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Antibiotic discovery: history, methods and perspectives

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Abstract

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

1. Introduction

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

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

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

2. The history of antibiotics

2.1. Antibiotic resistance is in fact very ancient

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

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

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

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

2.2. Historic of the antibiotic discovery

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

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

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

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

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

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

3. Chemical nature of antimicrobial agents

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

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

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

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

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

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

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

4. Methods for the discovery of antibiotics

4.1. Culture-based approaches

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

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

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

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

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

4.2. Discovering antimicrobial effects from already known compounds

Some antibiotics were discovered years before they were used (fidaxomycine, daptomycin, or linezolid). Several million of chemical compounds are known in chemical databases and could provide a potential source of antibiotics [32]. Moy *et al*. have tested the activity against *E. faecalis* of more than 6,000 chemical compounds and 1,136 natural 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 Lipinsks'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.

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

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

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

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

4.5.CRISPR Cas9

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

5. Conclusion and perspective

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

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

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

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

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Table legend

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

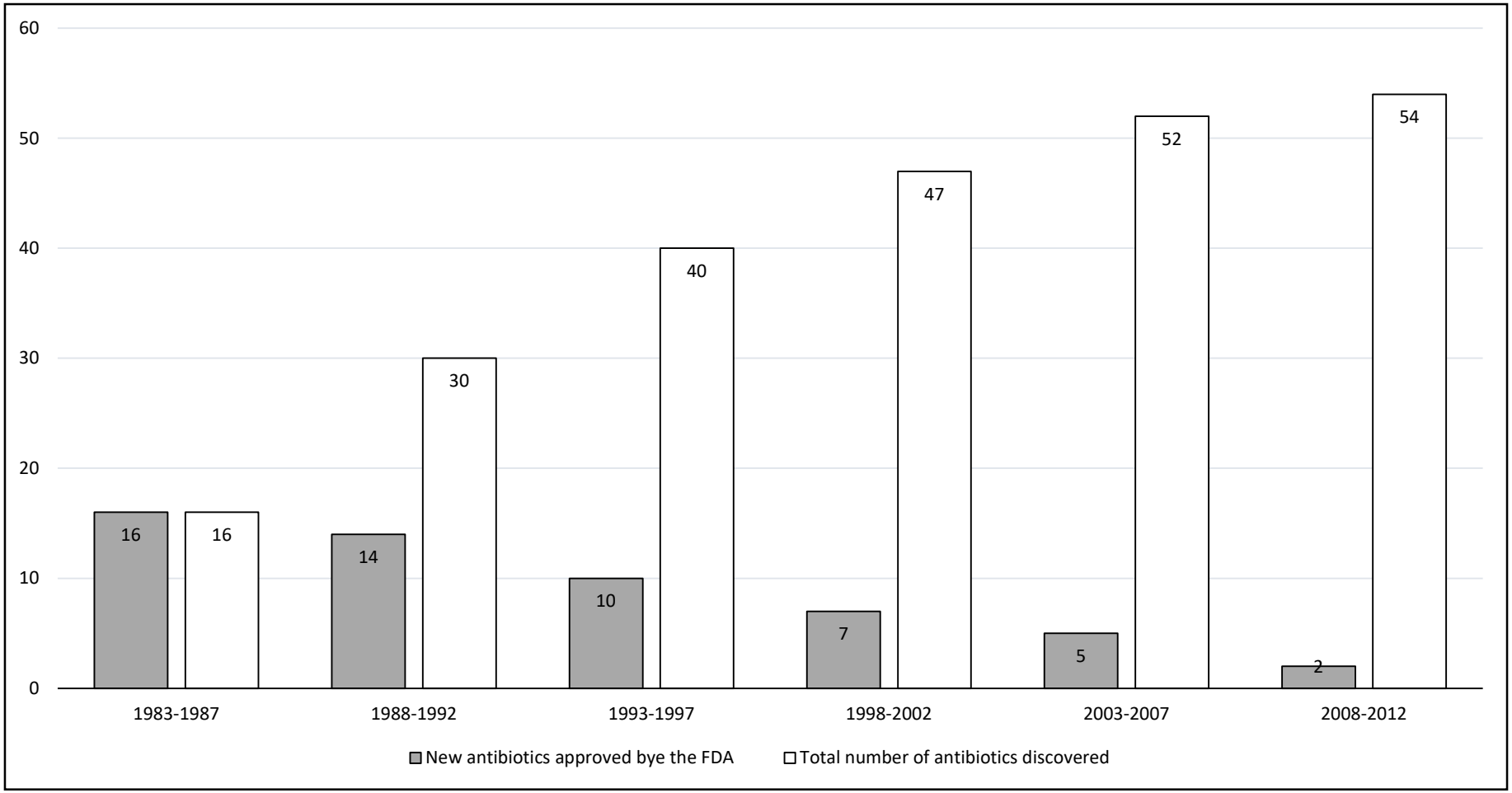
Table 2. Clinical trials involving antimicrobial peptides.

Figure legend

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

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

Figure 3. Liquid culture approaches highlight inhibition between two bacterial species.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1983-1987
1988-1992
1993-1997
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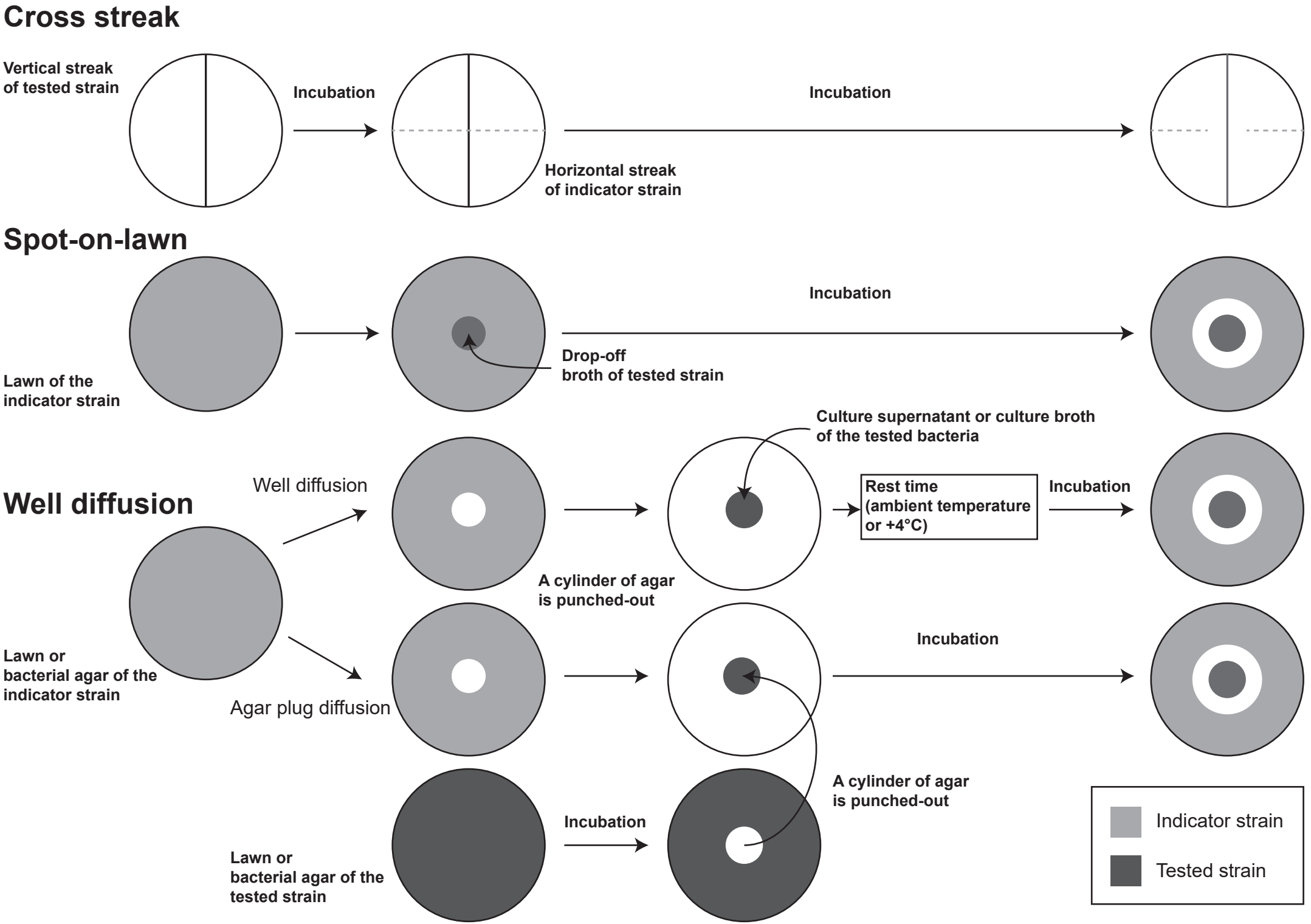
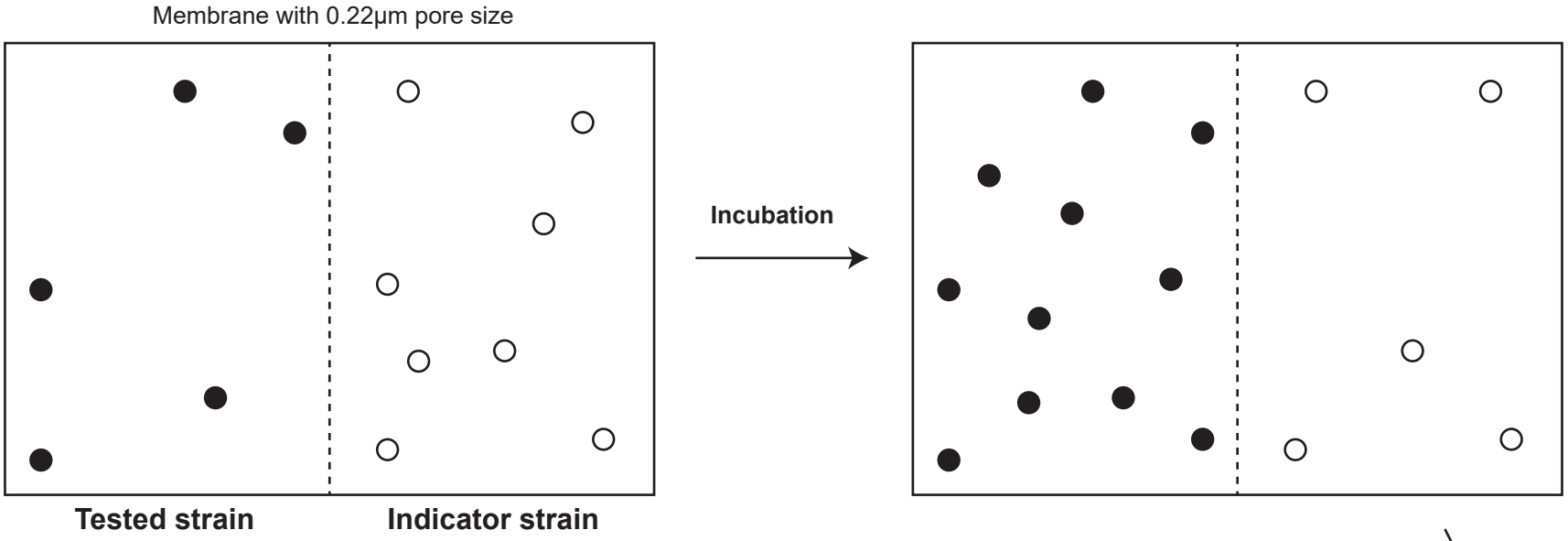
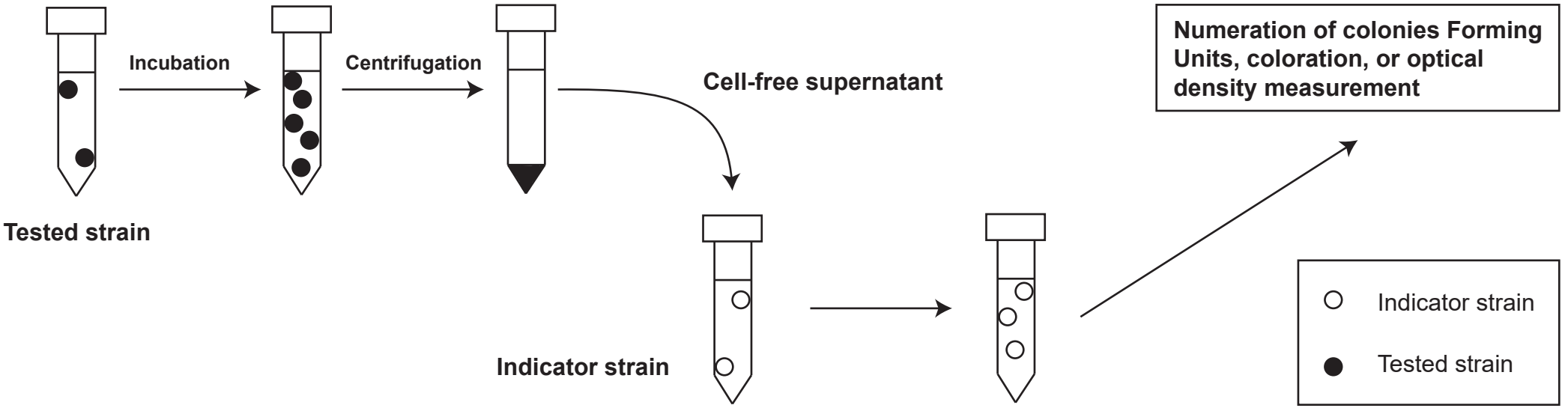
2003-2007
2008-2012
New antibiotics approved bye the FDA
Total number of antibiotics discovered


Cross streak
Vertical streak of tested strain
Incubation
Horizontal streak of indicator strain
Incubation
Spot-on-lawn
Lawn of the indicator strain
Drop-off broth of tested strain
Incubation
Well diffusion
Culture supernatant or culture broth of the tested bacteria
Rest time (ambient temperature or +4°C)
Incubation
Well diffusion
A cylinder of agar is punched-out
Lawn or bacterial agar of the indicator strain
Agar plug diffusion
Incubation
A cylinder of agar is punched-out
Incubation
Lawn or bacterial agar of the tested strain
Indicator strain
Tested strain

Co-culture in liquid broth



Supernatant



Antibiotic discovery: history, methods and perspectives

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References: 98

Abstract

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

1. Introduction

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

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

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

2. The history of antibiotics

2.1. Antibiotic resistance is in fact very ancient

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

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

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

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

2.2. Historic of the antibiotic discovery

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

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

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

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

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

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

3. Chemical nature of antimicrobial agents

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

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

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

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

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

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

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

4. Methods for the discovery of antibiotics

4.1. Culture-based approaches

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

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

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

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

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

4.2. Discovering antimicrobial effects from already known compounds

Some antibiotics were discovered years before they were used (fidaxomycine, daptomycin, or linezolid). Several million of chemical compounds are known in chemical databases and could provide a potential source of antibiotics [32]. Moy *et al*. have tested the activity against *E. faecalis* of more than 6,000 chemical compounds and 1,136 natural 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 Lipinsks'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.

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

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

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

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

4.5.CRISPR Cas9

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

5. Conclusion and perspective

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

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

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

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

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Table legend

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

Table 2. Clinical trials involving antimicrobial peptides.

Figure legend

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

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

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