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

3 **András Fodor[1,*,$], Birhan Addisie Abate[2], Péter Deák[1], László Fodor[3], Michael G. Klein[4,&] László Makrai[3],**
4 **Josephat Muvevi[5], and Dávid Vozik[6,#]**
5
6 [1]Department of Genetics, University of Szeged, H-6726 Szeged, Középfasor 52; ([$]Present mail address:
7 5651 Fredericksburg Road, Wooster, OH 44691, USA); E-mail (András Fodor): csingicsangi@gmsil.com;
8 fodorandras@yahoo.com; E-mail (Péter Deák): deakp@bio.u-szeged.hu
9
10 [2]Ethiopian Biotechnology Institute, Agricultural Biotechnology Directorate; E-mail: Birhan Addisie
11 birhanaddisie@gmail.com
12
13 [3]Department of Microbiology and Infectious Diseases, University of Veterinary Medicine, 1581 Budapest, P.O. Box
14 22, Hungary; E.mail (László Fodor): Fodor.Laszlo@univet.hu; E.mail (László Makrai): Makrai.Laszlo@univet.hu
15
16 [4]Department of Entomology, The Ohio State University, Madison Avenue, Wooster, OH-44691, USA; ([&]Present
17 mail address: O. Box 1104, Heber, AZ 85928, USA). E.mail: klein10@osu.edu

18 [5]Plant Biotechnology Research Division, Addis Ababa, Ethiopia; [4]National Cereals and Produce Board, P.O. Box
19 84696-80100, Mombasa, Kenya. E.mail: jmuvevi@gmail.com
20
21 [6]Research Institute on Bioengineering, Membrane Technology and Energetics, Faculty of Engineering, University of
22 Pannonia, Egyetem utca 10, 8200 Veszprém, H-8200, Hungary; ([#]Present mail address: Transdanubian Regional
23 Waterworks Co., Department of Technology, Siófok, Hungary); E.mail: vozikd@gmail.com
24
25
26 *Corresponding author: csingicsangi@gmail.com; fodorandras@yahoo.com, ORCID 0000- 0003-3495-00154
27 .
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29 **ABSTRACT**
30 The challenge posed by multi-drug resistance (MDR) of pathogenic organisms, spectacularly manifested in the 6
31 "ESKAPE" bacterium (two Gram-positive, four Gram-negative) species, should invoke new comprehensive strategies,
32 and needs cooperation of scientists with medical, veterinary and natural science background. This review is aimed at
33 informing newcomers, coming from the field of biology and genetics, about problems related to rapidly emerging, new
34 multi-drug resistant, pathogenic, bacteria. Unlike persistence, the antibiotic resistance is inherited. A functioning
35 "*resistance gene*" makes a susceptible organism resistant to a given antibiotic, encoding for polypeptides capable of
36 acting either as decomposing enzymes, or acting as trans-membrane pumps, or membrane structure components
37 capable of modifying the permeability implementing a «by pass» mechanism enabling the antibiotic molecule to reach
38 its cellular target(s). A functioning "*sensitivity gene*" encode for a polypeptide, capable (directly or indirectly) of
39 transferring toxic molecules into target cells, or of metabolizing non-transferable to transferable, or non-toxic
40 molecules to toxic derivatives. A gene of a normal function could act as a "sensitivity" gene in the presence of
41 antibiotics of chemical structures similar to the natural substrate of the gene product, (enzyme or binding/
42 trans-membrane protein). The Agrocin 84 story is a good example. Multi-drug resistance is a phenotypic consequence of
43 the sequential accumulation of mutations, and/or up-take of plasmids or genomic islands carrying resistance genes
44 from the environment *via* horizontal gene transfer, mediated by conjugative plasmid or bacteriophage carrying
45 mobile genetic elements. Both multi-drug resistance and collateral sensitivity are evolutionary products. Some revealed
46 evolutionary process and their Lamarckian and Darwinian interpretations are discussed. Toolkits of comparative
47 full-genome sequencing, genomics, experimental evolution and population genetics may provide perspectives for
48 overcoming the invincibility of multi-drug panresistance. The status of some recently emerging pathogenic bacterium
49 species with zoonic features and of veterinary background is also discussed.



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50 **KEYWORDS:** ESKAPE-bacteria; Persistence; Resistance; Intrinsic/Acquired/ Multidrug (MDR) and Pan –
51 Resistance; Genetic background; Experimental Evolution; Collateral sensitivity; Agrocin.
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98 **1. Introduction**

99 Multi-drug resistance (MDR) of pathogenic bacteria is an extremely complex field of life sciences that needs the
100 expertise of physicians, (docs, vets), microbiologist, biochemists, theoretical and preparative organic chemists,
101 bioinformatics, geneticists, and evolutionary biologists. This review is prepared first of all for geneticists and biologists
102 who are newcomers to life science background without clinical or veterinary experience. It is well known today, that
103 although the antibiotics are extremely important therapeutic tools in human, veterinary and even plant medicine,
104 their use has gradually become limited because of resistance problems. The phenomenon of antibiotic-resistance was
105 first discovered as early as 1940 [1]. Whenever pathogenic microorganisms are exposed to the selective pressure of
106 antimicrobials, either in the laboratory, medicine, or agriculture, it is favorable for the development, survival and
107 spread of resistant clones [2]. Resistance means non-susceptibility to given antibiotics. When an isolate of a given
108 pathogenic bacterium is resistant to more than one antibiotic, the options for antibiotic therapy of the disease caused by
109 this pathogen is decreased. The emergence of antibiotic multi-resistance in pathogenic bacteria has become alarming in
110 the recent decades. As an example, 1481 patients died in Hungarian hospitals in 2016. In 174 of those deaths,
111 infection was the cause of death, or was involved in it, according to the (Hungarian) National Epidemiological
112 Center. Last year, 4830 MDR infections were reported, compared with 4187 in 2015 and 3998 in 2014. Mostly urinary
113 tract infections occurred, followed by infected wounds, blood vessel infections, and hospital-related pneumonia. A
114 majority of the patients were above 60 [3].

115 Infections caused by multi-resistant bacteria have dramatically increased not only in Hungary, but all over the
116 world, invoking an enormous public concern. There are not human clinical, [4-7] but zoonic [8] and veterinary [9-
117 15], as well as plant health aspects [16-18] come forward alarmingly.

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

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

127 When reporting a radical and continuous decrease in the number of new antibiotics in the market, Canadian authors
128 [23] asked in 2005:

129 - "*Where are all the new antibiotics*?"

130 Eleven-years later, a late answer appeared in '*Nature*':

131 - "*Antibiotics" (are) "right under our nose"*! [24].

132 It is good news and may be true. But unfortunately they still have not been in the market; at least not in the required
133 numbers. Their number seems to be much less than needed for really effective control of multi-resistant pathogens
134 [4]. In the period 2003-2007 only 5 new antibiotics appeared. In 2009 there were 16 new molecules listed as being in
135 clinical trials phase, with only 2 in the pipeline; including 3 glycopeptides, 4 quinolones, 2 oxazolidinones, 2β-
136 lactams, 1β-lactamase inhibitor, 1 trimethoprim, 1 macro-ketolide, 1 streptogramin, and 1 glycyl-cycline [25].

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

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

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

146 (1) A short overview of the list of the most significant bacterial pathogens which cause the most striking examples of
147 MDR outbreaks;

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

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149 resistant pathogen bacteria around us;

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

## 152 2. The "Card Game" of antibiotics research scientists and antibiotic resistance. New antibiotic drugs 153 invoke new resistances; it is just question of time.

### 154 2.1. Multi-drug resistance: Definitions and nomenclature (Based on phenotype and origin of multi-drug 155 resistance)

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

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

### 179 2.2. The "ESKAPE Club" of omnipotent multi-drug resistant bacterium species

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

186 The present list (without the fungus *Aspergillus*), includes 6 bacterium species called the ESKAPE Pathogen
187 Bacterium Species list. The name of the six letters involve the initials of genus names of these bacteria: ***E**nterococcus*
188 *faecium, **S**taphylococcus aureus, **K**lebsiella pneumoniae, **A**cinetobacter baumannii, **P**seudomonas aeruginosa*, and
189 ***E**nterobacter* [32]. The explanation: these groups of bacteria may produce omniresistant (panresistant) pathogen
190 strains, against which there is "NO DRUG" (no protecting antibiotics), and therefore there is "NO ESKAPE" [32].

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

195 This internationally accepted list which has been refreshed yearly, and should be considered still authentic, but
196 probably will be expanding soon, and may not be considered as complete. This authentic list has been renewing
197 from time-to-time [4], and has been expected to be expanded. The *Clostridium* genus for instance, which provides
198 examples of MDR pathogens, (in *C. difficile*: see [33, 34]; in *C. perfringens:* see [35]), is not included, but is a potential
199 candidate. Similarly, the *Salmonella* genus has not been included in the ESKAPE list, despite alarming publications
200 related with signs of MDR pathogen evolution in this taxonomic group [36-40].

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201 This subsection is restricted only to the species "officially" registered in the ESKAPE "club" and tries to draw the
202 attention of the respected Reader to some new candidates.

203 **2.2.1. Methicillin resistant *Staphylococcus aureus*, MRSA**

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

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

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

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

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

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

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

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

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

244 **2.2.2. Extended spectrum β-lactamase (ESBL) producing Enterobacteriaceae and *Klebsiella pneumoniae***

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

250 From an evolutionary point of view, probably the most important discovery is that the extended spectrum β-
251 lactamase background exerts an unexplained, but demonstrated positive, kind of channelizing effect on the

252 evolutionary mechanisms, leading to the appearance of other antibiotic resistant mutants, at a significantly higher
253 degree than in the normal population [54, 55].

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

261 The biochemical background is the increasing number of β-lactamase enzymes with enlarged substrate specificities.
262 The substrate range includes cephalosporins (cefotaxime and ceftriaxone) [57-59], a monobactam (aztreonam) [60-62],
263 the amino-penicillin combinations [63], ampicillin sulbactam (a lactamase inhibitor) [64-67], the ureido-penicillins [68,
264 69], including piperacillin [70], tazobactam [71-73], temocillin and piperacillin/tazobactam [74], and ceftolozane-
265 tazobactam [75]. Furthermore, the appearance of new enzymes is not associated with the loss of ability to hydrolyze
266 the earlier-lactams, such as ampicillin. The prevalence of ESBL production among *E. coli* and *Klebsiella* species is
267 variable [31, 4].

268 To overcome ESBL problems, carbapenem antibiotics were developed. They are relatively resistant to hydrolysis by
269 most β-lactamases. In some cases they act either as “slow substrate” β-lactamases inhibitors, and are capable of
270 binding to penicillin binding proteins. This “value-added feature” of inhibiting β-lactamases serves as a major
271 rationale for expansion of this class of β-lactams [76]. Carbapenem antibiotics including imipenem (a Gram-
272 negative cell-wall synthesis interrupting molecule which binds to penicillin-binding proteins); cilastatin (a human
273 enzyme dehydropeptidase in the human kidney, inhibiting imipenem degradation); meropenem [77, 78] and ertapenem
274 [79-82] have served as a putative last line of defense against multi-drug-resistant Gram-negative organisms.

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

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

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

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

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

295 **2.2.3. *Pseudomonas aeruginosa*, a pan-genomic hot bed of multi-drug resistance**

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

300 **2.2.3.1. Taxonomy**

301 *Pseudomonas aeruginosa* (Class: Gammaproteo-bacteria; Family: Pseudomonadaceae; Phylum: Proteobacteria) is a
302 common Gram-negative, rod-shaped, bacterium species that can cause disease in plants, animals, and humans. It is a
303 ubiquitous, invasive, pangenomic, Gram-negative, opportunistic, pathogen, (Wikipedia).

304 **2.2.3.2. A short list of intrinsic and acquired antibiotic resistances**

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

316 **2.2.3.3. Incomplete list of diseases and pathomechanisms**

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

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

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

338 **2.2.3.4. Genetic background**

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

347

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

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358 genomics data from comparative sequence analysis of all available drug-resistant *P. aeruginosa* genomes.

359 Despite this impressive and powerful approach, the authors declared this year that the usefulness of the
360 interpretation of a predicted resistome must remain limited, because of the heterogeneity and the occurrence of gene
361 "identity discrepancies" between strains. The situation might be improved by incorporating a transcriptomic,
362 proteomic, and/or metabolomic, component into the database [115].

363 The reviewers wondered whether it would ever be possible to forecast invoked defense mechanisms to *in silico*
364 designed antimicrobial peptides on the basis of information provided by full-genome sequences.

365 **2.2.4. *Acinetobacter baumannii*, the "queen" of multi-drug resistant bacteria**

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

374 **2.2.4.1. Taxonomy**

375 Domain: Bacteria; Kingdom: Eubacteria; Phylum: Proteobacteria; Class: Gammaproteo-bacteria; Family: Order:
376 Pseudomonadales; Family: Moraxellaceae; Genus: *Acinetobacter* (see: Wikipedia).

377 The *Acinetobacter* species are rod-shaped, glucose-non-fermentative, non-motile, non-fastidious, catalase- positive,
378 oxidative-negative, aerobic, Gram-negative coccobacilli. *Acinetobacter baumannii* is the only member of the
379 Moraxellaceae to lack cytochrome C oxidases, and it can cause serious diseases in plants, animals and humans
380 [118].

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

384 **2.2.4.2. An incomplete list of diseases caused by**
385 ***A. baumannii***

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

395 **2.2.4.3. Virulence factors**

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

399 **2.2.4.4. Short evolutionary history of the global spread of multi-drug resistant lines of *A. baumannii***

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

402 The species *A. baumannii* used to have a low phylogenetic diversity, providing a severe evolutionary bottle-neck
403 through which a micro-evolutionary tree of many branches has emerged since the late 1980s [120, 128]. Other
404 species of *Acinetobacter* are soil-inhabiting organisms,
405 while *A. baumannii* is almost exclusively isolated from hospital environments [129], veterinary clinics [130] and
406 especially from intensive care units of hospitals [131]. Just like *P. aeruginosa* and other bacterium species, the
407 resistance in *A. baumannii* is also the phenotypic consequence of genetically encoded biochemical procedures,
408 namely, the presence and activity of decomposing enzymes, changed expression of porins, reduced permeability,

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409 and constitutive expression of active multi-substrate efflux systems [132]. Similar to *P. aeruginosa*, *A. baumannii* also
410 has been armored with both "intrinsic" and "acquired" (taken-up) resistance mechanisms [120, 133].

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

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

419 Colistin [141] was found to be a reliably effective drug *in vitro* against MDR, resulting in a renaissance to polymixins
420 [142-145]. It had previously been withdrawn from commerce because of serious *in vivo* side effects [146, 147] even
421 if these side effects could partially be compensated clinically [148, 149]. In a short amount of time there have been
422 reports on colistin resistance as well [139, 150-154].

423 **2.2.4.5. Antibiotic resistance mechanisms present in *A. baumannii***

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

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

433 **2.2.4.5.1. β-lactamases**

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

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

443 Since 2006, 18 Class B β-lactamases have been described or re-described, including NDM-1 [161] and NDM-3
444 [162], discovered in 2016. There is only 1 new Class A β-Lactamase, called AmpC [163, 164].

445 Forty nine Class D (OXA) β-Lactamases have also been described from *A. baumannii*; 4 of them - (OXA-239, OXA-
446 72, OXA-51, and OXA-253) - were discovered or re-described in the last year [165-170].

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

450 **2.2.4.5.2. Aminoglycoside-modifying enzymes**

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

454 *Aminoglycoside acetyltransferases* are represented by 5 enzymes: AAC3 [172], aaC1, aac2, AAC (6'), aacA4 [173-
455 178].

456 *Aminoglycoside adenyltransferases* are represented by 4 enzymes: ANT (2″), (aadB), [172], ANT (3″) and (aadA1)
457 [174, 177].

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458 *Aminoglycoside phosphotransferases* are represented by 3 enzymes: PH (3′), (aphA1) [179] and APH (3″) [163].

459 **2.2.4.5.3. Efflux pumps**

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

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

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

470 The major facilitator efflux pump superfamily is represented by TetA [193], TetB [194], and CmlA [195], CraA
471 [196] (responsible for tetracycline and chloramphenicol resistances, respectively), AmvA (mediating resistance
472 towards different classes of molecules of antibacterial activity, such as disinfectants, detergents, and dyes,
473 furthermore erythromycin, acriflavine, benzalkonium chloride, and methylviologen) [197], and finally, AbaF
474 (responsible for fosfomycin resistance) [198].

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

478 The EmrAB-TolC efflux pump is also present in *A. baumannii* and responsible for resistance to netilmicin,
479 tobramycin and imipenem [201]. A1S-1535 confers resistance to gentamicin, kanamycin, chloroxylenol,
480 oxytetracycline, 1, 10-phenanthroline, and chloramphenicol [202]. A1S-2795 is responsible for resistance to the
481 sulphonamide sulfathiazole, and ABAYE-0913 is associated with resistance to chloramphenicol and fusidic acid
482 [202].

483 **2.2.4.5.4. Altered permeability resulting in antibiotic resistance in A. *baumannii***

484 Porins which form channels that allow transport of molecules across the outer membrane of Gram-negative bacteria,
485 resulted in carbapenem resistance in *A. baumannii* [128]. Carbapenem resistance could be a phenotypic consequence
486 of reduced expression of some porins, such as Omp22-33 [203], or CarO [204, 205]. Imipenem resistance could be the
487 phenotypic consequence of the loss of Omp29, producing OXA-51-like, or OXA-23-like, carbapenemases [206].
488 Aztreonam, chloramphenicol, and nalidixic acid resistance is related with OmpA [207]. OmpA and CarO have recently
489 been reported as being associated with antibiotic resistance through physical interactions with OXA-23 carbapenemase
490 [208].

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

495 Aminoglycoside resistance could be the phenotypic consequences of the 16S rRNA methylase (ArmA) activity, found in
496 several pathogen isolates. It always coexists with OXA-type carbapenemases such as OXA-23 [178, 210-215].
497 Quinolone resistance could be the phenotypic consequence of modifications in GyrA – coding structure gene (GyrA
498 is one subunit of DNA gyrase), and that of ParC (one subunit of topoisomerase IV), in epidemiologically unrelated
499 *A. baumannii isolate*s [216]. Tetracycline resistance, determined by TetM, is thought to act through ribosomal
500 protection [217]. Trimethoprim resistance has been found in nosocomial MDR *A. baumannii* isolates and is
501 supposed be a phenotypic consequence of the action of dihydrofolate reductases (DHFR and FolA) [166, 218, 219].

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

503 **2.2.4.5.5. Biofilm formation**

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

507 **2.2.4.6. Genome plasticity and evolution of antibiotic resistance: International clonal lineages**

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

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

523 Originally three predominant pathogen clones (called 'international clonal lineages', ICLs) were known as being
524 responsible for hospital outbreaks worldwide. ICL1 and ICL2 have been known as of MDR phenotypes. Since then,
525 four more have been identified and listed at the *A. baumannii* MLST database, which is publicly available at
526 http://pubmlst.org/abaumannii/. At present at least 6, (if not more), major ICLs have been distributed worldwide
527 [225].

528 The genome of a representative ICL1 (AYE) strain includes 52 genes associated with resistance to antimicrobial
529 drugs, and 45 of them are localized in an 86 kb resistance island called AbaR1. ABAR1 is also present in other *A.*
530 *baumannii* strains, but with a much smaller size. It has been noted that the presence of an extraordinary 22 gene-
531 cassette coding for transposases and insertion sequences may be responsible for the acquisition of resistance genes into
532 the AbaR1 island of the AYE ICL1-type strain. Almost half are orthologous to coding sequences of *Pseudomonas*
533 [120].

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

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

539 **2.2.5. *Enterococci*: The Gram-positive "Vanguards" of the "MDR movement"**

540 **2.2.5.1. Taxonomy and general description**

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

549 The MDR enterococci are important nosocomial pathogens, and a growing clinical challenge, because they developed
550 full resistance against practically all traditional antimicrobials used in clinical practices, due to a large number of
551 genetic strategies [228]. The MDR enterococci display a wide repertoire of antibiotic resistance mechanisms, including
552 modification of drug targets, inactivation of therapeutic agents, overexpression of efflux pumps, and a sophisticated cell
553 envelope adaptive response that promotes survival in the human host and nosocomial environments [228]. MDR
554 enterococci strains are well adapted to survive in the gastrointestinal tract, and can become the dominant flora under
555 antibiotic pressure, predisposing the severely ill and immune-compromised patients, to other invasive infections
556 [229]. The excellent review of Gilmore [230] provides the authentic historic background and the most up-to-date
557 information and references about the known details, including the magic genetic and sophisticated biochemical
558 background of resistance mechanisms towards the different antibiotics for the enterococci.

559 Briefly, the history is as follows: streptococcal infections were successfully treated by the introduction of penicillin
560 to the clinical practice. However, the enterococci respond reluctantly to penicillin due to an inherent tolerance to the
561 killing action of these compounds [231]. In this pioneer work six penicillin-binding proteins (PBPs) were detected
562 in clinical isolates of each one of three group D streptococci: *E. (Streptococcus) bovis*, *E. faecalis* and *E. faecium*. *E.*
563 *faecium* is the most penicillin-resistant species of group D streptococci. When the authors examined in whole
564 organisms, they found that the PBPs of *E. faecium* showed lower affinities for the antibiotic than those of *E. faecalis*
565 (intermediate penicillin resistance), which in turn were of lower affinity than those of the penicillin-sensitive *E.*
566 *bovis* [231].

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

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

584 **2.2.5.2. Resistance mechanisms acquired and/or performed by *enterococci***

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

594 **2.2.5.2.1. Resistance mechanisms to penicillin and ampicillin**

595 This subject had recently been reviewed by Miller *et al*. [228]. The most important factor is that resistance toward
596 cell-wall active antibiotics is not restricted to intrinsic lactamase activity [244], but several others factors [245] related
597 to structural or expression-rate changes of penicillin-binding proteins: over-production low-affinity binding [246], or
598 mutations affecting the structure or regulation of a penicillin-binding protein [247]. Publications appearing since
599 2014 confirm differential penicillin-binding protein 5 (PBP5) levels in the *E. faecium* clades with different levels of
600 ampicillin resistance [248]. The genome-wide identification of ampicillin resistance determinants in *E. faecium*
601 revealed that although mutations in the low-affinity penicillin-binding protein PBP5 have played an important, but not
602 exclusive, role for ampicillin resistance in this species [249], the existence of additional resistance determinants has
603 been suggested. The authors constructed a high-density transposon mutant library for *E. faecium*, and developed a
604 transposon mutant tracking approach termed Microarray-based Transposon Mapping (M-TraM). This approach led to
605 the identification of a compendium of *E. faecium* genes that contribute to ampicillin resistance. These genes are part
606 of the core genome of *E. faecium*, indicating a high potential for it to evolve towards β-lactam resistance [249].
607 Furthermore, they validated their M-TraM results by adapting a Cre-lox recombination system to construct targeted,
608 marker-less, mutants. They confirmed the role of 4 more genes in ampicillin resistance by the generation of targeted
609 mutants, and further characterized these mutants with regard to their resistance to lysozyme. They showed that *ddc*P, a
610 gene predicted to encode a low-molecular-weight penicillin-binding protein with D-alanyl-D-alanine
611 carboxypeptidase activity, was essential for high-level ampicillin resistance. Furthermore, deletion of ddcP sensitized
612 *E. faecium* to lysozyme and abolished membrane-associated D, D-carboxypeptidase activity [249]. We consider this

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

615 **2.2.5.2.2. Resistance mechanisms of *Enterococci* to cephalosporins**

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

625 **2.2.5.2.3. Resistance mechanisms of *Enterococci* to glycopeptide antibiotics such as vancomycin and teicoplanin**

626 The glycopeptide antibiotics vancomycin and teicoplanin bind to the peptidyl-D-$Ala_4$-F-$Ala_5$ extremity of
627 peptidoglycan precursors, and cause inhibition by steric hindrance of the elongation of both glycan chains, by
628 glycosyl-transferases, and the cross-linking of stem peptides by D, D-trans-peptidases [243]. The L, D-trans-
629 peptidases use acyl donors, and they contain a stem tetra-peptide ending in D-$Ala_4$ that does not bind to these
630 antibiotics. A novel peptidoglycan cross-linking enzyme changing the terminal amino acids of the peptidoglycan
631 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-
632 Ser) results in resistance to glycopeptide antibiotic. This and other possible resistance mechanisms were also
633 considered [255].

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

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

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

641 The authors produced a strain (called M9); containing 79 relevant mutations obtained through 9 selections steps from
642 900 generations. At the end of each selection step they isolated a respective multi-mutant strain, and named them
643 M1–M9. The parental strain D344S M9 was completely sensitive, while M9 was fully resistant to ampicillin,
644 vancomycin and tetracycline. They found that the *ddC* locus was not affected through 4 selection steps, and mutant
645 strains M1–M4 remained sensitive to each of the three antibiotics, although the resistance to ampicillin seemed to be
646 gradually growing. The $5^{th}$ selection step was critical, resulting in M512, fully resistant to ampicillin, but still
647 sensitive to tetracycline, and showing a moderate vancomycin resistance. The *dd*C gene was inactivated. In the next 3
648 generations, the resistance towards vancomycin and tetracycline gradually grew, and M8 was fully resistant to
649 vancomycin.

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

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

662 These results suggest that the fluxes through central metabolic pathways, including glycolysis, might be profoundly
663 remodeled in mutant set of M9. This is an indirect confirmation of the recent report on *E. faecalis* mutants hyper-
664 susceptible to β-lactam antibiotics [252], indicating that existence of nutritional control of antibiotic resistance is

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665 based on a connection between CroR and the phosphotransferase system (PTS)system. As for the peptidoglycan
666 synthesis, they found that 4 of the 20 enzymes, committed to peptidoglycan biosynthesis, were affected by amino
667 substitutions. None of the substitutions had any obvious role in the activation of the L, D-transpeptidation pathway.

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

670 **2.2.5.2.4. Resistance mechanisms to antibiotics that interfere with protein synthesis**

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

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

679 Cross-resistance with all macrolides is a result of the modification of the 23S rRNA target. Resistance to
680 tetracyclines and glycylcyclinesis is mediated by multiple genes, but follows two general strategies: efflux of the
681 antibiotics and ribosomal protection. Mutations in the genes, *gyr*A and *par*C, (present in *E. faecium* and *E. faecalis*,
682 but absent from *E. gallinarum* and *E. casseliflavus*) affect the quinolone resistance-determining regions, which
683 presumably alter the binding affinity of the antibiotic quinolones, the target enzymes (DNA gyrase and
684 topoisomerase IV) that are responsible for DNA supercoil relaxation [53, 228, 261]. Rifampicin resistance arises from
685 a variety of mutations in the *rpo*B gene that encodes for the β-subunit of the RNA polymerase [228]. Trimethoprim
686 and sulfamethoxazole (inhibitors of bacterial enzymes involved in the folate synthesis pathway) are ineffective *in*
687 *vivo* to enterococci, because they are those extreme rare bacteria which can utilize exogenous sources of folate [228,
688 53].

689 **2.3. Zoonic and veterinary pathogen candidates for the "ESCAPE Club"**

690 **2.3.1. *Mycoplasma bovis***

691 *Mycoplasma bovis* is a worldwide pathogen that is the causative agent of pneumonia, mastitis, arthritis, and a
692 variety of other symptoms in cattle [262]. As a result, it is responsible for significant economic losses [263]. The
693 pathogens in the *Mycoplasma* species are members of the class *Mollicutes*, and comprise the simplest life form that
694 can replicate independently from the host. *Mycoplasma* spp. have no cell wall, and they have a limited number of
695 metabolic pathways. The greatly reduced genome size and coding capacity of *Mycoplasma* spp., makes them a good
696 model for genetic studies. *Mycoplasma* spp. are rather fast-evolving bacteria, pathogenic against humans and
697 animals. However, their importance is often underestimated. *Mycoplasma bovis* is a major cause of calf pneumonia,
698 mastitis and arthritis, and is intrinsically resistant to antibiotics acting on cell wall or folate synthesis [262], but the
699 antimicrobial protein synthesis inhibitor classes are active against it [264]. Tetracyclines and spectinomycin
700 primarily bind to the 30S subunit of the ribosome, whereas macrolides, lincosamides, phenicols, and pleuromutilins
701 are mycoplasmastatic antibiotics acting on the 50S ribosomal subunit, preventing the mechanisms of transpeptidation
702 and translation [265]. Expanded-spectrum fluoroquinolones, such as enrofloxacin, danofloxacin and marbofloxacin,
703 have anti-mycoplasmacidal effects by acting on topoisomerases that inhibit the DNA synthesis of bacteria [266].
704 Among the few antimicrobials licensed for treatment of *M. bovis*, there is increasing evidence for resistance [254, 265-
705 268].

706 As for the genetic background, a point mutation in the *par*C gene resulted in decreased susceptibility to
707 fluoroquinolones in *M. bovis* [265], and amino acid substitutions in *Gyr*A and *Par*C resulted in fluoroquinolone
708 resistant phenotypes [269]. Surprisingly enough, 16S rRNA gene mutations have been associated with decreased
709 susceptibility to tetracycline in *M. bovis* [270]. *Mycoplasma bovis* was detected in 32/45 bovine respiratory infection
710 outbreaks at beef farms in 8 provinces in China [271]. The isolates were susceptible, or had medium sensitivity, to
711 ciprofloxacin, enrofloxacin and doxycycline, but 13 of the 32 were resistant to macrolides. A point mutation at the 23
712 *rrn*A operon in domain V of 23S rRNA seems to be responsible for the macrolide resistance phenotype in *M. bovis*
713 [271]. Antibiotic susceptibility profiles of *M. bovis* strains isolated from cattle in Hungary were determined [272]. The
714 growth of many *M. bovis* strains was not inhibited by gentamicin, spectinomycin, florfenicol or lincomycin. The
715 most effective antibiotics tested *in vitro* were the fluoroquinolones: danofloxacin, enrofloxacin, and marbofloxacin.
716 But, there were 3 of the 35 Hungarian field strains for which the fluoroquinolone MICs were high [272].

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717 Fluoroquinolone-resistant mutants were also selected *in vitro* for danofloxacin, enrofloxacin and marbofloxacin and
718 each showed complete cross-resistance with the others. The respective mutations responsible for high macrolide,
719 lincomycin, florfenicol, and pleuromutilin antibiotic MICs were mapped into genes encoding 23S rRNA [273].

720 **2.3.2. *Bacillus anthracis***

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

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

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

758 The susceptibility of 29 *B. anthracis* bovine strains, collected in Hungary between 1933 and 2014 was tested against
759 10 antibiotics with commercially available minimum inhibitory concentration (MIC) test strips [281]. All strains were
760 susceptible to amoxicillin, ciprofloxacin, clindamycin, doxycycline, gentamicin, penicillin, rifampicin, and vancomycin.
761 Intermediate susceptibility to erythromycin and cefotaxime was detected in 17.2% (5/29) and 58.6% (17/29) of the
762 strains, respectively. Correlations were not observed between the isolation date, location, host species, genotype, and
763 antibiotic susceptibility profile of the strain. A similar study in Cameroon showed that Bovine *B. anthracis* isolates
764 from there showed a strong homogeneity, and they belong, together with strains from Chad, to a cluster Aβ, which
765 appears to be predominant in western Africa [282]. However, one strain that belongs to a newly defined clade (D)
766 and cluster (D1) was penicillin resistant.

767 **2.3.3. *Francisella tularensis***

768 *Francisella tularensis* is a fastidious, Gram-negative bacterium, a highly contagious zoonotic agent, and the
769 causative agent of the fatal disease, tularemia. Tularemia may occur in six well-recognized clinical forms in humans:

770 ulceroglandular; glandular; oculoglandular; oropharyngeal; pneumonic; and typhoid, or septicemic, tularemia. The [283]
771 *F. tularensis subsp. holarctica* (type B) is found throughout the Northern Hemisphere, and is the only endemic
772 subspecies found in Europe [284]. Lagomorphs, rodents, European brown hares (*Lepus europaeus*), and voles
773 (*Microtus arvalis*), serve as the primary mammalian reservoir hosts. Annual number of tularemia cases in humans is
774 well correlated with the yearly biologic cycle (March-February) for hares and hematophagous arthropods, such as
775 ticks, which play a role as vectors and hosts [285]. The antibiotics of choice in the treatment of tularemia are
776 aminoglycosides, quinolones, chloramphenicol, or tetracyclines.

777 Fortunately enough, there is no sign of the occurrence of multi-drug resistant *F. tularensis* strains, but resistances to
778 the same antibiotics used in its treatment are known in other bacteria, so acquired resistance could be forecast.
779 Furthermore, aminoglycosides, quinolones, chloramphenicol and tetracyclines are important, bearing in mind the
780 side effects and probability to replace them. This is why it is important to be informed about the general picture of the
781 antibiotic susceptibility of local populations. A resent screen in Hungary provided a satisfying result [286]. Twenty-
782 nine *F. tularensis* strains isolated between 2003 and 2010 from free-ranging European brown hares, and a captive
783 patas monkey (*Erythrocebus patas*), were collected from different parts of Hungary.

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

788 **2.3.4. *Escherichia coli***

789 Commensal strains of *E. coli*, as versatile residents of the lower intestine, are also repeatedly challenged by
790 antimicrobial pressures during the lifetime of their host. As a consequence, commensal strains acquire resistance
791 genes, and/or develop resistant mutants in order to survive and maintain microbial homeostasis in the lower intestinal
792 tract. Commensal *E. coli* strains are regarded as indicators of the antimicrobial load of their hosts. The recent review
793 [8] described the historic background of the origin, appearance and transfer mechanisms of antimicrobial resistance
794 genes into original animal - commensal intestinal *E. coli* with comparative information on their pathogenic
795 counterparts. The most efficient mechanism used by *E. coli* against different antimicrobial-based efflux pumps, and
796 mobile resistance mechanisms carried by plasmids and/or mobile genetic elements are known. For a while, these
797 mechanisms cannot protect *E. coli* against fabclavine (Fodor *et al*., in preparation). The emergence of hybrid
798 plasmids, both resistance and virulent, among *E. coli* is of additional public concern. Co-existence and co-transfer of
799 these “bad genes” in this huge and most versatile *in vivo* compartment may represent an increased public health risk
800 in the future. The significance of MDR commensal *E. coli* seem to be highest in the food animal industry, which
801 may function as a reservoir for intra- and interspecific exchange, and a source for spread of MDR determinants
802 through contaminated food to humans. Thus, the potential of MDR occurring in these commensal bacteria living in
803 animals used as sources of food (as meat, eggs, milk) should be a concern from the aspect of public health, and it
804 needs to be continuously monitored in the future by using the toolkit of molecular genetics [8]. In fact, that pessimistic
805 theory has been demonstrated. The first pilot study on the prevalence of verocytotoxin-producing (VTEC) *E. coli*
806 and of MDR/ESBL *E. coli* in illegally imported food products of animal origin, suggests that these strains could
807 represent reservoirs for dissemination of potentially new types of pathogenic and MDR *E. coli* in Europe [287].

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

819

820 **3. Evolutionary aspects**

821 **3.1. Prelude: Lamarck and Darwin**

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

837 **3.2. Tolerance, persistence & resistance**

838 The efforts of better understanding, and attempts at understanding either the intrinsic or environmental conditions behind
839 the evolution of antibiotics resistance have a long scientific history [289-293].

840 A new and promising approach to search for potent antibiotics, which may not provoke resistance in pathogen targets,
841 was initiated by the "rediscovery" of an old observation, made by Joseph W. Bigger [294] in 1944. He found
842 survivors in an antibiotic (penicillin)-treated bacterium (*Staphylococcus*) cell population without any inheritable
843 genetic change. The surviving subpopulation of the antibiotic-exposed cells, which showed the phenomenon of
844 "non-inherited antibiotic resistance" [295], had been in a metabolically dormant physiological state, meaning that
845 the metabolic rate was slowed down to the lowest possible level, and thus were non-dividing. This surviving
846 subpopulation has been considered as epigenetic variant cells and Kim Louis speaks about them as "persister cells"
847 [296, 297], or "persisters" [298]. The cells of bacterial biofilms also have strong multi-drug tolerance [299], causing
848 almost as serious a problem in health care as does the multi-drug resistance. The potential of using persistent cells as
849 targets of novel candidate antimicrobials was first realized by Lewis [296].

850 The "quasi-Lamarckian" definition of persistence as "non-inherited antibiotic resistance" [295] inspired and
851 motivated antibiotics research, since several laboratories turned to studying the phenomenon of persistence as a kind
852 of resistance. These scientific efforts made it possible to discover that the danger posed by the existence of
853 multiresistant *P. aeruginosa* has been enhanced by the emergence of new isolates producing high levels of persister
854 cells in patients with cystic fibrosis [99], and biofilms with this disease [100].

855 We found the best definition in the literature for distinguishing between resistance and tolerance in the excellent
856 paper of Friedman *et al*. [300], who declared that "Resistance" makes it possible for a microorganism to grow in the
857 constant presence of the antibiotic, provided that the concentration of the antibiotic is not too high. 'Tolerance'
858 allows a microorganism to survive antibiotic treatment, even at high antibiotic concentrations, as long as the
859 duration of the treatment is limited'. The persistent cells are not resistant, but a persistent cell mass is tolerant.

860 The biochemical mechanism behind persistence has mostly been revealed in the last 7 years. A second messenger
861 molecule, ppGpp, plays a key role by mediating in activating Type I and II Toxin-Antitoxins [301, 302]. However,
862 the evolution of tolerance is much less understood than that of resistance.

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

870 In the first, the authors followed the evolution of *E. coli* populations under intermittent exposures to rather high
871 concentrations of ampicillin, still comparable to therapeutic doses, and separated by intervals in fresh medium
872 [300]. They found the cultures became tolerant to ampicillin by acquiring mutations that extended their lag phase (i.e.,
873 the period before exponential growth is resumed after the stationary phase), without any change in the sensitivity to
874 the antibiotics, indicated by the minimal inhibiting concentration, MIC. A higher value of MIC indicated a stronger

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875 resistance. The authors characterized the evolved strains from the aspects of both resistance and tolerance. They
876 found that each selected strain adapted by specific mutations, which were ultimately fixed in their evolved
877 populations. They monitored the phenotypic changes at both the population and single-cell levels. They found the first
878 adaptive change to antibiotic stress was the development of tolerance through a major adjustment in the distribution
879 of the single-cell lag-time, without a change in resistance. Surprisingly enough, they observed that the lag time of
880 bacteria before starting to propagate again was optimized to match the duration of the antibiotic-exposure interval. The
881 authors identified the mutants, and named them "tolerance by lag" (*tbl*), of target genes involved in this "antibiotic-
882 driven" phenotype. It benefited from the whole genome-sequencing of each evolved strain, and restored the respective
883 wild-type alleles. They concluded that a "better understanding of lag-time evolution as a key determinant of the
884 survival of bacterial populations under high antibiotic concentrations", could lead to new approaches to block the
885 evolution.

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

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

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

921 We concluded that the hypothesis is based on increasing evidence suggesting that persistence triggered and enabled
922 by a network of intracellular stress responses can accelerate the processes of adaptive evolution beyond shedding
923 light on the basis of persistence. Those persisters could be an evolutionary reservoir from which resistant organisms
924 can emerge [307] is probably correct, but there is not any direct genetic link between tolerance and resistance so far.

925 Whatever was the philosophy behind it, the first antibiotic (teixobactin) found in a screen of uncultured bacteria, which
926 kill Gram-positive pathogens without detectable resistance, has been discovered in Professor Kim Lewis' Laboratory
927 [308]. Teixobactin inhibits cell wall synthesis by binding to a highly conserved motif of lipid II (a precursor of
928 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 **3.3. Evolution of antibiotic resistance and collateral sensitivity (Is antibiotic resistance evolution a two-**
938 **way street?)**

939 **3.3.1. Morbidostat and experimental evolution of intrinsic antibiotic multiresistance**

940 It is obvious that antibiotic resistance is an evolutionary process, based on sequential accumulation of multiple
941 mutations, under selective conditions. This part of the evolution can experimentally be studied, or even recapitulated.
942 The genetic variability in nature, or in a hospital, could be enlarged by horizontal gene transfer mediated by
943 compatible plasmids harboring mobile genetic elements and antibiotic resistant cassettes. To study the gradual
944 evolutionary processes, Toprak *et al.* [309] developed a selection device, the 'morbidostat', which is capable of
945 continuous monitoring of the growing and evolving bacterial population, under dynamically regulated antibiotic
946 concentrations. The morbidostat is suitable for carrying out experimental evolution studies on bacteria to
947 recapitulate genetic and molecular events of developing antibiotic resistance. The evolution of resistance in *E. coli*
948 towards several antibiotics, such as those of doxycycline, trimethoprim, chloramphenicol was reconstructed. Their
949 experimental protocol covered about 3 weeks, and they found that resistance levels toward a compound increased
950 substantially during this period. By using whole-genome sequencing of the evolved strains, they identified
951 mutations both specific to resistance to a particular drug, and shared in resistance to multiple drugs [309].

952 With trimethoprim, resistance evolved in the expected stepwise manner, through mutations restricted to the genes
953 encoding for the enzyme dihydrofolate reductase (DHFR). By sequencing of DHFR over time, they found parallel
954 populations evolved, with similar mutations, and mutations being acquired in a similar order. However, chloramphenicol
955 and doxycycline resistances evolved smoothly through diverse combinations of mutations in different genes which
956 were involved in transcription, translation and membrane transport.

957 The reviewer is attempted to play with the idea of whether similar experiments with persistent
958 *E. coli* strains under morbidostat conditions would, or would not, have resulted in similar conclusions. We would not
959 expect too much difference in the process of evolution, concerning either the case of trimethoprim, or that of
960 chloramphenicol and doxycycline resistance.

961 As we saw, in the "war" against antibiotic resistance, we have just been losing battle after battle. As mentioned before,
962 carbapenems used to be considered powerful chemical tools to overcome ESBL resistance, until the appearance of
963 carbapenem resistance in the Enterobacteriaceae. Considering the efficacy of intimate immunity in nature, it was
964 hoped that resistance toward antimicrobial peptides might cause fewer problems than is the case for antibiotics with
965 other chemical structures. As discussed above, colistin is an antimicrobial peptide which has been used for a
966 relatively long time for the treatment of multi-drug-resistant, Gram-negative, bacterial infected, clinical patients. It
967 has had moderate success, usually in combination with a carbapenem. However, expectations concerning colistin seem to
968 be evaporating too. Unfortunately, colistin resistance has been evolving at the clinical level [310]. This turned out to
969 be a real evolutionary product, based on multiple epistatic interrelations, not simply an accumulation of mutations at
970 one, or a very few, locus. The evolution of colistin resistance was a more-than-one step process, requiring mutation in
971 at least five independent loci synergistically, creating the resistant phenotype. As a wonderful example of justifying
972 the validity of the classical Mendelian genetics, strong and unambiguous intergenic epistasis seems to limit the
973 number of possible evolutionary pathways for antibacterial peptide resistance. Not only epistasis, but suppressor
974 mechanisms (mutations in transcriptional regulator genes) are also essential for the evolution of antimicrobial
975 peptides. These dominant suppressor mutations serve then as kind of "nodes potentiating further steps in the
976 evolutionary process leading to higher resistance" by increasing/channelizing the effects of other mutations, see
977 https://www.ncbi.nlm.nih.gov/
978 pubmed/27694971, (available publicly) [310].

979 **3.3.2. Experimental evolution of intrinsic antibiotic resistance and collateral sensitivity**

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980 Collateral sensitivity is a phenomenon that occurs when a new appearing antibiotic resistance is accompanied with a
981 loss of a previous resistance to another drug. Collateral sensitivity has been detected before (see [48]), but it has
982 neither been evolutionarily interpreted nor systematically studied like in this study [311] discussed below.

983 A completely new research line has been initiated at the Synthetic and Systems Biology Unit, Institute of
984 Biochemistry, Biological Research Center, Szeged, Hungary, aiming at experimental reconstruction of the evolution
985 of mechanisms of collateral sensitivity. This kind of research may lead to an option of reusing previously effective
986 antibiotics to which resistance had been developed, and consequently, those antibiotics were withdrawn from clinical
987 application. This research line was catalyzed by the surprising discovery that
988 the evolution of new antibiotic resistance has frequently been accompanied by losing resistance to other antibiotics
989 [311]. Working with *E. coli*, the authors ran large-scale laboratory evolutionary experiments and found that
990 populations adapted to aminoglycosides have an especially low fitness in the presence of several other antibiotics.
991 They sequenced the whole-genome of each of their laboratory-evolved aminoglycoside-resistant strains, and
992 demonstrated multiple mechanisms underlying aminoglycoside resistance, including a reduced proton-motive force
993 (PMF) through the inner membrane. They suggested that, as a pleiotropic consequence, these mutations diminished
994 the activity of PMF-dependent antibiotics efflux pumps (such as the AcrAB transporter), resulting in hypersensitivity
995 toward other antibiotics. We believe that this Hungarian discovery is of science historic value, allowing the fight
996 against resistance development to new antibiotics, to be compensated by "reactivating" some previously used ones. We
997 consider that the discovery of collateral sensitivity has an extremely great theoretical and practical impact, but
998 probably relevant only for mutation-based, "intrinsic" resistances [311].

### 999 3.4. MDR Revolution in genus *Enterococcus*

1000 *Enterococcus cecorum*, a normal commensal intestinal inhabitant, is increasingly responsible for outbreaks of
1001 arthritis and osteomyelitis in chickens worldwide. However, since 2002, *E. cecorum* has increasingly been
1002 recognized as a causative pathogen of enterococcal spondylitis (ES) [312-324].

1003 Enterococcal spondylitis is a specific manifestation of *E. cecorum*-associated diseases, in which increased flock morbidity
1004 and mortality result from chronic infection involving the free thoracic vertebra and adjacent notarium or synsacrum.
1005 Birds affected with ES have hind-limb paresis of variable severity, due to spinal cord compression caused by the
1006 chronic inflammation [319-322]. Birds often develop characteristic clinical signs as they near market weight [308,
1007 323]. To date, ES has been reported in both breeding and meat production flocks in several U.S. states, including
1008 Pennsylvania, Washington, North Carolina, South Carolina, Arkansas, Mississippi, Alabama, and California [319,
1009 321].

1010 ES has also been documented in broiler chickens in different countries all over the globe, in Belgium [312] in
1011 Canada [314], in Hungary [325], in Poland [326], in the Netherlands [327], and the UK [328]. Clinical
1012 presentations, gross findings and epidemiology are similar to those found in American broiler flocks [321].

1013 While the pathogenesis of ES remains poorly understood, recent evidence suggests that the increased incidence of
1014 enterococcal-associated disease in poultry may be due to horizontal spread of dominant clones of *E. cecorum* that
1015 exhibit increased pathogenicity [327, 328]. A recent study was directed at investigating the genetic relatedness and
1016 antimicrobial resistance of isolates recovered from spondylitis lesions and caeca of affected/ unaffected birds from
1017 geographically and temporally distinct outbreaks of ES in the southeastern United States [327]. ES outbreaks from
1018 2007 to 2011 were investigated in North Carolina (15 flocks, 13 farms, and four integrators), South Carolina (one
1019 flock, one farm, one integrator) and Alabama (six flocks, six farms, one integrator). From these 22 epidemiologically
1020 distinct outbreaks, 326 isolates of *E. cecorum* were recovered. Isolates from spinal lesions and caeca of affected birds
1021 (cases) and caeca of unaffected birds (controls) were genotyped using pulsed-field gel electrophoresis and compared with
1022 each other [327]. Phenotyping used GenIII MicroPlate™ (Biolog; Hayward, CA, USA), microbial identification plates,
1023 and antimicrobial sensitivity testing. Isolates from spinal lesions were incapable of mannitol metabolism, and the
1024 majority of these isolates were genetically clonal. In contrast, caecal isolates from control birds varied in their
1025 ability to metabolize mannitol, and were genetically diverse. Isolates from both case and control birds had high levels
1026 of antimicrobial resistance. These findings indicate that the increase in *E. cecorum*-associated diseases in the
1027 southeast United States is due to the emergence of new clones with increased pathogenicity and multi-drug
1028 resistance [329].

1029

## 1030 4. "Dialectics" of resistance and sensitivity: The agrocin 84 story in the genus *Agrobacterium*

1031 So far, we have discussed resistance genes, and resistance mutations, the existence and expression of which make
1032 the originally sensitive organism resistant to a given compound. The product of the mutant gene causing resistance

1033 can be located either on the chromosome, or on a newly taken-up plasmid, harboring it either as part of a mobile
1034 genetic element, or genomic island. But, there are examples of the opposite as well, when the existence and expression
1035 of a "sensitivity gene" makes the originally resistant organism sensitive to a given antibacterial compound. The
1036 protein product of such a sensitivity gene may catabolize a harmless molecule to a harmful derivative, or change the
1037 structure of the originally impermeable membrane to be permeable to the compound, or to block the multi-drug
1038 pumping activity. Alternatively, a sensitivity gene may code for a protein which is able to bind and transfer a toxic
1039 product into the cell, which it could not otherwise enter. This binding protein may play a role in the normal
1040 metabolism of the given cell.

1041 A wonderful example is the sensitivity/resistance (S/R) phenotype in different *Agrobacterium* strains to Agrocin 84. It
1042 is one of the most studied molecules of the group of plant biocontrol molecules called agrocins [330]. This "Trojan
1043 horse antibiotic" that controls the plant tumor called crown gall [331] is an adenine nucleotide antibiotic, produced
1044 by and discovered in, an avirulent Biovar 2 strain of
1045 *A. radiobacter* 84.

1046 Three *Agrobacterium* species have been identified so far: *A. tumefaciens*, also called Biovar I; *A. radiobacter*, Biovar
1047 2; and *A. rhizogenes*, Biovar 3 [332]. Each species includes virulent and avirulent strains. The virulent strains induce
1048 specific tumors, characterized by their secondary metabolites called opines. Depending on the type of opines, the virulent
1049 *Agrobacterium* strains of each species could be determined to be in the nopaline (NO), octopine (OCT), or agropine
1050 (AGR) opine group. Each *Agrobacterium* strain is capable of inducing a special opine-synthetizing tumor, and each of
1051 them is capable of catabolizing the respective opine, (although the respective opine was synthesized by the tumorous
1052 plant cells, and not the bacterium). Agrocin 84 was first identified as a trypsin and a pepsin-resistant small peptide
1053 with a molecular weight of 2,500. It was published as being built up of six different amino acids, including 9
1054 molecules of glutamine or glutamic acid, and seven molecules of serine. It inhibited DNA, RNA and protein synthesis
1055 as well as amino acid transport of the virulent, susceptible, *A. tumefaciens* (H38-9) strain [333]. Agrocin 84 is toxic
1056 to several other but not all, *Agrobacterium* strains. Agrocin 84 inhibits those virulent, tumor-causing, *Agrobacterium*
1057 strains (called NOP strains), which induce nopaline-synthetizing tumor cells. The respective tumor-inducing
1058 *Agrobacterium* strains carry nopaline catabolizing genes on their respective (Ti), or hairy-root inducing (Ri), plasmid. If
1059 the plasmid carrying nopaline catabolizing genes was removed (cured) from the Agrocin 84-sensitive NOP strains,
1060 they became resistant to Agrocin 84.

1061 The explanation of this phenomenon is that the agrocinopine gene, which has a normal metabolic function in the
1062 nopaline biosynthesis in the wild-type strain, also pleiotropically functions as a "sensitivity" gene, making the wild-
1063 type NOP *Agrobacterium* strains sensitive to Agrocin 84.

1064 For a better understanding, it is important to know that *A. tumefaciens* NOP strains, such as strain C58 [334],
1065 induced crown gall tumors to produce not only nopaline, but other opines, called agrocinopines A and B, as well.
1066 Agrocinopine A has a normal function in NOP strains, where it is the inducer of Ti plasmid conjugal transfer in the
1067 strain [335]. It turned out that Agrocin 84 and agrocinopines A, the precursor of agrocinopine B, are transported by
1068 the same uptake system.

1069 Consequently, mutations causing constitutive transfer of pTiC58 show the pleiotropic phenotype of super-sensitivity
1070 to Agrocin 84, while Agrocin 84-resistant mutants of *A. tumefaciens* A208 do not transport agrocinopine A.

1071 Other *Agrobacterium* strains harboring non-NOP, pTiBo542 plasmids (which induce L, L, succinamopine and
1072 agropine producing tumors), the cells which also synthetize agrocinopines, but of a different type (agrocinopines C,
1073 the precursor of agrocinopine D), are resistant to Agrocin 84, but could be made sensitive by pretreatment with
1074 agrocinopine C, [330].

1075 For more details, see [336-345].
1076

1077 **5. Closing remarks**

1078 In the "card game" of the antibiotics and invoked resistances many reliable investigators consider polymixin
1079 (colistin) and vancomycin as the respective last "trump" against Gram-negative and Gram positive resistant
1080 pathogens, forecasting that the appearance Gram-negative isolates of colistin-resistance and that of Gram-positive
1081 isolates of vancomycin resistance means the end of the heroic "age of antibiotics".

1082 The authors of this review have reservation for accepting this pessimistic view. We suppose that the "card game"
1083 between new antibiotics and invoked resistances has not been finished yet. The genetic sources of both intrinsic and
1084 acquired resistances in the bacteria seem to be non-exhaustible. This fact justifies 'hands-up' pessimism. Fortunately
1085 enough, the number of theoretically possible QSAR-designed antimicrobial peptides also seems to be unlimited, or

at least extremely high. This fact justifies rational optimism, hoping that there is a real chance to overcome newly appearing resistances by discovering and introducing new, properly designed antimicrobial peptides at least for a long time. Therefore, we are expecting new antimicrobial peptides (either of natural or of synthetic origin) as new trumps as in the "card game" of science and bacterial multi-drug resistance.

We propose that the options provided by the natural and synthetic antimicrobial peptides will offer new solutions. The QSAR-designed synthetic antimicrobial peptides, and the non-ribosomal (NRP) peptides, especially those produced by entomopathogenic nematode symbiotic bacteria (*Xenorhabdus, Photorhabdus*) provide abundant gold mines for antibiotics of novel modes of action (see our next Review).

As a very personal epilog, let us explain why we, practically newcomers, did undertake to put this review together. Our team has been working on natural antimicrobial peptides produced by entomopathogenic nematode symbiotic bacteria, and have recently started to cooperate with a team of veterinary scientists in order to materialize this conception. This review is our first joint venture.

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## CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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