Role of type III secretory system and related exotoxins in infections induced by an opportunistic pathogen *Pseudomonas aeruginosa*

E. Kiseleva[1] and **G. Novik**[2]

[1] The Institute of Bioorganic chemistry, National Academy of Sciences of Belarus, Acad. Kuprevicha 5/2, 220141 Minsk, Republic of Belarus
[2] The Institute of Microbiology, National Academy of Sciences of Belarus, Acad. Kuprevicha 2, 220141 Minsk, Republic of Belarus

The T3SS is a needle-like nanomachine that injects virulence proteins (exotoxins) directly into eukaryotic cells to initiate infection. Fore effector proteins of *P. aeruginosa*, ExoS, Exo T, Exo U, and ExoY, modulate a variety of cellular functions for the pathogen's benefit via their enzymatic activity. *P. aeruginosa* is invasive or cytotoxic to host cells, depending on the T3SS effectors encoded. We pay special attention to data related to biochemical activities and cellular targets of T3SS effectors, and known molecular mechanisms leading to cytotoxicity and blocking of phagocytosis, two most proven consequences of enzymatic activity of fore exoenzymes.

Keywords: *Pseudomonas aeruginosa*; T3SS, ExoS, ExoT, ExoU, ExoY, virulence factors

1. Epidemiology and pathogenesis of *Pseudomonas aeruginosa* infection

1.1 Pseudomonades are opportunistic pathogens in debilitated patients

Members of the genus *Pseudomonas* are found in soil, decaying organic matter, vegetation and water but are uncommon in humans as part of the normal microbiota. Though healthy people can carry pseudomonades without having any problems, the bacteria are recognized as serious opportunistic pathogens in immunocompromised patients, *i. e.* persons having an immune system that has been impaired by evoked diseases (e.g. AIDS [1]), inherited diseases that affect the immune system [2] or medical treatment [3, 4].

Infection resulting from *Pseudomonas aeruginosa* is the most common. Other pseudomonades*, viz., Pseudomonas putida* [5, 6], *Pseudomonas fluorescens* [7, 8], *Pseudomonas stutzeri* [9, 10] *Sphingomonas paucimobilis* (formerly known as *Pseudomonas paucimobilis*) [11, 12], and *Delftia acidovorans* (formerly known as *Pseudomonas acidovorans*) [13, 14] are rare in humans.

P. aeruginosa is the most common cause of respiratory failure in cystic fibrosis (CF) patients. CF is genetic disease caused by mutations in the CF transmembrane conductance regulator (CFTR) gene [15], which encodes a protein whose primary function is regulation of ions conductance in and out of cells. The lungs of individuals with CF are colonized and infected by bacteria from an early age via thick, sticky mucus. In CF pacients, *P. aeruginosa* does not invade the bronchial tissue, but remains in the airways forming a biofilm. The phenotype, known as "mucoid" pseudomonades, is rarely seen in people that do not have CF [16]; it develops resistance to commonly used antibiotics and drives the vast majority of morbidity encountered in this disease [17].

1.2 Community acquired infections

Since *P. aeruginosa* is common in the environment, especially in water [18, 19], the bacterium is a cause of community acquired infections. They include folliculitis (due to hot tubs, whirlpools, or contaminated sponges) [20, 21], puncture wound osteomyelitis [22, 23], otitis externa in swimmers [24, 25], corneal ulcers, keratitis associated with soft contact-lens wear [26], pneumonia [27, 28], endocarditis (mainly in intravenous drug abusers) [29], peritonitis infection in patients undergoing continuous ambulatory peritoneal dialysis [30, 31], and community-acquired bacteremia and pneumonia (mainly in patients with AIDS) [32, 33].

1.3 Nosocomial infections

P. aeruginosa is responsible for an increasing proportion of infections acquired in the modern hospital setting (nosocomial infections), especially in intensive care units (ICU) and in urology patients [34], and has held a nearly unchanged position in the rank order of pathogens causing ICU infections during the last 4 decades [35].

The application of molecular typing methods allowed identifying ICU tap water as a significant source of exogenous *P. aeruginosa* isolates [36]. Hospital reservoirs for the microorganism include respiratory equipment, bronchoscopes, antiseptics, disinfectants, soaps, sinks and faucets, and physiotherapy/hydrotherapy pools. Horizontal transmissions between patients have long been considered the most frequent source of *P. aeruginosa* infections, including direct patient contact and spread from patient to patient on the hand of hospital personnel [35]. Specific interaction between

© FORMATEX 2015

both patient colonization pressure and selective antibiotic pressure is the most relevant factor for *P. aeruginosa* acquisition in an ICU [37].

P. aeruginosa has a remarkable capability to colonize certain subgroups of patients. Risk factors in ICU include length of stay; mechanical ventilation, acute respiratory failure or source of infection in respiratory tract; widespread use of antibiotics; use of invasive medical devices, *e.g.* central vein catheter and urinary catheters; and wounds from surgery or from burns [38, 39]. In centers for disease control and prevention, risk factors of *P. aeruginosa* infection are some diagnostic procedures, *e.g.* transrectal ultrasound-guided prostate biopsies [40] and endoscopic retrograde cholangiopancreatography [41].

1.5 Antibiotic resistance

Of all the infections caused by *P. aeruginosa*, bacteremia (an invasion of the bloodstream by bacteria) is one of the most severe [42]. *P. aeruginosa* has been consistently associated with the highest case-fatality rate for any of the bacteraemic gram-negative infections [43]. The major risk factor associated with an increased mortality rate in patients with *P. aeruginosa* bacteremia is antimicrobial resistance [44, 38]. A number of mechanisms of antibiotic resistance in *P. aeruginosa* have been studied, including: (*i*) AmpC type β-lactamases, enzymes (EC 3.5.2.6) that provide resistance to β-lactam antibiotics by breaking a common element in their molecular structure, *viz.*, a four-atom ring, and thereby deactivating the molecule's antibacterial properties [45]; (*ii*) downregulation of the outer membrane protein OprD, a carbapenem-specific porin [46]; (*iii*) multi-drug efflux pumps [47]; (*iv*) ability of the organism to form a biofilm, *i.e.* community of microorganisms embedded in extracellular polymeric substances matrix [48, 49]; (*v*) possible transfer of a 16S rRNA methylase gene from *Actinomycetes* [50].

1.6 Virulence factors

It is surprising, that pseudomonades are not more common pathogens, considering their ubiquitous presence, ability to grow in virtually any environment, resistance to many antibiotics and virulence factors. The latter means specific substances (cell-associated and extracellular) that enable a microorganism to establish itself on or within a host and enhance its potential to cause disease via adhesion, colonization, invasion, inhibition of immune response and ability to cause tissue damage. Virulence in *P. aeruginosa* is both multifactorial and combinatorial, the result of a pool of pathogenicity-related genes that interact in various combinations in different genetic backgrounds [51].

In accordance with data base 'Virulence factors of pathogenic bacteria' (http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Pseudomonas), main virulence factors of *P. aeruginosa* include those implicated in adherence (flagella, type IV pili, lypopolysaccharides), antiphagocytosis (alginate) and iron uptake (siderophores pyochelin and pyoverdine). Besides, virulence factors include three secretion systems (type III secretion system (T3SS) for secretion of fore exotoxins, type VI secretion system known also as a hexameric protein (Hcp1) secretion island I (HSI-I) and type II secretion system known also as Xcp secretion system). In addition, virulence factors include toxins proper (ExoA (ADP-ribosyltransferase secreted by type II secretion system), highly related ADP-ribosyltransferases ExoS and ExoT, phospholipase ExoU and adenylate cyclase ExoY (all are secreted by T3SS), phospholipases C (PlcH, PlcN and PlcB) known as haemolysins and toxic pigment pyocyanin. The list of virulence factors is completed by proteases (alkaline protease secreted by type I secretion system; serine protease LasA and zinc metalloproteinase LasB, both secreted by type II secretion system), and biosurfactants rhamnolipids. Two different types of regulation systems control the expression of the majority of these virulence factors: the two-component transcriptional regulatory system and the quorum sensing system.

T3SS and related effector toxins, *viz.*, ExoS, ExoT, ExoU and ExoY, are the subjects of the review. We pay special attention to data, related to biochemical activities and cellular targets of T3SS effectors, and molecular mechanisms leading to cytotoxicity and blocking of phagocytosis, two most proven consequences of enzymatic activity of fore exoenzymes.

2. T3SS

Six different classes of secretion systems have been described in Gram-negative bacteria, five of them are in *P. aeruginosa* [52].

The type III secretion system (T3SS) is a needle-like nanomachine that injects virulence proteins (exotoxins) directly into eukaryotic cells to initiate infection. Injected exotoxins initiate and maintain infection by manipulating host cell biology, such as cell signaling, secretory trafficking, cytoskeletal dynamics, and the inflammatory response. T3SS is essential for survival and pathogenesis of many Gram-negative bacteria, including *Pseudomonas*, *Salmonella*, *Escherichia*, *Shigella*, *Yersinia*, and *Chlamydia* spp.

This complex macromolecular machine works in a highly regulated manner and can manipulate the host cell in many different ways. Since *P. aeruginosa* is an opportunistic pathogen in humans, it is unlikely that the T3SS evolved as the result of pressures to survive within host. The natural target of the *P. aeruginosa* T3SS is unclear. Probably, this system may have evolved to ward off environmental predators (*e.g.* amoebae [53] that inhabit the soil and water), and broad

© FORMATEX 2015

conservation of targeted substrates across eukaryotic organisms resulted in a system that also is active against human cells.

The current knowledge of the structure and function of the *P. aeruginosa* T3SS is summarized in reviews of Galle M. et al. [54] and Hauser A.R. [55]. In addition, T3SS recognition by the immune system was reviewed by Galle M. et al. [54].

The *P. aeruginosa* T3SS consists of five functional parts [55]:

(*i*) numerous proteins that comprise the secretion machine itself (the needle complex);

(*ii*) three translocator proteins, PopB, PopD, and PcrV, that comprise the targeting apparatus translocating secreted proteins into host cells;

(*iii*) fore regulatory proteins, ExsA, ExsC, ExsD, and ExsE, that regulate the secretion process;

(*iv*) chaperones, small bacterial cytosolic proteins that assist the assembly and operation of the T3SS; class I, class II and class III chaperones assist the pore forming proteins, the needle-like structure, and the effector toxins, respectively;

(*v*) fore effector proteins (exotoxins), ExoS, ExoT, ExoU, and ExoY, that are actually injected into host cells.

The needle complex (also known as the injectisome), is composed of a basal body spanning both bacterial membranes and the periplasm, and an external needle protruding from the bacterial surface. A set of three translocator proteins is required to allow translocation of exotoxins from the bacterium to the host cell cytoplasm. These translocators are themselves secreted by the needle complex and involved in the formation of a translocation pore (also known as the translocon) in the host cell membrane. The two hydrophobic translocators, PopB and PopD, insert into the target cell membrane. The hydrophilic translocator, PcrV, forms a complex on the distal end of the injectisome needle, the tip complex, and serves as an assembly platform for the two hydrophobic translocators [56]. Importantly, though much of the T3SS mediated damage is caused by the translocated exotoxins, there is increasing evidence that insertion of the needle complex itself can contribute to host cell injury [57].

Linkage of T3SS transcription to protein secretion is achieved through the interactions of four regulatory proteins: ExsA (that directly binds the promoters of T3SS genes), 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 T3SS 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 T3SS genes [55]. The detailed discussion of transcriptional regulation of the *P. aeruginosa* T3SS is in review of Yahr T.L. and Wolfgang M.C. [58].

The signal that triggers the expression of T3SS genes is commonly known as direct host cell contact sensed by the T3SS needle, but the exact signaling mechanism is not known [54]. It was shown that induction of T3SS is a part of *P. aeruginosa* response to limiting oxygen stress in host. Microaerobic induction of the T3SS is dependent on global anaerobic stress response regulator Anr that triggers gene expression of a range of target genes, including the response regulator *narL*. Microaerobic induction of the T3SS is mediated through direct NarL transcriptional repression of the small RNAs, *rsmY* and *rsmZ*, which allow the global post-transcriptional regulator RsmA protein to regulate the T3SS [59]. The mechanisms that may control type 3 secretion are reviewed by Barison N. et al. [60].

Recent evidences suggest that the proton motive force is the primary energy source for T3SS; possible mechanisms wherewith the proton motive force is converted to protein export were discussed in review of Lee P.C. and Rietsch A. [61].

2.1 Distribution of secretion toxins-encoding genes in nosocomial/environmental isolates and relative contributions of toxins to virulence of *P. aeruginosa*

Effector proteins secreted by T3SS have been shown to contribute significantly to the virulence of *P. aeruginosa* in multiple *in vivo* animal models [62 - 69]. Furthermore, analysis of *P. aeruginosa* clinical isolates has also shown a clear correlation between expression of T3SS effectors and increased disease severity and patient mortality [70]. Interestingly, despite the importance of T3SS in dictating clinical outcomes and promoting disease in animal models of infections, clinical isolates often do not express T3SS *in vitro* and can cause disease in a T3SS-independent manner [71].

Commonly accepted, that acute *P. aeruginosa* infections are associated with planktonic or free-floating cells, high virulence and fast growth. Conversely, chronic infections are associated with the biofilm mode of growth, low virulence and slow growth that resembles that of planktonic cells in stationary phase. Acute bacterial infections are associated with motility and cytotoxicity via T3SS, while chronic infections are linked to reduced virulence. In other words, biofilm formation and T3SS are reciprocally regulated, and virulence factors related to acute infection may be incompatible with biofilm formation.

It was shown recently [72] that biofilms are more similar to planktonic cells in exponential phase than to those in stationary phase. Moreover, biofilms express T3SS, whereas planktonic cells do not. Thus, biofilm formation and T3SS are not mutually exclusive in *P. aeruginosa*. In other study [66], comparison of strains from the same CF patient isolated at the initial onset of *P. aeruginosa* infection and more than a decade later was performed. It was shown that initial strains were more cytotoxic than chronic strains that have evolved to reduce T3SS. Moreover, constitutive

expression of genes for T3SS restored ExoS secretion but did not always reestablish cytotoxicity, which suggests that CF strains accumulate a number of mutations to reduce bacterial toxicity to the host.

In study of Feltman et al. [73] southern hybridization analyses and PCR were performed on over 100 *P. aeruginosa* isolates (clinical isolates obtained from urine, endotracheal, blood and wound specimens, from the sputum of CF patients, and environmental isolates) to determine the distribution of secretion toxins-encoding genes. Each isolate contained the *popB* gene that was used as a marker for the large chromosomal locus encoding the T3SS proteins. The *exoS*, *exoU* and *exoY* genes were detected in 72%, 28% and 89% isolates, respectively. An inverse correlation was noted between the presence of the *exoS* and *exoU* genes since all isolates except two contained either *exoS* or *exoU* but not both. No significant difference in *exoS*, *exoU* or *exoY* prevalence was observed between clinical and environmental isolates or between isolates cultured from different disease sites except for CF respiratory isolates. CF isolates harbored the *exoU* gene less frequently and the *exoS* gene more frequently than did isolates from other sites of infection. These results suggest that the *P. aeruginosa* T3SS is present in nearly all clinical and environmental isolates but that individual isolates and populations of isolates from distinct disease sites differ in their effector genotypes.

In other study [74] multiplex PCR was performed on the 85 *P. aeruginosa* isolates (68 nosocomial and 17 environmental isolates) to detect the secretion toxins-encoding genes. The prevalence of the genes among all isolates was as follows; *exoT* - 100%, *exoS* - 40%, *exoY* - 83.5% and *exoU* - 62.4%. No significant differences in *exoS*, *exoY* and *exoU* prevalence were observed between nosocomial and environmental isolates or between isolates from different sites of infection. *ExoS* and *exoU* genes were mutually exclusive; almost all isolates contain either *exoS* without *exoU* or *exoU* without *exoS*.

While almost no strain encodes or secretes all four effectors, the commonly found combinations of ExoU/ExoT or ExoS/ExoT provides redundant and failsafe mechanisms to cause mucosal barrier injury, inhibit many arms of the innate immune response, and prevent wound repair [75]. The data are consistent with other studies showing that the T3SS phenotype of most clinical isolates of *P. aeruginosa* is ExoS–/$ExoT_{low}$/$ExoU_{low}$/ExoY– or $ExoS_{low}$/$ExoT_{low}$/ExoU–/$ExoY_{low}$. The latter phenotype tends to be more invasive but less cytotoxic in the lung [76 - 78].

Comparison of the relative contributions of ExoU, ExoS and ExoT effector toxins to mortality, bacterial persistence in the lung, and dissemination in a mouse model of acute pneumonia indicated that secretion of ExoU had the greatest impact, ExoS had an intermediate effect, and ExoT had a minor effect [64]. The data are consistent with those obtained in an analogous animal model [66]. In strain PAK, ExoS was identified as the major cytotoxin required for colonization and dissemination during infection. ExoT conferred protection of tissue culture cells from T3SS -dependent lysis, while ExoY had little effect on cytotoxicity. ExoU was over 100-fold more cytotoxic than ExoS [66].

ExoS, ExoT, ExoY, and ExoU all possess enzymatic activities that disrupt host cellular physiology and prevent bacterial clearance by host defense mechanisms. Commonly accepted that each of three effectors, ExoS, ExoT, and ExoY, alter mammalian cell morphology in culture without causing a loss of cell viability. ExoU is the most toxic and destructive effector that causes rapid necrotic death in many cell types [55]. Cooperation between ExoS, ExoT and ExoU does not translate into a synergistically significant enhancement of disease severity in a mouse model of pneumonia; indeed, *in vitro* cytotoxicity and anti-internalization activities were not enhanced when effector proteins were secreted in combinations rather than alone [65].

2.2 Structure of T3SS exotoxins, their enzymatic activity and consequences for target human cells

2.2.1 ExoS and Exo T

ExoS is a 49 kDa protein, bifunctional toxin with GTPase activating protein (GAP) activity and ADP-ribosyltransferase (ADPRT) activity. Besides two domains responsible for enzymatic activity, ExoS contains the C-terminal chaperone binding site, membrane localization domain and N-terminal cofactor binding site. Human cofactor for ExoS activation known as FAS, factor activating ExoS, belongs to 14-3-3 protein family. Substrates for GAP activity are small GTPases, RhoA, Rac1, and Cdc42; substrates for ADPRT activity are ezrin, radixin, moesin, vimentin, cyclophilin A, apolipoprotein A1 and numerous members of small GTFases (Rho family, Ras, Rac1, Cdc42; Rab family, Rabs 1, 3, 5, 7, 8, and 11; Ras family, RalA, Rap1, Rap2) [55].

ExoT is a 53 kDa protein that demonstrates 76% homology with ExoS. Similarly to ExoS, ExoT has both GAP and ADPRT activities and use the cofactor for activation that belongs to 14-3-3 protein family [55]. The GAP activity of ExoT is directed against substrates identical to that of ExoS. Substrates for ADPRT activity of ExoT are Crk (CT10 (chicken tumor virus number 10) regulator of kinase) adaptor proteins, Crk-I and Crk-II [55].

Interestingly, ExoS and ExoT share 76% amino acid identity but ADP-ribosylate different substrates. It was shown by protein modeling and site-directed mutagenesis that regions B, C and E of ExoS are necessary and sufficient to recognize ExoS targets, whereas analogous regions of ExoT are necessary but not sufficient to recognize ExoT targets, such as the Crk proteins. A specific Crk recognition motif on ExoT was defined as region A [79]. The data can explain how ExoS and ExoT recognize their unique substrates.

Human cofactors for ExoS/ExoT activation, 14-3-3 proteins, serve as regulatory factors that modulate a wide variety of cellular functions, including cell cycle progression, protein trafficking, and apoptosis [80]. These cofactors are

 © FORMATEX 2015

absolutely required for ADPRT activity of ExoS and ExoT, which implies that their ADPRT activity is inhibited until exotoxins are injected in the host cell cytosol to prevent them from harming the bacteria.

For each exotoxin, a consequence of enzymatic activity depends on biological function of substances accepted as substrates. For this reason, we indicate the biological function of the above mentioned substrates of ExoS and ExoT and describe in details some of the known mechanisms explaining how ExoS and ExoT enzymatic activity results in disturbance of human cell morphology and functions.

Regulatory GTPases, RhoA, Rac1, and Cdc42 (substrates of ExoS and ExoT GAP activity) are the three most common members of the Rho family. They maintain the organization of the actin cytoskeleton [81]. In addition, Rho, Rac and Cdc42 are implicated in the regulation of epithelial apical junctional complex that plays a role in barrier function of epithelia [82]. Moreover, Rho GTPases control multiple cellular processes through their ability to bind to downstream effectors, which leads to diverse parallel signaling pathways [83]. In fact, over 60 targets of the three common Rho GTPases have been found [84].

These regulatory GTPases normally switch between an active GTP-bound form and an inactive GDP-bound form; the former binds effector(s) and the latter releases the bound effector(s). In this way, small GTPases transduce an upstream signal to a downstream effector(s) [81]. Importantly, regulatory GTPases are implicated in two distinct mechanisms of phagocytosis: type I mechanism, used by the immunoglobulin receptor, is mediated by Cdc42 and Rac, and type II mechanism, used by the complement receptor, is mediated by Rho [85]. The ExoS/ExoT GAP domain biases the switch towards inactive form of GTPases, which leads to disruption of the actin cytoskeleton in phagocytes and results in decreased internalization of *P. aeruginosa*. Thus, GAP activity of ExoS/ExoT is important in preventing phagocytosis of the pathogen.

Substrates of ExoS ADPRT activity are more diversible [55]. Ezrin, radixin and moesin, known as ERM proteins, are closely related members of the band 4.1 superfamily that crosslink the actin cytoskeleton to several transmembrane proteins [86]. Vimentin is a type III intermediate filament protein that comprises the cytoskeleton along with tubulin-based microtubules and actin-based microfilaments [87]. Cyclophilin A is peptidyl prolyl cis-trans isomerase that regulates protein folding and trafficking [88]. Apolipoprotein A1 has a specific role in lipid metabolism and is the major protein component of high density lipoprotein in plasma [89]. All small GTPases identified as targets of ExoS ADPRT activity belong to Ras superfamily. The Ras family regulates gene expression and cell proliferation, the Rho family regulates cytoskeletal reorganization and cell morphology, the Rab family regulates vesicle trafficking [81, 90].

Substrates of ExoT ADPRT activity, Crk adaptor proteins, play an important role during cellular signaling by mediating the formation of protein complexes; Crk-activated signal transduction cascades include the modulation of cell adhesion, cell migration and immune cell responses [91], in particular, immune responses mediated by T, B and natural killer cells [92]. Moreover, CrkI and CrkII function as key signaling integrators for migration and invasion of cancer cells [93].

In theory, ADP-ribosylation (the addition of one or more ADP-ribose moieties to a protein) alters the function of substrate protein dramatically and is involved in many cellular processes, including cell signaling, DNA repair, gene regulation and apoptosis [94].

Next, we give two examples to illustrate how ADPRT activity of ExoS and ExoT leads to disruption of the cytoskeleton; moesin and Crk adaptor proteins are used as substrates, respectively.

A possible mechanism explaining the role of ExoS ADPRT activity in modification of the actin cytoskeleton was proposed by Maresso A.W. et al. [95]. In the study, moesin was identified as a target of ExoS in cultured cells. It was shown that ExoS ADP-ribosylates moesin at three C-terminal arginines, which clusters Thr558, the site of phosphorylation of the protein by kinase C and Rho kinase. Phosphorylation of moesin and other ERM proteins at the conserved threonine residues leads to disruption of interactions between N and C domains and exposure of the high affinity F-actin binding site, which promotes F-actin binding. ADP-ribosylated moesin becomes a poor target for phosphorylation by two kinases that results in actin cytoskeleton reorganization and cell rounding.

The ADP ribosylation of identified ExoS substrates results in inhibition of DNA synthesis, endocytosis and vesicular trafficking, and apoptotic-like cell death [55].

Functional mechanisms explaining the role of Crk adaptor proteins in actin cytoskeleton dynamics were elucidated in study of Sato M. et al. [96]. Crk proteins comprise SH2 and SH3 domains that are components of the integrin signaling pathway leading to Rac1 and Rap1 functions. Crk binds and activates abelson murine leukemia viral oncogene homolog 1 (Abl) kinase, a protein implicated in cell differentiation, cell division, cell adhesion, and stress response. It induces increased tyrosine phosphorylation of Crk-associated substrate, p130Cas, a docking protein that plays a central coordinating role for tyrosine-kinase-based signaling related to cell adhesion. The phosphorylated p130Cas then binds the Crk SH2 domain, while dedicator of cytokinesis, DOCK180, a protein involved in intracellular signaling, binds the Crk SH3 domain, resulting in ternary complex p130Cas-Crk-DOCK180. Formation of the complex is sufficient to activate Rac1 and localize it to focal adhesion (FA) sites, the sub-cellular structures that connect the cell cytoskeleton to the extracellular matrix and mediate the regulatory effects of a cell in response to environment [97]. The Arg20 located within the SH2 domain of CrkI was identified as the site of ADP-ribosylation by ExoT. ADP-ribosylated CrkI failed to bind upstream signaling molecule p130Cas. Thus, the ADP-ribosylation of Crk uncouples integrin signaling by direct inhibition of the binding of Crk to FA proteins, that results in inhibition of cell migration and cell rounding [98, 99].

© FORMATEX 2015

Both domains of ExoT cooperate to inhibit mammalian cytokinesis. GAP-mediated inhibition of cytokinesis occurs early, likely as a consequence of its inhibitory effect on RhoA. The domain of ExoT with ADPRT activity inhibits late steps of cytokinesis by blocking syntaxin-2 localization to the midbody (a transient structure that connects two daughter cells at the end of cytokinesis), an event essential for completion of cytokinesis [100]. Thus, each of ExoT enzymatic domains inhibits cytokinesis in a kinetically, morphologically, and mechanistically distinct manner. It results in delayed wound healing [101], which allows *P. aeruginisa* to invade through breaches in the mucosal barriers.

It was shown that ADPRT activity of ExoT is both necessary and sufficient to cause apoptosis in HeLa cells via activation of the mitochondrial/cytochrome c-dependent apoptotic pathway [102]. Similarly, the virulence of ExoS was largely dependent on its ADP-ribosyltransferase activity; the GAP activity of this protein had only a minor effect *in vivo*. It was proven by comparison of infection with isogenic *P. aeruginosa* mutants secreting wild-type ExoS, ExoS defective in GAP activity and ExoS defective in ADPRT activity [64].

In sum, both GAP and ADPRT activities of ExoS/ExoT lead to disruption of the actin cytoskeleton and apoptosis-like cell death. The apoptosis induced by ExoT occurs later than ExoS-induced apoptosis [102]. Impact of ADPRT activity on toxic properties of ExoS/ExoT is more substantial than such of GAP activity. In addition, ADPRT activity of ExoS mediates cytotoxicity while such of ExoT interferes mainly with host cell phagocytic activity. Since phagocytes are targets of ExoT GAP activity, this exotoxin is important in preventing phagocytic uptake of *P. aeruginosa*.

Mutations in the gene encoding ExoS were used to differentiate the mechanisms underlying the effects of ExoS enzymatic activities on *P. aeruginosa* internalization and ExoS translocation in model of highly migratory T24 epithelial cells [103]. Inactivation of ExoS GAP activity allowed *P. aeruginosa* to be internalized and secrete ExoS within T24 cells, but as with wild-type ExoS, translocation of ExoS was limited in association with disruption of actin anchoring. Inactivation of ExoS ADPRT activity resulted in significantly enhanced ExoS translocation by bacterial cells that remained extracellular, and this occurred in conjunction with maintenance of actin-plasma membrane association. Infection with *P. aeruginosa* expressing ExoS lacking both GAP and ADPRT activities resulted in the highest level of T3SS translocation, and this occurred in conjunction with the entry and alignment of *P. aeruginosa* and ExoS along actin filaments. The data suggest that ExoS ADPRT activity contributes to the opportunistic nature of *P. aeruginosa* infection through its ability to exert cytotoxic effects that interrupt ExoS translocation and *P. aeruginosa* internalization, which in turn limit the *P. aeruginosa* infectious process.

In other study was shown that invasive *P. aeruginosa* can enter epithelial cells wherein they mediate formation of plasma membrane bleb-niches for intracellular compartmentalization using ADPRT activity of ExoS [104]. The mutants lacking the capacity to induce bleb-niches are located in perinuclear vacuoles and their viability is suppressed by vacuolar acidification. Indeed, the ADP-ribosylation domain of ExoS is important for intracellular replication of *P. aeruginosa* [105]. In sum, T3SS effectors can participate in pathogenesis without translocon-mediated translocation across host membranes and ADPRT activity of ExoS contributes to *P. aeruginosa* pathogenesis within epithelial cells.

Moreover, the pathogenesis of *P. aeruginosa* keratitis in ExoS- and ExoT-producing strains is almost entirely due to their ADPR activities, which subvert the host response by targeting the antibacterial activity of infiltrating neutrophils [106]. Indeed, the ADP-ribosyltransferase activity of ExoS was responsible for inhibition of phagocytosis in mouse model of pneumonia, which led to bacterial persistence and decreased host survival [69]; blocking the activity of ExoS may have potential therapeutic benefit.

More recently it was shown that ExoT induces potent apoptosis in host epithelial cells in a manner that primarily depends on its ADPRT activity. The mechanism underlying apoptosis includes ExoT/ADPRT disruption of focal FA sites, and activation of p38 (mitogen-activated protein kinase, MAPK) and JNK (c-Jun N-terminal kinase, other member of MAPK family), two kinases that regulate apoptosis. It interferes with integrin-mediated survival signaling and leads to atypical anoikis mediated by the Crk adaptor protein [107]. Crk is generally believed to be a component of FA but it is not required for FA assembly or for host cell survival *per se*. However, when Crk is modified by ExoT, it transforms to a cytotoxin that induces anoikis by disrupting FA sites and interfering with integrin survival signaling. This is the first example whereby a bacterial toxin exerts its cytotoxicity by subverting the function of an innocuous host cellular protein and turning it against the host cell.

Interestingly, ExoS not only has a T3SS contact-dependent intracellular function in cytotoxicity but can also interact as a soluble extracellular protein with T cells and monocytes [108, 109]. This results in T cell apoptosis and proinflammatory cytokine and chemokine production by activated monocytes [110, 111]. ExoS has been reported to activate monocytic cells via a MyD88-dependent pathway, using both TLR2 and TLR4; TLR2 and TLR4 activities were localized to C-terminal and N-terminal domains of ExoS, respectively [112]. In contrast, T3SS-mediated injection of ExoS in macrophages negatively regulates *P. aeruginosa* induced proteolytic maturation and secretion of the proinflammatory cytokine IL-1 [113], indicating that ExoS has different immune regulatory functions dependent on its location. Moreover, ExoS deficiency also switched the mode of macrophage death from apoptosis to pro-inflammatory pyroptosis [113].

One more function of ExoS is support of translocation of endogenous *P. aeruginosa* from the colonized intestinal tract that is considered the most important reservoir of the bacterium in ICU. It was shown that the translocation is mediated by the binding of ExoS to an Na,K-ATPase regulator, FXYD3. In turn, Na,K-ATPase controls the structure and barrier function of tight junctions in epithelial cells. Thus, ExoS impairs the defense function of tight junctions

 © FORMATEX 2015

against bacterial penetration [114]. ExoS has been linked to a higher incidence of dissemination to the bloodstream in patients with hospital-acquired pneumonia [115]. It was shown that phagocytic cells, primarily neutrophils, were targeted for injection with ExoS early during infection, but type I pneumocytes became increasingly injected at later time points. The increased size of discrete regions designated as fields of cell injection (FOCI) was associated with enhanced disruption of the pulmonary-vascular barrier and increased bacterial dissemination into the blood, both of which were dependent on the ADPRT activity of ExoS [115].

Unexpected result was that ExoT targets six proteins that are required for survival and proliferation of tumour cells [116], which suggests that ExoT is a possible chemotherapeutic candidate for the treatment of cancer resistant to current chemotherapeutic drugs.

2.2.2 ExoU

ExoU is a 74 kDa protein. Besides potation-like domain responsible for phospholipase A2 activity, ExoU contains the N-terminal chaperone binding site and C-terminal membrane localization domain. Indeed, ExoU contains the bridging domain that consists of two subdomains; the first part of the bridging domain precedes the catalytic region and the second part connects phospholipase and membrane-binding domains, the latter of which displays specificity to phosphatidylinositol 4,5-bisphosphate, PI(4,5)P2 [117]. ExoU shaperone, SpcU, maintains the N-terminus of ExoU in an unfolded state, required for secretion. The phospholipase domain carries an embedded catalytic site whose position within ExoU does not permit direct interaction with the bilayer, which suggests that ExoU must undergo a conformational rearrangement in order to access lipids within the target membrane [117].

ExoU has been shown to carry phospholipase activity with broad substrate specificity which relies on an essential catalytic dyad (Ser142/Asp344) [118 - 120], as is the case for other phospholipases. Enzymes with phospholipase A2 activity hydrolyze the sn-2 ester bond of phospholipids, major components of host cell membranes, and thus play a role in membrane disruption, fatty acid release, and in many cases, signal transduction [121-123]. Indeed, enzymatic activity of ExoU leads to production of lysophospholipids, metabolites that modify the function of membrane proteins including ion channels [124].

Initially, the Cu^{2+}, Zn^{2+}-superoxide dismutase (SOD1) was identified as a cofactor for ExoU activation, though its enzymatic activity was not required [125]. It was shown later, that ExoU was activated by ubiquitylated SOD1 and other ubiquitylated mammalian proteins, and ubiquitin was recognized as a true activator of ExoU [126]. A model for complex formation between ExoU and monoubiquitin was proposed by Anderson DM. et al. [127]. ExoU becomes ubiquitinated on Lys178 (in catalytic domain), a modification which has a small effect on its intracellular turnover rate [128]. Both transfection experiments and infection of eukaryotic cells with ExoU-secreting bacteria show that ExoU ubiquitination results in its co-localization with endosomal markers. This could reflect an attempt of the infected cell to target ExoU for degradation in order to protect itself from its toxic action [117]. The PI(4,5)P2 was identified as another important coactivator of ExoU [129]. PI(4,5)P2 works synergistically with ubiquitin to greatly enhance the phospholipase A2 activity of ExoU. Distinct residues of ExoU were critical for activation by PI(4,5)P2 and by ubiquitin, indicating that these factors activate ExoU by discrete mechanisms. In support of the biological relevance of PI(4,5)P2 coactivation, a yeast mutant with reduced PI(4,5)P2 levels was less susceptible to the cytotoxic activity of ExoU. Further elucidation of the structural mechanism of ExoU activation may provide a rational approach to the design of inhibitors that can diminish tissue damage during infection by ExoU-producing strains of *P. aeruginosa*.

Unlike ExoS, when administered extracellularly, ExoU is unable to intoxicate epithelial cells in culture, even in the presence of the cofactor. Injection or transfection of ExoU is necessary to observe the acute cytotoxic response. Biochemical approaches indicated that ExoU is capable of utilizing PI(4,5)P2 as a substrate. In eukaryotic cells, PI(4,5)P2 is mainly located in the cytoplasmic side of the plasma membrane and anchors adaptor proteins that are involved in cytoskeletal structures, focal adhesions, and plasma membranes. Time-lapse fluorescent microscopy analyses of infected live cells demonstrated that in the early phases of infection ExoU intoxication correlated with intracellular damage, such as disruption of focal adhesions, cytoskeletal collapse, actin depolymerization, and cell rounding. At later time points, a membrane blebbing phenotype was prominent prior to the loss of the plasma membrane integrity [130].

ExoU enzymatic activity results in rapid lysis of many mammalian cell types. Isogenic mutants of *P. aeruginosa* secreting catalytically inactive ExoU were noncytotoxic and did not cause acute lung injury or death of the infected mice, as opposed to wild-type bacteria [131]. In a mouse model of acute pneumonia, delaying expression of exoU enhanced clearance of bacteria and survival of infected mice [68]. In other study [132] it was shown that *P. aeruginosa* persists in the lungs of acutely infected mice by an ExoU- and neutrophil-dependent mechanism. The recruited phagocytes were identified as a major target of ExoU intoxication. Seemingly, inhibition of the ability of these cells to eradicate pathogen (i.e. immunosupression) contributes to both the high mortality and the frequency of polymicrobial infections associated with *P. aeruginosa* human acute pneumonia.

© FORMATEX 2015

2.2.3 ExoY

ExoY is a 41 kDa protein with adenylate cyclase activity. Substrate of ExoY is ATP; host cofactor for activation still is not identified. Injection of ExoY into mammalian cells results in an elevation of intracellular 3',5'-cyclic adenosine monophosphate (cAMP) concentration [78] and differential expression of multiple genes, including many known to be regulated by cAMP. This leads to disruption of the actin cytoskeleton [129], increased endothelial permeability [134] and inhibition of bacterial uptake by host immune cells [132]. Such activities would be predicted to lead to more severe disease.

The immune effector cells appear to be the primary target of ExoY and other known bacterial adenylate cyclase toxins, which facilitate the survival of the pathogenic bacteria in the host [135].

Another target of ExoY is edothelial cells. ExoY was identified as an edema factor that chronically impairs endothelial cell barrier integrity following lung injury [136]. In this *in vitro* study, microtubule breakdown and interendothelial cell gap formation caused by ExoY were detected, and gaps were not repaired even 3-5 days after removal of bacteria.

Since ExoY increases the cytoplasmic levels of cAMP [78], it decreases endothelial barrier integrity in accordance with the concept of cAMP compartmentalization proposed by Sayner SL [137]. Cyclic AMP generated in the subplasma membrane compartment acts through two ubiquitously expressed intracellular cAMP receptors, the classic protein kinase A/cAMP-dependent protein kinase (PKA/cAPK) and the recently discovered exchange protein directly activated by cAMP/cAMP-regulated guanine nucleotide exchange factors (Epac/cAMP-GEF) [138], to tighten cell adhesions, strengthen cortical actin, reduce actomyosin contraction, and decrease permeability. Confining cAMP within the subplasma membrane space is critical to its barrier-protective properties. When cAMP gains access to the cytosolic compartment, or when soluble adenylyl cyclases generate cAMP within the cytosolic compartment, this second messenger activates established cytosolic cAMP signaling cascades to perturb the endothelial barrier through PKA-mediated disruption of microtubules.

Other mechanism is hyperphosphorylation of endothelial Tau proteins mediated by intracellular cAMP. It leads to accumulation of insoluble Tau proteins dissociated from microtubules and results in large inter-endothelial gaps and increased permeability in pulmonary microvascular endothelial cells [139, 140].

In sum, the disruption of epithelial/endothelial barriers by effector toxins allows *P. aeruginosa* to disseminate into the bloodstream and pro-inflammatory mediators to leak into the systemic circulation, which leads to septic shock [141]. Indeed, T3SS enhances the pro-inflammatory response, for example by triggering IL-1α maturation [113], and this leads to acute lung injury. The death or impairment of alveolar neutrophils and macrophages mediated by exotoxins leads to impairment of bacterial clearance from the lungs [132]. When *P. aeruginosa* are not eradicated from the airway, there is a greater opportunity for T3SS to produce and/or inject more effector toxins into the host cells, which results in characteristic symptoms of pneumonia induced by *P. aeruginosa*. This explains why T3SS-expressing bacterial strains are associated with high mortality and bad prognosis.

3. Conclusion

The T3SS is a needle-like nanomachine to inject virulence proteins (exotoxins) directly into eukaryotic cells to initiate infection. Fore effector proteins of *P. aeruginosa* modulate a variety of cellular functions for the pathogen's benefit. *P. aeruginosa* is invasive or cytotoxic to host cells, depending on the T3SS effectors encoded. ExoS is a 49-kDa form of exoenzyme S, a bifunctional toxin that exerts ADPRT and GAP activity to disrupt the actin cytoskeleton, disturb endocytosis and cell proliferation, induce apoptosis and mediate formation of plasma membrane bleb-niches for intracellular compartmentalization of the bacterium. ExoT, a 53-kDa protein with 75% sequence homology to ExoS, also exerts ADPRT and GAP activity to interfere with cell morphology and phagocytic activity of host immune cells. ExoY is a nucleotidal cyclase that increases the intracellular levels of cyclic adenosine and guanosine monophosphates, resulting in edema formation. ExoU, which exhibits phospholipase A2 activity is a major pathogenic cytotoxin that causes alveolar epithelial injury and macrophage necrosis. The central role of T3SS effectors in pathogenesis of *P. aeruginosa* infection makes them great targets for novel antimicrobial strategies. Antibody-mediated blocking of proteins involved in translocation of T3SS exotoxins or inhibition of the enzymatic activity of exotoxins have the potential to improve clinical outcome.

References

[1] Panis C, Matsuo T, Reiche EM. Nosocomial infections in human immunodeficiency virus type 1 (HIV-1) infected and AIDS patients: major microorganisms and immunological profile. Braz J Microbiol. 2009;40(1):155-62.

[2] Baro M, Marín MA, Ruiz-Contreras J, de Miguel SF, Sánchez-Díaz I. *Pseudomonas aeruginosa* sepsis and ecthyma gangrenosum as initial manifestations of primary immunodeficiency. Eur J Pediatr. 2004;163(3):173-4.

[3] Hakki M, Limaye AP, Kim HW, Kirby KA, Corey L, Boeckh M. Invasive *Pseudomonas aeruginosa* infections: high rate of recurrence and mortality after hematopoietic cell transplantation. Bone Marrow Transplantat. 2007;39(11):687–93.

 © FORMATEX 2015

[4] Zeglen S, Wojarski J, Wozniak-Grygiel E, Siola M, Jastrzebski D, Kucewicz-Czech E, et al. Frequency of *Pseudomonas aeruginosa* colonizations/infections in lung transplant recipients. Transplant Proc. 2009;41(8):3222-4.
[5] Liu Y, Liu K, Yu X, Li B, Cao B. Identification and control of a *Pseudomonas* spp (*P. fulva* and *P. putida*) bloodstream infection outbreak in a teaching hospital in Beijing, China. Int J Infect Dis. 2014;23():105–8.
[6] Thomas BS, Okamoto K, Bankowski MJ, Seto TB. A lethal case of *Pseudomonas putida* bacteremia due to soft tissue infection. Infect Dis Clin Pract (Baltim Md). 2013;21(3):147–213.
[7] Hsueh P-R, Teng L-J, Pan H-J, Chen Y-C, Sun C-C, Ho S-W, et al. Outbreak of *Pseudomonas fluorescens* bacteremia among oncology patients. J Clin Microbiol. 1998;36(10):2914-7.
[8] Gershman MD, Kennedy DJ, Noble-Wang J, Kim C, Gullion J, Kacica M, et al. Multistate outbreak of *Pseudomonas fluorescens* bloodstream infection after exposure to contaminated heparinized saline flush prepared by a compounding pharmacy. Clin Infect Dis. 2008;47(11):1372-9.
[9] Malhotra S, Singh K. *Pseudomonas stutzeri* associated conjunctivitis. Indian J Pathol Microbiol. 2008;51(4):572.
[10] Ceri M, Ortabozkoyun L, Altay M, Unverdi S, Kurultak I, Huddam B, et al. Peritonitis due to *Pseudomonas stutzeri*, an organism that may be difficult to culture. Perit Dial Int. 2010;30(4):484-6.
[11] Lin JN, Lai CH, Chen YH, Lin HL, Huang CK, Chen WF, et al. *Sphingomonas paucimobilis* bacteremia in humans: 16 case reports and a literature review. J Microbiol Immunol Infect. 2010;43(1):35-42.
[12] Toh HS, Tay HT, Kuar WK, Weng TC, Tang HJ, Tan CK. Risk factors associated with *Sphingomonas paucimobilis* infection. J Microbiol Immunol Infect. 2011;44(4):289-95.
[13] Kam S-K, Lee W-S, Ou T-Y, Teng S-O, Chen F-L. *Delftia acidovorans* bacteremia associated with ascending urinary tract infections proved by molecular method. J Clin Exp Med. 2012;4(3)180-182.
[14] Mahmood S, Taylor KE, Overman TL, McCormick MI. Acute infective endocarditis caused by *Delftia acidovorans*, a rare pathogen complicating intravenous drug use. Clin Microbiol. 2012;50(11):3799-800.
[15] Wang Y, Wrennall JA, Cai Z, Li H, Sheppard DN. Understanding how cystic fibrosis mutations disrupt CFTR function: from single molecules to animal models. Int J Biochem Cell Biol. 2014;52:47-57.
[16] Saiman L. Microbiology of early CF lung disease. Paediatr Respir Rev. 2004;5(Suppl A): S367–69.
[17] Campódonico VL, Gadjeva M, Paradis-Bleau C, Uluer A, Pier GB. Airway epithelial control of *Pseudomonas aeruginosa* infection in cystic fibrosis. Trends Mol Med. 2008;14(3):120–33.
[18] Mena KD, Gerba CP. Risk assessment of *Pseudomonas aeruginosa* in water. Rev Environ Contam Toxicol. 2009;201:71-115.
[19] Tirodimos I, Arvanitidou M, Dardavessis L, Bisiklis A, Alexiou-Daniil S. Prevalence and antibiotic resistance of *Pseudomonas aeruginosa* isolated from swimming pools in northern Greece. East Mediterr Health J. 2010;16(7):783-7.
[20] Yu Y, Cheng AS, Wang L, Dunne WM, Bayliss SJ. Hot tub folliculitis or hot hand-foot syndrome caused by *Pseudomonas aeruginosa.* J Am Acad Dermatol. 2007;57(4):596-600.
[21] Alsedà M, Godoy P, Gardeazabal J, Borràs MÀ. [Community outbreak of *Pseudomonas aeruginosa* folliculitis associated with a spa]. Med Clin (Barc). 2014;142(2):86-7. Spanish.
[22] Eidelman M, Bialik V, Miller Y, Kassis I. Plantar puncture wounds in children: analysis of 80 hospitalized patients and late sequelae. Isr Med Assoc J. 2003;5(4):268-71.
[23] Chachad S, Kamat D. Management of plantar puncture wounds in children. Clin Pediatr (Phila). 2004;43(3):213-6.
[24] Roland PS, Stroman DW. Microbiology of acute otitis externa. Laryngoscope. 2002;112(7 Pt 1):1166–77.
[25] Wang M-C, Liu C-Y, Shiao A-S, Wang T. Ear problems in swimmers. J Chin Med Assoc. 2005;68(8):347–352.
[26] Willcox MD. Management and treatment of contact lens-related *Pseudomonas* keratitis. Clin Ophthalmol. 2012;6:919-24.
[27] Hatchette TF, Gupta R, Marrie TJ. *Pseudomonas aeruginosa* community-acquired pneumonia in previously healthy adults: case report and review of the literature. Clin Infect Dis. 2000;31(6):1349-56.
[28] Von Baum H, Welte T, Marre R, Suttorp N, Ewig S; CAPNETZ study group. Community-acquired pneumonia through *Enterobacteriaceae* and *Pseudomonas aeruginosa*: diagnosis, incidence and predictors. Eur Respir J. 2010;35(3):598-615.
[29] Sousa C, Botelho C, Rodrigues D, Azeredo J, Oliveira R. Infective endocarditis in intravenous drug abusers: an update. Eur J Clin Microbiol Infect Dis. 2012;31(11):2905-10.
[30] Siva B, Hawley CM, McDonald SP, Brown FG, Rosman JB, Wiggins KJ, et al. *Pseudomonas* peritonitis in Australia: predictors, treatment, and outcomes in 191 cases. Clin J Am Soc Nephrol. 2009;4(5):957–64.
[31] Lui SL, Yip T, Tse KC, Lam MF, Lai KN, Lo WK. Treatment of refractory *Pseudomonas aeruginosa* exit-site infection by simultaneous removal and reinsertion of peritoneal dialysis catheter. Perit Dial Int. 2005;25(6):560-3.
[32] Fichtenbaum CJ, Woeltje KF, Powderly WG. Serious *Pseudomonas aeruginosa* infections in patients infected with human immunodeficiency virus: a case-control study. Clin Infect Dis. 1994;19(3):417-22.
[33] Arancibia F, Bauer TT, Ewig S, Mensa J, Gonzalez J, Niederman MS, et al. Community-acquired pneumonia due to gram-negative bacteria and *Pseudomonas aeruginosa*: incidence, risk, and prognosis. Arch Intern Med. 2002;162(16):1849-58.
[34] Suarez C, Peña C, Arch O, Dominguez MA, Tubau F, Juan C, et al. A large sustained endemic outbreak of multiresistant *Pseudomonas aeruginosa*: a new epidemiological scenario for nosocomial acquisition. BMC Infect Dis. 2011;11():272.
[35] Wunderink RG, Mendoza DL. Epidemiology of *Pseudomonas aeruginosa* in the intensive care unit. In: Rello J, Kollef, M, Díaz E, Rodríguez A, editors. Infectious diseases in critical care. Berlin: Springer-Verlag Berlin Heidelberg; 2007. p. 218-25.
[36] Trautmann M, Lepper PM, Haller M. Ecology of *Pseudomonas aeruginosa* in the intensive care unit and the evolving role of water outlets as a reservoir of the organism. Am J Infect Control. 2005;33(5 Suppl 1):S41-9.
[37] Boyer A, Doussau A, Thiébault R, Venier AG, Tran V, Boulestreau H, et al. *Pseudomonas aeruginosa* acquisition on an intensive care unit: relationship between antibiotic selective pressure and patients' environment. Crit Care. 2011;15(1):R55.
[38] Vitkauskienė A, Skrodenienė E, Dambrauskienė A, Macas A, Sakalauskas R. *Pseudomonas aeruginosa* bacteremia: resistance to antibiotics, risk factors, and patient mortality. Medicina (Kaunas), 2010;46(7):490-5.
[39] Rello J, Lisboa T. Respiratory infections in patients undergoing mechanical ventilation. Lancet Respir Med. 2014;2(9):764–74.
[40] Gurbuz C, Canat L, Atis G, Caskurlu T. Reducing infectious complications after transrectal prostate needle biopsy using a disposable needle guide: is it possible? International Braz J Urol. 2011;37(1):79-86.

© FORMATEX 2015

[41] Classen DC, Jacobson JA, Burke JP, Jacobson JT, Evans RS. Serious *Pseudomonas* infections associated with endoscopic retrograde cholangiopancreatography. Am J Med. 1988;84(3 Pt 2):590-6.
[42] Papagheorghe R Bloodstream infections in immunocompromised hosts. Roum Arch Microbiol Immunol. 2012;71(2):87-94.
[43] Kang CI, Kim SH, Park WB, Lee KD, Kim HB, Kim EC, et al. Bloodstream infections caused by antibiotic-resistant gram-negative bacilli: risk factors for mortality and impact of inappropriate initial antimicrobial therapy on outcome. Antimicrob Agents Chemother. 2005;49(2):760-6.
[44] Joo EJ, Kang CI, Ha YE, Kang SJ, Park SY, Chung DR, et al. Risk factors for mortality in patients with *Pseudomonas aeruginosa* bacteremia: clinical impact of antimicrobial resistance on outcome. Microb Drug Resist. 2011;17(2):305-12.
[45] Gupta V. Metallo beta lactamases in *Pseudomonas aeruginosa* and *Acinetobacter* species. Expert Opin Investig Drugs. 2008;17(2):131-43.
[46] Pai H, Kim JW, Kim J, Lee JH, Choe KW, Gotoh N. Carbapenem resistance mechanisms in *Pseudomonas aeruginosa* clinical isolates. Antimicrob Agents Chemother. 2001;45(2):480-4.
[47] Poole K. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. J Mol Microbiol Biotechnol. 2001;3(2):255-64.
[48] Wei Q, Ma LZ. Biofilm matrix and its regulation in *Pseudomonas aeruginosa*. Int J Mol Sci. 2013;14(10):20983-1005.
[49] Mah TF, Pitts B, Pellock B, Walker GC, Stewart PS, O'Toole GA. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. Nature. 2003;426(6964):306-10.
[50] Yokoyama K, Doi Y, Yamane K, Kurokawa H, Shibata N, Shibayama K, et al. Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*. Lancet. 2003;362(9399):1888-93.
[51] Lee DG, Urbach JM, Wu G, Liberati NT, Feinbaum RL, Miyata S, et al. Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial. Genome Biol. 2006;7(10):R90.
[52] Bleves S, Viarre V, Salacha R, Michel GP, Filloux A, Voulhoux R. Protein secretion systems in *Pseudomonas aeruginosa*: A wealth of pathogenic weapons. Int J Med Microbiol. 2010;300(8):534-43.
[53] Matz C, Moreno AM, Alhede M, Manefield M, Hauser AR, Givskov M, et al. *Pseudomonas aeruginosa* uses type III secretion system to kill biofilm-associated amoebae. ISME J. 2008;2(8):843–52.
[54] Galle M, Carpentier I, Beyaert R. Structure and function of the Type III secretion system of *Pseudomonas aeruginosa*. Curr Protein Pept Sci. 2012;13(8):831-42.
[55] Hauser AR. The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. Nat Rev Microbiol. 2009;7(9):654-65.
[56] Mueller CA, Broz P, Cornelis GR. The type III secretion system tip complex and translocon. Mol Microbiol. 2008;68(5):1085-95.
[57] Audia JP, Lindsey AS, Housley NA, Ochoa CR, Zhou C, Toba M, et al. In the absence of effector proteins, the *Pseudomonas aeruginosa* type three secretion system needle tip complex contributes to lung injury and systemic inflammatory responses. PLoS One. 2013;8(11):e81792.
[58] Yahr TL, Wolfgang MC. Transcriptional regulation of the *Pseudomonas aeruginosa* type III secretion system. Mol Microbiol. 2006;62(3):631–40.
[59] O'Callaghan J, Reen FJ, Adams C, O'Gara F. Low oxygen induces the type III secretion system in *Pseudomonas aeruginosa* via modulation of the small RNAs rsmZ and rsmY. Microbiology. 2011;157(Pt 12):3417-28.
[60] Barison N, Gupta R, Kolbe M. A sophisticated multi-step secretion mechanism: how the type 3 secretion system is regulated. Cell Microbiol. 2013;15(11):1809-17.
[61] Lee PC, Rietsch A. Fueling type III secretion. Trends Microbiol. 2015;23(5):296-300.
[62] Allewelt M, Coleman FT, Grout M, Priebe GP, and Pier GB. Acquisition of expression of the *Pseudomonas aeruginosa* ExoU cytotoxin leads to increased bacterial virulence in a murine model of acute pneumonia and systemic spread. Infect Immun. 2000;68(7): 3998–4004.
[63] Lee EJ, Cowell BA, Evans DJ, Fleiszig SM Contribution of ExsA-regulated factors to corneal infection by cytotoxic and invasive *Pseudomonas aeruginosa* in a murine scarification model. Invest Ophthalmol Vis Sci. 2003;44(9):3892–8.
[64] Shaver CM, Hauser AR. Relative contributions of *Pseudomonas aeruginosa* ExoU, ExoS, and ExoT to virulence in the lung. Infect Immun. 2004;72(12):6969–77.
[65] Shaver CM, Hauser AR. Interactions between effector proteins of the *Pseudomonas aeruginosa* type III secretion system do not significantly affect several measures of disease severity in mammals. Microbiology. 2006;152(Pt 1):143-52.
[66] Lee VT, Smith RS, Tümmler B, Lory S. Activities of *Pseudomonas aeruginosa* effectors secreted by the type III secretion system *in vitro* and during infection. Infect Immun. 2005; 73(3):1695-1705.
[67] Le Berre R, Nguyen S, Nowak E, Kipnis E, Pierre M, Quenee L, et al. Relative contribution of three main virulence factors in *Pseudomonas aeruginosa* pneumonia. Crit Care Med. 2011;39(9):2113-20.
[68] Howell HA, Logan LK, Hauser AR. Type III secretion of ExoU is critical during early *Pseudomonas aeruginosa* pneumonia. MBio. 2013;4(2):e00032-13.
[69] Rangel SM, Logan LK, Hauser AR. The ADP-ribosyltransferase domain of the effector protein ExoS inhibits phagocytosis of *Pseudomonas aeruginosa* during pneumonia. MBio. 2014;5(3):e01080-14.
[70] Roy-Burman A, Savel RH, Racine S, Swanson BL, Revadigar NS, Fujimoto J, et al. Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. J Infect Dis. 2001;183(12):1767–74.
[71] Toska J, Sun Y, Carbonell DA, Foster AN, Jacobs MR, Pearlman E, et al. Diversity of virulence phenotypes among type III secretion negative *Pseudomonas aeruginosa* clinical isolates. PLoS One. 2014;9(1):e86829.
[72] Mikkelsen H, Bond NJ, Skindersoe ME, Givskov M, Lilley KS, Welch M. Biofilms and type III secretion are not mutually exclusive in *Pseudomonas aeruginosa*. Microbiology. 2009;155(Pt 3):687-98.
[73] Feltman H, Schulert G, Khan S, Jain M, Peterson L, Hauser AR. Prevalence of type III secretion genes in clinical and environmental isolates of *Pseudomonas aeruginosa*. Microbiology. 2001;147(Pt 10):2659–69.

 © FORMATEX 2015

[74] Gawish AA, Mohammed NA, El-Shennawy GA, Mohammed HA. An investigation of type 3 secretion toxins encoding-genes of *Pseudomonas aeruginosa* isolates in a University Hospital in Egypt. J Microbiol Infect Dis. 2013;3(3):116-22.
[75] Engel J, Balachandran P. Role of *Pseudomonas aeruginosa* type III effectors in disease. Curr Opin Microbiol. 2009;12(1):61-6.
[76] Finck-Barbancon V, Goranson J, Zhu L, Sawa T, Wiener-Kronish JP, Fleiszig SM, et al. ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. Mol Microbiol. 1997;25(3):547–57.
[77] Fleiszig SM, Wiener-Kronish JP, Miyazaki H, Vallas V, Mostov KE., Kanada D, et al. *Pseudomonas aeruginosa*-mediated cytotoxicity and invasion correlate with distinct genotypes at the loci encoding exoenzyme S. Infect Immun. 1997;65(2):579–86.
[78] Yahr TL, Vallis AJ, Hancock MK, Barbieri JT, Frank DW. ExoY, an adenylate cyclase secreted by the *Pseudomonas aeruginosa* type III system. Proc Natl Acad Sci U S A. 1998;95(23):13899–904.
[79] Sun J, Maresso AW, Kim JJ, Barbieri JT. How bacterial ADP-ribosylating toxins recognize substrates. Nat Struct Mol Biol. 2004;11(9):868-76.
[80] Fu H, Subramanian RR, Masters SC. 14-3-3 proteins: structure, function, and regulation. Annu Rev Pharmacol Toxicol. 2000;40:617-47.
[81] Takai Y, Sasaki T, Matozaki T. Small GTP-binding proteins. Physiol Rev. 2001;81(1):153-208.
[82] Samarin S, Nusrat A. Regulation of epithelial apical junctional complex by Rho family GTPases. Front Biosci (Landmark Ed). 2009;14:1129-42.
[83] Schwartz M. Rho signalling at a glance. J Cell Sci. 2004;117(Pt 23):5457-8.
[84] Etienne-Manneville S, Hall A. Rho GTPases in cell biology. Nature. 2002;420(6916): 629–35.
[85] Caron E, Hall A. Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. Science. 1998; 282(5394):1717-21.
[86] Arpin M, Chirivino D, Naba A, Zwaenepoel I. Emerging role for ERM proteins in cell adhesion and migration. Cell Adh Migr. 2011;5(2):199-206.
[87] Low RB, Woodcock-Mitchell J, Mitchell JJ, Arnold J, Absher PM. Synthesis of cytoskeletal and contractile by cultured IMR-90 fibroblasts proteins. J Cell Biol. 1985;101(2):500-5.
[88] Nigro P, Pompilio G, Capogrossi MC. Cyclophilin A: a key player for human disease. Cell Death Dis. 2013;4:e888.
[89] Frank PG, Marcel YL. Apolipoprotein A-I: structure-function relationships. J Lipid Res. 2000;41(6):853-72.
[90] Munemitsu S, Innis M, Clark R, McCormick F, Ullrich A, Polakis P. Molecular cloning and expression of a G25K cDNA, the human homolog of the yeast cell cycle gene CDC42. Mol Cell Biol. 1990;10(11):5977–82.
[91] Feller SM. Crk family adaptors-signalling complex formation and biological roles. Oncogene. 2001;20(44):6348-71.
[92] Liu D. The adaptor protein Crk in immune response. Immunology and Cell Biology. (2014);92, 80–89.
[93] Rodrigues SP, Fathers KE, Chan G, Zuo D, Halwani F, Meterissian S, et al. CrkI and CrkII function as key signaling integrators for migration and invasion of cancer cells. Mol Cancer Res. 2005;3(4):183-94.
[94] Althaus FR, Richter C. ADP-ribosylation of proteins. Enzymology and biological significance. Mol Biol Biochem Biophys. 1987;37:1-237.
[95] Maresso AW, Deng Q, Pereckas MS, Wakim BT, Barbieri JT. *Pseudomonas aeruginosa* ExoS ADP-ribosyltransferase inhibits ERM phosphorylation. Cell Microbiol. 2007;9(1):97-105.
[96] Sato M, Maruoka M, Takeya T. Functional mechanisms and roles of adaptor proteins in abl-regulated cytoskeletal actin dynamics. J Signal Trans. 2012;2012:Article ID414913, 13 p.
[97] Chen CS, Alonso JL, Ostuni E, Whitesides GM, Ingber DE. Cell shape provides global control of focal adhesion assembly. Biochem Biophys Res Commun. 2003;307(2):355–61.
[98] Sun J, Barbieri JT. *Pseudomonas aeruginosa* ExoT ADPribosylates CT10 regulator of kinase (Crk) proteins. J Biol Chem. 2003;278(35):32794-800.
[99] Deng Q, Sun J, Barbieri JT. Uncoupling Crk signal transduction by *Pseudomonas* exoenzyme T. J Biol Chem. 2005;280(43):35953-60.
[100] Shafikhani SH, Engel J. *Pseudomonas aeruginosa* type III secreted toxin ExoT inhibits host-cell division by targeting cytokinesis at multiple steps. Proc Natl Acad Sci U S A. 2006;103(42): 15605–10.
[101] Garrity-Ryan L, Shafikhani S, Balachandran P, Nguyen L, Oza J, Jakobsen T, et al. The ADP ribosyltransferase domain of *Pseudomonas aeruginosa* ExoT contributes to its biological activities. Infect Immun. 2004;72(1):546-58.
[102] Shafikhani SH, Morales C, Engel J. The *Pseudomonas aeruginosa* type III secreted toxin ExoT is necessary and sufficient to induce apoptosis in epithelial cells. Cell Microbiol. 2008;10(4):994-1007.
[103] Bridge DR, Martin KH, Moore ER, Lee WM, Carroll JA, Rocha CL, Olson JC. Examining the role of actin-plasma membrane association in *Pseudomonas aeruginosa* infection and type III secretion translocation in migratory T24 epithelial cells. Infect Immun. 2012;80(9):3049-64.
[104] Heimer SR, Evans DJ, Stern ME, Barbieri JT, Yahr T, Fleiszig SM. *Pseudomonas aeruginosa* utilizes the type III secreted toxin ExoS to avoid acidified compartments within epithelial cells. PLoS One. 2013;8(9):e73111.
[105] Hritonenko V, Evans DJ, Fleiszig SM. Translocon-independent intracellular replication by *Pseudomonas aeruginosa* requires the ADP-ribosylation domain of ExoS. Microbes Infect. 2012;14(15):1366-73.
[106] Sun Y, Karmakar M, Taylor PR, Rietsch A, Pearlman E. ExoS and ExoT ADP ribosyltransferase activities mediate *Pseudomonas aeruginosa* keratitis by promoting neutrophil apoptosis and bacterial survival. J Immunol. 2012;188(4):1884-95.
[107] Wood S, Goldufsky J, Shafikhani SH. *Pseudomonas aeruginosa* ExoT Induces Atypical Anoikis Apoptosis in Target Host Cells by Transforming Crk Adaptor Protein into a Cytotoxin. PLoS Pathog. 2015;11(5):e1004934.
[108] Bruno TF, Woods DE, Storey DG, Mody CH. Recombinant *Pseudomonas* exoenzyme S and exoenzyme S from *Pseudomonas aeruginosa* DG1 share the ability to stimulate T lymphocyte proliferation. Can J Microbiol. 1999;45():607-11.
[109] Epelman S, Neely GG, Ma LL, Gjomarkaj M, Pace E, Melis M et al. Distinct fates of monocytes and T cells directly activated by *Pseudomonas aeruginosa* exoenzyme S. J Leukoc Biol. 2002;71(3):458-68.

© FORMATEX 2015

[110] Bruno TF, Woods DE, Mody CH. Exoenzyme S from *Pseudomonas aeruginosa* induces apoptosis in T lymphocytes. J Leukoc Biol. 2000;67(6):808-16.
[111] Epelman S, Bruno TF, Neely GG, Woods DE, Mody CH. *Pseudomonas aeruginosa* exoenzyme S induces transcriptional expression of proinflammatory cytokines and chemokines. Infect Immun. 2000;68(8):4811-4.
[112] Epelman S, Stack D, Bell C, Wong E, Neely GG, Krutzik S, et al. Different domains of *Pseudomonas aeruginosa* exoenzyme S activate distinct TLRs. J Immunol. 2004;173(3):2031-40.
[113] Galle M, Schotte P, Haegman M, Wullaert A, Yang HJ, Jin S, et al. The *Pseudomonas aeruginosa* Type III secretion system plays a dual role in the regulation of caspase-1 mediated IL-1beta maturation. J Cell Mol Med. 2008;12(5a):1767-76.
[114] Okuda J, Hayashi N, Okamoto M, Sawada S, Minagawa S, Yano Y, et al. Translocation of *Pseudomonas aeruginosa* from the intestinal tract is mediated by the binding of ExoS to an Na,K-ATPase regulator, FXYD3. Infect Immun. 2010;78(11):4511-22.
[115] Rangel SM, Diaz MH, Knoten CA, Zhang A, Hauser AR. The role of ExoS in dissemination of *Pseudomonas aeruginosa* during pneumonia. PLoS Pathog. 2015;11(6):e1004945.
[116] Goldufsky J, Wood S, Hajihossainlou B, Rehman T, Majdobeh O, Kaufman HL, et al. *Pseudomonas aeruginosa* exotoxin T induces potent cytotoxicity against a variety of murine and human cancer cell lines. J Med Microbiol. 2015;64(Pt 2):164-73.
[117] Gendrin C, Contreras-Martel C, Bouillot S, Elsen S, Lemaire D, Skoufias DA, et al. Structural basis of cytotoxicity mediated by the type III secretion toxin ExoU from *Pseudomonas aeruginosa*. PLoS Pathog. 2012;8(4):e1002637.
[118] Sato H, Frank DW. ExoU is a potent intracellular phospholipase. Mol Microbiol. 2004;53(5):1279–90.
[119] Rabin SDP, Hauser AR. Functional regions of the *Pseudomonas aeruginosa* cytotoxin ExoU. Infect Immun. 2005;73(1):573–82.
[120] Phillips RM, Six DA, Dennis EA, Ghosh P. In vivo phospholipase activity of the *Pseudomonas aeruginosa* cytotoxin ExoU and protection of mammalian cells with phospholipase A2 inhibitors. J Biol Chem. 2003;278(42): 41326–32.
[121] Murakami M, Taketomi Y, Sato H, Yamamoto K. Secreted phospholipase A2 revisited. J Biochem. 2011;150(3):233–55.
[122] Ghosh M, Tucker DE, Burchett SA, Leslie CC. Properties of the group IV phospholipase A2 family. Prog Lipid Res. 2006;45(6):487–510.
[123] Burke JE, Dennis EA Phospholipase A2 structure/function, mechanism, and signaling. J Lipid Res. 2009;50(Suppl):S237–42.
[124] Lundbaek JA, Andersen OS. Lysophospholipids modulate channel function by altering the mechanical properties of lipid bilayers. J Gen Physiol. 1994;104(4):645-73.
[125] Sato H, Feix JB, Frank DW. Identification of superoxide dismutase as a cofactor for the *Pseudomonas* type III toxin, ExoU. Biochemistry. 2006;45(34):10368-75.
[126] Anderson DM, Schmalzer KM, Sato H, Casey M, Terhune SS, Haas AL et al. Ubiquitin and ubiquitin-modified proteins activate the *Pseudomonas aeruginosa* T3SS cytotoxin, ExoU. Mol Microbiol. 2011;82(6):1454-67.
[127] Anderson DM, Feix JB, Monroe AL, Peterson FC, Volkman BF, Haas AL et al. Identification of the major ubiquitin-binding domain of the *Pseudomonas aeruginosa* ExoU A2 phospholipase. J Biol Chem. 2013;288(37):26741-52.
[128] Stirling FR, Cuzick A, Kelly SM, Oxley D, Evans THE. Eukaryotic localization, activation and ubiquitinylation of a bacterial type III secreted toxin. Cell Microbiol. 2006;8(8):1294–1309.
[129] Tyson GH, Hauser AR. Phosphatidylinositol 4,5-bisphosphate is a novel coactivator of the *Pseudomonas aeruginosa* cytotoxin ExoU. Infect Immun. 2013;81(8):2873-81.
[130] Sato H, Frank DW. Intoxication of host cells by the T3SS phospholipase ExoU: PI(4,5)P2-associated, cytoskeletal collapse and late phase membrane blebbing. PLoS One. 2014;9(7):e103127.
[131] Pankhaniya RR, Tamura M, Allmond LR, Moriyama K, Ajayi T, Wiener-Kronish JP, et al. *Pseudomonas aeruginosa* causes acute lung injury via the catalytic activity of the patatin-like phospholipase domain of ExoU. Crit Care Med. 2004;32(11):2293-9.
[132] Cowell BA, Evans DJ, Fleiszig SM. Actin cytoskeleton disruption by ExoY and its effects on *Pseudomonas aeruginosa* invasion. FEMS Microbiol. Lett. 2005;250():71–6.
[133] Sayner SL, Frank DW, King J, Chen H, VandeWaa J, Stevens T. Paradoxical cAMP-induced lung endothelial hyperpermeability revealed by *Pseudomonas aeruginosa* ExoY. Circ Res. 2004;95():196–203.
[134] Ahuja N, Kumar P, Bhatnagar R. The adenylate cyclase toxins. Crit Rev Microbiol. 2004;30(3):187-96.
[135] Stevens TC, Ochoa CD, Morrow KA, Robson MJ, Prasain N, Zhou C, et al. The *Pseudomonas aeruginosa* exoenzyme Y impairs endothelial cell proliferation and vascular repair following lung injury. Am J Physiol Lung Cell Mol Physiol. 2014;306(10):L915-24.
[136] Sayner SL. Emerging themes of cAMP regulation of the pulmonary endothelial barrier. Am J Physiol Lung Cell Mol Physiol. 2011;300(5):L667-78.
[137] Cheng X, Ji Z, Tsalkova T, Mei F. Epac and PKA: a tale of two intracellular cAMP receptors. Acta Biochim Biophys Sin (Shanghai). 2008;40(7):651–62.
[138] Ochoa CD, Alexeyev M, Pastukh V, Balczon R, Stevens T. *Pseudomonas aeruginosa* exotoxin Y is a promiscuous cyclase that increases endothelial tau phosphorylation and permeability. J Biol Chem. 2012;287(30): 25407–18.
[139] Balczon R, Prasain N, Ochoa C, Prater J, Zhu B, Alexeyev M, et al. *Pseudomonas aeruginosa* exotoxin Y-mediated tau hyperphosphorylation impairs microtubule assembly in pulmonary microvascular endothelial cells. PLoS One. 2013;8(9):e74343.
[140] Kurahashi K, Kajikawa O, Sawa T, Ohara M, Gropper MA, Frank DW, et al. Pathogenesis of septic shock in *Pseudomonas aeruginosa* pneumonia. J Clin Invest. 1999;104(6):743-50.
[141] Diaz MH, Shaver CM, King JD, Musunuri S, Kazzaz JA, Hauser AR. *Pseudomonas aeruginosa* induces localized immunosuppression during pneumonia. Infect Immun. 2008;76(10):4414-421.

 © FORMATEX 2015