

# **Anti-virulence strategies to target bacterial infections**

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## **Abstract**

Resistance of important bacterial pathogens to common antimicrobial therapies and the emergence of multidrug-resistant bacteria is increasing at an alarming rate and constitutes one of our greatest challenges in the combat of bacterial infection and accompanied diseases. Given the current shortage of effective drugs, lack of successful prevention measures and only a few new antibiotics in the clinical pipeline demands the development of novel treatment options and alternative antimicrobial therapies. Our increasing understanding of bacterial virulence strategies and the induced molecular pathways of the infectious disease provide novel opportunities to target and interfere with crucial pathogenicity factors or virulence-associated traits of the bacteria while bypassing the evolutionary pressure on the bacterium to develop resistance. In the past decade, numerous new bacterial targets for anti-virulence therapies have been identified, and structure-based tailoring of intervention strategies as well as screening assays for small molecule inhibitors of such pathways were successfully established. In this chapter, we will take a closer look at the bacterial virulence-related factors and processes that present promising targets for anti-virulence therapies, recently discovered inhibitory substances and their promises and discuss the challenges and problems that have to be faced.

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# 1 Introduction - Challenges in fighting bacterial infections

Although prevention and treatment of infectious diseases has improved over the last decades due to the widespread use of vaccines and anti-infectives and the development of infection control measurements, bacterial infections are still a major cause of morbidity and mortality worldwide. In particular global spreading of antibiotic resistance genes and their acquisition by clinically relevant bacterial pathogens constitutes a serious public health problem. In the European Union alone, about 3 million healthcare-associated infections were reported in 2004 leading to an approximate 50,000 deaths (McHugh et al. 2010). A report published by the Centre for Disease Control and Prevention (CDC) in 2013 estimates that more than 2 million infections and 23,000 deaths annually are caused by antibiotic-resistant bacteria in the USA alone and lists the top 18 drug resistant pathogens considered a threat in the USA (Control 2013). These pathogens are categorized into three threat classes, urgent, serious and concerning and include the clinically most relevant pathogens *Enterococcus faecium*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species summarized as ESKAPE pathogens (Rice 2008) to emphasize that they efficiently “escape” the effects of common antibacterial drugs. This list also includes *Clostridium difficile*, *Neisseria gonorrhoeae*, carbapenem- and Extended Spectrum  $\beta$ -Lactam-resistant *Enterobacteriaceae* (ESBL), drug-resistant *Campylobacter*, *Salmonella* and *Shigella*, and *Streptococcus pneumoniae* (Control 2013).

At a time, when antibiotic and multidrug-resistance is becoming increasingly common in the clinic, new treatments are urgently needed to circumvent high mortality rates due to untreatable infections. Increasing effort is currently being put into the discovery and development of new resistance-breaking antibiotics and some novel antimicrobial agents are currently in the preclinical and clinical pipeline (Hesterkamp 2015). However, we do not yet know whether this classical approach will succeed to identify new agents with activities against these pan-resistant pathogens in the foreseeable future. Recent work on the occurrence of antibiotic resistance determinants in bacterial populations further demonstrated that non-pathogenic bacteria can be identified in the environment that are already resistant to recently developed antimicrobial drugs (D'Costa et al. 2006; Martinez et al. 2009). For instance, many antibiotic-producing microorganisms encode resistance genes to the antibiotics they

synthesize for self-protection (Hopwood 2007). In addition, many of the acquired antibiotic resistance genes are carried on mobile genetic elements such as transposons, integrons, and plasmids, which are frequently transferred to other bacteria of the same or related species by horizontal gene transfer. Evidence exists that the transfer of resistance determinants between commensal bacteria and pathogens, in particular in the intestinal tract is extensive, leading to the rapid spread of resistance in bacterial populations and communities (Aarestrup 2005; Allen et al. 2010; Salyers et al. 2004). Moreover, an alarmingly high prevalence of antibiotic resistant bacterial strains has been reported in both, domestic and wild animals as well as in the environment. Prophylactic use of antibiotics in agricultural settings and in feed/water in the animal food production has significantly enhanced the evolution and global spread of antibiotic resistances (Allen et al. 2010; Berendonk et al. 2015; Canton 2009). These findings are daunting as bacteria can rapidly catch up or are even on par with the development of new chemical entities and the danger that we re-enter an apocalyptic pre-antibiotic era is looming on the horizon.

Consequently, novel intervention strategies are required to respond to current antimicrobial resistance and anticipate evolving resistance mechanisms (Häussler 2015/same issue). One compelling approach to antibiotic therapy is the development of anti-virulence strategies, by which only virulence-, but not survival/fitness-relevant traits are targeted. In other words, the anti-infective drug interferes with pathogenicity mechanisms, in particular properties of the bacteria that cause disease. This targeted intervention effectively disarms the pathogen and enables its clearance by the host immune system, but contrary to common antibiotic therapies it is not bacteriostatic (inhibiting bacterial growth) or bacteriocidal (killing bacteria). Active agents acting in this fashion, the so-called '**patho- or virulence blockers**' alleviate the pressure on the pathogen to develop resistance by solely affecting pathogens expressing the targeted pathogenicity factor (Baron 2010; Beckham and Roe 2014; Cegelski et al. 2008; Clatworthy et al. 2007; Escaich 2008; Lee and Boucher 2015; Lynch and Wiener-Kronish 2008; O'Connell et al. 2013; Rasko and Sperandio 2010; Zambelloni et al. 2015). Consequently, they neither damage nor modify the composition of the natural host microbiota, a process, which is increasingly recognized to facilitate the development, progress and persistence of chronic inflammatory diseases and other morbidities such as diabetes.

## 2 Potential Virulence Targets and Strategies

Recent efforts in the development of anti-virulence therapies are directed to target various factors or mechanisms of pathogens that are crucial to initiate an infection and cause disease. This includes interference with various pathogenicity factors promoting cell adhesion, cell invasion, intracellular replication and damage of host tissues, biofilm formation and maintenance (Fig. 1), stress adaptation and metabolic functions important to adapt to the different host environments, mechanisms to evade or overcome the host immune defense, and control systems regulating the expression of virulence-relevant genes.

The development of novel technologies, such as *in vivo* transcriptome analysis by RNA-Seq (dual RNA-Seq) and transposon-directed insertion site sequencing (TraDIS) now enables us to identify *in vivo* active genes and gene functions that are crucial for the survival of pathogens in certain tissues (Chaudhuri et al. 2013; Langridge et al. 2009; Westermann et al. 2012). The discovery of the bottlenecks of an infection will allow us to develop anti-infectives that particularly target fundamental virulence mechanisms (the Achilles heel) of a pathogen. Especially approaches that (i) target classical virulence factors, such as adhesins/invasins, (ii) inhibit pathogen-induced host signaling disruption by toxins, effectors and immune-modulators, (iii) manipulate microbial signal transduction and regulation, or (iv) interfere with functions required for bacterial survival and/or persistence during the infection appear promising for the development of new therapeutics against infections (Fig. 2).

### 2.1 Denying access: Adherence and invasion inhibitors

Upon entering the host, bacterial pathogens must travel to their respective site of infection in order to initialize the disease process. Once the bacteria reach the site of infection, bacterial cell surface structures and appendages such as **pili/fimbriae** and **afimbrial adhesins** detect and interact tightly with specific host cell receptors to adhere to the host cell (Thanassi et al. 2012). Cell attachment enables bacteria to withstand host mechanical and immunological clearance and is crucial for the initiation of an infection. Furthermore, adhesion is essential for the pathogen to get into close proximity with the host cell surface, a process required for the formation and activation of secretion systems (Thanassi et al. 2012). Agents targeting bacterial adherence e.g. by inhibition of **pili/fimbriae** or **adhesin formation** would not only

deny access to host tissues, they would also promote rapid clearance of the bacteria and avoid the release and translocation of tissue-damaging factors (e.g. toxins) (Krachler and Orth 2013). As the adhesion structures are further specific to the pathogen and will mediate only attachment to host cells that express the corresponding receptor, they are potential targets for new anti-infectives.

Pili or fimbriae are hair-like, multi-subunit bacterial cell surface protrusions that facilitate bacterial adhesion and aid colonization. They are important virulence factors in a wide range of bacterial pathogens, including *E. coli*, *Salmonella*, *Yersinia*, *Haemophilus*, *Pseudomonas* and *Klebsiella* (Krachler and Orth 2013). Many pili structures are assembled in the bacterial cell via the chaperone-usher pathway. In this pathway, the pilin subunits reach the periplasm via the Sec pathway, where they interact with the chaperone, which aids the folding of the pilin subunit's immunoglobulin fold (Li et al. 2004). The chaperone-pilin complex traffics to bacterial outer-membrane usher proteins, where the pilus is formed by incorporation of the pilin subunit into the growing pilin fiber resulting in the liberation of the chaperone (Barnhart et al. 2003).

Bicyclic 2-pyridones and N-substituted amino acid derivatives were discovered as potential pilicides (inhibitors of pili formation/biogenesis) that target conserved regions on the chaperone and competitively inhibit the binding of the chaperone to the pilin subunits. They were shown to inhibit biogenesis of type 1 and P pili of uropathogenic *E. coli* (UPEC), the major cause of urinary tract infections, which decrease UPEC binding to bladder cells by 90% (Pinkner et al. 2006). Furthermore, they also inhibit hemagglutination and biofilm formation in *E. coli* strains (Pinkner et al. 2006). While none of the substances affect bacterial growth, pilicides may have broad-spectrum activity as both, the chaperone structure and the chaperone-usher pathway are highly conserved among bacteria.

The interaction of pili or fimbriae with host cell surface receptors is often mediated through binding of the terminal pilin subunits and usually involves specific sugar or peptide moieties at the receptor (Ofek et al. 2003). Therefore, competitive inhibition of bacterial binding by tailoring anti-adhesive compounds to the specific receptor can be a promising strategy for anti-adhesion therapies. For instance, FimH or PapG are bacterial lectins that are located at the tip of type 1 or P pili of UPEC and they are one of the bacterium's main virulence factors, responsible for colonization, invasion of host bladder epithelial cells and biofilm formation (Wright and Hultgren 2006).

FimH recognizes mannosylated receptors on the host cell surface. Initial studies showed that monovalent mannose derivatives display rather weak inhibitory effects (Firon et al. 1987), but recent use of multivalent compounds with increased binding activity is promising success (Han et al. 2010). Biphenyl mannosides were shown to be 200,000 fold more potent than the originally tested monovalent mannose. The substances are orally available and demonstrate an overall low toxicity (Han et al. 2012; Hartmann et al. 2012). Additionally, mouse models indicate that biphenyl mannosides decrease colonization levels significantly (Klein et al. 2010). Alternatively, **adhesin binding** to glycosylated receptor proteins can be inhibited by mucins. Mucins are glycoproteins of the mucus that mimic the glycosylation pattern found on host cells receptors. Purified Muc1, a mucin derived from cow milk that is highly glycosylated, can selectively inhibit binding of Gram-negative pathogens such as *E. coli* and *Salmonella* (Parker et al. 2010). It can also limit infection with *Helicobacter pylori* (Linden et al. 2009). Muc1 does, however, have a less pronounced effect on Gram-positive bacteria (Parker et al. 2010). Another very effective option to block cell adhesion by a particular bacterial adhesin is the application of synthetic peptides that mimic the epitope or use of monoclonal antibodies raised against the binding epitope that interacts with the host cell receptor. This strategy has been successfully applied to the streptococcal antigen (SA) I/II, an adhesin of *Streptococcus mutans* responsible for bacterial binding to host salivary receptors. A synthetic peptide and an antibody directed against a binding epitope of SA I/II have been shown to prevent colonization of the oral cavity by *S. mutans* (Lehner et al. 1985; Ma et al. 1989).

Another option to inhibit bacterial adhesion is interference with the biogenesis and presentation of the host receptor. Many bacterial adhesins and toxins bind to host glycosphingolipids. Blocking of the ceramide-specific glycosyltransferase that catalyzes the formation of a glycosphingolipid precursor has been used to successfully diminish the amount of colonization of UPEC in cell culture and mouse studies (Svensson et al. 2003). Also, depletion of glycosphingolipids using enzyme replacement therapy was successfully used to treat systemic salmonellosis (Margalit et al. 2002).



## 2.2 Fighting biofilms and chronic infections

More than 80% of all microbial infectious diseases, which are difficult to treat involve the formation of biofilms (Romling and Balsalobre 2012). Therefore, **biofilms** and **quorum sensing** implicated in the control of biofilm formation constitute other important treatment targets.

### Interference with cell-cell communication - quorum sensing

Quorum sensing was discovered in the early 1970s as a means of cell-cell-communication between bacteria of a species. It allows the cells to react to environmental changes/stresses as a group by coordinating gene expression according to the local cell population density (LaSarre and Federle 2013). Quorum sensing is a three-step process, in which the bacteria produce small signal molecules, so-called autoinducers, that are released from the cell and detected by membrane-bound or cytoplasmic receptors (Ng and Bassler 2009). When bacterial numbers are low, the concentration of autoinducers is also low and the molecules dissipate in the environment. If, however, the concentration of bacteria reaches a threshold level, they come into contact with the released autoinducer molecules, inferring that they have neighbors. This, in turn, induces formation of biofilms, antibiotic resistance and expression of multiple pathogenicity factors (Deep et al. 2011). By regulating genes that are mainly associated with virulence and persistence, quorum sensing poses an important target for anti-virulence therapies. Different strategies identified for its inhibition include the **blockage of signal production**, interference with or **degradation of signal molecules** (signal dissemination), and **disruption of the signal reception and response** (Hentzer and Givskov 2003; LaSarre and Federle 2013; Lu et al. 2014a,b).

One important strategy to interfere with quorum sensing is to prevent the production of autoinducers. Gram-negative bacteria generate autoinducer molecules such as acyl-homoserine lactone (AHL) from S-adenosyl methionine (SAM) by proteins homologous to LuxI of *Vibrio fischeri*. This knowledge was exploited for the generation of quorum sensing inhibitors. For instance, analogues of SAM (e.g. S-adenosyl-homocysteine) were shown to inhibit AHL synthesis in *P. aeruginosa* (Parsek et al. 1999), and analogues of AHLs resulted in inhibition of the expression of quorum sensing regulated genes (Smith et al. 2003b) and, subsequently, biofilm formation

(Smith et al. 2003a). Furthermore, substances blocking regulators of AHL synthesis were identified (Park et al. 2015; Soheili et al. 2015; Lu et al., 2014a,b). Gram-positive bacteria such as *S. aureus* produce autoinducing peptides that require cleaving for activation and recognition. Administration of inhibitory autoinducer peptides to mice during the initial stages of *S. aureus* infection was shown to inhibit *S. aureus*-induced abscess formation (Wright et al. 2005). In addition, diverse small molecule inhibitors of biofilm formation have been identified. These include hydroxycarbin-type flavonolignans and streptorubin B for *S. aureus* and cyclosporine and valsopodar, for *Streptococcus* biofilm inhibition (Aggarwal et al. 2015; Suzuki et al. 2015; Vimberg et al. 2015).

Several species of bacteria also produce enzymes that are capable of inactivating AHL molecules. *Bacillus subtilis* produces an acyl-homoserine lactonase, which hydrolyses the lactone ring of AHL molecules, resulting in ablation of AHL function (Dong et al. 2000). Interestingly, tobacco plants expressing this AHL-lactonase show an enhanced resistance to *Erwinia* infection suggesting a broad-spectrum function of these enzymes (Dong et al. 2002; Dong et al. 2001). Moreover, AHL acylases, which cleave AHL after the N-terminal acyl, inactivating AHL molecules in a substrate specific manner have been identified in different strains of *Pseudomonas* and *Ralstonia* (LaSarre and Federle 2013) and are also of interest as anti-quorum sensing agents.

Another possibility is the blockage of AHL signaling by competitive inhibitors that prevent AHL binding to its LuxR-type receptor (Hentzer and Givskov 2003). Several AHL analogues with modified acyl side chains were identified with agonistic activities (Chhabra et al. 1993; Kline et al. 1999; Smith et al. 2003b), and halogenated furanones of marine algae were shown to modulate quorum-sensing regulators at a post-transcriptional level (Hentzer and Givskov 2003; Hentzer et al. 2002; Manefield et al. 1999).

Cyclic-di-GMP (c-di-GMP) is a widely used second messenger that plays a crucial role in bacterial biofilm formation as it stimulates the biosynthesis of adhesins and exopolysaccharide matrix components and prevents bacterial motility. C-di-GMP is synthesized by diguanylate cyclase (DGC), which is considered to be an attractive drug target. Recent research led to the identification of a catechol-containing sulfonohydrazide compound that inhibits the DGC PleD (Fericola et al. 2015).

## Prevention and resolution of biofilms

Biofilms form once bacteria sense a sufficiently high population density in their vicinity. This alters their gene expression and induces the secretion of a mix of polysaccharides, proteins and extracellular DNA. These secreted substances interact to form an extracellular matrix for the pathogens. Once the biofilm has been formed and bacteria are embedded in the matrix, the targeting of these bacteria becomes difficult. The formation of a biofilm increases bacterial resistance to exogenous stresses such as antibiotics, UV damage, acidity, metal toxicity and to host immune clearance and phagocytosis (Costerton et al. 1999; Hall-Stoodley et al. 2004; Romling and Balsalobre 2012). An additional disadvantage of biofilm formation in terms of treatment is that growth in biofilms aids the occurrence of processes that lead to the acquisition of inheritable resistance traits, such as horizontal gene transfer and adaptive mutations (Madsen et al. 2012). Growth within biofilms also raises the probability of bacterial persistence and antibiotic tolerance as bacteria within the biofilm alter their metabolism (Lewis 2005; 2008). This may lead to resistance to bacterial clearance, enabling the bacteria to regrowth within the host when the conditions become more hospitable. To target biofilm formation to inhibit persistence and recurrent infection, substances are being investigated that can inhibit secretion of biofilm components, biofilm matrix formation or destroy or resolve existing biofilm matrices.

Strategies to target bacteria within the context of a mature biofilm or to prevent biofilms from forming include the induction of bacterial motility, inhibition of host cell adhesion (see also 2.1, Erhardt, 2015/same issue) and/or the initiation of cell dispersal. Under natural circumstances, molecules inducing cell dispersal are produced by bacteria within the biofilm, allowing some cells to detach and infect new cells or surfaces. *B. subtilis* produces D-amino acids that result in disruption of amyloid fibers, which link bacteria within the biofilm (Kolodkin-Gal et al. 2010). Exogenous treatment of biofilms with low levels of D-amino acids results in the disruption of mature biofilms and inhibition of biofilm formation by *B. subtilis* (Kolodkin-Gal et al., 2010). Norspermidine is another molecule produced by bacteria and plants that targets polysaccharides present in the matrix. It disperses biofilms formed by *B. subtilis* and was further shown to inhibit formation of biofilms by *E. coli* and *S. aureus*. Administration of a combination of D-amino acids and norspermidine enhances their activity against mature biofilms (Kolodkin-Gal et al. 2012). Moreover,

nitric oxide or the addition of the nitric oxide donor sodium nitroprusside (SNP) induce dispersion of *P. aeruginosa* biofilms, and co-treatment of biofilms with SNP and antibiotics increases the efficacy of the antibiotics (Barraud et al. 2006). Nitric oxide was suggested to reduce biofilms by stimulating c-di-GMP-degrading phosphodiesterases, decreasing local c-di-GMP levels (Barraud et al. 2009). Furthermore, DNase I can degrade the extracellular DNA present in the biofilm matrix (Okshevsky et al. 2015; Qin et al. 2007), interfering with its formation and stability as shown for *Bordetella pertussis* (Conover et al. 2011), *Listeria monocytogenes* (Harmsen et al., 2010), and *Campylobacter jejuni* (Brown et al. 2015). A bacterial glycoside hydrolase, named Dispersin B, has been isolated from *Actinobacillus actinomycescomitans*, which was shown to disrupt mature *Actinobacillus* biofilms (Kaplan et al. 2004) and to inhibit *S. aureus* biofilms when added exogenously to the cells (Izano et al., 2008){Kaplan, 2004 #195}. Furthermore, the use of lytic bacteriophages to treat bacteria within a biofilm has been investigated and it was shown that using a phage engineered to express Dispersin B could lead to complete dispersal of *E. coli* biofilms (Lu and Collins 2007) targeting even potential persister cells.

### **2.3 Interference with global virulence control (regulation of virulence gene expression)**

Bacteria tightly control the production of energy-consuming pathogenicity factors and virulence-associated traits to avoid unnecessary energy expenses and optimize their biological fitness. Recent advances in our understanding of virulence regulation have identified many control circuits and networks implicating many different often conserved sensory and regulatory components acting at the transcriptional or post-transcriptional level that could be targeted. This includes bacterial sensory and signal transduction molecules, global and specific transcriptional regulators and RNA-based regulatory mechanisms. A major advantage of **targeting signal transduction and regulatory mechanism** is that these control systems are specific for bacteria and not present in eukaryotic host cells.

## Two-component systems involved in virulence

Environmental cues, which are important for pathogenesis and the biological fitness of bacterial pathogens during infection, are sensed by ubiquitous, highly conserved two-component systems. They constitute of a membrane-bound histidine sensor kinase, which activates a corresponding cytoplasmic response regulator by phosphorylation. In particular the **two-component systems** EnvZ/OmpR, RcsB/RcsC, PhoP/PhoQ, BarA/SirA, CpxR/CpxS, AgrC/AgrA and QseC/QseB of bacterial pathogens have been well characterized and shown to control complex gene networks important for virulence in response to temperature, osmolarity, nutrients, secondary metabolites and ions (Altier et al. 2000; Arya and Princy 2013; Clarke 2010; Forst and Roberts 1994; Groisman and Mouslim 2006; Vogt and Raivio 2012; Weigel and Demuth 2015). Moreover, studies exist that inhibitors of bacterial two-component systems worked in animal models and blocked pathogenesis of important pathogens (Rasko et al. 2008; Stephenson et al. 2000; Wilke et al. 2015; Worthington et al. 2013). One prominent example is the small molecular inhibitor Savirin, which reduces the expression of AgrCA-regulated genes in *S. aureus*, affecting its virulence, but not its survival and has no impact on the commensal *Staphylococcus epidermidis* (Sully et al. 2014). Another promising approach has identified a small molecule LED209, which prevents autophosphorylation of the sensor kinase QseC without influencing bacterial growth or inducing cell cytotoxicity. The QseC kinase contributes to virulence in a number of gram-negative pathogens and has been studied extensively in enterohemorrhagic *E. coli*. Here, QseC phosphorylates three transcription factors, QseB (regulates flagella and motility genes by binding the master regulator *flhDC*), QseF (activates Stx production), and KdpE (binds to *ler*, the regulator of EHECs main pathogenicity island LEE). Furthermore, QseC knockout strains of *Salmonella* and *Francisella* were shown to be attenuated in animal models. Strikingly, the inhibitor LED209 prevents the expression of the EHEC pathogenicity island LEE and Shiga toxin 2 without triggering an SOS response in EHEC, a reaction, which has been shown to result in the activation of Shiga toxin expression. On the contrary, a decrease in Stx2 expression could be observed (Curtis et al. 2014; Rasko et al. 2008).

### **Global transcriptional regulators of virulence**

A growing number and range of **global transcriptional regulators** of virulence have been identified which are highly conserved among bacteria, but specific for prokaryotic gene expression control, and which adjust co-expression of host-adapted metabolic processes, stress adaptation to host stresses and virulence factors. Targeting of these regulators is an attractive concept as rapid adjustment to continuously changing environments during the course of an infection is a prerequisite for successful persistence in the host and the development of the infectious disease. Among the most promising drug targets are transcriptional regulators, such as the cAMP repressor protein (Crp) of Gram-negative bacteria, the equivalent catabolite control protein (CcpA) of Gram-positive bacteria, the carbon storage regulator (CsrA/RsmA, see also below), and AraC-type activators (e.g. RhaR). They are produced by both Gram-negative and Gram-positive of bacteria which all adjust expression of important virulence-relevant processes (e.g. toxin production) in response to available nutrients in the infected tissues (Brautaset et al. 2009; Bruckner and Titgemeyer 2002; Deutscher et al. 2005; Heroven et al. 2012; Romeo et al. 2013; Skredenske et al. 2013; Vakulskas et al. 2015). The strength of this approach is supported by a recent study demonstrating that the phenylpropanoid anethole, which influences the virulence regulatory cascade by overproduction of Crp suppressed toxigenic *V. cholerae*-mediated fluid accumulation in ligate ileum of rabbits (Zahid et al. 2015).

### **Regulatory and sensory RNAs**

Novel deep-sequencing-based strategies have discovered an unprecedented level of complexity of transcriptional networks by identification of **small *trans*-acting regulatory RNAs** (sRNAs), **antisense RNAs** and sensory RNAs, such as **RNA thermometers** and **riboswitches**. Many of these RNA-based control elements were shown to influence expression of virulence-relevant processes (Oliva et al. 2015; Papenfort and Vogel 2014). Several regulatory RNAs (e.g. Qrr RNAs, CsrB/C and RsmZ/Y RNAs) have redundant functions and are used to fine-tune the regulation of pathogenicity, stress adaptation and/or metabolic genes by sequestration of regulatory proteins, hindrance of translation and/or control of RNA degradation (Feng et al. 2015; Heroven et al. 2012; Vakulskas et al. 2015). Redundancy and distinct regulatory mechanisms hamper the development of anti-regulatory RNA inhibitors. However, mechanistic insights further revealed that the function of many regulatory

RNAs is governed by highly conserved, global RNA-binding proteins, such as the RNA chaperone Hfq and CsrA/RsmA (Heroven et al. 2012; Lucchetti-Miganeh et al. 2008; Oliva et al. 2015; Papenfort and Vogel 2014; Vakulskas et al. 2015). Hfq is known as the central mediator of sRNA-based gene regulation in bacteria as it establishes dynamic interactions of a wide range of RNA molecules and manipulates translation and degradation of many mRNAs important for pathogenesis (Chao and Vogel 2010; Vogel and Luisi 2011). In fact, *hfq* knock-out derivatives of many important pathogens (e.g. *Francisella*, *Neisseria*, *Legionella*, *Salmonella*, *Yersinia*, *Listeria*) showed a severe growth defect and were drastically attenuated in animal infection models (Oliva et al. 2015). Another well-characterized RNA-binding protein implicated in the control of multiple regulatory RNAs and a large set of virulence-linked traits is the CsrA/RsmA protein. This RNA-binding regulator is highly conserved among bacteria, but it is not produced in archaea and eukaryotes. It predominantly controls RNA translation and degradation by binding to A(N)GGA motifs within the 5'-untranslated region of the mRNA targets (Duss et al. 2014). The CsrA/RsmA-controlled network is very versatile and includes target mRNAs implicated in the control of cell morphology, motility, biofilm formation, multiple stress responses and crucial virulence factors/regulators (i.e. secretion systems and secreted effectors, adhesins and invasins). Accordingly, *csrA/rsmA* mutants of many pathogens are avirulent or strongly attenuated (Heroven et al. 2012; Lucchetti-Miganeh et al. 2008; Vakulskas et al. 2015). Furthermore, multiple regulatory RNAs of pathogenic *Enterobacteriaceae* implicated in the control of virulence functions are controlled by the global transcriptional regulator Crp (see above) in response to the available nutrients in the medium. Strikingly, transcriptional profiling using RNA-Seq recently revealed Crp as a master regulator of 50% of all identified small RNAs in *Y. pseudotuberculosis* which are reprogrammed by Crp in response to temperature (Nuss et al. 2015). The major impact of Hfq, CsrA/RsmA and Crp on the expression of the virulence phenotype of many pathogens makes these global regulators also to promising targets for anti-virulence strategies.

RNA riboswitches and thermometers represent another type of regulatory RNA elements. They are predominantly located in the 5'-untranslated regions of target mRNAs and comprise complex RNA structures (e.g. stem-loops, which include the ribosome-binding site and/or the start codon in base-pairing of the stem structure) that sense and react to thermal or biochemical signals by conformational changes,

which mainly affect translation of the downstream gene(s) (Kortmann and Narberhaus 2012). Prominent examples are the RNA thermometers controlling the expression of major virulence regulators, such as PrfA of *Listeria monocytogenes* (Johansson et al. 2002), LcrF/VirF of *Yersinia* (Bohme et al. 2012), and the cholera toxin regulator ToxT of *Vibrio cholerae* (Weber et al. 2014). Additionally, three RNA thermosensors were described to be essential for *Neisseria meningitidis* resistance against immune killing (Loh et al. 2013).

Several virulence-related metabolic functions are also controlled by riboswitches - metabolite-binding mRNA or part of regulatory RNA structures (Mellin et al. 2014; Peselis and Serganov 2014; Serganov and Nudler 2013). Utilization of ethanolamine and propandiol, byproducts of rhamnose and fucose fermentation by the intestinal microbiota, is controlled by riboswitches in response to vitamin B12 binding. This leads to the synthesis of short or longer regulatory RNAs, which differentially modulate transcription of the *pdu* and *eut* genes (Mellin et al. 2014; Mellin et al. 2013; Toledo-Arana et al. 2009). Moreover, *trans*-regulatory riboswitches can function as small regulatory RNAs to link metabolic and virulence control. Several potential S-adenosyl-methionine (SAM)-binding riboswitches were identified in enteric pathogens and two of them - SreA and SreB - were shown to bind the 5'-untranslated region of the *prfA* transcript encoding the master virulence regulator of *L. monocytogenes* (Toledo-Arana et al. 2009). Rapidly increasing numbers of crucial RNA thermometers and riboswitches involved in virulence control may warrant the design of potential RNA-based inhibitors, e.g. RNA fragments that interfere with these crucial RNA elements or show perfect complementarities to the ribosomal binding site and the start codon of crucial virulence regulators.

## 2.4. Preventing host damage and development of the disease

Toxin-producing pathogens exhibit the severest effect on their host. The most serious clinical symptoms associated with infectious diseases are the results of severe tissue damage, cellular malfunction or destruction caused by **bacterial exotoxins** such as botulinum, cholera, diphtheria, anthrax, tetanus and Shiga toxins (Henkel et al. 2010; Schmitt et al. 1999). A deletion of the toxin gene(s) generally disarms the bacteria and results in avirulence without harming their overall biological fitness. This makes



these virulence factors ideal targets for new inhibitors, and multiple approaches are currently being followed to prevent toxin-mediated damages of the host.

### Targeting exotoxin trafficking or function

Exotoxins are bacterial virulence factors that are actively released into the surrounding environment from the bacterium during its growth, commonly via bacterial type II secretion systems (Henkel et al. 2010) and/or outer membrane vesicles (Kulp and Kuehn 2010; Kunsmann et al. 2015). As toxins remain outside the cell for a period of time prior to binding of their specific target cells, the toxins themselves make for good therapeutic targets, for example for **competitive inhibitors** or **neutralizing antibodies**. Once released into the environment, the toxins traffic through the host until they recognize and bind to specific host cells receptors. Interaction of the toxin with its receptor induces the uptake of the receptor from the cell surface by endocytosis. Once inside the host cell cytosol, the trafficking and destination of toxins varies according to their specificity. Some toxins are activated at neutral pH, others require the acidification of the endosome for activation or trafficking to a particular compartment (Henkel et al. 2010; Schmitt et al. 1999). Knowledge of the route of action of a specific toxin now enables us to develop new **toxin-trafficking inhibitors**.

For many exotoxins screening assays have been designed to identify small molecule compounds that inhibit the action of the particular exotoxin. For instance, several potent inhibitors have been identified for the *Bacillus anthracis*-encoded anthrax toxin, a multimeric toxin consisting of three proteins, the protective antigen (PA), the edema factor (EF) and the lethal factor (LF) (Montecucco et al. 2004; Nestorovich and Bezrukov 2014; Tonello et al. 2002). The PA binds to the host cell surface, where it gets cleaved and forms multimeric channels in the host cell membrane through which the EF and the LF can enter the cell. The EF, an adenylate cyclase, raises the level of cAMP within the host cell, leading to edema (Baldari et al. 2006; Dell'Aica et al. 2004). The LF, a zinc-metalloprotease, which has been shown to be critical for infection, disrupts host MAP kinase signaling pathways. Initial screening of a library of known zinc-metalloprotease inhibitors identified a potent sulfonamide derivative, anthrax LF inhibitor 40 ((2R)-2-[(4-fluoro-3-methylphenyl)sulfonylamino]-N-hydroxy-2-(tetrahydro-2H-pyran-4-yl)acetamide), which was further shown to be effective in several animal models (Xiong et al. 2006). The compound

binds competitively within the active site of LF and was found to be efficient in prophylactic therapy as well as for therapeutic treatment when used in combination with classic antibiotics (Shoop et al. 2005). Other screening assays using small molecule libraries and application of a mixture-based peptide library approach identified additional small molecule inhibitors and peptide analogs, which show competitive inhibition of anthrax LF (Bannwarth et al. 2012; Goldman et al. 2006; Kim et al. 2011; Panchal et al. 2004; Shoop et al. 2005; Turk et al. 2004). Furthermore, some polyphenols such as catechin-gallate (CG) and epigallocatechin-3-gallate (EGCG) abundantly identified in green tea were found to exert a strong inhibition of the LF proteolytic activity (Dell'Aica et al. 2004). Several non-competitive/exosite-targeting inhibitors that prevent LF function were also identified (Bannwarth et al. 2012; Kuzmic et al. 2006). Similarly, antitoxins targeting the EF enzymatic activity have been identified of which the most potent inhibitors interact with the catalytic site of the protein (Nestorovich and Bezrukov 2014).

As the PA subunit of the anthrax toxin is responsible for LF and EF delivery and represents the major antigen for toxin-neutralizing antibodies, it has been the most important target for preventive and therapeutic measures. Several attempts have been made to: (i) prevent PA binding to its host cell receptors (ATR/TEM8, CMG2) by blocking the receptor-binding domain of PA or the cell receptors or by the design of soluble polyvalent peptide analogues which compete with the natural receptors for PA-binding (Cryan et al. 2013; Cryan and Rogers 2011; Rogers et al. 2012; Scobie et al. 2005), (ii) to prevent endocytosis of the toxin by blocking pH-dependent cell entry and endosomal trafficking, or (iii) to prevent PA-promoted translocation of the LF and the EF. The most potent inhibitors include liposome-functionalized multiple copies of the AWPLSQLDHSYN peptide that binds and neutralizes the LF (Basha et al. 2006), dominant negative derivatives of the PA that co-assemble with wild-type PA, and the small molecule inhibitor (3-aminopropylthio)- $\beta$ -cyclodextrin, which disrupts proper PA channel formation and/or blocks its activity to translocate the LF and the EF (Karginov et al. 2006; Nestorovich and Bezrukov 2014).

Shiga toxin and Shiga-like toxin are found in *Shigella* and Shiga toxin-producing *E. coli* strains (EHEC). In EHEC pathogenesis, Shiga toxin has been identified as the main virulence factor responsible for bloody diarrhea, destruction of red blood cells and platelets and the development of hemolytic-uremic syndrome (HUS) resulting in severe kidney and neurological damage (Greener 2000; Kaplan et al. 1998; Tarr et al.

2005). Shiga toxin is an AB<sub>5</sub> toxin, of which the B subunit promotes binding to Gb3 glycolipid receptors most commonly found on kidney cells, but also on thrombocytes and neuronal cells (Boyd and Lingwood 1989; Kaplan et al. 1998). Several groups have designed strategies to interfere with receptor binding of the Shiga toxin. Sugars mimicking the Gb3 receptor have been designed in previous studies with varying success at neutralizing the free toxin (Kitov et al. 2000; Nishikawa et al. 2005; Trachtman et al. 2003). Despite toxin binding and removal, clinical symptoms were not significantly reduced. In another approach, glycan-encapsulated gold nanoparticles have been used to display ligands for Shiga toxin in an attempt to bind the free toxin. The nanoparticles were able to limit Vero cell cytotoxicity in response to Shiga toxins 1 and 2, but were unable to neutralize certain Shiga toxin 2 variants (Kulkarni et al. 2010). Furthermore, C-9, an inhibitor of glycosylceramide synthase, was applied to down-regulate Gb3 expression and prevented the cytotoxic effect of Stx2 on Vero cells (Silberstein et al. 2011). Using a rat model of infection, C-9 protected animals against Shiga toxin 2-associated disease and both, prophylactic as well as therapeutic treatment of rats with C-9 decreased expression of Gb3 receptors and the development of a disease phenotype (Kulkarni et al. 2010).

### **Inhibition of exotoxin synthesis**

The two main virulence factors of *V. cholerae* are cholera toxin (CT) and toxin co-regulated pilus (TCP). CT is another AB<sub>5</sub> toxin of which the catalytically active A subunit activates host cell G proteins upon uptake into the host cell. This in turn leads to the activation of adenylate cyclase, increasing the concentration of cAMP in intestinal epithelial cells, inducing strong secretory diarrhea (Field 2003). The specific transcription factor ToxT is known to directly activate the expression of both, cholera toxin (*ctxAB*) and *tcp* genes. The small molecule viristatin was found to interfere with the homodimerization of the ToxT N-termini and thus blocks CT and TCP production resulting in reduced colonization of *V. cholerae* in mice (Hung et al. 2005; Shakhnovich et al. 2007). Unfortunately, Viristatin-resistant *toxT* mutants containing a single amino acid substitution in the N-terminus have already been isolated (Hung et al. 2005).

### **Antibody-mediated exotoxin neutralization**

A very effective strategy to block toxin function and prevent the deleterious effect of very aggressive exotoxins is the treatment with antibodies that specifically bind and neutralize the toxin. One example for a highly potent neurotoxin that can be efficiently treated with neutralizing antibodies is the *Clostridium botulinum*-produced botulinum toxin. In the case of infections of adults, the US Centers for Disease Control supply an antitoxin that contains horse antibodies raised against type A, B and/or E strains of the neurotoxin. For treatment of children, the FDA approved a drug containing anti-botulinum toxin antibodies produced from human (marketed as human botulism immune globulin (BabyBIG)) (Arnon et al. 2006). Due to the high cost of the antibodies, a new equine alternative of the antibody is being tested (Vanella de Cuetos et al. 2011).

In addition, monoclonal antibodies binding directly to the PA subunit of the anthrax toxin and thus preventing its interaction with host cells were developed which were shown to protect rats and chimpanzees against *B. anthracis* infections (Chen et al. 2011).

Furthermore, antibodies targeting Shiga toxin have been isolated from rabbits immunized with a fusion protein consisting of an epitope of the B subunit of heat-labile toxin (LT) of enterotoxigenic *E. coli* (ETEC) and the A subunit of Shiga toxin (Stx) fused to the fimbrial protein FaeG. The produced antibodies were able to inhibit the adhesion of *E. coli* to enterocytes and neutralize Shiga toxin (Zhang and Zhang 2010). Similarly, a fusion protein of two different Shiga toxin antigens as well as the bacterial cell surface protein intimin was used to immunize mice and shown to result in the production of anti-Stx and anti-intimin antibodies. Mice immunized with this fusion protein were immune to lethal doses of EHEC (Gu et al. 2011).

### **Targeting of secretion systems**

Secretion systems are used by the bacterium to translocate virulence factors (effectors) directly into the host cytosol. Several types of secretion systems have a strong association with disease. The secretion systems most innately connected to pathogenesis are the type III (T3SS) and type IV (T4SS) secretion systems. T3SSs are needle-like structures with a high similarity to flagella and T4SSs are evolutionary related to bacterial conjugation systems. Both secretion systems are highly conserved in structure between the pathogens that employ them. They span the bacterial inner and outer membrane and connect to the host cell. Here, they form a pore in the

host cell membrane enabling the pathogen to translocate virulence proteins directly into the host cell cytosol in which they can interfere with cell signaling pathways in favor of bacterial persistence (Chandran 2013; Galan and Wolf-Watz 2006; Hueck 1998; Trokter et al. 2014; Waksman and Orlova 2014).

The genes for T3SSs and T4SSs are only found in pathogenic bacteria and are usually encoded within mobile genetic regions that are associated with virulence (pathogenicity islands). T3SSs are found in more than 25 species of Gram-negative pathogens including pathogenic *Chlamydia*, *E. coli*, *Salmonella*, *Shigella*, *Yersinia*, and *Pseudomonas* (Coburn et al. 2007; Galan and Wolf-Watz 2006; Schroeder and Hilbi 2008). T4SSs are associated with virulence in pathogens such as *Legionella*, *Bartonella*, *Helicobacter*, *Coxiella* and *Brucella* (Nagai and Kubori 2011; Voth et al. 2012). As the structures and functions of the secretion systems themselves show a high conservation between the different strains of bacteria, so does their synthesis and assembly. Furthermore, the proteins that make up the secretion systems are exposed on the bacterial cell surface, making them accessible. Taken everything into consideration, a number of different potential targets present themselves: synthesis of needle components, assembly of the secretion system, interaction with the host cell, and secretion/translocation of the substrates. Due to the high similarity of the secretion systems, it is likely that inhibitors can be found which target not only one but several different pathogens at once. Furthermore, as only pathogenic bacteria express these types of secretion systems, non-pathogenic bacteria will not be targeted. Additionally, as secretion system inhibition does not influence the overall survival of the bacterium, the selective pressure to develop resistance is low.

The design and use of special secretion test assays, i.e. fusion of the  $\beta$ -lactamase gene to the translocation signal of effectors secreted by T3SSs, GFP-labeled chaperone and tagged effector labeling, allowed the identification of different natural compounds and chemical inhibitors that block the function of T3SSs of different important pathogens (Baron 2010; Izore et al. 2011; Keyser et al. 2008; Marshall and Finlay 2014; McShan and De Guzman 2015; Pan et al. 2009; Tsou et al. 2013). The glycolipids Caminoside A, B, and C isolated from the marine sponge *Caminus sphaeroconia* were the first T3SS inhibitors. They block the secretion of the effector EspB, a protein that makes up part of the translocon of the *E. coli* T3SS, by varying degrees. Caminoside B appears to be the most potent inhibitor of this class, however, the cellular target remains unknown (Linnington et al. 2002). The Kirromycin derivative

Aurodox, isolated from *Streptomyces*, inhibited T3SS-mediated hemolysis of red blood cells in response to incubation with enteropathogenic *E. coli* (EPEC). It was further shown to decrease the secretion of the effectors EspB, EspF and Map without affecting bacterial growth. Furthermore, Aurodox protected mice against infection with a lethal dose of *Citrobacter rodentium*, the mouse homologue of EPEC. The mode of action of Aurodox is still unknown, however, it was suggested that it might interact with transcriptional regulators (Kimura et al. 2011). Guadinomines A and B are *Streptomyces*-produced natural compounds with a strong activity against the T3SS of EPEC as shown by inhibition of T3S-induced hemolysis. The mode of action of these compounds is also unknown (Duncan et al. 2014; Iwatsuki et al. 2008). The insecticidal, actinomycete-derived respiratory chain inhibitor Piericidin A1 and its closely related derivative Mer-A2026B were also shown to act against T3SS of *Y. pseudotuberculosis*, inhibiting the secretion and translocation of the *Yersinia* virulence proteins (Yops) into host cells (Duncan et al. 2014). A series of thiazolidinone inhibitors were discovered to inhibit both the *Salmonella* and the *Yersinia* T3SSs. However, they seem to also block T2SS and type IV pili, most likely due to an inhibition of the common outer membrane protein secretin (Felise et al. 2008). The polyphenol (-)-hopeaphenol is a natural product that also decreases Yop secretion in *Y. pseudotuberculosis* without affecting Yop expression. The compound is also active against *Pseudomonas*, but the exact target of (-)-hopeaphenol is still unknown (Zetterstrom et al. 2013). The salicylidene acylhydrazides (SAHs) are by far the best-studied chemical substances targeting T3SSs. They are effective against the T3SS of a range of pathogens including *Salmonella* Typhimurium (Hudson et al. 2007; Negrea et al. 2007), *Y. pseudotuberculosis* (Nordfelth et al. 2005), *Chlamydia* (Bailey et al. 2007; Muschiol et al. 2006; Slepkin et al. 2007; Wolf et al. 2006), *E. coli* (Tree et al. 2009) and *Shigella* (Veenendaal et al. 2009). The mode of action seems to be via the inhibition of needle subunit secretion or assembly, but recent analysis further demonstrated that SAHs significantly repressed the expression of main regulators of the T3SS machinery and modulate the function or activity of several protein targets (Layton et al. 2010; Tree et al. 2009; Wang et al. 2011). An involvement in iron chelation (Layton et al. 2010; Slepkin et al. 2007) and a role for the metabolism (Wang et al. 2011) are also debated. In addition to these inhibitors, several other virulence-blockers have been identified that inhibit transcription factors important for the expression of the T3SS in different pathogens. For instance, N-hydroxybenzi-

imidazole derivatives block T3SS expression in *Pseudomonas* and were found to prevent T3SS expression of *Yersinia* by inhibition of the MarA-type transcriptional activator LcrF/VirF of *Yersinia* and ExsA of *Pseudomonas* (Bowser et al. 2007; Garrity-Ryan et al. 2010; Harmon et al. 2010; Kim et al. 2009). Furthermore, salicylanilides were identified as inhibitors of LcrF expression (Kauppi et al. 2003), and the small molecule inhibitor SE-1 inhibits the master T3SS regulator VirF of *Shigella* (Koppolu et al. 2013).

Another very promising approach includes the use of antibodies directed against the tip complex of the secretion systems, e.g. PcrV for the *Pseudomonas* T3SS. A PcrV-specific antibody of KaloBios Pharmaceuticals is in clinical phase II and already showed that it is non-immunogenic and safe in pharmacokinetic studies (Francois et al. 2012).

The *Brucella abortus* protein VirB8 is an essential component of the T4SS and indispensable for its assembly. B8I-2 was identified to inhibit the dimerization of VirB8 as well as the interaction with other VirB proteins (Paschos et al. 2011). The compound markedly reduced *virB* transcription and subsequently VirB protein levels, but it also strongly reduced the intracellular survival of *Brucella* in macrophages (Paschos et al. 2011). Another study identified three substances to inhibit VirB11, the crucial ATPase of the T4SS of *H. pylori*, which were named CHIR-1, -2 and -3. All were shown to inhibit the secretion of CagA into cells *in vitro* which resulted in a strong reduction of *H. pylori* colonization in mice (Hilleringmann et al. 2006).

## Sortases

Gram-positive bacteria use enzymes named sortases to present proteins such as pilins or glycoproteins on their surface. In addition, pathogens such as *S. aureus* use these proteins to display their virulence factors (Cascioferro et al. 2014). This presentation is essential, as sortase A (*srtA*) mutant strains are impaired in their ability to cause infection in the mouse model (Jonsson et al. 2002). Sortase A mutants in *Streptococcus suis* and *L. monocytogenes* also show a marked reduction in pathogenicity (Bierne et al. 2002; Vanier et al. 2008). The conservation, essentiality and widespread use of sortase by pathogens suggest that compounds, which inhibit their activity, will function as potent anti-infective agents (Cascioferro et al. 2015). The surface proteins anchored to the cell wall by *S. aureus* SrtA include virulence factors that play key roles in the infection process by promoting nutrient acquisition from the

host, bacterial adhesion and immune evasion. Among the first sortase inhibitors were peptidomimetic molecules, the small molecule inhibitor diarylacrylonitriles and flavonols such as morin (Cascioferro et al. 2015). More recently, the compound (2-(2,3-dihydro-1*H*-perimidin-2-yl)-phenoxy)-acetic acid was found to inhibit SrtA without affecting bacterial growth (Chan et al. 2013; Zhang et al. 2014). It also protects mice from bacteremia (Zhang et al. 2014). Furthermore, aryl beta-amino(ethyl) ketone (AAEK) was identified as a sortase inhibitor in a high-throughput screen and selected for further investigation on the basis of its marked effect on sortases of staphylococci and bacilli (Maresso et al. 2007).

## 2.5. Antiresistance drugs

The development and use of anti-resistance drugs that are administered together with known antibiotics, to circumvent acquired resistance mechanisms of bacterial pathogens, opens a door to extending the lifespan of known antibiotics.

### Resistance against antibiotics

#### $\beta$ -lactamase inhibitors

$\beta$ -lactam antibiotics have been widely used for almost 80 years. They are bactericidal compounds that act by inhibiting cell wall synthesizing enzymes found only in bacteria (Kong et al. 2010). The continuous use of this family of antibiotics has given rise to the extensive proliferation of bacterial  $\beta$ -lactamases, which hydrolyze the  $\beta$ -lactam ring of the antibiotic, rendering the bacteria resistant to antibiotics of the carbapenem family (Palzkill 2013). A combination therapy of  $\beta$ -lactamase inhibitors as an adjuvant to suppress resistance with a  $\beta$ -lactam antibiotic increases the efficacy and the spectrum of the antibiotic. Successful combinations of antibiotics and  $\beta$ -lactamase inhibitors include clavulanic acid, sulbactam or tazobactam and penicillins (Carlier et al. 2014; Totir et al. 2007). Avibactam, a non-beta-lactam bicyclic diazabicyclooctane with no antibiotic activity, forms reversible bonds with several beta-lactamases including *K. pneumoniae* carbapenemase and ESBL or AmpC overexpressing strains (Castanheira et al. 2014; Coleman et al. 2014). Similar modes of action have been described for Merck MK-7655, a piperidine analogue used with imipenem (Blizzard et al. 2014).



## Efflux pump inhibitors

Overexpression of efflux pumps to expel toxic compounds such as antibiotics from the cell is a common resistance strategy found in bacteria. With the added advantage that uptake of antibiotics into Gram-negative bacteria is slowed down by decreased permeability of the bacterial outer membrane, the bacteria are less susceptible to efflux pump substrates even those with poor affinity, as the rate of efflux usually exceeds that of influx (Nikaido and Pages 2012). Discovering compounds that selectively inhibit the bacterial efflux pumps may lead to the development of therapies that could restore sensitivity to antibiotics. Several bacterial efflux pumps have been studied in detail and would make for promising therapeutic targets.

Resistance-Nodulation-Division (RND) efflux pumps are encoded by Gram-negative *Enterobacteriaceae* and *Pseudomonas*. They transport a variety of toxic substances from the bacterial cell, including antibiotics such as fluoroquinolones,  $\beta$ -lactams, tetracyclines, and oxazolidinones, mediating intrinsic resistance of the bacteria to these substances (Piddock et al. 2006; Nikaido and Pages 2012). Phenylalanyl arginyl  $\beta$ -naphthylamide (PA $\beta$ N) was discovered in a high-throughput screen for molecules that sensitize efflux pump-overexpressing *P. aeruginosa* to levofloxacin (Renau et al. 1999). PA $\beta$ N inhibits the efflux pumps of several bacterial pathogens such as *E. coli*, *S. typhimurium*, *K. pneumoniae*, and *Campylobacter* sp. with divergent efficacy. It was shown that while PA $\beta$ N sensitizes *Pseudomonas* to antibiotics such as levofloxacin, erythromycin and chloramphenicol, it showed little effect for enhancing the susceptibility to carbenicillin, suggesting that its activity is strongly dependent on the antibiotic (Lomovskaya et al. 2001). It was therefore suggested that PA $\beta$ N is a competitive inhibitor that binds within the substrate-binding pocket used by a specific set of antibiotics. Interestingly, PA $\beta$ N was found to increase the permeability of the cellular membrane (Lomovskaya et al. 2001) in addition to its function as an efflux pump, adding increased uptake into the bacterial cell to a reduction of export, increasing its potency. Naphtylpiperazines (NMPs) were identified as potentiators of levofloxacin in RND efflux pump-overexpressing *E. coli* (Bohnert and Kern 2005). Addition of NMP to cells increases the intracellular concentration of levofloxacin. It further affects the susceptibility of the bacteria to other antibiotic substances such as rifampicin and chloramphenicol. NMP is able to reverse multi-drug resistance in clinical *E. coli* isolates (Kern et al. 2006) and shows some effects against multi-drug resistant *K. pneumoniae* (Schumacher et al. 2006) and *A.*

*baumannii* (Pannek et al. 2006). However, NMP is ineffective in increasing antibiotic susceptibility of *P. aeruginosa* (Coban et al. 2009). Notably, antisense peptide nucleic acids (PNAs) are synthetic homologues of nucleic acids that bind complementary DNA and RNA sequences with very high specificity (Paulasova and Pellestor 2004). They can be used as antisense peptides to specifically inhibit the gene expression of efflux pump genes. This approach was employed to decrease the expression of the *C. jejuni* RND efflux pump CmeABC, resulting in a significant increase of susceptibility to antibiotic treatment (Jeon and Zhang 2009; Mu et al. 2013). NorA efflux pumps are found in Gram-positive pathogens such as *S. aureus*. They confer resistance to antibiotics including those of the family of fluoroquinolones. Capsaicin, an alkaloid has recently been shown to potently reduce the resistance of *S. aureus* to ciprofloxacin and inhibit the efflux of ethidium bromide *in-vitro* (Kalia et al. 2012).

## **Environmental stress resistance**

### **Depletion of iron**

Pathogenic bacteria are exposed to multiple host-associated stresses directed to prevent proliferation and eliminate the invaders. Among the most important stresses is the depletion of iron from the blood and lymph systems by specific iron-complexing molecules. To overcome this problem bacteria synthesize a plethora of iron-chelating siderophores for iron uptake, which are essential for virulence (Miethke & Marahiel 2007). Several attempts were made to identify compounds that prevent siderophore synthesis and transport. A salicylsulfamoyl adenosine and other nucleoside bi-substrate analogous were found to prevent the early step of siderophore synthesis of *Yersinia* and *Mycobacterium* (Ferrerias et al. 2005; Miethke and Marahiel 2007; Neres et al. 2008).

### **Resistance against reactive oxygen and nitrogen species (ROS, NOS)**

In a similar manner, virulence factors can be targeted which are essential to promote resistance against reactive oxygen or nitrogen species used by innate immune cells to destroy bacteria. Enzymes which have been successfully targeted by inhibitors are staphyloxanthin, a *S. aureus* pigment with antioxidant activity, which is blocked by phosphosulfonate, and the *Mycobacterium tuberculosis* factor DltA essential to resist NOS intermediates which are targeted by rhodanine analogs (Escaich 2010).

### **Interference with bacterial host resistance mechanisms**

Components of the innate immunity, such as complement factors, antimicrobial peptides (e.g. defensins) and professional phagocytes (e.g. neutrophils, macrophages) circulating in the blood, lymph system and tissues are able to efficiently eliminate invading pathogens. Lipopolysaccharides (LPS) of the outer membrane of Gram-negative bacteria are required for the resistance to complement and cationic antimicrobial peptides (CAMPs). Modification of LPS to decrease surface charges (e.g. by addition of amino-arabinose or heptoses) and inhibition of several enzymes of LPS biosynthesis have been identified as treatment strategies (Desroy et al. 2013; Escaich 2010). Bacterial resistance to CAMPs is linked to an increase in D-alanylation of the lipoteichoic acids in the cell wall of staphylococci and streptococci (Peschel et al. 1999; Saar-Dover et al. 2012). Furthermore, inhibitors of D-alanylation enzymes have been identified, some of which were also shown to reduce bacteremia (Escaich 2010; Santa Maria et al. 2014).

## **3 Challenges and problems associated with virulence blockers**

The large variety of newly identified natural compounds, structural analogs, mimetic peptides and antibodies that act as inhibitors of crucial virulence traits over the last ten years has demonstrated that anti-virulence strategies can be successfully applied to combat bacterial infections. However, there are still many challenges and problems for the development of anti-virulence drugs, which will have to be addressed in the near future.

Recent development of target-based high-throughput screening or rational drug design with chemical and natural compound libraries or structural analogs has furthered the field and facilitated the identification of inhibitors. However, for many inhibitors the demonstration that inhibition of a particular virulence strategy leads to an inhibition of the bacterial infection *in vitro* and the validation of inhibitor activities in animal models is still missing.

Another important issue concerns the mode of action of many compounds. Although several effective inhibitors of certain virulence traits (e.g. T3SS-mediated translocation of bacterial effectors) have been identified, the precise molecular

mechanism of the inhibition and the exact targets are often unclear. Moreover, some of the identified inhibitor classes (i.e. SAHs) seem to have multiple molecular targets, which contribute to the inhibitory effect of the compounds, and other inhibitors are active on more than one species.

One major challenge in the development of successful anti-virulence strategies is the general redundancy of many crucial virulence mechanisms of pathogens. Bacterial pathogens usually expose multiple adhesive surface structures with distinct cell receptor specificities, which allow binding and colonization of different tissues during the infection cycle. Furthermore, alternative infection routes are used by several pathogens to disseminate into deeper tissues. Hence, the development of a successful universal adherence inhibitor is rather difficult. Nonetheless, certain adhesion structures have been identified as hallmark requirements for specific pathogens, which are promising targets for drug discovery and development. Other examples are bacterial effector proteins, which are injected into host cells by T3SS or T4SSs to manipulate host cells and circumvent immune responses. Many bacteria that cannot evade detection by the receptors encode multiple effector proteins, which are able to specifically modify the host innate immune response in their favor. Pathogenic *Yersinia* species encode 5 effector proteins YopH, YopE, YopJ/YopP, YopT and YopM (Bliska et al. 2013), and in EPEC and enterohemorrhagic *E. coli* (EHEC) at least seven translocated effector proteins (NleB1, NleC, NleD, NleE, NleF, NleH2, and Tir) are known to modulate different aspects of the pro-inflammatory and apoptotic cell signaling pathways (Wong et al. 2011). In *Legionella pneumophila*, which encodes a staggering amount of at least 300 effector proteins, the redundancy of effector function is consequently much higher (Pearson et al. 2015). In addition, many host-adapted metabolic processes, ion/nutrient uptake systems and regulatory circuits are redundant and elimination of one is almost fully compensated by upregulation or activation of (an) alternative pathway(s).

Crucial pathogenicity traits are often only expressed at distinct sites and at certain time points when they are required during the infection to prevent immune responses and balance their energy budget to optimize their fitness. Hence, detailed knowledge about the tempo-spatial expression pattern and the role of the potential targets is required for the design/development of effective anti-virulence drugs.

Over the past years an increasing number of reports demonstrated that certain crucial virulence traits are only expressed by a certain ratio of the bacterial population

at any time during the infection (Burton et al. 2014; Claudi et al. 2014; Diard et al. 2013; Helaine et al. 2014; Manina et al. 2015; Putrins et al. 2015; Sturm et al. 2011). The presence of genetically identical but phenotypically heterogeneous subpopulations is advantageous for a pathogen to survive within a fluctuating environment with varying nutrient, ion and stress conditions. It allows the bacteria to prepare themselves for uncertainties (bet-hedging) and to divide a biological task into different subtasks executed by the different subpopulations (division of labor) (Ackermann 2015; Avery 2006; Smits et al. 2006). Furthermore, certain pathogen subsets reside in diverse tissue microenvironments and biofilms as a response to local conditions and molecular interactions, and this has disparate consequences on the expression of virulence-relevant traits. Differential expression of certain virulence traits in individual subpopulations can result in a failure to control the infection foci by identified inhibitors or in selective targeting of pathogen subsets.

Even if the selective pressure of virulence blockers is less than that induced by antibiotics, the appearance of resistance against anti-virulence drugs may result in pathogens that have an advantage over the remaining population. This allows for (involuntary) selection for these better-adapted pathogens over time. Many crucial virulence factors are located on mobilizable DNA elements, such as plasmids and phages. Rapid exchange of mutated gene-variants of a targeted virulence trait by horizontal DNA transfer could result in the rapid evolution of drug-resistant bacterial populations. Application of combinational therapies may be a good option to suppress these better-adapted pathogens at the moment they appear.

## **4 Conclusions and outlook**

The present review takes a close look at current anti-virulence strategies and identified classes of novel virulence blockers and illustrates the promising advances made in our attempts to develop alternatives to antibiotic therapies. To fully exploit this strategy it is imperative that we improve our understanding of the molecular mechanisms and the consequences of host-pathogen interactions, as many crucial virulence-associated processes remain unclear. Future research in this field will also be aided by the increasing number of crystal structures of crucial pathogenicity factors allowing directed drug design for high-throughput screening assays. This will

not only facilitate structure-function analyses and optimization of identified inhibitors, it will also allow an exploitation of other less well-characterized adhesion structures and secretion systems (T6SS, T7SS).

The loss of interest in antimicrobial drug development by large pharmaceutical companies and the urgent need for alternatives have triggered science funding agencies around the world to support projects that advance the development of alternatives to antibiotics. This generated a boost for academic researchers and small biotechnology enterprises to pursue different strategies with the goal to find alternative solutions for future applications. However, the following pipeline for the development of novel antibiotics is often not available. There is an urgent task to provide test systems to study pharmacokinetics/dynamics parameters and evaluate the efficacy in animal models. In particular the specificity, safety and tolerability of the compounds need to be assessed, an appropriate formulation for delivery must be developed and set-ups must be installed that help transfer successful virulence blockers into broad-scale clinical trials. A major task will be to convince the pharmaceutical industry to participate and support this mission early on.

Another important aspect is that an effective application of virulence blockers requires a rapid and precise diagnosis of infectious agents in the clinics (Häussler 2015/ same issue). This will include a more detailed profiling of the pathogen and its virulence traits and demands a more patient-specific, personalized analysis of the responsible disease agent. Such advancements are not only attractive due to their potential to combat resistant pathogens, they also help to improve the use of standard antibiotics and offer the possibility to use both antibiotics and virulence blockers in a synergistic therapy approach to minimize selection of resistant variants.

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## Figure legend

### Figure 1:

Schematic representation of the infection processes that can be targeted by anti-virulence drugs. Bacterial pathogens coordinate highly complex and organized temporal and special events to colonize and to disseminate to different sites within the host. They produce special pathogenicity factors to adhere to, invade, persist and in some case replicate within host cells. Many pathogens are also able to form and replicate with a biofilm-like bacterial community on and/or within cells. Knowledge about the molecular mechanisms will allow the interference with colonization by (i) inhibition of adhesin biosynthesis and function, (ii) inhibition of invasion, persistence and proliferation in cells or outside cells in distinct host tissues.

### Figure 2:

Schematic overview of the targets of current anti-virulence strategies designed for Gram-negative bacterial pathogens. Pathoblockers can target toxin function, quorum and environmental parameter sensing, gene regulation by global or specific regulators, sensory or regulatory RNAs, cell adhesion and invasion-promoting surface structures, and bacterial secretion systems (i.e. T3SS, T4SS).