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Review

The antibiotic crisis: How bacterial predators can help

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SUMMARY

Discovery of antimicrobials in the past century represented one of the most important advances in public health. Unfortunately, the massive use of these compounds in medicine and other human activities has promoted the selection of pathogens that are resistant to one or several antibiotics. The current antibiotic crisis is creating an urgent need for research into new biological weapons with the ability to kill these superbugs. Although a proper solution requires this problem to be addressed in a variety of ways, the use of bacterial predators is emerging as an excellent strategy, especially when used as whole cell therapeutic agents, as a source of new antimicrobial agents by awakening silent metabolic pathways in axenic cultures, or as biocontrol agents. Moreover, studies on their prey are uncovering mechanisms of resistance that can be shared by pathogens, representing new targets for novel antimicrobial agents. In this review we discuss potential of the studies on predator-prey interaction to provide alternative solutions to the problem of antibiotic resistance.

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Contents

1. Introduction	2547
2. Predatory bacteria: what are they? How do they kill?	2548
3. BALOs as biocontrol agents	2549
4. Myxobacteria as sources of new antibiotics, bioactive products and lytic enzymes	2551
5. Summary and outlook	2553
Declaration of Competing Interest	2554
CRediT authorship contribution statement	2554
Acknowledgments	2554
Appendix A. Supplementary data	2554
References	2554

Abbreviations: AR, antibiotic resistance; ARB, antibiotic-resistant bacteria; ARG, antibiotic-resistant gene; MDRB, multi-drug resistant bacteria; WHO, World Health Organization; HGT, horizontal gene transfer; SM, secondary metabolite; BALOs, *Bdellovibrio* and like organisms; OSMAC, one strain many compounds; BGC, biosynthetic gene cluster; NRPS, nonribosomal peptide synthetase; PKS, polyketide synthase; OMV, outer membrane vesicle.

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1. Introduction

Antibiotics revolutionized medicine after the serendipitous discovery of penicillin by Fleming in the 1920s [1], the subsequent development of synthetic antimicrobials [2], the establishment of useful platforms for screening and isolation of antibiotic-

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producing microorganisms [3], and the development of the pharmaceutical industry. These antibacterial drugs have saved millions of lives, not only by combating fatal infectious diseases, but also by enabling physicians to make advances in surgery, in organ transplantation, in cancer chemotherapy, and in the use of artificial devices [4,5]. For these reasons, the discovery and use of antibiotics in public health is one of the main pillars of modern medicine, considered one of the great achievements of the 20th century.

Antibiotics are natural products produced by microorganisms or their semisynthetic derivatives. These compounds have been a feature of the environment for a long time, so that bacteria have needed to evolve some forms of antibiotic resistance (AR) to survive. For instance, bacteria inactivate antibiotics by producing enzymes that modify them, but they can also alter their target of action, or prevent the antibiotics from accumulating, either because they are expelled using efflux pumps or by altering the permeability of the membrane [6,7]. The antibiotic-resistant genes (ARGs) are favored in nature due to the selective pressure of natural production of antibiotics by bacteria. However, the use, overuse, or misapplication of these drugs, not only for therapeutic purposes, but also in animal feed and agriculture, have added an additional selective pressure and accelerated the ability of bacteria to evolve and, consequently, led to the inevitable emergence and spread of AR [8–11]. Furthermore, bacteria can collect multiple resistance mechanisms, and this fact has promoted the appearance of the so-called superbugs or MDRB (multi-drug resistant bacteria), which are strains that have acquired resistance to a wide variety of antibiotics. The World Health Organization (WHO) has issued the priority pathogens list with thirteen human pathogens that have developed high levels of resistance across the world [12,13]. Members of the so-called ESKAPE pathogens constitute an especially urgent threat to human health [14].

The first step in AR transmission is the transfer of ARGs of environmental bacteria through mobile genetic elements by horizontal gene transfer (HGT) to human or animal commensal bacteria or pathogens, generating resistant clones carrying ARGs that can then be spread in the environment [15,16]. The interconnection between humans, animals and their ecosystems leads to the transmission of resistance. Although AR in hospitals or health centers represents a major concern, the dissemination is not restricted to clinical settings. Antibiotics eliminated by humans or animals are discharged into urban, agricultural or farm waste water, and can be incorporated by food crops through irrigation with those contaminated waters, increasing the selective pressure [17]. Moreover, waste water treatment plants, which receive a great variety of bacteria, including antibiotic-resistant bacteria (ARB) from various sources, act as reservoirs and offer convenient conditions for the interaction and exchange of ARGs by HGT, contributing to a broader dissemination [18]. Other types of industrial contaminants, such as metals, can contribute to AR, since many resistance mechanisms that protect against them, such as multidrug efflux pumps, also confer resistance to antibiotics [19–21]. Finally, physical forces contribute to their dissemination. The hyper-connection of the current world promotes the worldwide spread of AR. This has serious consequences, not only in the treatment of clinical and/or veterinary infections, but also in maintaining the balance of microbial communities across the biosphere.

Currently there are 700,000 deaths per year due to ARB. It is estimated that by 2025 many of the current antibiotics will be ineffective [22], and that by 2050 superbugs may cause 10 million deaths annually worldwide [23]. The problems derived from AR in healthcare could cost the world a trillion USD per year in healthcare, which would lead to a reduction of 2% to 3.5% in gross domestic product [23,24].

Many public health agencies and economic and political institutions across the world recognize that AR is one of the great chal-

lenges for the 21st century. Many experts consider that we are initiating the post-antibiotic era, in which we will see the return of epidemics prevalent during the pre-antibiotic era [5]. But the antibiotic crisis that we have experienced in the last decade is caused not only by the appearance of MDRB, but also by the scarcity of resources dedicated to the search for new products, and, in this scenario, the pipeline for the discovery of new antibiotics is running dangerously low [4,5,12,24–27]. Since the development of resistance is inevitable, even if we make good and sparing use of antibiotics, the search for new antibiotics and alternative therapies is critical. The WHO, in a recent report, proposes several guidelines to effectively deal with AR to avoid drifting back to a pre-antibiotic era. These leading strategies, along with procedures to reduce antibiotic pressure and transmission of AR and ARGs, are: i) developing new ideas that question pre-existing forms of knowledge; ii) protecting research from purely commercial determinants; iii) creating new interpretations and strategies to address resistance; and iv) reorientating research to better understand the role of bacteria and human-microbe relationships [22]. In this context, laboratories around the world are developing new therapeutic approaches for reducing the AR burden, which include microbiota transplant procedures to displace ARB, genetic engineering to interrupt resistance genes (such as editing based on CRISPR/Cas), metagenomic engineering, phage cocktails against ARB, vaccines against pathogens, antimicrobial peptides, the use of metals (Cu, Ag, Ga), development of molecules that interfere with the ability of bacteria to communicate and group together in antibiotic-resistant biofilms, the search for new antimicrobial products, and the use of bacteria as therapeutic agents [16,29].

Predatory bacteria play an essential role in these new lines of research [16,28–31]. The fact that these micropredators are designed to lyse and kill other bacteria, including MDRB and those pathogens that form biofilms, has led to them being proposed as a reasonable antibiotic alternative to be explored. The aim of this review is to collect and discuss the advances in the use of predatory bacteria in this fight against AR, either as biocontrol agents (“living antibiotics”) or as biological resources for innovative antimicrobial products (“microfactories”).

2. Predatory bacteria: what are they? How do they kill?

Predatory bacteria use other bacteria or yeasts as a food source; hence, they are able to actively hunt and kill their neighbors to later consume their macromolecules as nutrients. Various studies have shown that these predators are widely distributed in many natural and artificial environments (such as artificial microcosms, experiments in laboratory, waste waters treatment plants, or aquaculture plants) where they play an important role not only in maintaining microbial diversity, but also in shaping ecosystems [32–39].

In the context of fighting AR, predatory bacteria in nature contribute to limiting the spread of ARGs in different environments, because they can prey upon ARB and degrade their DNA [40,41], reducing the ARG pools and their spread to other bacteria by HGT via conjugation or transformation [42]. Moreover, they can also limit the possibility of the transfer of ARGs via transduction by preying on bacteria targeted by phage-resistant bacteria [43].

Bacterial hunting strategies can be categorized in two major groups: epibiotic and endobiotic predation [44]. In the epibiotic strategy, predators attack and consume the prey from the outside. Two different subgroups can be differentiated within this group: epibiotic “solitary hunters”, which, strongly attached to the prey cell envelope, suck their contents out before dividing into two daughter cells; and the epibiotic “cooperative hunters”, where individual cells cooperate within the community by sharing a com-

bination of diffusible hydrolytic enzymes and secondary metabolites (SMs), including antibiotics, that kill and decompose the prey in a non-species-specific manner before consuming the macromolecules [44]. Whether hunting is truly cooperative or merely communal is not clear [45]. In the epibiotic lone-hunter subgroup the most studied genera are *Micavibrio*, *Vampirococcus* and *Vampirovibrio*, and the species *Bdellovibrio exovorus*, [32]. The cooperative (or communal) hunting strategy subgroup includes bacteria whose mode of attack is based on lysis at a distance that does not require contact with the prey, such as the genus *Streptomyces* [46,47], and those that require close proximity to the prey cells such as myxobacteria [44,48–52].

The endobiotic strategy is used by lone predatory bacteria that actively move to encounter their prey, stick to their outside, perforate and modify the prey cell wall by secreting hydrolytic enzymes, and penetrate the periplasmic or the cytoplasmic space, devouring it from within. Once the cycle is finished, predators move on to attack neighboring cells. A representative species of this group is *Bdellovibrio bacteriovorus* [32,53–55].

Biochemical and microbiological studies carried out in several laboratories and analyses of the genomes and transcriptomes during predation (predatosomes) of obligate epibiotic predators such as *Micavibrio aeruginosavorus*, *Vampirovibrio chlorellavorus*, and *B. exovorus* [56–58], and endobiotic *B. bacteriovorus* and other BALOs (*Bdellovibrio* and like organisms), have revealed that they are able to synthesize and secrete an unusual number of hydrolytic proteins, such as lipases, glycanases, peptidases and proteases, which are probably involved in damaging and digesting prey cell structures [53,58–61]. They are, however, non-competent antibiotic producers [62], since their killing strategies are based on the active search and recognition of the prey, the specific binding to the external structures, and its degradation. This particular way of life has led researchers to mainly focus on its possible application in alternative live therapies in which the predators' whole cells can be used to kill or control the growth of other ARB; that is, the use of predators in bacterial therapy as biocontrol agents [53,61].

However, the cooperative facultative epibiotic predators, which include genera of the two most important groups of bacterial SM and hydrolytic enzyme producers, Actinobacteria and myxobacteria, are by far the most promising resources for innovative bioactive natural products. Within Actinobacteria, the genus *Streptomyces* has been thoroughly investigated for many years for the isolation of antibiotics [63–65], but it has only recently been described as being a bacterial predator that kills other soil bacteria by secreting hydrolytic enzymes and SMs far away from the prey [46,47]. Myxobacteria include broad prey range predators such as *Myxococcus xanthus*, *Myxococcus flavescens*, *Myxococcus virescens*, *Myxococcus macrosporus*, *Corallococcus coralloides*, *Stigmatella aurantiaca*, *Chondromyces apiculatus* and *Chondromyces crocatus* [44]. Compared with *Bdellovibrio* and BALOs, which exhibit a restricted prey spectrum, myxobacteria are able to kill a broad range of bacteria, including clinically relevant species [66–78]. This is probably due to their capacity to secrete a plethora of lytic metabolites which, acting either in isolation or synergistically, are able to attack a wide variety of prey. They are able to produce cell wall-degrading enzymes, lipases, nucleases, polysaccharidases, proteases and, unlike many other antibiotic producers, various classes of antibiotics, some of which are hardly ever found as microbial SMs [79–82]. Although they have been underestimated in the past, in the last decade they have emerged as excellent “microfactories”, with a high capacity to produce an unlimited number of previously unknown promising antimicrobials [82].

The use of epibiotic predators in alternative therapies in medicine or as biocontrol in different fields should be done in such a way that they contribute to the fight against MDRB, without

increasing the accumulation of antibiotics in the environment that may generate AR.

3. BALOS as biocontrol agents

Endobiotic predators, such as *B. bacteriovorus*, other BALOs, and some lone hunters that follow an epibiotic strategy, such as *M. aeruginosavorus*, are generating interest for the treatment of intractable, antibiotic-resistant infections. Multiple studies, using human cells or serum, or animal models such as chicks, zebrafish, rabbits, guinea pigs, mice, and rats, and many in vitro studies have shown that predator whole cells or their enzymes can be used as therapeutic agents and as alternative or complementary applications in biological control [83–89] (Fig. 1 and Supplementary Table 1).

The potential of these predatory bacteria as a preferred or supplementary treatment of multidrug-resistant bacterial infections is supported by a range of evidence.

First, predators such as *Bdellovibrio* and *Micavibrio* have the ability to kill human Gram-negative pathogens such as *Acinetobacter*, *Aeromonas*, *Bordetella*, *Burkholderia*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Listonella*, *Morganella*, *Proteus*, *Salmonella*, *Serratia*, *Shigella*, *Vibrio*, *Yersinia*, *Helicobacter pylori*, and *Legionella* that have acquired, or are at risk of acquiring, resistance to antibiotics [86,90–95]. These predators also reduce the bacterial burden of drug-resistant members of the ESKAPE group such as *K. pneumoniae*, *A. baumannii* [86,96–98] or *P. aeruginosa* [99], *Stenotrophomonas maltophilia* [100] and other MDRB Gram-negative clinical pathogens such as *Shigella* [101]. The ability to kill all these Gram-negative bacteria makes BALOs to postulate as an alternative to fight against most of the MDRB identified as a critical priority by WHO. However, there are also several Gram-positive bacteria in this list, what makes interesting to explore the capacity of BALOs to kill them. In fact, it has recently been reported that *B. bacteriovorus* seems to attack *S. aureus* cystic fibrosis isolates by using an unusual epibiotic strategy [99].

These predators may also alter the commensal microbiota, and it is known that they increase the number of Gram-positive species in the gut microbiome of chickens and rats [94,102]. The consequences of this imbalance on animal health remain to be investigated. Iebba et al. [103] reported that *Bdellovibrio* is present and abundant in the human gut microbiota populations of healthy individuals and suggested that the use of predatory bacteria as probiotics could restore the balance in the intestinal ecosystem to control dysbiotic events. However, studies by Shatzkes et al. [102] on the effect on rat gut microbiota showed mixed effects, since they found that intrarectal inoculation of *Bdellovibrio* contributes to health, while *M. aeruginosavorus* has potential adverse consequences. Therefore, although further studies are required to decipher the consequences of changes in the microbiota due to the administration of predatory bacteria, it is worth bearing in mind that antibiotics also may have adverse effects on commensal bacteria.

Second, these predators are able to inhibit the formation of, or reduce, preformed Gram-negative and even Gram-positive biofilms through a plethora of secreted enzymes, particularly proteases and nucleases [86,92,104–108]. Biofilms can be found in many natural environments and on industrial equipment, waste water treatment plants, hospital surfaces, health care settings and instruments. Bacteria are able to persist as reservoirs within these multicellular structures, and biofilms inside the host allow pathogens to overcome innate immune defenses. This capacity to disrupt biofilms of relevant pathogens and to remove environmental and industrial biofilms opens the way to using these predators

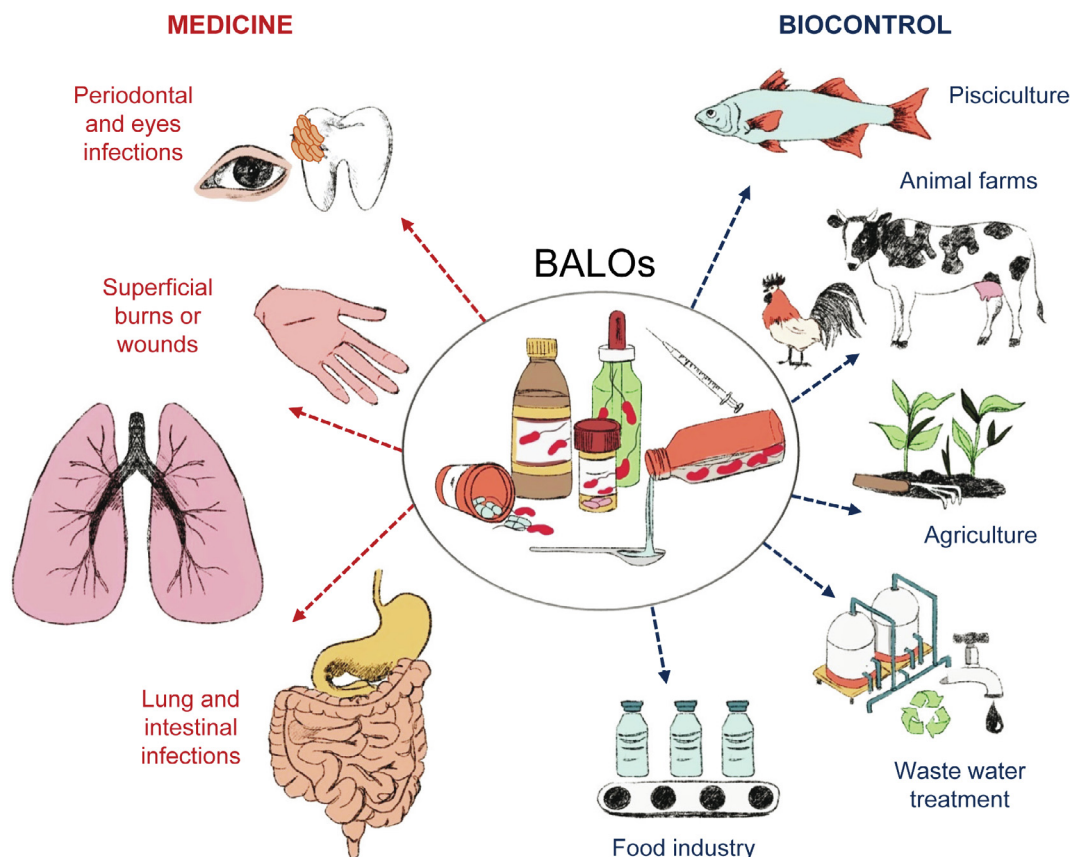


Fig. 1. Applications of *Bdellovibrio* and BALOs in medicine and as biocontrol agents.

or their enzymes not only in medicine, but also in many biocontrol processes that will be discussed below [100,109,110].

Third, topical application, ingestion, injection, or intranasal inoculation of whole cells have no apparent cytotoxicity, neither in terms of pathological effects, nor in terms of diminution in cell viability in *in vitro* cell culture models [111–113] or *in vivo* animal models [94,96,101,102,113–116].

Fourth, they do not incite a systemic or sustained immune response, most likely due to the special structure of their lipopolysaccharide, which lacks the typical negatively charged phosphate groups, resulting in only low binding affinity to the lipopolysaccharide receptors in human immune cell pathogens [101,117]. They are passively engulfed by macrophages and are able to persist inside these cells over 24–48 h as non-replicative forms, although they retain their predatory competence. Although they do not affect host cell viability, they stimulate moderate cytokine responses [111–113,115]. Predator persistence inside macrophages for sufficient time to prey on pathogens opens the way to using predatory bacteria to eliminate intracellular pathogens, such as *Salmonella*, *Klebsiella* or *Francisella* species. Moreover, the relatively benign occupancy of macrophages by *Bdellovibrio* could prevent other intracellular pathogens from entering. However, this passive uptake suggests that macrophages might present predatory bacteria to antibody-forming cells and, consequently, humans could develop immune reactions against them after repeated exposures [30]. In fact, Raghunathan *et al.* [118] detected, although at low levels, IgG and IgM antibodies against two *B. bacteriovorus* strains in more than 90% of human serum samples from a biobank, suggesting that the predatory treatment could be used only once. On the other hand, experiments in immune-compromised zebrafish inoculated with *B. bacteriovorus* HD100 demonstrated that

cooperation between the host immune system and bacterial predation is important, and it must be taken into consideration in order to maximize antibacterial therapy benefits [101]. Although a lot of progress has been made in recent years, more research is needed to understand not only the interactions and fate of these predatory bacteria within human immune cells, but also to elucidate the processes involved in their uptake by phagocytic cells, how long bacteria survive in the host cells, and the mechanisms induced by the predatory bacteria.

Fifth, *Bdellovibrio* is an aerobic predator, conditioning it to be an effective treatment in aerobic environments such as superficial burns or wounds, eyes and lungs [85,96,119,120]. Nevertheless, this predator is able to tolerate microaerophilic conditions, which widens its areas of use to treating gastrointestinal and periodontal infections [83,85,121–124].

Sixth, one of the main advantages of using these predators as therapeutic agents is the failure of prey bacteria to develop induced resistance against predation. The main reason for this is that these predators are not antibiotic producers, and their killing mechanisms do not target specific receptor proteins that can evolve resistance. However, predation can weaken due to different adaptations arising in the prey bacteria. For instance, transient resistance has been observed in some prey similar to bacterial persistence [83,125]. As a consequence, *Bdellovibrio* predation never completely kills off the attacked population. To resolve this difficulty, the combined use of *B. bacteriovorus* and antibiotics in a co-therapy has been proposed [85]. This combined therapy has the additional advantage of expanding the individual killing spectra of both the antibiotics and the predator. Im *et al.* [126] have confirmed that a combination of *Bdellovibrio* and violacein is effective against Gram-positive bacteria, multidrug-resistant patho-

gens, and Gram-positive and negative mixed species populations. However, for an efficient development of these co-therapies it is necessary to determine the correct predator-antibiotic combination to increase the antimicrobial efficiency, while avoiding development of drug resistance. With this in mind, researchers are making efforts to determine the sensitivity of *Bdellovibrio* against antibiotics [127], and more research must be carried out to determine its ability to grow on antibiotic-inactivated cells and the AR mechanisms developed by the predator. Recently, experiments using *Bdellovibrio* and prey-specific bacteriophages eradicated *E. coli* when preyed upon by both predators, demonstrating the potential of this innovative co-therapy as a future alternative antibacterial treatment that would reduce the selection for single predator-specific resistance [128].

And seventh, there is a lack of uptake of prey genetic material during predation, and it has been shown that, even after regular repeated high exposure to the prey genome, there is no incorporation of any major pathogenicity island(s) or of any potentially virulent genes from the prey [129]. The exchange of genetic material would have an additional negative impact because prey could also incorporate ARGs, which could contribute to the spread of resistance. The absence of DNA interchange further strengthens the possibility of using these bacteria in the future as a safe alternative in the fight against AR.

Besides clinical uses (Fig. 1, Supplementary Table 1), BALOs are also being explored as a feasible alternative to antibiotics as biocontrol agents in horticulture, aquaculture, livestock farming and food processing. In addition, they are being assayed for their application in other biotechnological processes such as waste water treatment, extraction of bioplastics, reduction of horizontal transfer of ARGs, and other environmentally-friendly applications in which the reduction of unwanted bacteria would be positive [43,85,130–132] (Fig. 1, Supplementary Table 1).

The use of epibiotic predators as biocontrol agents remains largely unexplored. However, the ability of myxobacteria (besides their predatory behaviors) to glide on solid surfaces, produce a wide variety of antibiotics, and form myxospores, make these bacteria excellent, commercially viable candidates for the biocontrol of phytopathogenic bacteria and fungi [69,73,133–135] (Supplementary Table 1).

4. Myxobacteria as sources of new antibiotics, bioactive products and lytic enzymes

Although *Bdellovibrio* and BALOs are mainly being explored within the concept of whole cell therapy, other investigations are being carried out that aim to make use of the large amount of bacterially destructive lytic enzymes that predators employ to kill their prey [59,61,130,131,136,137]. Those applications include some interesting uses such as tools for antibody analysis or for controlling the spread of ARGs in mixed microbial communities through the degradation of cell-free DNA or inactivation of phage particles, thus limiting two of the genetic DNA transfer processes that dictate the outcome of spread: transformation and transduction [43].

For the discovery of new antimicrobial agents, cooperative predators are the most promising bacteria. In the past, the producers of antibiotics and other bioactive products were mainly isolated from soil and tested by traditional diffusion methods using culture filtrates or extracts [4,138]. The advent of the post-genomic era, with the increase in genome sequencing and annotation, next-generation sequencing technologies, modern proteomic, transcriptomic and metabolomic tools, and scientific and technological advances in genetics, biology, robotics, chemistry, metabolic engineering, and bioinformatics, has amplified the range of

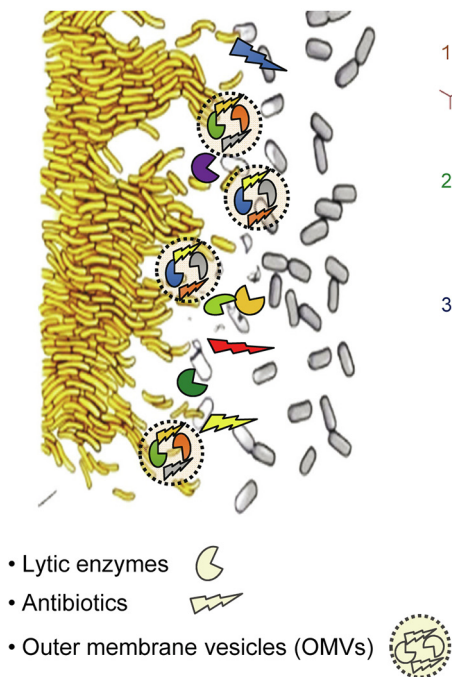
methodologies used for the isolation and identification of producers and has opened new doors to the identification of their “antibioticomes”, defined as the specific subset of microbial natural products with antibiotic activity [65,139–141].

Although isolation of new bacterial producers is one of the priority lines in antibiotic discovery, another approach is the use of new technologies to exploit the knowledge accumulated over the years about well recognized “microfactories”. The term “OSMAC” (one strain many compounds) was introduced at the beginning of this century [142], and it refers to the fact that a single bacterial strain is capable of producing a diverse collection of structurally different SMs. Bacteria never produce the full collection of compounds encoded by their genomes at the same time under a specific environmental condition, since this would be energetically costly. To avoid unnecessary costs, they modify their transcriptomes, proteomes, and metabolomes to survive under changing conditions. For that reason, biosynthesis pathways are firmly controlled by complex regulatory networks that respond to internal and/or external signals, many of which are still unknown. The OSMAC approach focuses on the systematic alteration of culturing parameters, such as medium components (salts, amino acids, carbon source), temperature, pH, culture aeration, type of culture vessel, etc., to imitate in the laboratory those natural environments that optimize the production of useful compounds [143]. The detailed study of every single step of the complex metabolic pathways for the biosynthesis of SMs, their regulative crosstalk, and the signals that activate the regulatory bacterial sensors is facilitating the discovery of novel products from one bacterium or fungus [144]. Myxobacteria are perfect candidates to be considered from the OSMAC point of view [82,142]. The Helmholtz-Center for Infection Research in Germany has a collection of myxobacteria with more than 9,000 strains, and more than 1,000 biosynthetic gene clusters (BGCs) associated with myxobacteria are deposited in the antisMASH database (antismash.secondarymetabolites.org).

M. xanthus DK1622, besides being a predator, is also a model organism for studying prokaryotic development. Over many years, numerous groups have accumulated a deep knowledge about the production of SMs and lytic enzymes, multicellular behavior, intra- and extracellular signaling, movement mechanisms, adaptations to changing environments, etc. [145]. This predator holds a large genome with an unprecedentedly high number of regulatory mechanisms [146–148] and an outstanding biosynthetic capacity for degradative enzymes and SM production, including 18 nonribosomal peptide synthetases (NRPS), 22 polyketide synthases (PKS), and six mixed PKS/NRPS, which concurs well with its predatory lifestyle [146,149]. Analyses using the antisMASH server calculate that those clusters correspond to a total of 14.5% of its 9.1 Mb genome [150]. Several of these natural products have been isolated [80,82,151–158] (Fig. 2) and even expressed in heterologous hosts [159]. However, many BGCs are not yet assigned to their corresponding hypothetical SMs, probably because some of these genes remain silenced (cryptic genes) or are expressed at low levels, yielding low quantities of the respective compounds in the growth conditions assayed [82,149,160]. The same situation is observed in *Streptomyces*, a genus notorious for its ability to produce a large quantity and variety of antibiotics and SMs [65,161]. *Streptomyces* genomes are also large and typically contain more than 20 BGCs dedicated to specialized metabolism such as NRPSs and PKSs. In the case of *Streptomyces coelicolor* A3, these enzymes are found in up to 27 clusters, which represent 10.6% of its 8.7 Mb genome [162,163]. Only a small fraction of them are transcriptionally active under laboratory conditions and most of their products have never been characterized [161,164].

This huge untapped biosynthetic potential makes the unlocking of the cryptic pathways of those cooperative predators another good strategy for antibiotic discovery. It is reasonable to think that

A Predator’s attack mechanisms



B Myxobacterial antibiotics production

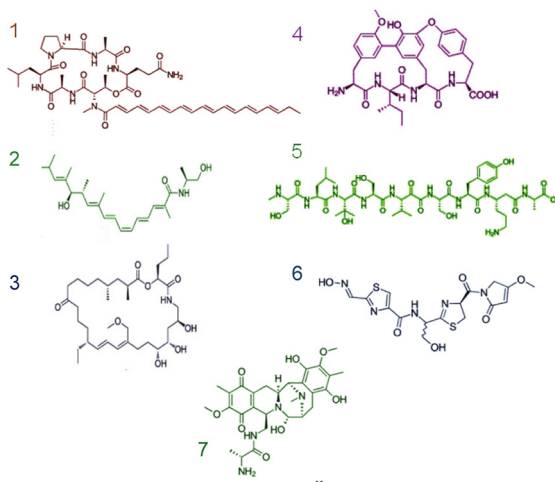
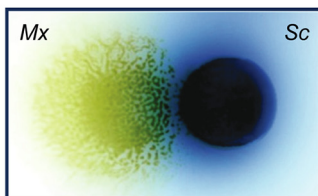


Fig. 2. The cooperative predator *M. xanthus* as a source of antibiotics and new products. A. During predation, *M. xanthus* secretes lytic enzymes, antibiotics and other secondary metabolites, some of them included in OMVs. B. Antibiotics and bioactive products identified in *M. xanthus* species. 1: myxochromide; 2: myxalamid; 3: myxovirescin TA; 4: cittilin A; 5: myxoprincomide; 6: althiomycin; 7: saframycin Mx1.

A Induction of new antibiotics

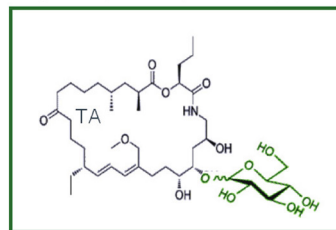
- Overproduction of actinorhodin



- Awakening of genes for new antibiotics (*Streptomyces*)
 - Bacillaene (*B. subtilis*)

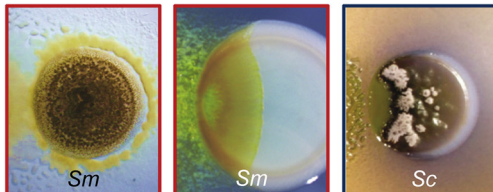
B Novel antibiotic resistance mechanisms

- TA glucosylation (*B. thuringensis*)



C Other defense mechanisms

- Galactoglucan - Melanin - Development



- Biofilm formation (*E. coli*)
 - Spore formation (*S. coelicolor*)
 - Megastructures (*B. subtilis*)

Fig. 3. Defense mechanisms in the prey with biotechnological applications. A. Silenced antibiotics are induced in the prey during the predatory process. In the pictures, *M. xanthus* (Mx, predator) induces in *Streptomyces coelicolor* (Sc, prey) the blue antibiotic actinorhodin, in solid and liquid media. B. Novel antibiotic resistance mechanism have been discovered in *B. thuringensis*: myxovirescin TA glucosylation. C. Other physical/chemical defenses mechanisms induced by *M. xanthus* predation in different bacteria. Galactoglucan (left picture) and melanin (middle picture) protect *Sinorhizobium meliloti* from predation. *M. xanthus* induces development in *Streptomyces* (right picture). Pictures from panel A and right picture from panel C are reproduced from Pérez et al. (2011) Microb Biotechnol 4: 175–183. Left picture in panel C is adapted from Muñoz-Dorado et al., (2016) Front Microbiol 7: 781. Middle picture in panel C is from Contreras-Moreno et al. (2020) Front Microbiol 11: 94. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

one of the ecological roles of antibiotics in nature is to be used as weapons against susceptible bacteria during predatory processes and, in fact, some of them have been shown to be involved in predation [46,165]. It has been recently found that although the genes responsible for SM biosynthesis in *M. xanthus* are expressed during growth, their expression increases during development [148]. These expression profiles suggest that SMs are used to protect developing cells from other microbes in the soil, to defend spores inside the fruiting bodies or to release nutrients from prey to promote germination [148]. It is also realistic to infer that production of such metabolites by predators might be regulated by contact with and/or proximity to the prey. Therefore, it would be useful to design efficient screening methods in the presence of a variety of pathogens for the induction of novel antibiotics against them.

But interspecies cross talk can also trigger the production by prey of specific bioactive compounds for defensive purposes and other resistance mechanisms to counterattack such as biofilm formation [166], spore differentiation, or induction of new ARGs [167] (Fig. 3). Co-cultivation experiments, followed by deep analysis of the influence on the secondary metabolome, are gaining increasing attention and are already yielding exciting results. Some studies have demonstrated that the presence in cultures of other bacteria stimulates *Streptomyces* to produce some SMs [144,164]. Most of these pairwise interactions have been assayed with other related Actinobacteria or *Bacillus*. Moreover, *M. xanthus* is able to induce the overproduction of the blue antibiotic actinorhodin while preying on *S. coelicolor* (Fig. 3A) [168]. Other results involving the production of SMs during predatory relationships are the induction of bacillaene by *B. subtilis* [76,169], or the biosynthesis of melanin by *S. meliloti* to protect against predation by *M. xanthus* (Fig. 3) [77,170].

The first transcriptomic study during a predatory process of *M. xanthus* against *E. coli* [171] indicated that *M. xanthus* does not perceive live prey as food, and that the prey shows extensive transcriptomic changes when co-cultured with the predator. Moreover, *M. xanthus* supernatants and secreted outer membrane vesicles (OMVs, see below) also induced changes in the expression of large numbers of prey genes. Another transcriptomic analysis confirmed the actinorhodin overproduction observed in *Streptomyces* by Pérez et al [168], and confirmed that it is iron competition during this interaction which leads to the activation of actinorhodin BGC plus 21 other SM BGCs [172] (Fig. 3A). This is a good example of how basic research on bacterial predator-prey interaction is helping to discover new ways to activate cryptic biosynthetic pathways and is improving our comprehension of awakening mechanisms.

Another aspect to be considered in the search for innovative applications for predators are the Gram-negative OMVs. OMVs are spherical portions of the bacterial outer membrane with a diameter of ~20–250 nm, which represent an alternative to general secretion systems. The myxobacterial OMVs contain a highly concentrated dose of hydrolytic enzymes and molecules associated with antibiotic activity, and are able to deliver these compounds to distant and inaccessible locations, enabling more efficient epibiotic cooperative killing of prey [50,51,173,174]. The OMVs can be used as shuttles to deliver hydrophobic compounds with any desired properties (Fig. 2). For instance, they have been confirmed to be good adjuvants in vaccine delivery [175]. The myxobacterial OMVs have recently been explored as natural antimicrobial carriers able to deliver these products to target cells. They are able to kill some pathogenic bacteria such as *E. coli* or *P. aeruginosa* [176,177]. Recently, they have also been confirmed to be efficient tools for fighting intracellular bacterial pathogens such as *S. aureus*. Those infections are difficult to treat because several classes of antibiotics

are unable to reach the pathogen, and higher concentrations and a longer therapy time are needed. In these assays, OMVs from *Cystobacter velatus* and *Sorangineae* species exhibit low toxicity in various cell lines and primary immune cells, and are able to take them up into infected cells [177,178]. From these results it is possible to envision the exploitation of OMVs from myxobacteria as novel therapeutic delivery systems to combat bacterial infections. The reports on *M. xanthus* OMV proteomics show that they are enriched (along with toxic proteins and antibiotics) in hypothetical proteins of unknown function which remain to be studied in depth [173,176,179,180]. Moreover, a recent proteomic study of the OMV cargo from several *M. xanthus* strains seems to indicate that genetically similar strains of myxobacteria have diverse OMV proteomes [51,181], giving rise to new research challenges.

Finally, it has been shown that myxobacterial predatory activity varies depending on the prey [77,78,182]. Moreover, Sutton et al. [183], using genome-wide association studies with 29 myxobacterial strains, identified different numbers of predation genes depending on the prey organisms. They suggested that the broad prey range of myxobacteria seems to be a consequence of the accumulation of arrangements of prey-specific predation genes, rather than possession of a set of general antibiotic genes. These results clearly indicate that the awakening of cryptic genes should be assayed in the presence of pathogens against which we are looking for a drug, since they may reinforce the positive results of the strategy of testing other bacteria as prey and assaying new products against resistant bacteria.

5. Summary and outlook

Development of AR by many pathogens is one of the main challenges for researchers in order to avoid a new era in which the number of deaths from infectious diseases that were thought to be under control increases to a level that represents an unacceptable health and economic cost. Finding new alternatives to kill these pathogens requires a focus on various different strategies, and one of these promising strategies is learning about the behaviors of bacterial predators and prey in nature. Understanding the strategies developed by predatory bacteria during evolution is helping researchers to emulate and use them in different antimicrobial approaches. Research on the use of predators as living antibiotics is yielding promising results, and although more research is required to demonstrate their efficacy *in vivo*, they represent a serious alternative to be considered. Currently, finding structural genes and regulatory elements involved in predation and the induction of silenced and cryptic genes or BGCs during these dynamic predatory processes is not only starting to help in the discovery of new antibiotics with novel mechanisms of action and new classes of bioactive natural products, but also facilitating the establishment of the best laboratory conditions for optimal scaled-up industrial production of metabolites. Finally, from the studies of the antagonistic predator-prey relationships, researchers are learning about the attack strategies used by the hunters, which are opening innovative research lines such as the use of OMVs as antimicrobial carriers. The mechanisms developed by the prey to defend itself are also proving useful in finding out about new determinants of AR. All of the studies reviewed here about bacterial predators are not only opening new doors for the discovery of new antibiotics, but also harnessing the enormous potential of bacterial predators as whole cell therapeutic agents. Taken together, these studies will help to prepare us for a near future when many antibiotics fail to treat MDRB. However, they require more support from government initiatives, along with changes in

regulation to pave the way for valuable, efficacious, highly targeted, pathogen-specific antimicrobial therapies.

Declaration of Competing Interest

None.

CRedit authorship contribution statement

Juana Pérez: Writing - original draft, Conceptualization. **Francisco Javier Contreras-Moreno:** Writing - review & editing. **Francisco Javier Marcos-Torres:** Writing - review & editing. **Aurelio Moraleda-Muñoz:** Writing - review & editing, Funding acquisition. **José Muñoz-Dorado:** Writing - original draft, Funding acquisition, Conceptualization.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.csbj.2020.09.010>.

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