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Adenylate cyclase activity of *Pseudomonas aeruginosa* ExoY can mediate bleb-niche formation in epithelial cells and contributes to virulence

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ABSTRACT

We previously showed that ADP-ribosylation (ADP-r) activity of ExoS, a type III secreted toxin of *Pseudomonas aeruginosa*, enables bacterial replication in corneal and respiratory epithelial cells and correlates with bacterial trafficking to plasma membrane blebs (bleb-niche formation). Here, we explored another type III secreted toxin, ExoY, for its impact on intracellular trafficking and survival, and for virulence *in vivo* using a murine corneal infection model. Chromosomal or plasmid-mediated expression of *exoY* in invasive *P. aeruginosa* (strain PAO1) enabled bacteria to form and traffic to epithelial membrane blebs in the absence of other known effectors. In contrast, plasmid expression of any of four adenylate cyclase mutant forms of *exoY* did not enable bleb-niche formation, and bacteria localized to perinuclear vacuoles as for effector-null mutant controls. None of the plasmid-complemented bacteria used in this study showed ADP-r activity in the absence of ExoS and ExoT. In contrast to ADP-r activity of ExoS, bleb-niche formation induced by ExoY's adenylate cyclase activity was not accompanied by enhanced intracellular replication. *In vivo* results showed that ExoY-adenylate cyclase activity promoted *P. aeruginosa* corneal virulence in susceptible mice. Together the data show that adenylate cyclase activity of *P. aeruginosa* ExoY, similarly to the ADP-r activity of ExoS, can mediate bleb-niche formation in epithelial cells. While this activity did not promote intracellular replication *in vitro*, ExoY conferred increased virulence *in vivo* in susceptible mice. Mechanisms for bleb-niche formation and relationships to intracellular replication and virulence *in vivo* require further investigation for both ExoS and ExoY.

1. Introduction

Pseudomonas aeruginosa is a versatile opportunistic pathogen ubiquitously present in water and soil and is recognized as a leading cause of nosocomial pneumonia, respiratory infection in cystic fibrosis, burn wound and urinary catheter infections, and ocular infections (e.g. microbial keratitis) [1–4]. While the pathogenesis of *P. aeruginosa* infection is complex, involving multiple virulence mechanisms, many recent studies have shown the importance of Type III Secretion System (T3S) in virulence through the manipulation of mammalian cell function [5,6].

For the *P. aeruginosa* T3S system, four known effector proteins are delivered into host cells to modify cell function and/or viability; ExoS, ExoT, ExoU, and ExoY. Both ExoS and ExoT have dual enzymatic activity: N-terminal Rho-GTPase activating protein activity and C-terminal ADP ribosyl-transferase activity which exert cytopathic effects on host cells [7], and demonstrated contributions to virulence [8,9]. The only known activity of ExoY is adenylate cyclase activity [10]. The virulence contributions are unclear even though ExoY causes the retraction and rounding of host cells [11,12], and inhibits epithelial cell invasion by *P. aeruginosa* [13]. ExoU is a powerful phospholipase which kills epithelial cells [14,15], and also contributes to virulence [9,16,17], but is not encoded by invasive *P. aeruginosa* that survive intracellularly such as strain PAO1 [15,18].

Recently we reported that strains of *P. aeruginosa* expressing ExoS, ExoT and ExoY induce the formation of membrane bleb-niches in epithelial cells correlating with bacterial intracellular survival and replication [19]. Without these effectors, intracellular

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bacteria traffic to perinuclear vacuoles which label with the late endosomal marker LAMP-3, and do not thrive [19], suggesting a "default" trafficking pathway exists that inhibits *P. aeruginosa* replication, from which bacteria must escape to survive and then presumably traffic to bleb-niches. Subsequently, we showed that the ADP-ribosylation domain of ExoS was sufficient to enable both bleb-niche formation and intracellular survival [20]. However, we also noted in that study that mutants in both *exoS* and *exoT* (i.e. expressing only ExoY) conferred a low level capacity to form bleb-niches, particularly if the incubation time was extended [20].

Here, we tested the hypothesis that the adenylate cyclase activity of ExoY could mediate bleb-niche formation and intracellular survival/replication in epithelial cells, and could contribute to virulence *in vivo* in the absence of other known T3S system effectors. The results showed roles for the adenylate cyclase activity in bleb-niche formation in epithelial cells *in vitro* and in virulence *in vivo*, but not in intracellular survival in cultured epithelial cells, suggesting that these capacities are not always related.

2. Results

2.1. P. aeruginosa ExoY mediates bleb-niche formation in corneal epithelial cells

Real time phase-contrast microscopy was used to compare bleb-niche formation in cultured human corneal epithelial cells (hTCEpi) after 8 h of infection with wild-type *P. aeruginosa strain* PAO1, an isogenic effector-null mutant (PAO1Δ*exoS*Δ*exoT*Δ*exoY*), and a double effector mutant that expresses ExoY, but not ExoS or ExoT (PAO1Δ*exoS*Δ*exoT*Δ) (Fig. 1). Uninfected epithelial cells appeared healthy with small perinuclear vacuoles present in their cytoplasm (Fig. 1A). As expected from previous studies [19], cells infected with wild-type *P. aeruginosa*, displayed numerous membrane bleb-niches containing motile bacteria swimming at a speed observable in real time (Fig. 1B). Cells infected with the triple (known effector-null) mutant or PAO1Δ*exoS*Δ*exoY* (expresses only ExoT) showed no bleb-niche formation with bacteria localized to perinuclear vacuoles (Fig. 1C, data not shown, respectively). In contrast, cells infected with *P. aeruginosa* mutants expressing only ExoY (mutants in both *exoS* and *exoT*) showed a combination of the above phenotypes; with some bacteria localized within perinuclear vacuoles (Fig. 1D), and others demonstrating real-time observable swimming motility in plasma membrane blebs (Fig. 1E). Thus ExoY, in the absence of ExoS or ExoT, can enable *P. aeruginosa* to form and traffic to bleb-niches.

2.2. ExoY mediation of bleb-niche formation requires adenylate cyclase activity

The only known enzymatic action of ExoY is adenylate cyclase (AC) activity. To determine if AC activity is required for ExoY to induce membrane bleb-niche formation, triple effector mutants of *P. aeruginosa* (PAO1Δ*exoS*Δ*exoT*Δ*exoY*) each complemented with one of four different mutants forms of *exoY* lacking adenylate cyclase activity (pUCP*exoY*K81M, pUCP*exoY*K88I, pUCP*exoY*D212N and pUCP*ExoY*D214N) were compared to mutants complemented with native adenylate cyclase active *exoY* (pUCP*exoY*) (Fig. 2). As expected numerous bleb-niches were observed in cells infected with bacteria complemented with the native form of *exoY* (Fig. 2C and H, middle panel). In contrast, cells infected with bacteria expressing adenylate cyclase inactive forms of ExoY did not display bleb-niches containing bacteria (Fig. 2H, middle panel), and intracellular bacteria were found to be in perinuclear vacuoles, similar to the cells infected with the vector control (Fig. 2D–G and H, right panel). Quantitative differences between *exoY*-complemented bacteria and *exoY*-adenylate cyclase domain mutant-

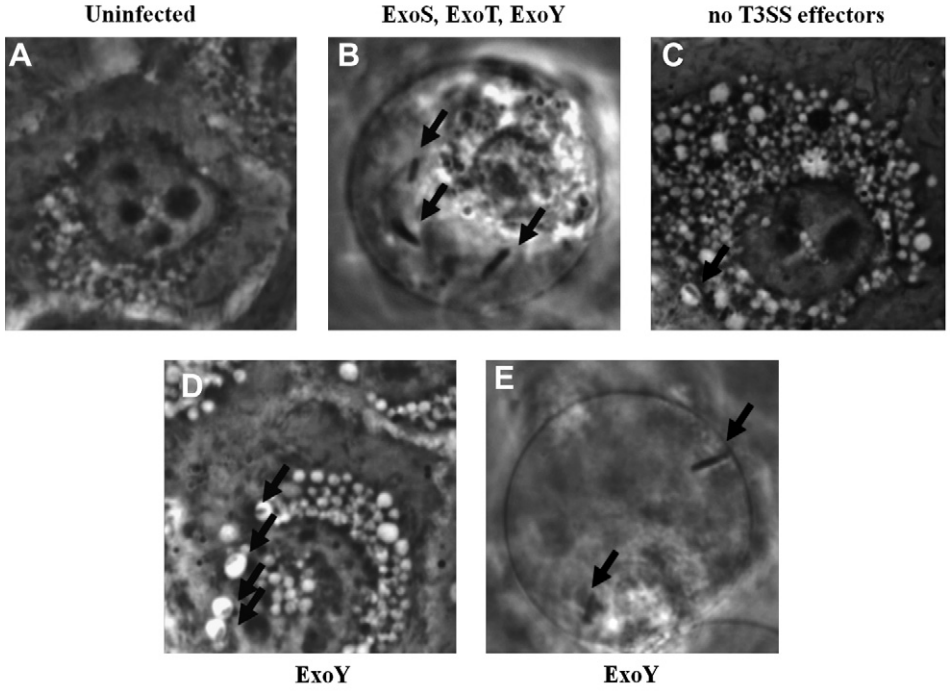

Fig. 1. Phase-contrast microscopy of human corneal epithelial cells showing the intracellular localization of *P. aeruginosa* PAO1 versus various T3SS mutants at 8 h after inoculation with ~2×10^7 cfu bacteria. (A) Uninfected cells. (B) Wild-type PAO1 (positive control expressing ExoS, ExoT and ExoY) localized to bleb-niches as expected (arrows). (C) A negative control mutant lacking all known effectors (PAO1Δ*exoS*Δ*exoT*Δ*exoY*) localized to perinuclear vacuoles. (D and E) Bacteria expressing only ExoY (PAO1Δ*exoS*Δ*exoT*) displayed both phenotypes, i.e. bacteria were found in both perinuclear vacuoles (D) and bleb-niches (E). Magnification 1000×. Images representative of three independent experiments.