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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*.

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

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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 *et al.*, 2007, Juan *et al.*, 2017, Klockgether & Tummler, 2017, Newman *et al.*, 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 *et al.*, 2016, Juan *et al.*, 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), that exert cytopathic effects on host cells and contribute to virulence (Lee *et al.*, 2003, Barbieri & Sun, 2004, Lee *et al.*, 2005, Vance *et al.*, 2005, Deng & Barbieri, 2008). 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 (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, 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).

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 Ca^{2+}), 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 % CO_2 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 ~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   ~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 ~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 seconary antibody (Bio-Rad) and

116 chemiluminescence were used for detection of bound primary antibody. Experiments were

117 repeated at least twice.

118

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

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, ΔΔCq

140 method). Data were expressed as mean ± standard deviation (SD), and significance of

6

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 appreaed 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. aerugionsa* strain PAO1 after expoure 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 supernatnants 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 that 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

11

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

Competing interests

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

293

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.

298

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400

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 ºC 1 h, 100 ºC, 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 oberved 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.