

The arsenal of pathogens and antivirulence therapeutic strategies for disarming them

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Abstract: Pathogens deploy an arsenal of virulence factors (VFs) to establish themselves within their infectious niche. The discovery of antimicrobial compounds and their development into therapeutics has made a monumental impact on human and microbial populations. Although humans have used antimicrobials for medicinal and agricultural purposes, microorganism populations have developed and shared resistance mechanisms to persevere in the face of classical antimicrobials. However, a positive substitute is antivirulence therapy; antivirulence therapeutics prevent or interrupt an infection by counteracting a pathogen's VFs. Their application can reduce the use of broad-spectrum antimicrobials and dampen the frequency with which resistant strains emerge. Here, we summarize the contribution of VFs to various acute and chronic infections. In correspondence with this, we provide an overview of the research and development of antivirulence strategies.

Keywords: virulence factors, antivirulence therapeutics, biofilms, regulation, *Escherichia coli*, quorum sensing, persister cells

The ascent and collapse of conventional antimicrobials

In 1929, Alexander Fleming described a method for isolating *Bacillus influenzae* using penicillium mold broth filtrate, which he termed penicillin.¹ In his report, Fleming emphasized penicillin's use in the bacteriological laboratory and briefly mentioned its potential implications for medicine. Ironically, in that first report, Fleming recommended taking advantage of the innate penicillin resistance of gram-negative bacteria to promote their isolation and identification.¹ This basic scientific discovery heralded in the golden age of antimicrobials.

Although penicillin launched the age of antimicrobials, prontosil, a sulfonamide discovered by Gerhard Domagk working for IG Farben (Bayer) in 1935, was the first antimicrobial manufactured for therapeutic application.^{2,3} *Neisseria* and *Streptococcus* infections were among the first to be widely treated with sulfonamides.^{2,4,5} Penicillin was not available for clinical use until the 1940s, when Ernst Chain and Howard Florey developed a method allowing for mass production.⁶

The abundant use of antibiotics that followed in hospitals and agricultural industry initiated the surfacing of antimicrobial-resistant strains. For example, sulfonamide-resistant *Neisseria gonorrhoeae* isolates became wide spread in the clinical setting shortly after World War II.⁵ Penicillin replaced sulfonamides for treatment, leading to the emergence of penicillin resistance within the next 15–20 years.^{7–9} Gradually, *N. gonorrhoeae* developed resistance to the vast majority of other classes of antibiotics, including aminoglycosides, tetracycline, macrolides, and most recently fluoroquinolones.¹⁰

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In 2007, the United States Centers for Disease Control and Prevention announced cephalosporins as the only remaining option for treating *N. gonorrhoeae*.¹¹ Strains with reduced susceptibility to the cephalosporins cefixime and ceftriaxone have since emerged.¹¹ Over the past few decades, only one new antimicrobial class was discovered.¹² The next generation of antimicrobials and therapeutic strategies must withstand the force of microbial evolution; unless, we wish to confront another cohort of “super-bugs”.

Antivirulence therapies (AVTs) that target virulence factors (VFs) or their production/regulation constitute an alternative approach to classical antimicrobials. This is in contrast to current cidal or static antimicrobials that disrupt pathogen growth, thereby selecting for mutations that would allow resistant populations to take over.¹³ The aim of many AVTs is to tax bacterial metabolism to a point that pathogens are incapable of paying the “fitness cost” associated with a prospective infectious niche. In addition, commensal microbes are unlikely to produce the targets of AVTs, thus reducing the collateral damage imposed to the host’s microbiota by current antimicrobials. However, AVTs require a greater level of forethought in their design and understanding of microbial pathogenesis, which of course come with a greater fiscal burden. In certain cases, effective implementation of AVTs requires rapid diagnostics, which is far from being a new priority in medical research. In this review, we focus on microbial VFs and the latest progress in developing AVTs against them.

A battle of the ages: common virulence traits and antivirulence strategies aimed to combat them

Establishing residency through adherence

Upon introduction to a niche, pathogens are faced with the challenge of remaining “on location” long enough to establish infection. Microorganisms can be swept away by shear forces applied from the movement of biological fluids and host cell cilia. Pathogens can use various extracellular structures for mediating adherence to host cells. Enteropathogenic *Escherichia coli* (EPEC) rely on bundle-forming pili (BFP) and EspA filaments for microcolony formation and early attachment to intestinal epithelial cells.^{14–16} Volunteers, who ingested wild-type EPEC, exhibited frequent bouts of diarrhea; in contrast, their luckier counterparts, who ingested *bfp* mutant strains, had negligible symptoms.¹⁷ EPEC, enterohemorrhagic *E. coli* (EHEC), and their murine model counterpart *Citrobacter rodentium* can form attaching and effacing lesions during infection. These pathogens use type 3

secretion systems (T3SSs) to inject the intimin receptor (Tir) into the host cell.^{18–20} Translocated Tir becomes embedded into the host cell and serves as a bacterial docking mechanism via its interaction with the bacterial intimin protein.^{18–20}

Extraintestinal pathogenic *E. coli* adhere to host cell niches via the use of pili that assemble by the chaperone usher pathway (CUP) system.^{21–27} The proteins adorning the tips of CUP pili are specialized adhesins with stereochemical specificity to distinct moieties depending on the CUP pilus. For example, the adhesin of type 1 pili (Fim), FimH, mediates adherence to the bladder epithelial cells by binding with glycoproteins on the host cell’s surface.^{23,28} The pyelonephritis-associated pili (*pap*) operon codes for the P pili components; P pili are capped by the PapG adhesin, which binds specifically to the α -D-Galp-(1→4)- β -D-Galp disaccharide moieties and mediates kidney colonization.^{29–35}

Antiadherence strategies to prevent bacterial colonization

Strategies aiming to prevent bacterial adherence can target the regulatory systems governing adhesin expression, disrupt the secretion of adhesins and their assembly, or block the binding between the bacterial adhesin and host receptor. In EPEC, a vaccine against BFP is in development, inspired by production of antibodies to the BfpA component, in children naturally infected with EPEC.^{36–38} Research delineating the composition and biogenesis of BFP has elucidated the occurrence of two BfpA variants, termed α or β .³⁹ The minor subunits BfpJ, BfpI, and BfpK are also found incorporated in the external BFP filament.⁴⁰ A vaccine including both BfpA versions and Bfp minor units will likely confer a wider range of protection. The capacity of an EPEC vaccine against BFP subunits to elicit a protective and memorable immune response is untested at this time.

Another strategy targeting adhesins is the rational design of host receptor mimicking saccharides, which is heavily sought after for uropathogenic *E. coli* (UPEC) infections. The vital role of PapG and FimH in mediating adherence to the uroepithelium makes them prime candidates for antivirulence targeting. Analysis of the binding requirements between PapG and the galabiose receptor guided the design of a PapG inhibitor with a superior aptitude for binding to the host receptor than the natural galabiose receptor; the ability of this PapG inhibitor or derivatives to outcompete the galabiose receptor and consequently disrupt UPEC adherence in vivo is a priority for further development.⁴¹ The same strategy is even further along in development for targeting FimH of UPEC’s type 1 pili. FimH inhibitors, or mannosides, are analogous

to the FimH receptors and sequester available FimH from binding. In murine models, a mannoside was effective in reducing the bladder bacterial burden during acute and chronic cystitis; furthermore, administration of the mannoside made a trimethoprim–sulfamethoxazole-resistant UPEC strain vulnerable to trimethoprim–sulfamethoxazole treatment.^{42,43} CocrySTALLIZATION of FimH with mannosides facilitated the engineering of a new mannoside with improved affinity for FimH and increased availability in urine, which further reduced bladder bacterial burden compared to the predecessor compound.⁴⁴

Mannosides have recently been used to target the FimH of adherent-invasive *E. coli* (AIEC) associated with Crohn's disease (CD) and *E. coli* isolated from osteoarticular (hip replacement) infections.⁴⁵ In a CD murine model, transgenic CEABAC10 mice, which express the human CECAM6 mannosylated receptor, infected with AIEC LF82, were treated with monovalent mannosides post-infection.⁴⁶ Two mannosides designated 1A-HM and 1CD-HM reduced the bacterial fecal load by ~2.5 and 3 logs, respectively; AIEC LF82 associated with the ileal and colonic mucosa dropped below the limit of detection and mirrored the uninfected mice.⁴⁶ Furthermore, treatment with 1A-HM and 1CD-HM severely diminished colitis and inflammation.⁴⁶

Disrupting the biogenesis of adherence factors is another promising AVT. Larzabal et al⁴⁷ synthesized two small peptides corresponding to the C-terminal coiled-coil domain of the EPEC T3SS translocator EspA. These coiled-coil peptides blocked polymerization of EspA disrupting proper T3SS function and formation of attaching and effacing lesion on HEp-2 cells by EPEC and EHEC.^{47,48} C57Bl/6 mice inoculated with *C. rodentium* pretreated with both coiled-coil peptides suffered no damage to their colonic epithelium compared to their untreated counterparts.⁴⁸

In UPEC, nonpeptidic, pyridone molecules have been designed to bind specifically to the periplasmic chaperone protein, which escorts CUP pili subunits across the periplasmic space.⁴⁹ The application of chemical synthesis platforms enabled the generation of large-scale 2-pyridone libraries and analysis of various chemical substitutions impacting pilicide potency.^{50,51} Recently, one pilicide, ec240, was shown to act on the transcriptional level repressing transcription of the *fim* operon.⁵² Ec240 is still in the early stages of preclinical development and its direct target(s) remain unknown.

Evasion of the innate immune system and invading the host

Initially, pathogens confront innate immune system barriers, including lysozyme and immunoglobulin A (IgA), and

antimicrobial peptides (AMPs).^{53–57} Bacteria, such as *Yersinia pestis* (the causative agent of pneumonic and bubonic plague) and *Moraxella catarrhalis* (one of the major causes of otitis media), encode lysozyme inhibitors to combat damage by lysozyme.^{58,59} *Y. pestis* mutants with deletion of the *ivy* gene, which encodes for a lysozyme inhibitor, were attenuated in *Y. pestis* bubonic and primary pneumonic plague rodent models.⁵⁹ Wild-type and Δ *ivy* *Y. pestis* strains exhibited indistinguishable virulence potential in FVB lysozyme M-deficient mice, which cannot produce antimicrobial lysozyme LysM.⁵⁹ In the mucosa, IgA helps maintain balance between the host and their microbiota, as well as, dissuade potential pathogens from invading the mucosa.⁵⁴ *Streptococcus pneumoniae* IgA1 protease cleaves IgA1 heavy chain and prevents IgA-dependent killing by human neutrophils in vitro.⁶⁰ In agreement with this in vitro IgA1 protease activity, mice passively immunized with a human monoclonal IgA1 antibody had enhanced protection from intranasal challenge with an IgA1 protease mutant compared to mice challenged with wild-type *S. pneumoniae*.⁶⁰ To counter AMP assault, some pathogens express proteins that proteolytically cleave the AMPs; examples include the EHEC OmpT, *C. rodentium* CroP, and *Y. pestis* Pla, which are homologues outer membrane proteases of the omptin family.^{61–64} On the other hand, *Haemophilus influenzae* imports AMPs from the periplasm, using the ABC transporter SapA, and degrades them for use as nutrient resource.⁶⁵

In addition to defense mechanisms mounted against innate immune barriers, pathogens can actively manipulate the host immune response. *Salmonella enterica* ser. Typhimurium is equipped with two T3SSs encoded on separate *Salmonella* pathogenicity islands (SPI-1 and SPI-2).^{66–68} The SPI-1-encoded T3SS is required for invasion of intestinal epithelial cells, and the SPI-2 system to manipulate phagolysosome fate and promote *Salmonella*'s intracellular survival.^{68–70} *S. enterica* secretes TlpA and SseL effector proteins that restrict a nuclear factor κ B-mediated inflammatory response and promote bacterial intracellular survival.^{71,72} *Yersinia pseudotuberculosis* has a similar anti-inflammatory virulence strategy, the T3SS effector YopJ deubiquitinates I κ B α , thereby preventing formation of free nuclear factor κ B that would induce a proinflammatory response.⁷³

Supporting the immune system and impeding bacterial invasion

An immune boosting therapeutic approach that is regaining popularity is the administration of exogenous AMPs or compounds that bolster the production of endogenous AMPs.

AMP drugs can potentially be used in combination with compounds that derail AMP resistance mechanisms that are common among pathogens.⁷⁴ *Y. pestis* Pla and *Shigella flexneri* IcsP omptins are required for dissemination within the host.^{75–79} The small peptide inhibitor aprotinin is the first inhibitor identified to be capable of inhibiting multiple omptin proteases and provides the basis for the construction of future therapeutic agents that can disrupt the role of omptin proteases in AMP resistance of multiple pathogens.⁸⁰ More in-depth research is required to understand the numerous AMP resistance mechanisms before an AVT-supporting AMP is successfully composed.

Salicylidene acylhydrazides inhibit the translocation of effector proteins through the T3SSs of both *S. enterica* ser. Typhimurium and *Y. pseudotuberculosis* in vitro.^{81,82} Importantly, salicylidene acylhydrazides also inhibited invasion of epithelial cells by *S. enterica* ser. Typhimurium in vitro.^{82,83} These compounds are also effective against *Chlamydia trachomatis* and *S. flexneri* T3SSs.^{84,85} However, the mechanism by which these compounds disrupt different T3SSs remains to be fully elucidated.

Nutrient scavenging

Nutrient acquisition is exceedingly difficult when pathogens have to compete with their fellow microbes and their host begins an attempt to starve them out. Bacterial niche competition can even occur within the same species; *E. coli* strains MG1655, HS, Nissle 1917, and EDL933 compete with one another for various resources during colonization of the murine intestinal lumen.^{86–88} In the intestinal lumen of specific pathogen-free C57Bl/6 mice, commensal *E. coli* outcompete *C. rodentium*.⁸⁹ Although prevented from colonizing the lumen, *C. rodentium* invades the intestinal mucosa and infects the epithelial cells inaccessible to commensals.⁸⁹

The host's systems are highly reluctant to give up their needed nutrients, such as Fe, and keep them well guarded.⁹⁰ Host immune cells secrete lipocalin 2 that binds to the *E. coli* siderophore enterochelin disrupting *E. coli*'s acquisition of Fe.⁹¹ In a *Staphylococcus aureus* murine infection model, neutrophil calprotectin restricts bacterial growth within abscess by limiting the level of available Mn²⁺ and Zn²⁺.⁹⁰ To survive in their ornery host, pathogens have developed intensive means for gathering their provisions. Pathogens are adept at scavenging valuable essential metals such as Fe, Zn, and Mn. Accumulation of Fe can be toxic to bacteria and requires strict regulation. When intracellular Fe is abundant, the ferric uptake regulator (Fur) binds Fe and represses the transcription of Fe acquisition systems, thereby avoiding

Fe overload and toxicity.⁹² As the Fe level drops, iron-free Fur poorly binds DNA and the once repressed systems become derepressed and more easily transcribed.⁹² Fur also represses the small noncoding RNA (sRNA) RyhB, which negatively regulates the expression of genes whose products utilize or store Fe.⁹³ *S. enterica* ser. Typhi *fur* mutants were defective in their invasion of epithelial and macrophage cells; additionally, *S. enterica* ser. Typhi requires Fur for in vitro intracellular macrophage survival.⁹⁴ UPEC CFT073 Δ *ryhB* and Δ *ryhB* Δ *fur* mutants are attenuated in murine bladder infection, while Δ *fur* is not.⁹⁵ In the absence of RyhB, the corresponding UPEC CFT073 *rhyB* mutant secreted lower levels of siderophores.⁹⁵ *S. aureus* is equipped with iron-regulated surface determinants that allow for the harvesting of Fe from the host's hemoglobin.^{96,97} *S. aureus* scavenges hemoglobin, extracts, and transfers heme to the cytoplasmic IsdG, a heme monooxygenase, which liberates Fe from heme for use by the bacteria.^{96,98} If too much heme is imported or synthesized by *S. aureus*, the heme sensory two-component system (HssRS) is activated and mediates the upregulation of the heme response transporter (HtrAB), an efflux pump, to avoid heme toxicity.^{99–101}

Starving the enemy

A particularly interesting therapeutic approach to infections is to reestablish healthy microbiota to compete with the disruptive pathogen. After a patient's gut microbiome is disrupted, *Clostridium difficile* no longer faces resource competition and takes the opportunity to take over the intestine.¹⁰² The modern fecal microbiota transplant (FMT), initially investigated in the treatment of pseudomembranous enterocolitis in 1958, involves the retrieval of the microbiota from a donor's fecal sample and placed into the intestinal tract of a recipient patient.¹⁰³ FMT has become popular in recent years for treating *C. difficile* recurrent infections.¹⁰⁴ FMT allows restoration of the microbiota and microbe–microbe competitive interactions. Simplifying the transplantation process and development toward donor pools is likely to contribute to the expansion of FMT use in coming years.^{105,106} The capacity of FMT to treat various intestinal diseases is yet to be fully explored.

Another “starvation” strategy is the use of small molecules that can potentially be used to target iron uptake systems. Outer membrane transporters rely on energy from the ExbB-ExbD-TonB system to bring in siderophores.¹⁰⁷ High-throughput screening of a small molecule library revealed 16 compounds that were bacteriostatic against UPEC under iron-limiting growth conditions.¹⁰⁸ A *tonB* mutant was not

sensitive to two of these compounds, suggesting that they disrupted TonB activity.¹⁰⁸ These small molecules are still in the early stages of investigation.¹⁰⁸ Hma, IreA, IutA, and FyuA are siderophore receptors highly conserved across UPEC strains found in the bacterial outer membrane and surface exposed, which makes them ideal vaccine candidates.^{109,110} Intranasal immunization with Hma, IreA, IutA, or FyuA elicits a humoral antigen-specific response, and mice were protected against UPEC 1 week after immunization.^{109,110} A long-lasting response to Hma, IreA, and IutA remains undetermined at this point. FyuA immunization generated long-lived plasma cells indicating a potential for lasting memory and specifically protective against UPEC in pyelonephritis infection models.^{109,111}

Chemical probes are helpful tools for the molecular dissection of biological systems.¹¹² During their investigation of *S. aureus* HssRS activation by heme, Mike et al¹¹³ identified compound '882 that indirectly activated HssRS by promoting heme biosynthesis. Independent from HssRS activation, '882 was bacteriostatic under fermentative conditions and an '882 derivative, '373, significantly reduced the bacteria load within liver abscess of *S. aureus*-infected mice.¹¹³ Chemical modification of the parental '822 allowed for two different classes of derivatives with activity specific for HssRS activation or inhibitors of fermentative growth.¹¹⁴ While analysis of heme biosynthesis will progress with the usage of HssRS activators, the other new class of compounds represents a promising new scaffold for the development of therapeutics against *S. aureus* infections.

Microbial population dynamics

A pathogen's virulence is not solely reliant upon the genetic toolbox equipped within an individual cell. In certain cases, microorganisms will work together in a socialistic fashion to benefit the population at large. This survival strategy includes the formation of biofilms, intracellular bacterial communities (IBCs), and "division of labor" among subpopulations. Biofilms are an accumulation of sessile and diverse microbial populations held together by an extracellular matrix, which shields the microorganisms from antibiotic treatment.^{115,116} The extracellular matrix consists of an arrangement of various secreted biomolecules potentially including adhesive fibers, carbohydrate-binding proteins, polysaccharides, and extracellular DNA.^{115,116} *Candida albicans* and UPEC form biofilms on indwelling medical devices and pose serious health risks.^{116,117} During infection, UPEC enters superficial umbrella epithelial cells. Intracellular UPEC develops biofilm-like pods and replicates prior to release into the lumen to avoid the exfoliation

process of the urothelium.^{28,118,119} Upon exodus from the surface epithelial cell, UPEC infects additional surface cells, as well as, invading the exposed underlying epithelial cells.^{28,118,119} Bacteria invading the intermediate epithelium settle quiescent intermediate reservoirs (QIRs) and are safe from antibiotics that abolish the exposed extracellular.^{120,121} Weeks later, QIR bacteria can emerge from their fallout shelters and reinitiate the infectious cycle.^{121,122} IBC formation is not exclusively an UPEC-specific strategy; *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* also invade epithelial cells and form intracellular biofilm-like communities.^{123,124}

Within bacterial populations, organized subpopulations are phenotypically distinct and can carry out actions that benefit the entire bacterial community. *Y. pseudotuberculosis* cells on the peripheral region of a microcolony produce Hmp, a nitric oxide detoxifying enzyme, in response to toxic nitric oxide from the host.¹²⁵ The Hmp producing subpopulation prevents nitric oxide from penetrating the microcolony, and the protected centroid cells are spared the unnecessary *hmp* expression.¹²⁵ Originally recognized for their persistent survival in the presence of penicillin in the early 1940s, persister cells are genotypically identical to their kin yet metabolically dormant.¹²⁶ The minute persister subpopulation serves as a fail-safe to ensure survival in case of a catastrophic event, such as antibiotic clearance of the metabolically active cells.

Rising evidence indicates that bacterial signaling impacts subpopulation heterogeneity. *E. coli* cells sensing the presence of indole, which is produced under nutrient-limiting growth conditions, are more likely to enter a persister cell state.¹²⁷ *P. aeruginosa* uses quorum sensing (QS) to coordinate the persister subpopulation numbers during logarithmic phase.¹²⁸ Addition of cell-free spent media from stationary phase cultures or the addition exogenous QS autoinducers pyocyanine or *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC-HSL) substantially promoted persister cell formation of logarithmic *P. aeruginosa* cultures.¹²⁸ The autoinducer competence-stimulating peptide controls the transformational competency and biofilm formation of *S. pneumoniae* at low concentrations; however, at high concentrations competence-stimulating peptide stalls population growth through stimulating the ComD sensor.^{129–131} These studies highlight the dynamic role of QS in coordinating bacterial populations.

Disturbing the peace of biofilm populations

Biofilm formation allows bacteria to survive antibiotic treatment. Multiple factors are proposed to contribute to biofilm

antibiotic tolerance, including persister cell populations, slow growth, and poor antibiotic penetration. Within the past few years, development of platforms for high-throughput screening of chemical libraries has accelerated the discovery of compounds active against *C. albicans* biofilms.^{132,133} A family of diazapirodecane-based compounds was found particularly effective at inhibiting *C. albicans* biofilm formation.¹³⁴ Not only did the lead candidate, identified as compound 61894700, disrupt biofilm formation but also filamentation without affecting growth of planktonic cells.¹³⁴ Prophylactic treatment with compound 61894700 in an oral and invasive systemic candidiasis murine models reduced fungal filamentation and decreased disease severity.¹³⁴ Another strategy is to find compounds that sensitize biofilms to currently available drugs. Separately, 2-adamantanamine (AC-17) and azoles are not fungistatic, yet the combination of the two is fungicidal.¹³⁵ In vitro analysis reveals that AC-17-exposed *C. albicans* had disrupted ergosterol and hyphae production.¹³⁵ In a guinea pig cutaneous candidiasis model, treatment of subinhibitory fluconazole or AC-17 did not reduce the *Candida* tissue burden; nevertheless, dual treatment significantly reduced fungal burden.¹³⁵

UPEC QIR poses the additional challenge of passing the antimicrobial agents through the eukaryotic cells. Accelerating the uroepithelial cell turnover can circumvent this problem by exposing the intracellularly sheltered bacteria. Blango and colleagues¹³⁶ found that in a murine model resurgence of UPEC QIRs can be initiated by inducing exfoliation of bladder epithelial cells with chitosan. UPEC UTI89-infected mice that were treated with chitosan had a significant reduction in bacteria loads within the bladder 3 days posttreatment, compared to mice treated with antibiotics alone.¹³⁶ Chitosan treatment is likely to be found to lower the frequency of UPEC UTI recurrence.¹³⁶ The occurrence of UPEC QIR is reported in multiple clinical studies indicating that these intracellular biofilm-like communities are not specific to murine models.^{137–139} Furthermore, the presence of QIR in human UPEC UTIs validates AVTs, targeting this critical step in pathogenesis.

Biofilm tolerance is largely attributed to the presence of a dormant persister subpopulation.^{140,141} The persister subpopulation is associated with the recurrent *P. aeruginosa* infections of cystic fibrosis patients and long-term carriage of *Candida* by cancer patients prophylactically treated with topical chlorhexidine as part of their oral care.^{142,143} Therapeutics aimed at “resuscitating” persister cells are of particular importance for preventing recurrent infections. The protease ClpP is normally tightly controlled through ATPases;

however, in the presence of acyldepsipeptides, ClpP becomes active independent of ATPases.¹⁴⁴ Without proper regulatory control, ClpP nonspecifically degrades bacterial proteins resulting in autodestruction of the bacterial cell.¹⁴⁴ In lethal systemic murine infections of *E. faecalis* and *S. aureus*, the acyldepsipeptide (ADEP4) rescued 100% and 80% of the mice, respectively.^{144,145} Unlike rifampicin, ADEP4 eliminated ciprofloxacin surviving *S. aureus* persister cells in vitro, although a small population of *S. aureus* survived through *clpP* null mutations. A combination of rifampicin and ADEP4 effectively abolished *S. aureus* biofilms in vitro and *S. aureus* mouse thigh infections.¹⁴⁵ This is an example of a combinatorial approach to clear an infection through coordinated targeting of different subpopulations.

Instead of pursuing cells within a microbial population directly, the cells within the population can also be targeted indirectly through severing the intercellular communication systems by which the microbes synchronize their pathogenic efforts. *P. aeruginosa* coordinates biofilm fate and persister cell population through the synthesis and release of the extracellular autoinducer molecule 3OC-HSL.^{128,146} Production of both 3OC-HSL and *N*-butyryl homoserine lactone autoinducers is required for successful lung infection of *P. aeruginosa* in rodent models.^{147,148} A class of compound derived from marine algae *Delisea pulchra* called furanones disrupts the 3OC-HSL sensory system of *P. aeruginosa* and sensitized biofilms to tobramycin killing in vitro.¹⁴⁹ Administration of furanones C30 and C56 extended the survival time of mice with lethal pulmonary *P. aeruginosa* infections.^{148,149} By interfering with quorum sensory, furanones disrupt the expression of VFs. This disruption of VFs may potentiate the activity of conventional antibiotics that struggle to clear biofilms.

Regulatory systems of pathogens

To prevent excessive metabolic expenditure, microorganisms closely regulate their core metabolic processes, such as with the *fur* system previously discussed. Bacterial pathogens can monitor and react to their extracellular environments through two-component sensory systems (TCSs) comprised of a sensor kinase and a response regulator (RR). Typically, upon stimulation, the sensor kinase undergoes autophosphorylation and in turn phosphorylates and activates the RR. The QS *E. coli* (QseBC) TCS deviates from the customary signal transference cascade. Proper expression of the QseB regulon is tightly controlled through QseC.^{150,151} Deletion of *qseC* in UPEC UTI89 leads to virulence attenuation in a murine UTI model plus misregulation of curli, pili, flagella, and core

metabolic processes, whereas UPEC UTI $\Delta qseB$ and $\Delta qseBC$ do not display reduction in virulence.^{150,151} This contradiction to the conventional TCS model is explained by the ability of QseC to dephosphorylate QseB and allow expression of the QseB repressed genes.¹⁵¹ The PhoPQ TCS is critical for the regulation of VF of *S. enterica*, UPEC, *C. rodentium*, and *P. aeruginosa*.^{64,152–154} Stimulation of the sensor PhoQ by low cation concentrations, acidic pH, and AMP induces canonical activation of the regulatory PhoP.¹⁵⁵ *S. enterica* ser. Typhi strains with deletion of *phoPQ* were constructed for the development of live attenuated oral vaccine.^{156,157} Although these strains did not elicit a strong immunogenic response, they were profoundly attenuated in virulence.^{156,157} The reliance of *S. enterica* on PhoPQ during infection is attributed to the ability of PhoPQ to coordinate restructuring of the outer membrane.¹⁵⁵

sRNAs are an additional regulatory layer of pathogens to control their transcripts. *E. coli*, *P. aeruginosa*, *S. aureus*, and *Vibrio cholerae* can use sRNA to moderate their production of VFs.^{158–161} In *E. coli*, the master regulator *phoPQ* mRNA is regulated by MicA and GcvB, which are sRNAs stabilized by host factor Q.^{162,163} MicA and GcvB both regulate *phoP* mRNA translation to different extents, which is reflected in their different effects on the PhoP regulon.¹⁶³ *V. cholerae* and *Vibrio harveyi* have complex regulatory network to modulate their QS pathways. At low cell density and autoinducer concentrations, the QS receptors LuxPQ, LuxN, and CqsS transfer phosphate through LuxU to the RR LuxO.^{164–167} Phosphorylated LuxO promotes the transcription of the four quorum regulatory rna loci (*qrr1–4*).^{158,168,169} These Qrr sRNAs redundantly repress and activate the translation of the high cell density HapR and low cell density AphA master regulators, respectively.^{169–172} This regulatory system is reversed in high-density populations with high autoinducer production.^{169,173} The *Vibrio* QS regulatory network seems to allow prioritization of VF production. The Qrr sRNAs directly repress the large type 6 secretion system (T6SS) cluster, which encodes the secretory machinery, and indirectly represses T6SS effectors that are activated by HapR.¹⁷¹ The response of Qrr sRNAs genes, such as those encoding for T6SS, directly targeted is relatively more rapid compared to those indirectly regulated through HapR.^{171,172}

Communication breakdown

The disruption of genetic regulatory systems within pathogens poses an option to target multiple virulence systems with one blow and drive the microbe's virulence to ruin. Misregulation of VF genes results in attenuation. AVTs can

block signal detection or inhibit signal relay between the sensor and regulator. The compound LED209 prevents the autophosphorylation of the EHEC sensor QseC.¹⁷⁴ Although LED209 treatment failed to prevent EHEC from intestinal colonization in rabbits, LED209 treatment pre- and postinoculation of *S. enterica* ser. Typhimurium and *Franciaisella tularensis* modestly improved the survival rates of mice.^{174,175} LED209 demonstrates the feasibility of targeting virulence regulatory systems, and future LED209 derivatives may have improved bioavailability and efficacy during infection.^{174,175} The QseBC system cross-interacts with the PmrAB system associated with polymyxin resistance.¹⁷⁶ In the absence of QseC, PmrB favorably phosphorylates QseB, which would normally be dephosphorylated by QseC.¹⁷⁶ QseC and PmrB balance out the level of active QseB. This delicate harmonization is reflected during UPEC infection models. In a UPEC model for acute UTI, UPEC UTI89 $\Delta qseC$ forms significantly fewer IBCs than wild type, and deletion of *pmrB* in the UTI89 $\Delta qseC$ strain restores IBC abundance to wild-type levels.^{176,177} An AVT that promotes the buildup of QseB to diminish UPEC virulence by increasing phosphorylated QseB through enhancing the PmrB kinase and/or inhibiting QseC phosphatase activities would be ideal.¹⁷⁶

The *Vibrio* QS network contains multiple promising targets, including signal receptors and gene regulators.¹⁷⁸ A pro-QS strategy is proposed to disrupt the QS signaling cascade and expression of VF between low and high cell density population transitions.^{179,180} One class of compounds was found that inhibits the ATPase action of phosphorylated LuxO and consequently disrupted regulation of the downstream QS circuitry.¹⁷⁸ Analysis of the biosynthesis of the autoinducer CAI-1 identified the intermediate product Ea-CAI-1 as more potent agonist than CAI-1.¹⁸¹ Recently, stable derivatives of Ea-CAI-1 were developed. The most stable Ea-CAI-1 derivative, compound 18, is approximately tenfold more potent than CAI-1.¹⁷⁹ Compound 18 does not display toxicity to murine fibroblast cells or disrupt the growth of *V. cholerae*.¹⁷⁹ The ability of compound 18 to improve *V. cholerae* infection remains to be tested. These studies demonstrate the validity of prosignaling therapeutics strategies, promoting the suppression of VFs. These AVTs may also be applicable to other sensory systems, such as with the MerR-like transcriptional regulator BluR in *E. coli*. BluR is a repressor of *ymgA* and *ymgB*.¹⁸² In the presence of blue-light irradiation, BluF, a flavin binding sensor, binds and antagonizes BluR allowing for the transcription of *ymgA* and *ymgB*, which promote biofilm formation through the RcsCDB phosphorelay system.¹⁸² Blue-light irradiation was recently

shown to deter growth of *E. coli* in a strain and growth phase-dependent manner, as well as, induce a persister-like state in some of the cells, which may involve BluFR light sensing (Mitchell et al, unpublished data, 2016). In sum, the targeting of virulence regulation both at the transcriptional and translational levels is a very promising prospect that has many avenues open for exploration and development.

Conclusion and outlook

Commensal microorganisms maintain a delicate balance with their host. Pathogenic microorganisms acquire VF stochastically and via selective pressures and use them to misbehave in a niche-dependent manner. During the 20th century, classical antimicrobials have helped to strike back at pathogens; nevertheless, the pathogen menace is equipped with strategies to resist antimicrobials and return for their revenge. The widespread usage of antimicrobials awakened a force of resistance mechanisms, which we must confront to thwart the postantibiotic era we are facing. The quagmire of antibiotic resistance has reached such an extraordinary level that it has finally captured the interest of politicians. In 2014, US President Obama signed Executive Order 13676: Combating Antibiotic-Resistant Bacteria, which called for the formation of Task Force for Combatting Antimicrobial-Resistant Bacteria.¹⁸³ The plan of action outlined by the task force set in place under this executive order prioritizes the development of alternative antimicrobials, such as AVTs, and next-generation diagnostic tools.¹⁸⁴ We are at a critical point where necessity is motivating the development of new discovery platforms. Many questions remain to be answered. AVTs represent a new hope for starting a silver age of antibiotics.

Disclosure

The authors report no conflicts of interest in this work.

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