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Review Article

Striving for sustainable biosynthesis: discovery, diversification, and production of antimicrobial drugs in *Escherichia coli*

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New antimicrobials need to be discovered to fight the advance of multidrug-resistant pathogens. A promising approach is the screening for antimicrobial agents naturally produced by living organisms. As an alternative to studying the native producer, it is possible to use genetically tractable microbes as heterologous hosts to aid the discovery process, facilitate product diversification through genetic engineering, and ultimately enable environmentally friendly production. In this mini-review, we summarize the literature from 2017 to 2022 on the application of *Escherichia coli* and *E. coli*-based platforms as versatile and powerful systems for the discovery, characterization, and sustainable production of antimicrobials. We highlight recent developments in high-throughput screening methods and genetic engineering approaches that build on the strengths of *E. coli* as an expression host and that led to the production of antimicrobial compounds. In the last section, we briefly discuss new techniques that have not been applied to discover or engineer antimicrobials yet, but that may be useful for this application in the future.

Introduction

Antimicrobial-resistant (AMR) infections pose a serious threat to society [1], driving the need to discover and develop new antimicrobial molecules. Promising sources of such antimicrobials are naturally occurring chemicals produced by microbes, plants, and animals—the so-called natural products (NPs) [2]. Traditionally, the utilization of NPs has relied on their isolation from the native producers [3], but this carries several limitations. In brief, the major challenges of working with native producers are: (1) the uncultivability of many organisms under controlled conditions; (2) their slow growth compared with model organisms, and thus the limited availability of biomass for NP extraction; (3) the dependence of NP production on specific environmental stimuli; and (4) the lack of genetic tools to optimize NP production and generate derivatives.

To bypass these limitations, model organisms can be used as heterologous production hosts, often streamlining the discovery and characterization of NPs. Heterologous expression is achieved through cloning and overexpression of the genes encoding the underlying biosynthetic enzymes in a genetically tractable plant, fungus or bacterium. The use of heterologous hosts even allows the exploration of mutant variants or combinations of genes not found in nature [3]. Microbial hosts are often preferred over plants because they have shorter reproductive cycles and can be manipulated at a genetic level more easily. The strengths and weaknesses of each microbial host are extensively covered in other reviews [4,5].

Escherichia coli is one of the earliest and simplest microbial hosts to be used for heterologous expression of single genes and entire biosynthetic pathways (Figure 1a), particularly of bacterial origin [6]. For the expression of eukaryotic genes, *E. coli* is not as well suited, because it cannot splice introns and introduce post-translational modifications, and often does not support the proper folding of eukaryotic enzymes [7]. Furthermore, it may not provide the required cofactors or partner enzymes

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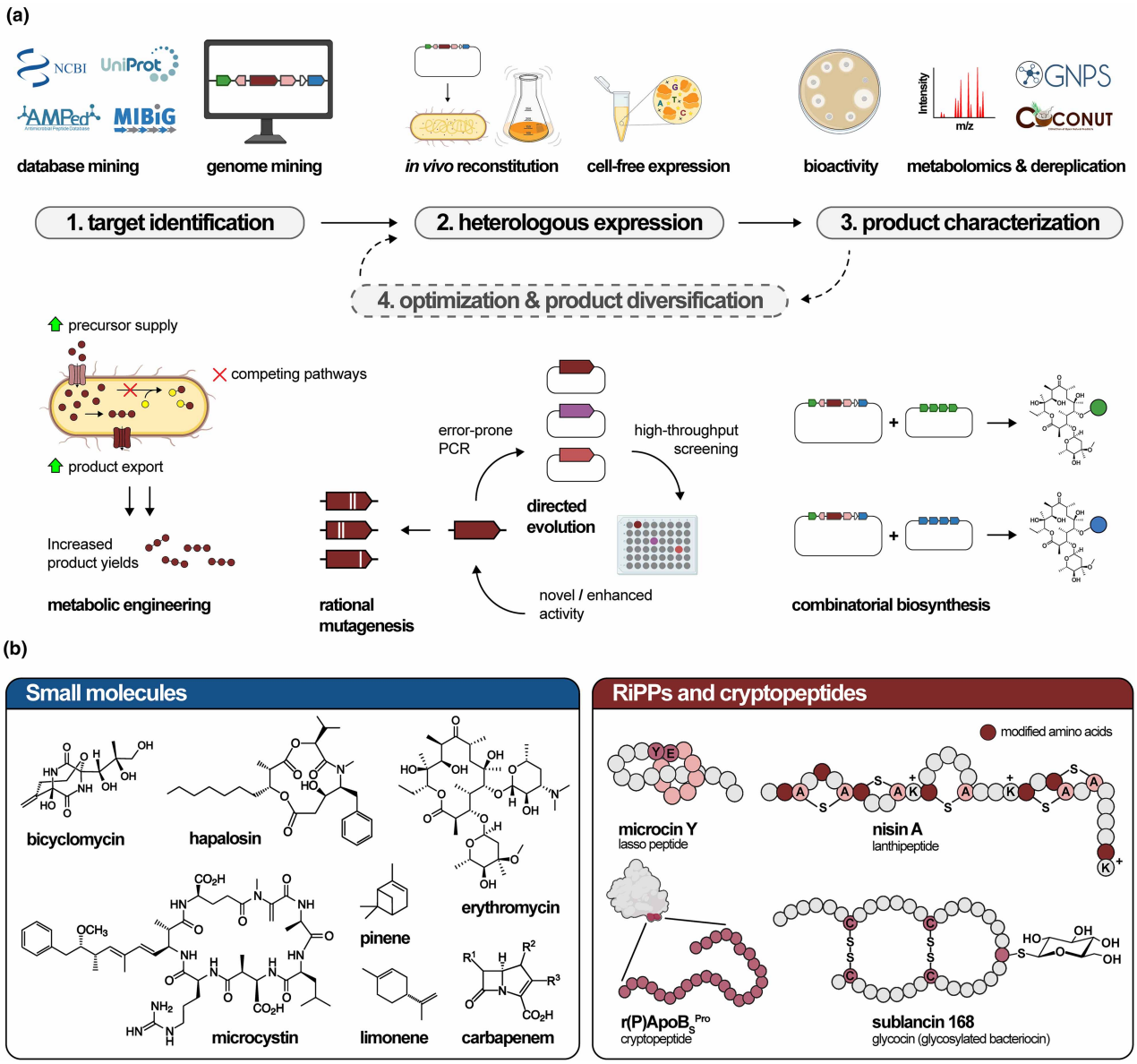

Figure 1. Overview of antimicrobial discovery and production in *E. coli*.

(**a**) Workflow for natural product discovery and production in *E. coli*-based heterologous platforms: a target BGC is selected via genome and/or database mining (step 1); the BGC is cloned into expression vectors for *E. coli* and either reconstituted and expressed in live cultures (*in vivo*) or in cell-free extracts (*in vitro*) (step 2); the engineered *E. coli* strains or their extracts can be used directly for plate bioassays to identify antimicrobial activity. Analytical chemistry techniques are used to analyze the metabolic profiles of such extracts for full chemical characterization and compound identification (step 3); (optional) strategies to optimize production yields and diversify the product, namely metabolic, enzyme engineering and combinatorial biosynthesis (step 4). (**b**) Examples of NPs with proven antimicrobial activity discovered or produced using *E. coli*. Figure created with BioRender.com.

(e. g, cytochrome P450 reductase) for enzymatic activity [8]. Many of these challenges can be overcome by carefully choosing the target enzymes, designing the expression constructs, and co-expressing important accessory proteins or pathways [8–10]. On the other hand, *E. coli* hosts have a high growth rate and the ability to yield large amounts of heterologous proteins. Furthermore, protocols for transformation with exogenous DNA and genetic manipulation in *E. coli* are well established and fast [11]. These features enable rapid clone--express–test cycles and high-throughput screening—fundamental advantages in the field of NP discovery and production. Indeed, *E. coli* has been successfully used to produce a wide range of bioactive NPs, including peptides, polyketides (PK), β-lactams, and more (Figure 1b, Table 1).

Table 1. Examples of NPs with antimicrobial activity produced in *E. coli*-based platforms Part 1 of 2

NP class	NP	*E. coli* platform	Titer (mg/L)	Genes/BGC expressed	Bioactivity	Reference
Bacteriocins	BM173, BM797, BM1029, BM1122, BM1556, BM1829, BMP11, BMP32	BL21(DE3) pLysS	/	/	Antibacterial (broad spectrum, including MDR strains of *Staphylococcus aureus*, *Cronobacter sakazakii* and *Salmonella*)	[109]
Bacteriocins	BtCspB	BL21(DE3)	20	*BtCspB*	Antibacterial (*Bacillus cereus* ATCC 10987). MIC: 12.5 μg/ml	[110]
Glycocins	Pallidocin, Hyp1, Hyp2	BL21(DE3)	0.15	*palAST*, *paldbAB*	Antibacterial (*Bacillus megaterium* DSM319, and some thermophilic bacteria). MIC range: 2.4 pM to 37 nM	[31]
Glycocins	Bacillicin CER074, bacillicin BAG2O, geocillicin, listeriocytocin	BL21(DE3)	/	Putative *sunAS*-like	Antibacterial (*E. coli* JM109, *Pseudomonas aeruginosa* PA01, MRSA, VRE, *Listeria monocytogenes* 4b F2365 and several *Bacillus* strains)	[47]
Glycocins	Sublancin derivatives	BL21 (DE3), SHuffle T7 Express	2	*sunAS*	Antibacterial (Gram-positive, including MRSA); not altered here	[19]
Glycocins	Enterocin 96	Lemo 21(DE3), KRX, BL21 (DE3), SHuffle T7 Express	25–30	ORF0422 ORF0417	Antibacterial (*L. monocytogenes*, *E. coli*, *Bacillus licheniformis*, *Vibrio cholerae*, *Bacillus halodurans*)	[21]
AMPs	Scyreprocin	BL21 (DE3)	/	Scyreprocin ORF	Antifungal, antibacterial (broad spectrum)	[111]
AMPs	Random combinatorial 20-mer library	*E. coli* W3110, WD101	/	**	Antibacterial (carbapenem-resistant *E. coli*, *Acinetobacter baumannii* Ab5075 and *P. aeruginosa* PA14)	[87]
AMPs	10 300 naturally occurring peptides	TOP10	/	**	Antibacterial (*E. coli* TOP10). MIC ≥ 0.5 μM	[88]
AMPs	Bottromycin A2 and derivatives	ET12567 (pUB307) for conjugation, GB05-red for Red/ET recombineering	1.15–11.5	BGC0000468	Antibacterial (MRSA, VRE); not measured here	[112]
AMPs	Oncocin and mutants	T7 Express LysY/Iq	/	oncocin V1M	Antibacterial (*E. coli* JW0013)	[90]
Cryptopeptides	(P)PAP-A3, (P) IMY25, (P)FLK22	BL21(DE3)	7–20	*pga3***	Anti-biofilm and antibacterial (broad spectrum, including MRSA and clinical isolates of *P. aeruginosa* and *L. monocytogenes*). MIC ≤ 10 μM	[25]
Cryptopeptides	r(P)ApoBL Pro, r(P) ApoBS Pro, r(P) ApoBL	BL21(DE3), BL21(DE3) pLysS	/	*apoB***	Antibacterial (broad spectrum, including drug resistant *Klebsiella pneumoniae*, *A. baumannii* and Staphylococci). MIC range: 2.5 –20 μM	[27]
Lanthipeptides	Haloduracin and analogs; lacticin 481	BL21 (DE3)	40–50	*halA2M2*, SPOmpA-*licP*; *lctAMT*	Antibacterial (*Lactococcus lactis* HP). MIC: 50 nM	[81]
Lanthipeptides	Lacticin 481; nisin analogs	BL21 (DE3), genomically recoded organism (GRO) C321.ΔprfA-T7RNAPΔrneΔompTΔlon1	0.2–0.8	*lctAM*; *nisABC*	Antibacterial (*Lactococcus lactis* HP). MIC range: 195–390 nM	[64]

Continued

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Table 1. Examples of NPs with antimicrobial activity produced in *E. coli*-based platforms Part 2 of 2

NP class	NP	*E. coli* platform	Titer (mg/L)	Genes/BGC expressed	Bioactivity	Reference
Lanthipeptides	Roseocin	BL21(DE3)	4	*rosA1A2M*	Antibacterial (Gram-positive: *L. monocytogenes* MTCC 839, *B. subtilis* MTCC 121, *S. aureus* MTCC 1430, MRSA, VRE, *P. aeruginosa* MTCC 1934, *E. coli* MTCC 1610 and *V. cholerae* MTCC 3904)	[23]
Lanthipeptides	Amylopeptins	BL21(DE3)	/	*amyA*(I–VII)*KC*	Antibacterial (*B. megaterium* R28). MIC: 9.0 μM	[24]
Lanthipeptides	Nisin analogs and mutants	BL21(DE3) cell extract, BL21 Rosetta (DE3)	6.5 ± 0.25	*lanA* (RL1–18), *nisBCP*	Antibacterial (*E. faecalis*, *Micrococcus luteus,* MRSA). MIC range: 0.037–74.75 μM	[18]
Lanthipeptides	Semisynthetic lipo-lanthipeptides	BL21(DE3)	/	*cinAM*	Antibacterial (MRSA, VRE, *A. baumannii, Shigella flexneri*, *K. pneumoniae*, *P. aeruginosa*). MIC range: 2–32 μM	[75]
Lasso peptides	Klebsidin and mutants	BW25113	/	*kleABC*	Antibacterial (*E. coli* and *K. pneumonia*). MIC range: 125–500 μM	[89]
Lasso peptides	Microcin Y	BL21	9.6 ± 4.5	*mcyABCD* (MccY)	Antibacterial (broad spectrum, including pathogenic isolates of *Salmonella* and *Shigella*). MIC range: 0.025–12.5 μM	[32]
Type I PKs	Erythromycin	TB3	/	*eryA(I–III)FGK*	Antibacterial; not altered here	[66]
Type I PKs	Erythromycin derivatives	BL21(DE3), BAP1	/	*eryC(I–VI)FGK, mtmDE*, *ermE, eryBV* + deoxysugar tailoring pathways	Antibacterial (broad spectrum, including erythromycin- and streptomycin-resistant *B. subtilis*). MIC range: 0.25–1 μg/ml	[74]
Type III PKs	Adipostatins A and B, several alkylresorcinols	ET12567/pUZ8002 for intergeneric conjugation, BL21(DE3) for expression	0.6–1.7	*adp*	Antibacterial (*S. aureus*, *B. subtilis*, *Enterococcus faecium*, *Staphylococcus epidermidis*, *K. pneumoniae*). MIC range: 1–64 μg/ml	[69]
NRP-PKs	[d-Asp3] microcystin-LR, microcystin-LR	BL21(DE3), BAP1, TB3	0.4–0.6	*mcyABCDEFGHIJ*	Antibacterial (*Mycobacterium tuberculosis*); not measured here. Inhibition of eukaryotic phosphatase type 2A	[113]
NRP-PKs	[d-Asp3, DMAdda5] microcystin-LR	GB05-Red for recombination, GB05-MtaA for expression	0.6	*mcyABCDEFGHI*	Antibacterial (*M. tuberculosis*); not measured here. Inhibition of eukaryotic phosphatase type 2A	[114]
NRP-PKs	Hapalosin	BAP1	/	BGC0001467	Reversal of multidrug resistance; not reported here	[39]
β-lactams	(5R)-carbapen-2-em-3-carboxylic acid (Car)	BL21(DE3)	54.1 ± 17.4	*carCBADE*	Antibacterial (*E. coli* BL21, autotoxicity)	[65]
2,5-diketo-piperazines	Bicyclomycin	S2060 for PACE experiments	0.6	*bcmABCDEFG*	Antibacterial; not altered here	[34]
Terpenoids	Limonene, α-pinene, β-pinene, bisabolene	BL21 Star (DE3) lysate	610	**	Antibacterial; not altered here	[52]

/ = not reported or not measurable; * = estimated; ** = sourced from a variety of organisms; *** = partial sequence; MIC = minimum inhibitory concentration; MRSA = methicillin-resistant *Staphylococcus aureus*; VRE = vancomycin-resistant Enterococci.

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In the workflow for NP discovery and production in *E. coli*, candidate biosynthetic genes or pathways are first selected from the genome of antimicrobial producer organisms (Figure 1a, step 1). Often, enzymes within a pathway are encoded by colocalized genes in so-called biosynthetic gene clusters (BGCs), which makes it possible to identify them with dedicated bioinformatic tools [12–15]. Once a target is selected, dedicated *E. coli* strains or cell-free extracts—optimized for NP production [9,10,16–18]—are used for overexpression (step 2) and characterization of the pathway products (step 3). *E. coli* is also an ideal host to optimize product yields through modulation of gene expression and enzyme performance by metabolic engineering, directed evolution, and combinatorial biosynthesis (optional step 4). In the following sections, we provide a summary of recent advances in the discovery and heterologous production of antimicrobials based on this workflow.

Heterologous production of antimicrobials in *E. coli*

Reconstitution of biosynthetic machineries *in vivo*

One of the simplest approaches for NP production in *E. coli* (Figure 1a, step 2) is to clone and overexpress a minimal set of genes of the target BGC. For example, the production of sublancin—an antimicrobial glycosylated RiPP (*r*ibosomally synthesized and *p*ost-translationally modified *p*eptide), or glycocin—was achieved by co-expressing the precursor peptide SunA and its cognate glycosyltransferase SunS [19]. As often done for this class of molecules [20], SunA was engineered with a fused His_6 tag for purification and a recognition site for the protease Xa. The latter allows the *in vitro* release of the bioactive form from the leader peptide after purification, thereby preventing toxicity in the host. This system was efficiently used for structure–activity relationship studies of sublancin to identify amino acids crucial for its antimicrobial bioactivity. Similarly, Choudhary and Rao expressed a mutant library of enterocin 96, a class II bacteriocin, as inactive fusion peptides with a pH-cleavable tag, enabling the rapid identification of a variant with broadened antimicrobial spectrum [21]. Another type of RiPPs, the lanthipeptides, require dedicated enzymes called lanthipeptide synthetases that generate the typical lanthionine bridge through dehydration and cyclization reactions [22]. To obtain the mature NP, the precursor peptide can be either processed *in vitro* with purified enzymes or co-expressed with the synthetase *in vivo*. The latter approach was used to discover roseocin, a two-component lanthipeptide with antimicrobial activity against a panel of Gram-positive bacteria (including important pathogens such as MRSA and VRE) [23], and amylopeptins, lanthipeptides with narrow-spectrum antibacterial activity [24].

A powerful approach that can be readily employed with *E. coli* is the use of fusion proteins to express antimicrobial peptides (AMPs) that would otherwise be toxic to the host. With this strategy, antibacterial cryptopeptides derived from human pepsinogen A3 [25] and apolipoprotein B were produced [26,27]. Cryptopeptides are small peptides that derive from the cleavage of precursor proteins, and often exhibit functions unrelated to that of their parent protein [28,29]. For both AMPs, the peptides were fused to a carrier through an acid-labile Asp-Pro linker, which allowed expression in *E. coli* as inclusion bodies at high yields. Once purified, the linker allows the chemically controlled release of the bioactive peptides. The peptides produced by Cesaro et al. showed potent antimicrobial and anti-biofilm activity against several pathogens belonging to the concerning ESKAPE group [27,30].

Complementary to the expression of the minimal set of genes, it is also possible to reconstitute entire BGCs. This approach was used to characterize the antimicrobial glycocin pallidocin from a thermophilic bacterium [31]. The entire *pal* BGC—including precursor peptide, tailoring enzymes, and transporter—was expressed under the control of an inducible promoter in *E. coli*, which successfully produced the glycosylated peptide in its bioactive form. Bioassays with purified pallidocin showed activity against several thermophilic bacteria and *Bacillus* sp. [31]. Analogously, the novel lasso peptide microcin Y from *Salmonella enterica* was discovered by overexpressing the entire MccY BGC in *E. coli* [32]. This yielded the fully bioactive form of microcin Y, which showed antibacterial activity against several Gram-positive and Gram-negative AMR bacteria, including pathogenic isolates of *Shigella* and *Salmonella* [32]. In addition to these numerous examples of AMPs, BGCs of other compound classes have been successfully reconstituted in *E. coli*, such as nonribosomal peptides [33], diketopiperazines [34] and type I PK [35]. Most recently three type II PK systems were functionally reconstituted and expressed in *E. coli* [36,37], which may lead to the development of antimicrobial PKs in the future.

Cloning entire BGCs via PCR amplification of the target genes and assembly into suitable vectors is time-consuming and can prove altogether unfeasible, especially with larger BGCs (>10 kb). To streamline the process, alternative methods have been developed. Direct pathway cloning (DiPaC) [38] relies on long-amplicon PCR and HiFi DNA assembly, where small to mid-sized clusters (up to ~25 kb) are amplified in one

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or more fragments and assembled *in vitro* into *E. coli* expression vectors. Among others, DiPaC was successfully used to reconstitute the BGC of phenazines from *S. fonticola*, and the BGCs of cyanobacterial nonribosomal peptides anabaenopeptins [38] and hapalosin [39], all of which possess valuable pharmacological properties. For larger BGCs, there are several methods based on *in vivo* homologous recombination that can be used to capture and mobilize the entire pathway directly from isolated genomic DNA [40–42]. Although they require complex workflows, it is advantageous that these methods do not require amplification steps, since BGCs are captured from genomic libraries. Recombination-based BGC capture has been successfully used to express BGCs up to 106 kb [41,43].

Despite the many successful examples discussed above, capturing and cloning entire BGCs might lead to unsuccessful transcription of the genes because their native regulatory systems may not function in the heterologous host. One possibility to overcome this is pathway refactoring [44], which typically involves the engineering of promoter and other regulatory regions to bypass the native regulatory system and achieve defined expression levels. Tools such as Golden Gate Cloning—a method for the simultaneous one-pot assembly of multiple DNA fragments into one single piece [45]—enable rapid refactoring of large pathways in a few steps [46]. This strategy is widely employed for the assembly of heterologous pathways in *E. coli* and was successfully used to rapidly discover four novel glycocins through overexpression of their refactored pathways in *E. coli*, two of which exhibited strong antibacterial activity against *Bacillus cereus*, a widespread foodborne pathogen [47].

Overall, the current genetic toolbox for *E. coli* is versatile, however, there remain challenges for its application for antimicrobial discovery. First, heterologous genes and NPs may be toxic to *E. coli*. Second, expression levels and solubility of the target enzymes might be suboptimal, preventing further characterization efforts. Both problems might be overcome with the use of cell-free expression systems.

E. coli-based cell-free platforms

E. coli-based cell-free protein synthesis (CFPS) was pioneered >60 years ago [48], yet only recently has it truly matured in terms of user-friendliness and reproducibility [49,50]. Apart from its applicability for the synthesis of toxic or unnatural proteins, CFPS offers possibilities for rapid *in vitro* BGC prototyping and modular pathway construction. For instance, candidate enzymes—even from different organisms—can be expressed using CFPS, and then combined in a mix-and-match fashion [51]. This approach was used to produce the industrially relevant terpenes limonene, pinene and bisabolene [52], as well as phytocannabinoid acids with therapeutic potential [53]. CFPS platforms can also be tailored to produce and characterize specific classes of NPs. For example, a customized cell-free platform expressing the biosynthetic machinery of nisin—one of the most studied lanthibiotics—was employed to rapidly prototype a series of putative lanthipeptides [18]. For that, the sequence of each candidate core peptide was fused to the nisin leader, so that the CFPS system would be able to directly process their bioactive form. In total, four novel lanthibiotics were identified, all of which showed activity against the indicator species *Micrococcus luteus*, and *Enterococcus faecalis*. Several other studies produced mature RiPPs with cell-free expression systems [54–58], yet bioactivity assays were not reported.

Since CFPS offers an open reaction system without the limitations of transport across membranes, it can be fully tailored to the specific application by providing cofactors, coenzymes, precursors, and other reaction components of choice.

Pathway optimization and product diversification

Metabolic engineering of the *E. coli* host

Metabolic engineering is a powerful tool to increase product titers and enable further modifications of the core scaffold in a heterologous host. It can be used to optimize the metabolic flux towards the desired product by enzyme engineering, removal of feedback inhibition, increasing precursor supply, blocking of competing pathways, and other techniques (Figure 1a, step 4) [59]. The large number of genome editing tools and techniques available for *E. coli* [60,61] allow quick and precise manipulation of this organism for biotechnological applications.

In the context of NP biosynthesis in *E. coli*, metabolic engineering has been used to build chassis strains such as HM0079 and BAP1, that are optimized to support the synthesis of complex NPs by expressing phosphopantetheine transferase, an enzyme crucial for the function of the polyketide synthases and nonribosomal peptide synthetases [9,10]. BAP1 and its derivatives are further optimized to support increased pools of building blocks for polyketide biosynthesis, in particular erythromycin and its analogs [62]. Also, genetically recoded

strains that allow the incorporation of non-canonical amino acids by amber stop codon repression into enzymes and RiPPs are intriguing chassis strains for NP biosynthesis [63]. With such a recoded strain Kakkar et al. [64] recently generated unnatural variants of the lantibiotics nisin and lacticin 481, demonstrating its applicability for the direct modification of peptide NPs.

Metabolic engineering is furthermore employed to alleviate specific pathway bottlenecks. In a recent effort, Shomar et al. [65] engineered a strain with elevated pools of precursors and increased β-lactam tolerance through the timed arrest of fatty acid synthesis (FAS). When FAS arrest is induced, *E. coli* cells stop growing—becoming tolerant to the β-lactams they produce—but remain metabolically active for many generations and are able to yield higher titers. This engineered strain can serve as a powerful platform for carbapenem production and diversification through the co-expression of tailoring enzymes.

Another example of classic metabolic engineering is how Fang et al. [66] increased the production of the glycosylated macrolide antibiotic erythromycin A by 400-fold by knocking out the pathways competing with deoxysugar incorporation. Notably, the large BGC (>50 kB) was cloned into two bacterial artificial chromosomes, each encoding a discrete stage (scaffolding or tailoring) of erythromycin biosynthesis. Such separation enables systematic manipulation of either stage of the biosynthetic process to generate new analogs.

Strategies to unlock new antimicrobial variants

Along with the search for novel antibiotics, a promising approach to combat AMR is the chemical diversification of known antimicrobial scaffolds. Heterologous expression in genetically tractable hosts such as *E. coli* facilitates the generation of new bioactive derivatives via precursor-directed biosynthesis, rational engineering, and combinatorial biosynthesis.

Precursor-directed biosynthesis exploits the inherent substrate promiscuity of many enzymes in NP pathways. The incorporation of alternative building blocks can thus be achieved by restricting defined nutrients and/or by feeding substrates of choice [67]. With the latter approach, new erythromycin analogs were generated by feeding synthetic building blocks that mimicked native biosynthesis intermediates [68]. Furthermore, expression in a heterologous host may already provide alternative building blocks, resulting in the production of analogs with altered biological activities. For example, the expression of a stand-alone actinobacterial type III polyketide synthase in *E. coli* resulted in the production of antimicrobial adipostatins A and B and nine additional alkylresorcinols, some with enhanced activity against AMR pathogens [69].

Rational mutagenesis of active site residues alters the substrate scope of an enzyme and enables the production of specific derivatives. For example, it was recently demonstrated that fluorinated building blocks can be incorporated into macrolide antibiotics by using engineered polyketide synthases [70,71]. Similarly, the lyngbyatoxin-producing nonribosomal peptide synthetase was engineered towards the production of pharmaceutically relevant indolactam variants [64,72,73].

Combinatorial biosynthesis enables the generation of NP analogs by genetic manipulation of biosynthetic pathways, for instance by mixing and matching of non-cognate enzymes. Fang et al. [74] diversified the bioactivity of erythromycin analogs by incorporating engineered deoxysugar pathways within their *E. coli* heterologous system. Three of these analogs were active against an erythromycin-resistant strain of *Bacillus subtilis*. Similarly, lipidation can be used to modulate the biological activity of peptide-based antimicrobials. Zhao et al. [75] employed a hybrid approach combining *E. coli*-based expression and processing of disulfide-containing peptides with *in vitro* lipidation. The resulting non-natural lipo-lanthipeptides exhibited bactericidal activity against several human pathogens through membrane disruption—a mode of action different from the parent peptides.

Combinatorial biosynthesis and random mutagenesis techniques have the potential to become one of the largest sources of new antimicrobial compounds. As they require efficient screening techniques, we will discuss these in detail in the following section.

Screening and characterization of antimicrobials in *E. coli*

Screening is a fundamental step in the discovery of new antimicrobials. Once a BGC has been successfully expressed, its products need to be characterized, ideally through a combination of bioactivity testing and analytical chemistry (Figure 1a, step 3). Characterization can be carried out at a single-colony level, where extracts or whole cells from clonal cultures are analyzed for NP production, or in bulk, where large libraries are screened in parallel with high-throughput techniques. In the following section, we will briefly discuss some of the most widely used methods with *E. coli*-based platforms (Figure 2).

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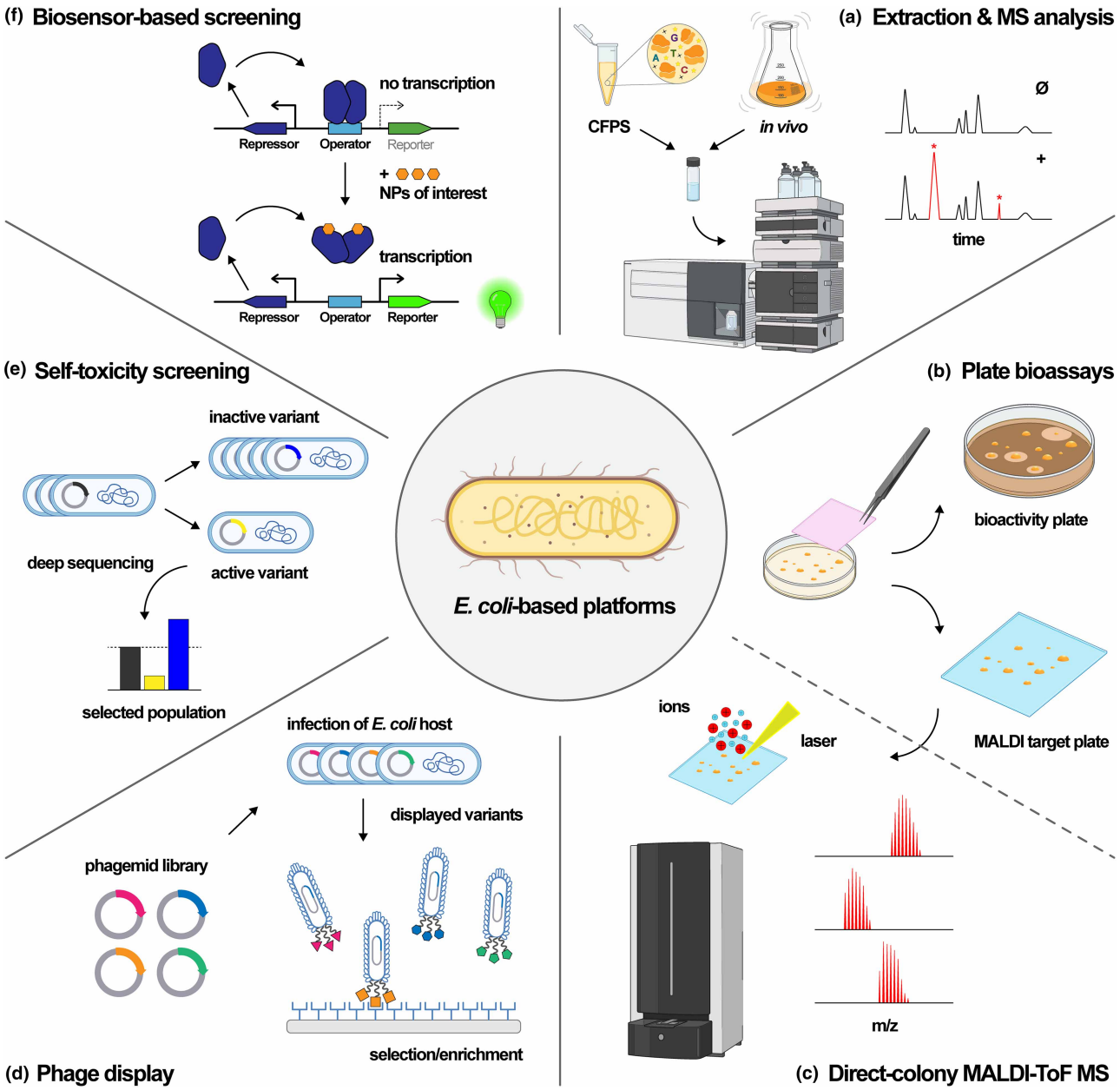

Figure 2. Strategies for screening and characterization of antimicrobial NPs in *E. coli*.

(**a–c**) single-colony approaches with (**a**) metabolomic profiling of *E. coli* extracts by mass spectrometry, (**b**) plate-based bioactivity screening of *E. coli* colonies possibly coupled to (**c**) direct-colony MALDI for metabolomic profiling. (**d–e**) parallel high-throughput approaches with d) phage display to enrich for peptide NPs with high affinity for a drug target protein, (**e**) self-toxicity screens selecting for peptide NPs active against Gram-negative bacteria and (**f**) biosensor-based screening to increase titers of antimicrobial NPs. Figure created with BioRender.com.

Single-colony screening

Nowadays, HPLC and MS are the standard techniques for the chemical identification and characterization of heterologously expressed NPs. Herein, extracts of *E. coli* strains (or CFPS reactions) expressing the target BGC are compared with the host background to identify newly produced molecules (Figure 2a). Scale-up of the fermentation and compound purification then allow structural characterization via high-resolution MS (HRMS) and NMR [76,77]. MS and NMR spectra enable the rapid identification of the produced NPs (dereplication) [78]. Complementary to that, agar plate bioassays (Figure 2b) are often used to screen *E. coli* transformants (or

their extracts) for antimicrobial activity—an approach largely unchanged over almost a century [79]. The main disadvantages of this method are the high limits of detection in terms of potency and quantity of the metabolites. Furthermore, plate bioassays do not provide any insight into the metabolic profile and therefore cannot be used for the chemical identification of bioactive compounds. Fortunately, plate bioassays can be easily combined with direct-colony MALDI-ToF MS [80,81] to overcome the latter, by replicating the bacterial colonies on a MALDI target plate (Figure 2b,c). This was elegantly showcased in a recent study on lanthibiotics [81], where researchers could directly link amino acid changes to the bioactivity of the peptide variants in a single experiment. Other MS techniques for direct metabolic profiling of live cultures exist [82,83], but they have not been used in combination with bioactivity assays yet.

Parallel high-throughput screening

One of the most common and powerful high-throughput techniques used in *E. coli* is phage display (Figure 2d), although it is only suitable for peptide NPs. Herein, a library of peptides—fused to a phage coat protein—is cloned into the host and the newly assembled virions will display the peptides of interest on their surface. By combining this step with systems such as ELISA or streptavidin-biotin–based co-immobilization, large libraries (>10^6 phages) can be screened against biological targets and enriched for variants with increased binding affinity [84]. This technique was applied to screen and enrich libraries of lanthibiotics using lipid II as binding target [85], as well as a library of artificial lanthipeptides that bind specifically to human urokinase plasminogen activator (uPA), an important factor in tumor progression [86].

Several new approaches treat bacterial death as the target phenotype, leveraging the dissonance between antibiotics discovery and bacterial host systems. Herein, a library of natural or synthetic AMP candidates is expressed in a bacterial host, and the grown cultures are sequenced to identify the least frequent—and thus the most potent—antimicrobial sequences (Figure 2e). *E. coli* can therefore serve as a chassis for the development of self-inhibition screening platforms for antibiotics against Gram-negative bacteria. Surface localized antimicrobial display (SLAY) was the first technique to apply this approach, screening 800 000 peptides in a single tube [87]. A library encoding random 20-mer peptides was expressed on the *E. coli* cell surface and the pool of clones was sequenced pre- and post-induction of expression. The initial screening revealed 7968 hits with potential antimicrobial activity. Interestingly, these peptides possessed an unprecedented range of physicochemical properties different from those of naturally occurring AMPs, which are commonly positively charged and hydrophobic. Of the 22 hits chosen for experimental validation, most proved to be active against *E. coli*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* in the low μM range. Similarly, Koch et al. [88] screened a library of 10 663 naturally occurring peptides with a self-inhibition screen, in which >1000 sequences were active. From these hits, 15 were chemically synthesized and 10 peptides inhibited *E. coli* at a minimal inhibitory concentration in the upper nM to low μM range. In addition, autotoxicity-based approaches were used to conduct comprehensive mutational studies of the lasso peptide klebsidin [89] and a proline-rich AMP, oncocin [90], providing new insight into their mechanism of action and enabling sequence-activity mapping.

Whole-cell biosensors also emerged as a powerful tool to detect the structure and report the titer of a given NP with high sensitivity and specificity [91]. The basic principle relies on metabolite-sensing proteins, such as allosteric transcription factors, which are activated upon binding of effector molecules and in turn control the expression of an actuator part (e.g. fluorescent reporters, regulatory switches, or selection markers) [92] (Figure 2f). A well-known example of a natural antibiotic biosensor is the erythromycin resistance repressor protein (MphR). This transcription factor controls the transcription of a gene cassette responsible for resistance to macrolide antibiotics and is derepressed by several macrolides with varying degrees of efficiency. Kasey et al. [93] transformed *E. coli* with libraries of rational and random MphR variants to reveal macrolide biosensors with altered inducer specificities. Contrary to wild-type MphR, some variants were induced by pikromycin and roxithromycin, revealing remarkable plasticity for engineering new sensitivities and selectivity in this transcription factor [93]. Li et al. [94] further shifted the specificity of MphR towards clarithromycin, while Miller et al. [95] improved its sensitivity towards erythromycin. One recent example of how biosensors can be applied to improving antibiotic production, is the work of Johnston et al. [34]. The authors exploited the sensitivity of the Rho-dependent λtR1 terminator to the antibiotic bicyclomycin to apply selective pressure in a phage-assisted continuous evolution experiment (PACE) and generate more efficient variants of the bicyclomycin pathway. Here, higher amounts of bicyclomycin led to increased production of infectious phage particles and thus PACE gradually selected for pathway variants that yielded higher titers of antibiotics. Similarly, d'Oelsnitz et al. [96]

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developed highly specific and sensitive alkaloid biosensors, which were directly employed to evolve an engineered simplified pathway for tetrahydropapaverine, an important precursor to pharmaceutical compounds. Biosensors may also be useful for the metabolic engineering of producer strains. The FapR-based biosensor engineered by Kalkreuter et al., for instance, detects malonyl-CoA and its C2-substituted derivatives, which serve as extender units in the synthesis of polyketide antibiotics such as merochlorin B, splenocin B and macrolides [97]. The utility of whole-cell biosensors for the selection and engineering of potent specialized antimicrobials is expected to expand as more transcription factors are being characterized.

Future directions

With the rapid development and increasing accessibility of high-quality genome sequencing and DNA synthesis, the field of NP discovery through genome mining and heterologous expression is moving quickly [98]. New pathway cloning strategies facilitate fast or even automated cloning and refactoring of BGCs for experimental characterization [38,42,99,100]. Several approaches have been used to assemble libraries of natural, engineered, or even combinatorial pathway variants [101–103], yet they remain to be applied for the discovery of antimicrobial NPs. Lastly, for the bioactivity screening step, the field is moving towards medium- and high-throughput approaches that enable parallel screening of larger pathway libraries. Herein, adaptations of the two approaches recently developed by Yang et al. [104] and Sarkar et al. [105] could be particularly interesting for antimicrobial or antiparasitic discovery. In both studies, *E. coli* was used as the expression host for a set of NP pathway variants, as well as the biological target protein(s). Inhibition of the target protein(s) was coupled to the survival of the host and thus enabled the rapid detection of bioactive NPs [104,105]. Furthermore, whole-cell biosensor approaches reporting antibiotic-induced stress reactions as used with other bacteria [106,107] could be further developed for applications in *E. coli*.

As already highlighted by the 'pressure test' commissioned by the U.S. Defense Advanced Research Projects Agency in 2018, *E. coli* and its cell-free lysates are exquisite platforms for the generation of target compounds, including antimicrobials [108]. This is particularly true for peptides, with many approaches and screening methods available that allow fast product characterization and massive parallelization. Since the expression of large biosynthetic machineries such as nonribosomal peptide synthetases and some types of polyketide synthases remains challenging, the further development of tailored host strains and cell-free platforms might offer solutions to expand the range of NPs produced with *E. coli*. Thus, it may gain even more importance for antimicrobial discovery and sustainable production in the future.

Perspectives

- New antimicrobial drugs are needed to combat emerging AMR pathogens. Antimicrobial drug development is deeply rooted in the discovery and exploitation of NPs.
- *E. coli* is a powerful and versatile workhorse for the heterologous expression of enzymes and biosynthetic pathways and has been heavily used to discover and produce numerous antimicrobial drugs.
- Recent advances in pathway cloning and refactoring, and new screening technologies, allow rapid and parallelizable prototyping of biosynthetic pathways in *E. coli*. This will accelerate the discovery of new leads for drug development and the sustainable biosynthesis of antimicrobial drugs.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Author Contributions

R.I., N.S. and K.H. collected literature. R.I. and N.S. wrote the manuscript. All authors edited the manuscript, read, and approved the final version.

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Abbreviations

AMPs, antimicrobial peptides; AMR, antimicrobial-resistant; BGCs, biosynthetic gene clusters; CFPS, cell-free protein synthesis; DiPaC, direct pathway cloning; FAS, fatty acid synthesis; NPs, natural products; PACE, phage-assisted continuous evolution experiment; PK, polyketides.

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