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On the road to structure-based development of anti-virulence therapeutics targeting the type III secretion system injectisome

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The type III secretion system injectisome is a syringe-like multimembrane spanning nanomachine that is essential to the pathogenicity but not viability of many clinically relevant Gram-negative bacteria, such as enteropathogenic *Escherichia coli*, *Salmonella enterica* and *Pseudomonas aeruginosa*. Due to the rise in antibiotic resistance, new strategies must be developed to treat the growing spectre of drug resistant infections. Targeting the injectisome via an 'anti-virulence strategy' is a promising avenue to pursue as an alternative to the more commonly used bactericidal therapeutics, which have a high propensity for resulting resistance development and often more broad killing profile, including unwanted side effects in eliminating favourable members of the microbiome. Building on more than a decade of crystallographic work of truncated or isolated forms of the more than two dozen components of the secretion apparatus, recent advances in the field of single-particle cryo-electron microscopy have allowed for the elucidation of atomic resolution structures for many of the type III secretion system components in their assembled, oligomerized state including the needle complex, export apparatus and ATPase. Cryo-electron tomography studies have also advanced our understanding of the direct pathogen-host interaction between the type III secretion system translocon and host cell membrane. These new structural works that further our understanding of the myriad of protein-protein interactions that promote injectisome function will be highlighted in this review, with a focus on those that yield promise for future anti-virulence drug discovery and design. Recently developed inhibitors, including both synthetic, natural product and peptide inhibitors, as well as promising new developments of immunotherapeutics will be discussed. As our understanding of this intricate molecular machinery advances, the development of anti-virulence inhibitors can be enhanced through structure-guided drug design.

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1. Introduction

Antimicrobial resistance (AMR) is a growing concern for the global population, predominantly in animal husbandry, aquaculture, hospitals, and developing countries. The World Health Organization has identified priority pathogens that are of particular concern for the development of novel antibiotics to circumvent AMR and multi-drug resistance (MDR).¹ These pathogens include the so-called *ESKAPE* bacteria: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. (*Escherichia coli*); as well as the Gram-negatives *S. enterica*, and *Shigella flexneri*.^{2–5} The efficacy of current antibiotics has declined due to the rise in MDR, leading to larger hurdles for the development of novel

antibiotics due to inevitable resistance, thus pushing the world closer to a pre-antibiotic era. Unfortunately, while still often attractive to both clinical and pharmaceutical interests, targeting vital, universal mechanisms that bacteria require for survival will most inevitably lead to antibiotic resistance and development of AMR and MDR bacterial infections.

On the other hand, virulence mechanisms that pathogenic bacteria use are often not required for their survival within the host environment and are arguably more promising, though often more species specific, targets for the development of anti-virulence compounds.⁶ The type III secretion system (T3SS) is a highly conserved virulence mechanism employed by several Gram-negative pathogens. They are responsible for a variety of human diseases such as enteric fever, sexually transmitted infections, and pneumonia. The T3SS is a syringe-like proteinaceous channel (often termed the injectisome) that spans the inner and outer membranes of the Gram-negative bacterial cell, projecting into the extracellular medium where it punctures the host cell membrane to form a continuous conduit for the delivery of protein

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virulence factors. Upon entry into the host cell, these virulence factors manipulate key functions of eukaryotic cells, leading to a disease phenotype.⁷

The main components of the T3SS (Fig. 1) that will be discussed in this review are: (1) the cytosolic sorting platform (ATPase complex, cytoplasmic ring and associated components), which drives the energy for transport of T3SS-targeted substrates; (2) the inner membrane (IM) localized export apparatus and associated components that assist with gating mechanisms prior to secretion; (3) the dual membrane spanning basal body including IM rings and outer membrane (OM) secretin, the continuous scaffold which encapsulates the export apparatus and (4) adjoining rod/needle; lastly, the (5) tip and pore-forming translocon that completes the channel path from the needle end into the host cell cytoplasm. Altogether the complex is composed of more than 20 unique proteins that form the multi-megadalton injectisome.^{8,9} While in the cytosol, T3S substrates often remain bound in a partially unfolded state to a cognate chaperone, a complex which promotes recruitment to the sorting platform and export gate, followed by passage through the needle lumen.¹⁰

The essential association of the T3SS to pathogenicity/virulence but not viability of the bacterium, as well as the intricate but relatively well conserved manner of assembly and function makes the T3SS an attractive target for antimicrobial design and development. Inhibitors have been designed to target several features of the T3SS including: gene regulation of the T3SS-encoding pathogenicity islands, the assembly hierarchy, substrate secretion and translocation.¹¹ Targeting these features may assist with reducing the virulence of several of the notoriously MDR Gram negative *ESKAPE* pathogens and beyond, allowing for the host's immune system to naturally clear the less-virulent bacteria.⁷ This review will focus on recent advances in small-molecule inhibitor development that target these T3SS characteristics, as well as recent advances of T3SS-targeted immunotherapy for treating pseudomonal pneumonia.

2. Update on the structure and function of the T3SS

A rapidly evolving field due to the recent advent of single particle cryo-EM methods to capture atomic resolution data, here we summarize the latest status of resolved structural components of the T3SS, with a focus on those which have promise for drug development or have been targeted previously. The unified secretion and translation nomenclature^{9,12} will be used for clarity; specific homologues will be highlighted when necessary.

2.1. Sorting platform: ATPase and associated components

Despite the potential for cross target effects due to the conservation of the virulence T3SS with the flagellar T3SS, the T3SS ATPase has to date been one of the most extensively targeted components of the virulence system, as discussed in

detail below. Transport of substrates through the T3SS is thought to require the contribution from both the proton-motive force (PMF) and ATP hydrolysis. The ATPase complex is composed of three main components: the ATPase (SctN) that hydrolyses ATP, the central stalk (SctO), which is predicted to both couple the ATPase to SctV^{13,14} and facilitate the transfer of chaperone/substrates to the export apparatus,^{15,16} and lastly the stator or peripheral stalk (SctL), anchoring the ATPase complex to the cytoplasmic ring (SctQ).¹⁷ A detailed review discussing the organization of these components can be found in Deng *et al.* (2017).⁹

The first atomic resolution structure of the T3SS ATPase in complex with the central stalk was recently reported.¹⁸ Previous crystallographic structures of the protomeric form^{19–22} have been determined for both the injectisome and flagellar T3SS's. However, the absence of the physiological oligomeric state and rod were unable to reveal the complexity and mechanistic details that the recent EscN (EPEC) structure has provided. The protomer consists of three major domains that are conserved among the T3SS ATPases of known structure: an N-terminal oligomerization domain, the central ATPase domain and the C-terminal domain. The N-terminal oligomerization domain forms a group of β -sheets, the ATPase domain contains the Rossmann fold characteristic of nucleotide binding proteins, and the C-terminal domain lines the central pore with four α -helices. The central stalk, SctO, forms a coiled-coil structural motif and sits within the central pore of the ATPase hexamer. The quaternary structure of the homohexameric ATPase is asymmetric, forming a large cleft between the first and sixth protomers due to the presence of a 'pivot point' between the N-terminal and ATPase domains, thus allowing for rigid movement of the ATPase and C-terminal domains. This is proposed to allow the ATPase to cycle through conformations that in turn lead to a proposed 6 ATP molecules hydrolysed per turn. Several characteristics of EscN, including the domain organization, C-terminal domain movement and active site architecture are shared with the F₁/V₁ rotary ATPases, previously proposed for both the T3SS injectisome and related flagellar T3SS ATPases.^{20,23}

The central stalk interacts *via* hydrophobic and electrostatic interactions, with charge pairs between the ATPase subunits interacting with the stalk in a rotational pattern upon conformational changes of the ATPase.¹⁸ Domain movement is proposed to allow for disruption of chaperone/substrate binding and may lead to the transfer of substrates to the export gate, mediated by the stalk.^{15,16} There is evidence to suggest that the chaperone/substrate complex interacts with the C-terminal domain of the ATPase,²¹ placing the complex at the export gate face of the ATPase.

2.2. Export apparatus

The export apparatus is composed of five distinct proteins: SctR, SctS, SctT, SctU and SctV, collectively facilitating a proposed structural framework for substrate gating. Recently, the isolated, recombinant structure of a complex of several

Fig. 1 Structural overview of the type 3 secretion system. SctA (SipB; PDB ID: 3NZZ), SctE (SipD; PDB ID: 3TUL), SctF (PrgI; PDB ID: 6DWB), SctC (InvG; PDB ID: 6DV6), *SctRST (FlpQR of flagellar T3SS; PDB ID: 6F2D; positioning based on ref. 28), SctJ and D (PrgK and H respectively; PDB ID: 6DUZ), SctD on cytoplasmic face (PrgH; PDB ID: 3J1W, Rosetta model), SctV (MxiA; PDB ID: 4A5P), SctW (SepL; PDB ID: 5C9E), SctU (SpaS; PDB ID: 3C01), SctN/O (EscN/O; PDB ID's: 6NJO/6NJP), MxiM (PDB ID: 1Y9L), EtgA (PDB ID: 4XP8) and OrgC (6CJD). Structures for which atomic models are not available are displayed as outlined structures based on sub-tomogram averaging: SctK,L,Q,V (EMD-8544; see inset), SctBE (EMDB not available⁷⁹). SctI is predicted to bridge SctRST to SctF²⁸ and SctA is predicted to bridge SctF to SctBE.⁷⁹ Though not discussed in text, an additional proposed component of the ATPase complex is SctK, a constituent of the cytosolic sorting platform that is suggested to be important for proper assembly of the sorting platform.^{136,137} This is a composite structure generated using Chimera, with reference to available structural data discussed in this review.

components of the flagellar T3SS export apparatus (FlpQR) was determined at 4.2 Å resolution *via* cryo-EM, showing them not to be composed of transmembrane helices as previously predicted, but remarkably to interact *via* coiled-coil interactions in a soluble form that was suggested to sit atop the IM rather than within.²⁴ Their observed interactions also illustrate a “closed” form with no apparent opening sufficient to passage unfolded proteins. However, Lea and colleagues proposed the potential for an iris-like opening involving helical rearrangements of the coiled-coil domains as a gating mechanism for both T3S-mediated flagellar export at the IM as well, by analogy, passage of effectors in the related T3S injectisome.²⁴ The export apparatus is also predicted to play a role in coupling PMF to the active transport of effectors, a necessary process for efficient secretion through the system and thus a very attractive target for drug development. In particular, PMF is suggested to rely on the nonomeric SctV component, unfortunately still largely uncharacterized at the atomic level. This statement is better supported by ref. 14

The core of the export apparatus consists of SctRST, which has a stoichiometry of 5R:4S:1T. The entirety of the complex forms a pseudohexameric, helical platform, predicted to allow for nucleation of the rod (SctI) and needle filament (SctF). The core complex interacts with SctU, an autoprotease, and the export gate, SctV, both components of the export apparatus.^{25,26} Prior predictions for the export apparatus structure suggested that it is located within the IM, however, the new FlpQR structure suggests rather that the export apparatus core is located within the ‘cup and socket’ region of the basal body in the periplasm, as described for the *Salmonella* injectisome basal body complex.²⁷ Though as of yet there are no high resolution structures of the export apparatus for the T3SS injectisome, a recent cryo-EM structure of the native *Salmonella* needle complex reveals the highest resolution to-date of the ‘cup and socket’ region and corroborates the observed organization of the FlpQR complex, further supporting the conservation between the flagellar and non-flagellar T3SS injectisomes.²⁸ The electrostatics of the inner lumen, or ‘atrium’, of the export apparatus reveals a highly electropositive surface, consistent with the flagella and hook lumens, and needle filament of the injectisome.^{24,28} The conservation of electropositive surface residues may suggest a mode of substrate interaction through the injectisome.

2.2.1. Autoprotease, SctU. The autoprotease plays a critical role in the T3SS assembly and secretion hierarchy. Monomeric SctU is located within the IM, consisting of a C-terminal cytoplasmic domain and an N-terminal periplasmic domain that is proposed to interact with the three core components of the export apparatus.^{24,29} SctU (SpaS in *Salmonella*) undergoes intein-like autocleavage at N258 (SpaS) within a conserved NPTH motif,^{30–32} an event that is required for proper hierarchical secretion of middle (translocon) and late (virulence factors) effectors.³³ A study performed by Feria *et al.* (2015) suggested that autocleavage occurs prior to incorporation into the injectisome, and *Salmonella* expressing only post-processed SpaS is capable of assembling WT-like

injectisomes.³³ Autocleavage incompetent strains (SpaS N258A) of *Salmonella* were unable to secrete middle and late substrates and exhibited a significant decrease in host-cell invasion. Similar outcomes have been observed in both EPEC³¹ and *Yersinia* homologues,³² with additional evidence suggesting that the post-processed form plays a role in chaperone docking.

The structure of the C-terminal cytoplasmic domain of SctU is known for several major pathogens,^{34–37} illustrating a common α/β -fold with a combination of antiparallel and parallel 5-stranded β -sheet.³⁰ From these structures, post-cleaved SctU adopts a more flexible conformation when compared to the pre-cleaved form. Corroborating snapshot structures of various NPTH motif mutants in EscU (EPEC) at very high resolution allowed for the elucidation of the intein-like autocleavage mechanism employed by this important secretion regulator.³⁰

2.2.2. Export gate, SctV. The fifth component of the export apparatus, SctV, is termed the export gate: a likely ring-forming homononamer localized within the IM of the basal body, with a cytoplasmic facing soluble domain.³⁸ SctV is involved in regulating substrate access to the secretion system through its proposed close interactions with components of the ATPase (SctN and SctO), SctU, and the gatekeeper SctW.^{39–42} Additionally, SctV has been suggested to interact with the switch regulator SctW and its cognate chaperone to prevent effector recruitment during translocon secretion.¹⁰ Two domains make up the structure of SctV, an N-terminal polytopic domain that spans the IM and a C-terminal cytoplasmic domain that, from cryo-ET analysis,¹⁷ localizes adjacent to the homohexameric ATPase. The crystallographic structure of the cytoplasmic domain has been determined from multiple T3S species showing a common fold.³⁹ The T3S injectisome of *Shigella* SctV (MxiA) crystallized as a homononamer, an oligomerization state supported in solution by size exclusion chromatography-multi-angle light scattering (SEC-MALS) and crosslinking studies.³⁸ Of the four subdomains that make up the cytoplasmic face of SctV, one subdomain shares similarities with the peripheral stalk of the A/V type ATPase and a second separate subdomain contains the canonical ring-building motif that has been observed as a structural feature of several T3SS ring forming components,³⁹ as well as that of other secretion systems.

2.3. Basal body and secretin

The T3SS injectisome basal body is the core dual membrane spanning conduit of the system, forming the structural foundation that facilitates subsequent steps in assembly. The basal body IM components (SctD and J) form nested 24-mer concentric rings that span into the periplasmic space,²⁷ and house the export apparatus constituents.²⁸ The outer membrane basal body component, the secretin, forms a 60-stranded double-walled β -barrel, decorated on the exterior face with a small, C-terminal α -helical pilotin-binding S-domain. An intimately associated and conserved ring-

building motif allows for initial rapid formation of the characteristic pentadecameric assembly. As the inner rod (SctI) assembles from the export apparatus, the needle (SctF) follows and is predicted to push through the periplasmic gate of the secretin, which protects the pathogen from solute leakage in the need-less, closed gate conformation.²⁸ A recent review on the structure and mechanism of the outer membrane secretin, which is conserved in several secretion systems, can be found in Majewski *et al.* (2018).⁴³

2.3.1. Lytic transglycosylase. In order for the OM secretin to couple to the IM rings of the basal body in the periplasm and subsequent assembly of the T3SS to proceed, there must presumably be localized and controlled clearing of the surrounding peptidoglycan layer. Several related systems, including type IV,⁴⁴ VI⁴⁵ and flagellated T3SS⁴⁶ are all known to use a small lytic transglycosylase to locally clear the peptidoglycan, allowing for efficient assembly of the inner and outer membrane components. Though T3SS-associated LT's have been identified in a few pathogens, only the EPEC LT has been shown to be vital in efficient assembly.⁴⁷ Knockouts of this LT, termed EtgA, lead to a severe effect in middle substrate secretion and a loss in haemolytic activity.⁴⁷ The structure of EtgA has been determined, illustrating conservation of key catalytic residues with related LT's, as well the distinct addition of a conserved catalytic residue present in lysozyme hydrolases.⁴⁸ The activity and stability of EtgA is enhanced in the presence of the inner rod component, EscI, suggesting a mechanism of localized regulation and sequential order of T3SS assembly.

2.3.2. Secretin pilotin. To allow for OM targeting of the T3SS secretin, an accessory lipoprotein – termed the pilotin – is employed to promote proper localization and assembly of the secretin.⁴⁹ Of the T3SS systems, pilotins have been characterized in *Salmonella* SPI-1 (InvH^{50,51}), *Pseudomonas aeruginosa* (ExsB⁴⁹), *Shigella flexneri* (MxiM⁵²) and *Yersinia* (YscW⁵³). Notably, EPEC/EHEC, *Salmonella* SPI-2 and *Chlamydia* spp. do not have identified pilotins at present.⁹ Pilotins contain a conserved N-terminal lipobox motif (V/LxxC), a marker for lipidation that allows for pilotin anchoring into the OM upon periplasmic localization.⁵⁴ Knockouts of the pilotin results in reduced secretion,⁵³ and in some cases a complete loss of T3SS function and virulence.⁵⁵

Structures of two T3SS-associated pilotins are known: MxiM⁵⁶ from *S. flexneri* and ExsB from *P. aeruginosa*.⁵⁴ The structure of MxiM is described as a 'cracked' barrel motif; a highly curved β -fold and an α -helix forms a pseudo β -barrel, forming a hydrophobic cavity that is proposed to bind lipids.⁵⁶ Based on NMR and crystallographic studies, a mechanism of secretin localization was proposed for the *Shigella* pilotin.⁵⁷ While in the periplasmic space and prior to interaction with its cognate secretin (MxiD for *Shigella*), the lipid anchor remains bound within this cavity, preventing premature OM localization. Upon interaction with the secretin, the lipid anchor becomes occluded from the cavity and free to interact with accessory proteins (presumably in a LolA-mediated pathway) for proper localization of the secretin–pilotin complex.

This is supported by the presence of lipid in MxiM prior to binding MxiD, and absence of lipid upon complex formation in the NMR models. The structure of ExsB from *P. aeruginosa* is distinct from MxiM and pilotins of other secretion systems (reviewed in Koo *et al.*, 2012).⁵⁸ The lack of structural conservation suggests that pilotins may use distinct mechanisms for secretin localization, though their importance in proper assembly cannot be understated.⁴⁹

2.4. Needle filament

After assembly of the export apparatus and basal body complexes, early substrates, composed of the inner rod (SctI) and needle filament (SctF), are recruited for consecutive assembly. Solid-state NMR structures of recombinantly produced *Salmonella* and *Shigella* SctF needle filaments have shown the overall architecture of both native and recombinant assemblies, made up of several hundred ~10 kDa protomers that polymerize into a helical tube with a diameter of ~8 nm and an inner lumen of ~1.5 nm to allow for transport of semi-unfolded substrates.^{59–62} Recent high-resolution cryo-EM structures of the *Shigella* and *Salmonella* native needle filaments reveal a highly conserved inner lumen, following a grooved spiral of electropositive and hydrophobic residues.^{28,60} Mutation of these residues in the *Shigella* homologue lead to changes in secretion and assembly of the needle filament,⁶¹ supporting the proposal that the combination of groove and electrostatics may provide a platform for unidirectional transport. Specific polar residues that line the surface were shown to be important for sensing host-cell contact (measured by haemolysis) and cellular invasion, further suggesting the role the needle filament may play in triggering the switch to late effector secretion.⁶¹ A similar study was performed on the *Yersinia* T3SS,⁶³ corroborating the *in vivo* and structural interpretations described previously.^{28,59,61}

Recently, the structure and function of the effector OrgC (*Salmonella*) was determined, representing a new component of the T3SS that was previously thought to act as a virulence factor.⁶⁴ OrgC is secreted with the early substrates (SctF, SctI and SctP) and upon secretion, is predicted to assist with needle filament initiation and assembly. OrgC knockouts lead to needle-less basal body complexes and in turn, a decrease in secretion of middle and late substrates. The interaction surface between OrgC and the *Salmonella* PrgI (SctF) needle was determined using NMR spectroscopy. Using dynamic light scattering (DLS) for measurement of needle formation, Kato and colleagues illustrated that in the absence of OrgC, needle polymerization of recombinant PrgI occurs much slower than when complemented with recombinant OrgC, leading to the proposal that OrgC is involved in acceleration of needle polymerization. The isolated structure of OrgC resembles a triangular prism composed of 4 α -helices with a poorly ordered C-terminal loop. Based on NMR models, the authors show that the C-terminal region of OrgC interacts with SctF (PrgI from *Salmonella*). Kato *et al.* note that the function and

topological organization of OrgC is reminiscent of the flagellar cap protein, FliD.⁶⁴

A single protomer of the SctF needle filament follows the coiled-coil theme that several T3SS substrates share.^{65–67} The conserved coiled-coil motif allows for an extended helical architecture that forms a stable structure that may provide resistance against the significant mechanical shearing forces that the needle and tip complex are exposed to within the host environment. A single protomer is described by a helix-turn-helix – constituting the coiled-coil – with the N-terminus decorating the outside of the filament and the C-terminus lining the central lumen.^{28,59,62} The mediating PxxP turn is highly conserved in all SctF homologues,^{28,61} with mutational analysis showing elevated effector secretion, suggesting that this structural motif may play a role in regulation of secretion.⁶¹ Notably, this common feature of the T3SS has been exploited for drug design of coiled-coil peptide inhibitors,^{68,69} as will be discussed below.

2.4.1. Inner rod. The inner rod, SctI, assembles *via* interactions with the export apparatus, forming a likely hexameric helical assembly, bridging the export apparatus to the needle filament.^{25,28} The structure of SctI has been difficult to obtain, in part due to its instability when expressed *via* recombinant methods.⁷⁰ Crosslinking studies suggest PrgJ (*Salmonella* SctI) likely interacts with both SpaP and SpaR (SctR and SctT, respectively) of the export apparatus, supporting the theory that the SctI rod is a continuum from the export apparatus to the needle filament.²⁹

Needle length is regulated by a ruler protein, SctP. A previous study linearly correlated the residue length of SctP with the length of the needle filament.⁷¹ SctP has been shown to bind to and inhibit autoproteolysis of SctU, leading to the proposed switch mechanism: upon achieving sufficient needle-length, SctP falls off of the complex and leads to SctU autocleavage and the coinciding trigger to middle and late substrates.^{36,72} The structure of PscP (SctP from *P. aeruginosa*) C-terminal globular domain contains a hyperstable α/β -fold, with an extended N-terminal region shown to be intrinsically disordered.³⁶

2.5. Tip and translocon

At the terminal end of the injectisome, the needle is capped with a homopentameric tip, SctA, followed by the translocon, an heterooligomeric complex of SctE and SctB.⁷³ Several crystal structures of the tip protein have been determined for major pathogens, revealing a conserved coiled-coil structure allowing for the continuum of the helical needle.^{67,73–76} One exception to this assembly occurs in the EPEC and EHEC T3SS, which has a short needle followed by the EspA helical filament.^{77,78} This flexible filament can reach extreme lengths of 700 nm and is proposed to provide additional protection against shearing forces experienced within the gut lumen.⁷⁷

The tip protein bridges the needle to the pore-forming translocon, completing the continuous channel from pathogen to host and allowing for virulence factor delivery. A re-

cent cryo-tomography study revealed the assembly of the translocon at the host membrane upon approach of the needle-tip complex.⁷⁹ The translocon contains two components: the major component (SctB), which contains a predicted two transmembrane segments and a coiled-coil domain; and minor component (SctE), consisting of one transmembrane segment, a coiled-coil domain and an amphipathic helix.^{80,81} Due to the intricate nature of the translocon assembly, reconstituted structures of the intact complex have been so far unattainable. Structures of the coiled-coil domains are known for the major translocators (SctB) SipB (*Salmonella*) and IpaB (*Shigella*),⁸² as well as the chaperone bound state for the *Aeromonas* homologue, AopB.⁸³

3. Inhibitor classes and their associated mechanism of inhibition

The T3SS injectisome is essential for virulence but not viability of the strictly bacterial pathogens that encode them. The T3SS flagellum, with orthologous components in the cytosol and inner membrane to that of the injectisome, does however promote bacterial growth and motility and thus the potential for cross over action of any developed antimicrobials must be considered. Many small molecule inhibitors of the T3SS have thus far been identified, targeting a variety of mechanisms such as: gene regulation and plasmid copy number, T3SS assembly, secretion and translocation. In terms of an anti-virulence strategy, an ideal drug candidate would exhibit no bactericidal properties, no cytotoxicity, and no effect on the related flagella system; inhibitors would disrupt the virulence mechanisms of pathogens without affecting their fitness or viability.⁸⁴ As they target specific virulence mechanisms that are not required for survival, these compounds reduce the selective pressure on pathogens to develop drug resistance. In addition, deleterious effects of antibiotic classes which block universal, essential cellular processes (for example cell wall biosynthesis by the β -lactam antibiotics such as penicillin) and thus more generalized disruption of the natural microbiota, would be minimized with pathogen-specific, anti-virulence compounds. It is therefore important to understand the mode of action a drug plays when it exhibits T3SS inhibitory activity.

Several inhibitors of the T3SS have been identified, with most exhibiting inhibition of bacterial growth or affecting motility and by extension, targeting of the T3SS flagellar system. However, natural product compounds have shown some promise in reducing effects on the natural microbiota, with preferred interactions occurring with the T3SS injectisome. As well, immunotherapy strategies that have been developed for use in *Pseudomonas* infections have yielded promising outcomes. Herein we discuss a brief background on well-characterized classes of T3SS inhibitors and detail new compounds that have recently been characterized and show promise in future studies. We focus here on compounds which specifically target the virulence T3SS mechanism.

However, we note that inhibitors targeting the orthologous flagellar system common to both pathogenic and non-pathogenic bacteria – and thus not anti-virulence *per se* – could nonetheless yield important information for optimization of more specific anti-T3SS compounds. Table 1 and Fig. 2 represent a summary of examples discussed below.

3.1. Synthetic small-molecule inhibitors

3.1.1. Salicylidene acylhydrazides. Salicylidene acylhydrazides (SAH) are one of the most well characterized small-molecule inhibitors of the T3SS. Despite their strong inhibitory effects on the T3SS injectisome action and virulence, SAH compounds often exhibit additional unwanted effects: they typically affect flagellar motility, can inhibit bacterial growth and many are cytotoxic in mammalian cells. We will focus on two well-studied compounds as examples, INP0007 and INP0010, which have been shown to inhibit several different T3SSs in diverse ways within distinct bacterial species.

INP0007 has been tested in several pathogens, illustrating conserved and divergent mechanisms of action. In *Salmonella enterica* serovar Typhimurium and *Yersinia pseudotuberculosis* serotype III (YPIII), INP0007 exhibits no observable cytotoxicity to the bacterial strains tested, nor to HeLa cells, as shown in multiple studies.^{85–87} However, the compound was found to severely affect growth of *Shigella flexneri* and could not be

tested for anti-virulence efficacy.⁸⁸ INP0007 did not inhibit motility in *Salmonella*, despite exhibiting dose-dependent inhibition of motility in YPIII and *Y. enterocolitica* strains.^{89,90} Therefore, the compound may be targeting conserved virulence and flagellar T3SS components in *Yersinia* spp. A dose-dependent decrease in effector secretion is observed in *Salmonella* for SipA and SopE, with a slight decrease in SipC (SctB) secretion.⁸⁵ Notably, INP0007 also exhibited a slight decrease in secretion of the flagellin protein H1, despite not significantly affecting motility.⁸⁶ In *Pseudomonas*, INP0007 exhibits an impressive IC₅₀ of 0.8 μM for T3SS mediated secretion.⁹¹ INP0007 was able to inhibit *Yersinia* middle and late substrate translocation into HeLa cells, again without affecting bacterial or HeLa cell growth.⁸⁷ In *Yersinia*, T3SS expression is sensitive to calcium concentrations: high extracellular concentrations inhibit expression and suppress activity, whereas low concentrations induce expression an increase activity of the T3SS.⁹² Specific gene knockouts, such as $\Delta lcrG$ (the chaperone for the tip protein, SctV, which acts as a negative regulator of secretion when uncoupled from the tip protein), mimic the low calcium response in *Yersinia* and are so-called 'Ca²⁺-blind' mutants, where the T3SS is constitutively active regardless of the calcium concentration. In both Ca²⁺ limiting conditions and a *Y. pseudotuberculosis* Ca²⁺ blind mutant (in the presence of Ca²⁺), INP0007 was still able to block effector secretion and translocation, thus concluding that Ca²⁺ does not affect INP0007 and that the compound

Table 1 Examples of inhibitors discussed within that main text. The inhibitor name is that which was used in the given references

Inhibitor	Class	Pathogen	Proposed mechanism	Mammalian cell model	Animal model	Ref. #
INP-0007	SAH	<i>Salmonella</i> , <i>Yersinia</i> , <i>Shigella</i> , <i>Pseudomonas</i> , <i>Chlamydia</i>	May target the basal body assembly; affects motility; cytotoxic in <i>Shigella</i>	Y	Y	85–88, 90, 91, 93
INP-0010	SAH	<i>Yersinia</i> , <i>Salmonella</i> , EPEC, <i>Shigella</i>	May target needle assembly; cytotoxic in <i>Shigella</i> and EPEC	Y (cytotoxic)	N	86, 88, 90, 94
C5631	Benzimidazole	<i>Pseudomonas</i> and <i>Yersinia</i>	Targets the AraC family of T3SS transcriptional regulators	Y	N	102
W1774182	N-Arylbenzylamines	<i>Chlamydia</i>	Targeting ATPase, SctN	Y	N	103
C9652	—	<i>Shigella</i>	Targeting ATPase, SctN	Y	N	105
MBX2359	Phenoxyacetamide	<i>Pseudomonas</i>	Needle-filament assembly	Y	Y	91, 108
Licoflavonol	Flavanoid	<i>Salmonella</i>	Reduced secretion and down-regulated transcription of translocon chaperone and the regulator <i>invF</i>	Y	N	110
(–)-Hopeaphenol	Flavanoid	<i>Yersinia</i> , <i>Chlamydia</i>	May covalently modify translocases (SctB and E); decreased middle and late substrate secretion	Y	N	112
Thymol	Phenol	<i>Salmonella</i>	Reduction in secretion of late effectors	Y	Y	135
Aurodox	Polyketide	EPEC, EHEC, <i>Citrobacter rodentium</i>	Repression of <i>ler</i> master regulator, independent of antibiotic properties	Y	Y	115, 116
R101SPM	Spermidine analogue	<i>Pseudomonas</i>	Inhibits spermidine dependent activation of <i>exsA</i> transcriptional regulator expression	Y	Y	119
CoilA and CoilB	Peptide	EPEC	Targets the EspA filament	Y	Y	68, 69
MEDI3902	Antibody	<i>Pseudomonas</i>	Anti-PcrV (SctA); blocks the tip protein from assembling	Y	Y (murine and human)	122–124

INP0007*
Multi-targeted

INP0010*
Needle assembly/multi-targeted

C5631
Transcription regulation

W1774182
ATPase

C9652
ATPase

MBX2359
Needle assembly

Licoflavonol
Unknown

(-)-hopeaphenol
Translocase secretion

Thymol
Late effector secretion

R101SPM
Gene regulation

Aurodox
Gene regulation

MEDI3902
Tip protein

Fig. 2 Examples of T3SS inhibitors. The proposed target for each inhibitor is listed below the name; a complete description of each inhibitor can be found in Table 1. A (*) indicates that the inhibitor targets both the virulence T3SS and flagellar T3SS. Chemical structures were generated using MarvinSketch. The structure of the antibody fragment (FAb) from MEDI3902 bound to a fragment of PcrV (PDB ID: 6CYF) was generated using Chimera.

appears to mimic Ca^{2+} inhibitory effects.^{87,89} Morgan *et al.* (2018) have recently shown that INP0007 decreased transcript levels of *lcrF*, the positive transcriptional activator of the *Yersinia* T3SS, and in turn led to a decrease in *lcrV* (SctV) as well as *yopH*, *yopE*, and *yopK* effectors, but exhibited no impact on *yscN* (SctN), *yscF* (SctF), or *yscD* (SctD).⁹⁰ The experiments were performed in low concentrations of Ca^{2+} , when the low calcium response is normally active and thus *lcrF* is highly expressed. These authors suggest that the mode of action for INP0007 is likely to disrupt assembly of the basal body as they exhibited a significant inhibition of YscD (SctD) puncta formation in *Y. pseudotuberculosis*, measured by GFP-tagged YscD. Most T3SS systems employ a feedback loop to help control resources: active secretion leads to upregulated transcription of T3SS-associated genes, whereas when secretion is blocked, expression is down regulated. Thus, the observed decrease in *lcrF* transcript levels is probably due to the T3SS positive feedback loop being disrupted in the secretion-inhibited state.⁹⁰

INP0007 exhibited a small reduction in haemolytic activity of *S. enterica*.⁸⁵ When the compound was preincubated with the pathogen, a significant decrease in T3SS-induced pathological changes was observed in treated calves; no change was observed when the drug was co-injected with *S. enterica*.⁸⁵ In *Chlamydia trachomatis*, INP0007 appears to inhibit the T3SS-dependent development cycle of the pathogen in HeLa cells, in turn leading to a decrease in secretion of the late effector, IncA.⁹³ The exact target of INP0007 remains to be shown; knowing the target of INP0007 may allow for structure–activity relationship studies to be initiated and potentially reduce the deleterious effects on the related flagellar system.

INP0010 shares a very similar scaffold to INP0007; however, the nitrobenzohydrazide group is substituted for a pyridine ring, ostensibly resulting in different mechanisms of inhibition. INP0010 severely depleted secreted levels of effector SipB (SctE) without affecting cytosolic levels in *S. enterica*, thus targeting secretion of the middle effector independent of gene expression.⁸⁶ When grown in the presence of INP0010, transcript levels of *hilA*, *sipC*, and *prgH* are depleted, thus transcriptionally silencing the *S. enterica* SPI-1 operon. Negrea *et al.* (2007) also showed that INP0010 is a better inhibitor of *S. enterica* invasion than INP0007. *S. enterica* growth was not affected, but intracellular yields of the pathogen in RAW264.7 cells (macrophage-like) were diminished with INP0010 treatment.⁸⁶ INP0010 exhibits toxicity to EPEC⁹⁴ and *S. flexneri*.⁸⁸

Morgan *et al.* (2018) additionally tested INP0010 in their experimental pipeline for determining the mechanism of T3SS inhibitors.⁹⁰ In contrast to INP0007, INP0010 had no effect on basal body assembly; however, YscF puncta formation (measured using immunofluorescence with anti-YscF) was inhibited, suggesting that the compound impacts needle assembly. INP0010 was found to be cytotoxic in host cells, thus translocation could not be screened. INP0010 exhibited the same pattern of change in transcript levels as INP0007. Addi-

tionally, INP0010 slightly inhibited motility and bacterial growth, suggesting deleterious effects with the conserved flagellar T3SS.⁹⁰

As stated above, the specific cellular target of SAH compounds are often not known. Wang *et al.* (2011) describe an elegant method to link SAH compounds to a polymer bead, Affigel-10, allowing an affinity-based pull down of cellular targets within lysates.⁹⁵ This method was used to identify the targets for the SAH inhibitor ME0055, which was found to interact with enzymes involved in metabolism and downstream regulation of both the virulence and flagellar T3SS. Optimizing the poor solubility of ME0055 yielded a different but more selective target, the major translocon component, EspD.⁹⁶ Overall, SAH compounds yield promise in targeting T3SS function; however, knowing more specifically the mechanism that is targeted by promising inhibitors, while minimizing inhibition of the orthologous flagellar T3SS, is vital for further development.

3.1.2. Benzimidazoles. Benzimidazole compounds have been shown to inhibit T3SS regulators in a few pathogens: VirF in *Shigella*,⁹⁷ LcrF in *Yersinia*,⁹⁸ and ExsA in *Pseudomonas*.⁹⁹ These regulators fall within the AraC family of DNA-binding proteins and exhibit a conserved mechanism of action, binding upstream of the T3SS-associated operons and acting as positive regulators to increase transcription upon host cell contact.¹⁰⁰ Previously, a structure–activity relationship study was established, screening the corresponding IC₅₀ (concentration of inhibitor whereby a 50% decrease in target response is observed) of several different substitutions on the benzimidazole scaffold for LcrF DNA-binding activity.¹⁰¹ Marsden *et al.* (2016) have characterized the inhibitory activity of several previously studied *N*-hydroxybenzimidazoles on DNA binding efficacy of the ExsA homologue, all exhibiting IC₅₀ values < 50 μM .¹⁰² By demonstrating a marked reduction in the corresponding *araC* homologue expression, each compound was shown to protect mammalian cells from T3SS infection in several pathogens at varying degrees amongst *P. aeruginosa*, *Yersinia pestis*, and *Vibrio parahaemolyticus*. As no structure of the DNA-binding domain for any of these homologues is known, the study probed the effect of mutating predicted binding pocket residues on the inhibitory activity of these compounds in order to identify key interacting residues.¹⁰² In addition, several benzimidazoles have been shown to be neither cytotoxic or bactericidal in nature.⁹⁹ The development of *N*-hydroxybenzimidazoles is promising but would benefit from structure-guided drug design optimizations when the structure of ExsA or of another homologue is eventually resolved.

3.1.3. Examples of other small molecule inhibitors. Several other classes of inhibitors are known to inhibit the T3SS with examples of a few most recently published studies will be discussed here. Employing virtual screening techniques for targets of known structure is often the initial stage for identifying promising inhibitors. Using the structure of EscN (SctN for EPEC; PDB ID: 2OBL), FliI (flagellar SctN; PDB ID: 2DPY) and a bovine F-type ATPase (PDB ID: 2JDI) as ATPase

templates, Grishin *et al.* (2018) screened a library of compounds that could potentially inhibit the *Chlamydia trachomatis* ATPase homologue, CdsN, which has no known structure but approximately 50% sequence identity with the SctN's of known structure.¹⁰³ The ATPase is the only conserved enzymatic activity of the T3SS apparatus itself, and is thus an attractive drug target.¹⁰⁴ Grishin and colleagues identified 16 top-scoring candidates in their virtual screening process, with 2 compounds exhibiting significant suppression of the *C. trachomatis* development cycle in mammalian cells at low μM concentrations without displaying significant toxicity in mammalian cells. Additionally, the two promising compounds completely blocked T3SS-mediated secretion of the IncA effector in *C. trachomatis* infected mammalian cells. The authors probed potential structure–activity relationships, but no improvements were found.¹⁰³ Case *et al.* (2018) identified 3 non-competitive inhibitors of Spa47 (SctN of *Shigella flexneri*) through virtual screening techniques and were predicted to bind at the interface between protomers based on modelling the oligomeric structure of Spa47 after a heterohexameric F-type ATPase.¹⁰⁵ Two inhibitors functioned *in vivo*, exhibiting almost no secretion without compromising *S. flexneri* or HeLa viability. All three compounds exhibited inhibition of the ATPase. The authors note that IpaD is still present in needle complexes, suggesting that the inhibitors are inhibiting secretion, potentially *via* an ATP dependent manner.¹⁰⁵ New knowledge from the recent cryo-EM structure of homohexameric EscN (SctN of EPEC discussed above) may assist with a more targeted approach to drug design against the ATPase.¹⁸

Phenoxyacetamide inhibitors have previously exhibited inhibitory properties towards the T3SS, with a few compounds displaying high nanomolar IC_{50} values.^{91,106,107} Optimized inhibitors show inhibition of T3SS secretion and translocation in *P. aeruginosa* HeLa cell infection models, while displaying minimal cytotoxicity. Bowlin *et al.* (2014) identified the target of these as PscF (SctF), the needle filament in *P. aeruginosa*, representing a novel target for drug discovery.⁹¹ Additionally, they identified mutations in PscF that were resistant to phenoxyacetamide inhibition. Berube *et al.* (2017) sought to test the efficacy of these compounds in *Pseudomonas* abscess formation in murine models.¹⁰⁸ Phenoxyacetamide derivatives decreased abscess formation and assisted with clearance of *P. aeruginosa* in host organisms. *P. aeruginosa* expressing phenoxyacetamide resistant *pscF* displayed no response to drug treatment in murine abscess models, corroborating the proposed T3SS target.¹⁰⁸

Dey *et al.* (2017) screened a few hundred drug-like compounds using surface plasmon resonance to measure potential interactions with the *S. flexneri* tip protein, IpaD (SctA).¹⁰⁹ As discussed previously, SctA is essential for virulence in all T3SS-utilizing pathogens with knockouts exhibiting a severe loss of T3SS function.⁷⁵ From these *in silico* studies. Four new compounds were identified to have potential inhibitory properties, all with varying scaffolds: methylquinoline, pyrrolidine-aniline, hydroxyindole and

morpholinoaniline. As the structure of monomeric IpaD is known, the authors used NMR to identify three binding pockets where these compounds interacted, though the actual inhibitory properties of these compounds remain to be shown.

An additional synthetic compound of interest is 2,2'-thiobis-(4-methylphenol), discussed in Jessen *et al.* (2014).¹³⁸ This small molecule exhibited inhibition of translocon secretion in *Yersinia pestis* and *pseudotuberculosis* strains, and *Pseudomonas aeruginosa*, without affecting the secretion of the tip protein. Notably, inhibition was only observed in a calcium depleted state, a trigger for positive feedback of the T3SS, indicating that a fully functional T3SS had to be present for inhibition to be observed. The authors illustrate that the *Yersinia* translocase YopD (SctB) specifically interacts with the compound, suggesting that the mechanism of inhibition is to prevent translocase assembly.

3.1.4. Natural product compounds as small molecule inhibitors. Several novel natural product compounds have recently been identified for their T3SS inhibitory properties. Plant-derived inhibitors often exhibit minimal cytotoxicity to mammalian cells and therefore are useful in further developing compounds for animal trials. Additionally, several commercially available antibiotics are derivatives of natural products.

A novel prenylated flavonoid derivative was identified as a *Salmonella* T3SS inhibitor, shown to inhibit secretion of effectors from the SPI-1 T3SS variant.¹¹⁰ The compound, licoflavonol, did not affect bacterial mobility or viability, both key properties of anti-virulence compounds. Guo *et al.* (2016) showed that licoflavonol down-regulated transcription of *sicA* and *invF*, as well as reduced secretion of several T3SS injectisome secreted effectors, mainly the translocon component SipC (SctB). SicA is the cognate chaperone for both translocon components, SipC and SipB (SctE), in *Salmonella*, whereas *invF* corresponds to a transcriptional regulator of specific SPI-1 genes. The results suggest that by downregulating expression of *sicA*, less chaperone is available for delivering SipC to the export gate for subsequent translocation. Although licoflavonol did not protect HeLa cells from *S. enterica* invasion, downstream secretion of virulence effectors was successfully blocked.¹¹⁰

Oligomers of the flavonoid resveratrol have also shown inhibitory activity towards multiple T3SS-utilizing Gram-negative pathogens. The compound (–)-hopeaphenol, a tetramer of resveratrol, was found to suppress expression and secretion of YopE (Rho-GTPase activating protein; inhibits the adaptive immune response (*e.g.* phagocytosis) in host cells to help *Yersinia* evade detection¹¹¹) and decrease secretion of YopH (phosphatase with many host protein targets) with reported IC_{50} values of $<10\ \mu\text{M}$;¹¹² both are secreted effectors and late substrates for the T3SS of *Yersinia*. The compound was not found to have an appreciative effect on bacterial growth, though effects on bacterial motility was not explored. Additionally, both translocon component YopD (SctB) expression and secretion were inhibited in a dose dependent

manner; notably, at concentrations $>13\ \mu\text{M}$ no secretion of YopD was reported, suggesting that (–)-hopeaphenol is targeting a specific mechanism of the T3SS to block secretion of middle and late effectors. In several T3SS systems, a feedback loop is employed, presumably as a measure to control resources.^{90,113} Active secretion leads to upregulated transcription of T3SS-associated genes, whereas when secretion is blocked, expression is down regulated. This may explain why residual expression is seen despite a lack of secretion. Zetterström *et al.* (2013) additionally show that (–)-hopeaphenol is an irreversible inhibitor of the T3SS and may covalently modify its target.¹¹² This has been illustrated for the flavonoid baicalein, which covalently modifies bacterial effectors (e.g. SipA) and translocases in *Salmonella*.¹¹⁴ (–)-Hopeaphenol exhibited similar activity towards clinical isolates of *P. aeruginosa* and prevented *C. trachomatis* internalization in HeLa cells.¹¹² The specific mechanism of action for (–)-hopeaphenol remains to be determined but is nonetheless a promising compound for future studies.

Thymol derivatives have also recently shown promise in *S. enterica* T3SS inhibition. Using a SipA effector- β -lactamase fusion reporter – a method often employed for monitoring inhibitor-induced changes in T3SS secretion – Zhang *et al.* (2018) showed a dose-dependent inhibition of secretion.¹³⁵ In addition, the compound protected mice against *S. enterica* mortality without cytotoxic effects (70% survival at day 9, compared to 0% for untreated), solidifying its efficacy in an animal model. Viability of bacteria was not detected at effective concentrations used within the study, though the authors note that at very high levels bactericidal properties are exhibited.

Aurodox, an antibiotic produced by *Streptomyces goldiniensis*, has additionally been shown to act as an anti-virulence compound that specifically represses the expression of *ler*, the master regulator of the locus of enterocyte effacement island (LEE) in EPEC/EHEC/*Citrobacter rodentium*.¹¹⁵ LEE encodes several vital virulence factors and structural components of the T3SS. Aurodox is a poor antibiotic against *Escherichia coli*, likely due to poor uptake across the dual membrane, but does have bactericidal activity in Gram-positive bacteria.¹¹⁶ Nonetheless, understanding the specific molecular mechanism of *ler* repression may allow for the development of more specific derivatives of aurodox with improved bioavailability in Gram-negatives. Indeed, aurodox has been shown to provide protection against *Citrobacter rodentium* colonization in mice by specifically inhibiting the T3SS.¹¹⁶ Notably, aurodox also does not trigger the SOS response that is typical of treatment with broad spectrum antibiotics.¹¹⁵ The SOS response in EHEC is responsible for the activation of expression of resident prophages, which contains the genes encoding Shiga toxin. Shiga toxin expressing strains are among the most virulent strains of EHEC and are responsible for some of the most devastating outbreaks and enterohemorrhagic disease outcomes.¹¹⁷

3.1.5. Derivatives of natural product compounds. In *P. aeruginosa*, ExsA is a transcriptional regulator of the T3SS,

binding upstream of all T3SS operons. *Pseudomonas* knock-outs of *exsA* exhibited attenuated T3SS virulence in mammalian cells, corroborating its vital role in T3SS regulation.² The ExsA gene is thought to be activated by endogenous spermidine signals from the host. Supporting this, upon exogenous addition of spermidine to *P. aeruginosa* culture, T3SS expression was induced in an ExsA-dependent mode of action.¹¹⁸ Spermidine uptake in *Pseudomonas* is driven by the Spu transporter complex and when knocked out, *exsA*-regulated expression of the T3SS is repressed, resulting in attenuated pathogenicity in mammalian cells. Recently, spermidine derivatives were screened for potential at blocking the Spu transporter and thereby blocking spermidine-induced activation of *exsA*.¹¹⁹ One compound was identified, R101SPM, displaying an MIC₅₀ of $<10\ \mu\text{M}$ and EC₅₀ (effective concentration of compound to exhibit a 50% decrease in response) of $10\ \mu\text{M}$, representing a promising lead compound. Efficacy in murine models was tested for protection against *P. aeruginosa* infection; mice treated with R101SPM had a 100% survival rate after 6 days when compared to a 20% survival rate of untreated mice. The compound effectively blocked expression of *exsA*.¹¹⁹ Of note, R101SPM displayed cytotoxic effects in HeLa cells at $>100\ \mu\text{M}$, well above the range of inhibitory activity. Other spermidine derivatives, spermatinamine and pseudoceramine B have also shown inhibition of T3SS secretion by *Yersinia*, though the mechanism of action is unknown.¹²⁰

3.2. Coiled-coil peptides

The highly conserved coiled-coil motif – found within several components of the T3SS – provides a promising scaffold for the development of competitive inhibitor peptides that may prevent the assembly of these structures. Building off of previous data,⁶⁸ Larzabal and colleagues (2019) sought to understand the mechanism of inhibition of these inhibitory peptides using molecular modelling techniques.⁶⁹ Using the truncated EspA structure from the chaperone (CesA)-substrate complex as their model, the authors used molecular dynamic simulations to design and evaluate peptides that may inhibit the oligomerization of the EspA filament (tip – SctA) structure. In their previous work, two promising peptides were developed, termed CoilA and CoilB, both exhibiting approximately 95% inhibition of EPEC-induced RBC haemolysis and protected murine models from colonic injury during *C. rodentium* (possesses the same T3SS-encoded pathogenicity island – LEE – as EPEC/EHEC) infection.⁶⁸ No evidence of toxicity was observed *in vivo*. These peptides possess identical sequences to coiled-coil regions present in EspA. In this recent study, they sought to understand the interaction of these inhibitory peptides using molecular modelling techniques and to optimize their previously developed peptides. Their new peptide inhibitors did not exhibit near the amount of inhibition as CoilA and B but provided promise for future development of peptide inhibitors against the T3SS virulence system. Notably, there is no atomic

resolution native, oligomerized structure of the EspA filament, which in the future, may present a new understanding for further optimization of inhibitors.

3.3. Immunotherapy

Immunotherapy in the prevention of T3SS assembly has proven promising in several studies.¹²¹ Recently, the results of the phase I clinical trial for an anti-PcrV monoclonal antibody have been published, yielding positive results leading into phase II for MEDI3902 (Clinical Trials identifier: NCT02696902).¹²² The phase I results showed limited toxicity and no adverse effects in subjects. The phase II trial has begun for MEDI3902 in testing its efficacy for treatment of ventilator-associated pneumoniae and is expected to be completed by 2020.

MEDI3902 is a bispecific monoclonal antibody that has shown efficacy in treating pneumonia in animal models caused by *Pseudomonas aeruginosa*.¹²³ The antibody targets both the tip protein (SctA) and the exopolysaccharide Psl in *Pseudomonas*, which is important in host-cell adherence. By binding to secreted SctA, the antibody successfully halts assembly of the tip protein and prevents the secretion, and therefore assembly of the translocases. Additionally, the crystal structure of a PcrV epitope bound to the fragment-antigen binding domain of MEDI3902 was published.¹²⁴ The authors were interested in the variance in sequence of PcrV in clinical isolates, and in turn mapped the variable regions on the co-crystal structure. The epitope region exhibited high conservation among clinical isolates and thus will be effective in treating a range of *Pseudomonas* pneumoniae cases. This example illustrates the importance of corroborating structure with function in drug design.

A second immunotherapy targeting PcrV has gone through clinical trials and unfortunately did not meet clinical efficacy after phase II trials.¹²⁵ The antibody, KB001-A, was tested for efficacy in treating chronic *Pseudomonas* infections in cystic fibrosis (CF) patients by measuring the time-to-need for antibiotics. The authors note that in patients with chronic infections, T3SS expressing *Pseudomonas* is present in only 11% of isolates, which may have led to its failure in clinical efficacy. The immunotherapy was deemed to be safe and well tolerated in CF patients¹²⁵ and may be useful in treatment of other pseudomonal nosocomial infections, such as septic shock, as shown *in vivo* by Sawa and colleagues.^{121,126}

4. Outlooks and conclusions

Several WHO priority pathogens use the T3SS virulence mechanism to evoke a disease state in humans. With recent advances in cryo-EM, several previously unknown structures have been determined, further elucidating the mechanism of assembly and function of this vital virulence mechanism. New native assembled structures have been published^{24,28,43} and may allow for structure-guided drug design of new therapeutics that specifically target the myriad of protein–protein interfaces in the T3SS. Moreover, new structural knowledge

may assist with optimization of previously identified inhibitors with unknown mechanisms of action.

The homohexameric but surprisingly asymmetric native structure of the EscN/O (SctN/O) complex provides the first insights into the rotary mechanistic workings of a T3SS ATPase.¹⁸ Previously, inhibitors against the ATPase of several T3SS's have been designed, based off of high throughput or virtual screening using homology modelling and notably in the absence of the central stalk component, which defined the observed asymmetry.^{103–105,127} As the active site(s) sits between adjacent protomers, the precise architecture provided by the novel atomic model of EscN/O may assist with optimization of previously designed inhibitors through SAR techniques.

Immunotherapy offers a promising avenue for T3SS-targeted drug development. The PcrV (SctA)-targeted antibody MEDI3902 represents one of the first T3SS targeted therapeutics to reach clinical trials.¹²² By co-crystallizing MEDI3902 and PcrV, Tabor *et al.* (2018) illustrated the high sequence conservation of the epitope region of PcrV among clinical isolates of *Pseudomonas*, thus supporting the potential of MEDI3902 in treating a range of pseudomonal pneumonia cases.¹²⁴ Additionally, T3SS-targeted cattle vaccines are a promising avenue within animal husbandry for reducing fecal shedding of *E. coli* O157:H7 (Econiche Corp, Belleville, Canada),¹²⁸ an important causative agent of the recent increase in global produce contamination.¹²⁹ The Econiche™ vaccine contains the secreted components of O157:H7: the tip protein EspA (SctA), the translocon component EspB (SctB) and the virulence effector Tir.¹³⁰ However, complications arise when vaccines are developed to target a specific strain or are used as a prophylactic.

In addition, there are numerous components of the T3SS that, to our knowledge, have never been targeted and yet represent crucial mechanisms that may be disrupted. Components discussed here include: the secretin pilotin, important in targeting the secretin to the OM during the late stages of T3SS assembly;⁴⁹ the lytic transglycosylase, which is important for the local clearance of peptidoglycan around the developing rod/filament, particularly in EPEC;⁴⁷ the autoprotease (SctU), responsible for switch regulation from early to middle and late substrates;³⁰ or other regulators, such as the needle-length regulator SctP.¹³¹

In this review, we focus on recently characterized inhibitors, but it is important to highlight previously identified compounds which target structures that have not been discussed in this section. For example, those which show promise in disrupting the assembly of the basal body complex, as described by the Miller group.^{132,133} High throughput screening identified a 2-imino-5-arylidene thiazolidinone¹³² and further optimization and characterization resulted in an inhibitor¹³³ with an IC₅₀ of 3 μM through monitoring effector secretion. Their data suggested that these compounds specifically interact with the basal body of the T3SS to inhibit its assembly, though this mechanism requires further validation. Notably, these inhibitors were

selective towards the virulence T3SS and not the orthologous flagellar system.^{132,133}

Going forward with identification of novel anti-virulence compounds, researchers can employ several methodologies that have been eluded to in this review. As the triple membrane spanning T3SS complex presents significant hurdles to achieve active and complete reconstitution in a membrane mimetic, screening strategies to date have typically involved a defined compound library, followed by *in vitro* screening of T3SS encoding cell cultures to monitor decreased secretion to the extracellular media. *In vitro* screening to monitor inhibitory effects on T3SS secretion in infected mammalian cells (such as the luciferase assay used by Zhang *et al.* (2018) as mentioned above) and infection models is also possible; however, this can be difficult to accurately accomplish.¹³⁵ Growth conditions should ideally emulate the pathogen's environment or lifestyle within the host, otherwise screening may result in false positives. For example, assay conditions should take into consideration whether a pathogen is invasive, such as in *Salmonella enterica* and *Chlamydia trachomatis*, or forms complex biofilms, as in the case of *Pseudomonas aeruginosa* (see Totsika *et al.* (2016) for a recent comprehensive review of further challenges and considerations in anti-virulence drug design).¹³⁴

As biochemical and structural information for specific targets of interest have accrued, *in vitro* assays have typically involved purification of the target, at least in part, followed by affinity pull down assays; however recently more sophisticated binding characterizations have begun to emerge. Grishin *et al.* (2018) used the homology model of the *Chlamydia trachomatis* ATPase to screen a virtual library of compounds that may target this family of enzymes.¹⁰³ With atomic resolution data now possible *via* cryoEM for the assembled T3SS ATPase,¹⁸ identification of precise interfaces of these new inhibitors and their structure-guided optimization is now on the horizon. Other recent examples include, Dey *et al.* (2017) who recombinantly expressed and purified the needle tip protein from *Shigella flexneri* (IpaD); they employed a high-throughput surface plasmon resonance screening strategy to monitor interactions of IpaD with 288 different compounds in a defined library.¹⁰⁹

Several identified inhibitors currently have no known mechanism of action, present inhibition of motility that results in bacterial cell death or are not suitable in mammalian model systems due to their inability to cross membranes to reach their target or general cytotoxicity. Morgan *et al.* (2018) presented a set of sequential experiments that can be performed to assist in identifying and validating area(s) of the T3SS that may be affected, covering gene regulation to effector translocation.⁹⁰ An ideal inhibitor would exhibit no bactericidal properties, no mammalian cell cytotoxicity, and no effect on the orthologous flagellar T3SS. These properties satisfy the anti-virulence strategy: inhibitors that disrupt virulence mechanisms of pathogens without affecting their motility or viability, reducing the selective pressure for resistance development and providing new alternatives to antibiotics.

Abbreviations

T3SS	Type 3 secretion system
WT	Wild-type
EPEC	Enteropathogenic <i>Escherichia coli</i>
EHEC	Enterohemorrhagic <i>Escherichia coli</i>
Cryo-EM	Cryo-electron microscopy
Cryo-ET	Cryo-electron tomography
NMR	Nuclear magnetic resonance
LT	Lytic transglycosylase
ATP	Adenosine triphosphate
PMF	Proton-motive force
SEC-MALS	Size exclusion chromatography multi-angle light scattering
QSAR	Quantitative structure–activity relationship
SAH	Salicylidene acylhydrazides
CF	Cystic fibrosis
MDR	Multi-drug resistant
AMR	Antimicrobial resistant
WHO	World Health Organization
IM/OM	Inner or outer membrane
LEE	Locus of enterocyte effacement
DLS	Dynamic light scattering
SPR	Surface plasmon resonance

Conflicts of interest

There are no conflicts to declare.

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References

- 1 Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics, http://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf, (accessed 20 September 2001).
- 2 A. Roy-Burman, R. H. Savel, S. Racine, B. L. Swanson, N. S. Revadigar, J. Fujimoto, T. Sawa, D. W. Frank and J. P. Wiener-Kronish, *J. Infect. Dis.*, 2001, **183**, 1767–1774.
- 3 H. Agaisse, *Lancet Infect. Dis.*, 2015, **15**, 867–868.
- 4 S. Makvana and L. R. Krilov, *Pediatr. Rev.*, 2015, **36**, 167–171.
- 5 S. Stefani, S. Campana, L. Cariani, V. Carnovale, C. Colombo, M. M. Lleo, V. D. Iula, L. Minicucci, P. Morelli, G. Pizzamiglio and G. Taccetti, *Int. J. Med. Microbiol.*, 2017, **307**, 353–362.

- 6 D. Maura, A. E. Ballok and L. G. Rahme, *Curr. Opin. Microbiol.*, 2016, **33**, 41–46.
- 7 B. K. Johnson and R. B. Abramovitch, *Trends Pharmacol. Sci.*, 2017, **38**, 339–362.
- 8 D. Büttner, *Microbiol. Mol. Biol. Rev.*, 2012, **76**, 262–310.
- 9 W. Deng, N. C. Marshall, J. L. Rowland, J. M. McCoy, L. J. Worrall, A. S. Santos, N. C. J. Strynadka and B. B. Finlay, *Nat. Rev. Microbiol.*, 2017, **15**, 323–337.
- 10 A. G. Portaliou, K. C. Tsois, M. S. Loos, V. Balabanidou, J. Rayo, A. Tsirigotaki, V. F. Crepin, G. Frankel, C. G. Kalodimos, S. Karamanou and A. Economou, *EMBO J.*, 2017, **36**, 3517–3531.
- 11 N. C. Marshall and B. B. Finlay, *Expert Opin. Ther. Targets*, 2014, **18**, 137–152.
- 12 A. G. Portaliou, K. C. Tsois, M. S. Loos, V. Zorzini and A. Economou, *Trends Biochem. Sci.*, 2016, **41**, 175–189.
- 13 T. Minamino, Y. V. Morimoto, N. Hara and K. Namba, *Nat. Commun.*, 2011, **2**, 1–9.
- 14 P.-C. Lee, S. E. Zmina, C. M. Stopford, J. Toska and A. Rietsch, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, E2027–2036.
- 15 Y. Akeda and J. E. Galán, *Nature*, 2005, **437**, 911–915.
- 16 Y. Yoshida, T. Miki, S. Ono, T. Haneda, M. Ito and N. Okada, *PLoS One*, 2014, **9**, 1–12.
- 17 B. Hu, D. R. Morado, W. Margolin, J. R. Rohde, O. Arizmendi, W. L. Picking, W. D. Picking and J. Liu, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, 1047–1052.
- 18 D. D. Majewski, L. J. Worrall, C. Hong, C. E. Atkinson, M. Vuckovic, N. Watanabe, Z. Yu and N. C. J. Strynadka, *Nat. Commun.*, 2019, **10**, 1–12.
- 19 J. L. Burgess, R. A. Burgess, Y. Morales, J. M. Bouvang, S. J. Johnson and N. E. Dickenson, *J. Biol. Chem.*, 2016, **291**, 25837–25852.
- 20 R. Zarivach, M. Vuckovic, W. Deng, B. B. Finlay and N. C. J. Strynadka, *Nat. Struct. Mol. Biol.*, 2007, **14**, 131–137.
- 21 S. E. Allison, B. R. Tuinema, E. S. Everson, S. Sugiman-Marangos, K. Zhang, M. S. Junop and B. K. Coombes, *J. Biol. Chem.*, 2014, **289**, 23734–23744.
- 22 X. Gao, Z. Mu, X. Yu, B. Qin, J. Wojdyla, M. Wang and S. Cui, *Front. Microbiol.*, 2018, **9**, 1–15.
- 23 T. Ibuki, K. Imada, T. Minamino, T. Kato, T. Miyata and K. Namba, *Nat. Struct. Mol. Biol.*, 2011, **18**, 277–283.
- 24 L. Kuhlen, P. Abrusci, S. Johnson, J. Gault, J. Deme, J. Caesar, T. Dietsche, M. T. Mebrhatu, T. Ganief, B. Macek, S. Wagner, C. V. Robinson and S. M. Lea, *Nat. Struct. Mol. Biol.*, 2018, **25**, 583–590.
- 25 S. Zilkenat, M. Franz-Wachtel, Y.-D. Stierhof, J. E. Galán, B. Macek and S. Wagner, *Mol. Cell. Proteomics*, 2016, **15**, 1598–1609.
- 26 J. S. Van Arnam, J. L. Mcmurry, M. Kihara and R. M. Macnab, *J. Bacteriol.*, 2004, **186**, 2495–2498.
- 27 L. J. Worrall, C. Hong, M. Vuckovic, W. Deng, J. R. C. Bergeron, D. D. Majewski, R. K. Huang, T. Spreter, B. B. Finlay, Z. Yu and N. C. J. Strynadka, *Nature*, 2016, **540**, 597–601.
- 28 J. Hu, L. J. Worrall, C. Hong, M. Vuckovic, C. E. Atkinson, N. Caveney, Z. Yu and N. C. J. Strynadka, *Nat. Commun.*, 2018, **9**, 1–11.
- 29 T. Dietsche, M. T. Mebrhatu, M. J. Brunner, P. Abrusci, J. Yan, M. Franz-Wachtel, C. Schärfe, S. Zilkenat, I. Grin, J. E. Galán, O. Kohlbacher, S. Lea, B. Macek, T. C. Marlovits, C. V. Robinson and S. Wagner, *PLoS Pathog.*, 2016, **12**, 1–25.
- 30 R. Zarivach, W. Deng, M. Vuckovic, H. B. Felise, H. V. Nguyen, S. I. Miller, B. B. Finlay and N. C. J. Strynadka, *Nature*, 2008, **453**, 124–127.
- 31 J. L. Thomassin, X. He and N. A. Thomas, *BMC Microbiol.*, 2011, **11**, 205.
- 32 I. Sorg, S. Wagner, M. Amstutz, S. A. Müller, P. Broz, Y. Lussi, A. Engel and G. R. Cornelis, *EMBO J.*, 2007, **26**, 3015–3024.
- 33 J. V. M. Faria, M. D. Lafebre, Y.-D. Stierhof, J. E. Galán and S. Wagner, *mBio*, 2015, **6**, 1–8.
- 34 G. T. Lountos, B. P. Austin, S. Nallamsetty and D. S. Waugh, *Protein Sci.*, 2009, **18**, 467–474.
- 35 J. E. Deane, S. C. Graham, E. P. Mitchell, D. Flot, S. Johnson and S. M. Lea, *Mol. Microbiol.*, 2008, **69**, 267–276.
- 36 J. R. C. Bergeron, L. Fernández, G. A. Wasney, M. Vuckovic, F. Reffuveille, R. E. W. Hancock and N. C. J. Strynadka, *J. Biol. Chem.*, 2016, **291**, 1676–1691.
- 37 V. A. Meshcheryakov, A. Kitao, H. Matsunami and F. Samatey, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 2013, **69**, 812–820.
- 38 P. Abrusci, M. Vergara-Irigaray, S. Johnson, M. D. Beeby, D. Hendrixson, P. Roversi, M. E. Friede, J. E. Deane, G. J. Jensen, C. M. Tang and S. M. Lea, *Nat. Struct. Mol. Biol.*, 2013, **20**, 99–104.
- 39 L. J. Worrall, M. Vuckovic and N. C. J. Strynadka, *Protein Sci.*, 2010, **19**, 1091–1096.
- 40 Q. Xing, K. Shi, A. Portaliou, P. Rossi, A. Economou and C. G. Kalodimos, *Nat. Commun.*, 2018, **9**, 1–9.
- 41 B. J. Burkinshaw, S. A. Souza and N. C. J. Strynadka, *Acta Crystallogr., Sect. F: Struct. Biol. Commun.*, 2015, **71**, 1300–1308.
- 42 W. Deng, H. B. Yu, Y. Li and B. B. Finlay, *J. Bacteriol.*, 2015, **197**, 1263–1275.
- 43 D. D. Majewski, L. J. Worrall and N. C. Strynadka, *Curr. Opin. Struct. Biol.*, 2018, **51**, 61–72.
- 44 P. L. Kohler, H. L. Hamilton, K. Cloud-Hansen and J. P. Dillard, *J. Bacteriol.*, 2007, **189**, 5421–5428.
- 45 B. Byun, K. V. Mahasenan, D. A. Dik, D. R. Marous, E. Speri, M. Kumarasiri, J. F. Fisher, J. A. Hermoso and S. Mobashery, *Sci. Rep.*, 2018, **8**, 1–8.
- 46 P. Zaloba, B. A. Bailey-Elkin, M. Derksen and B. L. Mark, *PLoS One*, 2016, **11**, 1–13.
- 47 E. García-Gómez, N. Espinosa, J. de la Mora, G. Dreyfus and B. González-Pedrajo, *Microbiology*, 2011, **157**, 1145–1160.
- 48 B. J. Burkinshaw, W. Deng, E. Lameignère, G. A. Wasney, H. Zhu, L. J. Worrall, B. B. Finlay and N. C. J. Strynadka, *J. Biol. Chem.*, 2015, **290**, 10406–10417.

- 49 C. Perdu, P. Huber, S. Bouillot, A. Blocker, S. Elsen, I. Attrée and E. Faudry, *Infect. Immun.*, 2015, **83**, 1789–1798.
- 50 A. M. Crago and V. Koronakis, *Mol. Microbiol.*, 1998, **30**, 47–56.
- 51 S. Daefler and M. Russel, *Mol. Microbiol.*, 1998, **28**, 1367–1380.
- 52 R. Schuch and A. T. Maurelli, *Infect. Immun.*, 1999, **67**, 1982–1991.
- 53 P. Burghout, F. Beckers, E. De Wit, R. Van Bortel, G. R. Cornelis, J. Tommassen and M. Koster, *J. Bacteriol.*, 2004, **186**, 5366–5375.
- 54 T. Izoré, C. Perdu, V. Job, I. Attree, E. Faudry and A. Dessen, *J. Mol. Biol.*, 2011, **413**, 236–246.
- 55 S. Raymond, R. C. Sandlin and A. T. Maurelli, *Mol. Microbiol.*, 1999, **34**, 675–689.
- 56 P. I. Lario, R. A. Pfuetzner, E. A. Frey, L. Creagh, C. Haynes, A. T. Maurelli and N. C. J. Strynadka, *EMBO J.*, 2005, **24**, 1111–1121.
- 57 M. Okon, T. F. Moraes, P. I. Lario, A. L. Creagh, C. A. Haynes, N. C. J. Strynadka and L. P. McIntosh, *Structure*, 2008, **16**, 1544–1554.
- 58 J. Koo, L. L. Burrows and P. Lynne Howell, *FEMS Microbiol. Lett.*, 2012, **328**, 1–12.
- 59 A. Loquet, N. G. Sgourakis, R. Gupta, K. Giller, D. Riedel, C. Goosmann, C. Griesinger, M. Kolbe, D. Baker, S. Becker and A. Lange, *Nature*, 2012, **486**, 276–279.
- 60 J.-P. Demers, B. Habenstein, A. Loquet, S. K. Vasa, K. Giller, S. Becker, D. Baker, A. Lange and N. G. Sgourakis, *Nat. Commun.*, 2014, **5**, 1–28.
- 61 R. Kenjale, J. Wilson, S. F. Zenk, S. Saurya, W. L. Picking, W. D. Picking and A. Blocker, *J. Biol. Chem.*, 2005, **280**, 42929–42937.
- 62 J. Verasdonck, D. K. Shen, A. Treadgold, C. Arthur, A. Böckmann, B. H. Meier and A. J. Blocker, *J. Struct. Biol.*, 2015, **192**, 441–448.
- 63 J. Torruellas, M. W. Jackson, J. W. Pennock and G. V. Plano, *Mol. Microbiol.*, 2005, **57**, 1719–1733.
- 64 J. Kato, S. Dey, J. E. Soto, C. Butan, M. C. Wilkinson, R. N. De Guzman and J. E. Galan, *eLife*, 2018, **7**, 1–23.
- 65 S. J. Daniell, R. M. Delahay, R. K. Shaw, E. L. Hartland, M. J. Pallen, F. Booy, F. Ebel, S. Knutton and G. Frankel, *Infect. Immun.*, 2001, **69**, 4055–4064.
- 66 A. D. Gazi, M. Bastaki, S. N. Charova, E. A. Gkougkoulia, E. A. Kapellios, N. J. Panopoulos and M. Kokkinidis, *J. Biol. Chem.*, 2008, **283**, 34062–34068.
- 67 C. K. Yip, B. B. Finlay and N. C. J. Strynadka, *Nat. Struct. Mol. Biol.*, 2005, **12**, 75–81.
- 68 M. Larzábal, E. Zotta, C. Ibarra, B. C. Rabinovitz, D. A. Vilte, E. C. Mercado and Á. Cataldi, *Int. J. Med. Microbiol.*, 2013, **303**, 9–15.
- 69 M. Larzábal, H. A. Baldoni, F. D. Suvire, L. M. Curto, G. E. Gomez, W. M. Da Silva, S. L. Giudicessi, S. A. Camperi, J. M. Delfino, A. A. Cataldi and D. Enriz, *J. Pept. Sci.*, 2019, **25**, 1–15.
- 70 D. Zhong, M. Lefebvre, K. Kaur, M. A. McDowell, C. Gdowski, S. Jo, Y. Wang, S. H. Benedict, S. M. Lea, J. E. Galan and R. N. De Guzman, *J. Biol. Chem.*, 2012, **287**, 25303–25311.
- 71 L. Journet, C. Agrain, P. Broz and G. R. Cornelis, *Science*, 2003, **302**, 1757–1761.
- 72 O. Ho, P. Rogne, T. Edgren, H. Wolf-Watz, F. Login and M. Wolf-Watz, *J. Biol. Chem.*, 2017, **292**, 3299–3311.
- 73 C. Epler, N. E. Dickenson, E. Bullitt and W. L. Picking, *J. Mol. Biol.*, 2012, **420**, 1–19.
- 74 S. Chaudhury, K. P. Battaile, S. Lovell, G. V. Plano and R. N. De Guzman, *Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun.*, 2013, **69**, 477–481.
- 75 S. Chatterjee, D. Zhong, B. A. Nordhues, K. P. Battaile, S. Lovell and R. N. De Guzman, *Protein Sci.*, 2011, **20**, 75–86.
- 76 S. Johnson, P. Roversi, M. Espina, A. Olive, J. E. Deane, S. Birket, T. Field, W. D. Picking, A. Blocker, E. E. Galyov, W. L. Picking and S. M. Lea, *J. Biol. Chem.*, 2007, **282**, 4035–4044.
- 77 K. Sekiya, M. Ohishi, T. Ogino, K. Tamano, C. Sasakawa and A. Abe, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 11638–11643.
- 78 S. J. Daniell, E. Kocsis, E. Morris, S. Knutton, F. P. Booy and G. Frankel, *Mol. Microbiol.*, 2003, **49**, 301–308.
- 79 D. Park, M. Lara-Tejero, M. N. Waxham, W. Li, B. Hu, J. E. Galán and J. Liu, *eLife*, 2018, **7**, 1–15.
- 80 F. B. Romano, Y. Tang, K. C. Rossi, K. R. Monopoli, J. L. Ross and A. P. Heuck, *J. Biol. Chem.*, 2016, **291**, 6304–6315.
- 81 W. Luo and M. S. Donnenberg, *J. Bacteriol.*, 2011, **193**, 2972–2980.
- 82 M. L. Barta, N. E. Dickenson, M. Patil, A. Keightley, G. J. Wyckoff, W. D. Picking, W. L. Picking and B. V. Geisbrecht, *J. Mol. Biol.*, 2012, **417**, 395–405.
- 83 V. S. Nguyen, C. Jobichen, K. W. Tan, Y. W. Tan, S. L. Chan, K. Ramesh, Y. Yuan, Y. Hong, J. Seetharaman, K. Y. Leung, J. Sivaraman and Y. K. Mok, *Structure*, 2015, **23**, 2022–2031.
- 84 S. Muhlen and P. Dersch, *Curr. Top. Microbiol. Immunol.*, 2015, **398**, 147–183.
- 85 D. L. Hudson, A. N. Layton, T. R. Field, A. J. Bowen, H. Wolf-Watz, M. Elofsson, M. P. Stevens and E. E. Galyov, *Antimicrob. Agents Chemother.*, 2007, **51**, 2631–2635.
- 86 A. Negrea, E. Bjur, S. E. Ygberg, M. Elofsson, H. Wolf-Watz and M. Rhen, *Antimicrob. Agents Chemother.*, 2007, **51**, 2867–2876.
- 87 R. Nordfelth, A. M. Kauppi, H. A. Norberg, H. Wolf-Watz and M. Elofsson, *Infect. Immun.*, 2005, **73**, 3104–3114.
- 88 A. K. J. Veenendaal, C. Sundin and A. J. Blocker, *J. Bacteriol.*, 2009, **191**, 563–570.
- 89 A. M. Kauppi, R. Nordfelth, H. Uvell, H. Wolf-Watz and M. Elofsson, *Chem. Biol.*, 2003, **10**, 241–249.
- 90 J. M. Morgan, H. N. Lam, J. Delgado, J. Luu, S. Mohammadi, R. R. Isberg, H. Wang and V. Auerbuch, *Front. Cell. Infect. Microbiol.*, 2018, **8**, 1–17.
- 91 N. O. Bowlin, J. D. Williams, C. A. Knoten, M. C. Torhan, T. F. Tashjian, B. Li, D. Aiello, J. Mecsas, A. R. Hauser, N. P. Peet, T. L. Bowlin and D. T. Moir, *Antimicrob. Agents Chemother.*, 2014, **58**, 2211–2220.

- 92 R. D. Perry, P. A. Harmon, W. S. Bowmer and S. C. Straley, *Infect. Immun.*, 1986, **54**, 428–434.
- 93 K. Wolf, H. J. Betts, B. Chellas-Géry, S. Hower, C. N. Linton and K. A. Fields, *Mol. Microbiol.*, 2006, **61**, 1543–1555.
- 94 J. J. Tree, D. Wang, C. McNally, A. Mahajan, A. Layton, I. Houghton, M. Elofsson, M. P. Stevens, D. L. Gally and A. J. Roe, *Infect. Immun.*, 2009, **77**, 4209–4220.
- 95 D. Wang, C. E. Zetterström, M. Gabrielsen, K. S. H. Beckham, J. J. Tree, S. E. Macdonald, O. Byron, T. J. Mitchell, D. L. Gally, P. Herzyk, A. Mahajan, H. Uvell, R. Burchmore, B. O. Smith, M. Elofsson and A. J. Roe, *J. Biol. Chem.*, 2011, **286**, 29922–29931.
- 96 R. Zambelloni, J. P. R. Connolly, A. Huerta Uribe, K. Burgess, R. Marquez and A. J. Roe, *Mol. Microbiol.*, 2017, **105**, 606–619.
- 97 V. Koppolu, I. Osaka, J. M. Skredenske, B. Kettle, P. S. Hefty, J. Li and S. M. Egan, *Infect. Immun.*, 2013, **81**, 4220–4231.
- 98 L. K. Garrity-Ryan, O. K. Kim, J. M. Balada-Llasat, V. J. Bartlett, A. K. Verma, M. L. Fisher, C. Castillo, W. Songsunthong, S. K. Tanaka, S. B. Levy, J. Mecsas and M. N. Alekshun, *Infect. Immun.*, 2010, **78**, 4683–4690.
- 99 M. C. Grier, L. K. Garrity-Ryan, V. J. Bartlett, K. A. Klausner, P. J. Donovan, C. Dudley, M. N. Alekshun, S. K. Tanaka, M. P. Draper, S. B. Levy and O. K. Kim, *Bioorg. Med. Chem. Lett.*, 2010, **20**, 3380–3383.
- 100 L. Schwiesow, H. Lam, P. Dersch and V. Auerbuch, *J. Bacteriol.*, 2016, **198**, 604–614.
- 101 O. K. Kim, L. K. Garrity-Ryan, V. J. Bartlett, M. C. Grier, A. K. Verma, G. Medjanis, J. E. Donatelli, A. B. Macone, S. K. Tanaka, S. B. Levy and M. N. Alekshun, *J. Med. Chem.*, 2009, **52**, 5626–5634.
- 102 A. E. Marsden, J. M. King, M. A. Spies, O. K. Kim and T. L. Yahr, *Antimicrob. Agents Chemother.*, 2016, **60**, 766–776.
- 103 A. V. Grishin, S. I. Luyksaar, L. N. Kapotina, D. D. Kirsanov, E. S. Zayakin, A. S. Karyagina and N. A. Zigangirova, *Chem. Biol. Drug Des.*, 2018, **91**, 717–727.
- 104 W. Swietnicki, D. Carmany, M. Retford, M. Guelta, R. Dorsey, J. Bozue, M. S. Lee and M. A. Olson, *PLoS One*, 2011, **6**, 1–13.
- 105 H. B. Case, D. S. Mattock and N. E. Dickenson, *Biochemistry*, 2018, **57**, 6906–6916.
- 106 D. Aiello, J. D. Williams, H. Majgier-Baranowska, I. Patel, N. P. Peet, J. Huang, S. Lory, T. L. Bowlin and D. T. Moir, *Antimicrob. Agents Chemother.*, 2010, **54**, 1988–1999.
- 107 J. D. Williams, M. C. Torhan, V. Neelagiri, C. Brown, N. O. Bowlin, M. Di, C. T. McCarthy, D. Aiello, N. P. Peet, T. L. Bowlin and D. T. Moir, *Bioorg. Med. Chem.*, 2015, **23**, 1027–1043.
- 108 B. J. Berube, K. R. Murphy, M. C. Torhan, N. O. Bowlin, J. D. Williams, T. L. Bowlin, D. T. Moir and A. R. Hauser, *Antimicrob. Agents Chemother.*, 2017, **61**, 1–15.
- 109 S. Dey, A. Anbanandam, B. E. Mumford and R. N. De Guzman, *ChemMedChem*, 2017, **12**, 1534–1541.
- 110 Z. Guo, X. Li, J. Li, X. Yang, Y. Zhou, C. Lu and Y. Shen, *Biochem. Biophys. Res. Commun.*, 2016, **477**, 998–1004.
- 111 S. Atkinson and P. Williams, *F1000Research*, 2016, **5**, 1–10.
- 112 C. E. Zetterström, J. Hasselgren, O. Salin, R. A. Davis, R. J. Quinn, C. Sundin and M. Elofsson, *PLoS One*, 2013, **8**, 1–12.
- 113 M. S. Francis, H. Wolf-Watz and Å. Forsberg, *Curr. Opin. Microbiol.*, 2002, **5**, 166–172.
- 114 L. K. Tsou, M. Lara-Tejero, J. RoseFigura, Z. J. Zhang, Y.-C. Wang, J. S. Yount, M. Lefebvre, P. D. Dossa, J. Kato, F. Guan, W. Lam, Y. Cheng, J. E. Galán and H. C. Hang, *J. Am. Chem. Soc.*, 2016, **138**, 2209–2218.
- 115 R. E. McHugh, N. O'Boyle, J. P. R. Connolly, P. A. Hoskisson and A. J. Roe, *Infect. Immun.*, 2019, **87**, 1–12.
- 116 K. Kimura, M. Iwatsuki, T. Nagai, A. Matsumoto, Y. Takahashi, K. Shiomi, S. Omura and A. Abe, *J. Antibiot.*, 2011, **64**, 197–203.
- 117 C. Frank, D. Werber, J. P. Cramer, M. Askar, M. Faber, M. Der Heiden, H. Bernard, A. Fruth, R. Prager, A. Spode, M. Wadl, A. Zoufaly, S. Jordan, M. J. Kemper, P. Follin, L. Müller, L. A. King, B. Rosner, U. Buchholz, K. Stark and G. Krause, *N. Engl. J. Med.*, 2011, **365**, 1771–1780.
- 118 L. Zhou, J. Wang and L. H. Zhang, *PLoS One*, 2007, **2**, 1–10.
- 119 C. Wang, X. Liu, J. Wang, J. Zhou, Z. Cui and L. H. Zhang, *Sci. Rep.*, 2016, **6**, 1–13.
- 120 S. Yin, R. A. Davis, T. Shelper, M. L. Sykes, V. M. Avery, M. Elofsson, C. Sundin and R. J. Quinn, *Org. Biomol. Chem.*, 2011, **9**, 6755–6760.
- 121 T. Sawa, E. Ito, V. H. Nguyen and M. Haight, *Hum. Vaccines Immunother.*, 2014, **10**, 2843–2852.
- 122 S. O. Ali, X. Q. Yu, G. J. Robbie, Y. Wu, K. Shoemaker, L. Yu, A. DiGiandomenico, A. E. Keller, C. Anude, M. Hernandez-Illas, T. Bellamy, J. Falloon, F. Dubovsky and H. S. Jafri, *Clin. Microbiol. Infect.*, 2019, **25**, 629.
- 123 H. N. Le, J. S. Quetz, V. G. Tran, V. T. M. Le, F. Aguiar-Alves, M. G. Pinheiro, L. Cheng, L. Yu, B. R. Sellman, C. K. Stover, A. DiGiandomenico and B. A. Diep, *Antimicrob. Agents Chemother.*, 2018, **62**, 1–12.
- 124 D. E. Tabor, V. Oganessian, A. E. Keller, L. Yu, R. E. McLaughlin, E. Song, P. Warrenner, K. Rosenthal, M. Esser, Y. Qi, A. Ruzin, C. K. Stover and A. DiGiandomenico, *J. Infect. Dis.*, 2018, **218**, 1983–1994.
- 125 R. Jain, V. V. Beckett, M. W. Konstan, F. J. Accurso, J. L. Burns, N. Mayer-Hamblett, C. Milla, D. R. VanDevanter and J. F. Chmiel, *J. Cystic Fibrosis*, 2018, **17**, 484–491.
- 126 P. Warrenner, R. Varkey, J. C. Bonnell, A. DiGiandomenico, M. Camara, K. Cook, L. Peng, J. Zha, P. Chowdury, B. Sellman and C. K. Stover, *Antimicrob. Agents Chemother.*, 2014, **58**, 4384–4391.
- 127 L. Bzdzion, H. Krezel, K. Wrzeszcz, I. Grzegorek, K. Nowinska, G. Chodaczek and W. Swietnicki, *Acta Biochim. Pol.*, 2017, **64**, 49–63.
- 128 K. J. Allen, D. Rogan, B. Brett Finlay, A. A. Potter and D. J. Asper, *Can. J. Vet. Res.*, 2011, **75**, 98–105.
- 129 Y. Wadamori, R. Gooneratne and M. A. Hussain, *J. Sci. Food Agric.*, 2017, **97**, 1396–1403.

- 130 K. Vande Walle, D. Vanrompay and E. Cox, *Vet. Immunol. Immunopathol.*, 2013, **152**, 109–120.
- 131 M. K. Nariya, J. Israeli, J. J. Shi and E. J. Deeds, *PLoS Comput. Biol.*, 2016, **12**, 1–14.
- 132 H. B. Felise, H. Nguyen, R. A. Pfuetzner, K. C. Barry, S. R. Jackson, M. Blanc, P. A. Bronstein, T. Kline and S. I. Miller, *Cell Host Microbe*, 2008, **4**, 325–336.
- 133 T. Kline, H. B. Felise, K. C. Barry, S. R. Jackson, H. V. Nguyen and S. I. Miller, *J. Med. Chem.*, 2008, **51**, 7065–7074.
- 134 M. Totsika, *Drug Delivery Lett.*, 2016, **6**, 30–37.
- 135 Y. Zhang, Y. Liu, J. Qiu, Z. Q. Luo and X. Deng, *Front. Microbiol.*, 2018, **9**, 149–154.
- 136 T. Morita-Ishihara, M. Ogawa, H. Sagara, M. Yoshida, E. Katayama and C. Sasakawa, *J. Biol. Chem.*, 2006, **281**, 599–607.
- 137 E. Soto, N. Espinosa, M. Díaz-Guerrero, M. Gaytán, J. Puente and B. González-Pedrajo, *J. Bacteriol.*, 2017, **199**, 1–19.
- 138 D. L. Jessen, D. S. Bradley and M. L. Nilles, *Antimicrob. Agents Chemother.*, 2014, **58**, 839–850.