Epithelial Cell Lysates Induce ExoS Expression and Secretion by Pseudomonas Aeruginosa

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

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Short title: Epithelial cell lysates induce *P. aeruginosa* ExoS

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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 bacteria cell pellets, that 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*.

**Key words:** *P. aeruginosa*, type three secretion system, epithelial cells, lysates, ExoS, ExoT
**INTRODUCTION**


Other effectors also contribute to virulence. ExoY, an adenylate cyclase, modulates the actin cytoskeleton (Cowell *et al.*, 2005, Hritonenko *et al.*, 2011), and reduces host inflammatory responses (He *et al.*, 2017, Jeon *et al.*, 2017). ExoU exerts potent phospholipase activity causing acute cytotoxicity (Finck-Barbancon *et al.*, 1997, Sato & Frank, 2004), but is not encoded by invasive strains of *P. aeruginosa*, e.g. PAO1 or PAK (Fleischig *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, Angus *et al.*, 2010, Hritonenko *et al.*, 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 *et al.*, 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 (hTCEpi) (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 et al., 2012). To prepare lysates, cells were grown on 12-well tissue-culture treated plates until confluent, then after 3 washes with 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 x g, 2 min), and supernatant (cell lysate) used for experiments. Cell lysates were freshly prepared for each experiment.

**Bacterial strains**
P. aeruginosa strains PAO1 and PAK were used along with their respective mutants in exoS (ΔexoS), exoT (ΔexoT) or both genes (ΔexoSΔexoT), and a pscC mutant of PAO1 (Table 1). A list Bacteria were cultured on trypticase soy agar (TSA) plates overnight at 37 °C, 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 x 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 x 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 3 washes with PBS-Tween (0.05 %), HRP-conjugated goat anti-rabbit seconary 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/](https://www.ebi.ac.uk/Tools/psa/emboss_water/)), with default parameters, from EMBL-EBI (The European Bioinformatics Institute).

### RNA extraction and qRT-PCR analysis

Bacteria were collected by centrifugation at 12,000 x g for 5 min at 4 °C, 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 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, ΔΔCq method). Data were expressed as mean ± 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

P. aeruginosa growth in cell lysates induces secretion of a ~50-51 kDa protein that binds anti-ExoS antibody

Growth of P. aeruginosa strain PAO1 for 16 h in epithelial cell lysates resulted in the appearance of ~50-51 kDa 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 (~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 (~53 kDa) in the culture supernatants (and very faintly in cell pellets) by the anti-ExoS antibody. ExoS and ExoT exhibit ~76 % amino acid sequence homology, although their ADPr domains differ (Barbieri & 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 pre-treated 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. aeruginosa 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 supernatants 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 \textit{exoS} gene in PAO1 at \(~20\%\) of levels triggered by the TSBi positive control. Although ExoT protein was not detected in culture supernatants of PAO1 after lysate exposure, lysates did induce some \textit{exoT} gene expression, \(~6\%\) of TSBi. As expected, no \textit{exoS} expression was detected for \textit{exoS} mutants, and \textit{exoT} expression in an \textit{exoS} mutant background was similar to that in PAO1 (\(~6\%\) of TSBi). In the \textit{exoT} mutant background, however, lysate induction of \textit{exoS} expression was increased to \(~42\%\) of TSBi versus \(~20\%\) of TSBi in wild-type PAO1 (Table 2). Although \textit{exoS} expression was higher in the \textit{exoT} mutant compared to PAO1 under baseline (non-inducing) conditions, there was no difference in \textit{exoS} expression between the \textit{exoT} mutant and PAO1 after TSBi-induction, suggesting that the absence of \textit{exoT} affected lysate induction of \textit{exoS}.

DISCUSSION

The type three secretion system of \textit{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 \textit{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 \textit{exoS} and \textit{exoT} effector gene mutants of \textit{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 \textit{exoS} and \textit{exoT} after 16 h, although induction was less effective (\(~20\%\) for \textit{exoS}, and \(~6\%\) for \textit{exoT}) of levels noted for the T3SS induction medium (TSBi). The significantly greater \textit{exoS} induction in an \textit{exoT} mutant of PAO1 compared to wild-type, suggested \textit{exoT} modulation of \textit{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). *P. 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 *et al*., 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 *et al.*, 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 that 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 & 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
\textit{exoT} gene expression induced by the lysates, which appear to have greater ability to induce
\textit{exoS}. Absence of this protein from supernatants of lysate grown \textit{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 \textit{exoT} or \textit{pscC} mutants after lysate growth. This finding
will require further investigation. Another interesting result that will need further exploration
is why lysate-induced \textit{exoS} expression was higher in an \textit{exoT} mutant compared to wild-type
PAO1. It is possible that \textit{exoT} negatively regulates \textit{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 \textit{exoS} expression in the \textit{exoT} mutant. Further studies
will also be needed to explore the apparent lack of accumulation of pellet-associated ExoS in
\textit{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 \textit{P. aeruginosa} T3SS. This leads to
secretion of a modified form of the T3SS effector ExoS, ExoS being a key component of \textit{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 \textit{P. aeruginosa} T3SS,
the mechanisms for differences in induction compared to standard \textit{in vitro} methods, and the
significance in the context of \textit{P. aeruginosa} intracellular survival and disease pathogenesis.
Competing interests

The authors have no conflicts of interest pertaining to the data presented in this study.

Author contributions

All authors were involved in planning the experiments. VH conducted experiments presented in Figures 1 and 2. MM conducted the experiments presented in Table 2. VH, MM, DE and SF wrote the manuscript.

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References


**Figure Legends**

**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.

**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.