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Epithelial cell lysates induce ExoS expression and secretion by *Pseudomonas aeruginosa*

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One sentence summary: Lysed epithelial cells induce expression of the *Pseudomonas aeruginosa* type three secretion system.

Editor: Kendra Rumbaugh

ABSTRACT

The type three secretion system (T3SS) is important for the intracellular survival of *Pseudomonas aeruginosa*. Known T3SS inducers include low Ca^{2+}, serum or host cell contact. Here, we used corneal epithelial cell lysates to test if host cytosolic factors could also induce the T3SS. Invasive *P. aeruginosa* strain PAO1 was exposed to cell lysates for 16 h, and expression of T3SS effectors determined by q-PCR and Western immunoblot. Lysate exposure reduced PAO1 growth (~5-fold) versus trypticase soy broth (TSB), but also resulted in appearance of a protein in culture supernatants, but not bacterial cell pellets, which reacted with antibody raised against ExoS. T3SS-inducing media (TSBi) caused the expression and secretion of ExoS and ExoT. Heat-treated lysates induced the protein; 1:3 diluted lysates did not. The protein that bound anti-ExoS antibody was found in supernatants of lysate-exposed *exoT* mutants, but not *exoS* or *pscC* mutants, suggesting a secreted form of ExoS, albeit slightly larger than that induced by TSBi. Lysate-exposed strain PAK expressed the same protein. Lysates caused PAO1 *exoS* and *exoT* gene expression, but only ~20% and ~6% of TSBi, respectively. T3SS induction by epithelial cell lysates could help explain T3SS expression by internalized *P. aeruginosa*.

Keywords: *P. aeruginosa*; type three secretion system; epithelial cells; lysates; ExoS; ExoT

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic bacterial pathogen capable of infecting multiple tissues and organ systems including respiratory and urinary tracts, burn wounds, blood (bacteremia) and the cornea (Evans, McNamara and Fleiszig 2007; Juan, Pena and Oliver 2017; Klockgether and Tummler 2017; Newman, Floyd and Fothergill 2017). The Type 3 Secretion System (T3SS) is important for the virulence of *P. aeruginosa*, and a promising target for new therapies (Hauser 2009; Anantharajah, Mingeot-Leclercq and Van Bambeke 2016; Juan, Pena

and Oliver 2017). The T3SS encodes a protein nano-syringe capable of injecting four effectors (ExoS, ExoT, ExoU, ExoY) directly into host cells. ExoS and ExoT share sequence homology and enzymatic activities (Rho-GAP and ADP-ribosyltransferase [ADPr] activity), which exert cytopathic effects on host cells and contribute to virulence (Lee *et al.* 2003, 2005; Barbieri and Sun 2004; Vance, Rietsch and Mekalanos 2005; Deng and Barbieri 2008). Other effectors also contribute to virulence. ExoY, an adenylate cyclase, modulates the actin cytoskeleton (Cowell, Evans and Fleiszig 2005; Hritonenko *et al.* 2011), and reduces host inflammatory responses (He *et al.* 2017; Jeon *et al.* 2017). ExoU exerts

Table 1. List of *P. aeruginosa* strains and T3SS mutants used.

Strain or plasmid	Relevant description	Source (citation)
PAO1	Wild-type *P. aeruginosa*. Expresses ExoS, ExoT, ExoY (invasive strain)	Dr Arne Rietsch, Case Western Reserve University (Vance, Rietsch and Mekalanos 2005)
PAO1Δ*pscC*	T3SS needle mutant. Unable to secrete effectors out of bacterial cells	
PAO1Δ*exoS*	Expresses ExoT, ExoY	
PAO1Δ*exoT*	Expresses ExoS, ExoY	
PAO1Δ*exoS*Δ*exoT*	Expresses ExoY	
PAK	Wild-type *P. aeruginosa*. Expresses ExoS, ExoT, ExoY (invasive strain)	Dr Stephen Lory, Harvard Medical School (Lee *et al.* 2005)
PAKΔ*exoS*	Expresses ExoT, ExoY	
PAKΔ*exoT*	Expresses ExoS, ExoY	
PAKΔ*exoS*Δ*exoT*	Expresses ExoY	

potent phospholipase activity causing acute cytotoxicity (Finck-Barbancon *et al.* 1997; Sato and Frank 2004), but is not encoded by invasive strains of *P. aeruginosa*, e.g. PAO1 or PAK (Fleiszig *et al.* 1997) which instead use ExoS to modulate host cell function.

Previously, we showed that *P. aeruginosa* uses the T3SS to survive and replicate inside host cells. Primarily ExoS mediates formation and bacterial occupation of membrane bleb-niches via its ADPr activity (Angus *et al.* 2008, 2010; Hritonenko, Evans and Fleiszig 2012). Intracellular *P. aeruginosa* can replicate, show swimming motility and express the T3SS within these blebs (Heimer *et al.* 2013), whose formation is osmotically driven (Jolly *et al.* 2015). Without a T3SS translocon, bacteria remain within perinuclear vacuoles (Angus *et al.* 2008), but can still use ExoS to survive and replicate intracellularly (Hritonenko, Evans and Fleiszig 2012). Indeed, vacuoles containing ExoS-expressing translocon mutants do not label with the late endosomal marker LAMP3 (Angus *et al.* 2008), and show reduced acidification (Heimer *et al.* 2013).

While the T3SS is clearly important for intracellular survival of *P. aeruginosa*, it remains unclear if the system is activated extracellularly or intracellularly or both in that context. Known triggers of the T3SS include bacterial exposure to low levels of divalent cations (e.g. low Ca^{2+}), serum and host cell contact (Iglewski *et al.* 1978; Vallis *et al.* 1999; Dasgupta *et al.* 2006). Here, we tested if exposure to the cytosol of host cells could induce the T3SS, by exposing invasive strains of *P. aeruginosa*, and their *exoS* and *exoT* mutants, to lysates of human corneal epithelial cells.

MATERIALS AND METHODS
Cell culture and lysate preparation

Human telomerase-immortalized corneal epithelial cells (Robertson *et al.* 2005) were grown at 37°C in 5% CO_2 in 75 mm plastic flasks with vented caps using serum-free keratinocyte growth medium (KGM-2) (Lonza, MD) supplemented with antibiotics (gentamicin, streptomycin, penicillin, fungizone) as previously described (Hritonenko, Evans and Fleiszig 2012). To prepare lysates, cells were grown on 12-well tissue-culture treated plates until confluent, then after three washes with phosphate-buffered saline (PBS) to remove growth medium, cells were lysed in PBS by three repeated freeze-thaw cycles (–80°C for 10 min, 5 min on ice). Cellular debris was removed by centrifugation (12 000× *g*, 2 min), and supernatant (cell lysate) used for experiments. Cell lysates were freshly prepared for each experiment.

Bacterial strains

Pseudomonas aeruginosa strains PAO1 and PAK were used along with their respective mutants in *exoS* (Δ*exoS*), *exoT* (Δ*exoT*) or both genes (Δ*exoS*Δ*exoT*), and a *pscC* mutant of PAO1 (Table 1). Bacteria were cultured on trypticase soy agar plates overnight at 37°C, and then resuspended into trypticase soy broth (TSB) to a concentration of ∼10^8 CFU/mL (absorbance at 650 nm of 0.1). Cell lysates were then inoculated with bacteria at a starting concentration of ∼10^3 CFU/mL, then incubated for 16 h at 37°C (3 mL volume, without shaking). TSB and KGM-2 were used as negative (non-inducing) controls, and T3SS-inducing medium (TSBi) as a positive control. TSBi consisted of TSB supplemented with 50 mM monosodium glutamate, 1% glycerol and 100 mM EGTA (pH adjusted to 7.0 with NaOH). In control experiments using *P. aeruginosa* strain PAO1, 16 h of growth in cell lysates at 37°C resulted in ∼10^8 CFU/mL, ∼5-fold lower on average than that found in TSB (or TSBi) (data not shown). This information was used to standardize bacterial numbers in other experiments.

Western immunoblot

After 16 h incubation with cell lysate, TSB, or TSBi, bacteria were pelleted by centrifugation (12 000× *g*, 5 min), and the supernatants and pellets examined for the presence of ExoS and ExoT by Western immunoblot. Samples were standardized according to bacterial numbers, and denatured in 2× SDS-PAGE sample buffer (Bio-Rad, CA), prior to resolving by SDS-PAGE (Bio-Rad). Proteins were transferred to nitrocellulose membranes using a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad). After blocking for 1 h with 5% skimmed milk in PBS, membranes were probed overnight at 4°C with polyclonal rabbit anti-ExoS affinity-purified antibody diluted 1:1000 in 5% skimmed milk in PBS. The anti-ExoS antibody was custom-made by New England Peptide (Gardner, MA) using a synthesized peptide corresponding to part of the ExoS ADP-r domain (amino acids 424 to 438). After three washes with PBS-Tween (0.05%), HRP-conjugated goat anti-rabbit secondary antibody (Bio-Rad) and chemiluminescence were used for detection of bound primary antibody. Experiments were repeated at least twice.

Amino acid homology

Alignment and comparison of amino acid homology between ExoS (424–438) and ExoT was performed using EMBOSS water (https://www.ebi.ac.uk/Tools/psa/emboss_water/), with default
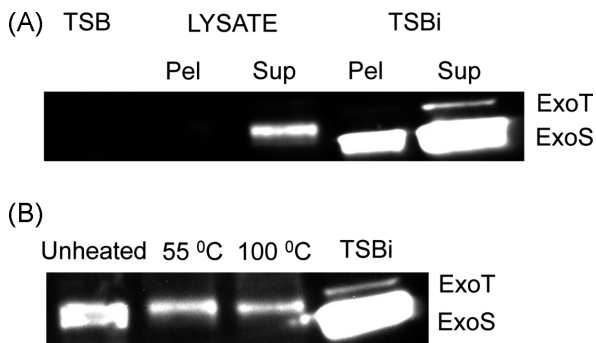
Figure 1. Western immunoblots of *P. aeruginosa* culture supernatants (Sup) and bacterial cell pellets (Pel) after growth for 16 h in TSB, corneal cell lysates or TSBi (**A**) *P. aeruginosa* PAO1 growth in corneal cell lysates resulted in the appearance of a protein in the culture supernatant recognized by anti-ExoS antibody. The protein was not associated with bacterial cell pellets. Growth in TSBi, but not TSB, resulted in detection of both secreted and bacterial cell-associated ExoS and ExoT. (**B**) Growth of *P. aeruginosa* PAO1 in heat-treated cell lysates (55°C 1 h, 100°C, 5 min) resulted in the continued appearance of the protein in culture supernatants. Gel loading was normalized to the number of bacteria present.

parameters, from EMBL-EBI (The European Bioinformatics Institute).

RNA extraction and real-time quantitative PCR analysis

Bacteria were collected by centrifugation at $12\,000\times g$ for 5 min at 4°C, and then resuspended in 800 μl of TRIzol (Invitrogen, CA). RNA was extracted with a Direct-zol RNA miniprep kit (Zymo Research, CA) according to manufacturer's instructions. RNA samples were treated with DNase (Roche, CA) for 2 h at 37°C, and further purified using the Direct-zol RNA miniprep kit (Zymo Research), including a second DNase treatment on the column for 30 min at room temperature, according to manufacturer's instructions. The cDNA was prepared using iScript reverse transcription supermix (Bio-Rad) with 1 μg of total RNA per reaction mixture. Real-time quantitative PCR (qRT-PCR) was performed on 50 ng of cDNA, amplified using LightCycler 96 DNA SYBR green I master mix (Roche). Specific primers for *exoT* (*exoT*-rtF: CGG TAG AGA GCG AGG TAA AGG, *exoT*-rtR: TAT AGA GAC CGA GCG CCA TC) and *exoS* (*exoS*-rtF: TCT CTA CAC CGG CAT TCA CTA C, *exoS*-rtR: CCT TGG TCG ATC AGC TTT TG) were designed using primer3 plus, and reactions monitored using a LightCycler 96 instrument and software (Roche). Transcript amounts under each condition were standardized to transcription level of an internal control gene [*proC* (Savli *et al.* 2003)], and compared with standardized expression in PAO1 grown in TSB (relative quantification, $\Delta\Delta$Cq method). Data were expressed as mean $\pm$ standard deviation (SD), and significance of differences between groups assessed with Student's t-Test. *P* values < 0.05 were considered significant. Experiments were repeated at least twice.

RESULTS

Pseudomonas aeruginosa growth in cell lysates induces secretion of a protein that binds anti-ExoS antibody

Growth of *P. aeruginosa* strain PAO1 for 16 h in epithelial cell lysates resulted in the appearance of a protein in culture supernatants that was detected with anti-ExoS antibody by Western immunoblot (Fig. 1A). The protein was not detected after bacterial growth in normal TSB (non-inducing media), or KGM-2 (not shown), and appeared slightly larger than ExoS

($\sim$49 kDa) found in both culture supernatant and bacterial pellets of bacteria grown in T3SS-inducing medium (TSBi) (Fig. 1A). Interestingly, the protein was not associated with bacterial cell pellets.

Growth in TSBi also appeared to result in the detection of ExoT ($\sim$53 kDa) in the culture supernatants (and very faintly in cell pellets) by the anti-ExoS antibody. ExoS and ExoT exhibit $\sim$76% amino acid sequence homology, although their ADPr domains differ (Barbieri and Sun 2004). The amino acid sequence of ExoS from 424–438 (used to generate the anti-ExoS antibody) was aligned and compared to ExoT, and showed 64.3% identity and 85.7% similarity. Thus, it was quite feasible that the anti-ExoS antibody could also detect ExoT.

Dilution of lysates (1:3), or storage at 4°C for 1 h, abolished expression of the protein that bound anti-ExoS antibody (data not shown), although the protein was still present when PAO1 was grown in lysates previously heated at 55°C for 1 h, or 100°C for 5 min (Fig. 1B), suggesting the involvement of a heat-stable host factor(s). In other experiments, prior to preparation of lysates, human corneal epithelial cells were pretreated for up to 12 h with bacterial antigens in sterile culture supernatants (prepared by filtration of overnight cultures of PAO1 in TSB). Antigen-pretreated lysates also induced the protein, but at a similar level to untreated lysates (data not shown).

Lysate-induction of the protein binding anti-ExoS antibody in culture supernatants of *P. aeruginosa exoT* mutants, but not *exoS* or *pscC* mutants, suggests a secreted form of ExoS

Gene knockout (deletion) mutants in *exoS* or *exoT* were used to help identify the protein expressed by *P. aeruginsa* strain PAO1 after exposure to cell lysates that bound anti-ExoS antibody. Effector mutants in another invasive strain PAK were also tested. After 16 h growth in cell lysates, the protein reactive with ExoS antibody was detected in culture supernatants, but not bacterial pellets, of *exoT* mutants and wild-type PAO1, but not at all for *exoS* mutants (Fig. 2A). The same result was found for culture supernatants of strain PAK and its effector mutants after 16 h growth in cell lysates (Fig. 2B), showing that PAO1 is not unusual in its response to the epithelial cell lysates. These data suggest that the protein represents a secreted form of ExoS albeit slightly larger than that induced by TSBi. Absence of this form of ExoS in culture supernatnans of a *pscC* mutant of PAO1 after lysate growth (Fig. 2C) suggested that the T3SS needle was required for its secretion, as for TSBi-induced ExoS secretion (Fig. 2D). As observed for wild-type bacteria and their *exoT* mutants, however, lysate growth did not result in pellet-associated ExoS in the *pscC* mutant. It was also observed that pellet-associated ExoS did not appear to accumulate in the *pscC* mutant after TSBi induction (Fig. 2D), suggesting that the T3SS needle may be needed for *exoS* expression.

Epithelial cell lysates induce *exoS* and *exoT* gene transcription in *P. aeruginosa* PAO1

Next, qPCR was used to determine the impact of the cell lysates on expression of *exoS* and *exoT* genes in strain PAO1 compared to TSB and TSBi controls (Table 2). Consistent with the results of Western immunoblot experiments, exposure to cell lysates for 16 h was found to induce expression of the *exoS* gene in PAO1 at $\sim$20% of levels triggered by the TSBi positive control. Although ExoT protein was not detected in culture supernatants of PAO1
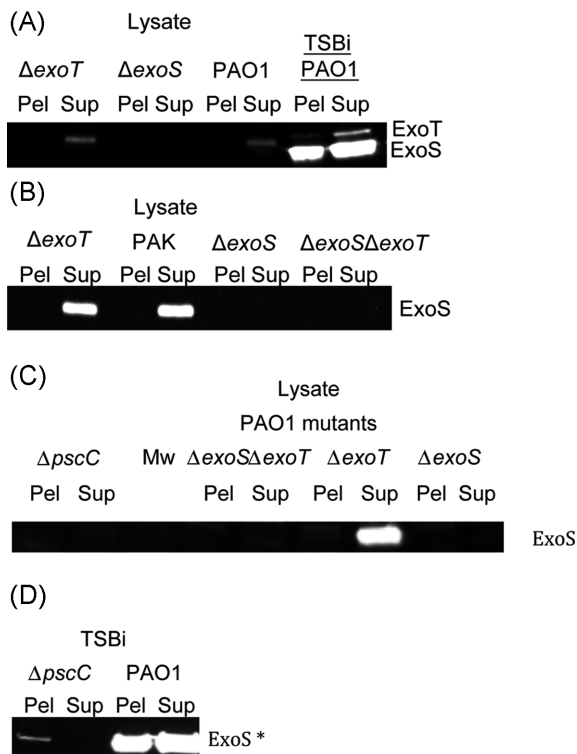
Figure 2. Western immunoblots of *P. aeruginosa* culture supernatants (Sup) and bacterial cell pellets (Pel) for (**A**) PAO1 and (**B**) PAK and their respective *exoS* and *exoT* mutants, and (**C, D**) PAO1 and its *pscC, exoS, exoT* and *exoSexoT* mutants after 16 h growth in corneal cell lysates or TSBi. Growth of PAO1 or PAK or their *exoT* mutants in corneal cell lysates induced the appearance of the protein recognized by anti-ExoS antibody, but only in culture supernatants. The protein was absent from culture supernatants and pellets of *exoS* mutants of each strain suggesting that it represented a form of ExoS. However, the protein was slightly larger than that induced in TSBi for which ExoT was also detected. This form of ExoS was not observed in lysate-grown in *pscC* mutants. TSBi induction was associated with a small amount of ExoS expression, but not secretion, in *pscC* mutants, and ExoT was not observed. TSBi induced both ExoS and ExoT expression and secretion in PAO1. Gel loading was normalized to the number of bacteria present. *ExoT merged with ExoS in panel D due to greater loading.

Table 2. Expression of *P. aeruginosa exoS* and *exoT* genes using q-PCR after exposure to human corneal epithelial cell lysates, TSB (negative control) or TSBi (T3SS-inducing medium) for 16 h. Gene expression was expressed as the mean ± SD relative to PAO1 in TSB.

P. aeruginosa genotype	Conditions	Relative gene expression mean ± SD	
		exoT	*exoS*
PAO1	Lysate	33.83 ± 1.27*	68.73 ± 1.39*
	TSB	1.00 ± 0.07	1.00 ± 0.07
	TSBi	590.10 ± 0.81	342.25 ± 32.33
ΔexoS	Lysate	35.00 ± 3.58*	0
	TSB	0.82 ± 0.01	0
	TSBi	553.00 ± 36.26	0
ΔexoT	Lysate	0	127.52 ± 4.79*,**
	TSB	0	2.15 ± 0.03
	TSBi	0	305.13 ± 19.74

*Significantly lower than that in TSBi, $P < 0.0001$, Student's t-Test.
**Significantly higher than lysate induction in PAO1, $P < 0.0001$, Student's t-Test.

after lysate exposure, lysates did induce some *exoT* gene expression, ∼6% of TSBi. As expected, no *exoS* expression was detected for *exoS* mutants, and *exoT* expression in an *exoS* mutant background was similar to that in PAO1 (∼6% of TSBi). In the *exoT* mutant background, however, lysate induction of *exoS* expression was increased to ∼42% of TSBi versus 20% of TSBi in wild-type PAO1 (Table 2). Although *exoS* expression was higher in the *exoT* mutant compared to PAO1 under baseline (non-inducing) conditions, there was no difference in *exoS* expression between the *exoT* mutant and PAO1 after TSBi induction, suggesting that the absence of *exoT* affected lysate induction of *exoS*.

DISCUSSION

The T3SS of *P. aeruginosa* is important for the intracellular survival of strains that express the effector ExoS. The results of this study show that 16 h exposure of ExoS-expressing (invasive) strains of *P. aeruginosa* to cell lysates prepared from cultured human corneal epithelial cells induced the expression of a protein in culture supernatants that bound anti-ExoS antibody. Studies of *exoS* and *exoT* effector gene mutants of *P. aeruginosa* suggested that the protein was a form of ExoS, albeit slightly larger than that induced by TSBi (normally ∼49 kDa). Consistent with these findings, lysates also induced the expression of T3SS genes encoding *exoS* and *exoT* after 16 h, although induction was less effective (∼20% for *exoS,* and ∼6% for *exoT*) of levels noted for the T3SS induction medium (TSBi). The significantly greater *exoS* induction in an *exoT* mutant of PAO1 compared to wild-type, suggested *exoT* modulation of *exoS* expression with lysate induction.

Known inducers of the *P. aeruginosa* T3SS include host-cell contact (Vallis *et al.* 1999), and induction can be reproduced *in vitro* by exposing bacteria to growth media containing chelating agents, e.g. nitrilotriacetic acid (Iglewski *et al.* 1978) or EGTA (McCaw *et al.* 2002) to lower the levels of divalent cations, especially Ca^{2+}. The latter mechanism operates via the regulatory chaperone protein ExsC in *P. aeruginosa* (Dasgupta *et al.* 2006). *Pseudomonas aeruginosa* contact with human serum can also induce the T3SS (Vallis *et al.* 1999). Here, cell lysates were prepared in PBS, were not treated with chelating agents to remove divalent cations and epithelial cells used to prepare lysates were grown in serum-free KGM-2. While preparation of KGM-2 does involve the inclusion of essential growth factors (added as a proprietary 'Bullet Kit') that may also be present in serum, control experiments indicated that PAO1 growth in KGM-2 did not induce the ∼50–51 kDa form of ExoS. Thus, it seems unlikely that lysate induction of T3SS genes and proteins observed in our study involved exposure to residual KGM-2-derived growth factors. However, it remains possible that some lysate components, e.g. DNA fragments, could provide sufficient chelation of Ca^{2+} and other divalent cations to induce the T3SS.

Another known factor for T3SS induction is host cell contact. While lysate preparation involved destroying intact host cells along with centrifugation to remove unlysed cells and cell debris, it remains possible that the lysates still contained factors that would usually activate the T3SS extracellularly upon host cell contact. However, permeabilization of host cells with bacterial pore-forming bacterial toxins abolishes the induction of *exoS* gene expression by host cell contact (Cisz, Lee and Rietsch 2008), suggesting that external host cell cues for T3SS induction require an intact cell membrane. Thus, our data suggest that lysates from human corneal epithelial cells may contain previously unrecognized host cytosolic factors that contribute to T3SS induction in *P. aeruginosa* after internalization. Alternatively, or additionally, low levels of cytosolic intracellular Ca^{2+}

could induce the same effect. However, either possibility would align with our previous studies showing that ExoS can mediate *P. aeruginosa* intracellular survival and replication after internalization (Angus *et al.* 2010; Hritonenko, Evans and Fleiszig 2012), and that intracellular *P. aeruginosa* exhibit an activated T3SS coinciding with their avoidance of acidified vacuoles (Heimer *et al.* 2013).

Our data provide clues as to the identity of the factor(s) within corneal epithelial cell lysates inducing effector gene and ExoS expression. Activity was retained after heat treatment sufficient to denature proteins, but inactivation occurred after mild dilution (1:3) or by storing cell lysates at 4°C for 1 h. This suggested involvement of factor(s) that induce the T3SS independently of protein structure or enzymatic activity, are present at low concentration, and which are inhibited by exposure to low temperatures.

Western immunoblot data using anti-ExoS antibody, *exoS* and *exoT* mutants of *P. aeruginosa*, and qPCR data showing induction of *exoS* gene expression by lysates, all suggested that the lysate-induced protein expressed by PAO1 and PAK is a form of ExoS. Why it was slightly larger than the expected size of ∼49 kDa could relate to auto-ADP ribosylation of ExoS (Sundin *et al.* 2001). For example, ExoS was ∼50 kDa from auto-ADP ribosylation of the GAP domain (Riese *et al.* 2002). However, it is also possible that the lysates induce a unique (and stable) complex of ExoS with another bacterial or host cell-derived factor, or that ExoS is modified directly by the lysate. While ExoS secretion via the T3SS involves interaction with a chaperone SpcS (Shen *et al.* 2008), a ∼13 kDa protein, the chaperone is not secreted via the T3SS needle. Moreover, ExoS-SpcS complexes would be larger than the protein detected, and would also dissociate under denaturing SDS-PAGE conditions used. Further studies will be needed to determine the mechanism for, and significance of, the size increase of the lysate-induced ExoS.

TBSi induction usually leads to detection of both ExoS and ExoT in both supernatants and bacterial pellets, as confirmed in this study. Further, T3SS induction events usually activate the whole system from injectisome to effectors (Yahr and Wolfgang 2006). Thus, it is surprising that the lysate-induced form of ExoS was present in culture supernatants, but not cell pellets, and that ExoT was not detected at all. Lack of ExoT detection may reflect the low levels of *exoT* gene expression induced by the lysates, which appear to have greater ability to induce *exoS*. Absence of this protein from supernatants of lysate grown *pscC* mutants of PAO1 suggested that the T3SS needle was required for its secretion, and it was not simply released by bacterial cell lysis. It is not clear, however, why this slightly larger form of ExoS was not pellet-associated in wild-type, nor in *exoT* or *pscC* mutants after lysate growth. This finding will require further investigation. Another interesting result that will need further exploration is why lysate-induced *exoS* expression was higher in an *exoT* mutant compared to wild-type PAO1. It is possible that *exoT* negatively regulates *exoS* induction, which it does not using conventional induction methods. Alternatively, the absence of ExoT could reduce competition for the chaperone SpcS resulting in greater *exoS* expression in the *exoT* mutant. Further studies will also be needed to explore the apparent lack of accumulation of pellet-associated ExoS in *pscC* mutants, a finding that suggests a potential role for the T3SS needle in regulating ExoS expression. A necessary first step in sorting out differences between induction strategies will be identification of the responsible factor(s) in the epithelial cell lysates.

In conclusion, our data suggest that the cytosol of human corneal epithelial cells contains heat-stable factor(s) that contribute to induction of the *P. aeruginosa* T3SS. This leads to secretion of a modified form of the T3SS effector ExoS, ExoS being a key component of *P. aeruginosa* survival after internalization by these cells. Further studies will be needed to determine the host factor(s) involved in host cell lysate induction of the *P. aeruginosa* T3SS, the mechanisms for differences in induction compared to standard *in vitro* methods and the significance in the context of *P. aeruginosa* intracellular survival and disease pathogenesis.

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