drug resistance, and the capability of *P. aeruginosa* to grow in a biofilm, which may enhance its ability to cause infections by protecting the bacteria from host defenses and chemotherapy [100]. Once established in the patient, *P. aeruginosa* can be especially difficult to treat.

2.2.3.4. Genetic background

The genome encodes a host of resistance genes, including multi-drug efflux pumps [107] and enzymes responsible for resistance to beta-lactam and aminoglycoside antibiotics. Consequently, the therapy against this Gram-negative pathogen is rather challenging, especially because of the need for unavailable novel antimicrobial therapeutics, and “the lost art of drug discovery” [108]. The resistance to multiple drugs is usually a result of a combination of different mechanisms in a single isolate. *Pseudomonas aeruginosa* is an example of that, because different mechanisms can jointly contribute to its multi-resistant phenotype [109] and multi-drug efflux systems [110, 111]. All of this makes *P. aeruginosa* extremely invasive. The rapidly increasing number of new *Pseudomonas* isolates of MDR, XDR and PDR phenotypes, severely reduces the antibiotic therapy options available [28].

The availability of whole-genome sequencing offers a challenging option for resistance surveillance via the resistome (i.e. the genes and mutations underlying antibiotic resistance), especially in bacteria species owning a large pan-genome, like *P. aeruginosa*. To help identify virulence-associated genes, antimicrobial resistance genes and other genomic features associated with pathogenicity and host adaptation, an international consortium was formed at the Laval University, Quebec, Canada (Institut de Biologie Intégrative et des Systèmes (IBIS), Université Laval, Québec, QC, Canada), to provide comparative genomic information and prognosis for clinical use. Their first publication on comparative genomics of isolates of a *P. aeruginosa* epidemic strain associated with chronic lung infections of cystic fibrosis patients [112] was followed by publication on comparative genomics and biological characterization of sequential *P. aeruginosa* isolates from persistent airway infections [113], and provided genomics data for clinical utilization [114]. Two years later, they provided an antimicrobial resistance prediction concluded from the comparative
The presence and activity of decomposing enzymes, changed expression of porins, reduced permeability, resistance in especially from intensive care units of species of written by Antunes.

2.2.4.4. Short evolutionary history of the global protein secretion systems [128]

The other Gram-negative opportunistic human pathogen bacterium species creating an extraordinarily large scale of MDR phenotypes is A. baumannii. This species is probably even a worse pathogen than P. aeruginosa, with an extremely large pan genome and heterogeneous accessory genome. It has a continuously enlarging set of virulence and resistance armament, as first reviewed by Bonomo and Szabó in the literature [89]. Since then A. baumannii has been considered as a threatening source of resistance coding genes transmittable via horizontal gene transfer. The WHO declared that A. baumannii is one of the most serious ESKAPE organisms that effectively escape the effects of antibacterial drugs [116]. Since then, the global evolution of multi-drug-resistant A. baumannii isolated has been carefully monitored, and clonal lineages have been revealed [117].

2.2.4.1. Taxonomy

Domain: Bacteria; Kingdom: Eubacteria; Phylum: Proteobacteria; Class: Gammaproteobacteria; Family: Moraxellaceae; Genus: Acinetobacter (see: Wikipedia).

The Acinetobacter species are rod-shaped, glucose-non-fermentative, non-motile, non-fastidious, catalase-positive, oxidative-negative, aerobic, Gram-negative cocobacilli. Acinetobacter baumannii is the only member of the Moraxellaceae to lack cytochrome C oxidases, and it can cause serious diseases in plants, animals and humans [118].

Since Acinetobacter species have been discovered, as avirulent group of bacteria (32 years ago) taxonomic relations of the species within the genus have also been clarified [119], and A. baumannii positioned itself as Enemy #1 virulent pathogen.

2.2.4.2. An incomplete list of diseases caused by A. baumannii

Several serious or fatal diseases including pneumonia, bacteremia secondary meningitis and endocarditis are on the “crime-list” of A. baumannii, and have been recently reviewed [120]. Infections, with high mortality rates, are ventilator-associated pneumonia and bloodstream infections [121]. Community acquired A. baumannii pneumonia is more fatal than nosocomial pneumonia, resulting in death within a week of diagnosis, and the mortality rates are as high as 50% [122]. Other nosocomial skin, soft tissue wound and urinary tract infections, as well as secondary meningitis, have also been reported as the consequence of A. baumannii infections [123-125]. The different A. baumannii strains could be scored according to their pathogenic potential [126, 127]. The differences in mortality are probably not independent of the expression of specific virulence factors and determinants [120].

2.2.4.3. Virulence factors

The large scale of virulence factors, contributing to A. baumannii pathogenesis, includes porins, capsule polysaccharides, lipopolysaccharides, phospholipases, outer membrane vesicles, metal acquisition systems, and protein secretion systems [128], which will not be discussed here.

2.2.4.4. Short evolutionary history of the global spread of multi-drug resistant lines of A. baumannii

To keep track, and not be lost in the jungle of relevant literature, we relied mainly on two excellent previous reviews written by Antunes [120] and Lee [128] with their associates, respectively, and used them as “beacon-of-lights”.

The species A. baumannii used to have a low phylogenetic diversity, providing a severe evolutionary bottle-neck through which a micro-evolutionary tree of many branches has emerged since the late 1980s [120, 128]. Other species of Acinetobacter are soil-inhabiting organisms, while A. baumannii is almost exclusively isolated from hospital environments [129], veterinary clinics [130] and especially from intensive care units of hospitals [131]. Just like P. aeruginosa and other bacterium species, the resistance in A. baumannii is also the phenotype consequence of genetically encoded biochemical procedures, namely, the presence and activity of decomposing enzymes, changed expression of porins, reduced permeability,
and constitutive expression of active multi-substrate efflux systems [132]. Similar to P. aeruginosa, A. baumannii also has been armored with both “intrinsic” and “acquired” (taken-up) resistance mechanisms [120, 133].

The increasing number of resistant pathogens is due to the appearance and spread of aminoglycoside-modifying enzymes, carbapenemases, ESBLs, or to changes in outer-membrane proteins, and penicillin-binding proteins [128]. The vast majority of new isolates are MDR, including resistance to carbapenem, which had previously been considered as capable of protecting against pathogenic MDR A. baumannii strains [134]. Multi-resistance has been spreading, with reports of carbapenem resistant Acinetobacter spp. in cattle and other animals [135-137], as well as in the Seine River in Paris, France [138].

Many new isolates are now resistant to all aminoglycosides, cephalosporins and fluoroquinolones [120]. The antimicrobial resistance mechanisms in A. baumannii have frequently been reviewed [133-136, 139, 140].

Colistin [141] was found to be a reliably effective drug in vitro against MDR, resulting in a renaissance to polymixins [142-145]. It had previously been withdrawn from commerce because of serious in vivo side effects [146, 147] even if these side effects could partially be compensated clinically [148, 149]. In a short amount of time there have been reports on colistin resistance as well [139, 150-154].

2.2.4.5. Antibiotic resistance mechanisms present in A. baumannii

Several strains of A. baumannii are highly resistant to most clinically available antibiotics [118]. Acinetobacter baumannii has a number of resistance mechanisms, including β-lactamases, aminoglycoside-modifying enzymes, efflux pumps, permeability defects, and modifications of target sites. The accumulation of several resistance mechanisms in A. baumannii has gradually decreased the number of antibiotic classes available to treat those infections in the clinical practice.

The most up-to-date reviews about the biology, virulent factors and antibiotic resistance problems demonstrate the extreme rapid evolution of multi-drug resistance of the species, allowing science to discover many new mechanisms and strategies. Herein we just make a short summary of the resistance mechanisms, mainly based on the two previous reviews [120, 128].

2.2.4.5.1. β-lactamases

Inactivation of β-lactams by β-lactamases is a major antibiotic resistance mechanism in A. baumannii. Based on sequence homology, β-lactamases are grouped into molecular classes, A, B, C, and D [155]. All four classes of β-lactamases were identified in A. baumannii. Recent studies have shown that it has natural competence to incorporate exogenous DNA, its genome has DNA of foreign origin at high frequencies and serum albumin enhances the natural competence of A. baumannii [156].

Since 2006, 17 Class A β-lactamases have been discovered and have been described (or re-described), including 6 discovered in the last year. They are GES-1 and GES-5 [157]; GES-11 [158]; KPC-2 and [159]; PER-1. The last, (similar to other pathogen bacteria such as Clostridium perfringens) is also an essential virulence factor needed to adhere A. baumannii cells to the target cell membrane [160].

Since 2006, 18 Class B β-lactamases have been described or re-described, including NDM-1 [161] and NDM-3 [162], discovered in 2016. There is only 1 new Class A β-Lactamase, called AmpC [163, 164]. Forty nine Class D (OXA) β-Lactamases have also been described from A. baumannii; 4 of them - (OXA-239, OXA-72, OXA-51, and OXA-253) - were discovered or re-described in the last year [165-170].

One report from India showed that blaOXA-51 and blaOXA-23 were present in all 103 carbapenem-resistant A. baumannii isolates, and almost 80% of the isolates had ISAba1 upstream of the blaOXA-23 gene, indicating the prevalence of the ISAba1 insertion [171].

2.2.4.5.2. Aminoglycoside-modifying enzymes

They are the major resistance mechanism in A. baumannii to neutralize aminoglycosides. They are all encoded by genes localized in mobile genetic elements in different isolates of the A. baumannii species [118].

Aminoglycoside acetyltransferases are represented by 5 enzymes: AAC3 [172], aaC1, aac2, AAC (6’), aacA4 [173-178].

Aminoglycoside adenyltransferases are represented by 4 enzymes: ANT (2’), (aadB), [172], ANT (3’) and (aadA1) [174, 177].
**2.2.4.5.3. Efflux pumps**

In *A. baumannii*, efflux pumps are associated with resistance against many different classes of antibiotics, such as tigecycline [180] and imipenem [181]. Loss of antibiotic resistance invoked by efflux pump inhibitors such as 1-(1-naphthylmethyl)-piperazine and carbonyl cyanide 3-chlorophenyl-hydrazone is a supporting evidence of the importance of efflux pumps [182].

As for efflux pumps, the resistance-nodulation-division efflux pump superfamily is represented by 3 members in *A. baumannii*. The AdeABC is responsible foraminoglycoside resistance [183], and for the reduced susceptibility to tigecycline [184] and to non-fluoroquinolone antibiotics [185]. The responsible gene is repressed in the wild type by the BaeSR two-component system [186, 187] but over-expressed in the respective (“inducer”) mutants [188].

AdeFGH [189] and AdeJK [190] are synergistically associated with tigecycline resistance [190]; AdeFGH and AdeJK expression is regulated by TetR-type transcriptional regulator AdeN [191, 192].

The major facilitator efflux pump superfamily is represented by TetA [193], TetB [194], and CmlA [195]; CraA [196] (responsible for tetracycline and chloramphenicol resistances, respectively), AmvA (mediating resistance towards different classes of molecules of antibacterial activity, such as disinfectants, detergents, and dyes, furthermore erythromycin, acriflavine, benzalkonium chloride, and methylviologen) [197], and finally, AbaF (responsible for fosfomycin resistance) [198].

Multi-drug and toxic compound extrusion family represented by AbeM is responsible for resistance to imipenem and fluoroquinolones [199]. Deletion mutants of the small multi-drug resistance family AbeS show increased sensitivities to different antibiotics [200].

The EmrAB-ToLC efflux pump is also present in *A. baumannii* and responsible for resistance to netilmicin, tobramycin and imipenem [201]. A1S-1535 confers resistance to gentamicin, kanamycin, chloroxylenol, oxytetracycline, 1, 10-phenanthroline, and chloramphenicol [202]. AIS-2795 is responsible for resistance to the sulphamamide sulfathiazole, and ABAYE-0913 is associated with resistance to chloramphenicol and fusidic acid [202].

### 2.2.4.5.4. Altered permeability resulting in antibiotic resistance in *A. baumannii*

Porins which form channels that allow transport of molecules across the outer membrane of Gram-negative bacteria, resulted in carbapenem resistance in *A. baumannii* [128]. Carbapenem resistance could be a phenotypic consequence of reduced expression of some porins, such as Omp22-33 [203], or CarO [204, 205]. Imipenem resistance could be the phenotypic consequence of the loss of Omp29, producing OXA-51-like, or OXA-23-like, carbapenemases [206].

Aztreonam, chloramphenicol, and nalidixic acid resistance is related with OmpA [207]. OmpA and CarO have recently been reported as being associated with antibiotic resistance through physical interactions with OXA-23 carbapenemase [208].

Alteration of target sites is the resistance mechanism materialized by modifications in antibiotic target sites in *A. baumannii*. Alteration of target sites seems to be a successful mechanism against almost all antibiotics tested so far. Imipenem resistance could be the phenotypic consequences of overexpression of altered penicillin-binding proteins (PPBs), which have a low affinity for imipenem [209].

Aminoglycoside resistance could be the phenotypic consequences of the 16S rRNA methylase (ArmA) activity, found in several pathogen isolates. It always coexists with OXA-type carbapenemases such as OXA-23 [178, 210-215].

Quinolone resistance could be the phenotypic consequence of modifications in GyrA – coding structure gene (GyrA is one subunit of DNA gyrase), and that of ParC (one subunit of topoisomerase IV), in epidemiologically unrelated *A. baumannii isolates* [216]. Tetracycline resistance, determined by TetM, is thought to act through ribosomal protection [217]. Trimethoprim resistance has been found in nosocomial MDR *A. baumannii* isolates and is supposed to be a phenotypic consequence of the action of dihydrofolate reductases (DHFR and FolA) [166, 218, 219].

Other Resistance Mechanisms are also discussed by Lee et al. (2017) [128].

### 2.2.4.5.5. Biofilm formation

Biofilm formation plays an important role not only in the immune evasion by *A. baumannii* [220], but persistence as well. Imipenem treatment of the imipenem-resistant *A. baumannii* isolate induces expression of important genes responsible for synthesis of type IV pili [221], the existence of which is needed for biofilm formation.

### 2.2.4.6. Genome plasticity and evolution of antibiotic resistance: International clonal lineages
At least 15 complete, and 180 draft, chromosomal \textit{A. baumannii} genomes, 31 plasmid and six bacteriophage sequences, have been available on the NCBI database, (see: http://www.ncbi.nlm.nih.gov) together with those of another species of the genus [120]. A bacterial species can be defined by its pan-genome, which consists of a core genome conventionally defined as those genes present in all isolates, and an accessory genome, which includes the genes absent from one or more isolates or unique to a given isolate. The spectacular progress in next-generation sequencing methods allows carrying on the pangenome sequence analysis, which is a new tool for redefining pathogenic bacteria species [222].

The whole pangenome of \textit{A. baumannii} consists of > 8800 orthologous coding sequences, and has exponentially been increasing as new genomes become available (an open pan genome), mainly due to unique accessory genomes of different isolates enriched with acquired genes of transport and of transcription regulation functions [223]. Strain-specific genes mainly encode hypothetical proteins, transposases and insertion sequences [224]. Genes associated with resistance to antimicrobial drugs were found in the species core and accessory genomes [224]. In the accessory genome, antimicrobial resistance genes were found in alien islands, and were often flanked by integrases, transposases, or insertion sequences [224], suggesting their possible acquisition by horizontal gene transfer from other \textit{Acinetobacter} strains of bacteria that colonize the same environment.

Originally three predominant pathogen clones (called ‘international clonal lineages’, ICLs) were known as being responsible for hospital outbreaks worldwide. ICL1 and ICL2 have been known as of MDR phenotypes. Since then, four more have been identified and listed at the \textit{A. baumannii} MLST database, which is publicly available at http://pubmlst.org/abau/annii/. At present at least 6, (if not more), major ICLs have been distributed worldwide [225].

The genome of a representative ICL1 (AYE) strain includes 52 genes associated with resistance to antimicrobial drugs, and 45 of them are localized in an 86 kb resistance island called AbaR1. ABAR1 is also present in other \textit{A. baumannii} strains, but with a much smaller size. It has been noted that the presence of an extraordinary 22 genes-cassette coding for transposases and insertion sequences may be responsible for the acquisition of resistance genes into the AbaR1 island of the AYE ICL1-type strain. Almost half are orthologous to coding sequences of \textit{Pseudomonas} [120].

The type strain (ACICU), representing the ICL2 global clone, also contains a homolog to AbaR1, but it is much smaller (seven antibiotic resistance coding genes). In this strain, drug resistance is more evenly distributed throughout the genome [120].

Based on the concentration of six housekeeping genes, \textit{A. baumannii} is of monophyletic origin [118, 120]. The monophyletic status of ICLs 1 and 2 have also been shown [226, 227].

\textbf{2.2.5. \textit{Enterococci}: The Gram-positive “Vanguards” of the “MDR movement”}

\textbf{2.2.5.1. Taxonomy and general description}

Species belonging to genus \textit{Enterococcus} (\textit{E. faecium}, \textit{E. faecalis} and \textit{E. gallinarum}) are Gram-positive, facultative anaerobes that once only lived as commensals in the gastrointestinal tract of a variety of organisms including humans. As for taxonomy, Domain: Bacteria; Kingdom: Eubacteria; Phylum: Firmicutes (this includes all Gram-positives and a few others); Class: Bacilli; Family: Enterococcaceae; Order: Lactobacillales; Genus: \textit{Enterococcus}. \textit{Enterococcus} is a large genus of lactic acid bacteria that are Gram-positive cocci that often occur in pairs or short chains, and are difficult to distinguish from streptococci on physical characteristics alone. The pathogenic strains cause infections, including urinary tract infection (UTIs), endocarditis, bacteriaemia, catheter-related infections, wound infections, and intra-abdominal and pelvic infections (Wikipedia).

The MDR enterococci are important nosocomial pathogens, and a growing clinical challenge, because they developed full resistance against practically all traditional antimicrobials used in clinical practices, due to a large number of genetic strategies [228]. The MDR enterococci display a wide repertoire of antibiotic resistance mechanisms, including modification of drug targets, inactivation of therapeutic agents, overexpression of efflux pumps, and a sophisticated cell envelope adaptive response that promotes survival in the human host and nosocomial environments [228]. MDR enterococci strains are well adapted to survive in the gastrointestinal tract, and can become the dominant flora under antibiotic pressure, predisposing the severely ill and immune-compromised patients, to other invasive infections [229]. The excellent review of Gilmore [230] provides the authentic historic background and the most up-to-date information and references about the known details, including the magic genetic and sophisticated biochemical background of resistance mechanisms towards the different antibiotics for the enterococci.
Briefly, the history is as follows: streptococcal infections were successfully treated by the introduction of penicillin to the clinical practice. However, the enterococci respond reluctantly to penicillin due to an inherent tolerance to the killing action of these compounds [231]. In this pioneer work six penicillin-binding proteins (PBP)s were detected in clinical isolates of each one of three group D streptococci: E. (Streptococcus) bovis, E. faecalis and E. faecium. E. faecium is the most penicillin-resistant species of group D streptococci. When the authors examined in whole organisms, they found that the PBPs of E. faecium showed lower affinities for the antibiotic than those of E. faecalis (intermediate penicillin resistance), which in turn were of lower affinity than those of the penicillin-sensitive E. bovis [231].

It was later found that the addition of streptomycin (discovered in 1944) [232] to penicillin, produced synergistic activity improving recovery from enterococcal infective endocarditis [233]. This synergistic effect was seen despite the fact that enterococci are also inherently less susceptible to streptomycin than many other Gram-positive bacteria. Thus, the combination of a cell-wall active agent (i.e., ampicillin/penicillin) plus an aminoglycoside became the standard of care for deep-seated enterococcal infections, and this combination is still used to the present day [234]. However, the seeds of the modern MDR enterococci were already being sown. Comparative genomics showed that the MDR E. faecium belonged to a genetic clade (Clade 1) that separated evolutionarily from animal-adapted E. faecium at about the same time penicillin and streptomycin were introduced into clinical use [235]. Clade A1 is capable of taking out intensively mobile genetic elements, resulting in alterations in hyper-mutability that lends E. faecium a remarkable genome plasticity, which is a selective virtue under multiple selective pressure conditions. The remarkable increase in the use of antimicrobials in clinical medicine in the latter half of the 20th century provided the selective environment for these microorganisms to evolve by recruiting a variety of antibiotic resistance determinants [228].

Unlike S. aureus, they have a unique capability to recruit antibiotic resistance determinants, and maintain not only one, but a variety of gene clusters encoding the biochemical machinery for resistance to different antibiotics, including vancomycin [236-240]. They also serve as a donor of resistance gene clusters, providing resistance to different antibiotics, including vancomycin, to other pathogenic microorganisms such as MRSA [241, 242].

### 2.2.5.2. Resistance mechanisms acquired and/or performed by enterococci

As referred above, provisioning of antibiotic resistance can be materialized by enzymatic detoxification, efflux, decreased cell wall permeability (that is decreased affinity for the target for the antibiotic), and bypass of the target. For β-lactams, detoxification of the antibiotics by β-lactamas is widespread in nearly all bacterial phyla. In Gram-negative bacteria, β-lactamase production is frequently associated with reduced permeability of the outer membrane and efflux. However, in the Gram-positive ones, this permeability barrier does not exist, and resistance is often due to production of targets displaying a lower affinity for the respective antibiotics [53]. The fourth bypass mechanism has been identified for the first time in an ampicillin mutant hunt experiment with E. faecium [243]. In these mutants, the classical targets of β-lactams, the high-molecular-weight penicillin-binding proteins (PBPs) are replaced by a l, d-trans-peptidase (LDT), which catalyzes the essential cross-linking step of peptidoglycan synthesis.

### 2.2.5.2.1. Resistance mechanisms to penicillin and ampicillin

This subject had recently been reviewed by Miller et al. [228]. The most important factor is that resistance toward cell-wall active antibiotics is not restricted to intrinsic lactamase activity [244], but several others factors [245] related to structural or expression-rate changes of penicillin-binding proteins: over-production low-affinity binding [246], or mutations affecting the structure or regulation of a penicillin-binding protein [247]. Publications appearing since 2014 confirm differential penicillin-binding protein 5 (PBP5) levels in the E. faecium clades with different levels of ampicillin resistance [248]. The genome-wide identification of ampicillin resistance determinants in E. faecium revealed that although mutations in the low-affinity penicillin-binding protein PBP5 have played an important, but not exclusive, role for ampicillin resistance in this species [249], the existence of additional resistance determinants has been suggested. The authors constructed a high-density transposon mutant library for E. faecium, and developed a transposon mutant tracking approach termed Microarray-based Transposon Mapping (M-TraM). This approach led to the identification of a compendium of E. faecium genes that contribute to ampicillin resistance. These genes are part of the core genome of E. faecium, indicating a high potential for it to evolve towards β-lactam resistance [249]. Furthermore, they validated their M-TraM results by adapting a Cre-lox recombination system to construct targeted, marker-less, mutants. They confirmed the role of 4 more genes in ampicillin resistance by the generation of targeted mutants, and further characterized these mutants with regard to their resistance to lysozyme. They showed that ddcP, a gene predicted to encode a low-molecular-weight penicillin-binding protein with D-alanyl-D-alanine carboxypeptidase activity, was essential for high-level ampicillin resistance. Furthermore, deletion of ddcP sensitized E. faecium to lysozyme and abolished membrane-associated D, D-carboxypeptidase activity [249]. We consider this...
Fodor A. et al (2018) Multidrug resistance in bacteria...a review (Preprint)

study to be of historic importance, by allowing the building of a broadly applicable platform for functional genomic-based studies in E. faecium.

2.5.2.2. Resistance mechanisms of Enterococci to cephalosporins

This resistance mechanism can be achieved by reducing their binding capacities [250]. This subject had also recently been thoroughly reviewed by Miller et al. [228]. In bacteria, several regulatory pathways controlled by bacterial two-component regulatory systems (TCS), similar to that of the CroRS two-component system in E. faecalis [251], may also be associated with the intrinsic resistance to cephalosporins. Experimental proof of the existence of a nutritional control mechanism of antibiotic resistance mediated by the phosphotransferase system, and a two-component signaling system, has recently been published [252]. A third protein, IreB, a Ser/Thr kinase substrate also plays a role [253]. Since then, one publication came out confirming the role of an inducible, two-component, signaling system, in the cephalosporin resistance of E. faecalis [254].

2.5.2.3. Resistance mechanisms of Enterococci to glycopeptide antibiotics such as vancomycin and teicoplanin

The glycopeptide antibiotics vancomycin and teicoplanin bind to the peptidyl-D-Ala$_2$-F-Ala$_3$ extremity of peptidoglycan precursors, and cause inhibition by steric hindrance of the elongation of both glycan chains, by glycosyl-transferases, and the cross-linking of stem peptides by D, D-trans-peptidases [243]. The L, D-trans-peptidases use acyl donors, and they contain a stem tetra-peptide ending in D-Ala$_4$ that does not bind to these antibiotics. A novel peptidoglycan cross-linking enzyme changing the terminal amino acids of the peptidoglycan precursor from D-Ala-D-Ala to D-alanine-D-lactate (d-Ala-d-Lac) or, sometimes to D-alanine-D-serine (D-Ala-D-Ser) results in resistance to glycopeptide antibiotic. This and other possible resistance mechanisms were also considered [255].

The literature related to the resistance mechanisms of enterococci to glycopeptide antibiotics had recently been reviewed thoroughly [228.] Since then, however, two important discoveries have been published.

First, the novel membrane protein called VanJ is considered to confer resistance to teicoplanin [256] and vancomycin [257].

Second, a whole-genome sequence has been performed [53] to identify the complete set of mutations occurring during selective pressure of elevated dose of antibiotics. This extremely impressive study, carried out by Sacco and his associates, is probably worth a little more detailed discussion. (It should be taught in student courses).

The authors produced a strain (called M9); containing 79 relevant mutations obtained through 900 generations. At the end of each selection step they isolated a respective multi-selectant strain, and named them M1–M9. The parental strain D344S M9 was completely sensitive, while M9 was fully resistant to ampicillin, vancomycin and tetracycline. They found that the ddc locus was not affected through 4 selection steps, and mutant strains M1–M4 remained sensitive to each of the three antibiotics, although the resistance to ampicillin seemed to be gradually growing. The 5th selection step was critical, resulting in M512, fully resistant to ampicillin, but still sensitive to tetracycline, and showing a moderate vancomycin resistance. The ddc gene was inactivated. In the next 3 generations, the resistance towards vancomycin and tetracycline gradually grew, and M8 was fully resistant to vancomycin.

The whole-genome sequencing procedure, comparing the parental (D344S) and the various mutant M9 strains was carried out by Illumina single read sequencing technology. The Illumina library preparation (genomic DNA sample prep kit v1) and sequencing followed standard protocols developed by the supplier. They found that the genome of mutant M9s differed from that of the parental strain E. faecium D344S by a total of 79 mutations. Sanger sequencing was performed to confirm the presence of the 79 mutations in M9, and to assign each of the mutations to one of the nine selection steps used to obtain mutant M9.

The authors found that among the mutations detected in M9, 65 were nonsynonymous mutations. Assignment of the corresponding proteins in functional classes revealed sequence alterations in eight proteins involved in transcription regulation, including CroR, a response regulator of a two-component regulatory system that contributes to intrinsic β-lactam resistance in the enterococci by an unknown mechanism. Nonsynonymous mutations also affected two sensor kinases, suggesting that regulatory circuits involving two-component regulatory systems are affected in response to the acquisition of ampicillin and glycopeptide resistance.

These results suggest that the fluxes through central metabolic pathways, including glycolysis, might be profoundly remodeled in mutant set of M9. This is an indirect confirmation of the recent report on E. faecalis mutants hypersusceptible to β-lactam antibiotics [252], indicating that existence of nutritional control of antibiotic resistance is
Based on a connection between CroR and the phosphotransferase system (PTS) system. As for the peptidoglycan synthesis, they found that 4 of the 20 enzymes, committed to peptidoglycan biosynthesis, were affected by amino substitutions. None of the substitutions had any obvious role in the activation of the L, D-transpeptidation pathway.

An important observation is that the substitutions did not involve enzymes that recognize the peptide stems of peptidoglycan precursors [53].

2.2.5.2.4. Resistance mechanisms to antibiotics that interfere with protein synthesis

Enterococci display intrinsic tolerance to aminoglycosides [258]. Mutations in genes encoding the 23S rRNA, which is an important part of the drug-binding site at the ribosome, are the most common mechanisms for linezolid resistance [228, 259].

The streptogramins/macrolides/lincosamides are a mixture of pristinamycin derivatives, streptogramin A (dalfopristin) and B quinupristin, which are effective against E. faecium, but not E. faecalis. E. faecalis has the respective chromosomally located gene (for lincosamide and streptogramin A resistance), which encodes for a putative protein with an ATP-binding cassette motif of transporter proteins, but not the transmembrane region that would be expected for an efflux pump [228, 260].

Cross-resistance with all macrolides is a result of the modification of the 23S rRNA target. Resistance to tetracyclines and glycyclines is mediated by multiple genes, but follows two general strategies: efflux of the antibiotics and ribosomal protection. Mutations in the genes, gyrA and parC, (present in E. faecium and E. faecalis, but absent from E. gallinarum and E. casseliflavus) affect the quinolone resistance-determining regions, which presumably alter the binding affinity of the antibiotic quinolones, the target enzymes (DNA gyrase and topoisomerase IV) that are responsible for DNA supercoil relaxation [53, 228, 261]. Rifampicin resistance arises from a variety of mutations in the rpoB gene that encodes for the β-subunit of the RNA polymerase [228]. Trimethoprim and sulfamethoxazole (inhibitors of bacterial enzymes involved in the folate synthesis pathway) are ineffective in vivo to enterococci, because they are those extreme rare bacteria which can utilize exogenous sources of folate [228, 53].

2.3. Zoonic and veterinary pathogen candidates for the “ESCAPE Club”

2.3.1. Mycoplasma bovis

Mycoplasma bovis is a worldwide pathogen that is the causative agent of pneumonia, mastitis, arthritis, and a variety of other symptoms in cattle [262]. As a result, it is responsible for significant economic losses [263]. The pathogens in the Mycoplasma species are members of the class Mollicutes, and comprise the simplest life form that can replicate independently from the host. Mycoplasma spp. have no cell wall, and they have a limited number of metabolic pathways. The greatly reduced genome size and coding capacity of Mycoplasma spp., makes them a good model for genetic studies. Mycoplasma spp. are rather fast-evolving bacteria, pathogenic against humans and animals. However, their importance is often underestimated. Mycoplasma bovis is a major cause of calf pneumonia, mastitis and arthritis, and is intrinsically resistant to antibiotics acting on cell wall or folate synthesis [262], but the antimicrobial resistance synthesis inhibitor classes are active against it [264]. Tetracyclines and spectinomycin primarily bind to the 30S subunit of the ribosome, whereas macrolides, lincosamides, phenicols, and pleuromutilins are mycoplasmatic antibiotics acting on the 50S ribosomal subunit, preventing the mechanisms of transpeptidation and translation [265]. Expanded-spectrum fluoroquinolones, such as enrofloxacin, danofloxacin and marbofloxacin, have anti-mycoplasmatic effects by acting on topoisomerases that inhibit the DNA synthesis of bacteria [266]. Among the few antimicrobials licensed for treatment of M. bovis, there is increasing evidence for resistance [254, 265-268].

As for the genetic background, a point mutation in the parC gene resulted in decreased susceptibility to fluoroquinolones in M. bovis [265], and amino acid substitutions in GyrA and ParC resulted in fluoroquinolone resistant phenotypes [269]. Surprisingly enough, 16S rRNA gene mutations have been associated with decreased susceptibility to tetracycline in M. bovis [270]. Mycoplasma bovis was detected in 32/45 bovine respiratory infection outbreaks at beef farms in 8 provinces in China [271]. The isolates were susceptible, or had medium sensitivity, to ciprofloxacin, enrofloxacin and doxycycline, but 13 of the 32 were resistant to macrolides. A point mutation at the 23 rRNA operon in domain V of 23S rRNA seems to be responsible for the macrolide resistance phenotype in M. bovis [271]. Antibiotic susceptibility profiles of M. bovis strains isolated from cattle in Hungary were determined [272]. The growth of many M. bovis strains was not inhibited by gentamicin, spectinomycin, florfenicol or lincomycin. The most effective antibiotics tested in vitro were the fluoroquinolones: danofloxacin, enrofloxacin, and marbofloxacin.

But, there were 3 of the 35 Hungarian field strains for which the fluoroquinolone MICs were high [272].
Fluoroquinolone-resistant mutants were also selected in vitro for danofloxacin, enrofloxacin and marbofloxacin and each showed complete cross-resistance with the others. The respective mutations responsible for high macrolide, lincomycin, florfenicol, and pleuromutilin antibiotic MICs were mapped into genes encoding 23S rRNA [273].

2.3.2. Bacillus anthracis

Bacillus anthracis, the bacterium of Koch and Pasteur, is the etiologic agent of anthrax, a common disease of livestock and, occasionally, of humans. It is the only obligate pathogen within the genus Bacillus. Bacillus anthracis is a Gram-positive, endospore-forming, rod-shaped, bacterium, (Wikipedia). It causes extremely severe zoonoses, posing a serious threat to both public and animal health [274]. Bacillus anthracis belongs to the B. cereus group of bacteria. Infection with this bacterium can occur through the skin, gastrointestinal tract, or respiratory apparatus, following contact, ingestion, or inhalation of spores, respectively. The fluoroquinolones (FQs) are first-line antibiotics for the treatment of B. anthracis infection, and as a result, FQ resistance is a major concern for medical treatment following anthrax as a bioterrorism tool [275]. FQs act as broad-spectrum bactericidal antibiotics by inhibiting type II DNA topoisomerase, DNA gyrase (GyrA and GyrB), and type IV DNA topoisomerase (ParC and ParE). The mechanism responsible for FQ resistance has been well documented with bacteria, in which frequent mutations of topoisomerase genes have been identified in the designated quinolone resistance-determining region (QRDR) [276]. A recent detailed study to determine the basis for quinolone action and resistance was undertaken by Alfred et al. [277]. They compared the B. anthracis topoisomerase IV of the wild-type, and the GrlA (S81F) and GrlA (S81Y) of the quinolone-resistant mutants, in the presence or absence of quinolones and a related quinazolinedione, to determine the effects on these enzymes. Ser81 is believed to anchor a water-Mg (2+) bridge that coordinates quinolones to the enzyme through the C3/C4 keto acid. Consistent with this hypothesized bridge, ciprofloxacin required increased Mg (2+) concentrations to support DNA cleavage by GrlA (S81F) topoisomerase IV.

The three enzymes displayed similar catalytic activities in the absence of drugs. However, the resistant mutations decreased the affinity of topoisomerase IV for ciprofloxacin and other quinolones, diminished quinolone-induced inhibition of DNA religation and reduced the stability of the enzyme-quinolone-DNA ternary complex. Wild-type DNA cleavage levels were generated by mutant enzymes at high quinolone concentrations, suggesting that increased drug potency could overcome resistance. 8-Methyl-quinazoline-2,4-dione, which lacks the quinolone keto acid, and presumably does not require the water-Mg (2+) bridge to mediate protein interactions, was more potent than other quinolones against wild-type topoisomerase IV. Moreover, it maintained high potency and efficacy against the mutant enzymes, effectively inhibited DNA religation and formed stable ternary complexes.

In fact, reports have also suggested a possible contribution of multi-drug efflux pumps to FQ resistance in B. anthracis [278]. The genome-wide screening for novel genetic variations associated with ciprofloxacin resistance in B. anthracis resulted in the discovery of 2 strains showing resistance, or intermediate resistance, to ciprofloxacin (CIP) by a stepwise selection procedure with increasing CIP concentrations [279, 280]. Fifteen genetic variations were identified between the parental and CIP-resistant strains by next-generation sequencing. Nonsynonymous mutations in the quinolone resistance-determining region (QRDR) of the type II DNA topoisomerase were identified in the resistant strain, but not in the intermediate-resistant strain. The authors discovered a novel “mutation hot spot” (GBAA0834) that leads to the increased expression of multi-drug efflux systems for CIP resistance. Such disruptive mutations appear to be more easily acquired than those in an essential gene, such as that encoding type II DNA topoisomerase. Such an intermediate-resistant phenotype could increase a cell population under CIP-selective pressure, and might promote the emergence of highly resistant isolates [279].

The susceptibility of 29 B. anthracis bovine strains, collected in Hungary between 1933 and 2014 was tested against 10 antibiotics with commercially available minimum inhibitory concentration (MIC) test strips [281]. All strains were susceptible to amoxicillin, ciprofloxacin, clindamycin, doxycycline, gentamicin, penicillin, rifampicin, and vancomycin. Intermediate susceptibility to erythromycin and ceftoxime was detected in 17.2% (5/29) and 58.6% (17/29) of the strains, respectively. Correlations were not observed between the isolation date, location, host species, genotype, and antibiotic susceptibility profile of the strain. A similar study in Cameroon showed that Bovine B. anthracis isolates from there showed a strong homogeneity, and they belong, together with strains from Chad, to a cluster Aβ, which appears to be predominant in western Africa [282]. However, one strain that belongs to a newly defined clade (D) and cluster (D1) was penicillin resistant.

2.3.3. Francisella tularensis

Francisella tularensis is a fastidious, Gram-negative bacterium, a highly contagious zoonotic agent, and the causative agent of the fatal disease, tularemia. Tularemia may occur in six well-recognized clinical forms in humans:
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ulceroglandular; glandular; ocuoglandular; oropharyngeal; pneumonic; and typhoid, or septicemic, tularemia. The [283] F. tularensis subsp. holarctica (type B) is found throughout the Northern Hemisphere, and is the only endemic subspecies found in Europe [284]. Lagomorphs, rodents, European brown hares (Lepus europaes), and voles (Microtus arvalis), serve as the primary mammalian reservoir hosts. Annual number of tularemia cases in humans is well correlated with the yearly biologic cycle (March-February) for hares and hematophagous arthropods, such as ticks, which play a role as vectors and hosts [285]. The antibiotics of choice in the treatment of tularemia are aminoglycosides, quinolones, chloramphenicol, or tetracyclines.

Fortunately enough, there is no sign of the occurrence of multi-drug resistant F. tularensis strains, but resistances to the same antibiotics used in its treatment are known in other bacteria, so acquired resistance could be forecast.

Furthermore, aminoglycosides, quinolones, chloramphenicol and tetracyclines are important, bearing in mind the side effects and probability to replace them. This is why it is important to be informed about the general picture of the antibiotic susceptibility of local populations. A recent screen in Hungary provided a satisfying result [286]. Twenty-nine F. tularensis strains isolated between 2003 and 2010 from free-ranging European brown hares, and a captive patas monkey (Erythrocebus patas), were collected from different parts of Hungary.

Each isolate belonged to F. tularensis subsp. holarctica, phylogenetic group B.13. Each strain was susceptible to those antibiotics which have commonly been used in therapy, such as aminoglycosides, gentamicin, streptomycin, tetracycline, doxycycline, quinolones, ciprofloxacin, levofloxacin, and chloramphenicol, and in addition tigecycline and rifampicin. Naturally, they were resistant to erythromycin and linczolid.

2.3.4. Escherichia coli

Commensal strains of E. coli, as versatile residents of the lower intestine, are also repeatedly challenged by antimicrobial pressures during the lifetime of their host. As a consequence, commensal strains acquire resistance genes, and/or develop resistant mutants in order to survive and maintain microbial homeostasis in the lower intestinal tract. Commensal E. coli strains are regarded as indicators of the antimicrobial load of their hosts. The recent review [8] described the historic background of the origin, appearance and transfer mechanisms of antimicrobial resistance genes into original animal - commensal intestinal E. coli with comparative information on their pathogenic counterparts. The most efficient mechanism used by E. coli against different antimicrobial-based efflux pumps, and mobile resistance mechanisms carried by plasmids and/or mobile genetic elements are known. For a while, these mechanisms cannot protect E. coli against fabclavine (Fodor et al., in preparation). The emergence of hybrid plasmids, both resistance and virulent, among E. coli is of additional public concern. Co-existence and co-transfer of these “bad genes” in this huge and most versatile in vivo compartment may represent an increased public health risk in the future. The significance of MDR commensal E. coli seem to be highest in the food animal industry, which may function as a reservoir for intra- and interspecific exchange, and a source for spread of MDR determinants through contaminated food to humans. Thus, the potential of MDR occurring in these commensal bacteria living in animals used as sources of food (as meat, eggs, milk) should be a concern from the aspect of public health, and it needs to be continuously monitored in the future by using the toolkit of molecular genetics [8]. In fact, that pessimistic theory has been demonstrated. The first pilot study on the prevalence of verocytotoxin-producing (VTSEC) E. coli and of MDR/ESBL E. coli in illegally imported food products of animal origin, suggests that these strains could represent reservoirs for dissemination of potentially new types of pathogenic and MDR E. coli in Europe [287].

The latest British simulation experiments have supported this prognosis [288]. The authors developed an in vitro chemostat system to approximate the chicken caecal microbiota, simulated colonization by an MDR Salmonella pathogen and examined the dynamics of transfer of its MDR plasmid, harboring several genes, including the extended-spectrum beta-lactamase blaCTX-M1. They also evaluated the impact of cefotaxime administration on plasmid transfer and microbial diversity. Bacterial community profiles, obtained by culture-independent methods, showed that Salmonella inoculation resulted in no significant changes to bacterial community alpha and beta diversity, whereas administration of cefotaxime caused significant alterations to both measures of diversity, which largely recovered. MDR plasmid transfer from Salmonella to commensal E. coli was demonstrated by the polymerase chain reaction (PCR) technique, and whole-genome sequencing of isolates purified from agar plates containing cefotaxime. Transfer occurred in seven E. coli sequence types at high rates, even in the absence of cefotaxime, with resistant strains isolated within 3 days [288].

3. Evolutionary aspects

3.1. Prelude: Lamarck and Darwin
The Lamarckian evolution theory declares that “acquired characteristics can be transferred from parents to offspring”. Although scientific facts have not ever supported its validity, this theory has been able to reincarnate at any time. Whenever new evolutionary processes are discovered, there are new attempts to make the Lamarckian concept justified, that is, that acquired traits are inheritable. The actual debate usually ends up with realizing that inheritance is always DNA-related, because everything that was transferred from parents to progeny was coded in the DNA (except for RNA viruses). If a phenotypic trait, either intrinsic or acquired, is encoded in the DNA, it is inheritable. This mini-review unfortunately could not avoid facing and conflicting with the Lamarckian concept, but we think that these little collisions could, or more accurately should, be clarified. One collision point is related to the term of “acquired resistance”, the consequence of taking up a plasmid with a mobile element harboring an antibiotic resistance cassette. It is naturally “acquired” and also inherited. This is a proof of the Darwinian (and not the Lamarckian) evolutionary theory, since the newly, acquired/inherited traits, such as the antibiotic resistance based on enlarged genetic variability due to the up-taken resistance plasmid, and selective conditions such as the presence of the respective antibiotic are involved. The other collision point is inherited and non-inherited antibiotic “resistance”. As we see below, this debate has also been finished with victory of the evolutionary theory of Darwin over that of Lamarck, but the debate itself finally resulted in a revolutionary development in antibiotic research.

3.2. Tolerance, persistence & resistance

The efforts of better understanding, and attempts at understanding either the intrinsic or environmental conditions behind the evolution of antibiotics resistance have a long scientific history [289-293]. A new and promising approach to search for potent antibiotics, which may not provoke resistance in pathogen targets, was initiated by the “rediscovery” of an old observation, made by Joseph W. Bigger [294] in 1944. He found survivors in an antibiotic (penicillin)-treated bacterium (Staphylococcus) cell population without any inheritable genetic change. The surviving subpopulation of the antibiotic-exposed cells, which showed the phenomenon of “non-inherited antibiotic resistance” [295], had been in a metabolically dormant physiological state, meaning that the metabolic rate was slowed down to the lowest possible level, and thus were non-dividing. This surviving subpopulation has been considered as epigenetic variant cells and Kim Louis speaks about them as “persisters cells” [296, 297], or “persisters” [298]. The cells of bacterial biofilms also have strong multi-drug tolerance [299], causing almost as serious a problem in health care as does the multi-drug resistance. The potential of using persistent cells as targets of novel candidate antimicrobials was first realized by Lewis [296]. The “quasi-Lamarckian” definition of persistence as “non-inherited antibiotic resistance” [295] inspired and motivated antibiotics research, since several laboratories turned to studying the phenomenon of persistence as a kind of resistance. These scientific efforts made it possible to discover that the danger posed by the existence of multiresistant P. aeruginosa has been enhanced by the emergence of new isolates producing high levels of persister cells in patients with cystic fibrosis [99], and biofilms with this disease [100]. We found the best definition in the literature for distinguishing between resistance and tolerance in the excellent paper of Friedman et al. [300], who declared that “Resistance” makes it possible for a microorganism to grow in the constant presence of the antibiotic, provided that the concentration of the antibiotic is not too high. “Tolerance” allows a microorganism to survive antibiotic treatment, even at high antibiotic concentrations, as long as the duration of the treatment is limited. The persistent cells are not resistant, but a persistent cell mass is tolerant. The biochemical mechanism behind persistence has mostly been revealed in the last 7 years. A second messenger molecule, ppGpp, plays a key role by mediating in activating Type I and II Toxin-Antitoxins [301, 302]. However, the evolution of tolerance is much less understood than that of resistance.

There were two extremely important publications that came out of the Laboratory of Dr. Nathalie Q. Balaban (Racah Institute of Physics, The Sudarsky Center for Computational Biology and the Center for NanoScience, Edmond J. Safra Campus, The Hebrew University, Jerusalem, Israel) on comparing the evolutionary processes of antibiotic resistance and tolerance [300, 303]. These led to the recent discovery that the mechanisms of tolerance and resistance are not simply “mechanistically distinct” [304], but somehow are also interrelated, since tolerance frequently precedes resistance. These two outstanding experiments are definitely worthwhile to discuss in a little more detail.

In the first, the authors followed the evolution of E. coli populations under intermittent exposures to rather high concentrations of ampicillin, still comparable to therapeutic doses, and separated by intervals in fresh medium [300]. They found the cultures became tolerant to ampicillin by acquiring mutations that extended their lag phase (i.e., the period before exponential growth is resumed after the stationary phase), without any change in the sensitivity to the antibiotics, indicated by the minimal inhibiting concentration, MIC. A higher value of MIC indicated a stronger
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resistance. The authors characterized the evolved strains from the aspects of both resistance and tolerance. They
found that each selected strain adapted by specific mutations, which were ultimately fixed in their evolved
populations. They monitored the phenotypic changes at both the population and single-cell levels. They found the first
adaptive change to antibiotic stress was the development of tolerance through a major adjustment in the distribution
of the single-cell lag-time, without a change in resistance. Surprisingly enough, they observed that the lag time of
bacteria before starting to propagate again was optimized to match the duration of the antibiotic-exposure interval. The
authors identified the mutants, and named them “tolerance by lag” (tlg), of target genes involved in this “antibiotic-
driven” phenotype. It benefited from the whole genome-sequencing of each evolved strain, and restored the respective
wild-type alleles. They concluded that a “better understanding of lag-time evolution as a key determinant of the
survival of bacterial populations under high antibiotic concentrations”, could lead to new approaches to block the
evolution.

In the second effort to learn whether persistent and resistant phenotypes were genetically somehow coupled, another
excellent in vitro evolution experiment was carried out [303]. The authors of this paper applied a lower dose of
ampicillin, which was still comparable to the therapeutic dose, separated by intervals in fresh medium, and had a
fixed residual level during growth. They continued daily intermittent exposures until resistance was established as
defined by clinical standards (MIC values). Starting with 3 different E. coli strains, they found that 11 of the 14
cultures reached an MIC at least seven-fold greater than the MIC of the respective ancestral strains. They carried on
following the evolutionary process leading to ampicillin resistance. The results were analyzed by a mathematical
population-genetics model. The authors wanted to know if tolerant strains were able to evolve antibiotic resistance
quicker than others. This analysis provided a scientific confirmation of the hypothesis that tolerance facilitates the
subsequent evolution of resistance. The authors declared that “tolerance mutations pave the way for the rapid
subsequent evolution of resistance”. Consequently, they speculated that “Preventing the evolution of tolerance may
offer a new strategy for delaying the emergence of resistance”. This discovery has been motivating scientists, who
are eager to answer the question of whether and why tolerance really invites resistance [304], and if it does, how
antibiotics research could benefit from that.

Our (maybe a little bit impertinent, but Darwinian) interpretation is that this “pave” must be considered as a
channelization condition, rather than connected or interrelated genetic mechanisms. This interpretation seems to have been
indirectly confirmed by the powerful genetic analysis [305] based on the construction and (Tn-Seq) sequencing
analysis of a highly saturated transposon library covering a majority of the genes and promoter regions of E. coli,
and exposing stationary-phase cultures to a lethal dose of gentamicin. The survivors of the gentamicin exposure
seem to show that tolerance to amino-glycosides could be a pleiotropic phenotype of the disruption of much more
than one distinct pathway, without changing the MIC to gentamicin. Amino acid auxotrophs, including serine,
threonine, glutamine, and tyrosine auxotrophs were also found to exhibit strongly decreased tolerance to
gentamicin, which cannot be restored by supplying the corresponding amino acids to the culture. The activation of
motility and amino acid biosynthesis also contributes to the formation of persisters tolerant to gentamicin [305], but
no direct evidence was presented confirming any link between the evolution of antibiotic resistance and tolerance.

Experiments to discover the genetic background in Salmonella enterica serovar Typhimurium resulted in the isolation
of several extremely persistent mutants, and revealed the discovery of a shpAB gene [306]. The mutants showed a
great increase in the survival rate after ampicillin exposure. Genetic analysis revealed that shp is a newly
discovered, toxin-antitoxin, module. The high-persistence phenotype was attributed to a nonsense mutation in the 3’
end of the shpB gene encoding an antitoxin protein. The high persistence depends on the presence of Lon protease.
The results of this interesting experiment indirectly explain why it was previously possible to isolate highly persistent
mutants from Salmonella [306], and also demonstrated that the presence of a cell persister phenotype, independently
of their genotype, serves as channelizing the conditions of, rather than determining genetic effects leading to evolution
of antibiotic resistance. Again, no evidence of any common genetic background of the antibiotic resistant and persist
cells could be demonstrated [306].

We concluded that the hypothesis is based on increasing evidence suggesting that persistence triggered and enabled
by a network of intracellular stress responses can accelerate the processes of adaptive evolution beyond shedding
light on the basis of persistence. Those persisters could be an evolutionary reservoir from which resistant organisms
can emerge [307] is probably correct, but there is not any direct genetic link between tolerance and resistance so far.
Whatever was the philosophy behind it, the first antibiotic (teixobactin) found in a screen of uncultured bacteria, which
kill Gram-positive pathogens without detectable resistance, has been discovered in Professor Kim Lewis’ Laboratory
[308]. Teixobactin inhibits cell wall synthesis by binding to a highly conserved motif of lipid II (a precursor of
peptidoglycan), and lipid III (a precursor of cell wall teichoic acid).
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929 So far neither any S. aureus, nor any Mycobacterium tuberculosis mutants were found to be resistant to teixobactin.
930 The authors are sure of being on a pathway leading to developing antibiotics which lack possible evolution to
931 resistance. Teixobactin is active exclusively against Gram-positive, but not against Gram-negative, bacteria.
932 In fact, teixobactin is the only antibiotic in the literature without detectable resistance. It is a NRP (non-ribosomal),
933 peptide-like, enzymatically synthetized, molecule. The teixobactin gene cluster has only been predicted [308].
934 This publication was commented on by several authors (published as #65059 in the same issue of Nature) [308].
935 Draper (2015) warned that a broader variety of resistance mechanisms might be expected to be revealed in future
936 clinical settings, such as the appearance of a special reductase.
937
938 3.3. Evolution of antibiotic resistance and collateral sensitivity (Is antibiotic resistance evolution a two-
939  
940 3.3.1. Morbidostat and experimental evolution of intrinsic antibiotic multiresistance
941 It is obvious that antibiotic resistance is an evolutionary process, based on sequential accumulation of multiple
942 mutations, under selective conditions. This part of the evolution can experimentally be studied, or even recapitulated.
943 The genetic variability in nature, or in a hospital, could be enlarged by horizontal gene transfer mediated by
944 compatible plasmids harboring mobile genetic elements and antibiotic resistant cassettes. To study the gradual
945 evolutionary processes, Toprak et al. [309] developed a selection device, the ‘morbidostat’, which is capable of
946 continuous monitoring of the growing and evolving bacterial population, under dynamically regulated antibiotic
947 concentrations. The morbidostat is suitable for carrying out experimental evolution studies on bacteria to
948 recapitulate genetic and molecular events of developing antibiotic resistance. The evolution of resistance in E. coli
949 towards several antibiotics, such as those of doxycycline, trimethoprim, chloramphenicol was reconstructed. Their
950 experimental protocol covered about 3 weeks, and they found that resistance levels toward a compound increased
951 substantially during this period. By using whole-genome sequencing of the evolved strains, they identified
952 mutations both specific to resistance to a particular drug, and shared in resistance to multiple drugs [309].
953 With trimethoprim, resistance evolved in the expected stepwise manner, through mutations restricted to the genes
954 encoding for the enzyme dihydrofolate reductase (DHFR). By sequencing of DHFR over time, they found parallel
955 populations evolved, with similar mutations, and mutations being acquired in a similar order. However, chloramphenicol
956 and doxycycline resistances evolved smoothly through diverse combinations of mutations in different genes which
957 were involved in transcription, translation and membrane transport.
958 The reviewer is attempted to play with the idea of whether similar experiments with persistent
959 E. coli strains under morbidostat conditions would, or would not, have resulted in similar conclusions. We would not
960 expect too much difference in the process of evolution, concerning either the case of trimethoprim, or that of
961 chloramphenicol and doxycycline resistance.
962 As we saw, in the “war” against antibiotic resistance, we have just been losing battle after battle. As mentioned before,
963 carbapenems used to be considered powerful chemical tools to overcome ESBL resistance, until the appearance of
964 carbapenem resistance in the Enterobacteriaceae. Considering the efficacy of intimate immunity in nature, it was
965 hoped that resistance toward antimicrobial peptides might cause fewer problems than is the case for antibiotics with
966 other chemical structures. As discussed above, colistin is an antimicrobial peptide which has been used for a
967 relatively long time for the treatment of multi-drug-resistant, Gram-negative, bacterial infected, clinical patients. It
968 has had moderate success, usually in combination with a carbapenem. However, expectations concerning colistin seem to
969 be evaporating too. Unfortunately, colistin resistance has been evolving at the clinical level[310]. This turned out to
970 be a real evolutionary product, based on multiple epistatic interrelations, not simply an accumulation of mutations at
971 one, or a very few, locus. The evolution of colistin resistance was a more-than-one step process, requiring mutation in
972 at least five independent loci synergistically, creating the resistant phenotype. As a wonderful example of justifying
973 the validity of the classical Mendelian genetics, strong and unambiguous intergenic epistasis seems to limit the
974 number of possible evolutionary pathways for antibacterial peptide resistance. Not only epistasis, but suppressor
975 mechanisms (mutations in transcriptional regulator genes) are also essential for the evolution of antimicrobial
976 peptides. These dominant suppressor mutations serve then as kind of “nodes potentiating further steps in the
977 evolutionary process leading to higher resistance” by increasing/channelizing the effects of other mutations, see
979
980 3.3.2. Experimental evolution of intrinsic antibiotic resistance and collateral sensitivity
Collateral sensitivity is a phenomenon that occurs when a new appearing antibiotic resistance is accompanied with a loss of a previous resistance to another drug. Collateral sensitivity has been detected before (see [48]), but it has neither been evolutionarily interpreted nor systematically studied like in this study [311] discussed below.

A completely new research line has been initiated at the Synthetic and Systems Biology Unit, Institute of Biochemistry, Biological Research Center, Szeged, Hungary, aiming at experimental reconstruction of the evolution of mechanisms of collateral sensitivity. This kind of research may lead to an option of reusing previously effective antibiotics to which resistance had been developed, and consequently, those antibiotics were withdrawn from clinical application. This research line was catalyzed by the surprising discovery that the evolution of new antibiotic resistance has frequently been accompanied by losing resistance to other antibiotics [311]. Working with *E. coli*, the authors ran large-scale laboratory evolutionary experiments and found that populations adapted to aminoglycosides have an especially low fitness in the presence of several other antibiotics. They sequenced the whole-genome of each of their laboratory-evolved aminoglycoside-resistant strains, and demonstrated multiple mechanisms underlying aminoglycoside resistance, including a reduced proton-motive force (PMF) through the inner membrane. They suggested that, as a pleiotropic consequence, these mutations diminished the activity of PMF-dependent antibiotics efflux pumps (such as the AcrAB transporter), resulting in hypersensitivity toward other antibiotics. We believe that this Hungarian discovery is of science historic value, allowing the fight against resistance development to new antibiotics, to be compensated by “reactivating” some previously used ones. We consider that the discovery of collateral sensitivity has an extremely great theoretical and practical impact, but probably relevant only for mutation-based, “intrinsic” resistances [311].

### 3.4. MDR Revolution in genus *Enterococcus*

*Enterococcus cecorum*, a normal commensal intestinal inhabitant, is increasingly responsible for outbreaks of arthritis and osteomyelitis in chickens worldwide. However, since 2002, *E. cecorum* has increasingly been recognized as a causative pathogen of enterococcal spondylitis (ES) [312-324].

Enterococcal spondylitis is a specific manifestation of *E. cecorum*-associated diseases, in which increased flock morbidity and mortality result from chronic infection involving the free thoracic vertebra and adjacent notarium or synsacrum. Birds affected with ES have hind-limb paresis of variable severity, due to spinal cord compression caused by the chronic inflammation [319-322]. Birds often develop characteristic clinical signs as they near market weight [308, 323]. To date, ES has been reported in both breeding and meat production flocks in several U.S. states, including Pennsylvania, Washington, North Carolina, South Carolina, Arkansas, Mississippi, Alabama, and California [319, 321].

ES has also been documented in broiler chickens in different countries all over the globe, in Belgium [312] in Canada [314], in Hungary [325], in Poland [326], in the Netherlands [327], and the UK [328]. Clinical presentations, gross findings and epidemiology are similar to those found in American broiler flocks [321].

While the pathogenesis of ES remains poorly understood, recent evidence suggests that the increased incidence of enterococcal-associated disease in poultry may be due to horizontal spread of dominant clones of *E. cecorum* that exhibit increased pathogenicity [327, 328]. A recent study was directed at investigating the genetic relatedness and antimicrobial resistance of isolates recovered from spondylitis lesions and caeca of affected/ unaffected birds from geographically and temporally distinct outbreaks of ES in the southeastern United States [327]. ES outbreaks from 2007 to 2011 were investigated in North Carolina (15 flocks, 13 farms, and four integrators), South Carolina (one flock, one farm, one integrator) and Alabama (six flocks, six farms, one integrator). From these 22 epidemiologically distinct outbreaks, 326 isolates of *E. cecorum* were recovered. Isolates from spinal lesions and caeca of affected birds (cases) and caeca of unaffected birds (controls) were genotyped using pulsed-field gel electrophoresis and compared with each other [327]. Phenotyping used GenIII MicroPlate™ (Biolog; Hayward, CA, USA), microbial identification plates, and antimicrobial sensitivity testing. Isolates from spinal lesions were incapable of mannitol metabolism, and the majority of these isolates were genetically clonal. In contrast, caecal isolates from control birds varied in their ability to metabolize mannitol, and were genetically diverse. Isolates from both case and control birds had high levels of antimicrobial resistance. These findings indicate that the increase in *E. cecorum*-associated diseases in the southeast United States is due to the emergence of new clones with increased pathogenicity and multi-drug resistance [329].

### 4. “Dialectics” of resistance and sensitivity: The agrocin 84 story in the genus *Agrobacterium*

So far, we have discussed resistance genes, and resistance mutations, the existence and expression of which make the originally sensitive organism resistant to a given compound. The product of the mutant gene causing resistance...
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can be located either on the chromosome, or on a newly taken-up plasmid, harboring it either as part of a mobile genetic element, or genomic island. But, there are examples of the opposite as well, when the existence and expression of a “sensitivity gene” makes the originally resistant organism sensitive to a given antibacterial compound. The product of such a sensitivity gene may catabolize a harmless molecule to a harmful derivative, or change the structure of the originally impermeable membrane to be permeable to the compound, or to block the multi-drug pumping activity. Alternatively, a sensitivity gene may code for a protein which is able to bind and transfer a toxic product into the cell, which it could not otherwise enter. This binding protein may play a role in the normal metabolism of the given cell.

A wonderful example is the sensitivity/resistance (S/R) phenotype in different Agrobacterium strains to Agrocin 84. It is one of the most studied molecules of the group of plant biocontrol molecules called agrocin 

Three Agrobacterium species have been identified so far: A. tumefaciens, also called Biovar 1; A. radiobacter, Biovar 2; and A. rhizogenes, Biovar 3 [332]. Each species includes virulent and avirulent strains. The virulent strains induce specific tumors, characterized by their secondary metabolites called opines. Depending on the type of opines, the virulent Agrobacterium strains of each species could be determined to be in the nopaline (NO), octopine (OCT), or agropine (AGR) opine group. Each Agrobacterium strain is capable of inducing a special opine-synthetizing tumor, and each of them is capable of catabolizing the respective opine, (although the respective opine was synthesized by the tumorous plant cells, and not the bacterium). Agrocin 84 was first identified as a trypsin and a pepsin-resistant small peptide with a molecular weight of 2,500. It was published as being built up of six different amino acids, including 9 molecules of glutamine or glutamic acid, and seven molecules of serine. It inhibited DNA, RNA and protein synthesis as well as amino acid transport of the virulent, susceptible, A. tumefaciens (H38-9) strain [333]. Agrocin 84 is toxic to several other but not all, Agrobacterium strains. Agrocin 84 inhibits those virulent, tumor-causing, Agrobacterium strains (called NOP strains), which induce nopaline-synthetizing tumor cells. The respective tumor-inducing Agrobacterium strains carry nopaline catabolizing genes on their respective (Ti), or hairy-root inducing (Ri), plasmid. If the plasmid carrying nopaline catabolizing genes was removed (cured) from the Agrocin 84-sensitive NOP strains, they became resistant to Agrocin 84.

The explanation of this phenomenon is that the agrocinopine gene, which has a normal metabolic function in the nopaline biosynthesis in the wild-type strain, also pleiotropically functions as a “sensitivity” gene, making the wild-type NOP Agrobacterium strains sensitive to Agrocin 84.

For a better understanding, it is important to know that A. tumefaciens NOP strains, such as strain C58 [334], induced crown gall tumors to produce not only nopaline, but other opines, called agrocinopines A and B, as well. Agrocinopine A has a normal function in NOP strains, where it is the inducer of Ti plasmid conjugal transfer in the strain [335]. It turned out that Agrocin 84 and agrocinopines A, the precursor of agrocinopine B, are transported by the same uptake system.

Consequently, mutations causing constitutive transfer of pTiC58 show the pleiotropic phenotype of super-sensitivity to Agrocin 84, while Agrocin 84-resistant mutants of A. tumefaciens A208 do not transport agrocinopine A.

Other Agrobacterium strains harboring non-NOP, pTiBo542 plasmids (which induce L, L, succinamopine and agropine producing tumors), the cells which also synthetize agrocinopines, but of a different type (agrocinopines C, the precursor of agrocinopine D), are resistant to Agrocin 84, but could be made sensitive by pretreatment with agrocinopine C, [330].

For more details, see [336-345].

5. Closing remarks

In the “card game” of the antibiotics and invoked resistances many reliable investigators consider polymixin (colistin) and vancomycin as the respective last “trump” against Gram-negative and Gram positive resistant pathogens, forecasting that the appearance Gram-negative isolates of colistin-resistance and that of Gram-positive isolates of vancomycin resistance means the end of the heroic “age of antibiotics”.

The authors of this review have reservation for accepting this pessimistic view. We suppose that the “card game” between new antibiotics and invoked resistances has not been finished yet. The genetic sources of both intrinsic and acquired resistances in the bacteria seem to be non-exhaustible. This fact justifies ‘hands-up’ pessimism. Fortunately enough, the number of theoretically possible QSAR-designed antimicrobial peptides also seems to be unlimited, or
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1086 at least extremely high. This fact justifies rational optimism, hoping that there is a real chance to overcome newly
1087 appearing resistances by discovering and introducing new, properly designed antimicrobial peptides at least for a long
1088 time. Therefore, we are expecting new antimicrobial peptides (either of natural or of synthetic origin) as new trumps
1089 as in the “card game” of science and bacterial multi-drug-resistance.
1090 We propose that the options provided by the natural and synthetic antimicrobial peptides will offer new solutions.
1091 The QSAR-designed synthetic antimicrobial peptides, and the non-ribosomal (NRP) peptides, especially those
1092 produced by entomopathogenic nematode symbiotic bacteria (Xenorhabdus, Photorhabdus) provide abundant gold
1093 mines for antibiotics of novel modes of action (see our next Review).
1094 As a very personal epilog, let us explain why we, practically newcomers, did undertake to put this review together.
1095 Our team has been working on natural antimicrobial peptides produced by entomopathogenic nematode symbiotic
1096 bacteria, and have recently started to cooperate with a team of veterinary scientists in order to materialize this
1097 conception. This review is our first joint venture.
1098
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1113
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1115 The authors declare that the research was conducted in the absence of any commercial or financial relationships that
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1118 REFERENCES
1121 3. Kulsits, S. 2017, Magyar Nemzet, September 17, 2017 [In Hungarian]; www.althir.org; 2017-09-26 (English); see
1122 also on https://translate.googleusercontent.com/translate_c?depth=1&hl=en&prev=search&url=translate.google.co
1123 m&sl=hu&sp=nmt4&u=http://althir.org/&usg=ALkJrhiLDYxecod43VSTXDbwlPPgRm3eQ).
1126 Chemother., 53, 2522.
1129 7. Exner, M., Bhattacharya, S., Christiansen, B., Gebel, J., Groncy-Bermes, P., Hartemann, P., Heeg, P., Ilschner, C.,
1130 Kramer, A., Larson, E., Merkens, W., Miécke, M., Oltmanns, P., Ross, B., Rotter, M., Schmithausen, R. M.,
1131 Sonntag, H. G. and Trautmann, M. 2017, GMS Hyg. Infect. Control, 12, Doc05; Published online 2017 Apr 10. DOI:
1132 10.3205/ dgkh000290 PMCID: PMC5388835.
Fodor A. et al (2018) Multidrug resistance in bacteria...a review (Preprint)
Fodor A. et al (2018) Multidrug resistance in bacteria...a review (Preprint)

Fodor A. et al (2018) Multidrug resistance in bacteria...a review (Preprint)

Fodor A. et al (2018) Multidrug resistance in bacteria...a review (Preprint)

Fodor A. et al (2018) Multidrug resistance in bacteria...a review (Preprint)


Fodor A. et al. (2018) Multidrug resistance in bacteria...a review (Preprint)

254. Courvalin, P. 2006, Dig. Liver Dis., 38(Suppl. 2), S261.
265. Piddock, L. J. 1999, Drugs, 58(Suppl. 2), S11.
Fodor A. et al (2018) Multidrug resistance in bacteria...a review (Preprint)