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The Type III Secretion System of *Pseudomonas aeruginosa*: Infection by Injection

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Preface

The Gram-negative bacterium *Pseudomonas aeruginosa* utilizes a complex type III secretion apparatus to inject effector proteins into host cells. The configuration of this secretion machinery, the activities of the proteins that are injected by it, and the consequences of this process to infection are now being elucidated. This Review summarizes our current knowledge of *P. aeruginosa* type III secretion, including the secretion and translocation machinery, the regulation of this machinery, and the associated chaperones and effector proteins. The features of this interesting secretion system have important implications to the pathogenesis of *P. aeruginosa* infections and to other type III secretion systems.

Introduction

Pseudomonas aeruginosa is a major cause of health-care associated infections, including pneumonia and infections involving the urinary tract, wounds, burns, and the bloodstream ¹. A second population of patients prone to acquire *P. aeruginosa* infection is those with cystic fibrosis ². Like many other Gram negative bacteria, *P. aeruginosa* manipulates eukaryotic host cells by using a type III secretion system (T3SS). This T3SS forms complex needle-like machines on the bacterial surface that function in a highly regulated manner to transport proteins into host cells.

In this review, I will describe the T3SS of *P. aeruginosa*. Current knowledge of the components of this system will be discussed, with special emphasis on the effector proteins injected by this system. Recent studies in humans and progress in the development of therapeutic agents designed to inhibit the *P. aeruginosa* T3SS will be summarized.

The *P. aeruginosa* type III secretion system

To establish infections, *P. aeruginosa* employs a broad arsenal of virulence determinants ³, of which its T3SS has been the focus of much recent attention ⁴. Thirty-six genes encoded in five operons clustered together in the *P. aeruginosa* chromosome are involved in the biogenesis and regulation of the type III delivery machine. At least six other genes scattered around the chromosome encode effector proteins and their chaperones. This complex regulon can be divided into five parts (Fig. 1A): proteins that comprise the needle complex that transports substrates from the bacterial cytosol to the extracellular environment; proteins that translocate secreted proteins into host cells; proteins that regulate the secretion process; chaperone proteins that facilitate secretion of their cognate partners, and proteins that are injected into host cells, called effector proteins.

The needle complex

The needle complex is the supramolecular structure responsible for transport of specific type III proteins from the bacterial cytosol to the extracellular environment, moving them past the barriers of the bacterial cytoplasmic membrane, the peptidoglycan layer, and the outer membrane. The *P. aeruginosa* needle complex appears to be similar to the more thoroughly characterized needle complexes of *Yersinia* spp., *Salmonella enterica*, and *Shigella flexneri* and consists of two parts: a multi-ring base and a needle-like filament (Fig. 1A). The needle-like filament, which is comprised of subunits of the protein PscF^{5, 6}, is thought to be a conduit through which secreted factors move and to also serve as a sensor for host cell contact. These structures are 60–120 nm long and 6–10 nm wide (Fig. 1B), similar in dimensions to the needle-like filaments of *Shigella flexneri*, *Salmonella enterica*, and *Yersinia enterocolitica*^{7–10}. Relatively little work has been done to characterize the remaining components of the *P. aeruginosa* needle complex, although some functional assignments can be inferred from the closely related *Yersinia* type III needle complex (see Box 1). For example, PscN is thought to be an ATPase that powers the *P. aeruginosa* secretion system and is regulated by PscL^{11, 12}. PscC is a secretin-like protein that likely oligomerizes with the help of the lipoprotein PscW to form a channel through the bacterial outer membrane^{13, 14}. Based on studies of its *Yersinia* homolog, PscP is likely a molecular ruler that dictates the needle length¹⁵. PscJ is predicted to be a lipoprotein component of the basal substructure of the needle complex¹⁶. Although some progress has been made, considerable effort will be required to fully understand the complex mechanism by which the needle complex performs its role in the secretion process.

Box 1

Evolution of the *P. aeruginosa* type III secretion system

T3SSs share a common ancestor with flagellar systems¹⁶³. But whereas the phylogenies of the flagellar systems follow those of their source organisms, the clusters of genes encoding the T3SSs do not, indicating interspecies horizontal transfer¹⁶⁴. The *P. aeruginosa* T3SS belongs to the Ysc family of type III systems, which includes the Yop system of *Yersinia* spp., the Asc system of *Aeromonas salmonicida*, the Lsc system of *Photobacterium luminescens*, the Vcr system of *Vibrio parahaemolyticus*, the Bsc system of *Bordetella* spp., and the Dsc system of *Desulfovibrio vulgaris*¹⁶⁵.

The selective pressures driving the evolution of the *P. aeruginosa* T3SS are unclear. *P. aeruginosa* is an opportunistic pathogen in humans, meaning that it usually only infects individuals who are immunocompromised in some way¹. Thus most infections are “iatrogenic,” the consequence of procedures and treatments of modern health care. Even individuals with cystic fibrosis rarely lived long enough to acquire *P. aeruginosa* prior to the advent of modern antimicrobial therapies¹⁶⁶. For this reason, it is unlikely that the *P. aeruginosa* T3SS evolved as the result of pressures to survive within humans. What then is the natural target of the *P. aeruginosa* T3SS? *P. aeruginosa* is an environmental bacterium that must avoid or survive predation by the many organisms that inhabit the soil and natural bodies of water, and its T3SS may have evolved for this purpose. In support of this notion is that the majority of environmental *P. aeruginosa* isolates have functional T3SSs¹⁶⁷ and that ExoU, ExoS, ExoT, and ExoY each facilitate killing of environmental amoebae during co-cultivation^{168, 169}. Thus this system may have evolved to ward off environmental predators, and broad conservation of targeted substrates across eukaryotic organisms resulted in a system also active against human cells.

The translocation apparatus

The translocation apparatus is a proteinaceous membrane pore that accepts effector proteins secreted by the needle complex and delivers them across the host cell plasma membrane (Fig. 1A). The translocation process is quite efficient, with less than 0.1% of secreted effector proteins escaping to the extracellular milieu¹⁷. Like the T3SSs of *Yersinia*, *Salmonella* and *Shigella* spp., the T3SS of *P. aeruginosa* uses three proteins for translocation: PopB, PopD, and PcrV^{18–20}.

PopB and PopD are themselves secreted by the type III needle complex and, in a poorly understood manner, interact with each other and the host cell membrane to form the translocation pore 2.8–6.0 nm in diameter^{18–22}. Recombinant PopB and PopD were sufficient for the formation of oligomers and ring-like structures in lipid vesicles in vitro²¹, although PcrV was required for pore formation when these two proteins were injected through the needle complex into the plasma membranes of fibroblasts and red blood cells²³. PcrV is also secreted by the T3SS and is necessary for translocation but does not appear to be itself part of the pore^{23, 24}. It may instead form a multimeric scaffold at the tip of the type III secretion needle, as it has been shown to do when artificially expressed as part of the related *Yersinia enterocolitica* T3SS²⁵. This scaffold may then facilitate the assembly of the PopB/PopD translocation pore within the host cell plasma membrane. Alternatively, PcrV may link the needle complex to preformed PopB/PopD pores, forming part of a continuous conduit through which effector proteins move from the bacterial cytosol to the host cell cytosol²⁶.

The role of the translocation apparatus may not be limited to transport of effector proteins; the translocation pore by itself may be sufficient to cause the death of host cells^{20, 23, 27, 28}, either directly through pore-mediated increases in membrane permeability^{20, 23, 27, 29} or indirectly through the activation of broad cellular defense responses, as has been observed with the T3SSs of other bacterial pathogens³⁰. Death of the intoxicated cell in this context may be a protective mechanism of the host rather than a virulence mechanism of the bacterium. Details of such pathways are beginning to emerge. For example, a *P. aeruginosa* type III secretion/translocation apparatus (in the absence of any of the known effector proteins) is sufficient to trigger the activation of caspase-1 by the inflammasome via Ipaf, a Nod-like receptor (NLR) family member and cytosolic sensor of bacterial products^{31–34}. As has been shown with *Salmonella* type III and *Legionella* type IV secretion, the formation of the translocation pore may facilitate entry of flagellin into the cytosol, where it is recognized by Ipaf, although this remains controversial with *P. aeruginosa* type III secretion^{31–34}. Activation of the inflammasome and caspase-1 results in IL-1 β and IL-18 production and pyroptosis^{20, 32, 33}, a form of cell death that is proinflammatory and has features of both apoptosis and necrosis³⁵. Induction of pyroptosis may explain previous reports of T3SS-dependent but effector-independent death of phagocytes^{20, 23, 27, 28}. Since bacterial burdens were higher after 12 hr of intraperitoneal infection in Ipaf^{-/-} mice, inflammasome activation appears to be protective and may be an important aspect of the innate immune response against *P. aeruginosa*³².

Regulation of type III secretion

Type III secretion in *P. aeruginosa* is regulated at two levels: transcription of T3SS genes and initiation of secretion itself. These levels are linked in that transcription is induced upon activation of the secretion process,^{4, 36} which allows type III secretion components to be produced at high levels when most needed: following contact with host cells.

At its most proximal level, transcription of T3SS genes is controlled by ExsA, a member of the AraC/XylS family of transcriptional regulators. ExsA binds the promoters of T3SS genes, including its own promoter, in a region of DNA that extends through the putative -35 RNA

polymerase binding site and 18 nucleotides upstream^{37, 38}. This region includes an adenine-rich sequence at -55 that is sometimes referred to as an ExsA consensus element^{37, 38}. An elaborate “catch and release” mechanism involving three additional proteins, ExsC, ExsD, and ExsE, is employed to link ExsA-mediated transcriptional activation to secretion (Fig. 1C). Under conditions that prevent secretion, ExsA is bound by the “anti-activator” ExsD, which inhibits ExsA-dependent transcription³⁶. ExsC, an “anti-anti-activator,” has the potential to disrupt the ExsD-ExsA interaction by directly binding and sequestering ExsD³⁹ but under non-secreting conditions is prevented from doing so by its higher binding affinity for ExsE^{40, 41}. The coupling of transcription with secretion is achieved by the export ExsE through the type III needle complex into either the medium or the cytosol of host cells during secretion⁴². Thus under conditions that promote secretion, ExsE levels within the bacterium are depleted. The relative paucity of ExsE frees ExsC to bind ExsD, allowing unimpeded transcriptional activation of type III secretion genes by ExsA. The net result of this regulatory cascade is that production of T3SS proteins, including needle complexes and effector proteins, is maintained at low basal levels until contact with host cells activates secretion, which rapidly increases expression of T3SS genes.

Two global regulatory systems, both of which respond to poorly defined environmental stimuli, are known to work through ExsA to regulate transcription of T3SS genes: intracellular 3',5'-cyclic AMP (cAMP) and the Gac regulatory system. In the cAMP pathway, the inner membrane protein adenylate cyclase CyaB responds to inducing signals by increasing intracellular cAMP levels⁴³. cAMP likely allosterically regulates Vfr, a global regulatory protein with homology to the *Escherichia coli* cAMP receptor protein (CRP)⁴³. cAMP and Vfr, together with ExsA, regulate expression from T3SS promoters in a poorly understood manner. In the Gac system, the two-component sensors RetS and LadS transduce environmental signals to modulate the two-component GacA/GacS system, which in turn regulates expression of T3SS genes. RetS primes *P. aeruginosa* for acute infections by inducing T3SS genes and normal piliation but repressing genes that promote biofilm growth, whereas LadS acts in a reciprocal manner, down-regulating type III secretion during chronic infections (Box 2)^{44–47}. The reader is referred to a recent review for a more detailed discussion of transcriptional regulation of the *P. aeruginosa* T3SS⁴⁸.

Box 2

P. aeruginosa type III secretion in chronic infections

Whereas *P. aeruginosa* type III secretion plays a dominant role in acute infections, emerging evidence suggests that it may actually be selected against in chronic infections, such as those afflicting individuals with CF. Many CF patients have antibodies against type III effector proteins¹⁷⁰, suggesting that these factors are expressed at some point during infection, but *P. aeruginosa* strains gradually lose the ability to secrete type III proteins over time in the CF airways^{27, 151, 171}. This phenomenon may occur through a number of different mechanisms. *P. aeruginosa* isolates from chronically infected CF patients often have a mucoid phenotype that results from overexpression of the exopolysaccharide alginate. In these strains, alginate is often overproduced because of mutations within the *mucA* gene¹⁷², which also result in repression of type III secretion genes¹⁷³. A second mechanism is the RetS/LadS/GacA/GacS regulatory system (described in the “Regulation of type III secretion” section), which allows *P. aeruginosa* to switch from an acute to a chronic infection mode. Finally over a period of years in the CF airways, mutations accumulate in genes essential for type III secretion, such as *cyaB*, *vfr*, and *exsA*¹⁷⁴. Thus multiple mechanisms cause the attenuation of type III secretion during CF infections, underscoring the strength of the selection against this system. It may be that secretion of such destructive

toxins is not compatible with bacterial persistence in the airways for years or that T3SS proteins constitute attractive targets for the host immune system.

Triggering of secretion is complex and less well understood, but the physiological stimulus is contact with host cells while the most commonly utilized laboratory signal is exposure to calcium-depleted medium^{49, 50}. Low calcium concentrations alone are insufficient to trigger secretion but must be accompanied by the presence of certain amino acids, such as glutamate⁵¹. This is likely due to a role played by the citric acid cycle in type III secretion. For example, mutations in the genes encoding citrate synthetases, pyruvate dehydrogenase, and GltR, the regulator of the glucose transporter, all prevented induction of type III secretion^{43, 51, 52}. In fact, metabolic stress in general modulated the ability of *P. aeruginosa* to secrete type III proteins⁵³. Metabolically unfavorable conditions may reduce overall secretion by decreasing the percentage of bacterial cells able to assemble functional secretion machines on their surface⁵¹.

Chaperones

Some but not all type-III-secreted proteins have cognate chaperones to which they bind prior to secretion. These chaperones may facilitate storage of their cognate protein partner in the bacterial cytosol and their appropriate delivery to the secretion apparatus⁵⁴. SpsS (formerly called Orf1) serves as a chaperone for both ExoS and ExoT and is required for maximal secretion of these proteins (Fig. 1A, Table 1)^{55, 56}. Likewise SpsU is a chaperone for ExoU⁵⁷. No chaperone has yet been identified for ExoY. Chaperones also exist for type-III-secreted proteins other than effector proteins. For example, PcrH is a chaperone for the translocation proteins PopB and PopD²¹, PscE and PscG are chaperones for PscF, the structural subunit of the needle complex⁵⁸, and ExsC is a chaperone for the regulatory protein ExsE^{40, 41}.

The Effectors

Despite extensive characterization by a number of laboratories, only four effector proteins of the *P. aeruginosa* T3SS have been identified: ExoS, ExoT, ExoU, and ExoY (Table 1). Thus the *P. aeruginosa* T3SS appears to have fewer effector proteins than all other well characterized T3SSs. For example, the *Yersinia* Yop T3SS has 6 effector proteins, the *Salmonella* SPI1 system ≥ 13 , the *Salmonella* SPI2 system ≥ 10 , and the *Shigella* system ≈ 25 . This relative dearth of effector proteins is especially surprising given the wide spectrum of hosts and environments with which *P. aeruginosa* is associated but may reflect the large number of other types of secretion systems encoded by this bacterium. Alternatively, the small number of effector proteins may have been evolutionarily balanced by their activity against an extremely broad range of eukaryotic organisms.

While virtually all *P. aeruginosa* strains harbor the genes encoding the type III secretion machinery, most strains do not carry a complete set of effector-encoding genes^{59, 60}. In isolates from acute infections, the *exoS* gene is found in 58–72% of isolates, the *exoU* gene in 28–42%, the *exoY* gene in 89%, and the *exoT* gene in 92–100%. For reasons that are not entirely clear, nearly all strains have either the *exoS* or the *exoU* gene but not both. As described below, the secreted effector proteins define the phenotype of a strain during infection^{60, 61}. For example, ExoS-secreting strains cause delayed cell death with features of apoptosis, whereas ExoU secretion is associated with rapid and robust host cell lysis.

ExoS

ExoS is a bi-functional toxin with both GTPase activating protein (GAP) activity and adenosine diphosphate ribosyl transferase (ADPRT) activity (Table 1). This effector protein has a

modular domain structure that underscores the complexity of its interactions with host cells (Fig. 2).

As with other type III effector proteins, the extreme amino-terminus of ExoS carries the information for targeting to the type III secretion apparatus. Although no consensus sequence has been identified, the first 15 amino acids of ExoS presumably encode the information necessary for secretion through the type III apparatus⁴. Residues 15–51 are believed to comprise a binding site for the ExoS chaperone SpsS⁶².

Residues 51–72 contain a symmetrical leucine-rich motif that forms the membrane localization domain (MLD)⁶³, which is responsible for the initial transient localization of ExoS to the plasma membrane of the host cell (Fig. 3)⁶⁴. Subsequent trafficking to the Golgi and endoplasmic reticulum in the perinuclear region of the cell occurs in association with markers for Rab5-containing early endosomes and Rab6- and Rab9-containing late endosomes^{64, 65}. Of note, localization of ExoS by the MLD to these membrane compartments is essential for efficient modification of its substrates^{66, 67}.

Residues 96–233 of ExoS form a GAP domain that targets Rho, Rac, and Cdc42^{68, 69}, small GTPases that maintain the organization of the actin cytoskeleton. These regulatory GTPases normally switch between an active GTP-bound form and an inactive GDP-bound form, but the ExoS GAP domain biases the switch towards the ADP-bound inactive form, leading to disruption of the actin cytoskeleton^{68, 69}. This domain has functional and structural similarity with other type III effector proteins such as YopE of *Yersinia* spp. and StpP of *Salmonella enterica*^{70, 71}. The three-dimensional structure of the ExoS GAP domain bound to Rac1 indicates that ExoS uses an arginine finger (Arg146) to stabilize the Rac1 transition state and maintain intrinsic GTPase activity⁷². This in turn results in GTP hydrolysis to GDP, causing the GDP-bound form of Rac1 to predominate^{69, 73} and leading to inactivation of Rac signaling⁷⁴. The transient disruption of the actin cytoskeleton by the GAP activity of ExoS is associated with cell rounding and decreased internalization of *P. aeruginosa* by certain cell types, suggesting a role in preventing phagocytosis^{75–77}.

The ADPRT domain of ExoS resides between residues 233 and 453⁷⁸. Residues 418 to 429 comprise a binding site for a eukaryotic cofactor that is necessary for activation of ADPRT activity (but not GAP activity⁷⁹)⁸⁰. This cofactor was initially designated Factor Activating ExoS (FAS) but was later shown to be a 14-3-3 protein^{81, 82}. These proteins serve as regulatory factors that modulate a wide variety of cellular functions, including cell cycle progression, protein trafficking, and apoptosis. Similar to other 14-3-3 ligands, ExoS binds within a conserved amphipathic groove of 14-3-3 but does so in the opposite orientation of most ligands and utilizes unique intermolecular interactions⁸³. Deletion of the 14-3-3 binding site renders ExoS unable to interact with its cofactor and results in the loss of ADPRT activity and its consequences to mammalian cells⁸³. Thus the requirement for a host cell cofactor prevents this potent weapon from being turned against *P. aeruginosa* itself by precluding activation until after ExoS is injected into the host cell.

Following injection and activation, the ADPRT activity of ExoS has a number of adverse effects on the host cell, including cell death, actin cytoskeletal disruption associated with cell rounding, and inhibition of DNA synthesis, vesicular trafficking and endocytosis^{74, 84–86}. The irreversible disruption of the cytoskeletal structure may contribute to a reduction in cell-cell adherence, which in turn may facilitate *P. aeruginosa* penetration through epithelial barriers^{74, 84, 86}. Cell death has features of apoptosis although loss of cell membrane integrity, more characteristic of necrosis, is also observed^{84, 87}. It has been postulated that the killing of host immune cells by ExoS may allow *P. aeruginosa* to persist in the presence of a vigorous host immune response, although the cell types targeted by ExoS *in vivo* are unclear. Alternatively,

this form of cell death may be similar to pyroptosis and may be the culmination of a host cell response to the presence of ExoS aimed at eliminating intoxicated cells.

Defining the substrates modified by ExoS that lead to these pathogenic changes has been the focus of much effort and has been complicated by the promiscuous nature of the ADPRT activity of this toxin. It catalyzes the transfer of ADP-ribose to many different host proteins (Table 1)^{85, 88–97}, and the challenge has been to link ADP-ribosylation of specific substrates to the observed host cell phenotypes. Two substrates of special interest are Ras and the ezrin, radixin and moesin (ERM) family of proteins. Since Ras signaling affects cellular proliferation, survival, and cytoskeletal structure, this small GTPase has been an attractive candidate for the pathogenically relevant target of ExoS. Modification of Arg41 of Ras results in inhibition of GDP to GTP exchange normally catalyzed by the guanine nucleotide exchange factor (GEF) of Ras⁹⁸. This in turn disrupts Ras signaling by preventing association of Ras with its downstream effector, Raf-1^{99–101}. Over-expression of constitutively active Ras or the protein kinase B/Akt, a downstream component of the Ras signaling pathway, prevented ExoS-mediated cell killing, indicating that ExoS modification of Ras is biologically important¹⁰². ExoS lacking the MLD localized within the cytosol and failed to appropriately ADP-ribosylate membrane-associated Ras, demonstrating the importance of ExoS localization within cells (Fig. 3)¹⁰³. The ERM family of proteins⁹⁶ bind actin and play an important role in many actin processes, including motility, phagocytosis, adhesion, and the maintenance of cell shape. ADP-ribosylation of ERM proteins prevents their phosphorylation, resulting in inactivation¹⁰⁴. In support of the biological relevance of this modification, dominant active-moesin prevented the cell rounding normally associated with ExoS intoxication¹⁰⁴. Thus the ADPRT activity of ExoS appears to target a number of distinct cell factors and pathways to compromise the host cell.

ExoT

ExoT shares 76% amino acid identity with ExoS, and is also a bifunctional toxin with amino-terminal GAP activity and carboxy-terminal ADPRT activity (Table 1). Although not as thoroughly studied, the domain structure of ExoT appears to be similar to that of ExoS in that residues 1–50 likely encode secretion and chaperone binding information and residues 51–72 are thought to encode a MLD (Fig. 2)⁶².

Similar to ExoS, ExoT residues 78–235 contain GAP activity towards Rac, Rho, and Cdc42^{105, 106}. This activity appears to be very similar to that of the N-terminus of ExoS and causes reversible disruption of the actin cytoskeleton, which manifests as cell rounding, cell detachment, and inhibition of cell migration and phagocytosis^{76, 77, 106}. The GAP activity of ExoT also contributes to inhibition of cytokinesis through inactivation of Rho^{107, 108}.

Also similar to ExoS, residues 235–457 of ExoT encode an ADPRT domain that requires binding of the host cell cofactor 14-3-3 for activation¹⁰⁹. However unlike ExoS, ExoT ADP-ribosylates a distinct and limited number of host proteins (Table 1). In particular, ExoT modifies CT10-regulator of kinase (Crk)-I and Crk-II, adaptor proteins¹¹⁰. Hybrid protein experiments and structural modeling indicated that one small α -helix region (“region A”) present in ExoT but absent in ExoS conferred specificity for Crk¹¹¹. ExoT ADP-ribosylates Crk-I at Arg20, which interferes with Crk signaling by preventing association of Crk-I with focal adhesion proteins p130^{cas} and paxillin. This has multiple effects on cells, including disruption of signaling to Rac1 and blockage of cell division at the stage of cytokinesis^{107, 108}. Thus the GAP and ADPRT activities of ExoT work together to alter the actin cytoskeleton and to inhibit cell migration, adhesion, and proliferation. The net effect of these activities is to block phagocytosis and disrupt epithelial barriers to facilitate bacterial dissemination^{27, 28, 76, 112}. In addition, the enzymatic activities of ExoT have been linked to delays in wound healing^{113, 114}, which may augment the opportunistic capacity of *P. aeruginosa* by allowing

it to more fully exploit breaches in mucosal barriers. Although initially reported to have no effect on cell viability, ExoT does cause apoptosis-like cell death predominantly through its ADPRT activity at later time points (10 hours, compared to 2–5 hours for ExoS-mediated killing)²⁹. Apoptosis may be a consequence of inhibition of Crk signaling, which has been linked to this form of cell death¹¹⁵.

Multiple studies have demonstrated a pathogenic role for ExoT, but its contribution to disease in mammalian models has been modest relative to ExoU and ExoS^{27, 28, 76, 112}. ExoT forms a complex with its substrate Crk and a Crk ligand, the E3 ubiquitin ligase Cbl-b¹¹⁶, leading to polyubiquitination of ExoT by Cbl-b and subsequent proteasomal degradation. Interestingly, Cbl-b deficient mice were much more susceptible to infection with an ExoT⁺ *P. aeruginosa* strain than an ExoT⁻ *P. aeruginosa* strain, although wild-type mice differed only slightly in this regard¹¹⁶. Thus Cbl-b functions as a host-defense molecule by facilitating the degradation of ExoT.

ExoU

ExoU is a potent phospholipase capable of causing rapid cell death in eukaryotic cells (Table 1). Although not yet demonstrated, the secretion signal directing ExoU to the type III secretion apparatus is assumed to be within the first 15 residues, based on findings from other effector proteins (Fig. 2). A binding domain for the chaperone of ExoU, SpcU, lies within residues 3 through 123⁵⁷.

Immediately adjacent to the chaperone binding site, between residues 107 and 357, is a patatin-like domain that encodes phospholipase A₂ (PLA₂) activity^{117, 118}. Patatin-like domains are found in several other well-characterized PLA₂ enzymes, such as human calcium-independent PLA₂ (iPLA₂) and human cytosolic phospholipase A₂ (cPLA₂)¹¹⁹. PLA₂ enzymes hydrolyse the ester bond of the acyl group at the *sn*-2 position of phospholipids, resulting in release of free fatty acids and lysophospholipids. In actuality, the lipase activity of ExoU has a broad range of substrates, including phospholipids, lysophospholipids, and neutral lipids^{117, 118, 120}. Sequence alignment with well characterized PLA₂ enzymes and mutational analysis suggest that ExoU has a serine-aspartate catalytic dyad involving Ser142 and Asp344^{117, 118, 121}. Subsequent to the identification of the patatin-like domain in ExoU, similar domains have been identified in a large number of bacterial proteins¹²².

Like other *P. aeruginosa* type III effector proteins, ExoU requires a eukaryotic cofactor for activation^{117, 118}. Fractionation of eukaryotic cell extracts yielded Cu²⁺, Zn²⁺-superoxide dismutase (SOD1) as one such factor¹²³. Interestingly SOD1 enzymatic activity was not necessary for this effect, suggesting that SOD1 activated ExoU by other mechanisms such as inducing a conformational change or linking ExoU to other factors or its phospholipid substrate. The domain of ExoU that binds SOD1 has not yet been defined.

The C-terminal half of ExoU contains no recognizable homology or motifs but is essential for killing^{117, 121, 124, 125}. A portion of this part of ExoU, residues 550–683, is now known to contain a MLD that functions to target ExoU to the plasma membrane of the host cell (Fig. 3)^{124, 126}. The remaining portion of the C-terminal half of ExoU (residues 358 to 549) may also play an important role in the mechanism of this toxin, since 5-amino-acid insertions in this region also destroyed cytotoxicity¹²¹.

Another interesting aspect of ExoU is that it is ubiquitinated. Once inside the host cell, two ubiquitin molecules are added to lysine 178 of this effector protein. Although ubiquitination had a modest effect on ExoU turnover within cells, it did not appreciably alter toxicity or localization¹²⁴. Thus the significance of this modification to the pathogenicity of ExoU remains unclear.

The net result of intoxication with ExoU is death of the host cell. Unlike the cell killing caused by ExoS and ExoT, death is characterized by rapid (within 1–2 hours) loss of integrity of the plasma membrane, consistent with necrosis^{127, 128}. ExoU killing may be directed against phagocytes as well as epithelial barriers, thus promoting bacterial persistence and dissemination^{27, 112, 129–131}.

ExoY

ExoY is a secreted adenylate cyclase (Table 1)¹³². Two domains of ExoY are similar to corresponding domains of the extracellular adenylate cyclases of *Bordetella pertussis* (CyaA) and *Bacillus anthracis* (Edema factor)¹³² and act together to bind ATP (Fig. 2). ExoY also requires a host cell cofactor for full enzymatic activity, although the identity of this factor is not known¹³². Injection of ExoY into mammalian cells results in an elevation of intracellular cAMP concentration and differential expression of multiple genes, including many known to be regulated by cAMP^{132, 133}. This leads to disruption of the actin cytoskeleton^{132, 134}, inhibition of bacterial uptake by host cells¹³⁵, and increased endothelial permeability¹³⁶. Such activities would be predicted to lead to more severe disease, but this has not been uniformly observed^{27, 28}, and the significance of ExoY in infection remains unclear.

P. aeruginosa type III secretion and disease

Although not required for infection^{61, 137}, the *P. aeruginosa* T3SS enhances disease severity in a number of animal models, including acute pneumonia^{27, 127, 128, 138}, keratitis¹³⁹, bacteremia²⁸, peritonitis¹¹⁶, burn infections¹⁴⁰, and gut-derived sepsis in the setting of neutropenia¹⁴¹. The exact role of type III secretion in the pathogenesis of these infections is less clear, but a model is beginning to emerge. This discussion will focus on acute pneumonia, since the largest amount of data is available for this model system. Following inoculation into the airways, the first lines of defense faced by *P. aeruginosa* are the bronchial and bronchiolar epithelia. Intact polarized epithelia are relatively resistant to *P. aeruginosa* adherence and invasion, which may explain the opportunistic nature of this bacterium. In contrast, wounded or disrupted epithelia are quite susceptible to *P. aeruginosa* binding and injection of type III proteins^{142–144}. Thus *P. aeruginosa* may exploit naturally occurring breaches in epithelial barriers or those caused by trauma from endotracheal tubes to gain a foothold in the lungs. The antagonistic effects of ExoT towards wound healing may facilitate the ability of the bacterium to take advantage of these vulnerabilities. Upon contact with sentinel alveolar macrophages, ExoS and ExoU may delay or skew the early phases of the inflammatory response by blocking the inflammasome-mediated production of IL-1 β and IL-18^{32, 33}. Eventually, however, a brisk inflammatory response is induced^{130, 131}. ExoU, which is homologous to cPLA₂, may stoke the inflammatory furnace by generating large amounts of arachidonic acid that in turn leads to increased production of pro-inflammatory eicosanoids¹⁴⁵. The ensuing recruitment of excessive numbers of neutrophils and macrophages to the lungs causes collateral damage to host tissues¹⁴⁵ but fails to eradicate the bacteria, perhaps because these phagocytic cells are killed or otherwise impaired by ExoS, ExoT, and ExoU upon contact with *P. aeruginosa* bacteria^{19, 27, 28, 130, 146}. The neutralization of phagocytes also causes a localized immunosuppression, predisposing to superinfection by other pathogens, as has been observed experimentally and clinically^{130, 147}. Failure to eradicate *P. aeruginosa* from the lungs allows subsequently secreted type III effector proteins and other toxins to injure pulmonary tissues, which results in the signs and symptoms of pneumonia. Lung compliance is adversely affected by decreased synthesis of the pulmonary surfactant phosphatidylcholine and an influx of protein-rich fluid into the alveoli following disruption of epithelial and endothelial barriers within the lungs by ExoS, ExoT, and ExoU^{146, 148–150}. Loss of integrity of these barriers also allows bacterial dissemination into the bloodstream^{27, 112, 128, 131} and the leakage of proinflammatory cytokines such as TNF- α from the lungs into the systemic circulation, leading

to reductions in mean arterial pressure, cardiac output, and base excess, all indicators of septic shock¹³¹. The net result is increased mortality associated with infection by T3SS⁺ strains^{27, 61, 112, 146, 151}.

Human studies and therapeutic interventions

In addition to the abundance of evidence in animal models supporting a role for type III secretion in disease, several studies directly link this virulence system to worse outcomes in human infections. Culture of *P. aeruginosa* isolates with functional T3SSs has been linked to increased mortality, persistence, relapse, and higher bacterial burdens in acutely colonized or infected patients^{137, 151–153}. Given its important role in pathogenesis, this system is an attractive target for therapeutic intervention. Much effort has been directed towards PcrV, which is accessible at the tip of the needle complex²⁵ and required for translocation of effector proteins^{23, 24}. Active or passive immunization using PcrV is protective in animal models of infection^{19, 154}, apparently by direct blockade of injection of type III proteins into host cells¹⁹. Engineered human anti-PcrV Fab fragments have now been generated and should facilitate the clinical use of this approach¹⁵⁵. Phase I/II clinical studies are ongoing to evaluate the usefulness of PcrV-specific antibodies in the settings of mechanically ventilated patients and individuals with cystic fibrosis (http://www.kalobios.com/kb_pipeline.php).

By residing within cells both before and after secretion, effector proteins remain sheltered from a humoral immune response, and immunization with these proteins is at best minimally effective¹⁹. For this reason, cell-permeable small molecule inhibitors have been sought to neutralize ExoU and ExoS. Inhibitors of the PLA₂ activity of ExoU and the ADPRT activity of ExoS have been identified and shown to provide protection in models of infection^{156, 157}. Likewise inhibitors of ExsA, the activator necessary to express type III secretion genes, are currently under development¹⁵⁸. Targeting patients most likely to benefit from such therapies will require rapid and accurate identification of the effector proteins secreted by *P. aeruginosa* isolates. For this purpose, several assays capable of rapidly determining the genetic and phenotypic repertoire of effector proteins of *P. aeruginosa* clinical isolates have been devised^{159–161}. Thus in a relatively short period of time, remarkable progress has been made in developing interventions to protect patients from the toxic effects of *P. aeruginosa* type III secretion.

Concluding remarks

The T3SS is a powerful weapon wielded by *P. aeruginosa* to enhance the pathogenic process. A complex array of environmental signals are integrated to tightly control the injection of ExoS, ExoT, ExoU, and ExoY into host cells, where they are targeted to specific compartments, are activated by host cell cofactors, and manipulate a number of host cell processes. The net result is impairment of the innate immune response and compromise of tissue barriers leading to bacterial dissemination and sepsis.

Despite rapid progress, substantial gaps remain in our understanding of the *P. aeruginosa* T3SS, and these are the focus of ongoing efforts. Do additional effector proteins exist? Given the extensive variation between *P. aeruginosa* strains¹⁶², interrogation of a larger number of isolates may be necessary to uncover such proteins or at least exclude their existence. What are the additional regulatory systems that govern type III secretion and how are these systems integrated at the molecular level? What are the cell types targeted for type III injection in vivo, what are the consequences of injection to these cells, and how are these effects orchestrated to bring about enhanced disease severity? Active investigation of these and many other remaining questions promises to reveal novel aspects of bacterial pathogenesis in the years to come.

Online summary

- The *Pseudomonas aeruginosa* type III secretion system consists of five functional parts: (i) proteins that comprise the secretion machine itself, which shall be referred to as the needle complex; (ii) proteins that translocate secreted proteins into host cells; (iii) proteins that regulate the secretion process; (iv) proteins that bind secreted proteins to facilitate the secretion process, called chaperone proteins, and (v) proteins that are actually injected into host cells, called effector proteins.
- Only four effector proteins are known to be secreted by the *P. aeruginosa* type III secretion system: ExoS, ExoT, ExoU, and ExoY.
- ExoS is a bifunctional toxin with GTPase activating protein and ADP-ribosyltransferase activities. These activities lead to disruption of the actin cytoskeleton and apoptosis-like cell death.
- ExoT is 76% identical to ExoS and also has both GTPase activating protein and ADP-ribosyltransferase activities. However the ADP-ribosyltransferase activity of ExoT is directed against different substrates than that of ExoS. These activities lead to disruption of the actin cytoskeleton and apoptosis-like cell death.
- ExoU has phospholipase A₂ activity that results in rapid lysis of many mammalian cell types.
- ExoY is an adenylate cyclase. Its role in pathogenesis is less clear.

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Glossary

Inflammasome

a multiprotein cytoplasmic complex that mediates the activation of caspase-1 to promote the maturation of IL 1- β and IL-18. Activation may also lead to a form of cell death termed “pyroptosis.” The inflammasome itself may be activated by NOD-like receptors in response to intracellular microbial triggers

Flagellin

The protein monomer that forms the hollow filament of the bacterial flagellum

AraC/XylS transcriptional regulators

A large family of transcriptional activators defined by a 100-amino-acid region of sequence similarity. These proteins regulate diverse bacterial functions including catabolism, stress response, and virulence

cAMP receptor protein

A regulatory protein that, in the presence of cAMP, binds tightly to a specific DNA sequence in the promoters of a subset of bacterial genes, modulating their transcription. Also known as catabolite gene activator protein (CAP)

Adenylate cyclase

An enzyme that catalyzes the conversion of ATP to cAMP and pyrophosphate

Two-component signal transduction systems

Signal transducing proteins that are commonly used by bacteria to couple environmental stimuli to response mechanisms. They consist of sensor and response regulator components that are linked by phosphorelays

GTPase activating protein

A member of a family of regulatory proteins that stimulate the intrinsic GTPase activity of small signaling G proteins, which results in their inactivation

ADP ribosyltransferase activity

The enzymatic activity that covalently transfers an ADP-ribose group from nicotinamide adenine dinucleotide (NAD⁺) to a substrate protein, usually altering the activity of the substrate

Rab proteins

A family of GTPases that regulate many of the steps in membrane trafficking

E3 ubiquitin ligase

An enzyme that covalently attaches ubiquitin to lysines in substrate proteins. Single or multiple ubiquitin molecules may be attached to a single lysine and may target the substrate protein for degradation or altered cellular location or function

Lysophospholipids

Any phospholipid that is missing one of its two acyl chains

SOD1

A super oxide dismutase that binds copper and zinc ions and converts harmful free superoxide radicals to molecular oxygen and hydrogen peroxide

Arachidonic acid

A fatty acid that is freed from certain phospholipids by the catalytic activity of PLA₂ enzymes. Arachidonic acid is an important inflammatory intermediate that is converted into eicosanoids

Biography

Alan R. Hauser is an Associate Professor in the Departments of Microbiology/Immunology and Medicine at Northwestern University in Chicago, Illinois USA. He received his PhD from the University of Minnesota for studies on the pathogenesis of group A streptococcal infections. He did his postdoctoral studies at the University of California, San Francisco, with Joanne Engel on type III secretion in *Pseudomonas aeruginosa*. He continues to work on type III secretion as well as genetic determinants for strain-to-strain differences in virulence among *P. aeruginosa* isolates.

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Figure 1C

Figure 1. Overview of *P. aeruginosa* type III secretion

(A) The T3SS may be functionally divided into five components: the needle complex; the translocation apparatus, regulatory proteins, effector proteins, and chaperones. These five parts work together to inject effector proteins into host cells in a highly regulated manner. (C) Linkage of T3SS transcription to protein secretion is achieved through the interactions of four proteins: ExsA, ExsC, ExsD, and ExsE. When secretion is turned off, ExsE accumulates within the bacterium and binds ExsC, allowing ExsD to bind ExsA, and thus preventing transcription of type III secretion genes. When secretion is activated, the regulatory protein ExsE is secreted from the cell, allowing ExsC to bind ExsD. Sequestration of ExsD frees the transcriptional activator ExsA, resulting in unimpeded transcription of type III secretion genes. RNAP, RNA polymerase

Figure 2. Modular domains of ExoS, ExoT, ExoU, and ExoY

ExoS is a 453 amino acid bi-functional toxin that has both GAP and ADPRT activity. Arg146 is required for GAP activity and both Glu379 and Glu381 are required for efficient catalytic addition of the ADP-ribose moiety of NAD⁺ to substrate¹⁷⁵. Within the ADPRT domain, residues 418 to 429 comprise a binding site for the eukaryotic cofactor 14-3-3 that is necessary for activation of the ADPRT activity of ExoS. ExoT is a 457 amino acid protein that is closely related to ExoS. Arg149 is required for the GAP activity of ExoT⁷⁶ and residues Glu383 and Glu385 are crucial for its ADPRT activity¹⁷⁵. Residues 422–433 are thought to comprise the site of cofactor 14-3-3 binding. ExoU is a 687 amino acid protein that contains a patatin-like domain necessary for PLA₂ activity. Residues Ser142 and Asp344 are required for this activity^{117, 118, 121}. ExoY is a 378 amino acid adenylate cyclase. Residues Lys81, Lys88, Asp212, and Asp214 are required for its activity and are thought to be necessary for interactions between ExoY and ATP. See text for additional details. S, secretion signal; CBD, chaperone binding site; MLD, membrane localization domain; GAP, GTPase activating protein activity; ADPRT, ADP-ribosyl transferase activity; PLA₂, phospholipase A₂; CF, cofactor binding site. (Adapted from¹⁷⁶)

Figure 3. Localization of ExoS and ExoU following injection into mammalian cells

The N-terminal MLD of ExoS (shown in red) initially targets this effector protein to the plasma membrane and subsequently to early and late endosomes and finally to the endoplasmic reticulum (ER) and Golgi. Membrane localization is necessary for efficient ADP ribosylation of membrane-associated Ras. Following translocation into mammalian cells, ExoU is targeted to the plasma membrane by a C-terminal MLD. At this location, the enzymatic activity of ExoU is thought to cleave phospholipids within the plasma membrane. (Adapted from ⁶⁵)

Figure 4. Role of type III effector proteins in the pathogenesis of acute pneumonia

In early infection, *P. aeruginosa* exploits breaches in the epithelial mucosa, which is facilitated by ExoT-mediated inhibition of wound healing. ExoU and ExoS block IL-1 β and IL-18 production by alveolar macrophages, blunting or biasing the early inflammatory response. When subsequent development of an inflammatory response does occur, it is excessively amplified by ExoU-induced eicosanoid release and causes collateral damage to host tissues. However the recruited phagocytes are unable to eradicate *P. aeruginosa* because they are killed or impaired by ExoS, ExoT, and ExoU; the resulting paucity of functional phagocytes makes the lungs prone to superinfection by other bacteria. ExoS, ExoT, and ExoU also disrupt epithelial/endothelial barriers, allowing bacteria and proinflammatory cytokines to escape to the bloodstream, leading to bacteremia and septic shock, respectively. These same breaches allow protein-rich fluid to flow into the air spaces of the lung, which together with ExoU-mediated decreases in pulmonary surfactant, causes decreased lung compliance and oxygenation. AM=alveolar macrophage

Table 1Characteristics of *P. aeruginosa* T3SS effector proteins.

Effector protein	Size (kDa)	Chaperone	Substrates	Cofactor for activation
ExoS	49	SpcS	GAP activity: Rho, Rac, and Cdc42 ADPRT activity: ezrin, radixin, moesin, vimentin, cyclophilin A, IgG ₃ , apolipoprotein A1, Ras, Rac1, Cdc42, Rabs 1, 3, 5, 7, 8, and 11, RalA, Rap1, Rap2	14-3-3 proteins
ExoT	53	SpcS	GAP activity: Rho, Rac, and Cdc42 ADPRT activity: Crk-I, Crk-II, phosphoglycerate kinase	14-3-3 proteins
ExoU	74	SpcU	phospholipids, lysophospholipids, neutral lipids	SOD1
ExoY	41	?	ATP	?

GAP: GTPase activating protein

ADPRT: adenosine diphosphate ribosyl transferase

Crk: CT10-regulator of kinase

SOD1: Cu²⁺, Zn²⁺-superoxide dismutase

?: unknown

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