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DOI:
10.1016/j.chom.2019.06.004

Document Version
Peer reviewed version

Citation for published version (Harvard):
Theuretzbacher, U & Piddock, L 2019, 'Non-traditional antibacterial therapeutic options and challenges', *Cell Host & Microbe*, vol. 26, no. 1, pp. 61-72. https://doi.org/10.1016/j.chom.2019.06.004

Non-traditional antibacterial therapeutic options and challenges

Ursula Theuretzbacher[1] and Laura J.V. Piddock[2]*

[1]Centre for Anti-Infective Agents, Vienna, Austria

[2]Institute of Microbiology and Infection, University of Birmingham, Birmingham, West Midlands, B15 2TT, United Kingdom.

*Corresponding author Email: l.j.v.piddock@bham.ac.uk. Tel: +44 121 414 6966

Twitter: @LauraPiddock

Word count excluding references 6935

Summary (150 words)

The global challenges presented by drug-resistant bacteria infections has stimulated much activity in finding new treatments. This review summarizes the progress, and set-backs, of non-traditional approaches intent on circumventing bacterial drug-resistance. These approaches include targeting virulence via toxin production and virulence factor secretion, impeding bacterial adhesion to host cells and biofilm formation, interrupting/inhibiting bacterial communication and down regulating virulence. Other strategies include immune evasion, microbiome modifying therapies and the employment of phages as treatments or carriers. Finally, the prospects of nanoparticles, immunotherapy, antisense RNA and drug-resistance modulation approaches are discussed. The development of non-traditional treatments suffers similar challenges faced by developers of conventional antibiotics, however most of these new strategies have additional and considerable hurdles before it can be shown that they are safe and efficacious for patient use. For the foreseeable future, it is likely that most of these treatments, if approved, will be used in combination with antibiotics.

Key words: Anti-virulence, Quorum-sensing, Microbiome, Phage, Nanoparticles, Immunotherapy, Antisense RNA, non-traditional antimicrobials

Introduction

The global crisis of drug resistance, few new drugs to treat infections by the most resistant pathogens and the scientific challenges in discovery and early development of new antibiotics has inspired researchers to explore new ways to treat bacterial infections. The traditional antibiotic approach of treating infections is to find small molecules that either inhibit growth or kill Gram-positive or Gram-negative bacteria or both (broad-spectrum antibiotics). Non-traditional approaches explore many ways to influence the disease beyond inhibiting or killing

pathogens through small molecules. It should be noted that in this article, non-traditional does not imply an alternative therapy to antibiotics, which means replacing the use of these drugs.

Due to the wide diversity of research activities that could lead to the discovery of new therapies this article is not exhaustive. It focuses on the areas that are most frequently explored and have achieved some late preclinical or clinical experience. We have not discussed vaccines, devices, exclusively topical drugs or directly acting small molecule drugs such as potentiators of antibiotics including ß-lactamase and efflux inhibitors, combinations of small molecule drugs or conjugates or antimicrobial peptides. Instead, we have focused this article on the most promising non-traditional antibacterial treatments under development, their uses, and hurdles that must be overcome to provide safe and efficacious new medicines.

Anti-virulence

Anti-virulence approaches aim to inhibit the production or activity of Virulence Factors (VFs), typically these have no effect on bacterial growth *in vitro*. The number of VFs is growing (Virulence Factor Database, http://www.mgc.ac.cn/VFs/ (Dickey et al., 2017) and includes toxins, adhesins, quorum sensing molecules, virulence-dedicated secretion and regulation, siderophores, and immune evasion factors (Heras et al., 2015; Totsika, 2016). VFs are often species- or even strain-specific and variably conserved between and/or within a bacterial species. Furthermore, virulence gene expression may depend on the environment or site of infection or on the time course of a pathophysiological process. The complex biological variety constitutes a major challenge to translating early discovery to the clinical situation, especially as anti-VFs usually have a narrow spectrum of activity.

Numerous anti-virulence strategies are in the discovery or preclinical development phase (Garland et al., 2017). Progress in understanding pathogenesis and preclinical development efforts has progressed a few anti-virulence drugs to the clinical phase of development, and

some that block exotoxins have been approved as treatments. Here, we provide an overview of the anti-virulence strategies that are more advanced in development as a new treatment.

Toxins

Exotoxins are produced by pathogenic bacteria without which the microbe does not elicit symptoms in the infected individual. Therefore, they are obvious targets for anti-virulence therapies. Many bacterial toxins are released into the environment and are thus, amenable to antibody therapy. Antibodies approved for clinical use are active against toxins of *Clostridium botulinum*, *Bacillus anthracis* and *Clostridium difficile*. The recently FDA and EMA approved human monoclonal antibody (mAb) Bezlotoxumab (Merck) binds to the *C. difficile* toxin B and is indicated to prevent recurrent *C. difficile* infection (CDI) in at-risk adults.

Staphylococcus aureus harbors many VFs to facilitate tissue adhesion, immune evasion, and host cell injury. *S. aureus* α-toxin is a pore-forming toxin that plays an important role in staphylococcal infection. In general, exotoxins and surface-localized structures provide good targets for monoclonal antibodies (Table 1). The mAb suvratoxumab (MEDI4893, Medimmune) binds to and neutralizes *S. aureus* α-toxin (Yu et al., 2017). It is in Phase 2 clinical trials in colonized and mechanically ventilated patients to prevent ventilator-associated pneumonia (VAP) caused by *S. aureus*. Other companies are pursuing a similar strategy with α -toxin-binding mAbs (e.g. AR-301; Salvecin; tosatoxumab, Aridis). In contrast to suvratoxumab that is aimed at preventing symptomatic infection, Aridis plans to investigate AR-301 in a Phase 3 trial as an adjunctive therapy in combination with standard of care antibiotics to treat VAP caused by *S. aureus*. The monoclonal antibody preparation ASN100 (Arsanis), that neutralizes six *S. aureus* cytotoxins, failed to reach the primary endpoint in a Phase 2 study.

VF secretion

Type III secretion systems (T3SS) are complex structures embedded in the bacterial inner and outer membranes of many Gram-negative bacteria that are used to deliver virulence effector proteins into host cells and facilitate the establishment and dissemination of infections (Anantharajah et al., 2016; Deng et al., 2017). The interruption of toxin secretion or structural proteins of T3SS, especially the needle tip protein assembly is a focus of current research for small molecule inhibitors (Aiello et al., 2010; Gu et al., 2015, Fasciano et al, 2019) and mAb (Table 1). A bispecific mAb (MEDI3902, Medimmune) targets the *P. aeruginosa* T3SS needle tip protein PcrV to prevent T3SS mediated injection of toxins in host cells and Psl exopolysaccharide to prevent attachment of bacteria to epithelial cells (DiGiandomenico et al., 2014). MEDI3902 is in Phase 2 clinical development in mechanically ventilated patients for the prevention of VAP caused by *P. aeruginosa*. An earlier antibody approach did not progress to late stage clinical trials (Fasciano et al, 2019). Small molecules have the potential for a broader spectrum of activity than mAbs against T3SS, which are target specific and hence have a narrow spectrum.

Adhesion and biofilm formation

S. aureus expresses a number of cell surface adherence and immune evasion proteins, many of which are anchored to the cell wall by the transpeptidase sortase A (SrtA) enzyme (Cascioferro et al., 2015; Dickey et al., 2017). Several antibacterial drug discovery programs have found SrtA inhibitors that are believed to promote the disruption of the structure of mature biofilm; however, early preclinical projects have not advanced.

P. aeruginosa produces the surface polysaccharide alginate in response to environmental conditions. It enhances adhesion, biofilm formation and resistance to human leukocyte killing; this is most apparent in the environment of the affected lung in patients with CF. AR-105 is a

mAb directed at alginate and is currently being tested in a Phase 2 clinical trial as adjunctive treatment in mechanically ventilated patients with VAP caused by *P. aeruginosa*.

The first step of colonization of the bladder and artificial surfaces such as catheters is to avoid clearance during urine voiding by binding of bacteria via an available epithelial receptor (Spaulding and Hultgren, 2016). Therefore, recognition and attachment of the bacteria to the uroepithelium plays a key role in anti-virulence strategies that target uropathogenic *E. coli* (UPEC) and lower urinary tract infections (UTI). Adhesion is mediated through the expression of pili and their tip adhesin FimH that binds to mannosylated residues on the bladder epithelial surface (Spaulding et al., 2018). Several strategies to block adhesion have been pursued including developing mannose analogs that bind within the mannose-binding pocket of FimH and block pilus binding of FimH to host receptors and thus prevent attachment of UPEC (Maddirala et al., 2018) (Fimbrion). Other FimH inhibitors were effective in the mouse model, but it is not known if these animal models are predictive and whether data from them will translate into clinical efficacy (Kalas et al., 2018). UPEC colonize the intestinal space and establish a bacterial reservoir for infecting the urinary tract. The same adhesion mechanisms and binding principles apply to preventing selectively intestinal colonization of UPEC by treatment with mannosides. The oral and non-systemic small molecule drug EB8018 designed to block the FimH adhesin from overabundant Enterobacteriaceae (adherent-invasive *E. coli*) in Crohn's disease patients is in Phase 1 clinical studies (Enterome NCT03709628).

Bacterial communication

Quorum-sensing (QS) is a molecular communication system to synchronize the expression of certain genes affecting a global change in bacterial gene expression and cell physiology. There is a wide variety of signaling molecules that may serve as attractive and potentially broad-spectrum anti-virulence targets (Rémy et al., 2018). Various QS-interfering agents

(called quorum quenchers) including natural and synthetic compounds, enzymes and antibodies that target each step of the QS pathway have been described. These have been tested *in vitro* and *in vivo* (Defoirdt, 2018). *P. aeruginosa* has been studied most and serve as a model for targeting QS. The *P. aeruginosa* QS pathways LasR-LasI, RhlR-RhlI, IQS, MvfR have been explored in both acute and persistent infection models (Dickey et al., 2017). The *las* system controls LasB elastase, a pivotal VF in pseudomonal infection and target of drug discovery projects (Antabio). LasB is secreted at the site of infection, where it exerts a proteolytic action including broad tissue destruction and subtle action on components of the host immune system (Cathcart et al., 2011). The discovery of the role of the multiple VF regulator MvfR in mediating antibiotic tolerance and persister cell formation in *P. aeruginosa* inspired the research and discovery of inhibitors of MvfR (Maura et al., 2017) (Spero). In *S. aureus* QS is mainly regulated by the *agr* operon. Although several small molecule inhibitors have been described, none have advanced to the optimization phase of discovery (Salam and Quave, 2018). Based on considerable basic research, QS inhibiting strategies are increasingly included in discovery projects (Haque et al., 2018; Williams, 2017).

Counteracting Immune evasion

Many bacteria deploy factors to prevent detection by or to escape the host immune response. Therefore, strategies to neutralize such tactics are under development. Monoclonal antibodies targeting bacterial surface epitopes are hypothesized to increase bacterial clearance through enhancing antibody-dependent phagocytosis, and/or complement-mediated bactericidal activity, or via immune system-independent bacterial killing (Wang-Lin and Balthasar, 2018). The *S. aureus* Protein A (SpA) defends the bacterium against the adaptive immune response by resisting phagocytosis and inducing apoptosis of B cells. This protein also facilitates nasal colonization and cell adhesion (Hong et al., 2016). The mAb 514G3 neutralizes the SpA mediated immune evasion of *S. aureus* (Varshney et al., 2018). An on-going a Phase 1/2

clinical study to treat *S. aureus* blood stream infections as an adjunctive therapy is due to complete in June 2020 (XBiotech NCT02357966).

Regulating virulence

Clp proteases suppress the expression of multiple unrelated VFs in *S. aureus* by impacting on central processes such as virulence gene expression, cell wall metabolism, survival in stationary phase, and cell division. The simultaneous suppression of multiple VFs or pathways using small-molecule compounds is a promising approach to reducing the virulence of *S. aureus* (Gao et al., 2018). Many inhibitors have been discovered and are currently being optimized (Aviru). However, whether targeting a master regulator of virulence translates into clinically relevant benefits remains to be seen.

Advantages and Disadvantages of anti-virulence approaches

As anti-virulence drugs interact with non-essential targets, there is a general assumption that they do not select for resistance; however, this has been challenged (Allen et al., 2014). Experimental data revealed the emergence of resistance showing the complex evolution and resistance selection by some anti-virulence drugs; this was dependent on the importance to the pathogen of the targeted VF (Totsika, 2016). Whether such resistance will impact therapy and the potential of transmissible resistance is unknown (Rezzoagli et al., 2018).

There are numerous challenges to translating anti-virulence strategies to new treatments for patients (Table 2). Given the complexities of virulence systems, the likely specificity of single VFs, and the lack of research data, it is not surprising to find extremely long timelines for the preclinical development of anti-virulence approaches and the failure of clinical trials. Development of anti-virulence drugs requires an in-depth understanding of these factors and the roles that specific VFs have in disease processes. Traditional measurement of growth inhibitory activity, the Minimum Inhibitory Concentration (MIC) test, does not apply to anti-virulence drugs as by definition they do not inhibit growth or kill bacteria. In most cases,

alternative *in vitro* methods are not developed and the predictability of animal models for clinical outcome are poorly described.

Potential clinical indications for anti-virulence therapies are prevention of disease such as CDI, HAP/VAP and recurrent uncomplicated UTI. However, as some VFs are specific for a pathogen and rely upon of their expression, which is influenced by the condition of the patient and disease state, accurate diagnostic tests will be necessary to identify the pathogen and presence/expression of the targeted VF. Therefore, chronic or non-life-threatening infections are likely to be targeted as these will provide the opportunity to carefully select the patient, enabling a tailored patient-specific approach with less time pressure to start therapy. Most anti-virulence therapies are developed as adjunctive therapies in addition to standard therapy, usually antibiotics. This is the only ethically acceptable pathway in clinical practice in patients needing fast acting antibacterial therapy. This poses special challenges for drug development and clinical use as it is not possible to prove clinical efficacy of an adjunctive therapy with a conventional non-inferiority clinical trial design. Superiority design in a combination therapy versus stand-alone antibiotic therapy would be the most convincing way to show a clinically relevant effect of an add-on therapy. Selection of the appropriate indication, patient population, clinical endpoints and clinical trial sites are enormous challenges for late stage clinical studies. Relevant secondary endpoints may support the clinical therapy decisions (Maura et al., 2016).

Anti-virulence approaches will not replace antibiotics, thus may not contribute much to resolving the resistance problem and insufficient antibiotics pipelines. Nonetheless, they may complement the action of antibiotics; however, evidence that they provide benefit for patients in high-quality clinical trials is needed. Open discussion and analysis of failed clinical trials would enhance this field.

Microbiome modifying therapies

Recent advances in metagenomic, computational and synthetic biology tools inspired the revival of research into the human microbiome and has provided a deeper understanding of its interactions with the host (Wilson et al., 2019). Manipulating and engineering the human microbiome is an attractive option to prevent and resolve infection and so is generating considerable activity in academia and industry (Timmis et al., 2019) and attracts broad interest among public funders and private investors (Boers et al., 2016). The US National Microbiome Initiative had an important impact and advanced standards for the use of the next-generation technologies in metagenomic studies and generated quality-controlled data (Group et al., 2009). Not only bacterial microbiota but growing knowledge about the role of the human phageome has highlighted the impact of bacteriophages in shaping a healthy intestinal microbiome (Anonye, 2018; Paule et al., 2018; Rohde et al., 2018; Zuo et al., 2018). The gut microbiota is intricately connected to the host's immune system through a reciprocal developmental relationship. Specifically, the microbiome is critical for the appropriate development of the immune system, and in turn, the immune system helps modulate the microbiome community through a balance of pro- and anti-inflammatory pathways (Cammarota et al., 2017).

More than 10 small companies are developing microbiome therapies for infectious diseases (Table 3). So far, most experience has been gained on the impact of the intestinal microbiota on the physiology of *C. difficile* in the gut and recurrent CDI (Young, 2016). The underlying assumption is that rebuilding the microbiome after infection or preserving the microbiome to prevent infection will translate into clinical benefit. The strategies to restore an unbalanced microbiome are based on the experience of clinically successful transfers of a full natural microbiota in form of stool from healthy donors (Fecal Microbiome Transplantation, FMT) (Cammarota et al., 2017). This procedure has a typical cure rate of 90% and has encouraged

research groups to modify this principle and focus on production of stool banks, standardized products, engineering and oral delivery strategies.

Although no microbiome-modifying therapy has been officially approved, the US FDA allows FMT without filing an Investigational New Drug (IND) application exercising enforcement discretion when using the stool bank OpenBiome to treat *C. difficile* infection not responsive to standard therapy (FDA, 2016). FMT reintroduces a complete, stable community of gut microorganisms (Bakken et al., 2011; Borody and Campbell, 2012) and is the most advanced form of microbiota therapy with a large body of experience (Ooijevaar et al., 2018). Guidelines for clinical use are available (Mullish et al., 2018). In the UK, large clinical trials of FMT are underway (ISRCTN 74072945). Most experience has been gained so far with the application via enema but freeze-dried capsule-based formulations of the microbiota or mixture of spores from several bacteria isolated from healthy donor fecal samples are in clinical development (Baker et al., 2018). There are no high-quality studies available yet to show the efficacy and safety of the oral route (Iqbal, 2018). Two companies are currently conducting a randomized Phase 3 trial enrolling patients with recurrent CDI (Rebiotix NCT03244644, Seres NCT03183128), another company is enrolling patients in a Phase 2 trial to test a therapy that contains microbiota produced from pure, clonal bacterial cell banks (Vedanta NCT03788434) (Table 3).

Companies are also developing a wide range of microbiome strategies including rationally selected cocktails of bacteria or bacterial spores containing the "active components" of the complex microbiota (Khanna et al., 2016; Orenstein et al., 2016). The most extremely reduced approach is the use of a single non-toxic strain of *C. difficile* that is hypothesized to outcompete the toxigenic strains in the gut. Another approach is to assemble a synthetic microbiome comprising well characterized individual strains in pure cultures with standardized properties (Timmis et al., 2019). Other strategies build on the gastrointestinal

metabolic balance and subsequent changes made during disturbance and restoration to a healthy microbiota (Koropatkin et al., 2012). In another approach, genetically engineered bacteria produce antibacterial compounds that selectively remove key disease-causing species from the microbiota. Increasingly, bacteriophages or nanoparticles serve as vehicles to selectively target pathogenic bacteria or resistance and virulence determinants in the gut flora and thus can be used to manipulate the microbiota. Current approaches also target intestinal colonization with pathogenic bacteria such as carbapenem-resistant *Enterobacteriaceae* (CRE) in critically ill patients. However, it should be noted that there are conflicting data on the causality between CRE colonization and increased mortality in ICU patients (McConville et al., 2017). Studies with FMT to test for the effect of decolonization in high-risk patients were not conclusive (Huttner et al.; Relman and Lipsitch, 2018). Although the gut microbiota is the most common target for microbiome-modifying therapies, other concepts focus on manipulating the skin or lung microbiome. These approaches are at very early stages and correlations with the clinical situation are less clear.

The key challenges of developing new simplified microbiome therapies are the incomplete understanding of the complex genomic and phylogenetic diversity of the human microbiome. Indirect testing of hypotheses and statistical correlations may not prove pathophysiological causation. If innovative microbiome therapies beyond FMT and other complex microbiota strategies are to translate into clinical benefit a deeper understanding of the complex role of the microbiota in the pathogenesis of a specific disease is necessary. The extent to which the complexity of the therapeutic approach can be reduced while retaining efficacy remains to be seen. The high variability of the microbiome composition yields inconsistent and contradictory results that are difficult to interpret. Simple preclinical models may not be predictive. Selecting appropriate microbiota is important but proving that live bacteria constitute a coherent community and are incorporated into the recipient's gut and remain after

being ingested is similarly essential (Smillie et al., 2018). Additionally, the manufacture of live bacterial products is complex and expensive. There is no good rationale or model to support finding the appropriate or optimum dose and so doing reflects a trial and error strategy rather than characterizing an effective dose. Although microbiome treatments with complex microbiota for preventing recurrent CDI are in late stage clinical trials, the development of next generation treatments rely upon reducing unpredictable factors and filling the gaps in the basic understanding of underlying processes. Currently, reduction of the complexity of the microbiota substantially increases the risk of clinical failure.

Phages

Although therapy of bacterial infections with bacterial viruses (bacteriophages, phages) has been practiced in Eastern Europe for nearly a century, the interest in phage R&D has only gained traction elsewhere in the last 10-15 years as a response to the emergence of multidrug-resistant pathogens (Kortright et al., 2019). Synthetic biology and other modern tools have revived the field of phage research (Pires et al., 2016). They enable the modification of phages, the characterization and careful screening for and removal of genes coding for toxins and VFs to avoid the risk of transfer from one bacterium to another. In general, phages are regarded as safe because they do not infect mammalian cells.

There are at least 30 companies pursuing a treatment strategy that involves phages (table 4). Recent and ongoing trials focus on infections by *P. aeruginosa*, *S. aureus* and *E. coli.* Besides anecdotal case studies, case reports of compassionate use programs and poor quality non-controlled clinical studies, only one randomized placebo-controlled Phase 1/2 clinical trial with topical treatment of chronic otitis caused by *P. aeruginosa* has been successfully conducted (Wright et al., 2009). A recently completed Phase 1/2 clinical trial in infected burn wounds (phagoburn) failed to demonstrate efficacy and exemplifies the challenges described below when translating phage approaches to the clinical environment (Servick, 2016). A

Phase 1/2 trial with a *S. aureus* phage cocktail for i.v. administration is being prepared to start in 2019 (Ampliphi).

Phage therapy is characterized by its specificity to single bacterial species and usually to a subset of strains within that species (Kortright et al., 2019). To be active against >90% of strains within a bacterial species and to prevent rapid emergence of bacterial resistance to a single phage, mixtures (cocktails) of different phages, often more than 10 phages, are used for therapy. Phage resistance can evolve within hours, independently of the use of bacteriophage combinations. Although the combination of multiple phages in a cocktail compensates for a limited host range the increased complexity of such a cocktail not only dilutes the concentration of the individual phages but can promote potential unfavorable interactions between phages and cause manufacturing and quality control issues. This is the reason why companies try to reduce the number of phages in fixed cocktails and some produce mini-cocktails ($\leq$5 phages). The downside of such mini-cocktails may be a smaller host range. Currently, there is a trend towards patient specific cocktails based on libraries of pre-approved phages. Phage banks containing purified or pre-purified phages allow the quick assembly of patient-specific cocktails that contain only the most appropriate phages against the infecting bacterium. The choice of phages is based on new diagnostic tools that are not yet available in clinical practice. Based on modern genetic engineering tools, recent and current research focuses on engineered phages with improved or specific features (Barbu et al., 2016). In contrast to the above-mentioned strategies that use lytic phages, non-lytic phages are utilized as vehicles to express antibacterial proteins or genes (Krom et al., 2015).

There are several challenges to the clinical use of phage treatment. These include

1) the prerequisite of selecting appropriate phages to achieve an appropriate range of activity and prevent development of bacterial resistance. Such fixed phage cocktails need to consider bacterial isolates from different infections and geographic locations.

2) manufacturing phages under good manufacturing practices (GMP) and chemistry, manufacturing, and control (CMC) guidance. Some progress has been achieved when tackling the insurmountable challenge regarding CMC, especially production, stability, purity and quality control.

3) Considering phage biology in the design of phage treatment is a prerequisite of any successful approach (Bull and Gill, 2014). Unique pharmacokinetics (PK) and pharmacodynamics (PD) of phages means that dose finding processes are challenging. The immense size of phages when compared to small molecule antibiotics results in a wide range of PK challenges and is the reason why many sites of infection are not accessible by phages. Questions of basic PK such as distribution, dilution in the blood compartment, rapid clearance (Inchley, 1969), as well as kinetics of phage infection and other PK/PD characteristics are not well defined. Development of methods to access these parameters needs to proceed in parallel with clinical work to assess exposure and efficacy relationships. The concept of phage therapy is based on localized amplification in the presence of the specific susceptible bacteria. High bacterial loads are necessary for amplification and their localization is a complex pathophysiological issue (Rose et al., 2014). Though localized amplification is a key concept, the initially applied dose and the fate of the phages in the systemic circulation is not well understood. Bacterial loads and decreased availability of active phages in the circulation or localized infection sites may be responsible for a potentially slow onset of activity. The success of phage therapy ultimately depends on the optimal dose, dosing regimen, timing, formulation and administration, with PK and PD characterized for each phage or phage cocktail.

4) showing efficacy in clinical trials, and thus, gaining regulatory approval. Although case reports in compassionate use programs indicate the possibility of systemic use, due to access issues, topical delivery has been much more common. However, in this

setting, concomitantly used treatments (e.g. wound care products, disinfectants and antibiotic topical treatments) may affect the local stability of phages (Merabishvili et al., 2017). Inhalation treatment with phages, potentially also in combination with an antibiotic for specific indications such as CF seems to be feasible (Chang et al., 2018). Another aspect of phage therapy is their natural immunogenicity which is stimulated by bacteria (bacteria can highjack the innate immunity of hosts to inhibit phage). Interacting elements of adaptive and innate immunity are contributing to the clearance of phages with consequences on phage PK (Hodyra-Stefaniak et al., 2015). To use in patients, the immune response to phage therapy need to be assessed (Dąbrowska et al., 2014; Krut and Bekeredjian-Ding, 2018). At present, there are no high-quality data to show that phage therapy works routinely in clinical settings. The future and potential clinical use of phage therapeutics depends on the careful selection of phage-accessible infections and to be able to show a clinical benefit for patients (Harper, 2018). The current challenges of regulatory pathways, especially for patient-specific but also fixed phage cocktails include the need for constantly adjusting the preparation as the bacteria evolve and requires an appropriate legal and regulatory framework (Fauconnier, 2017; Pirnay et al., 2018) with recent progress in approval to conduct clinical trials.

5) developing and implementing appropriate diagnostics is essential to support use in patients.

For the most common bacterial infections it is unlikely that phage therapy will replace use of antibiotics (Rohde et al., 2018). However, synergy with antibiotics has been seen in vitro and in animal models (Oechslin et al., 2017). Therefore, phage therapy may be a promising adjunctive treatment in specific indications or salvage therapy for patients with infections that

have not responded to any other treatment. Convincing clinical efficacy in well-designed randomized controlled clinical trials needs to be demonstrated.

Phages as carriers

Genetically engineered non-replicative phages are designed to serve as specific nano-delivery vehicles and carry payloads that exert antibacterial activity beyond direct lysis of the cell (Krom et al., 2015). Synthetic biology approaches enable the use of a wide range of gene expression systems to target bacteria. Phage delivery systems are usually specific and so render the delivered therapeutics narrow spectrum and pathogen-specific. The delivered genes may be DNA sequence-independent and cause a rapid bactericidal effect (Phico) or utilize the clustered regularly interspaced short palindromic repeat (CRISPR) RNA-guided genome editing systems to engineer novel functions (Locus Biosciences, Eligo Bioscience) (Hatoum-Aslan, 2018). CRISPR Cas9 has developed into a new powerful technology to regulate gene expression in bacteria (Bikard et al., 2013). The RNA-guided nuclease Cas9 may serve as a sequence-specific antibacterial (Bikard et al., 2014) or may target a specific DNA sequence to inactivate antibiotic resistance or virulence genes (Nemesis Bioscience). Another CRISPR–Cas system is used to insert CRISPR-Cas3 constructs into the phage genome that specifically degrade the DNA of target bacterial cells (Locus Biosciences). The CRISPR/Cas mediated technology can also be used to modify phages and optimize favorable characteristics (Hatoum-Aslan, 2018).

As such phage vehicles are not self-replicative the dose must be very high to target bacteria in an infection. PK and dose finding are not well understood. New antibacterial approaches that use phages as carriers are faced with two big challenges, (1) the new technology (e.g. CRISPR-Cas) and (2) the phages themselves as discussed above. This doubles the risk and will need considerable time to progress to clinical studies to show a clinical benefit for patients.

Phage-derived products

Though phage-derived enzymes have been explored since the late 1990s, renewed interest emerged to address the current drug-resistance issues. Endolysins are the best studied phage-derived peptidoglycan-degrading enzymes. They are encoded by phages to liberate progeny phage from inside of infected bacterial cells, resulting in fast osmotic lysis and bacterial cell death (Fischetti, 2018). Endolysins are bacteriolytic on contact, independent of resistance pattern to conventional antibiotics, and are highly specific for a bacterial species or genus (Fernandes and São-José, 2018). Naturally, endolysins work from inside the cell but purified, recombinant lysins enabled enzymes are lytic from the outside. When developed as drugs, lysins must be stable, soluble and able to hydrolyze peptidoglycan from the outside. Numerous endolysins against Gram-positive bacteria have been studied in vitro and in animal models (Gutiérrez et al., 2018; Haddad Kashani et al., 2017). The opportunities to customize endolysin properties such as specificity, activity, stability and solubility are currently being explored and extensive protein engineering efforts have expanded (Oliveira et al., 2018). The first two products that have reached Phase 2 clinical development are recombinant lysins directed against *S. aureus* (SAL200/tonabacase from Roivant in-licensed from iNtRON Biotechnology (Kim et al., 2018) and CF-301/exebacase from Contrafect) (Schuch et al., 2014). A topically applied endolysin for inflammatory conditions due to *S. aureus* in atopic dermatitis is already available (Micreos Human Health BV). Another topical endolysin against staphylococci is being studied in Phase 1/2 clinical trials for nasal decontamination (GangaGen). In contrast to Gram-positive bacteria, the outer membrane of Gram-negative bacteria represents a difficult barrier to reach the peptidoglycan layer. Therefore, discovery of endolysins against Gram-negative bacteria has faced extensive challenges and projects are still in preclinical research (Bioharmony Therapeutics) (Briers and Lavigne, 2015). Based on protein engineering techniques some progress has been made but translating results into formal development programs requires more research (Lukacik et al., 2012; Schirmeier et al.,

2018). One approach for Gram-negative bacteria is fusing a natural antimicrobial peptide (AMP) to an endolysin (Artilysin) (São-José, 2018). Other options explored include combining endolysins with outer membrane-permeabilizing agents (Oliveira et al., 2018).

Via protein engineering, there is the potential to generate enzymes with several improved features; these include altered catalytic activities and binding specificities, solubility, and other physicochemical properties (Gutiérrez et al., 2018; São-José, 2018). Great progress has been achieved improving large scale production, purification, formulation, delivery, stability and acceptable shelf life. However, some studies have shown that in vitro antibacterial activity is not translated in vivo. It is unclear if discrepancies in antibacterial activity of some endolysins is due to the influence of bacterial cell growth conditions or growth stage (Oliveira et al., 2018). Pharmacokinetics are not well understood. Endolysins have a relatively short half-life which may be explained by proteolysis via plasma proteases and degradation of enzyme aggregates (Jun et al., 2017). PK/PD characteristics and dose finding are a new field for the first lysins in development. As lysins are proteins they are immunogenic in mammals. In vitro and animal studies with lysins have shown that non-neutralizing antibodies are generated (Pastagia et al., 2013), so could allow for repeated use in humans, but the development of antibodies has raised concern and requires further study (Jun et al., 2017). The potential for resistance to lysins is unknown as only simple serial passage experiments have been done so far. Although biologics are a growing part of authorized medicines (Cooper et al., 2016), the regulatory pathway of new antibacterial biologics still needs some clarification. Endolysins are likely to be suitable for classical clinical trials procedures due to their similarities with conventional antibiotics.

If developers succeed with defining an appropriate dosing schedule (beyond a single dose) as a basis for successful clinical studies for patients with confirmed infection due to drug-resistant pathogens, or who experience recurrent or relapse infections, endolysins may provide

an adjunctive therapy option. Endolysins are very large molecules and their distribution in the body is restricted to the bloodstream. Therefore, their clinical use will be limited to systemic infections or to infections with topical application. Synergistic activity with antibiotics and in vitro and in vivo (Schuch et al., 2014) and activity against biofilms may open opportunities to treat infections of infected implanted devices and endocarditis. In addition to human health, endolysin- based technologies are applied in many areas, including food safety, animal health and agriculture.

Other approaches

Recent advances in genome editing, gene regulation and systems biology has inspired a wide variety of other innovative discovery projects including ≥ 100 discovery and preclinical projects on approaches including nanoparticles, immunotherapy, anti-sense RNA, resistance modulation and removal of drug-resistance plasmids (Theuretzbacher et al., 2017).

Nanoparticles

Nanoparticles are 1–100 nm with ill-defined multiple simultaneous modes of action against Gram-positive and Gram-negative bacteria. Nanoparticles have been used for many years as antibacterial coatings for implantable devices and medicinal materials, wound dressings, bone cement, dental materials and vaccines (Wang et al., 2017). Several types of nanoparticles (especially liposomes) are currently available for drug delivery and extended release forms (Kwon et al., 2017). Nanoparticles have been studied as toxin binders in various infections including intestinal infections e.g. cholera (Das et al., 2018). One company is developing liposomes that mimic domains targeted by toxins so neutralizing many toxins, e.g. phospholipase C, pore-forming toxins, T3SS and can be used for a range of different infections (Combioxin, Phase 1/2) (Azeredo da Silveira and Perez, 2017). Nanoparticles also serve as delivery vehicle for synthetic oligonucleotides that function as transcription factor decoys and thus, control gene regulation (Mamusa et al., 2017) (Procarta). The potential of

nano-strategies as adjunctive therapy in addition to existing antibiotics is discussed in the recent review by Baptista et al (Baptista et al., 2018). Despite offering promising solutions, translational studies and development of nanoparticles for severe infections is in its infancy and several challenges remain.

Immunotherapy

Host-directed therapies utilize small-molecule drugs and proteins to target critical host signaling enzymes exploited by bacteria for their intracellular invasion, replication, and/or dissemination and virulence (Chiang et al., 2018; Pirofski and Casadevall, 2006). Potential immunomodulating therapeutics may encompass a great diversity of drug classes targeting a variety of biological processes that modify host cell function. This complexity of the immune response challenges the selection of suitable targets. Immunotherapeutics are thriving in other therapeutic areas but are relatively unexplored for bacterial infections (Baker et al., 2018). Like other non-traditional approaches, a clear correlation between the immunomodulating drug and clinical outcome of bacterial infection needs to be shown. Furthermore, if therapeutics stimulate the immune system, they may be associated with the risk of excessive inflammation leading to a cytokine storm or systemic inflammatory response syndrome (Chiang et al., 2018).

Nonetheless, some immunomodulating agents are being tested in preclinical or clinical trials. The most advanced drug, Reltecimod, is in Phase 3 clinical trials (Atox Bio). The short peptide immunomodulator attenuates excessive severe acute inflammation and is hypothesized to protect from superantigen toxins. It is tested in patients with necrotizing soft tissue infections in addition to the current standard of care (broad-spectrum antibiotics, wide surgical debridement, and supportive care). A phase 1/2 clinical trial of recombinant plasma gelsolin in community-acquired pneumonia has been completed (BioAegis Therapeutics). Plasma gelsolin is a highly abundant plasma protein in healthy individuals that enhances

macrophage activity and limits the excessive spread of inflammation. Its decline in a wide range of infections is correlated with poor clinical outcome (Self et al., 2018).

Antisense RNA

Antisense antimicrobial therapeutics are synthetic sequence-specific oligomers that silence expression of specific genes including essential, non-essential or resistance genes. (Sully and Geller, 2016). Broad functional genes are investigated as targets but still need to be validated. Many different chemical structures have been explored but all need a delivery system to penetrate bacterial cells. The most common approach is coupling antisense oligomers to cell-penetrating peptides (Sully and Geller, 2016). Such conjugates have not progressed into clinical trials. They face considerable challenges, including choice of target, potential emergence of resistance, carrier and translational issues but may benefit from advanced research to deliver improved approaches (Good and Stach, 2011).

Drug-resistance modulation

Resistance has inspired research to explore mechanisms to switch off drug-resistance without affecting bacterial growth as well as preventing horizontal gene transfer between bacteria. Several ways of silencing drug-resistance genes have been described, including CRISPR-Cas or synthetic oligomers (Good and Stach, 2011; Yosef et al., 2015). These methods aim at inactivating or deleting specific genes to re-establish the susceptibility of the bacteria to the antibiotic. The major challenge is the delivery of the genetic construct to and inside the bacteria (Vila, 2018). Commonly described delivery systems are phages, cell-penetrating peptides, nanoparticles or transmissible plasmids.

Although some technologies allow for simultaneous targeting of various drug-resistance genes, bacteria can express a great variety of different resistance mechanisms, requiring the identification of the target resistance mechanisms before administration. A few companies are

pursuing such resistance modulating approaches to re-sensitize bacteria to existing antibiotics and preventing horizontal drug-resistance-gene transfer in bacteria (see: phages as carriers).

Plasmid curing (recently reviewed by Buckner et al., 2018) is another approach, either by reducing transmission of plasmids to new bacterial hosts or reducing the stability of plasmids within bacterial cells. Agents that remove plasmids carrying antimicrobial resistance and/or virulence genes could be used to decolonize humans, animals and/or the environment of these transmissible elements. Adequate animal models to test these agents need to be developed. However, should there be a currently licensed drug that could be re-purposed for this role, analysis of drug-resistance surveillance data in patients taking such a drug and/or a clinical trial could be carried out.

Discussion

The increasing number of drug-resistant bacteria especially Gram-negative bacteria, the growing awareness of few new drugs and the scientific challenges to find novel antibiotics without cross-resistance to existing classes has stimulated discovery and early development of new antimicrobials. To contribute to re-stocking the pipelines with new treatments, non-traditional therapies have been proposed. However, it is very unlikely that they will replace the use of antibiotics as their use will mostly depend on concomitant use of active antibiotics (Czaplewski et al., 2016). Therefore, most non-traditional treatments will not solve the drug-resistance problem. Furthermore, the additional clinical benefit of such adjunctive therapies is unknown. It will be very difficult to show a meaningful clinical benefit in hospitalized patients for add-on therapies or preventive approaches for specific high-risk patient groups (the problem is to show that the new treatment works, rather than regulatory issues).

In addition to the R&D hurdles that antibiotics face, non-traditional antibacterials share some common challenges:

- Most anti-virulence and simplified microbiome approaches are indirect-acting strategies that do not inhibit or kill bacteria, and act by intervening or interacting with complex biological processes that may not be well understood. Furthermore, a causal relationship with clinical outcomes may not be known.
- Treatments that do not affect bacterial growth cannot use traditional MIC measurements that correlate reasonably well with outcome measures. Alternative in vitro tests need to be developed and validated. It remains unclear if current animal tests can predict outcome of non-traditional treatments in humans.
- The immense challenge of late stage clinical trials applies to all non-traditional therapies that must be administered with an active antibiotic. To demonstrate their efficacy and utility, until recently non-inferiority clinical studies (to show that the experimental treatment is no worse than the comparator) of adjuvants (active antibiotic with add-on therapy versus active antibiotic alone) were sufficient for regulatory approval. However, the FDA has recently indicated that they will prefer superiority studies (to show that the experimental treatment is better than standard of care) in relevant clinical endpoints for regulatory approval and future acceptance in clinical practice.
- In the case of biologics such as live bacterial preparations (Live biotherapeutic products), standardized and well characterized production processes as well as quality controls i.e. CMC requirements remain challenging, but progress is being made.
- Dose finding is a well characterized procedure in the antibiotic field and relies heavily on validated preclinical models that correlate PK and PD. Such predictive models are not usually available for non-traditional approaches and correlates to outcome effects may not be known. This is most apparent in the phage and microbiome fields.

- Many non-traditional therapies are pathogen-specific, or specific for a subset of strains of a species, or only active in a specific host niche or specific phase of infection. Such tailored approaches will require knowledge of underlying mechanisms and patient factors, and so high financial resources. Although most experts would agree that patient-specific antibacterial therapy is desirable, its translation into the clinical routine beyond highly specialized settings is unlikely to occur for many years. Therefore, without additional tailored diagnostics some of the therapies may not be useful.

Very few non-traditional therapies have advanced to late stage clinical trials. The most advanced non-traditional antibacterials are exotoxin targeting therapies, mostly mAbs (*C. difficile*, *S. aureus*). Similarly, microbiome therapies based on complex characterized microbiota are promising and likely to provide new options to treat or prevent CDI.

However, most therapies are in the discovery or preclinical development stages and so may not be available for at least 10 years. Commonly, non-traditional approaches require sophisticated diagnostics beyond pathogen identification and are patient-tailored approaches. The development and implementation of such specific companion diagnostics is further contributing to the challenges.

In conclusion whilst there is a considerable interest in the opportunities that non-traditional approaches will bring to treating bacterial infections, it is likely that effective treatments will be limited to healthcare settings with the best diagnostic and financial resources, and to healthcare systems that are able to financially support a strong growth of high-cost individual (personalized) medicines, and thus to high-income countries. The highest burden of drug-resistant infections is in babies and children in low-medium income countries. These are unlikely to have the resources for basic traditional antimicrobial treatments and in the near future extremely unlikely to be able to implement personalized medicine.

Author contributions

Ursula Theuretzbacher and Laura J.V. Piddock wrote this article.

Declaration of Interests

LJVP is currently seconded to the Global Antibiotic Research & Development Partnership. LJVP has no financial interests in any of the companies indicated in this article and has not received research funding to work on any of the approaches described herein. UT has no financial interests in any of the companies indicated in this article and has not received research funding to work on any of the approaches described herein.

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913 **Table 1. Monoclonal antibodies in clinical development**

Monoclonal antibody, company	Clinical development	Anti-virulence target	Indication
Suvratoxumab (MEDI4893), Medimmune	Phase 2	*S. aureus* α-toxin	Prevention of VAP caused by *S. aureus*
AR-301 (Salvecin, tosatoxumab), Aridis	Phase 3	*S. aureus* α-toxin	Adjunctive therapy for VAP caused by *S. aureus*
MEDI3902, Medimmune	Phase 2	*P. aeruginosa* T3SS needle tip protein PcrV and Psl exopolysaccharide	Prevention of VAP caused by *P. aeruginosa*
AR-105 (Aerucin), Aridis	Phase 2	*P. aeruginosa* alginate	Adjunctive treatment of VAP caused by *P. aeruginosa*
514G3, XBiotch	Phase 1/2	*S. aureus* Protein A	Adjunctive treatment of bloodstream infections caused by *S. aureus*
ASN-100, Arsanis	Phase 2	*S. aureus* α-toxin and five leukocidins	Failed to prove its effectiveness in high-risk, mechanically ventilated patients with *S. aureus* pneumonia

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915 For information about the status of R&D of antibodies, small molecules and other approaches
916 to new treatments, please see www.clincialtrials.gov or the developing organizations website.

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Table 2. Translational challenges of anti-virulence strategies

Challenge	Factor influencing translation
Selection of target	Selection of the most relevant anti-virulence target according to their distribution and contribution to disease
Coverage	VFs are not expressed uniformly in all strains. May differ geo-graphically and gene not present in all strains (genetic variation)
Effectiveness	May be effective only in specific disease states (e.g. chronic, dormant), a specific time point in the infectious process, at a specific infection site, or in specific patient groups (e.g. immunocompetent)
Diagnostics	Diagnostics beyond species identification may be necessary to guide use
Dose finding	Predictive models to support decisions to find the optimum dose are mostly lacking
Predictive models of efficacy	For most approaches there are no validated models that predict clinical outcome and preclinical studies may not be a meaningful guide to clinical development
Resistance	Resistance development has been shown for some anti-virulence approaches. It is not known how to predict the likelihood of developing anti-virulence drug resistance and any relevance in patients
Activity in patients	For some approaches it is not known if the selected approach has enough clinically relevant impact on the disease in humans
Clinical development	For adjunctive therapies non-inferiority studies will not prove that the therapy works in patients. Superiority studies are essential to show that the adjunctive therapy provides a benefit to patients. Superiority studies (and preventive studies) are very difficult to do.

922 **Table 3. Current approaches to manipulate the microbiome**

Approach	Indication	Comments
Transfer of human intestinal microbiota	Prevention of recurrent CDI	• FMT: Transfer of stool suspension from a donor via colonoscopy, enema, nasogastric routes or pills (Finch, Open Biome) • Fecal microbiota suspension: Standardized number of live bacteria from stool suspension from donors via enema or capsules, GMP produced (Rebiotix/Ferring) • Rationally selected microbiota: Well characterized selection of bacterial strains via capsules (Seres, Finch Therapeutics, Vedanta) • Spore suspension: Spores, fractionated from stool specimens from donors via capsules (Seres)
Synthetic microbiota	Intestinal, dermatological (e.g. atopic dermatitis), lung conditions (e.g. CF)	• Selected live bacteria producing specific metabolites or cocktail of secondary metabolites • Selected live bacteria from skin microbiota (MatriSys Bio) • Selected live bacteria for balancing the lung microbiota
Manipulating the metabolism of microbiota		Manipulating the metabolic balance through specific bacterial nutrients, e.g. Glycans (Kaleido)
Competition	Prevention of recurrent CDI, catheter associated UTI	• Non-toxinogenic *C. difficile* that is assumed to outcompete the toxic strain (Microbiotica) • Apathogenic E. coli introduced into the bladder via catheter coating (Atterx)
Engineering probiotics to deliver	Various indications (Bäumler and Sperandio, 2016)	• Engineered *Lactobacillus* to express bacteriocin against *P. aeruginosa* (inhaled, CF) and *C. difficile* (SciBac)

antibacterial proteins		• Engineered *Lactobacillus* to express SagA protein that promotes tolerance to enteric infections incl. *C. difficile* infection *(*Rise Therapeutics) • R-type bacteriocins against *C. difficile*
Prevention of disbalance of microbiome due to antibiotic therapy	Prevention of recurrent CDI	• Hydrolysing specific beta-lactam antibiotics in the gut (beta-lactamase, Synthetic Biologics, DaVolterra)
Decolonisation of MDR Gram-negative pathogens in high risk patients	Various indications	• Decolonisation of asymptomatic carriers with live bacteria consortia (e.g. *C. difficile*, MDR Gram-negative pathogens in high risk patients, *Salmonella* Typhi)

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925 **Table 4. Current approaches using phages**

Approach	Composition
Fixed phage cocktails	Fixed composition of lytic phages to achieve a broad host range of a bacterial species
Individualized phage cocktail	The lytic phages are stored individually in a phage bank with established QC. Only the best active phages based on rapid diagnostic tests are selected for an individual patient
Genetically engineered phages	Engineered phages with improved or specific characteristics
Genetically engineered non-replicating phages as vehicles	Engineered phages to express additionally antimicrobial peptides or protein toxins leading to rapid, nonlytic bacterial death. May deliver CRISPR CAS3 genes directly into bacteria
Phage products, e.g. endolysins	Natural or recombinant cell wall hydrolyzing phage-based enzymes. Endolysins against *S. aureus* are in clinical development

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