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Review

Antibiotic discovery: history, methods and perspectives

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ABSTRACT

Antimicrobial resistance is considered a major public-health issue. Policies recommended by the World Health Organization (WHO) include research on new antibiotics. No new class has been discovered since daptomycin and linezolid in the 1980s, and only optimisation or combination of already known compounds has been recently commercialised. Antibiotics are natural products of soil-living organisms. Actinobacteria and fungi are the source of approximately 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 owing to the difficulty of cultivating fastidious bacterial species under laboratory conditions. Various strategies, such as rational drug design, to date have not led 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 soil. Recently, many biosynthetic gene clusters were identified from human-associated microbiota, especially from the gut and oral cavity. For example, the antimicrobial lugdunin 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. Exploration of the repertoire of prokaryotes associated with humans using genome mining or newer culture approaches could be promising strategies for discovering new classes of antibiotics.

1. Introduction

Antimicrobial resistance is considered a major public-health concern by several international organisations as well as local agencies [1–3]. In fact, the US Centers for Disease Control and Prevention (CDC) assert 23 000 deaths each year in the USA related to antibiotic resistance, and some studies predict millions of deaths in the coming decades [4–6]. The United Nations has created a group in order to co-ordinate the fight against antimicrobial resistance [7]. Interestingly, 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 antimicrobial resistance appears far from these predictions [9]. One of the approaches used over the past decades to treat multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacteria was to break the vicious circle of β-lactams, enlarging the panel of antimicrobial agents commonly tested. It has been demonstrated that 'old' antibiotics (i.e. forgotten molecules) have remarkable efficacy against such isolates. For instance, minocycline, sulfadiazine and clofazimine are active against XDR *Mycobacterium tuberculosis* strains, similar to fosfomycin, colistin and minocycline against MDR Gram-negative bacterial isolates [10,11].

According to the World Health Organization (WHO), antimicrobial resistance control policies include rational use of antibiotics, in particular on farms, increased surveillance, and research and development for new tools and molecules [12]. Indeed, despite the rising number of available molecules (Fig. 1), the last new class of antibiotic discovered was daptomycin (1986), which was only approved in 2003 by the US 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 of new antibiotics recently marketed that belong to an already known class include oxazolidinones (tedizolid), lipoglycopeptides (dalbavancin) and cephalosporins (ceftaroline, ceftobiprole). Combination of improved molecules of an already known class is another example of recently commercialised new antibiotics, e.g. ceftolozane + tazobactam or ceftazidime + avibactam. Research and development of a totally new class of antibiotic appears to be 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 potential new approaches for the discovery of new classes of antibiotics.

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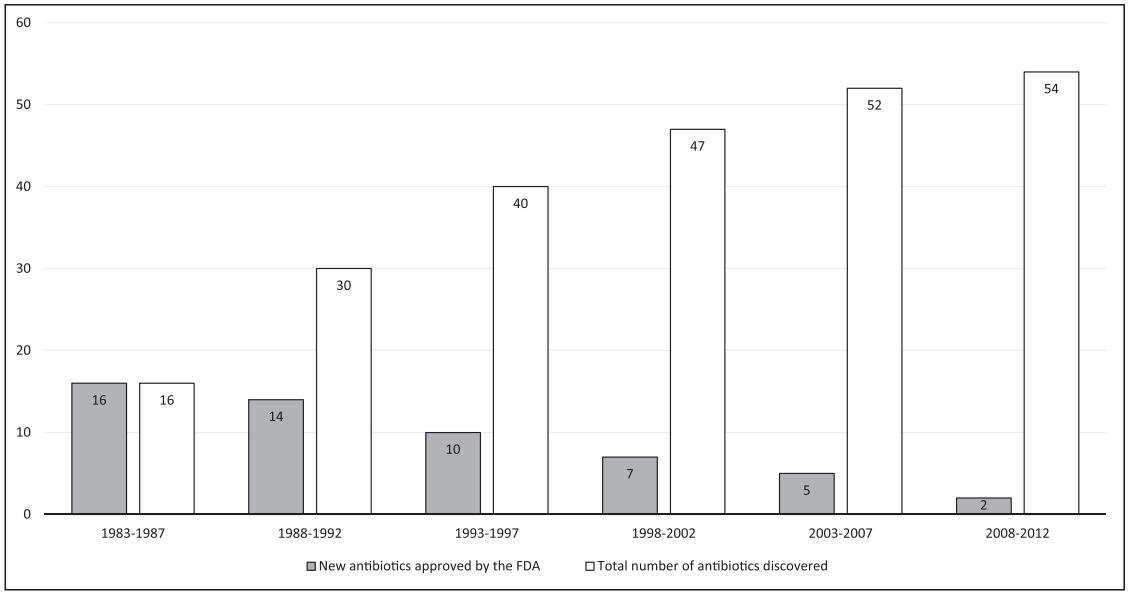

Fig. 1. Evolution of the number of antibiotics approved by the US Food and Drug Administration (FDA) compared with the total cumulative number of antibiotics available. Adapted from Boucher et al. [13].

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, micro-organisms have to fight against each other by producing antimicrobial substances, and have to develop resistance mechanisms to other antimicrobials [17]. Moreover, the species naturally producing antibacterials also have resistance genes to these antibacterials in order to avoid self-toxicity, located 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 antibiotics. 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 denote the collection of all of the antimicrobial resistance genes and their precursors in bacteria [20].

Interestingly, MDR bacterial species as well as resistance genes to antibiotics currently used have also been found from environmental archaeological samples. The bla_{OXA} genes that encode β-lactamases have been dated to several million years [21]. D'Costa et al. have found resistance genes to β-lactams, tetracyclines and glycopeptides from 30 000-year-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 antibiotic classes [24]. Resistance genes to β-lactams and glycopeptides were also found in the 5300-year-old gut microbiome of the mummy Ötzi [25]. Recently, 177 antimicrobial resistance genes belonging to 23 families (that represent all of 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 have also been found in bacteria isolated from clinical isolates [19]. For instance, Marshall et al. found orthologous genes of the *vanHAX* 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 appear to show that transfer of resistance genes from environmental producers in the soil to human pathogenic species is possible [28]. 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 suspected [29].

2.2. History of 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 have 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 *P. glaucum* extract. 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 micro-organisms, notably moulds, were published in the thesis works of Ernest Duchesne in 1897. He discovered the inhibition of *Escherichia coli* by *P. glaucum* 30 years before Fleming. Despite several observations of antagonisms between micro-organisms, no antimicrobial molecule was purified. The first antimicrobial molecules discovered were chemical compounds. In 1909, Paul Ehrlich discovered arsphenamine, an arsenic derivative active against *Treponema pallidum*, the agent of syphilis. This antibiotic was commercialised in 1911 under the name Salvarsan®, then Mapharsen®. In 1930, Gerhard Domagk discovered the antibiotic effects of sulphanilamide, a molecule synthesised 22 years before by Paul Gelmo [31]. This antibiotic was

marketed under the name Prontosil® 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 growth of the *Staphylococcus*. The molecule from *Penicillium notatum* has been purified and called penicillin. However, the industrial production of this antibiotic was performed only in 1940 by Howard Florey and Ernst Chain, using *Penicillium chrysogenum* [33]. Fleming also discovered lysozyme, an antibacterial enzyme [33]. In 1930, René Dubos discovered an enzyme from a soil-derived *Bacillus* that specifically decomposed *Streptococcus pneumoniae* type III capsular polysaccharide, with which 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 bacterial species [35]. Unfortunately, gramicidin showed too much toxicity for humans, except for local treatment [36]. In the USA, Selman Waksman was the first to perform a systematic research of the 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 1940s 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 revolutionised the treatment of tuberculosis (TB) and remains active against MDR-TB [42]. The pharmaceutical industry was inspired by the Waksman platform, which led to the discovery of all current antibiotics between the 1940s and 1970s. During this golden age, more than 20 classes of antibiotic were discovered from dozens of bacterial species and fungi (Table 1).

Despite recent commercialisation 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 are needed. After the culture approach through Waksman's platform, the industry turned to in vitro synthesis of new molecules based on knowledge of the known mechanism of action of antibiotics. Unfortunately, few new classes of antibiotic have been discovered; nitrofuran in 1953; quinolones in 1960; sulphonamides in 1961; and oxazolidinones in 1987. Modification and improvement of already known molecules has also been carried out. This is reflected by the commercialisation of linezolid in 2003 and daptomycin in 2001, although these molecules had been known since 1955 and 1986, respectively [32]. Recently, a new cephalosporin, named 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 abandon 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 5 left by 2015 [44]. More than 1200 antimicrobial peptides (AMPs) 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 approximately one-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. Frequently 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 into five groups of chemical molecules. The first, non-ribosomal peptides (NRP), are derived amino acids that are non-ribosomally synthesised. The second, polyketides (PK), are derived from acetyl coenzyme A or malonyl coenzyme A. NRP and PK represent ca. 50% of all current antibiotics (Table 1). The third are hybrids between NRP and PK, and the fourth are composed of several carbohydrate units substituted with amine groups (aminoglycosides). The final 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 AMPs are known from insects, mammals, plants and amphibians [47]. These peptides are usually classified as 'ribosomally synthesised and post-translationally modified peptides' (RiPPs), a subgroup of natural products [48].

3.1. Antibiotics

NRP and PK are synthesised by multi-enzymatic complexes, namely non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS), encoded by biosynthetic gene clusters (BGCs) [49]. These complexes are organised in modules including several domains. For example, the erythromycin synthase complex consists of three proteins and seven modules each containing three to six domains [49]. NRPS uses amino acids as substrate elongated by peptidic connection, whereas PKS uses acyl-coenzyme A as substrate elongated by Claisen condensation reaction [49]. This complex organisation allows the production of a great number of different products and therefore great diversity. For example, cyclosporine belongs to the NRP category [50]. Hybrid assembly lines that use both amino acids and acetyl coenzyme A, or fatty acid synthase (FAS), are also described [51]. NRP antibiotics comprise molecules such as β-lactams, daptomycin, lincomycin, polymyxins and vancomycin, whilst macrolides, mupirocin and tetracyclines belong to PK antibiotics. Rifampicin belongs to the hybrid NRP/PK antibiotics (Table 1).

3.2. Antimicrobial peptides (AMPs)

AMPs are broad-spectrum antibacterial molecules that were discovered in blood cells by Robert Skarnes in 1957 [52]. They are natural RiPP products that are small peptides (i.e. $<$100 amino acids). Natural peptides have multifunctional activity that participates in innate immunity in eukaryotic and prokaryotic cells [47]. AMPs are evolutionary well-conserved amphipathic molecules with hydrophobic and cationic amino acids [53]. They can be categorised according to their secondary conformation into group I (α helical), group II (β sheet), group III (mixed) and group IV (extended) [54]. They have been isolated from almost all living organisms from prokaryotes to vertebrates. Indeed, thousands of AMPs are known coming from insects, plants and amphibians [45,47]. The main known mechanisms of action are related to their cationic charge and amphipathic structure, responsible for disruption of the negatively charged bacterial cell membrane. AMPs can also interact with membrane-associated protein targets as well as intracellular targets following penetration into the bacterial cytoplasm [54]. Finally, AMPs may also have immunomodulatory effects on the innate immune system of the host [55].

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

Class	Antibiotic	Discovery	FDA approval	Organism	Synthesis pathway	Reference[a]
Aminoglycosides	Capreomycin	1960	1969	*Streptomyces capreolus*	Aminoglycoside	[1]
	Framycetin	1953	1955	*Streptomyces lavendulae*	Aminoglycoside	[2]
	Gentamicin	1963	1979	*Micromonospora purpurea*	Aminoglycoside	[3]
	Kanamycin	1957	1973	*Streptomyces kanamyceticus*	Aminoglycoside	[4]
	Natamycin	1957		*Streptomyces natalensis*	Aminoglycoside	[5]
	Neomycin	1949	1954	*Streptomyces fradiae*	Aminoglycoside	[6]
	Plazomicin	2009	2018		Semisynthetic	[7]
	Sisomicin	1970		*Micromonospora inyoensis*	Aminoglycoside	[8]
	Streptomycin	1943	1946	*Streptomyces griseus*	Aminoglycoside	[9]
	Tobramycin	1967	1975	*Streptomyces tenebrarius*	Aminoglycoside	[10]
Antituberculous drugs	Ethambutol	1961	1967		Synthetic	[11]
	Ethionamide	1956	1965		Synthetic	
	Isoniazid	1952	1952		Synthetic	[12]
	Pyrazinamide	1936	1952		Synthetic	[13]
β-Lactams	Carbapenem	1976	1986	*Streptomyces cattleya*	NRPS	[14]
	Cephalosporin	1948	1964	*Cephalosporium acremonium*	NRPS	Brotzu G, unpublished
	Monobactam	1981	1987	*Chromobacterium violaceum*	NRPS	[15]
	Penicillin	1928	1938	*Penicillium notatum, Penicillium chrysogenum*	NRPS	[16]
Carboxylic acid	Mupirocin	1971	1987	*Pseudomonas fluorescens*	PKS	[17]
Chloramphenicols	Chloramphenicol	1946	1948	*Streptomyces venezuelae*	Shikimate	[18]
Fosfomycin	Fosfomycin	1969	1989	*Streptomyces fradiae*	Carbon–phosphate	[19]
Glycopeptides	Dalbavancin	2002	2014		Semisynthetic	[20]
	Oritavancin	1996	2014		Semisynthetic	[21]
	Teicoplanin	1978	1987 [b]	*Actinoplanes teichomyceticus*	NRPS	[22]
	Vancomycin	1953	1958	*Amycolatopsis orientalis*	NRPS	[23]
Ketolides	Telithromycin	1997	2004		Semisynthetic, derived from macrolide	[24]
Lincosamides	Lincomycin	1963	1964	*Streptomyces lincolnensis*	NRPS	[25]
Lipopeptides	Daptomycin	1986	2003	*Streptomyces roseosporus*	NRPS	[26]
Macrolides	Erythromycin	1948	1951	*Streptomyces erythraeus*	PKS	Aguilar A & McGuire JM, unpublished
	Josamycin	1967		*Streptomyces narbonensis* var. *josamyceticus*	PKS	[27]
	Midecamycin	1975		*Streptomyces mycarofaciens*	PKS	[28]
	Spiramycin	1952	1955	*Streptomyces ambofaciens*	PKS	[29]
	Fidaxomicin	1975	2011	*Dactylosporangium aurantiacum* subsp. *hamdenesis*	PKS	[30]
Nitrofurans	Nitrofurantoin	1952	1953		Synthetic	[31]
Nitroimidazoles	Metronidazole	1960	1960	*Streptomyces* sp.	Semisynthetic	[32]
	Ornidazole	1975			Synthetic	[33]
Oxazolidinones	Linezolid	1987	2000		Synthetic	[34]
	Tedizolid	2008	2014		Synthetic	[35]
Polypeptides	Polymyxin	1947	1959	*Paenibacillus polymyxa*	NRPS	[36]
Quinolones	Delafloxacin	2000	2017		Synthetic	[37]
	Norfloxacin	1961	1968		Synthetic	[38]
	Nalidixic acid	1960	1967		Synthetic	[39]
Rifamycins	Rifampicin	1957	1958	*Streptomyces mediterranei*	Hybrid NRPS/PKS	[40]
Steroids	Fusidic acid	1962	1983	*Fusidium coccineum*	Terpene	[41]
Streptogramins	Streptogramin B	1953	1998	*Streptomyces graminofaciens*	NRPS	[42]
	Pristinamycin	1961		*Streptomyces pristinaespiralis*	NRPS	[43]
Sulfonamides	Sulfamethoxazole	1961	1961		Synthetic	[44]
		1968	1974		Synthetic	[45]
	Trimethoprim/sulfamethoxazole					
Tetracyclines	Chlortetracycline	1948	1952	*Streptomyces aureofaciens, Streptomyces rimosus*	PKS	[46]
	Eravacycline	2010			Synthetic	[47]
	Minocycline	1961	1971		Semisynthetic	[48]
	Tigecycline	1999	2005		Synthetic	[49]

FDA, US Food and Drug Administration; NRPS, ribosomal peptide synthetase; PKS, polyketide synthase.
[a] The references are given in the Supplementary references.
[b] First approved in Italy, then Europe, Asia and South America. Not approved by the FDA.

Bacteriocins are AMPs 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 (<5 kDa) that use the amino acid lanthionine and are therefore called lantibiotics; class II are non-modified, heat-stable, small peptides (<10 kDa) that do not use lanthionine; class III are large, heat-labile peptides (>30 kDa); and class IV are complex or cyclic peptides containing lipids or carbohydrates [56]. Bacteriocins inhibit closely related bacterial species [57]. For example, lacticin 3147 and nisin are lantibiotics that exhibit antibiotic activity against Gram-positive bacteria, notably methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci [58]. Nisin is the most well-known lantibiotic, largely used as a food preservative because of its activity against *Listeria monocytogenes*, MRSA and *S. 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 (APD) (http://aps.unmc.edu/AP/), 2478 AMPs are already known [61]. For all medical fields combined, 60 peptides have obtained FDA approval and 140 are in clinical trials, mainly for oncological and metabolic diseases [62]. Yet focusing on infectious diseases, only 12 naturally occurring AMPs have reached the stage of human clinical trials (Table 2). Among them, only three (LTX-109, LL-37 and nisin) demonstrated better efficacy than placebo, but none obtained 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 results in inhibition of *S. aureus* and *M. tuberculosis* [63]. Notably, this molecule uses the rare amino acid L-allo-enduracididine, which is challenging to synthesise therefore limiting its use despite efforts made to develop analogues [64]. Despite their narrow or broad spectrum of activity against human pathogens and easier bioengineering compared with NRPS or PKS, bacteriocins are not used in humans as antibiotics owing to several limitations.

The main limitations in the use of AMPs as antibiotics in clinical practice are their instability (proteolytic digestion, oxidation), high cost and low yield of production, short half-life and rapid elimination [60]. Notably, their low bioavailability following oral ingestion related to proteolytic degradation is a great obstacle. To solve these problems, production of analogues using rational drug design or nanoengineering is used to improve the pharmacokinetic properties. For instance, nanoengineering has increased the spectrum of nisin to Gram-negative bacterial species [65] and has allowed HPA3P^{HIS} to be highly effective against *Vibrio vulnificus* in a mouse model [66]. Use of nanoparticles also prolonged the stay in the stomach of pexiganan following oral administration, which reduced the concentration of pexiganan required in a mouse model of *Helicobacter pylori* eradication [67]. Another approach is the combination of bacteriocins with other antimicrobials in order to reduce the resistance risk and to increase antimicrobial potency [60]. Another matter of concern with AMPs is the risk of development of resistance against our own immunity peptides. For instance, pexiganan has been previously found to induce cross-resistance to human neutrophil defensin 1 [68].

4. Methods for the discovery of antibiotics

4.1. Culture-based approaches

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

The cross-streak method involves inoculation of the bacterial test strain vertically on an agar plate. The incubation time of the plate depends on the life cycle of the bacterial strain required to reach exponential phase, which is the moment where secondary metabolites are excreted. The indicator strain is then inoculated in a horizontal streak and the plate is incubated again (Fig. 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 third methods are the spot-on-the-lawn method and the well diffusion method. The spot-on-the-lawn method consists of depositing a drop of the test strain on a lawn of indicator strain [71]. Following incubation, an inhibition zone is looked for around the sediment. The well diffusion method is based on diffusion of antimicrobials through agar, which inhibits susceptible species. An agar plate is pooled with the indicator strain or is inoculated with a lawn of the indicator strain and then agar holes are punched out aseptically. Two main variants exist. The first is the agar plug diffusion method, which consists of removing a cylinder of agar from a plate previously inoculated with the test strain. This cylinder of agar is then placed into the hole of the indicator plate [72]. The second variant method consists of placing a liquid broth of the test strain or a growth supernatant in the hole [73]. Following an optional rest time at 4°C, the agar plate is incubated and the inhibition growth zone is measured (Fig. 2). Several variants have been developed, e.g. using stress conditions or iron chelators [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 test species and the indicator strain separated by a filter allowing the diffusion of nutrients but not the diffusion of cells. Following incubation, growth of the indicator bacterial strain is determined by numeration, coloration or optical density measurement [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 test bacteria to be grown under conditions different from those of the indicator strain (Fig. 3).

4.2. Discovering antimicrobial effects of already known compounds

Some antibiotics were discovered years before they were used (e.g. fidaxomicin, daptomycin and linezolid). Several million chemical compounds are known in chemical databases and could provide a potential source of antibiotics [32]. Moy et al. have tested the activity against *Enterococcus faecalis* of more than 6000 chemical compounds and 1136 natural products in the *Caenorhabditis elegans* animal model, discovering 16 molecules increasing survival of the nematodes [77]. The bottleneck remains the selection and high-throughput testing of these molecules.

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

Rational drug design consists of empirical synthesis of new molecules that are designed according to several rules to be well absorbed, non-toxic and active against a specific target [32]. The most recognised rules used by the industry are Lipinski's rules. Despite more than 10 million new molecules synthesised, only a few active molecules have reached the market, notably antituberculous drugs [32,37] (Table 1). This can be explained by the fact that antibiotics generally have poor economics [78].

Improvement of already known molecules is another strategy that can yield benefits. Modification of cephalosporins led to the development of cefiderocol, which demonstrates safety and tolerability in healthy subjects; clinical trials for the treatment of urinary tract infections are ongoing [79]. Another example is modification of the aminoglycoside sisomicin that led to the development of plazomicin [80]. This antibiotic was recently approved by the FDA and a small, phase 2 clinical trial found it had an efficacy comparable with that of levofloxacin in the treatment of urinary tract infections and acute pyelonephritis [81]. Although the potential use

Table 2
Clinical trials involving antimicrobial peptides (AMPs).

AMP	Natural product of:	Spectrum	Identifier[a]	Year	Phase	Administration	Indication	Results	Reference[b]
Pexiganan acetate (=MSI 78, Suponex™)	*Xenopus laevis* (African clawed frog)	Large, antitoxin activity	NCT01594762	2017	III	Topical cream 0.8%	DFI	No significant difference compared with placebo	[50]
			NCT01590758	2016	III	Topical cream 0.8%	DFI	No significant difference compared with placebo	[51]
			NCT00563433, NCT00563394	2007	III	Topical cream 1–2%	DFI	No significant difference compared with oral ofloxacin 400 mg	[52]
Iseganan (=IB367)	Porcine neutrophils	Large	NCT00118781	2005	II/III	Oral solution 9 mg	VAP	No significant difference compared with placebo	[53]
			NCT00022373	2004	III	Oral solution 9 mg	Oral mucositis	No significant difference compared with placebo	[54]
				2003	III	Oral solution 9 mg	Oral mucositis	No significant difference compared with placebo	[55]
				2004	III	Oral solution 9 mg	Oral mucositis	No significant difference compared with placebo	[56]
Omiganan (=MBI 226)	Bovine neutrophils	Large	NCT03091426	2017	II	Topical cream 1%, 1.75%, 2.5%	Atopic dermatitis	Work in progress	[57]
			NCT03071679	2017	I	Topical cream 1%, 2.5%	Healthy volunteers	Work in progress	[58]
			NCT02849262	2016	II	Topical gel 2.5%	Genital warts	Unknown	[59]
			NCT02456480	2015	II	Topical cream 1%, 2.5%	Atopic dermatitis	Unknown	[60]
			NCT02576847	2015	III	Topical cream	Rosacea	Work in progress	[61]
			NCT02596074	2015	II	Topical cream 2.5%	Vulval intraepithelial neoplasia	Work in progress	[62]
			NCT00608959	2010	III	Topical cream 1%	Skin antisepsis in healthy adults	No significant difference compared with chlorhexidine	[63]
			NCT00231153	2009	III	Topical cream 1%	Prevention of infection/colonisation of CVC	Significantly better than povidone–iodine for microbiologically-confirmed catheter infection, but not in clinical local catheter site infection	[64]
			NCT02571998	2015	II	Topical cream	Inflammatory acne vulgaris	Work in progress	[65]
Lytixar™ (=LTX-109, AMC 109)		Large	NCT01223222	2011	II	Topical cream 1%, 2%, 5%	Skin infection (Gram positive)	Unknown	[66]
			NCT01158235	2015	I/II	Topical cream 1%, 2%, 5%	Nasal MRSA decolonisation	Decolonisation significantly better than placebo	[67]
			NCT01803035	2014	II	Topical cream 1%, 2%	Impetigo	Unknown	[68]
hLF1-11	Human	Large	NCT00509834	2007	I/II	Intravenous 0.5 mg daily bolus for 14 days	Candidaemia	Unknown	[69]
			NCT00509847	2007	I/II	Intravenous 0.5 mg daily bolus for 10 days	*Staphylococcus epidermidis* bacteraemia	Unknown	[70]
			NCT00509938	2007	I/II	Intravenous 5 mg single dose	HSCT, bacterial infections and mycoses	Unknown	[71]
			NCT00430469	2007	I/II	Intravenous 0.5 mg for 10 days	Autologous HSCT recipients	Unknown	[72]
PXL01	Human		NCT01022242	2009	II	Local 0.5 mL	Flexor tendon surgery	No significant difference compared with placebo	[73]
			NCT00860080	2009	I	Local (intra-abdominal injection) 10, 20 and 40 mg	Healthy volunteers	Unknown	[74]
PAC-113	Human saliva	Narrow (fungus)	NCT00659971	2008	II	Topical mouthrinse 0.15%, 0.075%, 0.0375%	Oral candidiasis among seropositive individuals	Unknown	[75]
Novexatin (=NP-213)	Human	Narrow (fungus)	NCT02343627	2010	II	Topical brush-on treatment	Onychomycosis	Unknown	[76]
LL-37 (=CAP-18)	Human (epithelial cells)	Large		2013	I	Topical 0.5, 1.6 or 3.2 mg/mL twice weekly	Venous leg ulcers	0.5 mg was significantly better than placebo	[77]

(*continued on next page*)

Table 2 (*continued*)

AMP	Natural product of: Spectrum		Identifier[a]	Year	Phase	Administration	Indication	Results	Reference[b]
Gramicidin			NCT00990392	2009	I	Topical (gramicidin, polymyxin, bacitracin)	Prevention of infection/colonisation of central catheter	Withdrawn	[78]
				1980		Topical cream (triamcinolone acetonide, neomycin sulphate, nystatin and gramicidin)	Infected dermatoses	Less effective than topical cream (triamcinolone acetonide, neomycin sulphate and undecenoic acid)	[79]
				1982		Topical eye drops (neomycin–polymyxin–gramicidin)	Bacterial conjunctivitis	No differences versus trimethoprim–polymyxin	[80]
				1985		Topical ear drops (framycetin/gramicidin)	Acute external otitis	No differences versus oxytetracycline/hydrocortisone/polymyxin B	[81]
				1990		Topical ear spray (framycetin/gramicidin/dexamethasone)	Otitis externa	Significantly less effective than neomycin/dexamethasone	[82]
				2005		Topical eye drops (neomycin sulfate, polymyxin B sulfate and gramicidin)	Hordeolum	No significant difference compared with placebo	[83]
Gramicidin + polymyxin B (Polysporin®)			NCT00400595	2015	IV	Topical ointment	Prevention of catheter-related infections in patients treated with peritoneal dialysis	Not superior to mupirocin	[84]
				2009		Intranasal	Eradication of MRSA colonisation	Significantly less effective than mupirocin	[85]
NVB-302			NVB302/001[c]	2011	I	Unknown	*Clostridium difficile* infection	Unknown	[86]
Nisin	*Lactococcus lactis*	Gram-positive bacteria		2008		Topical cream (6 μg/mL)	Staphylococcal mastitis	Significantly better than placebo	[87]

DFI, diabetic foot infection; VAP, ventilator-associated pneumonia; CVC, central venous catheter; MRSA, methicillin-resistant *Staphylococcus aureus*; HSCT, haematopoietic stem cell transplantation.

[a] ClinicalTrials.gov ID (https://clinicaltrials.gov/ct2/home).

[b] All of the references are given in the Supplementary references.

[c] ICRCTN registry (http://www.isrctn.com).

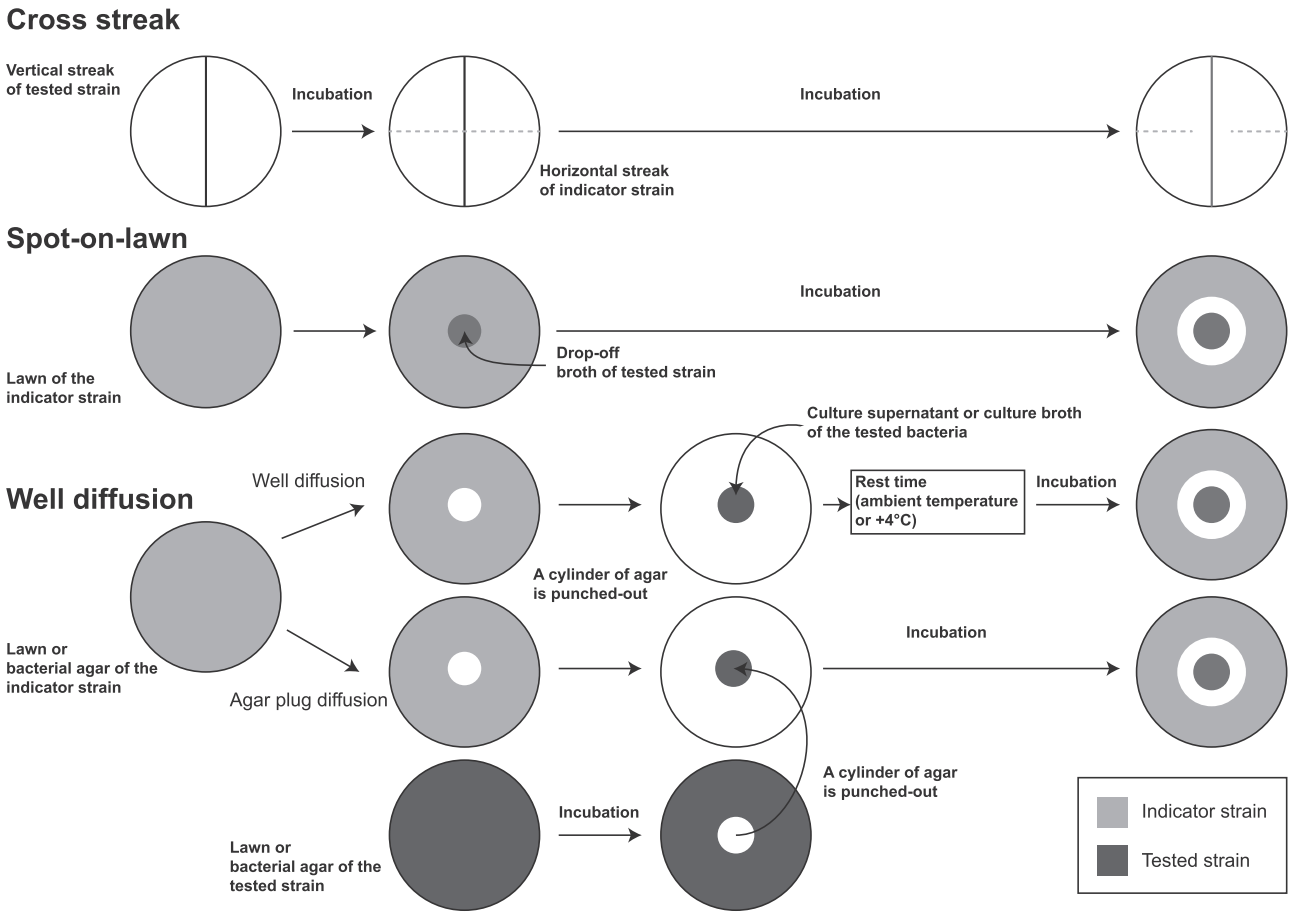

Fig. 2. Solid culture approaches highlight inhibition between two bacterial species.

of these antibiotics remains to determined, their mechanism of action is not novel and the appearance of resistance is expected in the same way as for their related antibiotic parent compound.

4.4. Genome mining

Secondary metabolites are encoded by biosynthetic gene clusters (BGCs). Thousands of prokaryotic genomes are available in sequence databases. These data have generated thousands of BGCs that potentially encode unknown molecules [82,83]. Despite the fact that many of them do not have any antimicrobial activity, little is known about them. Walsh et al. found 74 putative BGCs from 59 genomes from the Human Microbiome Project [84]. These BGCs belonged mainly to the Firmicutes, Proteobacteria and Bacteroidetes, and the most commonly putative bacteriocins encoded belonged to classes III and IV.

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

Recently, Hover et al. screened more than 2000 soil samples from various areas of the USA, searching for BGCs encoding the calcium-binding motif Asp-X-Asp-Gly [90]. This motif is related to calcium-dependent antibiotics such as lipopeptides for which the mechanism of action is not fully understood [91]. They found several clades of uncharacterised BGCs, 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 characterised in vitro. Malacidin A exhibited broad antibiotic activity against Gram-positive bacteria, notably MRSA, which was successfully confirmed in a rat model of skin wound infection. 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 protecting them from foreign genetic material that could be inserted by phages.

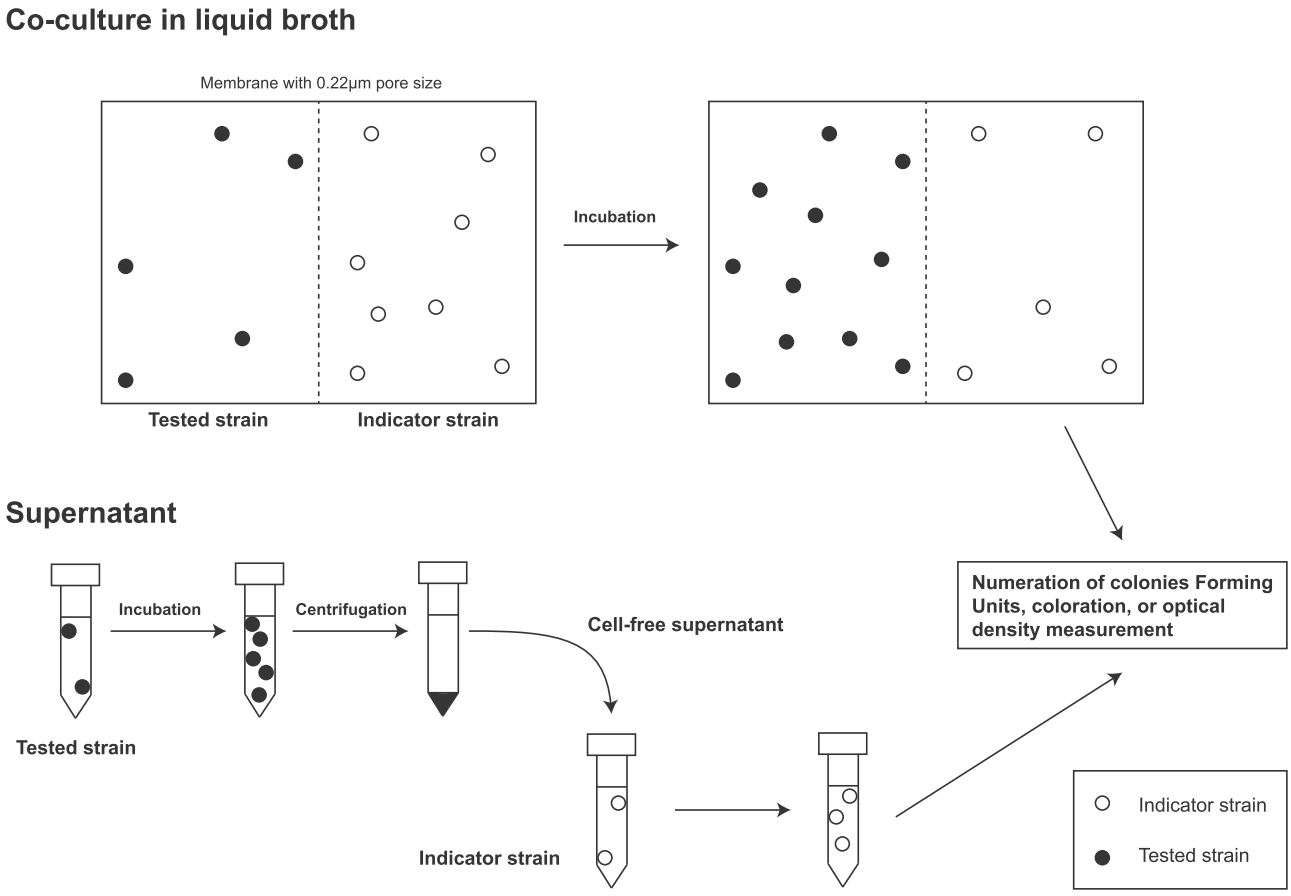

Fig. 3. Liquid culture approaches highlight inhibition between two bacterial species.

This immune system consists of restriction enzymes, toxin–antitoxin systems and the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas system [92]. Utilisation 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 genes bla_{SHV-18} and bla_{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 chromosomally or plasmidic [93]. The same approach has been successfully tested using a 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, showing efficacy comparable with that of mupirocin [94]. Other authors used genome editing technology to re-sensitise MDR cells. As an example, Kim et al. used CRISPR-Cas9 to target a conserved sequence of extended-spectrum β-lactamases (ESBLs), thus restoring the susceptibility of *E. coli* in their in vitro model [95].

5. Conclusions and perspective

More than three-quarters of all antibiotics currently used in human health are natural products or are derived from them. The discovery of antibiotics declined after the 1970s owing 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 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 from soil using a diffusion chamber [63].

The same approach is now possible for the human-associated microbiota. The nose is an example of an 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 conditions, Krismer et al. discovered lugdunin, a new antibiotic inhibiting the growth of *S. aureus* [75]. The gut microbiota is another microbiota of interest for antibiotic research. Indeed, the human gut has an average load concentration ranging from 10^4–hhh10^{12} CFU/mL from the duodenum to the colon. These species live in extreme competition, as they did before human colonisation 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 3118 BGCs including NRPS, RiPPs and PK 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 of BGCs in the human microbiota. They also found and purified lactocillin, a new thiopeptide antibiotic, isolated from the vaginal microbiota [96].

Study of the gut microbiota using culture methods was recently improved by culturomics, a novel approach consisting of multiple 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 1525 species, of which 247 were totally new [98]. Indeed, the new species described from the gut represent an opportunity for the search for novel antibiotics. The search for new antibiotics naturally synthesised by organisms living in complex ecosystems such as the gut microbiota, using the culture approach, appears to be the modern continuity of what has already worked in the past. Thus, if the study of antagonisms between environmental bacteria led to the discovery of a substantial proportion of antibiotic classes, such studies were rarely performed from human-derived microbiota.

In conclusion, the search for new antibiotic molecules is a key point among the strategies in the fight against antibiotic resistance. Recent advances both from 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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Competing interests

None declared.

Ethical approval

Not required.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2018.11.010.

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