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

2

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16 **ABSTRACT**

17

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

33

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

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41 INTRODUCTION

42

43 *Pseudomonas aeruginosa* is an opportunistic bacterial pathogen capable of infecting multiple
44 tissues and organ systems including; respiratory and urinary tracts, burn wounds, blood
45 (bacteremia), and the cornea (Evans *et al.*, 2007, Juan *et al.*, 2017, Klockgether & Tummeler,
46 2017, Newman *et al.*, 2017). The Type 3 Secretion System (T3SS) is important for the
47 virulence of *P. aeruginosa*, and a promising target for new therapies (Hauser, 2009,
48 Anantharajah *et al.*, 2016, Juan *et al.*, 2017). The T3SS encodes a protein nano-syringe capable
49 of injecting four effectors (ExoS, ExoT, ExoU, ExoY) directly into host cells. ExoS and ExoT
50 share sequence homology and enzymatic activities (Rho-GAP and ADP-ribosyltransferase
51 [ADPr] activity), that exert cytopathic effects on host cells and contribute to virulence (Lee *et*
52 *al.*, 2003, Barbieri & Sun, 2004, Lee *et al.*, 2005, Vance *et al.*, 2005, Deng & Barbieri, 2008).
53 Other effectors also contribute to virulence. ExoY, an adenylate cyclase, modulates the actin
54 cytoskeleton (Cowell *et al.*, 2005, Hritonenko *et al.*, 2011), and reduces host inflammatory
55 responses (He *et al.*, 2017, Jeon *et al.*, 2017). ExoU exerts potent phospholipase activity
56 causing acute cytotoxicity (Finck-Barbancon *et al.*, 1997, Sato & Frank, 2004), but is not
57 encoded by invasive strains of *P. aeruginosa*, e.g. PAO1 or PAK (Fleiszig *et al.*, 1997) which
58 instead use ExoS to modulate host cell function.

59 Previously we showed that *P. aeruginosa* uses the T3SS to survive and replicate inside host
60 cells. Primarily ExoS mediates formation and bacterial occupation of membrane bleb-niches
61 via its ADPr activity (Angus *et al.*, 2008, Angus *et al.*, 2010, Hritonenko *et al.*, 2012).
62 Intracellular *P. aeruginosa* can replicate, show swimming motility, and express the T3SS
63 within these blebs (Heimer *et al.*, 2013), whose formation is osmotically-driven (Jolly *et al.*,
64 2015). Without a T3SS translocon, bacteria remain within perinuclear vacuoles (Angus *et al.*,
65 2008), but can still use ExoS to survive and replicate intracellularly (Hritonenko *et al.*, 2012).

66 Indeed, vacuoles containing ExoS-expressing translocon mutants do not label with the late
67 endosomal marker LAMP3 (Angus *et al.*, 2008), and show reduced acidification (Heimer *et*
68 *al.*, 2013).

69 While the T3SS is clearly important for intracellular survival of *P. aeruginosa*, it remains
70 unclear if the system is activated extracellularly or intracellularly or both in that context.
71 Known triggers of the T3SS include bacterial exposure to low levels of divalent cations (e.g.
72 low Ca²⁺), serum, and host cell contact (Iglewski *et al.*, 1978, Vallis *et al.*, 1999, Dasgupta *et*
73 *al.*, 2006). Here, we tested if exposure to the cytosol of host cells could induce the T3SS, by
74 exposing invasive strains of *P. aeruginosa*, and their *exoS* and *exoT* mutants, to lysates of
75 human corneal epithelial cells.

76

77 **MATERIALS AND METHODS**

78

79 **Cell culture and lysate preparation**

80 Human telomerase-immortalized corneal epithelial cells (hTCEpi) (Robertson *et al.*, 2005)
81 were grown at 37 °C in 5 % CO₂ in 75 mm plastic flasks with vented caps using serum-free
82 keratinocyte growth medium (KGM-2) (Lonza, MD) supplemented with antibiotics
83 (gentamicin, streptomycin, penicillin, fungizone) as previously described (Hritonenko *et al.*,
84 2012). To prepare lysates, cells were grown on 12-well tissue-culture treated plates until
85 confluent, then after 3 washes with PBS to remove growth medium, cells were lysed in PBS
86 by three repeated freeze-thaw cycles (-80 °C for 10 min, 5 min on ice). Cellular debris was
87 removed by centrifugation (12,000 x g, 2 min), and supernatant (cell lysate) used for
88 experiments. Cell lysates were freshly prepared for each experiment.

89

90 **Bacterial strains**

91 *P. aeruginosa* strains PAO1 and PAK were used along with their respective mutants in *exoS*
92 (Δ *exoS*), *exoT* (Δ *exoT*) or both genes (Δ *exoS* Δ *exoT*), and a *pscC* mutant of PAO1 (Table 1). A
93 list Bacteria were cultured on trypticase soy agar (TSA) plates overnight at 37 °C, then
94 resuspended into trypticase soy broth (TSB) to a concentration of $\sim 10^8$ CFU/mL (absorbance
95 at 650 nm of 0.1). Cell lysates were then inoculated with bacteria at a starting concentration of
96 $\sim 10^3$ CFU/mL, then incubated for 16 h at 37 °C (3 mL volume, without shaking). TSB and
97 KGM-2 were used as negative (non-inducing) controls, and T3SS-inducing medium (TSBi) as
98 a positive control. TSBi consisted of TSB supplemented with 50 mM monosodium glutamate,
99 1 % glycerol, and 100 mM EGTA (pH adjusted to 7.0 with NaOH). In control experiments
100 using *P. aeruginosa* strain PAO1, 16 h of growth in cell lysates at 37 °C resulted in $\sim 10^8$
101 CFU/mL, ~ 5 -fold lower on average than that found in TSB (or TSBi) (data not shown). This
102 information was used to standardize bacterial numbers in other experiments.

103

104 **Western immunoblot**

105 After 16 h incubation with cell lysate, TSB, or TSBi, bacteria were pelleted by centrifugation
106 (12,000 x g, 5 min), and the supernatants and pellets examined for the presence of ExoS and
107 ExoT by Western immunoblot. Samples were standardized according to bacterial numbers, and
108 denatured in 2 x SDS-PAGE sample buffer (Bio-Rad, CA), prior to resolving by SDS-PAGE
109 (Bio-Rad). Proteins were transferred to nitrocellulose membranes using a Trans-Blot® SD
110 Semi-Dry Transfer Cell (Bio-Rad). After blocking for 1 h with 5 % skimmed milk in PBS,
111 membranes were probed overnight at 4 °C with polyclonal rabbit anti-ExoS affinity-purified
112 antibody diluted 1:1000 in 5 % skimmed milk in PBS. The anti-ExoS antibody was custom-
113 made by New England Peptide (Gardner, MA) using a synthesized peptide corresponding to
114 part of the ExoS ADP-r domain (amino acids 424 to 438). After 3 washes with PBS-Tween
115 (0.05 %), HRP-conjugated goat anti-rabbit secondary antibody (Bio-Rad) and

116 chemiluminescence were used for detection of bound primary antibody. Experiments were
117 repeated at least twice.

118

119 **Amino acid homology**

120 Alignment and comparison of amino acid homology between ExoS (424-438) and ExoT was
121 performed using EMBOSS water (https://www.ebi.ac.uk/Tools/psa/emboss_water/), with
122 default parameters, from EMBL-EBI (The European Bioinformatics Institute).

123

124 **RNA extraction and qRT-PCR analysis**

125 Bacteria were collected by centrifugation at 12,000 x g for 5 min at 4 °C, then resuspended in
126 800 µl of TRIzol (Invitrogen, CA). RNA was extracted with a Direct-zol RNA miniprep kit
127 (Zymo Research, CA) according to manufacturer's instructions. RNA samples were treated
128 with DNase (Roche, CA) for 2 h at 37 °C, and further purified using the Direct-zol RNA
129 miniprep kit (Zymo Research), including a second DNase treatment on the column for 30 min
130 at room temperature, according to manufacturer instructions. The cDNA was prepared using
131 iScript reverse transcription supermix (Bio-Rad) with 1 µg of total RNA per reaction mixture.
132 Real-time quantitative PCR (qRT-PCR) was performed on 50 ng of cDNA, amplified using
133 LightCycler 96 DNA SYBR green I master mix (Roche). Specific primers for *exoT* (*exoT*-rtF:
134 CGG TAG AGA GCG AGG TAA AGG, *exoT*-rtR: TAT AGA GAC CGA GCG CCA TC)
135 and *exoS* (*exoS*-rtF: TCT CTA CAC CGG CAT TCA CTA C, *exoS*-rtR: CCT TGG TCG ATC
136 AGC TTT TG) were designed using primer3 plus, and reactions monitored using a LightCycler
137 96 instrument and software (Roche). Transcript amounts under each condition were
138 standardized to transcription level of an internal control gene [*proC* (Savli *et al.*, 2003)], and
139 compared with standardized expression in PAO1 grown in TSB (relative quantification, $\Delta\Delta C_q$
140 method). Data were expressed as mean \pm standard deviation (SD), and significance of

141 differences between groups assessed with Student's t-Test. P values < 0.05 were considered
142 significant. Experiments were repeated at least twice.

143

144 **RESULTS**

145

146 ***P. aeruginosa* growth in cell lysates induces secretion of a ~50-51 kDa protein that binds** 147 **anti-ExoS antibody**

148 Growth of *P. aeruginosa* strain PAO1 for 16 h in epithelial cell lysates resulted in the
149 appearance of ~50-51 kDa protein in culture supernatants that was detected with anti-ExoS
150 antibody by Western immunoblot (Fig. 1A). The protein was not detected after bacterial growth
151 in normal TSB (non-inducing media), or KGM-2 (not shown), and appeared slightly larger than
152 ExoS (~49 kDa) found in both culture supernatant and bacterial pellets of bacteria grown in
153 T3SS-inducing medium (TSBi) (Fig. 1A). Interestingly, the protein was not associated with
154 bacterial cell pellets.

155 Growth in TSBi also appeared to result in the detection of ExoT (~53 kDa) in the culture
156 supernatants (and very faintly in cell pellets) by the anti-ExoS antibody. ExoS and ExoT exhibit
157 ~76 % amino acid sequence homology, although their ADPr domains differ (Barbieri & Sun,
158 2004). The amino acid sequence of ExoS from 424-438 (used to generate the anti-ExoS
159 antibody) was aligned and compared to ExoT, and showed 64.3 % identity and 85.7 %
160 similarity. Thus, it was quite feasible that the anti-ExoS antibody could also detect ExoT.

161 Dilution of lysates (1:3), or storage at 4 °C for 1 h, abolished expression of the protein that
162 bound anti-ExoS antibody (data not shown), although the protein was still present when PAO1
163 was grown in lysates previously heated at 55 °C for 1 h, or 100 °C for 5 min (Fig. 1B),
164 suggesting the involvement of a heat-stable host factor(s). In other experiments, prior to
165 preparation of lysates, human corneal epithelial cells were pre-treated for up to 12 h with

166 bacterial antigens in sterile culture supernatants (prepared by filtration of overnight cultures of
167 PAO1 in TSB). Antigen-pretreated lysates also induced the protein, but at a similar level to
168 untreated lysates (data not shown).

169

170 **Lysate-induction of the protein binding anti-ExoS antibody in culture supernatants of *P.***

171 ***aeruginosa* *exoT* mutants, but not *exoS* or *pscC* mutants, suggests a secreted form of ExoS**

172 Gene knockout (deletion) mutants in *exoS* or *exoT* were used to help identify the protein
173 expressed by *P. aeruginosa* strain PAO1 after exposure to cell lysates that bound anti-ExoS

174 antibody. Effector mutants in another invasive strain PAK were also tested. After 16 h growth

175 in cell lysates, the protein reactive with ExoS antibody was detected in culture supernatants,

176 but not bacterial pellets, of *exoT* mutants and wild-type PAO1, but not at all for *exoS* mutants

177 (Fig. 2A). The same result was found for culture supernatants of strain PAK and its effector

178 mutants after 16 h growth in cell lysates (Fig. 2B), showing that PAO1 is not unusual in its

179 response to the epithelial cell lysates. These data suggest that the protein represents a secreted

180 form of ExoS albeit slightly larger than that induced by TSBi. Absence of this form of ExoS in

181 culture supernatants of a *pscC* mutant of PAO1 after lysate growth (Fig. 2C) suggested that

182 the T3SS needle was required for its secretion, as for TSBi-induced ExoS secretion (Fig. 2D).

183 As observed for wild-type bacteria and their *exoT* mutants, however, lysate growth did not

184 result in pellet-associated ExoS in the *pscC* mutant. It was also observed that pellet-associated

185 ExoS did not appear to accumulate in the *pscC* mutant after TSBi induction (Fig. 2D),

186 suggesting that the T3SS needle may be needed for *exoS* expression.

187

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

189 Next, qPCR was used to determine the impact of the cell lysates on expression of *exoS* and

190 *exoT* genes in strain PAO1 compared to TSB and TSBi controls (Table 2). Consistent with the

191 results of Western immunoblot experiments, exposure to cell lysates for 16 h was found to
192 induce expression of the *exoS* gene in PAO1 at ~20 % of levels triggered by the TSBi positive
193 control. Although ExoT protein was not detected in culture supernatants of PAO1 after lysate
194 exposure, lysates did induce some *exoT* gene expression, ~ 6 % of TSBi. As expected, no *exoS*
195 expression was detected for *exoS* mutants, and *exoT* expression in an *exoS* mutant background
196 was similar to that in PAO1 (~ 6 % of TSBi). In the *exoT* mutant background, however, lysate
197 induction of *exoS* expression was increased to ~42 % of TSBi versus 20 % of TSBi in wild-
198 type PAO1 (Table 2). Although *exoS* expression was higher in the *exoT* mutant compared to
199 PAO1 under baseline (non-inducing) conditions, there was no difference in *exoS* expression
200 between the *exoT* mutant and PAO1 after TSBi-induction, suggesting that the absence of *exoT*
201 affected lysate induction of *exoS*.

202

203 **DISCUSSION**

204

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

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

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

241 align with our previous studies showing that ExoS can mediate *P. aeruginosa* intracellular
242 survival and replication after internalization (Angus *et al.*, 2010, Hritonenko *et al.*, 2012), and
243 that intracellular *P. aeruginosa* exhibit an activated T3SS coinciding with their avoidance of
244 acidified vacuoles (Heimer *et al.*, 2013).

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

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

264 TBSi-induction usually leads to detection of both ExoS and ExoT in both supernatants and
265 bacterial pellets, as confirmed in this study. Further, T3SS induction events usually activate the

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

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

290

291 **Competing interests**

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

293

294 **Author contributions**

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

298

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400

401 **Figure Legends**

402

403 **Figure 1. Western immunoblots of *P. aeruginosa* culture supernatants (Sup) and bacterial**

404 **cell pellets (Pel) after growth for 16 h in TSB, corneal cell lysates or TSBi (A) *P. aeruginosa***

405 PAO1 growth in corneal cell lysates resulted in the appearance of a protein in the culture

406 supernatant recognized by anti-ExoS antibody. The protein was not associated with bacterial

407 cell pellets. Growth in TSBi, but not TSB, resulted in detection of both secreted and bacterial

408 cell-associated ExoS and ExoT, (B) Growth of *P. aeruginosa* PAO1 in heat-treated cell lysates

409 (55 °C 1 h, 100 °C, 5 min) resulted in the continued appearance of the protein in culture

410 supernatants. Gel loading was normalized to the number of bacteria present.

411

412 **Figure 2. Western immunoblots of *P. aeruginosa* culture supernatants (Sup) and bacterial**

413 **cell pellets (Pel) for (A) PAO1 and (B) PAK and their respective *exoS* and *exoT* mutants,**

414 **and (C, D) PAO1 and its *pscC*, *exoS*, *exoT* and *exoSexoT* mutants after 16 h growth in**

415 **corneal cell lysates or TSBi. Growth of PAO1 or PAK or their *exoT* mutants in corneal cell**

416 lysates induced the appearance of the protein recognized by anti-ExoS antibody, but only in

417 culture supernatants. The protein was absent from culture supernatants and pellets of *exoS*

418 mutants of each strain suggesting that it represented a form of ExoS. However, the protein was

419 slightly larger than that induced in TSBi for which ExoT was also detected. This form of ExoS

420 was not observed in lysate-grown in *pscC* mutants. TSBi induction was associated with a small

421 amount of ExoS expression, but not secretion, in *pscC* mutants, and ExoT was not observed.

422 TSBi induced both ExoS and ExoT expression and secretion in PAO1. Gel loading was

423 normalized to the number of bacteria present. * ExoT merged with ExoS in Panel D due to

424 greater loading.