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1 **Antibiotic discovery: history, methods and perspectives**

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13 **Abstract**

14 Antibiotic resistance is considered a major public health issue. Policies recommended by the
15 World Health Organization include research on new antibiotics. No new class was discovered
16 since daptomycin and linezolid in the 1980s and only optimization or combination of already
17 known compounds has been recently commercialized. Antibiotics are natural products of soil-
18 living organisms. *Actinobacteria* and fungi are the source of about two thirds of the
19 antimicrobial agents currently used in human medicine; they were mainly discovered during
20 the golden age of antibiotic discovery. This era declined after the 1970s due to the difficulty
21 of cultivating fastidious bacterial species under laboratory conditions. Various strategies such
22 as rational drug design have not led to date to the discovery of new antimicrobial agents.
23 However, new promising approaches, e.g. genome mining or CRISPR Cas9 are now being
24 developed. The recent rebirth of culture methods from complex samples has, as a matter of
25 fact, permitted the discovery of teixobactin from a new species isolated from the soil.
26 Recently, many Biosynthetic Gene Clusters were identified from Human-associated
27 microbiota, especially from the gut and oral cavity. As an example, the antimicrobial
28 lugdunine was recently discovered in the oral cavity. The repertoire of human gut microbiota
29 has recently substantially increased, with the discovery of hundreds of new species. The
30 exploration of the repertoire of prokaryotes associated with human beings using genome
31 mining or newer culture approaches could be promising strategies for discovering new classes
32 of antibiotics.

33 1. Introduction

34 Antibiotic resistance is considered a major public health concern by several
35 international organizations and by local agencies [1–3]. As a matter of fact, the Centers for
36 Disease Control and Prevention claim 23,000 deaths each year in the US related to antibiotic
37 resistance and some studies predict millions of deaths in the coming decades [4–6]. The
38 United Nations then created a group in order to coordinate the fight against antibiotic
39 resistance [7]. Interestingly, the global mortality related to infectious diseases is decreasing
40 every year, from 10,7 million deaths in 2005 to 8,6 million in 2015 [8]. In addition, it was
41 recently shown that the current mortality due to antibiotic resistance seems far from these
42 predictions [9]. One of the approaches used over the past decade to treat Multi-Drug Resistant
43 (MDR) and Extremely-Drug Resistant (XDR) bacteria was to break the vicious circle of Beta-
44 lactams, enlarging the panel of antimicrobial agents commonly tested. It has been
45 demonstrated that “old” antibiotics (i.e. forgotten molecules) had remarkable efficacy against
46 such isolates. For instance, minocycline, sulfadiazine and clofazimine are active against XDR
47 tuberculosis strains, as for fosfomycin, colistin and minocycline against multi-drug resistant
48 Gram-negative isolates [10,11].

49 According to the World Health Organization, antimicrobial resistance control policies
50 include the rational use of antibiotics, in particular on farms, the increase of surveillance, and
51 the research and development for new tools and molecules [12]. Indeed, despite the rising
52 number of available molecules (**Figure 1**), the last new class of antibiotic discovered is
53 daptomycin (1986), which was only approved in 2003 by the Food and Drug Administration
54 (FDA) [13–15]. This fact confirms that antimicrobial agents found on the market in the last 30
55 years are associations or improvements of existing molecules. Examples are given of new
56 antibiotics recently marketed that belong to an already known class, such as oxazolidinones
57 (tedizolide), lipoglycopeptides (dalbavancin), or cephalosporins (ceftaroline, ceftobiprole).

58 Combination of improved molecules of an already known class is another example of recently
59 commercialized new antibiotics, as for example ceftolozane plus tazobactam, or ceftazidime
60 plus avibactam. The research and development of a totally new class of antibiotic appear as a
61 major issue. Herein, we propose to recall the history of antibiotic discovery, their structural
62 nature, and the methods that were used for their discovery. Finally, we review the potential
63 new approaches for the discovery of new classes of antibiotics.

64 **2. The history of antibiotics**

65 *2.1. Antibiotic resistance is in fact very ancient*

66 Most of the antibiotics currently used in human medicine are natural secretions of
67 environmental bacteria or fungi. Indeed, the majority of antibiotics currently used are derived
68 from *Streptomyces* isolated from soil samples [16]. In their natural environment,
69 microorganisms have to fight against each other by producing antimicrobial substances, and
70 have to develop resistance mechanisms to other antimicrobials [17]. Secondly, the species
71 naturally producing antibacterials also have resistance genes to these antibacterials in order to
72 avoid self-toxicity, within a biosynthetic antibiotic operon [18]. D’Costa et al. demonstrated
73 the presence of antibacterial resistance genes in an environment in which there was no innate
74 antibiotic. They first proposed the existence of a “reservoir of resistance determinants that can
75 be mobilized into the microbial community” [19]. Gerard D. Wright proposed the term
76 “resistome” to design the collection of all the antibiotic resistance genes and their precursors
77 in bacteria [20].

78 Interestingly, multi-drug resistant bacterial species as well as resistance genes to
79 antibiotics currently used were also found from environmental archaeological samples. The
80 OXA genes that encode beta-lactamases have been dated to several million years [21].
81 D’Costa *et al.* have found resistance genes to β -lactam, tetracyclines and glycopeptides from

82 30,000 years old permafrost samples [22]. Kashuba *et al.* have found several resistance genes
83 in the genome of a *Staphylococcus hominis* isolated from permafrost [23]. Of the 93 strains
84 cultured by Bhullar *et al.* from the 4 million-year-old Lechuguilla cave (New Mexico), 65%
85 of the species were resistant *in vitro* to three or four antibiotics classes [24]. Also, resistance
86 genes to β -lactam and glycopeptides were also found in the 5,300-year-old gut microbiome of
87 the mummy Ötzi [25]. Recently, 177 antibiotic resistance genes belonging to 23 families (that
88 represent all the mechanisms of resistance, i.e. mutation, efflux and antibiotic inactivation)
89 were found in the antibiotic naïve Mackay Glacier region [26].

90 Orthologous genes within mobile elements known from environmental bacteria were
91 also found in bacteria isolated from clinical isolates [19]. For instance, Marshall *et al.* found
92 orthologous genes of the *van HAX* cassette from the environmental species *Streptomyces*
93 *toyocaensis* and *Amycolatopsis orientalis* [27]. This cassette is responsible for the
94 glycopeptide resistance of *Enterococcus faecium*. Some experimental studies seem to show
95 that transfer of resistance genes from environmental producers in the soil to human
96 pathogenic species is possible [28]. The horizontal gene transfer of entire clusters of
97 resistance genes from the resistome to clinical strains under selective pressure related to the
98 human use of antibiotics is actually suspected [29].

99 2.2. *Historic of the antibiotic discovery*

100 Antibiotics were used for a long time before the advent of modern medicine. The
101 effects of bread on which filamentous fungi grew for the treatment of wounds and burns has
102 been known since ancient Egypt [30]. In the Middle Ages, healers in China and Greece used
103 musty textures to treat various ailments. In the 19th century, Sir John Scott Burden-Sanderson
104 noticed the absence of bacteria from a liquid growth culture covered with mould. In 1871,
105 Joseph Lister discovered the inhibitory effects of *Penicillium glaucum* on bacterial growth,

106 allowing him to cure a nurse's injury with *Penicillium glaucum* extracts. At the same time,
107 Louis Pasteur noticed that some bacteria could inhibit others. He discovered with his
108 colleague Jules François Joubert in 1877, while studying the growth of *Bacillus anthracis* in
109 urine samples, that it was inhibited when co-cultivated with "common" aerobic bacteria. In
110 1889, Jean Paul Vuillemin defined the word "antibiosis" as any biological relationship in
111 which "one living organism kills another to ensure its own existence". Several antagonisms
112 between microorganisms, notably moulds, were published in the thesis works of Ernest
113 Duchesne in 1897. He discovered the inhibition of *Escherichia coli* by *Penicillium glaucum*
114 thirty years before Fleming. Despite several observations of antagonisms between
115 microorganisms, no antimicrobial molecule was purified. The first antimicrobial molecules
116 discovered were chemical compounds. In 1909, Paul Ehrlich discovered the arsphenamine, an
117 arsenic derivative active against *Treponema pallidum*, the agent of syphilis. This antibiotic
118 was commercialized in 1911 under the name Salvarsan[®] then Mapharsen[®]. In 1930, Gerhard
119 Domagk discovered the antibiotic effects of sulphanilamide, a molecule synthesized 22 years
120 before by Paul Gelmo. This antibiotic was marketed under the name Protonsil[®] in 1935 and
121 was used by soldiers during World War II [32].

122 In 1928, Alexander Fleming accidentally discovered in his forgotten colonies of
123 *Staphylococcus aureus* that a fungus was inhibiting the growth of *Staphylococcus*. The
124 *Penicillium notatum* molecule has been purified and called penicillin. But the industrial
125 production of this antibiotic was performed only in 1940 by Howard Florey et Ernst Chain,
126 using *Penicillium chrysogenum* [33]. Fleming also discovered the lysozyme, an antibacterial
127 enzyme [33]. In 1930, René Dubos discovered an enzyme from a soil-derived *Bacillus* that
128 specifically decomposes *Streptococcus pneumoniae* type III capsular polysaccharide. With
129 this enzyme, he was able to treat mice with pneumococcal peritonitis [34]. Ten years later, he
130 isolated from *Bacillus brevis* the oligopeptide gramicidin that widely inhibited Gram-positive

131 species [35]. Unfortunately, gramicidin showed too much toxicity for humans, except for
132 local treatment [36]. In the US, Selman Waksman was the first to perform a systematic
133 research of antimicrobial activity of soil bacteria, particularly from *Streptomyces* members or
134 *Streptomyces*. He developed several culture techniques and strategies (“Waksman
135 platform”) in order to highlight antagonisms between bacterial species [37]. Using his
136 platform, he discovered in the 40’s several major antibiotics and antifungals, such as
137 actinomycin (from *Streptomyces* spp.) [38], streptomycin (from *Streptomyces griseus*) [39],
138 neomycin (from *Streptomyces fradiae*) [40], fumigacin (from *Aspergillus fumigatus*) and
139 clavacin (from *Aspergillus clavatus*) [41]. Actinomycin, neomycin and streptomycin are still
140 in use today. Moreover, streptomycin has revolutionized the treatment of tuberculosis, and is
141 still active against multi-drug resistant tuberculosis [42]. The pharmaceutical industry was
142 inspired by the Waksman platform, which led to the discovery of all current antibiotics
143 between the 1940s and the 1970s. During this golden age, 23 classes of antibiotic were
144 discovered from 19 bacterial species and 7 fungi (**Table 1**).

145 Despite recent commercialization for some, the last classes of antibiotic discovered are
146 from the 1980s. After 50 years of discoveries, no new classes have been found. Therefore,
147 new strategies were needed. After the culture approach through Waksman’s platform, the
148 industry turned to the *in vitro* synthetization of new molecules, based on knowledge of the
149 known mechanism of action of antibiotics. Unfortunately, few new classes of antibiotics have
150 been discovered, the nitrofurans in 1953, the quinolones in 1960, the sulphonamides in 1961 and
151 oxazolidinones in 1987. The modification and improvement of already known molecules has
152 also been carried out. This is reflected by the commercialization of linezolid in 2003 and
153 daptomycin in 2001, although these molecules had been known since 1955 and 1986,
154 respectively [32]. Recently, a new cephalosporin called cefiderocol was found to be active
155 against carbapenem-resistant Gram-negative bacteria [43]. Hemisynthetic compounds from

156 natural products were also developed, such as ketolides (derived from macrolides) or
157 metronidazole (derived from a natural product of *Streptomyces* sp.) (**Table 1**). But the lack of
158 return on investment and the emergence of resistance have led the industry to gradually let
159 down the research on antibiotics, preferring to invest in drugs for chronic diseases [32]. Of the
160 20 pharmaceutical companies that invested in antibiotic discovery in the 1980s, there were
161 only five left by 2015. [44]. More than 1,200 antimicrobial peptides were discovered from
162 various origins, from plants to invertebrates and animals, but none has been used as an
163 antibiotic [45]. In conclusion, the majority of antibiotics were discovered during the golden
164 age. Bacteria and fungi were the greatest producers. The genus *Streptomyces* is the source of
165 about half of the antimicrobial agents currently used in human medicine (**Table 1**).

166 **3. Chemical nature of antimicrobial agents**

167 Antimicrobial molecules are represented by a wide variety of chemical compounds.
168 Most of the time, they are natural products and secondary metabolites, implying that they are
169 not required for survival under laboratory conditions but still provide some advantages in the
170 environment [46]. Among the antimicrobial substances used in human medicine, it is possible
171 to classify antibiotics in five groups of chemical molecules. The first are derived amino acids
172 that are non-ribosomally synthesized (non-ribosomally synthesized peptides, NRP). The
173 second are acetyl coenzyme A or malonyl coenzyme A derived (polyketides, PK). NRP and
174 PK represent about 50% of all current antibiotics (**Table 1**). The third are hybrids between
175 NRP and PK and the fourth are composed of several carbohydrates units substituted with
176 amine groups (aminoglycosides). And finally, the last group is composed of various
177 molecules, such as terpenoids, fusidic acid or alkaloids such as metronidazole. In addition to
178 these molecules used as antibiotics, thousands of antimicrobial peptides are known from
179 insects, mammals, plants or amphibians [47]. These peptides are usually classified as

180 “ribosomally synthesized and post-translationally modified peptides” (RiPPs), a subgroup of
181 natural products [48].

182 *3.1. Antibiotics*

183 The NRP and PK are synthesized by multi-enzymatic complexes (non-ribosomal
184 peptides synthetase, NRPS and polyketides synthetase, PKS) encoded by biosynthetic gene
185 clusters [49]. These complexes are organized in modules including several domains. For
186 example, the erythromycin synthase complex consists of three proteins and seven modules
187 each containing three to six domains. [49]. The NRPS use amino acids as substrate elongated
188 by peptidic connection, whereas PKS use acyl-coenzyme A as substrate elongated by Claisen
189 condensation reaction [49]. This complex organization allows the production of a great
190 number of different products, and therefore a great diversity. For example, ciclosporin
191 belongs to the NRP category [50]. Hybrid assembly line that uses both amino acids and acetyl
192 coenzyme A, or fatty acid synthetases (FAS), are also described [51]. NRP antibiotics
193 comprise molecules such as β -lactams, daptomycin, lincomycin, polymyxins, and
194 vancomycin while macrolides, mupirocin and tetracyclines belong to polyketides antibiotics.
195 Rifampicin belongs to hybrid NRP/PK (**Table 1**).

196 *3.2. Antimicrobial peptides*

197 Antimicrobial peptides are broad spectrum antibacterial molecules that have been
198 discovered in blood cells in 1957 by Robert Skarnes [52]. They belong to natural products
199 that are small peptides (i.e. less than 100 amino acids) ribosomally synthesized with post-
200 translational modifications (RiPPs). Natural peptides have a multifunctional activity that
201 participates in innate immunity in eukaryotic and prokaryotic cells. [47]. Antimicrobial
202 peptides are evolutionary well-conserved amphipathic molecules with hydrophobic and
203 cationic amino-acids [53]. They can be categorized according to their secondary

204 conformation: group I (alpha helical), group II (beta sheet), group III (mixed) and IV
205 (extended) [54]. They have been isolated from almost all living organisms from prokaryotes
206 to vertebrates. Indeed, thousands of antimicrobial peptides are known and come from insects,
207 plants, or amphibians [45,47]. The main known mechanisms of action are related to their
208 cationic charge and amphipathic structure, responsible for the membrane disruption of the
209 negatively charged bacterial cell. Antimicrobial peptides can also interact with membrane-
210 associated protein targets, as well as intracellular targets after penetration into the bacterial
211 cytoplasm [54]. Finally, antimicrobial peptides can also have immunomodulatory effects on
212 the innate immune system of the host [55].

213 Bacteriocins are antimicrobial peptides that were first discovered in bacterial species.
214 They are used in the agro-alimentary industry as food preservatives and in veterinary
215 medicine [56]. Bacteriocins are separated into four groups: class I are small heat-stable post-
216 translationally modified peptides (<5kDa) which use the amino acid lanthionine, there are
217 therefore called lantibiotics; class II are not modified heat-stable small peptides (<10kDa)
218 which do not use lanthionine; class III are large heat-labile peptides (>30kDa) and class IV
219 are complex or cyclic peptides containing lipids or carbohydrates [56]. Bacteriocins inhibited
220 closely related species [57]. For example, lactacin 3147 and nisin are lantibiotics that
221 exhibited an antibiotic activity against Gram-positive bacteria, notably Meticillin-resistant
222 *Staphylococcus aureus* (MRSA) or Vancomycin-resistant *Enterococcus* [58]. Nisin is the
223 most famous lantibiotic, largely used as food preservative because of its activity against
224 *Listeria monocytogenes*, MRSA and *Streptococcus pneumoniae* [59]. Nisin is also used in
225 veterinary medicine under the name Wipe Out ® for the prevention of dairy mastitis [57]. The
226 synergistic effects of lantibiotics with antibiotics have also been demonstrated *in vitro* [60].

227 According to the Antimicrobial Peptide database, 2,478 antibacterial peptides are
228 already known [61]. All medical fields combined, 60 peptides have obtained the FDA

229 approval and 140 are in clinical trials, mainly for oncological and metabolic diseases [62].
230 Yet, when focusing on infectious diseases, only 12 naturally occurring antimicrobial peptides
231 have reached the stage of human clinical trial. (**Table 2**). Among them, only three (LYX-109,
232 LL-37 and nisin) demonstrated a better efficacy than placebo, but none obtained the FDA
233 approval for human application. Only gramicidin has received FDA approval for topical
234 application in association with polymyxin and neomycin for ophthalmic use. Teixobactin is a
235 new promising depsipeptide that provides an inhibition of *S. aureus* and *M. tuberculosis* [63].
236 This molecule uses notably the rare amino acid L-allo-enduracididine, which is challenging to
237 synthesize and therefore limits its use despite efforts made to develop analogues [64]. Despite
238 their narrow or broad spectrum of activity against human pathogens and easier bioengineering
239 compared to NRPS or PKS, bacteriocins are not used in humans as antibiotics due to several
240 limitations.

241 The main limitations in the use of antimicrobial peptides as antibiotics in clinical
242 practice are their instability (proteolytic digestion, oxidation), their high cost and low yield of
243 production, their short half-life, and their quick elimination [60]. Notably, their low
244 bioavailability after oral ingestion related to proteolytic degradation is a great bottleneck. To
245 solve these problems, production of analogues using rational drug design or nano-engineering
246 is used to improve the pharmacokinetic properties. For instance, nano-engineering has
247 increased the nisin spectrum to Gram-negative bacterial species [65] or allowed HPA3P^{HIS} to
248 be highly effective against *Vibrio vulnificus* in a mouse model [66]. The use of nanoparticles
249 also prolonged the stay in the stomach of pexiganan after oral administration, which reduces
250 the concentration of pexiganan required in a mouse model for *Helicobacter pylori* eradication
251 [67]. Another approach is the combination of bacteriocins with other antimicrobials in order
252 to reduce the resistance risk and to increase the antimicrobial potency [60]. Another matter of
253 concern with AMP is the risk of development of resistance against our own immunity

254 peptides. For instance, pexiganan have been previously found to induce cross-resistance to
255 human-neutrophil-defensine 1 [68].

256 **4. Methods for the discovery of antibiotics**

257 *4.1. Culture-based approaches*

258 In the 1940s, Selman Waksman systematically screened the soil bacteria for
259 antagonisms. Its culture-based approach is still in use today [69]. All the methods are based
260 on the same principle: showing an inhibition of a tested strain over an indicator strain closely
261 cultivated. The tested strain is the strain suspected to produce antimicrobials targeting the
262 strain used as indicator. Several techniques exist to detect antimicrobial activity, either in
263 solid or in liquid culture. There are three main methods regarding solid culture approaches:
264 the cross-streak method, the spot-on-the-lawn and the well diffusion method (**Figure 2**).

265 The cross streak is the inoculation of the bacterial strain tested vertically on the agar
266 plate. The incubation time of the plate depends on the life cycle of the bacterial strain required
267 to reach the exponential phase, which is the moment where secondary metabolites are
268 excreted. Then, the indicator strain is inoculated into horizontal streaks and the plate is
269 incubated (**Figure 2**) [70]. This technique is easy and powerful for screening but requires that
270 both bacterial strains have the same culture conditions (e.g., atmosphere, temperature and
271 growth duration).

272 The second and the third methods are respectively called “spot-on-the-lawn” and “well
273 diffusion method”. The “spot-on-the-lawn” method consists in depositing a drop of the tested
274 strain on a lawn of the indicator strain [71]. After incubation, an inhibition zone is searched
275 around the sediment. The “well diffusion method” is based on the diffusion of antimicrobials
276 through agar which inhibits sensitive species. An agar plate is pooled with the indicator strain
277 or inoculated with a lawn of the indicator and agar holes are punched out aseptically. Then,

278 two main variants exist. The first is the Agar Plug Diffusion method which consists in
279 removing a cylinder of agar from a plate previously inoculated with the tested strain. This
280 cylinder of agar is then placed into the hole of the indicator plate [72]. The second variant
281 method consists in placing a liquid broth of the tested strain or a growth supernatant in the
282 hole [73]. After an optional rest time at +4°C, the agar plate is incubated and inhibition
283 growth zones are measured (**Figure 2**). Several variants were developed, like using stress
284 conditions or iron chelator [74,75]. The main limitation of solid culture tests concerns
285 bacterial species that have different growth conditions or fastidious species.

286 Liquid culture-based approaches can solve this problem. Liquid broth co-culture has
287 been used since the existence of the Waksman platform [69]. This is the simultaneous culture
288 of the tested species and the indicator strain separated by a filter allowing the diffusion of
289 nutrients but not the diffusion of cells. Following incubation, the bacterial growth of the
290 indicator strain is determined by numeration, coloration or optical density [69]. Another
291 method is to add the growth supernatant of the test species previously filtered and
292 concentrated to a liquid culture of the indicator strain [76]. The latter method allows the
293 bacteria tested to be grown under conditions different from those of the indicator strain
294 (**Figure 3**).

295 4.2. *Discovering antimicrobial effects from already known compounds*

296 Some antibiotics were discovered years before they were used (fidaxomicin,
297 daptomycin, or linezolid). Several million of chemical compounds are known in chemical
298 databases and could provide a potential source of antibiotics [32]. Moy *et al.* have tested the
299 activity against *E. faecalis* of more than 6,000 chemical compounds and 1,136 natural
300 products in the *Caenorhabditis elegans* animal model, discovering 16 molecules increasing

301 the survival of animals [77]. The bottleneck remains the selection and the high throughput
302 testing of these molecules.

303 *4.3. Synthesis of new molecules and improvement of already known compounds*

304 The « Rational Drug Design » consists in the empirical synthesis of new molecules
305 that are designed according to several rules to be well-absorbed, non-toxic, and active against
306 a specific target [32]. The most famous rules used by the industry are Lipinski's rules.
307 Despite more than 10 million of new molecules synthesized, only a few active molecules have
308 reached the market, notably antituberculous drugs [32,37] (**Table 1**). This can be explained by
309 the fact that antibiotics have generally poor economics [78].

310 The improvement of already known molecules is another strategy that can yield
311 benefits. The modification of cephalosporin lead to the development of cefiderocol, that
312 demonstrated safety and tolerability in healthy subjects, and clinical trials for the treatment of
313 urinary tract infections are ongoing [79]. Another example is the modification of the
314 aminoglycoside sisomicin that leads to the development of plazomicin [80]. This antibiotic
315 was recently approved by the FDA and a small phase 2 clinical trial found it had an efficacy
316 comparable to that of levofloxacin in the treatment of urinary tract infections and acute
317 pyelonephritis [81]. If the potential use of these antibiotics remains to determine, their
318 mechanism of action is not novel, and the apparition of resistance is expected in the same way
319 as for their related antibiotic parent.

320 *4.4. Genome mining*

321 Secondary metabolites are encoded by Biosynthetic Gene Cluster (BGCs). Thousands
322 of prokaryotic genomes are available in sequence databases. These data have generated
323 thousands of BGCs that potentially encode unknown molecules [82,83]. Despite the fact that
324 many of them do not have any antimicrobial activity, little is known about them. Walsh et al.

325 found 74 putative BGC from 59 genomes from the Human Microbiome Project [84]. These
326 BGC belonged mainly to *Firmicutes*, *Proteobacteria* and *Bacteroidetes* and the most
327 commonly putative bacteriocins encoded belonged to classes III and IV.

328 Several approaches of genome mining are possible. The most used are sequence-based
329 approach, ecology-based genome mining, mode-of-action-based genome mining, or function-
330 based genome mining [83,85]. For instance, lichenicidin is a bacteriocin synthesized by
331 *Bacillus licheniformis* that was discovered using the mode-of-action genome mining
332 approach. The authors screened the databases for *LanM* genes, which are involved in the
333 biosynthesis of lantibiotics [86]. The identification of putative bacteriocins encoded by BGC
334 from the genome sequence is possible using bioinformatic algorithms [87]. Bacteriocins are
335 easily found using bioinformatic tools compared to NRPS or PKS. Tools such as BAGEL,
336 antiSMASH or PRISM are widely used for this purpose. These tools exploit two main
337 approaches [83]. The first consists in finding new congeners of already known scaffolds. This
338 approach is based on the homology comparison by the research of conserved domains
339 (anchors), as for the thio-template domain of NRPS and PKS [88]. Small structural changes of
340 the new homologue may result in a significant change in the activity of the product. The
341 second approach is more difficult and consists in finding new scaffolds. Predicting the
342 chemical structure and biological activity of a BGC informatic sequence is a real challenge
343 [83]. The main problem that remains is proving the functional activity of BGC [89]. Both
344 approaches often require the engineering expression of BGC from the native host or from a
345 heterologous host, which represents the main bottleneck of genome mining for the discovery
346 of new antibiotics.

347 Recently, Hover *et al.* have screened more than 2,000 soil samples from various areas
348 of the US, searching for BGCs encoding calcium-binding motif Asp-X-Asp-Gly [90]. This
349 motif is related to calcium-dependant antibiotics such as lipopeptides, for which the

350 mechanism of action is not fully understood [91]. They found several clades of
351 uncharacterized BGC, of which the most abundant was present in 19% of all samples. This
352 clade was named malacidins, the molecules were then synthesised, and their antibiotic activity
353 was characterized *in vitro*. The malacidin A exhibited a broadly antibiotic activity against
354 Gram-positive bacteria, notably MRSA, which was successfully confirmed in a skin-wound
355 infection rat model. In conclusion, genome mining is a promising approach for new antibiotic
356 discovery, despite the fact that the method is fastidious and time-consuming.

357 4.5. CRISPR Cas9

358 Bacteria and fungi have an immune system that protects them from foreign genetic
359 material that could be inserted by phages. This immune system consists of restriction
360 enzymes, toxin-antitoxin and Clustered Regularly Interspaced Short Palindromic Repeats
361 (CRISPR) Cas system [92]. The utilization of CRISPR Cas9 to design new antimicrobials
362 with a predetermined activity spectrum has been already performed with promising results.
363 Citorik et al. have developed RNA-guided nucleases that target the resistance gene *bla*_{SHV-18}
364 and *bla*_{NDM-1}. After transformation by plasmids and transduction by bacteriophages, the
365 authors observed a significant reduction in the number of *E. coli* containing the targeted
366 resistance gene, either chromosomal or plasmid [93]. The same approach has been
367 successfully tested using phagemid targeting the *S. aureus* methicillin resistance gene [94].
368 The same authors tested the use of bacteriophages in a mouse model of *S. aureus* skin
369 infection with an efficacy comparable to that of mupirocin [94]. Other authors used genome
370 editing technology to re-sensitize MDR cells. As an example, Kim *et al.* used CRISPR Cas9
371 to target a conserved sequence of Extended-Spectrum β -Lactamases, thus restoring the
372 susceptibility of *E. coli* in their *in vitro* model [95].

373

374 5. Conclusion and perspective

375 More than three quarters of all antibiotics currently used in human health are natural
376 products or derived from them. The discovery of antibiotics declined after the 1970s due to
377 the difficulty of cultivating bacterial species from soil under laboratory conditions. New
378 innovative culture approaches were then created thanks to the bloom of new molecular
379 methods. In this way, genome mining searching for new BGCs as well as CRISPR Cas9
380 technology are promising new approaches. Recently, the ability to rapidly identify bacterial
381 strains using Matrix Assisted Laser Desorption Ionisation Time of Flight (MALDI Tof) has
382 permitted the rebirth of culture. Therefore, new culture approaches trying to mimic the natural
383 environment were invented in order to grow fastidious species. This led to the discovery of
384 new bacterial species. For instance, Ling *et al.* discovered the new antibiotic teixobactin from
385 the new species *Eleftheria terrae* isolated in the soil using a diffusion chamber [63].

386 The same approach is now possible for the human-associated microbiota. The nose is
387 an example of ecological niche poor in nutrients in which the microbiota is probably in strong
388 competition [75]. Using a home-made nasal synthetic medium under iron-limited condition,
389 Krismer B *et al.* discovered lugdunin, a new antibiotic inhibiting *S. aureus* growth [74]. The
390 gut microbiota is another microbiota of interest for antibiotic research. Indeed, the human gut
391 has an average load concentration ranging from 10^4 to 10^{12} CFU/mL from the duodenum to
392 the colon. These species live in extreme competition, as they did before human colonization
393 where bacteria lived in a competitive world that led them to naturally develop many
394 antimicrobial products. Metagenomic analysis from the human gut microbiota found many
395 BGCs. In 2014, Donia *et al.* found 3,118 BGCs including NRPS, RiPPs and PKs in the
396 human microbiome. They also found 599 BGCs in the gut. Taken together with the oral
397 cavity, it is one of the richest sites in BGCs of the human microbiota. They also found and
398 purified lactocillin, a new thiopeptide antibiotic, isolated from the vaginal microbiota [96].

399 The study of the gut microbiota using culture methods was recently improved by
400 culturomics, a novel approach that consists in the multiplication of growth conditions [97].
401 This has led to the discovery of previously uncultivated species. With this approach, Lagier *et*
402 *al.* significantly increased in a couple of years the gut repertoire from 690 to 1,525 species, of
403 which 247 were totally new [98]. Indeed, the new species described from the gut represent an
404 opportunity for the research for novel antibiotics. The search for new antibiotics naturally
405 synthesized by organisms living in complex ecosystems like the gut microbiota, using the
406 culture approach, seems the modern continuity of what has already worked in the past. Thus,
407 if the study of antagonisms between environmental bacteria has led to the discovery of a
408 substantial proportion of antibiotic classes, such studies were rarely performed from human-
409 derived microbiota.

410 In conclusion, the research for new antibiotic molecules is a key point among the
411 strategies for the fight against antibiotic resistance. The recent advances from both culture-
412 dependant and culture-independent methods of exploration of complex ecosystems such as
413 soil or human-associated microbiota open a new era in antimicrobial research.

414

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417

418

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675

676 **Table legend**

677 **Table 1.** Discovery date, origin of the organism and synthesis pathway of antibiotics.

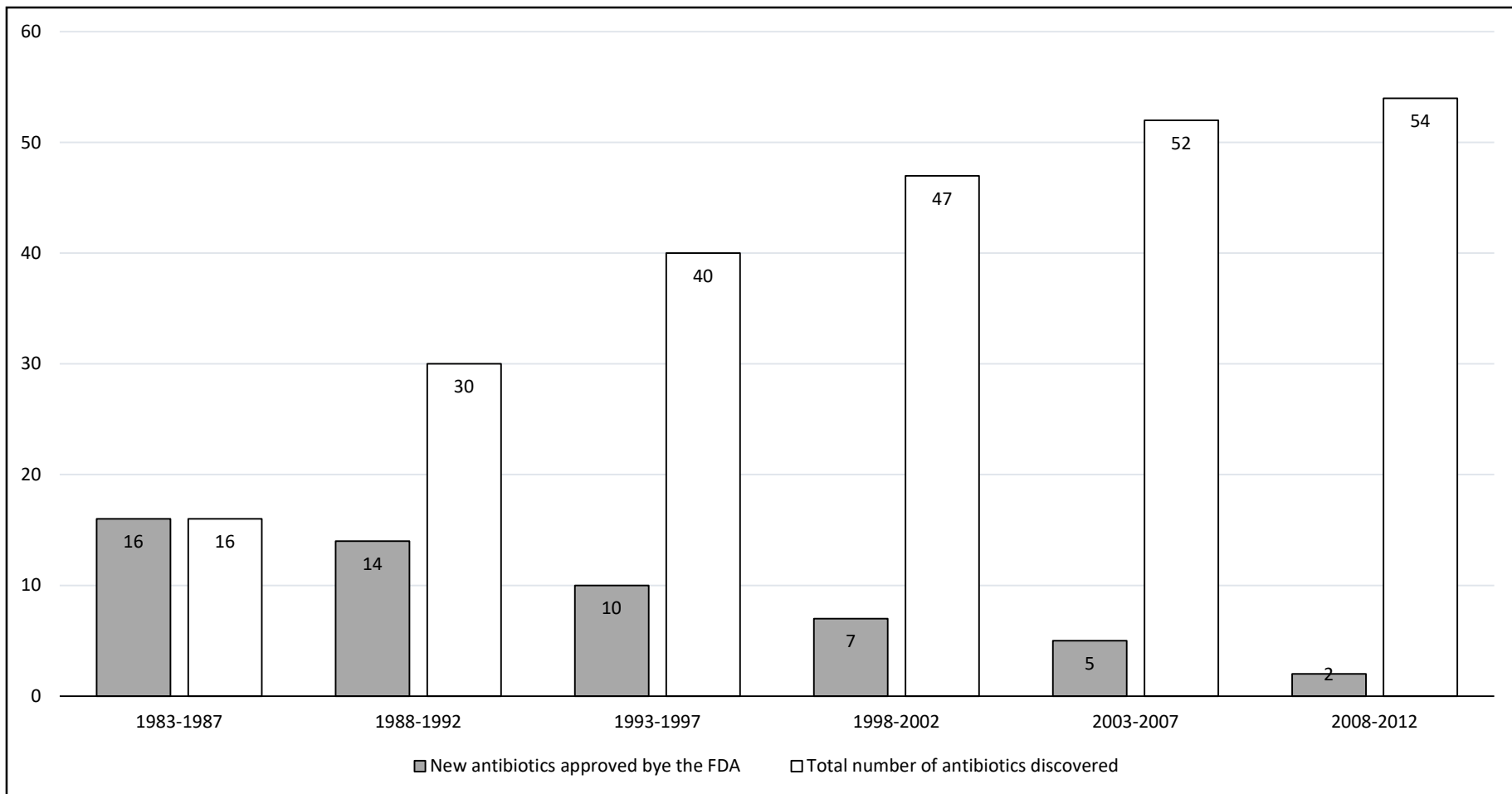
678 **Table 2.** Clinical trials involving antimicrobial peptides.

679 **Figure legend**

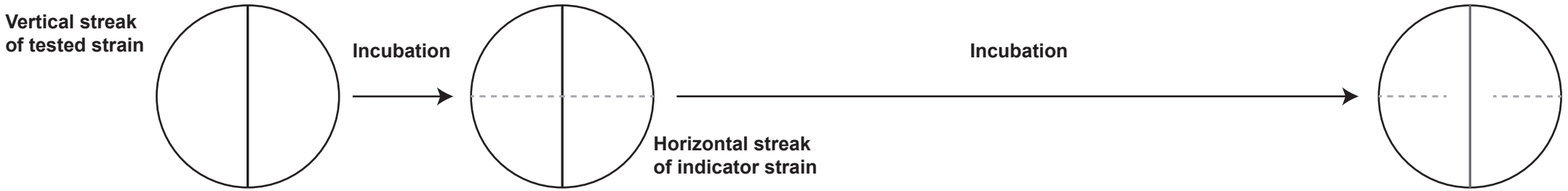
680 **Figure 1.** Evolution of the number of antibiotics approved by the US Food and Drug
681 Administration (grey) compared to the total cumulative number of antibiotics available (light
682 grey). Adapted from Boucher HW *et al.*, CID 2009.

683 **Figure 2.** Solid culture approaches highlight inhibition between two bacterial species.

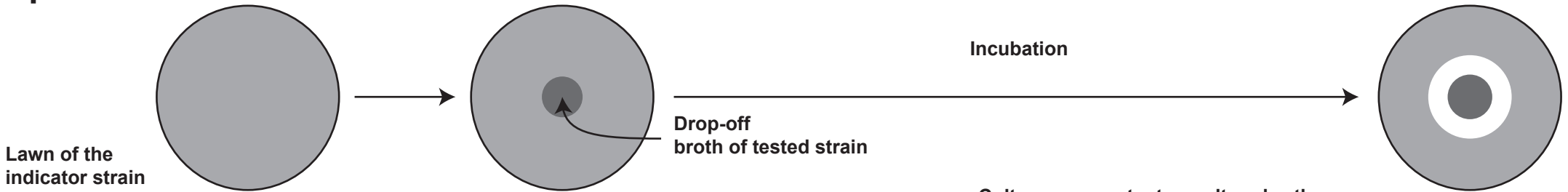
684 **Figure 3.** Liquid culture approaches highlight inhibition between two bacterial species.



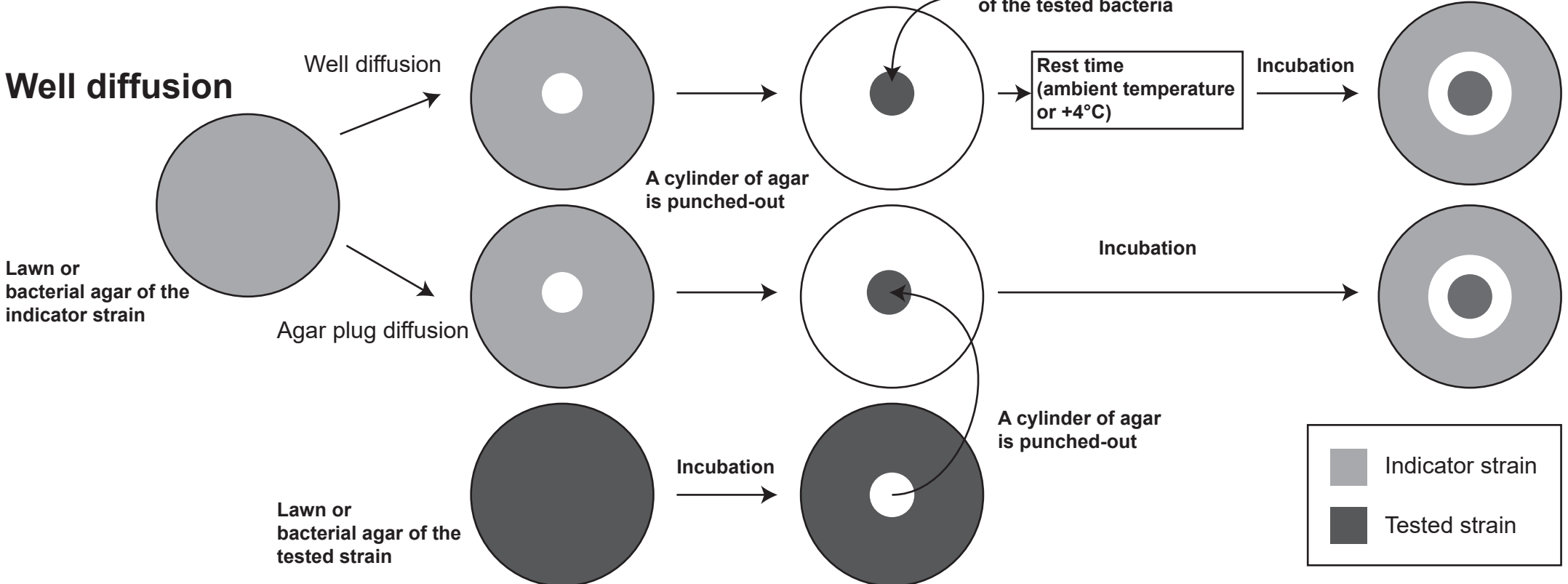
Cross streak



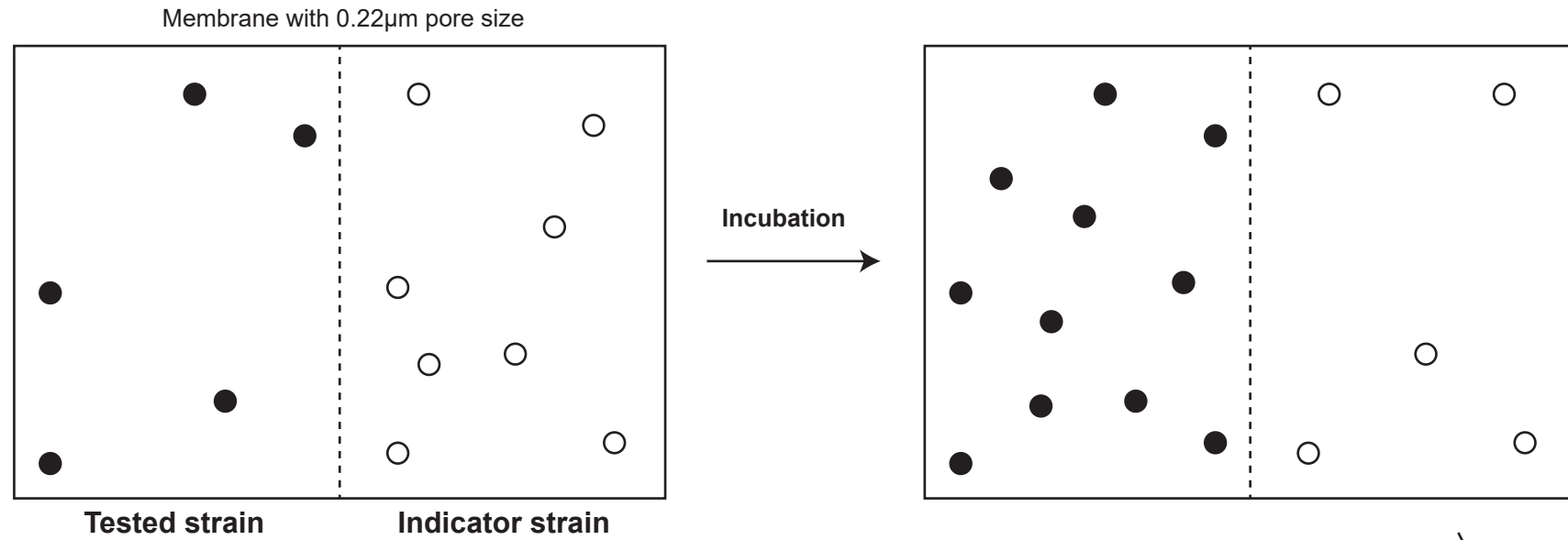
Spot-on-lawn



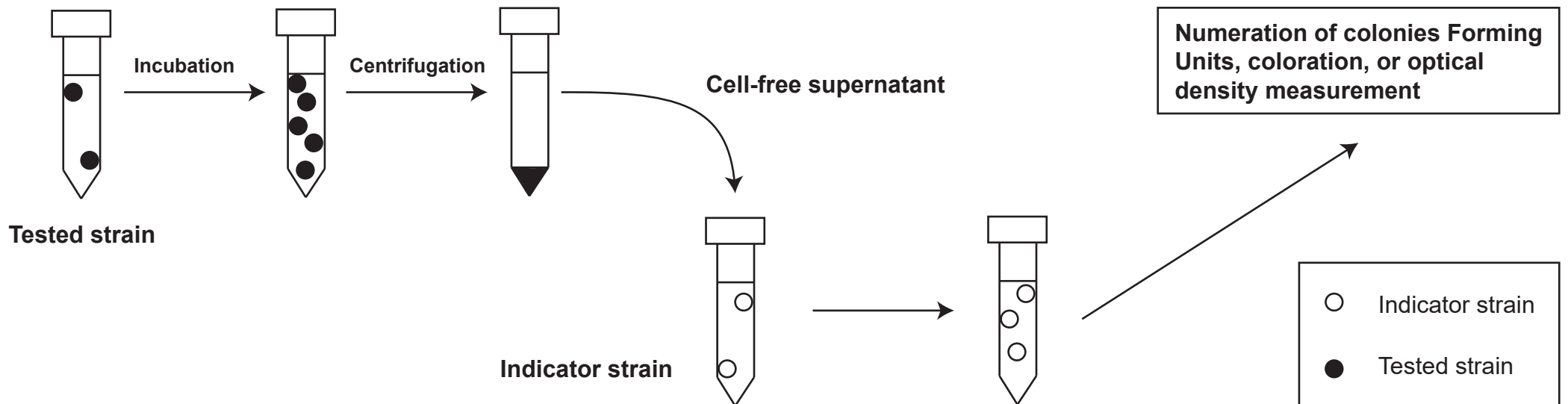
Well diffusion



Co-culture in liquid broth



Supernatant



1 **Antibiotic discovery: history, methods and perspectives**

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13 **Abstract**

14 Antibiotic resistance is considered a major public health issue. Policies recommended by the
15 World Health Organization include research on new antibiotics. No new class was discovered
16 since daptomycin and linezolid in the 1980s and only optimization or combination of already
17 known compounds has been recently commercialized. Antibiotics are natural products of soil-
18 living organisms. *Actinobacteria* and fungi are the source of about two thirds of the
19 antimicrobial agents currently used in human medicine; they were mainly discovered during
20 the golden age of antibiotic discovery. This era declined after the 1970s due to the difficulty
21 of cultivating fastidious bacterial species under laboratory conditions. Various strategies such
22 as rational drug design have not led to date to the discovery of new antimicrobial agents.
23 However, new promising approaches, e.g. genome mining or CRISPR Cas9 are now being
24 developed. The recent rebirth of culture methods from complex samples has, as a matter of
25 fact, permitted the discovery of teixobactin from a new species isolated from the soil.
26 Recently, many Biosynthetic Gene Clusters were identified from Human-associated
27 microbiota, especially from the gut and oral cavity. As an example, the antimicrobial
28 lugdunine was recently discovered in the oral cavity. The repertoire of human gut microbiota
29 has recently substantially increased, with the discovery of hundreds of new species. The
30 exploration of the repertoire of prokaryotes associated with human beings using genome
31 mining or newer culture approaches could be promising strategies for discovering new classes
32 of antibiotics.

33 1. Introduction

34 Antibiotic resistance is considered a major public health concern by several
35 international organizations and by local agencies [1–3]. As a matter of fact, the Centers for
36 Disease Control and Prevention claim 23,000 deaths each year in the US related to antibiotic
37 resistance and some studies predict millions of deaths in the coming decades [4–6]. The
38 United Nations then created a group in order to coordinate the fight against antibiotic
39 resistance [7]. Interestingly, the global mortality related to infectious diseases is decreasing
40 every year, from 10,7 million deaths in 2005 to 8,6 million in 2015 [8]. In addition, it was
41 recently shown that the current mortality due to antibiotic resistance seems far from these
42 predictions [9]. One of the approaches used over the past decade to treat Multi-Drug Resistant
43 (MDR) and Extremely-Drug Resistant (XDR) bacteria was to break the vicious circle of Beta-
44 lactams, enlarging the panel of antimicrobial agents commonly tested. It has been
45 demonstrated that “old” antibiotics (i.e. forgotten molecules) had remarkable efficacy against
46 such isolates. For instance, minocycline, sulfadiazine and clofazimine are active against XDR
47 tuberculosis strains, as for fosfomycin, colistin and minocycline against multi-drug resistant
48 Gram-negative isolates [10,11].

49 According to the World Health Organization, antimicrobial resistance control policies
50 include the rational use of antibiotics, in particular on farms, the increase of surveillance, and
51 the research and development for new tools and molecules [12]. Indeed, despite the rising
52 number of available molecules (**Figure 1**), the last new class of antibiotic discovered is
53 daptomycin (1986), which was only approved in 2003 by the Food and Drug Administration
54 (FDA) [13–15]. This fact confirms that antimicrobial agents found on the market in the last 30
55 years are associations or improvements of existing molecules. Examples are given of new
56 antibiotics recently marketed that belong to an already known class, such as oxazolidinones
57 (tedizolide), lipoglycopeptides (dalbavancin), or cephalosporins (ceftaroline, ceftobiprole).

58 Combination of improved molecules of an already known class is another example of recently
59 commercialized new antibiotics, as for example ceftolozane plus tazobactam, or ceftazidime
60 plus avibactam. The research and development of a totally new class of antibiotic appear as a
61 major issue. Herein, we propose to recall the history of antibiotic discovery, their structural
62 nature, and the methods that were used for their discovery. Finally, we review the potential
63 new approaches for the discovery of new classes of antibiotics.

64 **2. The history of antibiotics**

65 *2.1. Antibiotic resistance is in fact very ancient*

66 Most of the antibiotics currently used in human medicine are natural secretions of
67 environmental bacteria or fungi. Indeed, the majority of antibiotics currently used are derived
68 from *Streptomyces* isolated from soil samples [16]. In their natural environment,
69 microorganisms have to fight against each other by producing antimicrobial substances, and
70 have to develop resistance mechanisms to other antimicrobials [17]. Secondly, the species
71 naturally producing antibacterials also have resistance genes to these antibacterials in order to
72 avoid self-toxicity, within a biosynthetic antibiotic operon [18]. D’Costa et al. demonstrated
73 the presence of antibacterial resistance genes in an environment in which there was no innate
74 antibiotic. They first proposed the existence of a “reservoir of resistance determinants that can
75 be mobilized into the microbial community” [19]. Gerard D. Wright proposed the term
76 “resistome” to design the collection of all the antibiotic resistance genes and their precursors
77 in bacteria [20].

78 Interestingly, multi-drug resistant bacterial species as well as resistance genes to
79 antibiotics currently used were also found from environmental archaeological samples. The
80 OXA genes that encode beta-lactamases have been dated to several million years [21].
81 D’Costa *et al.* have found resistance genes to β -lactam, tetracyclines and glycopeptides from

82 30,000 years old permafrost samples [22]. Kashuba *et al.* have found several resistance genes
83 in the genome of a *Staphylococcus hominis* isolated from permafrost [23]. Of the 93 strains
84 cultured by Bhullar *et al.* from the 4 million-year-old Lechuguilla cave (New Mexico), 65%
85 of the species were resistant *in vitro* to three or four antibiotics classes [24]. Also, resistance
86 genes to β -lactam and glycopeptides were also found in the 5,300-year-old gut microbiome of
87 the mummy Ötzi [25]. Recently, 177 antibiotic resistance genes belonging to 23 families (that
88 represent all the mechanisms of resistance, i.e. mutation, efflux and antibiotic inactivation)
89 were found in the antibiotic naïve Mackay Glacier region [26].

90 Orthologous genes within mobile elements known from environmental bacteria were
91 also found in bacteria isolated from clinical isolates [19]. For instance, Marshall *et al.* found
92 orthologous genes of the *van HAX* cassette from the environmental species *Streptomyces*
93 *toyocaensis* and *Amycolatopsis orientalis* [27]. This cassette is responsible for the
94 glycopeptide resistance of *Enterococcus faecium*. Some experimental studies seem to show
95 that transfer of resistance genes from environmental producers in the soil to human
96 pathogenic species is possible [28]. The horizontal gene transfer of entire clusters of
97 resistance genes from the resistome to clinical strains under selective pressure related to the
98 human use of antibiotics is actually suspected [29].

99 2.2. Historic of the antibiotic discovery

100 Antibiotics were used for a long time before the advent of modern medicine. The
101 effects of bread on which filamentous fungi grew for the treatment of wounds and burns has
102 been known since ancient Egypt [30]. In the Middle Ages, healers in China and Greece used
103 musty textures to treat various ailments. In the 19th century, Sir John Scott Burden-Sanderson
104 noticed the absence of bacteria from a liquid growth culture covered with mould. In 1871,
105 Joseph Lister discovered the inhibitory effects of *Penicillium glaucum* on bacterial growth,

106 allowing him to cure a nurse's injury with *Penicillium glaucum* extracts. At the same time,
107 Louis Pasteur noticed that some bacteria could inhibit others. He discovered with his
108 colleague Jules François Joubert in 1877, while studying the growth of *Bacillus anthracis* in
109 urine samples, that it was inhibited when co-cultivated with "common" aerobic bacteria. In
110 1889, Jean Paul Vuillemin defined the word "antibiosis" as any biological relationship in
111 which "one living organism kills another to ensure its own existence". Several antagonisms
112 between microorganisms, notably moulds, were published in the thesis works of Ernest
113 Duchesne in 1897. He discovered the inhibition of *Escherichia coli* by *Penicillium glaucum*
114 thirty years before Fleming. Despite several observations of antagonisms between
115 microorganisms, no antimicrobial molecule was purified. The first antimicrobial molecules
116 discovered were chemical compounds. In 1909, Paul Ehrlich discovered the arsphenamine, an
117 arsenic derivative active against *Treponema pallidum*, the agent of syphilis. This antibiotic
118 was commercialized in 1911 under the name Salvarsan[®] then Mapharsen[®]. In 1930, Gerhard
119 Domagk discovered the antibiotic effects of sulphanilamide, a molecule synthesized 22 years
120 before by Paul Gelmo. This antibiotic was marketed under the name Protonsil[®] in 1935 and
121 was used by soldiers during World War II [32].

122 In 1928, Alexander Fleming accidentally discovered in his forgotten colonies of
123 *Staphylococcus aureus* that a fungus was inhibiting the growth of *Staphylococcus*. The
124 *Penicillium notatum* molecule has been purified and called penicillin. But the industrial
125 production of this antibiotic was performed only in 1940 by Howard Florey et Ernst Chain,
126 using *Penicillium chrysogenum* [33]. Fleming also discovered the lysozyme, an antibacterial
127 enzyme [33]. In 1930, René Dubos discovered an enzyme from a soil-derived *Bacillus* that
128 specifically decomposes *Streptococcus pneumoniae* type III capsular polysaccharide. With
129 this enzyme, he was able to treat mice with pneumococcal peritonitis [34]. Ten years later, he
130 isolated from *Bacillus brevis* the oligopeptide gramicidin that widely inhibited Gram-positive

131 species [35]. Unfortunately, gramicidin showed too much toxicity for humans, except for
132 local treatment [36]. In the US, Selman Waksman was the first to perform a systematic
133 research of antimicrobial activity of soil bacteria, particularly from *Streptomyces* members or
134 *Streptomyces*. He developed several culture techniques and strategies (“Waksman
135 platform”) in order to highlight antagonisms between bacterial species [37]. Using his
136 platform, he discovered in the 40’s several major antibiotics and antifungals, such as
137 actinomycin (from *Streptomyces* spp.) [38], streptomycin (from *Streptomyces griseus*) [39],
138 neomycin (from *Streptomyces fradiae*) [40], fumigacin (from *Aspergillus fumigatus*) and
139 clavacin (from *Aspergillus clavatus*) [41]. Actinomycin, neomycin and streptomycin are still
140 in use today. Moreover, streptomycin has revolutionized the treatment of tuberculosis, and is
141 still active against multi-drug resistant tuberculosis [42]. The pharmaceutical industry was
142 inspired by the Waksman platform, which led to the discovery of all current antibiotics
143 between the 1940s and the 1970s. During this golden age, 23 classes of antibiotic were
144 discovered from 19 bacterial species and 7 fungi (**Table 1**).

145 Despite recent commercialization for some, the last classes of antibiotic discovered are
146 from the 1980s. After 50 years of discoveries, no new classes have been found. Therefore,
147 new strategies were needed. After the culture approach through Waksman’s platform, the
148 industry turned to the *in vitro* synthetization of new molecules, based on knowledge of the
149 known mechanism of action of antibiotics. Unfortunately, few new classes of antibiotics have
150 been discovered, the nitrofurans in 1953, the quinolones in 1960, the sulphonamides in 1961 and
151 oxazolidinones in 1987. The modification and improvement of already known molecules has
152 also been carried out. This is reflected by the commercialization of linezolid in 2003 and
153 daptomycin in 2001, although these molecules had been known since 1955 and 1986,
154 respectively [32]. Recently, a new cephalosporin called cefiderocol was found to be active
155 against carbapenem-resistant Gram-negative bacteria [43]. Hemisynthetic compounds from

156 natural products were also developed, such as ketolides (derived from macrolides) or
157 metronidazole (derived from a natural product of *Streptomyces* sp.) (**Table 1**). But the lack of
158 return on investment and the emergence of resistance have led the industry to gradually let
159 down the research on antibiotics, preferring to invest in drugs for chronic diseases [32]. Of the
160 20 pharmaceutical companies that invested in antibiotic discovery in the 1980s, there were
161 only five left by 2015. [44]. More than 1,200 antimicrobial peptides were discovered from
162 various origins, from plants to invertebrates and animals, but none has been used as an
163 antibiotic [45]. In conclusion, the majority of antibiotics were discovered during the golden
164 age. Bacteria and fungi were the greatest producers. The genus *Streptomyces* is the source of
165 about half of the antimicrobial agents currently used in human medicine (**Table 1**).

166 **3. Chemical nature of antimicrobial agents**

167 Antimicrobial molecules are represented by a wide variety of chemical compounds.
168 Most of the time, they are natural products and secondary metabolites, implying that they are
169 not required for survival under laboratory conditions but still provide some advantages in the
170 environment [46]. Among the antimicrobial substances used in human medicine, it is possible
171 to classify antibiotics in five groups of chemical molecules. The first are derived amino acids
172 that are non-ribosomally synthesized (non-ribosomally synthesized peptides, NRP). The
173 second are acetyl coenzyme A or malonyl coenzyme A derived (polyketides, PK). NRP and
174 PK represent about 50% of all current antibiotics (**Table 1**). The third are hybrids between
175 NRP and PK and the fourth are composed of several carbohydrates units substituted with
176 amine groups (aminoglycosides). And finally, the last group is composed of various
177 molecules, such as terpenoids, fusidic acid or alkaloids such as metronidazole. In addition to
178 these molecules used as antibiotics, thousands of antimicrobial peptides are known from
179 insects, mammals, plants or amphibians [47]. These peptides are usually classified as

180 “ribosomally synthesized and post-translationally modified peptides” (RiPPs), a subgroup of
181 natural products [48].

182 *3.1. Antibiotics*

183 The NRP and PK are synthesized by multi-enzymatic complexes (non-ribosomal
184 peptides synthetase, NRPS and polyketides synthetase, PKS) encoded by biosynthetic gene
185 clusters [49]. These complexes are organized in modules including several domains. For
186 example, the erythromycin synthase complex consists of three proteins and seven modules
187 each containing three to six domains. [49]. The NRPS use amino acids as substrate elongated
188 by peptidic connection, whereas PKS use acyl-coenzyme A as substrate elongated by Claisen
189 condensation reaction [49]. This complex organization allows the production of a great
190 number of different products, and therefore a great diversity. For example, ciclosporin
191 belongs to the NRP category [50]. Hybrid assembly line that uses both amino acids and acetyl
192 coenzyme A, or fatty acid synthetases (FAS), are also described [51]. NRP antibiotics
193 comprise molecules such as β -lactams, daptomycin, lincomycin, polymyxins, and
194 vancomycin while macrolides, mupirocin and tetracyclines belong to polyketides antibiotics.
195 Rifampicin belongs to hybrid NRP/PK (**Table 1**).

196 *3.2. Antimicrobial peptides*

197 Antimicrobial peptides are broad spectrum antibacterial molecules that have been
198 discovered in blood cells in 1957 by Robert Skarnes [52]. They belong to natural products
199 that are small peptides (i.e. less than 100 amino acids) ribosomally synthesized with post-
200 translational modifications (RiPPs). Natural peptides have a multifunctional activity that
201 participates in innate immunity in eukaryotic and prokaryotic cells. [47]. Antimicrobial
202 peptides are evolutionary well-conserved amphipathic molecules with hydrophobic and
203 cationic amino-acids [53]. They can be categorized according to their secondary

204 conformation: group I (alpha helical), group II (beta sheet), group III (mixed) and IV
205 (extended) [54]. They have been isolated from almost all living organisms from prokaryotes
206 to vertebrates. Indeed, thousands of antimicrobial peptides are known and come from insects,
207 plants, or amphibians [45,47]. The main known mechanisms of action are related to their
208 cationic charge and amphipathic structure, responsible for the membrane disruption of the
209 negatively charged bacterial cell. Antimicrobial peptides can also interact with membrane-
210 associated protein targets, as well as intracellular targets after penetration into the bacterial
211 cytoplasm [54]. Finally, antimicrobial peptides can also have immunomodulatory effects on
212 the innate immune system of the host [55].

213 Bacteriocins are antimicrobial peptides that were first discovered in bacterial species.
214 They are used in the agro-alimentary industry as food preservatives and in veterinary
215 medicine [56]. Bacteriocins are separated into four groups: class I are small heat-stable post-
216 translationally modified peptides (<5kDa) which use the amino acid lanthionine, there are
217 therefore called lantibiotics; class II are not modified heat-stable small peptides (<10kDa)
218 which do not use lanthionine; class III are large heat-labile peptides (>30kDa) and class IV
219 are complex or cyclic peptides containing lipids or carbohydrates [56]. Bacteriocins inhibited
220 closely related species [57]. For example, lactacin 3147 and nisin are lantibiotics that
221 exhibited an antibiotic activity against Gram-positive bacteria, notably Meticillin-resistant
222 *Staphylococcus aureus* (MRSA) or Vancomycin-resistant *Enterococcus* [58]. Nisin is the
223 most famous lantibiotic, largely used as food preservative because of its activity against
224 *Listeria monocytogenes*, MRSA and *Streptococcus pneumoniae* [59]. Nisin is also used in
225 veterinary medicine under the name Wipe Out ® for the prevention of dairy mastitis [57]. The
226 synergistic effects of lantibiotics with antibiotics have also been demonstrated *in vitro* [60].

227 According to the Antimicrobial Peptide database, 2,478 antibacterial peptides are
228 already known [61]. All medical fields combined, 60 peptides have obtained the FDA

229 approval and 140 are in clinical trials, mainly for oncological and metabolic diseases [62].
230 Yet, when focusing on infectious diseases, only 12 naturally occurring antimicrobial peptides
231 have reached the stage of human clinical trial. (**Table 2**). Among them, only three (LYX-109,
232 LL-37 and nisin) demonstrated a better efficacy than placebo, but none obtained the FDA
233 approval for human application. Only gramicidin has received FDA approval for topical
234 application in association with polymyxin and neomycin for ophthalmic use. Teixobactin is a
235 new promising depsipeptide that provides an inhibition of *S. aureus* and *M. tuberculosis* [63].
236 This molecule uses notably the rare amino acid L-allo-enduracididine, which is challenging to
237 synthesize and therefore limits its use despite efforts made to develop analogues [64]. Despite
238 their narrow or broad spectrum of activity against human pathogens and easier bioengineering
239 compared to NRPS or PKS, bacteriocins are not used in humans as antibiotics due to several
240 limitations.

241 The main limitations in the use of antimicrobial peptides as antibiotics in clinical
242 practice are their instability (proteolytic digestion, oxidation), their high cost and low yield of
243 production, their short half-life, and their quick elimination [60]. Notably, their low
244 bioavailability after oral ingestion related to proteolytic degradation is a great bottleneck. To
245 solve these problems, production of analogues using rational drug design or nano-engineering
246 is used to improve the pharmacokinetic properties. For instance, nano-engineering has
247 increased the nisin spectrum to Gram-negative bacterial species [65] or allowed HPA3P^{HIS} to
248 be highly effective against *Vibrio vulnificus* in a mouse model [66]. The use of nanoparticles
249 also prolonged the stay in the stomach of pexiganan after oral administration, which reduces
250 the concentration of pexiganan required in a mouse model for *Helicobacter pylori* eradication
251 [67]. Another approach is the combination of bacteriocins with other antimicrobials in order
252 to reduce the resistance risk and to increase the antimicrobial potency [60]. Another matter of
253 concern with AMP is the risk of development of resistance against our own immunity

254 peptides. For instance, pexiganan have been previously found to induce cross-resistance to
255 human-neutrophil-defensine 1 [68].

256 **4. Methods for the discovery of antibiotics**

257 *4.1. Culture-based approaches*

258 In the 1940s, Selman Waksman systematically screened the soil bacteria for
259 antagonisms. Its culture-based approach is still in use today [69]. All the methods are based
260 on the same principle: showing an inhibition of a tested strain over an indicator strain closely
261 cultivated. The tested strain is the strain suspected to produce antimicrobials targeting the
262 strain used as indicator. Several techniques exist to detect antimicrobial activity, either in
263 solid or in liquid culture. There are three main methods regarding solid culture approaches:
264 the cross-streak method, the spot-on-the-lawn and the well diffusion method (**Figure 2**).

265 The cross streak is the inoculation of the bacterial strain tested vertically on the agar
266 plate. The incubation time of the plate depends on the life cycle of the bacterial strain required
267 to reach the exponential phase, which is the moment where secondary metabolites are
268 excreted. Then, the indicator strain is inoculated into horizontal streaks and the plate is
269 incubated (**Figure 2**) [70]. This technique is easy and powerful for screening but requires that
270 both bacterial strains have the same culture conditions (e.g., atmosphere, temperature and
271 growth duration).

272 The second and the third methods are respectively called “spot-on-the-lawn” and “well
273 diffusion method”. The “spot-on-the-lawn” method consists in depositing a drop of the tested
274 strain on a lawn of the indicator strain [71]. After incubation, an inhibition zone is searched
275 around the sediment. The “well diffusion method” is based on the diffusion of antimicrobials
276 through agar which inhibits sensitive species. An agar plate is pooled with the indicator strain
277 or inoculated with a lawn of the indicator and agar holes are punched out aseptically. Then,

278 two main variants exist. The first is the Agar Plug Diffusion method which consists in
279 removing a cylinder of agar from a plate previously inoculated with the tested strain. This
280 cylinder of agar is then placed into the hole of the indicator plate [72]. The second variant
281 method consists in placing a liquid broth of the tested strain or a growth supernatant in the
282 hole [73]. After an optional rest time at +4°C, the agar plate is incubated and inhibition
283 growth zones are measured (**Figure 2**). Several variants were developed, like using stress
284 conditions or iron chelator [74,75]. The main limitation of solid culture tests concerns
285 bacterial species that have different growth conditions or fastidious species.

286 Liquid culture-based approaches can solve this problem. Liquid broth co-culture has
287 been used since the existence of the Waksman platform [69]. This is the simultaneous culture
288 of the tested species and the indicator strain separated by a filter allowing the diffusion of
289 nutrients but not the diffusion of cells. Following incubation, the bacterial growth of the
290 indicator strain is determined by numeration, coloration or optical density [69]. Another
291 method is to add the growth supernatant of the test species previously filtered and
292 concentrated to a liquid culture of the indicator strain [76]. The latter method allows the
293 bacteria tested to be grown under conditions different from those of the indicator strain
294 (**Figure 3**).

295 4.2. *Discovering antimicrobial effects from already known compounds*

296 Some antibiotics were discovered years before they were used (fidaxomicine,
297 daptomycin, or linezolid). Several million of chemical compounds are known in chemical
298 databases and could provide a potential source of antibiotics [32]. Moy *et al.* have tested the
299 activity against *E. faecalis* of more than 6,000 chemical compounds and 1,136 natural
300 products in the *Caenorhabditis elegans* animal model, discovering 16 molecules increasing

301 the survival of animals [77]. The bottleneck remains the selection and the high throughput
302 testing of these molecules.

303 *4.3. Synthesis of new molecules and improvement of already known compounds*

304 The « Rational Drug Design » consists in the empirical synthesis of new molecules
305 that are designed according to several rules to be well-absorbed, non-toxic, and active against
306 a specific target [32]. The most famous rules used by the industry are Lipinski's rules.
307 Despite more than 10 million of new molecules synthesized, only a few active molecules have
308 reached the market, notably antituberculous drugs [32,37] (**Table 1**). This can be explained by
309 the fact that antibiotics have generally poor economics [78].

310 The improvement of already known molecules is another strategy that can yield
311 benefits. The modification of cephalosporin lead to the development of cefiderocol, that
312 demonstrated safety and tolerability in healthy subjects, and clinical trials for the treatment of
313 urinary tract infections are ongoing [79]. Another example is the modification of the
314 aminoglycoside sisomicin that leads to the development of plazomicin [80]. This antibiotic
315 was recently approved by the FDA and a small phase 2 clinical trial found it had an efficacy
316 comparable to that of levofloxacin in the treatment of urinary tract infections and acute
317 pyelonephritis [81]. If the potential use of these antibiotics remains to determine, their
318 mechanism of action is not novel, and the apparition of resistance is expected in the same way
319 as for their related antibiotic parent.

320 *4.4. Genome mining*

321 Secondary metabolites are encoded by Biosynthetic Gene Cluster (BGCs). Thousands
322 of prokaryotic genomes are available in sequence databases. These data have generated
323 thousands of BGCs that potentially encode unknown molecules [82,83]. Despite the fact that
324 many of them do not have any antimicrobial activity, little is known about them. Walsh et al.

325 found 74 putative BGC from 59 genomes from the Human Microbiome Project [84]. These
326 BGC belonged mainly to *Firmicutes*, *Proteobacteria* and *Bacteroidetes* and the most
327 commonly putative bacteriocins encoded belonged to classes III and IV.

328 Several approaches of genome mining are possible. The most used are sequence-based
329 approach, ecology-based genome mining, mode-of-action-based genome mining, or function-
330 based genome mining [83,85]. For instance, lichenicidin is a bacteriocin synthesized by
331 *Bacillus licheniformis* that was discovered using the mode-of-action genome mining
332 approach. The authors screened the databases for *LanM* genes, which are involved in the
333 biosynthesis of lantibiotics [86]. The identification of putative bacteriocins encoded by BGC
334 from the genome sequence is possible using bioinformatic algorithms [87]. Bacteriocins are
335 easily found using bioinformatic tools compared to NRPS or PKS. Tools such as BAGEL,
336 antiSMASH or PRISM are widely used for this purpose. These tools exploit two main
337 approaches [83]. The first consists in finding new congeners of already known scaffolds. This
338 approach is based on the homology comparison by the research of conserved domains
339 (anchors), as for the thio-template domain of NRPS and PKS [88]. Small structural changes of
340 the new homologue may result in a significant change in the activity of the product. The
341 second approach is more difficult and consists in finding new scaffolds. Predicting the
342 chemical structure and biological activity of a BGC informatic sequence is a real challenge
343 [83]. The main problem that remains is proving the functional activity of BGC [89]. Both
344 approaches often require the engineering expression of BGC from the native host or from a
345 heterologous host, which represents the main bottleneck of genome mining for the discovery
346 of new antibiotics.

347 Recently, Hover *et al.* have screened more than 2,000 soil samples from various areas
348 of the US, searching for BGCs encoding calcium-binding motif Asp-X-Asp-Gly [90]. This
349 motif is related to calcium-dependant antibiotics such as lipopeptides, for which the

350 mechanism of action is not fully understood [91]. They found several clades of
351 uncharacterized BGC, of which the most abundant was present in 19% of all samples. This
352 clade was named malacidins, the molecules were then synthesised, and their antibiotic activity
353 was characterized *in vitro*. The malacidin A exhibited a broadly antibiotic activity against
354 Gram-positive bacteria, notably MRSA, which was successfully confirmed in a skin-wound
355 infection rat model. In conclusion, genome mining is a promising approach for new antibiotic
356 discovery, despite the fact that the method is fastidious and time-consuming.

357 4.5. CRISPR Cas9

358 Bacteria and fungi have an immune system that protects them from foreign genetic
359 material that could be inserted by phages. This immune system consists of restriction
360 enzymes, toxin-antitoxin and Clustered Regularly Interspaced Short Palindromic Repeats
361 (CRISPR) Cas system [92]. The utilization of CRISPR Cas9 to design new antimicrobials
362 with a predetermined activity spectrum has been already performed with promising results.
363 Citorik et al. have developed RNA-guided nucleases that target the resistance gene *bla*_{SHV-18}
364 and *bla*_{NDM-1}. After transformation by plasmids and transduction by bacteriophages, the
365 authors observed a significant reduction in the number of *E. coli* containing the targeted
366 resistance gene, either chromosomal or plasmid [93]. The same approach has been
367 successfully tested using phagemid targeting the *S. aureus* methicillin resistance gene [94].
368 The same authors tested the use of bacteriophages in a mouse model of *S. aureus* skin
369 infection with an efficacy comparable to that of mupirocin [94]. Other authors used genome
370 editing technology to re-sensitize MDR cells. As an example, Kim *et al.* used CRISPR Cas9
371 to target a conserved sequence of Extended-Spectrum β -Lactamases, thus restoring the
372 susceptibility of *E. coli* in their *in vitro* model [95].

373

374 5. Conclusion and perspective

375 More than three quarters of all antibiotics currently used in human health are natural
376 products or derived from them. The discovery of antibiotics declined after the 1970s due to
377 the difficulty of cultivating bacterial species from soil under laboratory conditions. New
378 innovative culture approaches were then created thanks to the bloom of new molecular
379 methods. In this way, genome mining searching for new BGCs as well as CRISPR Cas9
380 technology are promising new approaches. Recently, the ability to rapidly identify bacterial
381 strains using Matrix Assisted Laser Desorption Ionisation Time of Flight (MALDI Tof) has
382 permitted the rebirth of culture. Therefore, new culture approaches trying to mimic the natural
383 environment were invented in order to grow fastidious species. This led to the discovery of
384 new bacterial species. For instance, Ling *et al.* discovered the new antibiotic teixobactin from
385 the new species *Eleftheria terrae* isolated in the soil using a diffusion chamber [63].

386 The same approach is now possible for the human-associated microbiota. The nose is
387 an example of ecological niche poor in nutrients in which the microbiota is probably in strong
388 competition [75]. Using a home-made nasal synthetic medium under iron-limited condition,
389 Krismer B *et al.* discovered lugdunin, a new antibiotic inhibiting *S. aureus* growth [74]. The
390 gut microbiota is another microbiota of interest for antibiotic research. Indeed, the human gut
391 has an average load concentration ranging from 10^4 to 10^{12} CFU/mL from the duodenum to
392 the colon. These species live in extreme competition, as they did before human colonization
393 where bacteria lived in a competitive world that led them to naturally develop many
394 antimicrobial products. Metagenomic analysis from the human gut microbiota found many
395 BGCs. In 2014, Donia *et al.* found 3,118 BGCs including NRPS, RiPPs and PKs in the
396 human microbiome. They also found 599 BGCs in the gut. Taken together with the oral
397 cavity, it is one of the richest sites in BGCs of the human microbiota. They also found and
398 purified lactocillin, a new thiopeptide antibiotic, isolated from the vaginal microbiota [96].

399 The study of the gut microbiota using culture methods was recently improved by
400 culturomics, a novel approach that consists in the multiplication of growth conditions [97].
401 This has led to the discovery of previously uncultivated species. With this approach, Lagier *et*
402 *al.* significantly increased in a couple of years the gut repertoire from 690 to 1,525 species, of
403 which 247 were totally new [98]. Indeed, the new species described from the gut represent an
404 opportunity for the research for novel antibiotics. The search for new antibiotics naturally
405 synthesized by organisms living in complex ecosystems like the gut microbiota, using the
406 culture approach, seems the modern continuity of what has already worked in the past. Thus,
407 if the study of antagonisms between environmental bacteria has led to the discovery of a
408 substantial proportion of antibiotic classes, such studies were rarely performed from human-
409 derived microbiota.

410 In conclusion, the research for new antibiotic molecules is a key point among the
411 strategies for the fight against antibiotic resistance. The recent advances from both culture-
412 dependant and culture-independent methods of exploration of complex ecosystems such as
413 soil or human-associated microbiota open a new era in antimicrobial research.

414

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417

418

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675

676 **Table legend**

677 **Table 1.** Discovery date, origin of the organism and synthesis pathway of antibiotics.

678 **Table 2.** Clinical trials involving antimicrobial peptides.

679 **Figure legend**

680 **Figure 1.** Evolution of the number of antibiotics approved by the US Food and Drug
681 Administration (grey) compared to the total cumulative number of antibiotics available (light
682 grey). Adapted from Boucher HW *et al.*, CID 2009.

683 **Figure 2.** Solid culture approaches highlight inhibition between two bacterial species.

684 **Figure 3.** Liquid culture approaches highlight inhibition between two bacterial species.