Microbial Cell Factories

REVIEW

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Metabolic engineering approaches for the biosynthesis of antibiotics

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Abstract

Background Antibiotics have been saving countless lives from deadly infectious diseases, which we now often take for granted. However, we are currently witnessing a significant rise in the emergence of multidrug-resistant (MDR) bacteria, making these infections increasingly difficult to treat in hospitals.

Main text The discovery and development of new antibiotic has slowed, largely due to reduced profitability, as antibiotics often lose effectiveness quickly as pathogenic bacteria evolve into MDR strains. To address this challenge, metabolic engineering has recently become crucial in developing efficient enzymes and cell factories capable of producing both existing antibiotics and a wide range of new derivatives and analogs. In this paper, we review recent tools and strategies in metabolic engineering and synthetic biology for antibiotic discovery and the efficient production of antibiotics, their derivatives, and analogs, along with representative examples.

Conclusion These metabolic engineering and synthetic biology strategies offer promising potential to revitalize the discovery and development of new antibiotics, providing renewed hope in humanity's fight against MDR pathogenic bacteria.

Keywords Antibiotics, Synthetic biology, Metabolic engineering, Actinomycetes, Biosynthetic gene cluster

Introduction

Antibiotics are one of the most revolutionary discoveries in the twentieth century that have significantly enhanced the lifespan of human beings. Before the discovery of penicillin, which is the first antibiotic to be discovered, infectious diseases accounted for high morbidity and mortality worldwide, resulting in a low average life expectancy of about 47 years [1]. One-third of Europe's population perished due to pandemics, such as the bubonic plague, from 1347 to 1350. Additionally, until the early 1910s, infectious diseases accounted for 25% of mortality, making them a major cause of death [2]. Penicillin was identified by Alexander Fleming through a serendipitous discovery that an active metabolite from the culture of *Penicillium notatum* inhibited the growth of a pathogen *Staphylococcus*. Afterward, the mortality rate due to infectious diseases has decreased sharply to less than 1%, and penicillin could save a lot of wounded soldiers from dying by infections during World War II [3]. Acknowledging the discovery of penicillin and the development of efficient bioprocesses for industrial-scale production, Fleming, Florey and Chain were awarded the Nobel Prize.

Ever since the first discovery of penicillin, numerous antibiotics have been discovered from nature, saving countless patients from infectious diseases. Antibiotics can be largely classified into chemicals [4], metal complexes [5], and peptides [6]. Of particular, the major categories of chemical antibiotics are β-lactams, penicillins, cephalosporins, monobactams, tetracyclines, and quinolones [7]. Antibiotics can also be classified according

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to their mechanism of action (MoA) such as inhibition of DNA replication, RNA synthesis, protein synthesis, cell wall biosynthesis, cell membrane biosynthesis, or fatty acid synthesis. The different MoAs of antibiotics can serve as a starting point for the discovery of new antibiotics (Table 1) [8].

While antibiotics are essential for treating infectious diseases, inevitable evolution of pathogens has led to the emergence of resistance towards antibiotics, mainly in hospitals. As a result of the emergence of these multidrug-resistant (MDR) bacteria, at least 1.27 million people have died globally, and in 2019, nearly 5 million deaths were reported. In the United States, over 2.8 million patients annually suffer from infections caused by MDR bacteria [9]. One of the major mechanisms for acquiring antibiotic resistance is horizontal gene transfer (HGT). Once genes involved in antibiotic resistance emerge, they can be easily and rapidly transferred to other bacteria through HGT [10]. Due to the lower profitability of antibiotic products resulting from the rapid emergence of new antibiotic-resistant pathogens, pharmaceutical companies have reduced their investment in antibiotic discovery, leading to a decreased pace of new antibiotic development. In particular, we are witnessing a severe natural antibiotic discovery void over the last couple of decades [11]. For these reasons, the development of novel strategies to discover and develop new antibiotics have been rapidly increasing.

This review focuses on antibiotic chemicals that can be produced from biological processes. Recent discovery of natural antibiotics as well as metabolic engineering and synthetic biology strategies for the development of novel antibiotic derivatives are discussed. The readers are guided to some excellent reviews on the discovery and development of new antibiotics as provided here [11–16].

Discovery of antibiotics produced from actinomycetes

Importance of actinomycetes in antibiotics discovery and production

As one of the most complex habitats for diverse microorganisms, soil provides a rich ecosystem known as the soil microbiome. Since the discovery of soil bacteria isolated from the roots of legumes [103], the microbial diversity within soil samples have been explored. It is estimated that the maximum number of operational taxonomic units (OTUs) in soil is 52,000 [104]. To survive and thrive in such a highly competitive environment, many soil bacteria have evolved to produce antibiotics that help them outcompete surrounding competitors [105]. Of particular, a significant proportion of antibiotics currently in use were isolated from actinomycetes, a group of filamentous gram-positive bacteria that contributes to the diversity of the soil microbial ecosystem. They are one of the prolific producers of natural products and antibiotics β-lactams, tetracyclines, rifamycins, aminoglycosides, macrolides, and glycopeptides. Therefore, they are widely utilized as chassis strains for the production of a wide array of antibiotics [106]. As shown in Fig. 1, new antibiotics were explosively discovered from actinomycetes during 1940s to 1960s, a period referred to as 'the golden era of antibiotic discovery' [107]. Despite the rapid decrease in the discovery of antibiotics from nature, the potential for new antibiotics still remains undiscovered within the genomes of actinomycetes (Fig. 1).

Among diverse strains within actinomycetes, the *Streptomyces* species are reported to produce the most antibiotics (up to 55%) that were discovered from 1945 to 1978. With advancements in sequencing technologies, the cost of sequencing has become more affordable, enabling the analysis of diverse microbial genomes. The availability of a larger volume of genome sequence data for various *Streptomyces* species has led to the discovery of novel antibiotics that could not be directly identified from nature. For example, *Streptomyces coelicolor*, *Streptomyces avermitilis*, *Streptomyces griseus*, and *Saccharopolyspora erythraea* each have more than 20 biosynthetic gene clusters (BGCs) encoding secondary metabolites in their genomes. This demonstrates the complex metabolic and regulatory pathways of *Streptomyces* species and highlights the wide range of secondary metabolites that can be produced in distinct cultural environments. Therefore, the chance of discovery of new antibiotics from the diverse set of actinomycetes are still high. New *Streptomyces* species capable of producing new antibiotics are still being discovered from soil, as exemplified by the recent discovery of a new type of actinomycete, *Streptomyces sp* SM01 [108, 109]. Despite the high potential for discovering new antibiotics, industrial-scale production is challenging due to low titers, productivity, and yields [12].

Actinomycetes chassis strains for heterologous production of antibiotics

As natural producers of diverse antibiotics, actinomycetes have been widely employed for the production of many antibiotics at the industrial-scale. While genetic manipulation of actinomycetes is challenging due to their GC-rich genomes and complex morphological and physiological characteristics [110], heterologous production of antibiotics in traditional model microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* are challenging due to their unfavorable metabolic and regulatory pathways for the expression of large BGCs containing many incompatible genetic elements. Therefore, a number of actinomycetes strains showing favorable

Table 1 Major antibiotics discovered from nature or synthesized from natural precursors

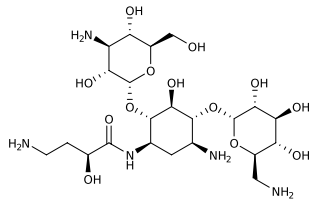
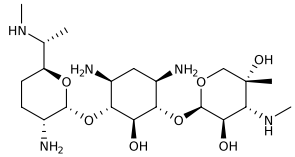
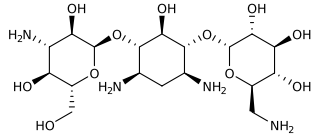
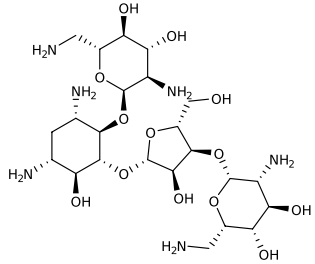
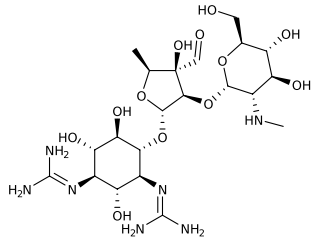
No	Antibiotics	Native producer	MoA	Structure	Refs
Class	Aminoglycosides				
1	Amikacin	Semisynthesis (Aminoglycosides)	Targeting 30S ribosomal subunit		[17]
2	Gentamicin C1	*Micromonospora purpurea*	Targeting 30S ribosomal subunit		[18]
3	Kanamycin	*Streptomyces kanamyceticus*	Inhibiting protein synthesis		[19]
4	Neomycin	*Streptomyces fradiae*	Inhibiting protein synthesis		[19]
5	Streptomycin	*Streptomyces griseus*	Inhibiting protein synthesis		[20]

Table 1 (continued)

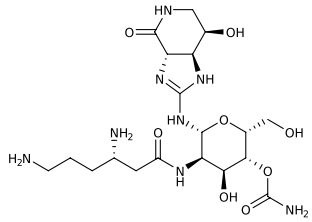
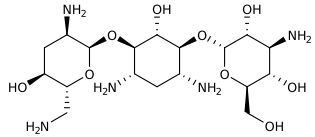
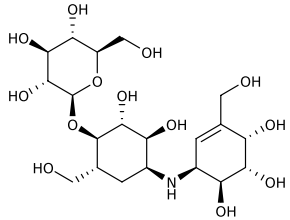
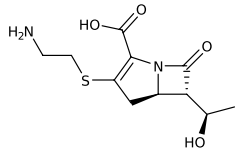
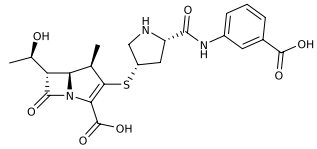
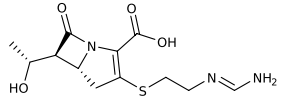
No	Antibiotics	Native producer	MoA	Structure	Refs
6	Streptothricin	*Streptomyces* (multiple sp.)	Inhibiting protein synthesis		[21]
7	Tobramycin	*Streptoalloteicus hindustanus*	Inhibiting protein synthesis		[22]
8	Validamycin	*Streptomyces hygroscopicus*	Not reported		[14]
Class	Carbapenems				
9	Thienamycin	*Streptomyces cattleya*	Inhibiting β-lactamase		[23]
10	Ertapenem	Semisynthesis (Thienamycin)	Targeting penicillin-binding proteins at cell wall		[24]
11	Imipenem	Semisynthesis (Thienamycin)	Targeting penicillin-binding proteins at cell wall		[24]

Table 1 (continued)

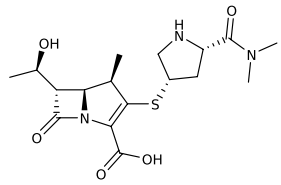
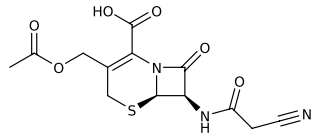
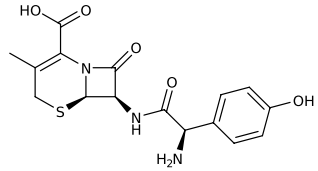
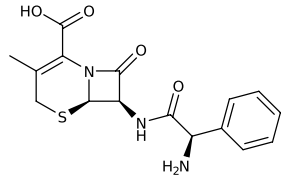
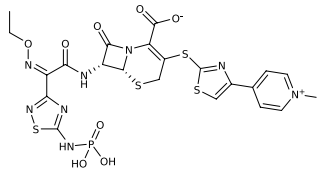
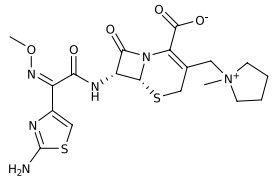
No	Antibiotics	Native producer	MoA	Structure	Refs
12	Meropenem	Semisynthesis (Thienamycin)	Inhibiting cell-wall biosynthesis		[25]
Class	Cephalosporins				
13	Cefacetrile	Semisynthesis (Derived from cephalosporin synthesized by *Cephalosporium acremonium*)	Targeting penicillin-binding proteins at cell wall		[12]
14	Cefadroxil	Semisynthesis (Derived from cephalosporin synthesized by *Cephalosporium acremonium*)	Targeting penicillin-binding proteins at cell wall		[26]
15	Cephalexin	Semisynthesis (Derived from cephalosporin synthesized by *Cephalosporium acremonium*)	Targeting penicillin-binding proteins at cell wall		[27]
16	Ceftaroline	Chemical Synthesis	Targeting penicillin-binding proteins at cell wall		[28]
17	Cefepime	Semisynthesis (Derived from cephalosporin synthesized by *Cephalosporium acremonium*)	Targeting penicillin-binding proteins at cell wall		[29]

Table 1 (continued)

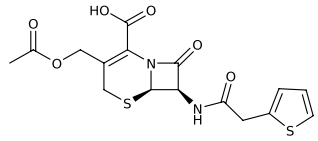
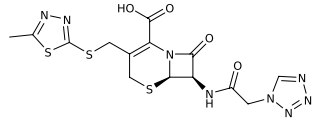
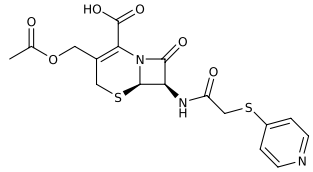
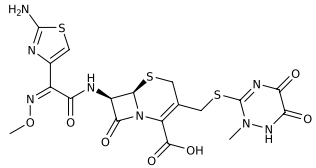
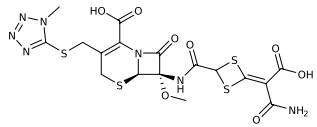
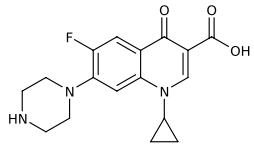
No	Antibiotics	Native producer	MoA	Structure	Refs
18	Cephalothin	Semisynthesis (Derived from cephalosporin synthesized by *Cephalosporium acremonium*)	Targeting penicillin-binding proteins at cell wall		[30]
19	Cefazolin	*Acremonium*	Targeting penicillin-binding proteins at cell wall		[31]
20	Cephapirin	*Acremonium*	Targeting penicillin-binding proteins at cell wall		[30]
21	Ceftriaxone	Semisynthesis (Derived from cephalosporin synthesized by *Cephalosporium acremonium*)	Targeting penicillin-binding proteins at cell wall		[32]
22	Cefotetan	Semisynthesis (Derived from cephalosporin synthesized by *Cephalosporium acremonium*)	Targeting penicillin-binding proteins at cell wall		[33]
Class	Fluoroquinolones				
23	Ciprofloxacin	Chemical Synthesis	Inhibiting DNA gyrase and topoisomerase IV		[34]

Table 1 (continued)

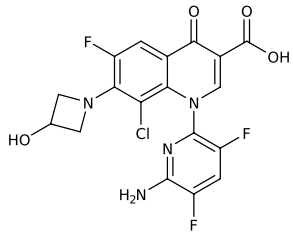
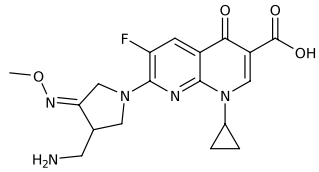
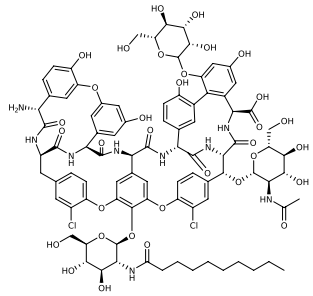
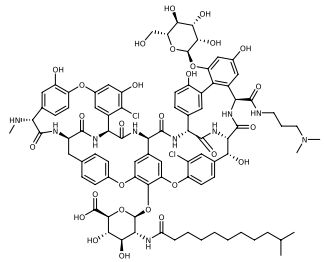
No	Antibiotics	Native producer	MoA	Structure	Refs
24	Delafloxacin	Chemical Synthesis	Inhibiting DNA gyrase		[35]
25	Gemifloxacin	Chemical Synthesis	Inhibiting DNA gyrase		[36]
Class	Non-ribosomal peptides				
26	Teicoplanin	*Actinoplanes teichomyceticus*	Inhibiting cell-wall biosynthesis		[37]
27	Dalbavancin	Semisynthesis (Teicoplanin)	Binding to D-alanyl-D-alanine residue of the growing peptidoglycan chain		[38]

Table 1 (continued)

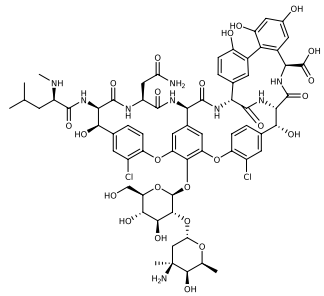
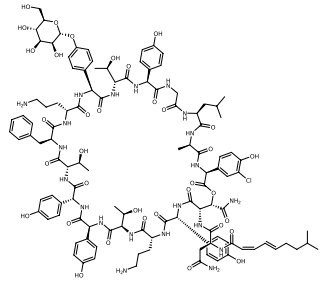
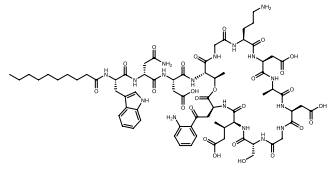
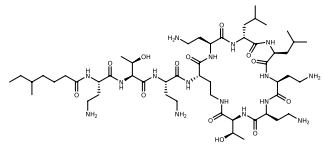
No	Antibiotics	Native producer	MoA	Structure	Refs
28	Vancomycin	*Streptomyces orientalis*	Inhibiting cell wall synthesis, Increasing cell membrane permeability, Inhibiting ribonucleic acid synthesis		[39]
29	Ramoplanin	*Actinoplanes* sp. ATCC 33076	Disrupting bacterial cell wall structure		[40, 41]
30	Daptomycin	*Streptomyces roseosporus*	Disrupting bacterial cell wall structure		[12]
31	Colistin	*Paenibacillus polymyxa*	Disrupting cell membrane		[42]

Table 1 (continued)

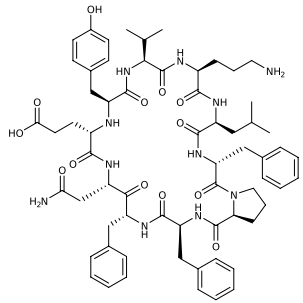
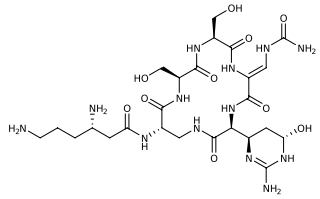
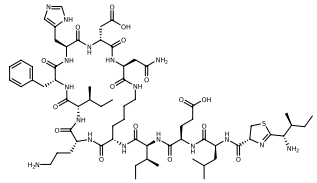
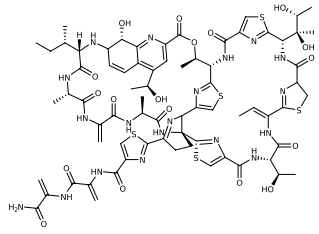
No	Antibiotics	Native producer	MoA	Structure	Refs
32	Gramicidin	*Bacillus brevis*	Forming ion channels that increase the permeability of the bacterial cell membrane		[43]
33	Viomycin	*Streptomyces* sp.11861	Inhibiting protein synthesis and 50S ribosomal subunits		[14]
34	Bacitracin A	*Bacillus subtilis*	Inhibiting dephosphorylation of isoprenyl pyrophosphate		[44]
35	Thiostrepton	*Streptomyces azureus*	Inhibiting protein synthesis		[45]

Table 1 (continued)

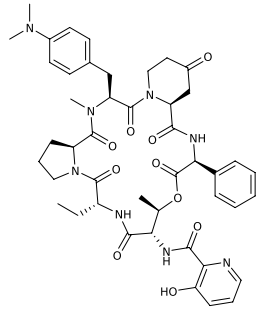
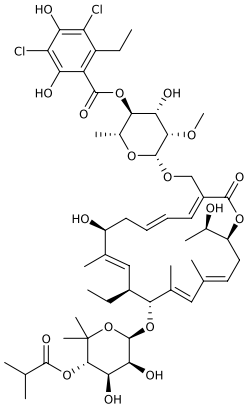
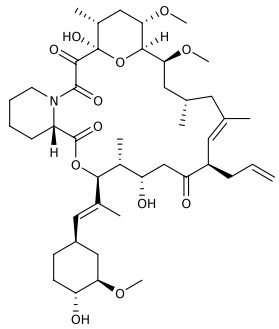
No	Antibiotics	Native producer	MoA	Structure	Refs
36	Pristinamycin IA	*Streptomyces pristinaespiralis*	Inhibiting protein synthesis		[46]
Class	Macrolides				
37	Fidaxomicin	*Dactylosporangium aurantiacum*	Inhibiting nucleic acid synthesis (RNA polymerase)		[47]
38	Tacrolimus	*Streptomyces tsukubaensis*	Inhibiting calcineurin phosphatase		[48]

Table 1 (continued)

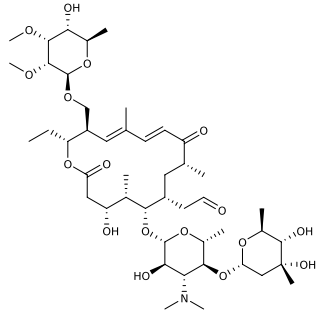
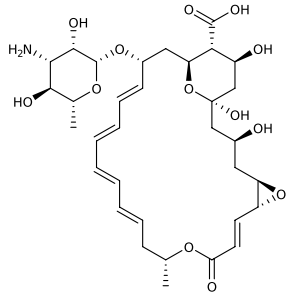
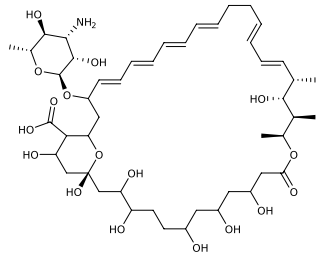
No	Antibiotics	Native producer	MoA	Structure	Refs
39	Tylosin	*Streptomyces fradiae*	Inhibiting protein synthesis		[49]
40	Natamycin (Pimaricin)	*Streptomyces* (multiple sp.)	Inhibiting mold and yeast growth		[19]
41	Nystatin	*Streptomyces noursei*	Inhibiting cell wall synthesis		[50]

Table 1 (continued)

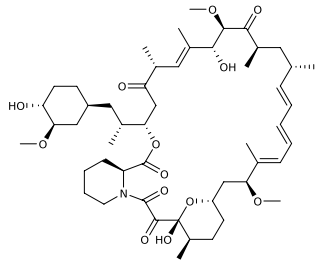
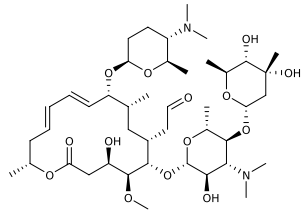
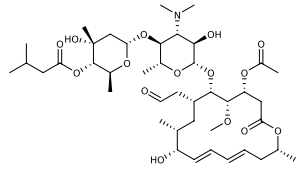
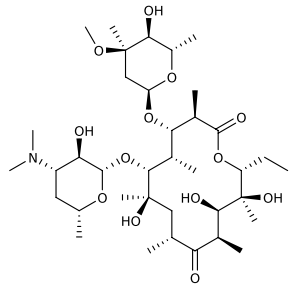
No	Antibiotics	Native producer	MoA	Structure	Refs
42	Rapamycin	*Streptomyces hygroscopicus*	Inhibiting protein synthesis		[51]
43	Spiramycin	*Streptomyces ambofaciens*	Inhibiting protein synthesis		[52]
44	Josamycin	*Streptomyces narbonensis*	Inhibiting protein synthesis		[53]
45	Erythromycin	*Saccharopolyspora erythraea*	Targeting 50S ribosomal subunit		[54, 55]

Table 1 (continued)

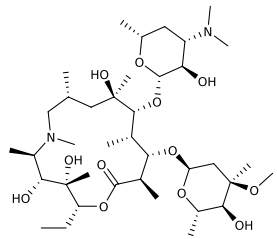
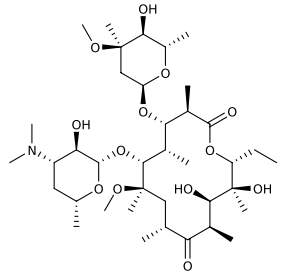
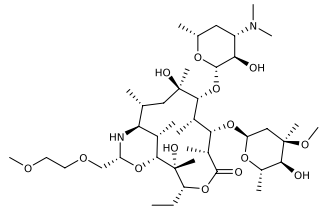
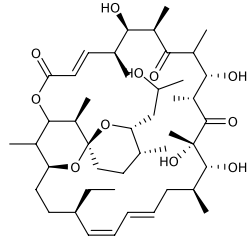
No	Antibiotics	Native producer	MoA	Structure	Refs
46	Azithromycin	Semisynthesis (Erythromycin)	Targeting 50S ribosomal subunit		[56]
47	Clarithromycin	Semisynthesis (Erythromycin)	Targeting 50S ribosomal subunit		[57]
48	Dirithromycin	Semisynthesis (Erythromycin)	Targeting 50S ribosomal subunit		[58]
49	Oligomycin	*Streptomyces avermitilis*	Inhibiting ATP synthesis		[59]

Table 1 (continued)

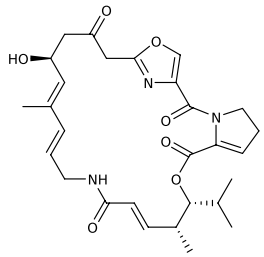
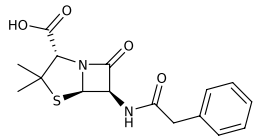
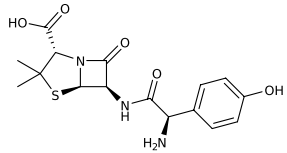
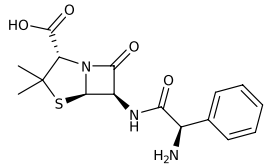
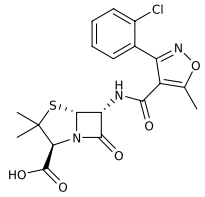
No	Antibiotics	Native producer	MoA	Structure	Refs
50	Pristinamycin IIA	*Streptomyces pristinaespiralis*	Inhibiting protein synthesis		[46]
Class	Penicillins				
51	Penicillin V	*Penicillium crysogenum*	Inhibiting cell wall synthesis		[60, 61]
52	Amoxicillin	Semisynthesis (Penicillin)	Targeting penicillin-binding proteins at cell wall		[62]
53	Ampicillin	Semisynthesis (Penicillin)	Targeting penicillin-binding proteins at cell wall		[63]
54	Cloxacillin	Semisynthesis (Penicillin)	Inhibiting cell wall synthesis		[64]

Table 1 (continued)

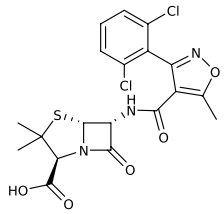
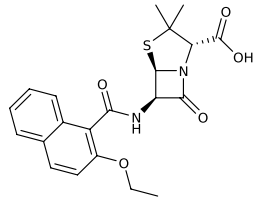
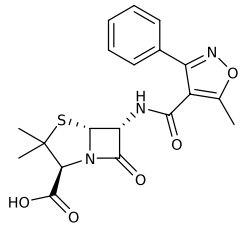
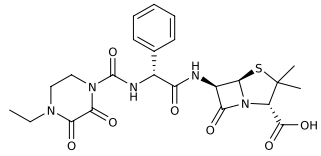
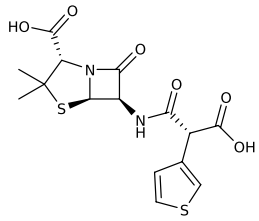
No	Antibiotics	Native producer	MoA	Structure	Refs
55	Dicloxacillin	Semisynthesis (Penicillin)	Inhibiting cell wall synthesis		[65]
56	Nafcillin	Semisynthesis (Penicillin)	Inhibiting cell wall synthesis		[64]
57	Oxacillin	Semisynthesis (Penicillin)	Inhibiting cell wall synthesis		[64]
58	Piperacillin	Semisynthesis (Penicillin)	Inhibiting cell wall synthesis		[66]
59	Ticarcillin	Semisynthesis (Penicillin)	Inhibiting cell wall synthesis		[66]
Class	Streptogramins				

Table 1 (continued)

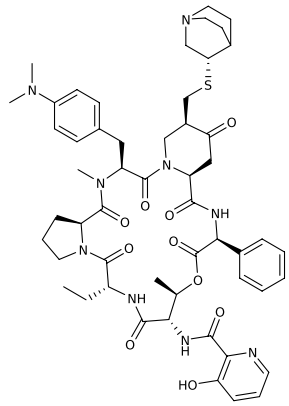
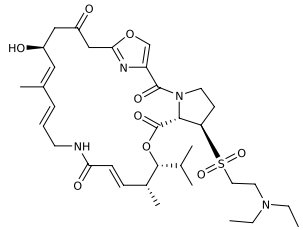
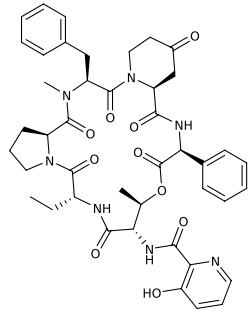
No	Antibiotics	Native producer	MoA	Structure	Refs
60	Quinupristin	Semisynthesis (Pristinamycin)	Inhibiting protein synthesis		[67]
61	Dalfopristin	Semisynthesis (Pristinamycin)	Inhibiting protein synthesis		[67]
62	Virginiamycin S1	*Streptomyces virginiae*	Inhibiting protein synthesis 23S ribosomal subunit		[68]

Table 1 (continued)

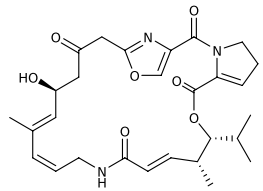
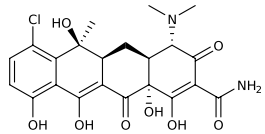
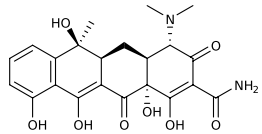
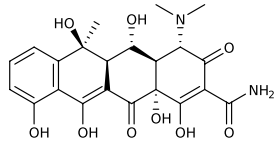
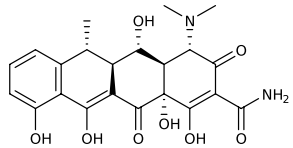
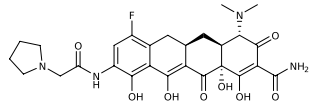
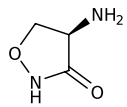
No	Antibiotics	Native producer	MoA	Structure	Refs
63	Virginiamycin M1	*Streptomyces virginiae*	Inhibiting protein synthesis		[69]
Class	Tetracyclines				
64	Chlortetracycline	*Streptomyces aureofaciens*	Targeting 30S ribosomal subunit		[70]
65	Tetracycline	Semisynthesis (Chlortetracycline)	Targeting 30S ribosomal subunit		[70]
66	Oxytetracycline	*Streptomyces rimosus*	Targeting 16S ribosomal subunit		[70]
67	Doxycycline	Semisynthesis (Chlortetracycline)	Targeting 30S ribosomal subunit		[71]
68	Eravacycline	Chemical Synthesis	Targeting 30S ribosomal subunit		[72]
Etc					
69	Seromycin	*Streptomyces orchidaceus*	Inhibiting cell wall synthesis		[73]

Table 1 (continued)

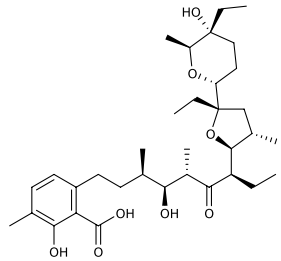
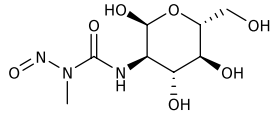
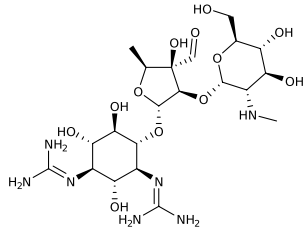
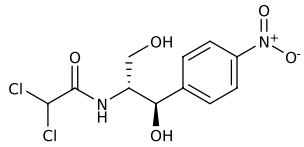
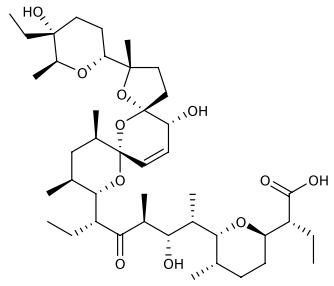
No	Antibiotics	Native producer	MoA	Structure	Refs
70	Lasalocid	*Streptomyces cinnamonensis*	Inhibiting cell growth		[74]
71	Streptozotocin	*Streptomyces acromogenes*	Damaging DNA		[75]
72	Spectinomycin	*Streptomyces spectabilis*	Inhibiting protein synthesis		[76]
73	Chloramphenicol	*Streptomyces venezuelae*	Targeting 50S ribosomal subunit		[14]
74	Salinomycin	*Streptomyces albus*	Accumulating and sequestering iron in lysosomes by interfere with ATP-binding-cassette transporters and Wnt/β-catenin		[77]

Table 1 (continued)

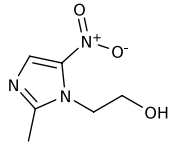
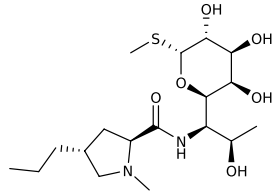
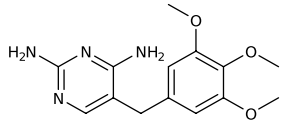
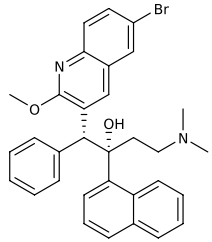
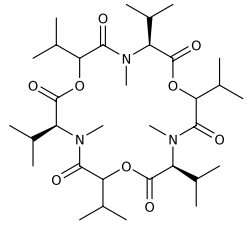
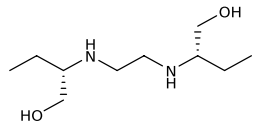
No	Antibiotics	Native producer	MoA	Structure	Refs
75	Metronidazole	Chemical Synthesis	Damaging DNA		[78]
76	Lincomycin	*Streptomyces lincolnensis*	Inhibiting protein synthesis		[79]
77	Trimethoprim	Chemical Synthesis	Inhibiting Folic Acid synthesis		[80]
78	Bedaquiline	Chemical Synthesis	Inhibiting proton pump		[81]
79	Fusafungine	*Fusarium lateritium*	Disrupting cell membrane		[82]
80	Ethambutol	Chemical Synthesis	Disrupting cell wall integrity		[83]

Table 1 (continued)

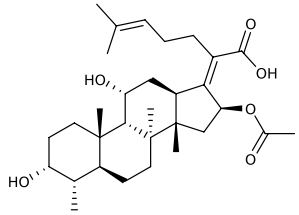
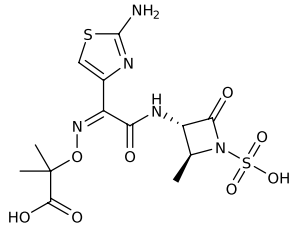
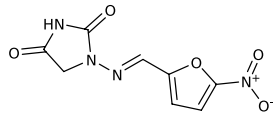
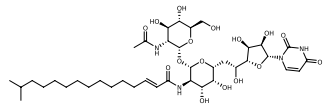
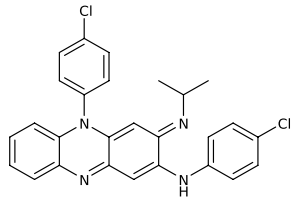
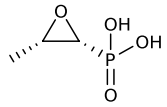
No	Antibiotics	Native producer	MoA	Structure	Refs
81	Fusidic acid	*Fusidium coccineum*	Inhibiting elongation factor G		[84]
82	Aztreonam	Semisynthesis (derived from Monobactam synthesized by *Acetobacter* sp.)	Targeting penicillin-binding proteins at cell wall		[85]
83	Nitrofurantoin	Chemical Synthesis	Inhibiting protein synthesis		[86]
84	Tunicamycin	*Streptomyces chartreusus*	Inhibiting the biosynthesis of N-linked glycoproteins		[87]
85	Clofazimine	Chemical Synthesis	Inhibiting DNA synthesis by binds to guanine bases		[88]
86	Fosfomycin	*Streptomyces fradiae*	Inhibiting MurA (UDP-GlcNAc-3-enolpyruvyltransferase)		[89]

Table 1 (continued)

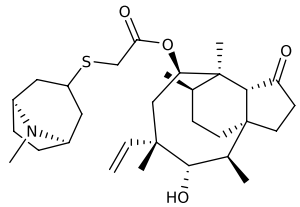
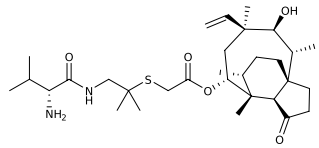
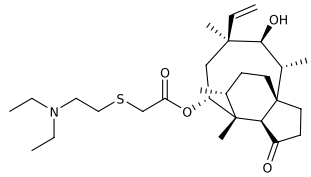
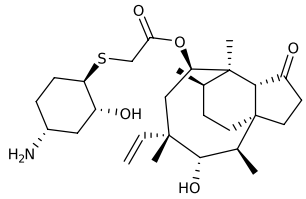
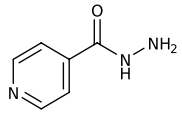
No	Antibiotics	Native producer	MoA	Structure	Refs
87	Retapamulin	Semisynthesis (Pleuromutilin)	Inhibiting protein synthesis		[90]
88	Valnemulin	Semisynthesis (Pleuromutilin)	Inhibiting protein synthesis		[90]
89	Tiamulin	Semisynthesis (Pleuromutilin)	Inhibiting protein synthesis		[90]
90	Lefamulin	Semisynthesis (Pleuromutilin)	Inhibiting protein synthesis		[90]
91	Isoniazid	Chemical Synthesis	Inhibiting the synthesis of mycolic acids		[91]

Table 1 (continued)

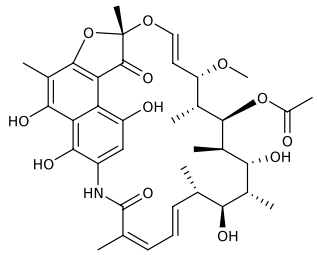
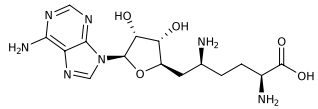
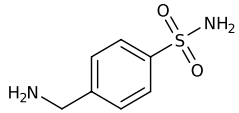
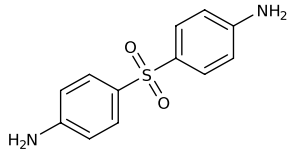
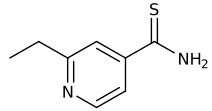
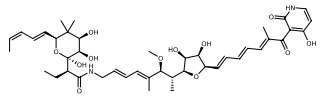
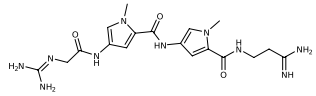
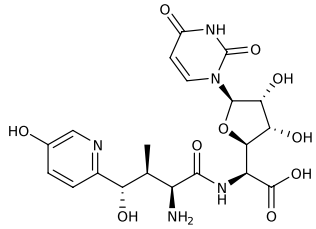
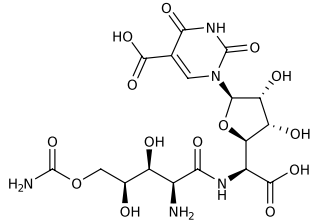
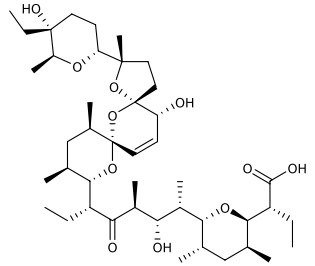
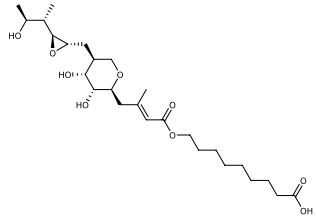
No	Antibiotics	Native producer	MoA	Structure	Refs
92	Rifamycin (Rifampicin)	*Amycolatopsis mediterranei*	Inhibiting RNA polymerase and protein synthesis		[92]
93	Sinefungin	*Streptomyces griseolus*	Inhibiting a pan-MTase		[93]
94	Mafenide	Chemical Synthesis	Inhibiting folic acid synthesis		[94]
95	Dapsone	Chemical Synthesis	Inhibiting dihydropteroate synthetase		[95]
96	Ethionamide	Chemical Synthesis	Inhibiting synthesis of mycolic acids		[96]
97	Kirromycin	*Streptomyces collinus*	Binding elongation factor Tu to shutdown translation		[97]
98	Netropsin	*Streptomyces ambofaciens*	Inhibiting DNA binding and transcription		[98]

Table 1 (continued)

No	Antibiotics	Native producer	MoA	Structure	Refs
99	Nikkomycin	*Streptomyces tendae*	Inhibiting chitin synthesis enzyme		[99]
100	Polyoxin (D)	*Streptomyces cacaoi*	Inhibiting fungal cell wall formation		[100]
101	Narasin	*Streptomyces aureofaciens*	Inhibiting IκBα phosphorylation		[101]
102	Mupirocin	*Pseudomonas fluorescens*	Inhibiting protein synthesis by binding to isoleucyl t-RNA synthetase		[102]

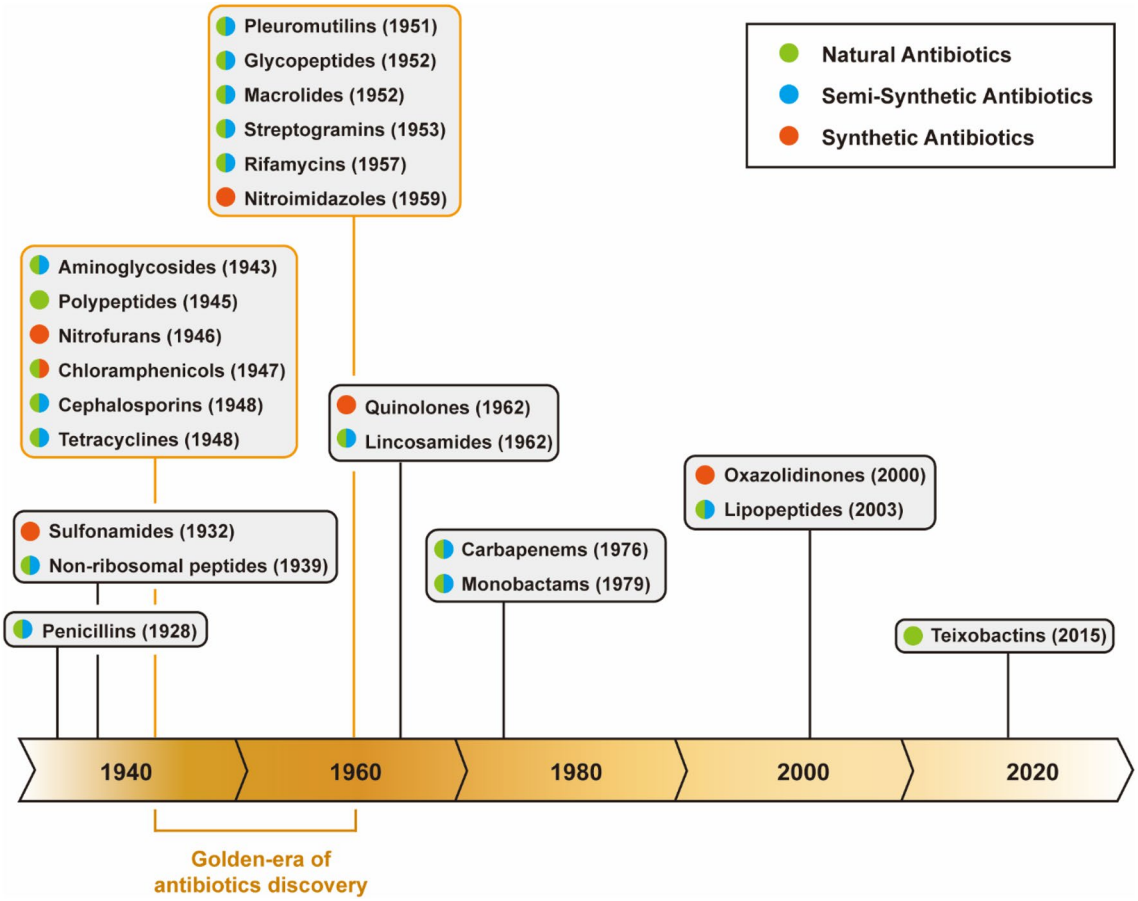


Fig. 1 The timeline of antibiotic discoveries approved for clinical use. Representative antibiotic classes are shown; left side of circle indicates the year of discovery for the first chemical belonging to the class. Right side of circle shows the proceeded development way for clinical use. Relevant standards are provided in parentheses. The 'Golden era' of antibiotic discovery (from the 1950s to the 1960s) is highlighted in yellow lines. Antibiotics are categorized as follows: green, natural antibiotics; blue, semi-synthetic antibiotics; orange, synthetic antibiotics

characteristics for heterologous BGC expression have been selected as chassis strains for antibiotics production. These chassis candidates have abundant pools of precursors and cofactors required for antibiotics production, relatively well established genome engineering tools, simpler growth conditions, compatible gene expression elements, and high genetic element transformation efficiency. Such strains include *S. coelicolor* A3(2), *Streptomyces albus*, *Streptomyces avermitilis* MA-4680, *S. albus* J1074, *Streptomyces lividans* TK24, and *Streptomyces venezuelae* [111, 112].

Among them, *S. albus* is one of the most commonly used chassis strains for the heterologous expression of diverse BGCs. The genome of *S. albus* (6.8 Mbp harboring 5.8 K genes in *S. albus* J1074) is one of the smallest among *Streptomyces* species, which allows for higher genetic stability when introducing heterologous BGCs [113, 114]. Growing and screening actinomycetes is a time-consuming and labor-intensive process, necessitating a rapid mutagenesis and screening strategy. Therefore, atmospheric and room temperature plasma, an effective mutagenesis method, was combined with ribosome engineering in the natural salinomycin producer *S. albus*, resulting in the generation of an overproducer that achieved twice the concentration of salinomycin [115]. *S. albus* has also been demonstrated as an efficient host for the production of complex terpenoids, making it particularly useful for the functional expression of tailoring enzymes including P450 for terpenoid modification [116]. To construct a more efficient chassis strain, 15 known BGCs were deleted, resulting in enhanced metabolic flux toward the desired products [117]. *S. coelicolor* is another important chassis strain for the efficient production of many secondary metabolites (e.g., actinorhodin, chloramphenicol, and congocidine). As with *S. albus*, BGCs encoding pathways for competing secondary metabolites were deleted, and mutations were introduced in genes encoding ribosomal components

(i.e., *rpoB* and *rpsL*) for the enhanced production of target chemicals [118]. Although engineering large BGCs in actinomycetes remains challenging, new synthetic biology tools and strategies are continuously being developed to facilitate the engineering of these highly potent hosts for industrial-scale antibiotic production.

Methods for screening new antibiotics

Phenotype screening

Despite the abundance of bacterial species in soil, only a small fraction (less than 1%) can be successfully cultured in the laboratory [119]. As a result, only a few of many natural products discovered in nature have been identified as antibiotics. To discover and identify antibiotics among many natural products, a primitive method 'phenotype screening' has been employed in the early days. Phenotype screening is an exploratory process that identifies chemicals with antibiotic properties by testing the viability of pathogens when treated with candidate chemicals, based on their observable effects on biochemical activities or MoA, without prior knowledge of the targets [120, 121].

Soil microbiome has been the primary source of antibiotics discovery, but recently, the human microbiome has been gaining interest due to the high chance of encountering pathogen invasions in the respiratory track. For example, a non-ribosomal peptide (NRP) antibiotic, lugdunin, was discovered by phenotype screening from 90 nasal Staphylococci. *Staphylococcus lugdunensis IVK28* found in the human nose was identified to produce lugdunin which can kill a representative pathogen *Staphylococcus aureus* [122].

Phenotypic screening based on target pathogen viability does not provide any insight into the biochemical targets of antibiotic candidates. Therefore, antimicrobial activity screening has shifted from viability tests to specific biochemical target inhibition approaches [123]. However, when the target pathogen (e.g., *Mycobacterium tuberculosis*) is difficult to test in the lab due to slow growth and biocontainment regulations, the target essential surrogate *E. coli* (TESEC) platform can be used instead [124]. The TESEC platform is constructed by the deletion of an essential gene in *E. coli* and replacing it with a functional analog from the target pathogen, linking bacterial growth to the activity of the target enzyme. In this study, high-throughput screening of antibiotic targets was performed in a TESEC platform for *M. tuberculosis* alanine racemase, leading to the identification of benazepril as an effective antibiotic against *M. tuberculosis* [124]. As such, phenotypic screening is still being actively used to discover new antibiotics as well as providing insights into the new MoA of new antibiotics [14].

Antibiotic discovery based on mechanisms of action

As discussed above, determining the MoA of an antibiotic is much more challenging than simple discovery of an antibiotic [125]. Some of the major biochemical targets of antibiotics are as follows: essential elements for cell survival, cell wall and cell membrane synthesis, cell membrane permeability, electron transport, purine and purine nucleotide synthesis, DNA synthesis, and protein synthesis [126]. In this subsection, methods for identifying MoAs of antibiotics are discussed.

Bacterial cytological profiling

As a rapid and powerful method for identifying the cellular pathways affected by antibiotics, bacterial cytological profiling (BCP) can distinguish between inhibitors that impact different cellular pathways as well as different targets within the same pathway [125]. Therefore, when similar imaging results are obtained by BCP when two different antibiotics are compared, their MoAs can be considered to be similar [127]. During BCP, bacteria are visualized through fluorescent dye staining methods such as DNA staining with DAPI, SYTOX, and ethidium bromide (EtBr), cell membrane staining with FM4-64, and cell wall staining with crystal violet and calcofluor white. For example, BCP was employed to identify an antibiotic peptide MciZ, which was shown to target FtsZ, a cell mitosis protein, in *Bacillus subtilis*. Upon treatment with MciZ, BCP shows that *B. subtilis* cells have shown phenotypes (e.g., undivided cells and abnormal Z-ring distribution) that could also be observed by treatment of FtsZ inhibitors. The loss of the Z-ring can be observed by the length of the DAPI-stained nucleoid, which becomes longer to fill in the area where the Z-ring is lost [128, 129].

In another example, BCP was employed to elucidate the MoA of the analogue of pan-assay interference compounds (PAINS), previously known to contain rhodamine. BCP, FM-6–64, SYTOX-green, and DAPI were employed as fluorescent markers. Upon treatment with the PAINS analogue, inhibition of cell wall synthesis and DNA replication was observed, achieved by repressing thymidylate kinase which is an enzyme responsible for synthesizing pyrimidine DNA bases. This inhibition manifested through filamentation and chromosomal replication defects, as indicated by fluorescence of BCP [130].

Flow cytometry

Flow cytometry is a tool that can quickly and accurately analyze individual microbial cells, even if they cannot be cultivated in the laboratory. Flow cytometry allows the analysis of various chemical and physical phenotypes

such as cellular type, viability, and gene expression using fluorescent markers [131]. For example, flow cytometry was employed to unveil the MoA of labdane diterpenes by labeling target bacteria with fluorescence to assess viability [132].

Flow cytometry is also useful for distinguishing live and dead cells by using SYTO9 which stains only live cells and PI which stains only dead cells. Live/dead cell assay by flow cytometry was used to find the best combinations of different antibiotics to enhance the antibacterial property. The combination of antimicrobial peptides, sphistin and sph_{12-38}, with antibiotics such as azithromycin and rifampicin, led to 85.93% reduction in viability of a representative gram-negative pathogen *Pseudomonas aeruginosa* [133].

CRISPRi

Clustered regularly interspaced short palindromic repeats interference (CRISPRi) employs a catalytically inactive Cas9 endonuclease to specifically repress the transcription of target genes, guided by single guide RNAs (sgRNAs) [134]. CRISPRi is employed to reveal the MoA of an antibiotic by repressing the antibiotic target gene candidates within a pathogen. Since the target genes are mostly essential genes in bacteria, their decreased expression would result in very low cell viability. For example, the MoA of peziculone was analyzed using CRISPRi. When genes involved in cell wall synthesis (i.e., *tagB* and *murB*), biofilm formation, and essential metabolic pathways (e.g., fatty acid biosynthesis and protein biosynthesis) were knocked down, the antibiotic sensitivity of *S. aureus* was increased [135]. In another example, the target gene of irresistin-16, a derivative of SCH-79797, was identified through both BCP and CRISPRi [136]. When essential genes involved in folate metabolism, *dfrA* (encoding dihydrofolate reductase) and *folC* (encoding dihydrofolate synthase), are knocked down in *B. subtilis*, the cell viability was observed to be highly sensitive to antibiotics treatment. Furthermore, irresistin-16 was shown to disrupt membrane integrity, as evidenced by BCP and flow cytometry [136]. As such, the MoA of antibiotics can be effectively elucidated by the combinations of different approaches.

Proteomics

Proteomics is a method for studying complex protein mixtures, such as bacterial lysates or clinical tissue samples containing several thousands of proteins. One of the most fundamental methods of analyzing the proteome of a bacteria is two-dimensional (2D) gel electrophoresis. Another method is iTRAQ®, which relies on the tagging of proteolytically cleaved peptides from different samples, with each sample conjugated to a different tag. The specific proteins found by either 2D gel electrophoresis or iTRAQ® can be subsequently elucidated and quantified by liquid chromatography-mass spectrometry (LC–MS) or matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF/MS), leading to the investigation of cellular pathways affected by antibiotic treatment [137, 138]. For instance, 2D gel electrophoresis and MS were employed to observe changes in protein production in *Acinetobacter baumannii* following treatment with the antibiotic sulbactam. As a result, the levels of essential proteins for cell survival, including the ATP-binding-cassette (ABC) transporter as well as the 30S and 50S ribosomal subunit proteins, were found to be reduced [139]. Also, the proteomic change of *S. aureus* after lactobionic acid treatment was investigated using iTRAQ®. The analysis of peptides tagged with the reporter marker iTRAQ® through LC–MS/MS revealed disruptions in the cell wall and the membrane integrity, as well as altered ABC transporter levels and cellular energy metabolism [140]. Such proteomic analysis will be useful to provide important insights about the MoA of new antibiotics.

Discovery and activation of cryptic BGCs for the biosynthesis of new antibiotics

Isolation of unculturable bacteria and recovery of products

As aforementioned, a significant number of unculturable bacteria remain elusive and have not yet been comprehensively analyzed. Many of these microorganisms are expected to be valuable for our lives since some of them could recycle various elements (e.g., carbon, nitrogen, and metals) from natural resources, and the others could produce a variety of natural products with unprecedented pharmaceutical activities. Therefore, it is important to isolate and recover these bacteria in order to elucidate the largely unexplored space of antibiotics from nature by reconstruction of the natural habitat as much as possible. This involves providing suitable stimuli such as temperature, osmosis, host conditions, chemical inducers or precursors, and specific interactions with other bacteria in the surroundings [141]. However, no matter how meticulously the culture environment is adjusted, many bacteria still fail to grow, making it challenging to point out the exact problem.

Influence and stimulation of neighboring bacteria can be an important factor for growing some unculturable bacteria. These neighboring bacteria can stimulate the growth of target bacteria by providing diffusible growth factors (e.g., siderophores, cAMP, and acyl-homoserine lactones) or through physical contact, although the exact mechanisms remain largely unknown. Therefore, co-culture with other bacteria found from the same environment might allow the target bacteria to grow in the

laboratory conditions. For example, *Bacillus marisflavi* requires a modified acyl-desferrioxamine siderophore as the growth factor produced from *Bacillus megaterium*, a helper bacterium inhabiting the same environment. This facilitated the utilization of oxidized iron as an essential nutrient, effectively regulating cell homeostasis and thus promoting growth of *B. marisflavi* [142]. In another study, co-culture of *Micromonospora* sp. with *Rhodococcus* sp. resulted in the production of keycin, a poly-nitroglycosylated anthracycline [143]. It turned out that the anthracycline backbone was first biosynthesized by *Micromonospora* sp., which was then modified to benzoxocin by *Rhodococcus* sp., and then further to keycin [143]. A notable feature of keycin is its distinct mechanism apart from that of most anthracyclines. However, the exact mechanism of keycin acting as an antibiotic remains elusive. Co-culture of fungal species can be also useful, as exemplified by the co-culture of two *Aspergillus* species from mangrove could produce a novel compound called aspergicin, which showed antibacterial activities towards *Bacillus proteus*, *E. coli*, *S. aureus*, and *B. subtilis* [144].

However, co-culture also has a number of limitations, including challenges in culturing helper bacteria, difficulties in precisely understanding bacterial interactions, and elucidation of suitable bacterial co-culture pairs. This led to the development of the isolation chip (iChip) which is a multichannel device that creates multiple sections divided by semi-permeable membranes in which only a single cell can be isolated. When iChips are buried in the soil, a single bacterial cell can be isolated in each section in the natural habitat, which can then be recovered in the lab. As bacteria are grown in the natural habitat through iChip, a striking 50% of all bacteria from a soil sample could be isolated [145]. As a case study, teixobactin, a newly discovered NRP antibiotic produced by *Eleftheria terrae*, was identified using the iChip method. Teixobactin shows significant bactericidal activity against MDR gram-positive pathogens without any resistance reported so far [146]. The authors are guided to additional studies on teixobactin in the following literature [147–150]. Interestingly, a new NRP antibiotic clovibactin could also be discovered from the same bacterial species *E. terrae* subspecies carolina [148]. Clovibactin was shown to block cell wall biosynthesis, with an unusual structure and MoA, showing no sign of resistance development as well.

Discovery of cryptic BGCs and genome mining

Despite efforts to culture "unculturable" bacteria, a majority of them still cannot be recovered in the lab. In this regard, metagenomics can be employed to comprehensively analyze large amounts of genomic data obtained directly from environmental samples, without the need to culture these elusive microorganisms [151]. The rapid advancement of sequencing technologies has enabled the accumulation of huge metagenomic data that include a significant volume of BGCs for secondary metabolite biosynthesis. As a representative example, *S. coelicolor* A3(2) was found to have about 7,825 genes involved in > 20 BGCs [108]. Also, 17 BGCs were identified in a marine actinomycete *Salinispora tropica*, most of which were novel, including the one responsible for the production of a polyene macrolactam salinilactam A [152]. The development of next-generation sequencing (NGS) technologies, along with recent advances in nanopore sequencing and single-cell sequencing, has enabled the rapid sequencing of the genomes of many *Streptomyces* strains. This has led to the discovery of a significant number of cryptic genes which are not expressed under normal culture conditions. In nature, these cryptic genes are activated only under specific environmental conditions to assist the survival of host cells in diverse environments.

To elucidate the cryptic genes and BGCs from metagenomic data, metagenome mining tools such as MG-RAST, IMG/M, EBI Metagenomics, SILVAngs, MEGAN, QIIME, and Mothur can be employed [153, 154]. For instance, metagenomic analysis was used to predict the genes encoding polyketide synthases and associated enzymes, leading to the discovery of new polyketides that potentially harbor antibacterial properties [151, 155, 156]. The antiSMASH (antibiotics & Secondary Metabolite Analysis Shell) pipeline was first developed in 2011 and is currently the most widely used bioinformatics tool for the prediction of BGCs and their possible products [157]. For example, antiSMASH was used to predict the cryptic BGCs within *Streptomyces globisporus* SP6C4, a bacterial strain known for its use in suppressing plant diseases. As a result, 15 BGCs were predicted, among which some were shown to produce secondary metabolites with antibacterial activities, providing valuable information for agricultural applications [158]. The antiSMASH pipeline can also be employed to comprehensively screen BGCs from a large volume of database. For example, all the high quality bacterial genomes from GenBank (~ 3000 as of 2014) were analyzed using antiSMASH to search NRPS-encoding gene clusters. As a result, 96 previously unidentified NRPs were predicted to be produced by the identified NRPSs, most of which were C-terminally cyclized peptides. Based on the common structural properties of these peptides, 171 synthetic peptides were designed, nine among which exhibited antibacterial activity against the ESKAPE pathogens (the group of major MDR bacteria including *Enterococcus faecium, S. aureus, Klebsiella pneumoniae, A. baumannii, P.*

aeruginosa and Enterobacter cloacae) and *M. tuberculosis* [159]. In another example, an NRP-PK hybrid, epifadin, was discovered through the isolation of the BGC by analyzing the genome of the nasal commensal bacterium *Staphylococcus epidermidis* IVK83 using antiSMASH [160]. Other metagenome mining tools are also available such as BAGEL [161], PRISM [162], RiPPER [163], and TOUCAN [164]. Such approaches enable the discovery of new antibiotics from the metagenomic data without the need to directly culture the bacteria.

Activation of cryptic BGCs

Genetic-level activation

After the elucidation of cryptic BGCs and the predicted products, activation of the cryptic BGCs from the host strain is required to produce, recover, and analyze the resulting chemicals. A representative method of activating BGCs in the native hosts is the insertion of strong constitutive promoters (e.g., ermE* and kasO*) in front of the BGCs by CRISPR/Cas9. For instance, in *Streptomyces viridochromogenes*, the insertion of the kasO* promoter upstream of the BGC encoding a type II polyketide synthase (PKS) resulted in the production of a previously unknown pentangular type II polyketide with a dihydrobenzo[α]naphthacenequinone core [165].

Other than direct activation of the biosynthetic pathway genes, pathway regulators can be employed to manipulate the expression of cryptic genes, as exemplified in *Streptomyces chattanoogensis* L10 [166]. By the overexpression of genes encoding three biosynthetic pathway regulators (ChaK, ChaK1, and ChaI) in *S. chattanoogensis*, the cryptic angucycline gene cluster could be activated, resulting in the successful production of new antibiotics, chattamycin A and B [166]. The large ATP-binding regulator (LAL) regulator family plays an important role in regulating the expression of genes related to type I modular PKSs, and thus can be found within the BGCs of various polyketide antibiotics such as avermectin, salinomycin, and staurosporine [167]. In another example, overexpressing *astG1* which encodes a LAL family regulator in *Streptomyces* sp. XZQH13 found from the cryptic ansatrienin BGC led to the production of hydroxymycotrienins A and thiazinotrienomycin G, previously unachievable ansatrienin antibiotics [168].

Another interesting approach is the deletion of BGCs responsible for the production of already known antibiotics instead of reinforcing the pathways related to unknown compounds [169]. For example, genes responsible for the production of two of the most frequently rediscovered antibiotics, streptothricin and streptomycin, were deleted from the genomes of 11 actinomycetes. As a result, previously unreported variants of antibiotics including thiolactomycin, amicetin, phenanthroviridin, and 5-chloro-3-formylindole could be discovered.

Activation of the cryptic BGC using chemical elicitors

Other than through direct activation of the cryptic BGCs at the genetic-level, they can be sometimes activated upon the addition of specific chemicals due to the complicated metabolic and regulatory pathways of actinomycetes. When bacteria are exposed to antibiotics at sub-inhibitory concentrations, a new secondary metabolite pathway can be activated to generate new products. This induction of BGCs mainly occurs at the transcriptional level and can even lead to various gene expression changes that can lead to many phenotypic changes other than those related to secondary metabolite production. For example, an antibiotic streptomycin was shown to induce the expression of a cryptic type II PKS-related BGC in *Microbispora* sp. BCCAGE54, leading to the production of tetarimycin B [170]. In addition, alteration of nutrient addition in the culture medium can lead to the induction of cryptic BGCs. A notable example is the discovery of coelichelin produced from *S. coelicolor* A3(2) by culturing the strain in an iron-deficient condition [171]. Since coelichelin serves as a siderophore, which helps the host cell to acquire iron, culturing the strain in the absence of iron would have led to the enhanced production of coelichelin to help the cell survive in an iron-deficient environment.

Some metabolites produced from neighboring microorganisms can also act as chemical elicitors for cryptic BGCs. Therefore, co-culture of multiple bacterial strains could lead to the observation of unprecedented metabolites that could not have been observed by culturing each bacteria individually. As an example, when *Micromonospora* sp. UR56 and *Actinokineospora* sp. EG49 were co-cultured, a number of new metabolites that were not produced by individually culturing the two bacterial strains could be found [172]. Remarkably, all of these products were found to have come from cryptic BGCs that would have been hidden until induced by the co-culture. Among the new chemicals, dimethyl phenazine-1,6-dicarboxylate, phencomycin, and tubermycin demonstrated antibacterial activities [172].

Reconstruction of the cryptic BGCs

For cryptic genes that are difficult to be expressed in native hosts, the genes can be cloned and introduced into heterologous hosts to identify the products that the BGCs can produce. Since it is often difficult to express actinomycetal BGCs in widely used model microorganisms such as *E. coli* or *S. cerevisiae*, a number of *Streptomyces* chassis strains (e.g., *S. coelicolor*, *S. lividans*, and *S. albus*) have been used for facile introduction of

actinomycetal cryptic BGCs. For example, as the cryptic ansamycin BGC (type I PKS) could not be activated in the marine actinomycete *Streptomyces seoulensis* A01 in the laboratory, the cryptic BGC cloned into a plasmid and was introduced in other *Streptomyces* hosts. As a result, successful heterologous production of ansaseomycins A and B was achieved using the actinomycetal chassis strains *S. lividans* SBT18 and *S. coelicolor* M1146 [173]. When the expression of cryptic BGCs in heterologous hosts is combined with genome mining tools (e.g., antiSMASH) for predicting cryptic BGCs from metagenomic data, the speed of discovering new antibiotics can be significantly enhanced.

Enhancing the production levels of antibiotics

Enhanced production of antibiotics by engineering actinomycetes

As previously discussed, actinomycetes are efficient microbial cell factories for the production of diverse antibiotics despite the difficulties of engineering. To facilitate the engineering process and to resolve such difficulties, several metabolic engineering and synthetic biology tools and strategies have been developed [174]. One of the most useful genome engineering tools is the CRISPR system [175, 176]. CRISPR-based genome engineering has allowed insertion and deletion of gene fragments, as well as editing of DNA and RNA bases. CRISPR has been recently adopted for facile engineering of actinomycetes to enhance their production capacities in order to better exploit these remarkable antibiotic producers. For instance, the genome of *S. erythraea* NRRL 23338, which produces erythromycin (a macrolide produced by type I PKS), was engineered by the CRISPR-Cas9 system [177]. As the expression levels of the *ery* cluster encoding the tailoring enzymes were low, which served as a significant bottleneck for erythromycin production, strong promoters were inserted in multiple loci within the *ery* cluster to enhance the transcription levels of the bottleneck genes. As a result, the erythromycin production was enhanced by six-fold when compared to the wild type strain [177]. Thus, the CRISPR-Cas9 system can be used to overexpress bottleneck genes to better streamline the metabolic flux towards the products. CRISPR-Cas9-based genome engineering is also useful for the deletion of major antibiotic pathways in order to redirect the metabolic flux towards other minor secondary metabolites, which would lead to previously undiscovered antibiotics. For example, the BGCs for the production of streptothricin or streptomycin were deleted in 11 actinomycete strains by using CRISPR-Cas9, leading to the discovery of previously unreported antibiotics including tiolactomycin, amicetin, and phenanthroviridin [169]. In addition, CRISPR can be used together with genetic elements of bacteriophage for the integration of large DNA fragments. For instance, the attachment and integration (Att/Int) system from bacteriophage ΦC31 was used together with CRISPR-Cas9 to integrate multiple copies of the large pristinamycin II BGC into the genome of *Streptomyces pristinaespiralis* [178]. This led to the production of 2.24 g/L of pristinamycin II by shake flask culture [178].

To avoid DNA double-strand breaks as well as the cytotoxic effects of Cas9, CRISPR-base editing system (CRISPR-BEST) was developed and was showcased in a non-model actinomycete *Streptomyces collinus* Tü365 [179]. The CRISPR-BEST system having a cytidine deaminase could manipulate the kirromycin biosynthetic pathway by inactivating the *kirN* gene by introducing stop codons within the gene. More recently, an improved base editing system (eSCBE3-ng-Hypa) with improved performance towards high GC DNA sequences, relaxed protospacer adjacent motif (PAM) requirement, and minimal off-target effects was developed in *Streptomyces* species [180]. The eSCBE3-ng-Hypa system was used to inactivate the competitive pathways within the *ave* BGC for enhanced production of avermectin B1a in *S. avermitilis* [180].

Given the complex metabolism and physiology of actinomycetes, it is often challenging to develop engineering strategies to enhance antibiotic production in these strains. Therefore, understanding the relationship between different metabolic and regulatory pathways and antibiotic production through omics analysis can help increase the production of diverse antibiotics. For example, comparative transcriptomic analysis was performed for *Actinosynnema pretiosum* ATCC 31280, an ansamitocin P-3 (AP-3) producer, to elucidate the cause for excessive mycelial fragmentation during fermentation [181]. As a result, the *APASM_4178* gene encoding a subtilisin-like serine peptidase was identified to be responsible for mycelial fragmentation. As mycelial fragmentation had a negative impact on the yield of AP-3, the *APASM_4178* gene led to increased cell growth as well as increased production of AP-3 by 43.65% [181]. In another study, metabolomic and transcriptomic analysis of *S. avermitilis* revealed that triacylglycerol (TAG) accumulated during cell growth was degraded during stationary phase, leading to increased metabolic flux towards acetyl-CoA, reducing equivalents, ATP, and thus polyketides [182]. As the *sco6196* gene was shown to be responsible for the degradation of TAG, overexpression of the gene in an industrial *S. avermitilis* A56 strain resulted in significantly enhanced production of avermectin B_{1a} (9.31 g/L) in a 180-m^3 fermenter [182]. Another method of enhancing the production of antibiotics is co-culture of several bacterial strains to activate core BGCs. Co-culture of *Vibrio coralliilyticus* and *Photobacterium galatheae* has

led to increased production of andrimid and holomycin by 4.3 and 2.7-fold, respectively, when compared to those produced by individually culturing each bacterial strain [183].

Antibiotic production from heterologous model microorganisms

Despite the capability of actinomycetes for the production of high-level antibiotics, they show several problems including difficulties in high-cell-density culture, cumbersome genome engineering, and complex metabolic and regulatory networks. Therefore, model microorganisms including *E. coli* and *S. cerevisiae* have been employed for the heterologous production of antibiotics. *E. coli* is especially known for its well-established genome-scale metabolic models, capability for high-cell-density culture, abundant genome engineering tools, and high growth rate [184, 185]. A classical example of antibiotics produced by metabolically engineered *E. coli* is erythromycin, which involves a type I PKS. By introducing dexoyerythronolide B synthase (DEBS) from *S. erythraea* into *E. coli*, 6-deoxyerythronolide (6-dEB), an aglycone precursor of erythromycin, was produced for the first time by a heterologous host [186]. Further increasing the metabolic flux towards the precursors (acetyl-CoA, propionyl-CoA, and methylmalonyl-CoA) as well as overexpression of genes encoding deoxysugar glycosyltransferase resulted in 4 mg/L of erythromycin A production [187]. Such examples showcase the capability of *E. coli* to produce macrolide antibiotics which require a mega-sized assembly line of enzymes such as type I PKS or NRPS. Compared with type I PKS, type II PKS had been rather difficult to express in heterologous hosts [188]. The recent identification of a type II PKS from *Photorhabdus luminescens* led to successful production of aromatic C16 polyketides in *E. coli* [189, 190]. *E. coli* capable of heterologous production of aromatic C16 polyketides was particularly useful for the production of non-natural derivatives, which can lead to the development of unprecedented new antibiotics. Carbapenem, a β-lactam antibiotic, could be also produced in *E. coli* by the introduction of the *carABCDE* BGC from *Pectobacterium carotovorum* as well as the removal of key feedback inhibition from glutamate 5-kinase (ProB) responsible for the conversion of glutamate to glutamyl 5-phosphate [191].

Other prokaryotic hosts have also shown to be suitable for antibiotics production. For example, *Corynebacterium glutamicum* is a gram-positive bacterium capable of efficiently producing food-grade products, but has suffered from reduced cell growth when supplemented with propionate, a precursor for propionyl-CoA and methylmalonyl-CoA. To address this issue, adaptive laboratory evolution was employed to improve the fitness of the host for polyketide production in the presence of propionate, resulting in an 18-fold increase in germicidin production compared to the wild-type *C. glutamicum* [192]. *Pseudomonas* species can also be employed for antibiotic production due to its high tolerance towards toxic chemicals, efficient metabolic pathway towards aromatic compounds, well-established genome engineering tools, as well as adaptability to industrial processes [193]; some *Pseudomonas* species are natural producers of polyketide antibiotics such as 2,4-diacetylphloroglucinol [194] or mupirocin [195]. As malonyl-CoA is a central metabolite for the production of a number of categories of secondary metabolites including polyketides and phenylpropanoids, *Pseudomonas taiwanensis* was engineered by the deletion of competing pathways, replacing the native 3-ketoacyl-ACP synthase II with that from *Pseudomonas putida*, and overexpression of acetyl-CoA carboxylase from *C. glutamicum* [196]. This resulted in the enhanced production of flaviolin, pinosylvin, and resveratrol, and the same strategy can also be applied to the production of antibiotics derived from malonyl-CoA.

Another important heterologous microorganism model for antibiotic production is *S. cerevisiae* due to its capability of functional expression of tailoring enzymes and well-established genome engineering tools, as well as adaptability to industrial application [197]. For example, penicillin, naturally produced by NRPS in *Penicillium chrysogenum*, was produced in *S. cerevisiae*. Co-expression of NRPS and NRPS activator genes (i.e., *pcbAB* and *npgA*) along with three additional genes (i.e., *pcbC*, *pclA*, and *penDE*) from *P. chrysogenum* resulted in the production of 70 ng/mL of benzylpenicillin production [198]. In another study, a type III PKS from *Aloe arborescens* was introduced in *S. cerevisiae* for the production of dihydrokalafungin, a precursor of the antibiotic actinorhodin [199]. Along with *S. cerevisiae*, the non-conventional yeast strain *Yarrowia lipolytica* can also be used for antibiotic production due to its ability to efficiently produce proteins, its high flux towards acetyl-CoA, and its capacity to accumulate high levels of lipids, which can dissolve hydrophobic chemicals at high concentrations [200]. Leveraging these advantages, *Y. lipolytica* was engineered by implementing a pyruvate bypass pathway and overexpressing PEX10, which is associated with β-oxidation, leading to a substantial production (35.9 g/L) of triacetic acid lactone (TAL) from glucose [201]. TAL produced from *Y. lipolytica* can be easily converted into the antibiotic pogostone and its analogs through a one-step chemical conversion [202]. The examples discussed above highlight the potential of engineering model microorganisms for efficient production of antibiotics, providing a

viable response to the current lack of high-performance antibiotic production platforms.

Diversification of antibiotics

Engineering PKSs and tailoring enzymes

Diversification of antibiotics by addition of functional groups, modification of the carbon skeleton, and other structural alterations, is an effective strategy for developing new antibiotics with unprecedented properties to combat emerging MDR pathogens [203]. Analogs and derivatives generated through diversification can also facilitate the discovery of novel MoA for treating MDR bacteria or even overcoming physical barriers, such as biofilms. To diversify antibiotics, chemical or biochemical reactions are applied to lead compounds. While chemical reactions have been widely employed for this purpose, the structural complexity of many antibiotics, such as fused polycyclic carbon skeletons and multiple stereocenters, poses significant challenges for chemical synthesis [204]. Sensitive functional groups further restrict reaction conditions to prevent degradation or loss of efficacy. Therefore, synthetic biology has emerged to engineer enzymes and microbial strains that can efficiently produce diverse analogs and derivatives of antibiotics.

To produce diverse derivatives of a lead compound, tailoring enzymes are often employed to add various functional groups. A notable example is the biosynthesis of novel anthraquinones, a group of polycyclic aromatic polyketides, by the introduction of tailoring enzymes in addition to a type II PKS in *E. coli* [189]. In this study, a type II minimal PKS from *P. luminescens* that are phylogenetically close to *E. coli* fatty acid synthases was employed for the efficient biosynthesis of the carbon chain of C16 aromatic polyketides in *E. coli*. The introduction of an *O*-methyltransferase to the PKS-harboring strain resulted in the production of a novel methylated anthraquinone termed neomedicamycin, and the introduction of a halogenase to the same strain resulted in the production of a novel chlorinated anthraquinone termed neochaetomycin (Fig. 2A). Particularly, since chlorinated anthraquinones are reported to show enhanced antimicrobial activities as exemplified by chlorinated emodin [205], the production of halogenated polyketide derivatives demonstrate the potential for the development and production of new antibiotics. Glycosylation is another important reaction for improving the property of a lead compound. For example, the introduction of a glycosyltransferase YjiC in an *E. coli* strain harboring a type I iterative PKS capable of producing an antimicrobial anthraquinone emodin, resulted in the production of an emodin glucoside [206]. The glucoside showed enhanced solubility and stability when compared with those of emodin. Another example is the production of glycosylated erythromycin derivatives. To achieve this, a promiscuous glucosyltransferase EryBV was employed to attach deoxysugars to 6-dEB, resulting in the production of glycosylated erythromycin derivatives [207]. Additionally, ErtBV was capable of glycosylating 6-dEB using diverse deoxysugars including D-allose, D-forosamine, L-noviose, and D-vicenisamine, allowing the production of diverse 6-dEB glycosides [208]. Such 'plug-and-play' mode of biosynthesis is useful for the production of diverse derivatives and analogs of a lead compound by the introduction of different combinations of biosynthetic enzymes, showing great potential for the development and production of novel compounds with unprecedented antimicrobial activities.

Due to the modular nature of PKS, engineering the PKS itself is another important strategy for the generation of diverse antibiotic derivatives. One notable example is the production of non-natural fluorinated erythromycin analogs. Fluorination is widely used for the chemical modification of drugs to give new pharmacokinetic properties [209, 210]. To produce fluorinated analogs of 6-dEB, the native extender unit for erythromycin biosynthesis, (2*S*)-methylmalonyl-CoA, should be replaced with fluoromalonyl-CoA. As the *cis*-AT within the modular PKS is primarily responsible for the gatekeeping of extender units, it was eliminated from the PKS, and a standalone *trans*-AT engineered for enhanced substrate selectivity towards fluoromalonyl-CoA was introduced instead (Fig. 2B) [211]. Fluoromalonyl-CoA could be produced within the cell by the supply of fluoromalonate, which was converted to fluoromalonyl-CoA by a malonyl-CoA synthetase MatB. In another study, the AT domain of the DEBS module 6 was exchanged with AT from 12 different PKSs, allowing the incorporation of diverse CoA units (e.g., ethylmalonyl-CoA, butylmalonyl-CoA, and benzylmalonyl-CoA) as extender units [212]. In addition to AT domain engineering, modifying other domains within type I PKS modules can improve the production of target chemicals by enhancing inter-modular interactions. For example, as the thioesterase (TE) domain plays a key role in substrate selectivity, site-directed mutagenesis of TE was employed to produce non-natural epimerized hexaketide [213].

Other than swapping domains within the assembly lines, introduction of point mutations at or near the active sites can also lead to the production of diverse compounds. For efficient and rapid editing of large NRPS assembly lines within natural producers, CRISPR-Cas9 gene editing tool was optimized, allowing the production of ten new lipopeptide variants of enduracidin with high yields [214]. Combinatorial engineering of PKS modules is not only used for the production of antibiotics, but

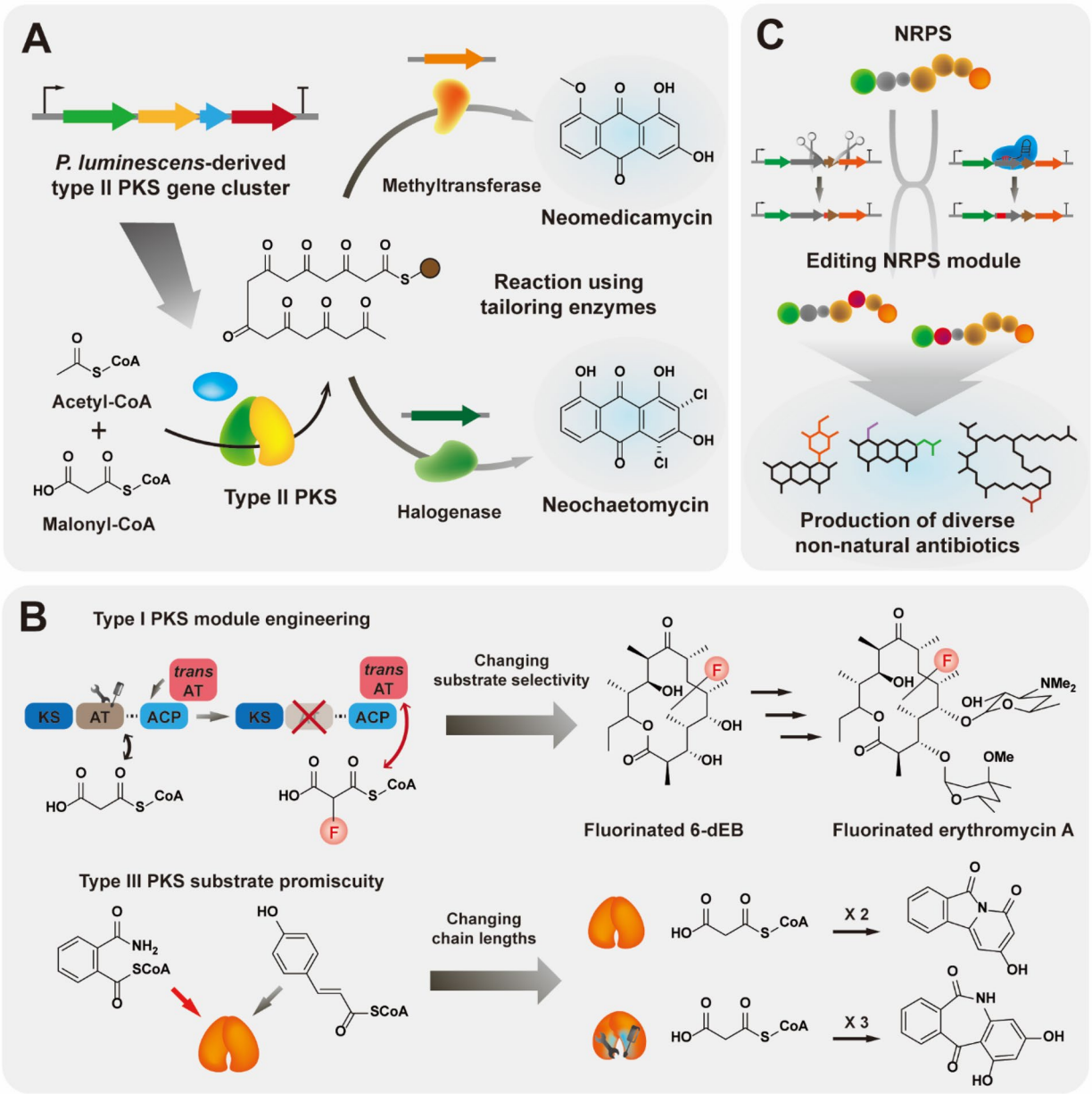


Fig. 2 Strategies for diversifying antibiotics. **A** Application of tailoring enzymes with type II PKS to diversify polyketide-based antibiotics. **B** Engineering of type I and III PKSs. Altering substrate-interacting domains in type I PKS changes starter/extender unit selectivity, facilitating the production of novel antibiotics. Production of fluorinated erythromycin using fluoromalonyl-CoA is shown as an example. For certain type III PKSs, substrate promiscuity can be employed to diversify products. Engineering the cavity near the active site of a type III PKS can alter the carbon chain lengths of the products. **C** As NRPS comprises functional domains, engineering these modular systems can lead to the production of a wide range of non-natural antibiotics

also for a diverse portfolio of chemicals, even including small chemicals (e.g., adipic acid, lactones, and ketones.) that have been conventionally produced from petroleum (Fig. 2C) [215–217].

Mutagenesis of type III PKSs is another effective strategy for diversifying antibiotics by virtue of their promiscuous substrate specificity [218–220]. For example, a type III PKS from *Huperzia serrata* (HsPKS) accepted a non-natural starter unit, 2-carbamoylbenzoyl-CoA, instead of *p*-coumaroyl-CoA, producing a new polycyclic alkaloid (2-hydroxypyrido[2,1-*a*] isoindole-4,6-dione) with two malonyl-CoA molecules

[221]. Expanding the active site cavity of HsPKS with the S348G mutation enabled the condensation of three malonyl-CoA molecules, producing another novel alkaloid (1,3-dihydroxy-5*H*-dibenzo[*b*,*e*]azepine-6,11-dione) [221]. Both non-natural alkaloids inhibited the formation of biofilm formation by MRSA. As discussed above, altering the cavity space for polyketide carbon chain elongation effectively diversifies polyketide chain lengths, as also shown in other type III PKS mutants [222, 223]. This shows the potential and versatility of engineering PKS for the production of diverse non-natural chemicals by engineered microbial cell factories.

Engineered PKS and tailoring enzymes are often introduced into heterologous microbial hosts, which frequently lack favorable intracellular conditions for the functional expression of these complex biochemical machineries. For example, as megasynthases such as type I PKSs are not always efficiently and solubly expressed in heterologous hosts due to their large sizes, the efficiency of expression of PKS-coding genes as well as the solubility of PKSs greatly affects the efficiency of polyketide biosynthesis. Also, when different modules within PKSs are recombined and engineered, protein folding and thus solubility can be affected, leading to alteration in the efficiency of polyketide biosynthesis. Therefore, monitoring the solubility and functionality of the engineered PKSs is important. In this regard, a biosensor that can monitor the abundance of misfolded and aggregated proteins was developed by employing promoters of *ibpA* and *fxs* that are observed to be highly expressed upon accumulation of misfolded proteins [224]. By the expression of the *mCherry* gene encoding a fluorescence protein under the *ibpA* promoter, the solubility of engineered PKSs could be easily monitored. Using the biosensor, a type I modular PKS harboring a hybrid acyltransferase domain showing high solubility and activity could be selected. The combined engineering of PKSs and tailoring enzymes could lead to the generation of a much larger number of new antibiotic candidates, potentially contributing to the development of new antibiotics with no existing resistance.

Semi-synthesis

While synthetic biology and enzyme engineering have been effective for producing diverse antibiotics and their derivatives, the range of reactions enzymes can catalyze still falls short of chemical synthesis. In this regard, semi-synthesis—combining biosynthesis with chemical reactions—can be employed. Semi-synthesis has been successful in developing new antibiotics from previously discovered classical antibiotics. Following the development of methicillin—the first semi-synthetic antibiotic derived from penicillin to combat β-lactamase-producing, penicillin-resistant bacteria—additional semi-synthetic antibiotics, such as ampicillin, amoxicillin, azithromycin, and tigecycline were subsequently developed [225]. One notable example is amoxicillin (a derivative of penicillin G), which is synthesized by attaching *p*-hydroxyphenylglycine to the amino group of 6-aminopenicillanic acid, the core structure of penicillin. This modification enhances the antibiotic's activity by inhibiting essential enzymes involved in the cross-linking of bacterial cell walls, resulting in a broader spectrum of antibiotic effects compared to penicillin [226]. Additionally, the production of arylomycin derivatives serves as another representative example. To diversify arylomycins, three moieties attached on the macrocyclic tripeptide core, an N-terminal lipopeptide tail, a C-terminal carboxylic acid, and two phenol groups, were modified. One of the derivatives, G0775 showed significanatly improved antibiotic effects against 49 MDR clinical strains of *E. coli* and *K. pneumoniae*, 16 MDR *A. baumannii* strains, 12 MDR *P. aeruginosa* strains, as well as methicillin-resistant *S. aureus* and *S. epidermidis* [227]. Another example is the production of derivatives of chelocardin, an atypical tetracycline produced by *Amycolatopsis sulphurea* [228]. Chemical modifications of amidochelocardin (2-carboxamid-2-deacetyl-chelocardin) through methylation, acylation, and halogenation resulted in the production of 22 different derivatives. Notably, fluorination at the C7 position led to the significant enhancement of antimicrobial activity (Fig. 3) [228].

Chemical modification can also lead to the enhancement of pharmacokinetic properties. For example, natamycin is an antibiotic with low toxicity, but its low bioavailability and solubility make it unsuitable for therapeutic use. To improve its solubility, natamycin was modified by attaching various diamines, among which the derivative containing an ethylenediamine moiety showed approximately a ten-fold increase in solubility and also two to eight-fold lower MIC [229]. Additionally, while natamycin lacks antibacterial effects at high concentrations, derivatives containing ethylenediamine or N-(2-fluorobenzyl) ethane-1,2-diamine moieties exhibited significant antibacterial effects.

Vancomycin has been one of the most effective antibiotics for treating complicated skin infections, bloodstream infections, endocarditis, bone and joint infections, and meningitis caused by methicillin-resistant *S. aureus* (MRSA), one of the most deadly pathogens. However, the emergence of vancomycin resistance has necessitated the development of vancomycin derivatives. To address this, a guanidinium motif was introduced at the vancosamine site in vancomycin, leading to the production of the lipoglycopeptide EVG7, which demonstrated superior antimicrobial activity compared to vancomycin

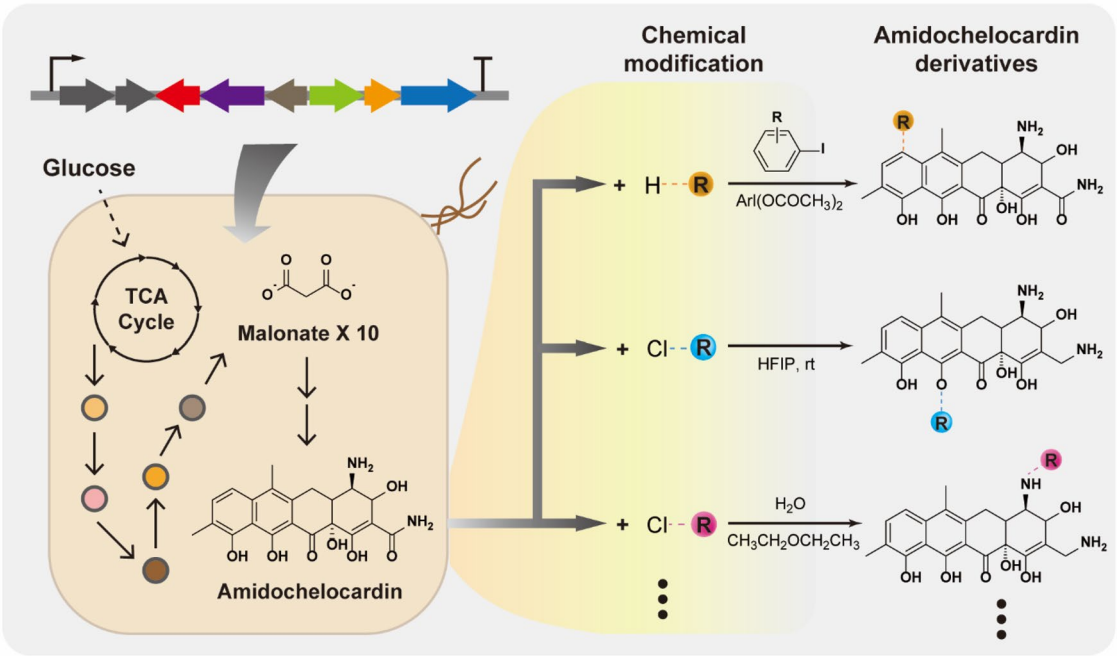


Fig. 3 Semi-synthesis for further diversification of antibiotics. Biosynthesis of atypical tetracycline, amidochelocardin, through metabolic engineering. Chemical modifications of amidochelocardin enable the production of diverse derivatives, some of which may exhibit enhanced antimicrobial properties

[230]. In addition, a biguanide-vancomycin conjugate, V – C6 – Bg-PhCl, showed superior antibacterial activities towards mycobacteria and the ESKAPE pathogens [231]. Derivatization of caprazamycin is another representative example of semi-synthetic antibiotics. Caprazamycin is an antibiotic derived from *Streptomyces* sp. MK 730-62F2, which is effective against several mycobacterial species as well as gram-positive and gram-negative bacteria. Acidic treatment of caprazamycins A-G led to the production of caprazene, which was further modified by adding alkylamide, anilide, and ester functional groups. Notably, while caprazene itself lost antibacterial activity, its derivatives restored and even showed higher antibacterial activities than the original caprazamycins [232]. Such examples demonstrate that diversification of known antibiotics to produce a series of non-natural antibiotics can be an effective strategy for combating MDR bacteria. For more in-depth studies on semi-synthetic antibiotics, readers are guided to the following literature [233, 234].

Conclusion

With the increasing threat posed by the emergence of MDR bacteria, the need for the discovery and development of novel antibiotics has grown. Although the rate of new antibiotic discovery had significantly slowed down after the 'golden era of antibiotic discovery' during 1940s ~ 1960s, new tools and strategies for the discovery and development of new antibiotics have continued to be reported in various fields, including biology, bioinformatics, synthetic biology, metabolic engineering, systems biology, and synthetic chemistry. As these tools and strategies have become more widely available, the discovery of new antibiotics directly from nature and the prediction of BGCs capable of producing new antibiotics through metagenome sequencing have become more common. As discussed in the above sections, culturing "unculturable" microorganisms for the discovery of new active compounds has greatly expanded the accessible chemical space in nature. Advances in single-cell sequencing technologies and genome mining tools have also allowed exploration of vast BGCs, which undoubtly harbor numerous BGCs capable of producing novel antibiotics with unprecendented properties [235]. Capturing such BGCs and introducing them into heterologous chassis microorganisms for producing them with large quantities has been made possible by advancements in synthetic biology strategies, including large gene cluster assembly, gene expression control, and extensive libraries of standardized biological parts. Activating cryptic BGCs in native producers has also proven useful for the discovery of new antibiotics. Such advancements have greatly facilitated the mining of "microbial dark matters" from

nature, which can be leveraged as antibiotics or serve as lead compounds for developing antibiotics [236].

Also, high performance bacterial strains capable of efficient production of antibiotics and their non-natural derivatives have been developed using synthetic biology, facilitating the translation of these new antibiotics to the clinic. State-of-the-art genome engineering tools, such as CRISPR, base editing, and prime editing, along with gene expression manipulation tools like CRISPRi and sRNA, have enabled the tailored construction of microbial cell factories capable of efficiently producing target antibiotics from renewable carbon sources in one-step bioprocesses [237]. With a defined target chemical and metabolic pathway, synthetic biology now enables significant increases in product titers. Improved understanding of metabolic and regulatory pathways in actinomycetes has also made it easier to manipulate these hosts, which are traditionally challenging to engineer but rich in secondary metabolites. Enzyme engineering has also played a crucial role in diversifying antibiotics to introduce new properties. Advanced enzyme and strain engineering strategies, such as automated directed evolution and machine learning-assisted metabolic engineering, will provide an expanded portfolio of reactions available for the synthesis of new antibiotics to combat MDR bacteria.

However, one notable challenge is that testing the efficacy of new antibiotics in animal models or clinical tests with human patients is difficult and time-consuming. This issue could potentially be addressed in the near future by employing 'organ-on-a-chip' technology, which mimics the environment of real organs within an animal or human. Using this technology will also allow for a deeper understanding of the MoA of new antibiotics and the physiological change in pathogens within the human body upon antibiotic treatment [238, 239]. Another challenge is that designing biosynthetic enzymes such as PKS or NRPS for the production of desired chemicals is still difficult. Recent advances in protein structure prediction and protein design models, such as AlphaFold3 [240], will eventually allow the creation of designable antibiotics using high-performance enzymes specifically designed to produce the target chemicals with desired chemical structures. The development of machine learning will not only facilitate the discovery of new BGCs from vast metagenomic data, but also allow for the design of antibiotics with specific chemical structures that can precisely interact with the target molecules within pathogens. Such designer antibiotics could be a game-changer in our fight against pathogenic bacteria. Recent applications of deep learning for the prediction of the antibiotic activity and cytotoxicity of millions of chemicals [241] showcase the potential of artificial intelligence (AI) in screening large libraries of antibiotic candidates [242, 243]. Other treatment options for combating MDR bacteria, such as antisense oligonucleotides, antimicrobial peptides, and microbiota-based therapeutics, are also being actively studied [244–246]. Continued efforts in developing innovative metabolic engineering and synthetic biology tools and strategies, combined with interdisciplinary collaboration in areas such as in silico enzyme modeling and semi-synthesis, will strengthen humanity's fight against infectious diseases.

Abbreviations

2D	Two-dimensional
6-dEB	6-Deoxyerythronolide
ABC	ATP-binding-cassette
AI	Artificial intelligence
AP-3	Ansamitocin P-3
Att/Int	Attachment and integration
BCP	Bacterial cytological profiling
BGCs	Biosynthetic gene clusters
CRISPR-BEST	CRISPR-base editing system
CRISPRi	Clustered regularly interspaced short palindromic repeats interference
DEBS	Dexoyerythronolide B synthase
EtBr	Ethidium bromide
HGT	Horizontal gene transfer
iChip	Isolation chip
LAL	Large ATP-binding regulator of the LuxR family
LC–MS	Liquid chromatography-mass spectrometry
MALDI-TOF/MS	Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry
MDR	Multidrug-resistant
MoA	Mechanism of action
MRSA	Methicillin-resistant *S. aureus*
NGS	Next-generation sequencing
NRP	Non-ribosomal peptide
OTUs	Operational taxonomic units
PAINS	Pan-assay interference compounds
PAM	Protospacer adjacent motif
PKS	Polyketide synthase
sgRNAs	Single guide RNAs
TAG	Triacylglycerol
TAL	Triacetic acid lactone
TESEC	Target essential surrogate *E. coli*

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Declarations

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