Cellular and Molecular Life Sciences (2018) 75:2719–2733
https://doi.org/10.1007/s00018-018-2827-7

REVIEW

Engineering microbes for targeted strikes against human pathogens

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Received: 18 December 2017 / Revised: 6 April 2018 / Accepted: 23 April 2018 / Published online: 7 May 2018
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Abstract
Lack of pathogen specificity in antimicrobial therapy causes non-discriminant microbial cell killing that disrupts the microflora present. As a result, potentially helpful microbial cells are killed along with the pathogen, altering the biodiversity and dynamic interactions within the population. Moreover, the unwarranted exposure of antibiotics to microbes increases the likelihood of developing resistance and perpetuates the emergence of multidrug resistance. Synthetic biology offers an alternative solution where specificity can be conferred to reduce the non-specific, non-targeted activity of currently available antibiotics, and instead provides targeted therapy against specific pathogens and minimising collateral damage to the host's inherent microbiota. With a greater understanding of the microbiome and the available genetic engineering tools for microbial cells, it is possible to devise antimicrobial strategies for novel antimicrobial therapy that are able to precisely and selectively remove infectious pathogens. Herein, we review the strategies developed by unlocking some of the natural mechanisms used by the microbes and how these may be utilised in targeted antimicrobial therapy, with the promise of reducing the current global bane of multidrug antimicrobial resistance.

Keywords Targeted therapy · Synthetic biology · Infectious pathogen · Live biotherapeutics · Microbiome · Phage engineering · Antimicrobial peptide · Antibiotic resistance

Introduction

Over the past decade, the fight against bacterial infections has intensified as the availability of effective antibiotics dwindles and the development of new classes of novel antibiotics is impeded with escalating difficulties. Furthermore, as the importance of commensal microbes for health-beneficial activity is increasingly recognised to be pertinent, antibiotics with broad-spectrum activity are becoming less desirable [1]. Therefore, the complex interactions within microbial populations required for health has to be taken into consideration in the development of antimicrobial therapy. The corollary for current antimicrobial therapy lies in novel strategies that can provide a selective removal of infectious opportunistic pathogens, without disturbing the healthy balance of neighbouring microflora (and microfauna). In doing so, it will also mitigate against the occurrence of antibiotic resistance, as exposure to antibiotics inevitably promotes selection pressures towards resistance, not only in the pathogen, but also in other commensal and dormant opportunistic pathogens.

Aided by a growing range of well-characterised genetic tools, synthetic biology enables the deliberate design and engineering of complex genetic constructs, paving the way for the implementation of novel functions and behavioural reprogramming of microbial cells [2]. The ability to introduce highly modular and programmable functionalities can be employed to generate new, effective antimicrobial strategies, particularly in the area of pathogen-specific detection and targeting mechanism [3]. For example, the molecular

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distinction between pathogens based on specific secreted or metabolised molecules can be exploited to confer pathogen selectivity in engineered microbes. Furthermore, selectivity can be achieved by introducing target-specific domains into antimicrobial peptides to augment pathogen-specific antimicrobial activity.

Traditional screening and development of antibiotics often involve the testing of antibiotic classes against a limited group of pathogens for their bactericidal activities. This overtly simplified testing platform overlooks the presence and possible involvement of microbial communities, or microbiota, in co-existence with the pathogens. With the advances in microbiome analyses, it has become evident that complex interspecies interactions exert dynamic control over the natural composition of microbes within a population. By identifying the natural activator or suppressor of pathogens and harnessing their intrinsic interactions, modulation of the microbiome can offer means to prevent or suppress the occurrence of pathogenic infections.

In addition, the rapidly expanding knowledge of the composition and function of the human gut microbiome has facilitated our quest to expand members of our microflora with potential health benefits, or probiotics. The extended range of beneficial microorganisms provides an excellent platform for the development of live antimicrobial therapy. Such microorganisms may even be engineered to enhance their beneficial functions, or express specific beneficial molecules. Under a new US regulatory framework, genetically modified microbes that are applied to prevent, treat or cure diseases or conditions in human beings are termed as live biotherapeutic products (LBPs) [4]. Taken together, engineered probiotics or LBPs can potentially be developed as an excellent live antimicrobial therapy that can exert specificity to discern targeting pathogens.

In this review, we discuss recent advances in novel antimicrobial strategies which incorporate pathogen selectivity by employing the latest advancements in programmable functionalities of microbial cells and expanded knowledge of microbiome. In particular, the approaches are reviewed based on the use of appropriate chassis (i.e. bacteria, phage and microbiome) to deliver different levels of pathogen targeting, in an attempt to develop a class of LBPs that can serve as antimicrobial therapy (Fig. 1).

Cell-mediated pathogen targeting

To augment current antibiotics that exhibit broad-spectrum activity, synthetic, natural or hybrid antimicrobial peptides have been developed to improve efficacy and confer specificity. Microbial cells innately produce a repertoire of antimicrobial molecules, such as bacteriocins, as a self-protection mechanism that helps the microorganisms to survive in their natural habitats. Bacteriocins are small, ribosomally

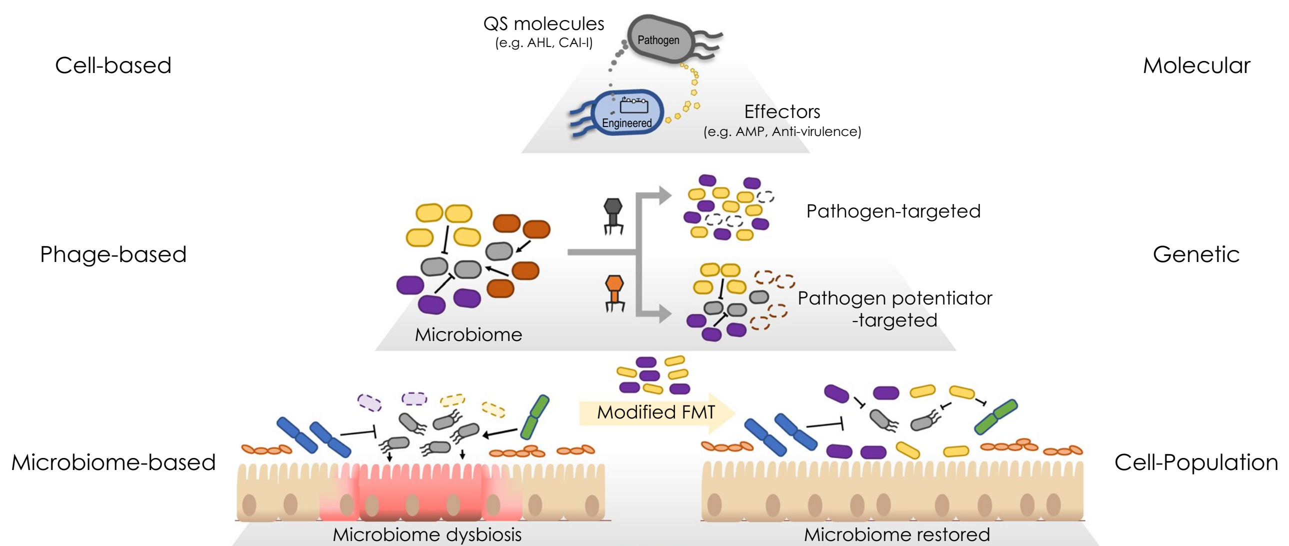

Fig. 1 Summary of pathogen targeting strategies broadly characterised at various levels of selectivity. The cellular level involves two-way communication between the engineered bacteria and pathogen where the engineered bacteria releases effector molecules in the presence of signal molecules from pathogens. The introduction of bacteriophages specifically eliminates the pathogen or pathogen potentiators based on genetic sequence of the target. The microbiome level approach involves the restoration of disrupted microbiome through the re-introduction of commensal strains to inhibit or modulate pathogen populations

synthesised peptides that are produced by bacteria to be active against other bacteria, most commonly targeting a narrow spectrum of species that are closely related to the producers [5]. This specificity is likely conferred by the protein recognition-mediated activity of the bacteriocins, as demonstrated by the lower concentrations required to achieve bactericidal effects compared to antibiotics. Based on this, adaptation to introduce target recognition sites into antimicrobial peptides can be used as a means to confer pathogen specificity. In addition to antimicrobial molecules, the microbes can also secrete various metabolites and molecules that serve as cell signalling molecules. They can sense and respond to their population density via self-produced diffusible molecules, termed quorum sensing (QS) molecules. Such a molecular-based approach has been employed against various infectious pathogens, exploiting their unique secretion molecules associated with the pathogen as an activator for downstream expression to execute antimicrobial activity against pathogens that are highly problematic in clinics.

Pathogen-specific 'sense-and-kill'

There are a number of studies on the development of cell-based novel biosensors and on the identification and development of effective antimicrobial peptides that are natural or synthetic to expand the list of available antibiotics for therapy. However, only a few studies have emerged from these pre-established disciplines to combine both biosensors and antimicrobial peptides to execute 'sense-and-kill' functionalities against human pathogens (Fig. 2). Herein, we review some of the emerging strategies taken in the field of synthetic biology that have been applied against selected human pathogens to perform highly selective and autonomous antimicrobial activities (summarised in Table 1).

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a common Gram-negative pathogen, which has gained notoriety for exhibiting multidrug resistance and causing serious hospital-acquired infections, ranging from severe sepsis, skin and soft tissue infections, to life-threatening pneumonia. Although the immunocompetent may succumb to *Pseudomonas* infections, immunocompromised hosts are particularly susceptible. The ability for *P. aeruginosa* to create a biofilm contributes to its virulence [6], as the biofilm forms a protective layer shielding the pathogen against traditional bactericidal interventions such as antibiotics.

Pseudomonas aeruginosa utilises specific QS molecules, typically acyl homoserine lactone (AHL) molecules for regulating bacterial gene expression [7], which in turn coordinates several phenotypic features of the pathogen. For example, secretion of extracellular polymeric substances can lay a foundation to form biofilm [8], which encapsulates the pathogen to confer antibiotic resistance and acts as a reservoir for recurrent infection. Hence, cellular circuits are designed to sense this specific QS molecule to seek and target *P. aeruginosa*. In Saeidi et al. [9], implementation of the quorum sensing circuit in *E. coli* to sense *N*-(3-oxododecanoyl) homoserine lactone produced by *P. aeruginosa* was coupled to an activation of Pyocin S5 and Lysis E7 protein production within *E. coli*. The selectivity of pyocin S5 against *P. aeruginosa* was validated previously [10], and the release of accumulated pyocin S5 was coordinated by the E7 lysis protein to result

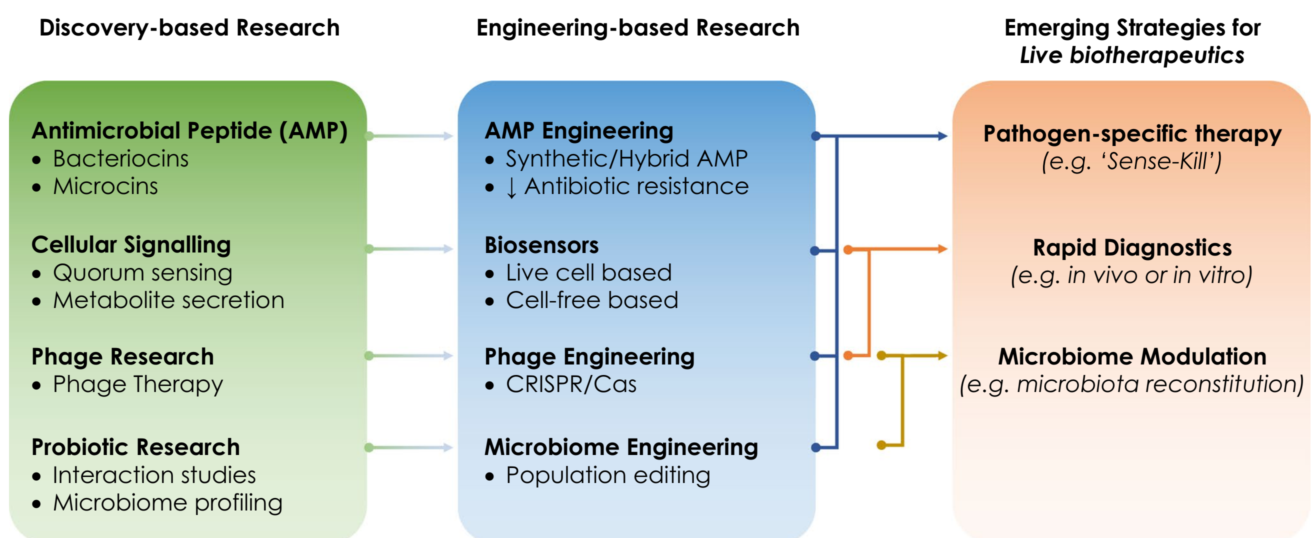

Fig. 2 Summary of progressing research culminating to combination strategies against pathogens

Table 1 Recent examples of engineered bacteria with selective antimicrobial activities

Target	Host	Mechanism	Result	References
Examples of engineering microbes for pathogen-specific activity: molecular level selectivity				
Pseudomonas aeruginosa	*E. coli, E. coli* Nissle	Synthesise and release antimicrobial proteins (e.g. recombinant pyocin and anti-biofilm enzymes) upon detection of acyl homoserine lactone (AHL) secreted by *P. aeruginosa*	Significantly reduced viable *P. aeruginosa* cells. Expression of anti-biofilm enzyme resulted in great reduction of mature biofilm and biofilm-encapsulated cells. Significant curative and prophylactic activity was demonstrated in mice gut infection model	[9, 11, 14, 16]
Vibrio cholerae	*E. coli*	Synthesise and release artilysins (Art-085) upon detection of CAI-I secreted by *V. cholerae*	A rapid and significant growth inhibition was achieved against *V. cholerae*, where 10^8 CFU was killed by activated *E. coli* within 1 h	[22]
Enterococcus faecalis/Enterococcus faecium	*L. lactis*	Synthesise and release cocktails of bacteriocins (enterocin A, hiracin JM79 and enterocin P) upon detection of the target	Significant growth inhibition was achieved against *E. faecalis* and a multidrug-resistant *E. faecium* strain, both by > 1000-fold	[23]
Salmonella spp.	*E. coli* Nissle	Synthesise and release microcin H47 upon detection of tetrathionate produced during inflammation. *E. coli Nissle* is further engineered to utilise tetrathionate to compete metabolically with *Salmonella*	Significant growth inhibition was observed against *S. typhimurium*. The metabolic competition resulted in reduced *S. typhimurium* fitness	[25]
Mycobacterium spp.	*E. coli*	Synthesise and release trehalose dimycolate hydrolase (TDMH) and listeriolysin O upon phagocytosis by macrophages	Significant reduction of mycobacterium in macrophage was observed when co-infected with engineered *E. coli* expressing TDMH and listeriolysin O	[27]
Examples of engineering bacteriophage for pathogen-specific activity: genetic level selectivity				
Enterohemorrhagic *E. coli* (EHEC) & carbapenem-resistant *Enterobacteriaceae*	*E. coli*-based filamentous phage M13	RNA-guided nucleases (RGNs) targeting antibiotic resistance genes (*eae*, ndm-1) delivered to target Enterobacteriaceae	Significantly increased the survival rate of EHEC infected *Galleria mellonella*, and re-sensitised pathogen towards antibiotic	[74]
Multidrug-resistant *S. aureus* (MRSA/VRSA)	*S. aureus*-based temperate phage ϕNM1	RGNs targeting methicillin resistance gene, *mecA*, delivered to target *S. aureus*	Targeted and specific reduction of methicillin-resistant *S. aureus* in skin infection model in mice that are infected with a mixture of targeted and non-targeted strains of *S. aureus*	[73]
Antibiotic-resistant *E. coli*	*E. coli*-based λ & T7 phage	RGNs targeting antibiotic resistance genes (*ndm-1, ctx-M-15*) delivered to target pathogen	Combination with T7 lytic phage selectively removed pathogens with resistance genes, resulting in enriched antibiotic-sensitive populations that were susceptible to treatment	[77]
Examples of engineering microbes for pathogen-specific activity: cell-population level activity				

Table 1 (continued)

Target	Host	Mechanism	Result	References
Clostridium difficile	Faecal samples from healthy donor/ reconstituted minimal bacteria consortium	Faecal transplant from a healthy donor or a simplified cocktail consisting of 4 or 6 different gut bacterial strains	Re-establishment of health-associated commensals, effectively displacing *C. difficile*, was demonstrated in a mouse model	[95, 103, 104]
H. pylori	*Lactobacillus* spp. *Bifidobacterium* spp.	Ingestion of probiotic/s given to: infected children OR to patients under triple therapy for *H. pylori* infection as adjuvant	Significant reduction in bacterial load and restored faecal Bifidobacteria in *H. pylori*-infected children when probiotic yogurt was given Meta-analysis of clinical trials of adjuvant probiotics demonstrated increased cure rates	[105] [102, 106]

in pathogen-activated and pathogen-specific killing activity. The efficiency was reported to be 99% in vitro, which also led to significant reduction in biofilm formation.

Gupta and colleagues [11] also exploited this QS molecule-based detection of *P. aeruginosa*, and went further by engineering pathogen-targeting bacteriocin. A chimeric combination of an *E. coli* bacteriocin, Colicin E3 [12] with the receptor and translocase domain of a Pseudomonad bacteriocin Pyocin S3 [13] was developed and termed CoPy. The chimeric bacteriocin allowed for the receptor and translocase domain to bind to cell surface receptors on *Pseudomonas*, facilitating the entry of *E. coli*-originating nuclease into the bacteria and consequent cell death. The secretion of CoPY was facilitated by adding an FlgM secretion tag to effect continual release of the antimicrobial peptide. Although the addition of FlgM reduced the activity of CoPy, antimicrobial activity was still effective, with an IC_{50} of 1 μM against *P. aeruginosa*, and pathogen growth inhibition in the vicinity of the engineered *E. coli* cells was clearly demonstrated.

Hwang et al. further described the use of a similar quorum sensing device in engineered *E. coli* to perform both cellular killing and biofilm degradation activity by incorporating the expression of bacteriocin (microcin S) together with nuclease (DNAseI) [14]. DNAse I targeted the extracellular DNA found within the biofilm matrix, thereby causing destabilisation of the biofilm. In combination, the engineered *E. coli* were able to target cells that were encapsulated, and protected within the biofilm. Furthermore, the specificity of the engineered *E. coli* towards *P. aeruginosa* was enhanced by rewiring the chemotaxis pathway of *E. coli*, where the expression of CheZ was placed under the control of a QS device. This cellular re-localisation in close proximity to the target pathogen enhanced the antimicrobial activity, as compared to non-migratory cells. This approach of engineering the directionality of bacterial swimming was also adopted in McKay et al. to demonstrate localisation of engineered *E. coli* in pyocyanin-rich region [15]. Although the work focuses on developing a controlled localisation, this can be used to target *P. aeruginosa*, as pyocyanin is a secondary metabolite produced and secreted by the pathogen. Recently, Hwang et al. further demonstrated the efficacy of an improved version of the engineered probiotic *E. coli* Nissle to treat or prevent *P. aeruginosa* infection in animal models [16]. While 77% clearance of *Pseudomonas* was observed when the engineered probiotic was administered to infected mice, prophylactic administration of the probiotic showed a more pronounced effect in preventing the onset of gut infection, demonstrating a possible clinical translation for the sense-kill strategy.

Vibrio cholerae

Vibrio cholerae is a Gram-negative pathogen responsible for epidemic outbreaks of severe diarrhoeal illnesses, particularly in post-calamity settings. Its key virulence factor lies in the production of a cholera toxin, which binds to the GM1 ganglioside receptors of enterocytes, activating a G-protein linked adenylate cyclase and increasing intracellular cAMP [17, 18]. This in turn leads to massive secretory diarrhoea and dehydration through increased efflux of chloride into the intestinal lumen [19]. The virulence of *V. cholerae* and the expression of the cholera toxin are dependent on the accumulation of secreted levels of QS molecules. On the similar premise of a quorum sensing device targeting *P. aeruginosa*, two different approaches that exploit the QS of *V. cholerae* have been explored. One approach is focused on modulating the virulence of *V. cholerae* by reducing the presence of QS molecules. Virulence-related gene expression and gut colonisation by *V. cholerae* is greatly dependent on the cell population, which is reflected by the level of an autoinducer molecule, cholera autoinducer 1 (CAI-1, (*S*)-3-hydroxytridecan-4-one) [20]. By engineering a probiotic cell that actively expresses CAI-1, the colonisation and induction of virulence by *V. cholerae* was suppressed, leading to prophylactic protection against *V. cholerae* infection in an infant mice infection study [21]. Due to the role of QS in *V. cholerae* virulence, this study demonstrates that CAI-1 can be used as mechanism for pathogen detection and decolonisation.

A more direct approach was reported by Jayaraman et al., where *E. coli* was engineered to sense the accumulation of CAI-I and subsequently promote expression of a novel class of antimicrobials called artilysins [22]. Artilysins (Art-085) is a fusion peptide consisting of outer membrane-targeting peptide sheep myeloid 29-amino acid antimicrobial peptide (SMAP-29) and the KZ144 endolysin. Art-085 functioned as both a pathogen-targeting peptide and a mechanism to mediate cellular release of expressed Art-085 itself, where modified Art-085 (YebF-Art-085) is secreted from *E. coli* in the presence of CAI-I to target the outer membrane of *E. coli* and promote cell lysis. The *E. coli* was also engineered to constitutively express Art-085, therefore, mediating the cell killing activity against *V. cholera* when the accumulated killer peptides were released upon lysis. The absence of CAI-I prevented lysis of *E. coli* and kept the Art-085 killer proteins within the intracellular space. The antimicrobial activity of the engineered *E. coli* was tested against *V. cholerae,* where the *V. cholerae*-induced supernatant of *E. coli* was able to effectively kill *V. cholerae,* with rapid suppression of *V. cholerae* colony forming units observed. The efficiency of the system was demonstrated where the number of viable cells fell below detection limit in 1 h post-exposure to the activated *E. coli* supernatant, highlighting the specificity of this sense-and-kill system.

Enterococcus species

Enterococcus faecalis and *Enterococcus faecium* are the main pathogenic enterococcal strains in the human gut, accounting for the majority of clinical infections. There is a growing concern with the increasing prevalence of vancomycin-resistant enterococci (VRE) particularly amongst nosocomial infections, prompting the need to seek novel effective antimicrobial strategies. Borrero et al. described the use of engineered *Lactococcus lactis* to sense *E. faecalis* via enterococcal sex pheromones cCF10, which in turn induced *L. lactis* vectors to express bacteriocins (enterocin A, enterocin P and hiracin JM79) that exhibited effective antimicrobial activity against *E. faecalis* [23]. A significant reduction in *E. faecalis* growth by up to 4–6 logarithmic units of colony forming unit (CFU) in the presence of cCF10-responsive *L. lactis* was observed within 2 h. In the absence of cCF10, reduction in enterococcal CFU was not significant between the recombinant *Lactococcus* strain and control in the first 3 h with differences observed only after 5–9 h of co-culture. To express antimicrobial peptides against *E. faecium*, which unlike *E. faecalis*, do not produce the enterococcal sex pheromone cCF10, the group used a lactococcal chloride-inducible promoter (CIP) that was activated by the physiological range of chloride concentrations found in human and mammalian gut [24]. In the presence of chloride, the engineered *L. lactis* was found to effectively reduce enterococcal growth when co-cultured with *E. faecum*. However, despite significant growth suppression by the *L. lactis*-expressed bacteriocins, enterococcal regrowth was observed after 10 h, suggesting a possible emergence of resistance towards the bacteriocins. This finding further highlights the importance of incorporating strategies to reduce bacterial resistance when developing new antibiotic technologies.

Salmonella species

The bacterial genus *Salmonella* causes a huge global burden of morbidity and mortality. In developed countries, non-typhoidal salmonellae predominantly cause a self-limiting diarrhoeal illness to mainly individuals with specific risk factors. While septic and focal infection incidents are rare in these countries, the occurrences of bacteraemia and associated cases of fatality reach 20–25% in areas such as sub-Saharan Africa. With the increasing occurrence of multidrug-resistant *Salmonella* which is driving the cost of antibiotics to be high, the need of an effective and affordable antimicrobial therapy is imperative.

To this end, *E. coli* Nissle was recently engineered to detect the presence of tetrathionate, a resultant metabolite when reactive oxygen species produced by the host reacts with luminal thiosulfate [25]. During inflammation, *Salmonella* utilises excess tetrathionate as a growth advantage over competing microbiota. This has been exploited as a pathogen-specific activation method to activate the expression of antimicrobial peptide microcin H47. In addition, the same ability to utilise tetrathionate has been engineered into Nissle, exerting direct competition over *Salmonella* while expressing the microcin that kills *Salmonella*. An in vitro evaluation has shown positive results, which would require further examination for translation. As this approach enables *E. coli* Nissle to outcompete *Salmonella* in addition to actively killing the pathogen, it can be more readily developed as a prophylactic agent, especially in areas with known high-risk factors, such as sub-Saharan Africa.

Cell-mediated targeting of immune-evasive pathogen: *Mycobacterium* species

Mycobacteria are fastidious intracellular organisms that undergo extreme slow growth. *Mycobacterium tuberculosis* is a pathogenic strain responsible for tuberculous pneumonia and meningitis, particularly in immunocompromised hosts. The challenge in treating tuberculosis stems from the bacteria's ability to lie dormant within infected macrophages, as well as the unique lipid-rich cell wall of the mycobacterium which is highly impermeable to conventional antibiotics [26]. Atanaskovic et al. [27] developed a non-pathogenic mycobacteria model using *Mycobacterium smegmatis* and studied the use of genetically engineered *E. coli* to serve as vectors to deliver trehalose dimycolate hydrolase (TDMH) [28], an enzyme potent enough to lyse the outer mycobacterial cell envelope. The *E. coli* also express listeriolysin O (LLO), a lytic protein which is able to lyse the phagosomal membrane [29]. The study showed that macrophages infected with *M. smegmatis* were able to phagocytose the *E. coli*. Upon phagocytosis, the production of TDMH and LLO-mediated killing of the intracellular mycobacteria were demonstrated and subsequent lysis of the infected macrophage was observed. The authors further demonstrated the ability of the engineered *E. coli* to limit the spread of mycobacteria within a pool of macrophages. At 3 h post-introduction of the engineered *E. coli*, the number of mycobacteria-infected macrophages significantly decreased compared to non-*E. coli*-treated macrophages. Furthermore, the expression of TDMH and LLO concertedly resulted in a significant decrease in mycobacterial counts by almost 99% in 6 h. Although the design currently lacks an inducible system that can drive autonomous activation as observed in aforementioned studies, current phagocytosis of engineered *E. coli* and subsequent release of its payload as it co-localises with the mycobacteria within the macrophage, provides a certain degree of spatial specificity that can be further exploited. More studies will also be required to ascertain the applicability of the model against pathogenic *M. tuberculosis*, and further development of the vectorised *E. coli* to deliver proteins to human cells can be explored for therapeutic applications.

Phage-mediated pathogen targeting

The discovery of bacteriophages demonstrates that specific targeting of pathogens exists in nature. Bacteriophages are obligate bacterial parasites which utilise their host's cellular machinery to replicate genetic material and propagate their progeny. Diverse in morphology and replication cycles, over 6000 bacteriophages and their specific bacterial targets are known [30]. As natural predators of bacteria, bacteriophages have been used for years in various food and industrial applications. However, using bacteriophages for therapeutic applications or phage therapy declined in the West with the prolific use and development of antibiotics [31]. Phage therapy was not well-received due to the lack of well-controlled trials and English-medium publications although it continued to be practiced in Eastern Europe [31]. In recent years, the problem of antibiotic resistance and rise of multidrug-resistant (MDR) pathogens spurred movement to venture into other alternative therapeutic strategies to control and clear infections. More on the history of bacteriophages and current developments in phage therapy can be found in a comprehensive review by Wittebole et al. [30].

Broadly, in terms of mechanism of action, bacteriophages (also referred to as phages hereafter) recognise their host through receptor recognition, then insert and replicate their genetic material for propagation. The packaging and transfer of genetic material by phages, occasionally of bacterial origin, have been found to contribute to horizontal gene transfer of advantageous adaptive genes such as antibiotic resistance genes—a process also known as transduction [32, 33]. The same processes can also be harnessed to package genes of interest to deliver antimicrobials for therapeutic applications. With increasing efforts to harness the unique delivery of genes and selective cell targeting mechanisms of phages, we highlight some recent, notable work showing the potential of phage therapy as a viable therapeutic alternative.

Phage therapy

With depleting options to tackle the problem of antibiotic resistance, phage therapy is gaining traction as a clinical alternative. The host-specific targeted nature of phage therapy is clinically significant as administered phages retain antimicrobial activity at the site of infection with minimal

disruption to commensal microflora. Current phage therapy research is advancing in three directions: (1) discovery and isolation of clinically effective bacteriophages, (2) engineering bacteriophages to deliver antimicrobials as adjuvant therapy and (3) engineering bacteriophages to expand the range of target host for increasing delivery flexibility.

Discovery and isolation of clinically effective bacteriophages

In the recent decade, several experimental efforts sought to isolate new bacteriophages and have shown translatable potential in controlling and reducing both Gram-positive and Gram-negative pathogens involved in various clinical conditions. Among the Gram-positive pathogens, *Staphylococcus aureus* is one major opportunistic pathogen implicated in many clinical infections [34]. Newly elucidated bacteriophages have demonstrated antibacterial and anti-biofilm effects, reducing inflammation and increasing survivability of murine models in systemic [35], implant [36] and diabetic-related infections [37]. Notably, phages were tested to be effective against MDR methicillin-resistant *S. aureus* (MRSA) strains [36, 37]. Bacteriophages isolated against *Enterococci* spp. have shown similar antibacterial and anti-biofilm properties [38, 39]. Recent work by Cheng et al. demonstrated that newly elucidated EF-P29 bacteriophage targeting vancomycin-resistant *E. faecalis* strains effectively reduced bacteraemia over 5 days and improved mice survivability, without major disruption to the gut microbiome [40]. Likewise, the clinical potential of bacteriophages against Gram-negative bacteria such as *Pseudomonas aeruginosa* [41–45], *Klebsiella pneumoniae* [46, 47], *E. coli* [48–50], *Shigella* [51] and *Acinetobacter baumannii* [52] is growing with many experimental phage therapies showing bactericidal effects. Significantly, the delivery of phage cocktails demonstrates the ability to tackle the problem of multidrug resistance, which arises from the presence of broad-spectrum antibiotic inactivating enzymes such as lactamases or biofilms that act as a physical barrier to reduce antibiotic efficacy [42, 45, 52–54]. Though pioneer clinical trials using phage therapy have not shown exceptional results in reducing infections [55], greater efforts are geared towards studying the relationship between bacteriophages and the microbiome to optimise conditions and realise the clinical potential of phage therapy [56].

Engineering bacteriophages as adjuvants

A second direction for phage therapy is engineering bacteriophages as adjuvants to complement current therapies such as antibiotic treatment. Intentional engineering of bacteriophages as co-treatments can specifically improve the efficacy of present treatments without causing unintended selection pressures that will lead to resistance. Lu and Collins first developed an antibiotic-enhancing M13 phage (ϕ*lexA3*) by overexpressing *lexA3*, which represses the DNA repair system of the host [57]. In vitro and in vivo delivery of ϕ*lexA3* in conjunction with quinolones effectively reduced the targeted bacteria and biofilm cells by significant orders of magnitude, ultimately improving survivability of murine models [57]. Two other studies using engineered bacteriophages to effectively deliver biofilm-degrading [58] and quorum sensing molecule-degrading enzymes [59] further demonstrated the possibility of using phages as antibiotic adjuvants.

Engineering bacteriophages for expanding host specificity range

Finally, some have ventured into engineering phages to be improved antimicrobial delivery vehicles of greater flexibility. Phage specificity limits the choice of antimicrobial agents to combat quickly evolving pathogens in clinical infections. An effective phage strategy developed for a certain pathogen may not be translatable to another due to limitations in phage recognition. To address this, two studies sought to improve and expand the target range of phages as flexible vehicles to develop antimicrobial strategies. In Ando et al., a yeast-based platform for tail fibre switching to propagate hybrid bacteriophages for different host targeting was developed [60]. T7 coliphages were engineered to target *Klebsiella* spp. and *Yersinia* spp., and population editing using these engineered phages was demonstrated in mixed bacteria populations to enhance the growth of the desired populations [60]. Yosef et al. further developed this idea of engineered T7 phages to develop a platform for optimised directed evolution to isolate specific hybrid phages with high transduction efficiency for different strains of bacteria [61]. These works expand the utility of phages and contribute to the development of clinical phage therapy.

However, there are still areas of concern surrounding this potential alternative. As observed in some works, one key area is the problem of resistance as bactericidal strategies present selective pressures that give rise to phage resistance mechanisms [62–64]. More on phage resistance mechanisms in bacteria can be found in the extensive review by Labrie et al. [65]. Another clinical concern is unwanted side effects due to the lysis of bacteria which leads to the release of endotoxins and bacterial contents, triggering inflammatory responses from the immune system [66]. These drawbacks call for a greater need to engineer strategies that circumvent these problems if phage therapy is to be a clinically viable alternative.

Sequence-specific phage therapy: CRISPR–Cas (RGNs)

As mentioned, there are inherent limitations of phage-mediated therapy, such as the event of phage resistance and potential unwarranted side effects. This has driven research to design phage-based antimicrobial strategies that prevent unwanted selection pressures and side effects, while achieving greater targeting specificity. Sequence-specific or nucleic-acid-based engineering and editing have recently been revolutionised with the discovery of the editing tool CRISPR–Cas. In light of these developments, we review some of the pioneering work that harness sequence-specific mechanisms and phage therapy.

Clustered, regularly interspaced, short palindromic repeats (CRISPR) and CRISPR-associated (Cas) protein nucleases form the first characterised bacterial adaptive immunity system against foreign DNA that may be acquired during horizontal gene transfer. Specifically, the Type II CRISPR–Cas system involves a single effector protein known as Cas protein endonuclease 9 (Cas9). Derived from *Streptococcus pyogenes*, Cas9 is guided by two interacting RNA molecules. The dual-RNA guide comprises a CRISPR RNA (crRNA) transcript containing target sequences (also known as protospacers) and a trans-activating crRNA (tracrRNA) which is involved in the processing of precursor crRNA molecules. The maturation of crRNA precursors followed by recognition of the protospacer adjacent motif (PAM) existing on the foreign DNA initiates DNA cleavage by the Cas9–dual RNA complex at target sequences through the introduction of double-stranded breaks [67]. With the elucidated mechanisms of the Type II CRISPR–Cas9 system, many genome-editing strategies and therapeutic applications have emerged to harness it as a biological editing tool [68–70]. The in vivo efficacy and potential of using CRISPR–Cas as an antimicrobial was first demonstrated to reduce target pathogenicity [71] and later, to discriminately eliminate target bacteria at the strain level in mixed populations [72]. These reinforced the prospect of using CRISPR–Cas as an antimicrobial in clinical settings which deal with complicated microbiome profiles.

Bolstered by these demonstrations, current research seeks to amalgamate the CRISPR–Cas system with phage therapy to develop highly specific and efficacious nucleic acid-based antimicrobials which can be delivered in a targeted and effective manner. Bikard et al. were one of the first groups to demonstrate the use of CRISPR–Cas9 sequence-specific targeting of *Staphylococcus aureus* delivered by means of bacteriophage [73]. Through phage delivery, the transduced CRISPR–Cas9 system specifically targeted the antibiotic-resistant gene, *mecA* and enterotoxin gene *sek* present. Their results showed discriminate targeting of antibiotic-resistant *S. aureus* in vitro and in vivo, simultaneously immunising antibiotic-sensitive *S. aureus* [73]. With growing concerns over the increase of methicillin- and vancomycin-resistant *S. aureus* (MRSA, VRSA), this work holds considerable significance for greater development in harnessing CRISPR–Cas9 system and phage delivery to combat the spread of antibiotic-resistant pathogens. Another pioneer group demonstrated this similarly in carbapenem-resistant *E. coli* and enterohemorrhagic *E. coli* O157:H7 (EHEC) [74]. Through a phagemid delivery system and crRNA design, Citorik et al. showed the flexibility of the CRISPR–Cas9 by targeting specific genes such as *eae*, which encodes the virulence gene intimin in EHEC and the New Delhi metallo-B-lactamase gene (*ndm-1*) [74]. Successful elimination of *E. coli* harbouring *ndm-1* was significant as the emergence and horizontal gene transfer of this carbapenemase-encoding gene can quickly give rise to resistance against broad-spectrum carbapenem antibiotics currently in clinical use, propagating MDR pathogens [75]. Effectively, this showed how the highly specific CRISPR–Cas system, delivered efficiently through phage, can target MDR pathogens and attenuate virulence.

In addition to tackling the problem of multidrug resistance, some work further ventured into re-sensitising bacteria to antibiotics through temperate phages. Before the elucidation of CRISPR–Cas9, Edgar et al. showed how temperate phages harbouring genetic constructs could re-sensitise streptomycin-resistant *E. coli* [76]. This approach provides advantage over other strategies of re-sensitisation that are plasmid mediated, as it has lower risk of horizontal gene transfer in clinical applications. This idea was further developed, harnessing CRISPR–Cas9 which allowed the freedom to target any gene of interest that could contribute to antibiotic resistance. Yosef et al. demonstrated how antibiotic-resistant bacteria could be re-sensitised and selected for in vitro [77]. Delivery of CRISPR–Cas by temperate phages could cleave plasmids or genomic sequences that conferred specific antibiotic resistance, re-sensitising the bacteria to antibiotics. Also, the nature of temperate phages to integrate packaged DNA into the host genome protects the host against lytic phages that may carry antibiotic resistance genes, thereby immunising native bacteria cells. As work on temperate phages centred mostly on *E. coli*, recent work seeks to apply this to other pathogens such as *S. aureus* for alternative antimicrobials [78].

However, the use of the CRISPR–Cas9 system is not limited to pathogenic bacteria and it has been cleverly extended to also target viral pathogens. Viral pathogens function by evading host immune response such as provirus and replicate to perpetuate their genetic material. The potential of CRISPR–Cas9 presents a potential solution to eliminate the source of viral replication, addressing some of the most difficult clinical problems currently. For example, Liao et al. demonstrated the use of CRISPR–Cas9 to target HIV

provirus in human pluripotent stem cells and primary T cells [79]. The work describes the use of stable Cas9 expression and multiplexed foreign DNA targeting approach which resulted in significantly reduced HIV-infected cells. Other human viral pathogens where CRISPR–Cas9 has been demonstrated to be effective include Hepatitis B virus [80], Epstein–Barr virus [81] and human papillomavirus [82].

Despite the various advantages offered by phage engineering and CRISPR–Cas9 system, there are potential limitations to be taken into consideration. Particularly, the efficiency of the system is largely dependent on the infectivity of the target cells. Furthermore, resistance mechanism towards the CRISPR–Cas9 system for targeted pathogen killing was observed where recipient cells managed to evade DNA cleavage by CRISPR–Cas9, albeit at low occurrences [70, 71]. Resistance against the CRISPR–Cas system still remains a challenge which requires further validation into the robustness of the system as an editing tool. Still, the system remains useful to be harnessed with phage therapy. While there is much enthusiasm to progress phage therapy, some fundamental links such as the interactions of bacteriophages with the microbiome remain to be elucidated. As the complete definition of the gut microbiome is still a work in progress, the effects of delivering phage cocktails in complex environments need to be well-examined to ensure clinical efficacy and viability.

Microbiome-mediated pathogen targeting

The human microbiome has been increasingly recognised as a key player in moderating the state of health and disease. Disruption of the normal gut microbiota has been implicated in the pathogenesis of many diseases, including infectious diseases [83]. The gut microbiota plays an important role in preventing infectious diseases in the host by providing colonisation resistance via direct and indirect mechanisms [84]. The gut microbiota can directly compete with pathogens for occupancy to attachment sites and nutrients, and also alter the host physiological conditions such as pH and metabolites [85]. Indirectly, it can stimulate the production of mucin which provides a protective layer over the epithelium and strengthen the gut barrier defence by augmenting epithelial cell signalling pathways and tight junctions [86]. Collectively, the gut microbiota produces molecules which can directly eliminate opportunistic pathogens and modulate host immune system [87, 88]. Therefore, it is possible to attribute particular compositions of microbiota as health- or disease-associated state due to their collective activities. As more comprehensive sequencing databases are established, the association can be made with higher confidence, where selective microbes required for restoration to 'normal' state can be deduced with accuracy. This approach can be applied against infectious pathogens, where commensal or probiotic strains that have relatively specific inhibiting activities can be used to outcompete or offer colonisation resistance against opportunistic pathogens at population level of intervention (Fig. 2).

Faecal microbial transplant (FMT) therapy

Microbiome-mediated pathogen targeting has been applied most successfully using faecal microbiota transplantation (FMT) to treat recurrent *Clostridium difficile* toxin colitis. One of the contributing factors in developing *C. difficile* toxin colitis is the use of broad-spectrum antibiotics which disrupts the normal gut microbiota. FMT involves transferring minimally processed faecal material of healthy donor to the patient with the aim to restore normal gut microbiota [89]. A systemic review showed that 91.2% experienced clinical resolution [90] and FMT is now an acceptable treatment for recurrent *C. difficile* infection. There are several reasons that could explain the success of FMT. One is that the microbiome from FMT competes with *C. difficile* for occupancy and nutrients. Second is that the production of bacteriocins could directly eliminate *C. difficile*. Third is that FMT restores secondary bile metabolism which inhibits germination of *C. difficile* spores [91]. However, there are concerns that FMT may transmit unwanted infections.

Other studies have attempted using more defined species of bacteria to compete with *C. difficile*. An open-label, single-arm trial using SER-109, which consists of 50 species of purified Firmicutes spores derived from healthy donors' stools, showed that 86.7% had no recurrence up to 8 weeks of study [92]. A phase 2 trial using non-toxigenic *C. difficile* strain M3 showed that successful colonisation with spore treatment was associated with lower recurrence of *C. difficile* infection (CDI). However, there are concerns that the non-toxigenic *C. difficile* strain could acquire toxins from toxigenic strains [93]. Another trial using synthetic stool mixture made from purified intestinal bacterial culture derived from a single healthy donor to treat two patients with recurrent CDI showed that they remained symptom free even after 6 months post-treatment [94]. As performing metagenomic analysis on stool samples is becoming increasingly affordable, information acquired has allowed to further refine these approaches of finding minimalistic and effective stool mixtures against recurrent CDI. In Buffie et al. [11, 95] bacterial operational taxonomic units (OTUs) associated with *C. difficile* infection resistance were identified based on a small cohort of patients with and without CDI following an antibiotic treatment. Out of the identified OTUs, *Clostridium scindens* was identified as being the most strongly associated with CDI resistance. Based on this discovery, transfer of a mixture consisting of the top 4 OTUs associated with *C. difficile* resistance, and transfer of *C. scindens* alone have shown to effectively ameliorate *C. difficile* infection in mice.

The mechanism by which *C. scindens* creates CDI resistance is investigated to be correlated to the synthesis of secondary bile acids which inhibit *C. difficile* growth in vitro. This example demonstrates that metagenomic analysis can facilitate in identifying microbes and the potential mechanisms to reconstitute disrupted microbiota and its function, thereby promoting colonisation resistance (CR) and target against opportunistic pathogens.

Probiotic therapy

The use of wild-type probiotics to treat various infectious diseases has also been studied extensively and it is another example of microbiome-mediated pathogen targeting. Probiotics can potentially outcompete pathogens, strengthen gut barrier function, produce antimicrobials such as bacteriocins and regulate the host's immunity [84]. Wild-type probiotics have been used to prevent and control enteric infections as shown by a systemic review and meta-analysis which demonstrated that the use of probiotics (including *Lactobacillus*, *Saccharomyces* and a mixture of probiotics) significantly reduced the risk of developing *C. difficile*-associated diarrhoea by 60.5% [96]. Shornicova et al. demonstrated that a single treatment with *Lactobacillus reuteri* in children with rotavirus gastroenteritis showed effective colonisation by the probiotic which resulted in reduction of watery stools in 24 h [97]. Subsequently, the use of various single or combinations of probiotic strains was explored with great interest for efficacy against enteric pathogens such as Shiga toxin-producing *E. coli* (STEC) and *Salmonella enterica* [98, 99]. Despite these efficacies, the mechanisms of activity of probiotics in mediating these beneficial effects are still obscure and have yet to be elucidated. As metagenomic analysis and culturing techniques improve to provide insights into microbial interactions, the role that probiotics play in restoring 'diseased' to 'healthy' state of gut can perhaps be more clearly understood.

Commensal gastric microbiome engineering

A large bacterial diversity found in the human stomach is challenging the dogma of *H. pylori* as the only bacterium able to survive in the acidic environment of the human stomach. Human stomach is proposed to harbour a distinct microbial ecosystem which is dominated by five major phyla: Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Fusobacteria [100]. However, data on functional interactions between the members of the microbial community in the stomach are scarce, and much less comprehensive than colonic microbiome data. Most of these studies are made in association with *H. pylori* infection and *H. pylori*-induced diseases, viewing the differences in gastric microbiota between patients with or without *H. pylori*. Although dysbiosis in the stomach induced by *H. pylori* infection is likely to contribute to gastric carcinogenesis, further studies are needed to support this hypothesis.

Isolated studies regarding gastric commensal microbes and potential effective probiotics against *H. pylori* are providing foundation for devising microbiome engineering approaches. For example, *Streptococcus mitis* is an aciduric bacterium considered to belong to the commensal oral microbiota but frequently detected in human stomach. It has been reported that *S. mitis* can induce a conversion of *H. pylori* into coccoid form and exert growth inhibition in vitro [101]. Furthermore, emerging evidence suggests *Lactobacillus* and *Bifidobacterium* species have inhibitory effects on *H. pylori* and can reduce the side effects of eradication therapies [102]. Despite the different antibiotic regimes approved against *H. pylori* infections, there are increasing rates of clarithromycin and levofloxacin resistance, which are reshaping the current treatment regime to include quadruple therapies for a longer duration (from 7 to 14 days). As the effects of antibiotic eradication treatment and probiotics on the composition of the gastric microbiota are becoming increasingly clear, the potential use of defined strains of probiotic to improve or complement current therapy against *H. pylori* infection will be promising.

Concluding remarks

We have reviewed recent examples of bacterial engineering approaches, exploring antimicrobial activities with pathogen selectivity at various levels of interventions (Table 1). Current antimicrobial therapy is faced with numerous limitations, such as lack of specific activity by currently available antibiotics and increasing prevalence of multidrug-resistant pathogens in clinics is depleting the available treatment options. Synthetic biology offers an alternative solution where specificity can be achieved to reduce the non-specific, off-target activity of antibiotic classes, and harness targeted therapy. With the advent of next-generation probiotics and live biotherapeutic product development, combined with changes in the climate of major regulatory authorities towards this new classes of therapeutic agents, a new generation of antimicrobial therapy may be available in the near future. However, as with every new therapy, there are inherent limitations and shortcomings. Although the approaches taken may limit antibiotic resistance, mechanisms that involve active cell killing, such as antimicrobial peptides and phage engineering, will inevitably apply selection pressures on pathogens to evolve. As the understanding of the dynamics of the microbiome evolves and metabolic interactions become more extensive, further strategies to suppress or subdue an activation of opportunistic pathogens at a highly selective manner can be achieved.

Acknowledgements This work was supported by the Summit Research Program of the National University Health System (NUH-SRO/2016/053/SRP/05), the Synthetic Biology Initiative of the National University of Singapore (DPRT/943/09/14), the U.S. Defense Threat Reduction Agency (HDTRA1-13-0037) and the Ministry of Defence of Singapore (MINDEF, RE2016-074). We thank Dr. Ping Han for her comments on the manuscript.

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