Title

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Journal

The Ocular Surface, 15(1)

ISSN

1542-0124

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Publication Date

2017

DOI

10.1016/j.jtos.2016.09.001

Peer reviewed

HHS Public Access

Author manuscript

Ocul Surf. Author manuscript; available in PMC 2018 January 01.

Published in final edited form as:

Ocul Surf. 2017 January ; 15(1): 88–96. doi:10.1016/j.jtos.2016.09.001.

Human Tear Fluid Reduces Culturability of Contact Lens Associated *Pseudomonas aeruginosa* Biofilms but Induces Expression of the Virulence Associated Type III Secretion System

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Abstract

Purpose—The type III secretion system (T3SS) is a significant virulence determinant for *Pseudomonas aeruginosa*. Using a rodent model, we found that contact lens (CL)-related corneal infections were associated with lens surface biofilms. Here, we studied the impact of human tear fluid on CL-associated biofilm growth and T3SS expression.

Methods—*P. aeruginosa* biofilms were formed on contact lenses for up to 7 days with or without human tear fluid, then exposed to tear fluid for 5 or 24 h. Biofilms were imaged using confocal microscopy. Bacterial culturability was quantified by viable counts, and T3SS gene expression measured by RT-qPCR. Controls included trypticase soy broth, PBS and planktonic bacteria.

Results—With or without tear fluid, biofilms grew to ~10^8 cfu viable bacteria by 24 h. Exposing biofilms to tear fluid after they had formed without it on lenses reduced bacterial culturability ~180-fold ($p<.001$). CL growth increased T3SS gene expression versus planktonic bacteria [5.46 ± 0.24-fold for T3SS transcriptional activitor *exsA* ($p=.02$), and 3.76 ± 0.36-fold for T3SS effector toxin *exoS* ($p=.01$)]. Tear fluid further enhanced *exsA* and *exoS* expression in CL-grown biofilms, but not planktonic bacteria, by 2.09 ± 0.38-fold ($p = 0.04$) and 1.89 ± 0.26-fold ($p<.001$), respectively.

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The authors have no commercial or proprietary interest in any concept or product described in this article.

Conclusions—Considering the pivitol role of the T3SS in *P. aeruginosa* infections, its induction in CL-grown *P. aeruginosa* biofilms by tear fluid might contribute to the pathogenesis of CL-related *P. aeruginosa* keratitis.

Keywords

biofilms; contact lenses; human tear fluid; *Pseudomonas aeruginosa*; type III secretion system

I. INTRODUCTION

More than 120 million individuals worldwide wear contact lenses for correction of refractive errors.[1] Yet contact lens wear poses a significant risk for corneal infection,[2] a serious eye disease that can result in permanent vision loss. Why *Pseudomonas aeruginosa* is the most common cause of lens-related corneal infection is not entirely clear,[3] but it is likely related to the unusually large number of genes devoted to survival, virulence and its regulation, and its capacity to adapt to diverse environments.[4–7]

P. aeruginosa is among the bacteria capable of forming robust biofilms. Biofilms are communities of surface-associated microbial cells in a heterogenous protective matrix of polysaccharides, proteins, and nucleic acids.[8–10] Biofilm growth can enhance bacterial tolerance to antimicrobial agents[11–13] and to host immune responses,[14–16] and involves changes in microbial metabolism, gene expression, and contributions from the matrix itself.[17] *P. aeruginosa* biofilms readily form on human contact lenses and in lens storage cases.[18]

Previously, we showed that *P. aeruginosa*-contaminated contact lenses reliably caused disease in otherwise healthy rat eyes after a period of ~8 days.[19] Since this timing was reduced to ~2 days when lenses from already infected rats were transferred to naïve rats, the data suggested that bacteria associated with the lens were primed for virulence during extended exposure to the ocular surface environment. Supporting this notion, lenses removed from rat eyes after disease onset consistently harbored mature biofilms containing ~$5x10^8$ cfu bacteria per lens.[19]

In this study, we tested the hypothesis that biofilm growth on a contact lens and subsequent exposure to tear fluid can trigger expression of virulence factors in *P. aeruginosa*. We focused on the type III secretion system (**T3SS**), because it is considered among the most significant virulence factors of this pathogen,[20–24] and we have shown that it can promote *P. aeruginosa* traversal across a susceptible corneal epithelium.[24, 25]

II. METHODS

A. Tear Collection

Basal tear fluid was collected from male and female healthy volunteers under a protocol approved by the Committee for the Protection of Human Subjects, University of California, Berkeley. This research followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all subjects, each of whom was informed of the nature of the study and potential consequences. Briefly, polyester capillary micropipettes (Drummond Scientific

Company; USA) were placed on the subject's ocular lateral canthus for approximately 20–30 min to collect tear fluid. Collected tears were frozen at -80 °C until used for experiments. Human tear fluid exhibits antimicrobial activity against susceptible strains of *P. aeruginosa* after pooling and freezing.[26]

B. Bacterial Culture

Pseudomonas aeruginosa strain PAO1 was used throughout the study and grown on trypticase soy agar (**TSA**) plates overnight at 37 °C.

Planktonic bacteria were obtained by inoculating 5 mL of trypticase soy broth (**TSB**) and incubating overnight at 37 °C with shaking. The overnight culture was then diluted 1:50 in TSB at 37 °C, and incubated with shaking until the optical density (**OD**) was 0.1 at 650nm (~10^8 CFU/mL) determined by a spectrometer. The bacterial solution was then diluted in TSB, PBS, or tear fluid to ~10^6 CFU/mL as required, and incubated at 35 °C for various times as specified in the Results section. TSB transferred from this dilution step (~1 % vol/vol) will likely contribute a nutrient source to the PBS and tear fluid samples. In control experiments, planktonic bacterial growth was evaluated by serial dilution and viable counts on TSA each day for 7 days. Bacterial viability was similar in both tear fluid and PBS at each time point ($p>.5$, one-way ANOVA with Dunn's Multiple post-hoc analysis), although as expected, both showed ~10-fold lower viability than TSB grown bacteria at each time point after inoculation (Figure 1).

Biofilms were formed on unworn contact lenses (omafilcon B 38%, H_2O 62 %) that had been previously soaked in 3 mL of PBS for 1–2 days to remove packaging solution. Lenses were punctured into 2 mm (diameter) discs and transferred to a 12-well tissue culture plate, each well containing 100 µl of planktonic bacteria at ~10^{15} cfu/mL, and incubated for 3 h at 37 °C with shaking. Contact lenses were washed once with PBS to remove non-adherent bacteria, then treated as follows. Some lenses were homogenized in 1 mL of PBS with Triton X-100 (0.25 % vol/vol) and initial adherent bacterial numbers enumerated by viable counts (initial bacterial adhesion), andthe remaining lenses were exposed to 100 µl of TSB, PBS or tear fluid for 24 h at 35 °C, after which respective fluids were replaced daily. TSB was used to control for the presence of nutrients in tear fluid, and PBS was used to match growth rates (Figure 1). Lenses from each condition were homogenized at Days 1, 3, and 5 to quantify biofilm formation.

For confocal microscopy of contact lens-associated biofilms, PAO1 was transformed with plasmid pSMC2 expresssing GFP (PAO1-GFP),[27] and growth media were supplemented with carbenicillin 300 µg/mL. Lenses were punctured as described above and transferred into sterile contact lens storage wells (5 lenses per well). Each well was filled with 2 mL TSB with carbenicillin, and inoculated with one colony of PAO1-GFP previously grown on TSA with carbenicillin for 18 h at 35 °C. The lens cases were incubated at 35 °C and the TSB with carbenicillin replaced daily for 7 days. Lenses were visualized with confocal microscopy at various time points as indicated.

C. Exposure of Preformed *P. aeruginosa* Biofilms on Contact Lenses to Tear Fluid

P. aeruginosa biofilms were formed on a subset of unworn contact lenses incubated in TSB for 7 days. Methods were as described above for confocal microscopy, but using *P. aeruginosa* PAO1 without GFP, and thus TSB without carbenicillin. After removal from the TSB, lenses were washed once with PBS, then exposed to 100 μl of TSB, PBS, or tear fluid and incubated at 37 °C for 24 h. As described above, both TSB and PBS were used as controls: TSB for the presence of nutrients in tear fluid, and PBS to match growth rates. After incubation, lenses were washed once with PBS, and numbers of viable bacteria on each lens were enumerated by homogenizing the lens in 500 μl of PBS containing Triton X-100 (0.25 % vol/vol) and performing viable counts. Bacteria dispersed from biofilm were enumerated by sampling 10 μl of supernatant in each well at the 24 h time point followed by viable counts.

D. Confocal Microscopy

Biofilm formation on contact lenses by *P. aeruginosa* strain PAO1-GFP was imaged by confocal microscopy. An uninoculated control lens was used to standardize imaging parameters, and lenses for imaging were prestained with Coomassie brilliant blue (5 % vol./vol.) prior to biofilm formation for contrast. Prior to imaging, lenses with biofilms were gently washed in 1 mL of PBS to remove loosely attached planktonic bacteria. PAO1-GFP biofilms were examined using a confocal laser scanning microscope (FV-1000; Olympus Optical Co. Ltd., Japan) and 488 nm and 533 nm lasers. Image J was used to compile z stack images into a 3-D or orthoslice displays.

E. Quantification of T3SS Gene Expression by Real Time (RT)-qPCR

Planktonic bacteria (*P. aeruginosa* strain PAO1) were prepared as described above. After the 1:50 dilution step, bacteria were incubated for 2 h at 37 °C until the OD was ~ 0.4. After centrifugation at 3,000 x g for 5 min, the supernatant was discarded and bacterial pellet resuspended in 2 mL of PBS or tear fluid and incubated for 5 h at 37 °C with shaking. Samples were then centrifuged again to obtain a bacterial pellet which was resuspended in RNA protect ™ for RNA extraction. Biofilm grown bacteria on unworn contact lenses were also prepared in TSB as described above, but using whole contact lenses. On day 7, the TSB was removed, lenses were washed once with PBS, and incubated in 2 mL of PBS or tear fluid for 5 h at 37 °C. Biofilm on the contact lenses was collected by vortexing the lenses in 2 mm glass beads with RNA protect ™.

Total RNA from either planktonic and biofilm grown PAO1 was extracted with the RNeasy Kit (Qiagen, Valencia, CA) according to manufacturer's protocol, and using Qiagen on-column DNase steps to remove any contaminating genomic DNA. RNA quality was assessed using a NanoDrop machine. The cDNA was transcribed with reverse transcriptase following manufacturer instructions (BioRad, CA). T3SS gene expression (*exsA* and *exoS*) was quantified by RT-qPCR. Reactions were carried out using the SsoFast EvaGreen Supermix (Biorad, CA) under these conditions: 95 °C for 30 s followed by 30 cycles of 95 °C for 5 s, 58 °C for 5 s, and a final melt curve at 72 °C with 2 sec/step. Primers used in the reactions were: 5’-CGA GGT CAG CAG AGT ATC GG-3’ (*exoS* forward) and 5’-GTA GAG ACC AAG CGC CAT CA-3’ (*exoS* reverse); 5’-CTG GCG AGT TGC TTT TCG

TC-3' (*exsA* forward) and 5'-ACG CTC GAC TTC ACT CAA CA-3' (*exsA* reverse); 5'-TCT AAG GAG ACT GCC GGT GA-3' (*ribosomal 16S* forward) and 5'-CAG CT GCG ATC CGG ACT AC-3' (*ribosomal 16S* reverse). Transcript levels of each gene were normalized to that of the internal control ribosomal 16S and expression calculated by using the 2- CT method.[28] Normalized fold expression was calcuated relative to control using BioRad CFX Manager version 1.6.541.1068. Controls without reverse transcriptase were also included.

F. Statistical Analysis

Data were expressed as a mean ± standard error of the mean (**SEM**) for each group. For viable counts of bacterial biofilms on contact lens discs, significance of differences between two groups at each time point was determined using the Mann-Whitney U test. For other experiments, the significance of differences between groups was determined using Student's t-test for two groups, or one-way ANOVA with Dunn's Multiple post-hoc analysis for multiple-groups. P values of <.05 were considered significant. All experiments were repeated at least three times.

III. RESULTS

A. *P. aeruginosa* Biofilm Formation on Contact Lenses

Contact lens discs (2 mm) incubated with *P. aeruginosa* PAO1-GFP formed substantial surface biofilms at 24 h as shown by 3D confocal imaging of progressive biofilm formation (Figure 2A). Orthoslice imaging revealed minimal bacterial penetration into the contact lens material at 24 h (Figure 2B). From 48 to 72 h, however, bacterial biofilm showed increasing penetration into the lens material (Figure 2B). Homogenization of biofilm-coated contact lens discs revealed similar numbers of culturable bacteria at 4 h and 8 h post-inoculation (4 h mean = $4.9 \times 10^5 \pm 1.3 \times 10^5$ CFU: versus 8 h mean = $3.0 \times 10^5 \pm 1.2 \times 10^5$ CFU, p>.05, Mann-Whitney U test). The number of bacteria recovered increased significantly by 24 h (Day 1 mean = $2.2 \times 10^8 \pm 8.5 \times 10^7$ CFU, p<.001, Mann-Whitney U test versus 8 h time point). Although greater penetration of biofilm into the lens material was observed from 48-72 h (Figure 2B), the number of recovered bacteria remained consistent from Day 1 to Day 7 (Day 7 mean = $2.6 \times 10^8 \pm 1.14 \times 10^8$ CFU).

B. Tear Fluid Does Not Affect *P. aeruginosa* Biofilm Formation, but Reduces the Number of Bacteria Culturable From Preformed Biofilms

Contact lens discs were incubated with *P. aeruginosa* for 5 days in the presence of human tear fluid, then compared to PBS and TSB controls. Tear fluid- and PBS-formed biofilms yielded similar number of culturable bacteria after both 1 and 3 days, each significantly less than TSB-formed biofilms (Figure 3A;p<.05 one-way ANOVA with Dunn's Multiple post-hoc analysis). By day 5, there were significantly more culturable biofilm bacteria than on Day 1 and Day 3 for both TSB and tear fluid (Figure 3A; p<.05 one-way ANOVA with Dunn's Multiple post-hoc analysis). While there were still more culturable bacteria recovered from TSB biofilms at Day 5 compared to PBS and tear fluid, differences between these groups were not significant.

We next exposed already-formed biofilms (i.e., grown in TSB for 7 days) to each of the three different media for 24 h (Figure 3B). The number of culturable bacteria continued to increase in TSB. However, tear fluid reduced the number of culturable bacteria in preformed *P. aeruginosa* biofilms by ~180-fold compared to the biofilms subsequently exposed to TSB ($p<.001$, one-way ANOVA with Dunn's Multiple post-hoc analysis), but by only ~ 4-fold compared to PBS controls ($p=.001$, one-way ANOVA with Dunn's Multiple post-hoc analysis).

For both tear fluid and PBS, some dislodgment of biofilm was noted after 24 h compared to TSB-exposed biofilms, which appeared more firmly attached to the lens material. To explore the role of dispersal in reducing the number of bacteria recovered from biofilms after tear fluid exposure, the incubation solution containing detached bacteria and their progeny was also examined. Significantly fewer detached bacteria were recovered after tear fluid exposure compared to controls (~2 log reduction compared to PBS, >3 log reduction compared to TSB, $p<.001$, one-way ANOVA with Dunn's Multiple post-hoc analysis; Figure 3B).

C. Tear Fluid Induction of T3SS Gene Expression in *P. aeruginosa* Biofilms

RT-qPCR was used to compare expression of two T3SS genes in *P. aeruginosa* biofilms vs. in planktonic bacteria, and after human tear fluid exposure. Expression of both *exsA* (encodes a transcriptional activator of the T3SS) and *exoS* (encodes an important T3SS effector toxin) was examined (Figure 4). Expression of both genes was significantly upregulated in *P. aeruginosa* biofilms associated with contact lens discs compared to planktonic bacteria (Figure 4A; $p=.02$ for *exsA*, $p=.01$ for *exoS*, t-Test). Moreover, exposure of the contact lens-associated *P. aeruginosa* biofilms to tear fluid (for 5 h) further induced expression of both T3SS genes as compared to control biofilms exposed only to PBS (Figure 4B, $p=.04$ for *exsA*, $p<.001$ for *exoS*, t-Test). Exposure of planktonic *P. aeruginosa* to tear fluid did not induce T3SS gene expression (1.04 ± 0.78-fold for *exsA*, and 0.95 ± 0.12-fold for *exoS* [$p=.25$ for each versus PBS controls, t-Test]).

IV. DISCUSSION

P. aeruginosa keratitis is a potentially blinding complication of contact lens wear, yet the mechanisms by which lens wear predisposes to the disease remains poorly understood. *P. aeruginosa* and many other bacterial species can form biofilms on contact lenses or in lens cases, a mode of growth that facilitates bacterial resistance to antimicrobials and phagocytosis by host cells, and can facilitate increased pathogenicity.[11,18,29–33]

Data collected in this study show that *P. aeruginosa* can form biofilms on clean unworn contact lenses in a range of nutritional conditions. Biofilms grown for 5 days in nutrient-rich culture medium (TSB), balanced salt solution containing few nutrients (PBS), and 100% concentrated human tear fluid (which contains factors with antimicrobial activities) all yielded similar numbers of culturable bacteria. This versatility is consistent with the large cadre of genes in the *P. aeruginosa* genome devoted to survival and adaptation to prevailing conditions.[4] Indeed, the kinetics of biofilm development, initially slower in tear fluid and PBS than in rich culture media, implicates adaptation. Biofilm formation even in the

presence of tear fluid aligns with our previously published results showing *P. aeruginosa* can resist and adapt to the antimicrobial effects of human tear fluid,[26] and that it can form biofilms on (and penetrate into) the surface of contact lenses worn by rats in vivo.[19] Others have shown that human tear fluid components can actually contribute to biofilm formation by *P. aeruginosa.*[34]

While the data showed that biofilm could form on lenses in the presence of tear fluid, tear fluid reduced the number of bacteria recoverable from biofilms that had been grown in TSB culture media. Some of this loss of culturability likely reflects a nonspecific shock response to a change in nutrient concentrations, given that exposure to PBS also reduced culturability. However, tear fluid caused a ~4-fold greater loss than did PBS, suggesting some specificity. The differences may not be a result of differential bacterial detachment, since there appeared to be more dispersal in PBS (and TSB) than there was in tear fluid. Growth of dispersed bacteria in TSB may complicate interpretation of dispersal data. However, tear fluid did not suppress *P. aeruginosa* culturability in suspension when compared with PBS (Figure 1). Thus, the mechanism for suppression in biofilms seems worthy of further investigation. In particular, it would be important to know if tear fluid exposure encourages biofilm *P. aeruginosa* associated with the contact lens to enter a Viable But Not Culturable (**VBNC**) and/or persister state,[35,36] phenotypes more resistant to antimicrobials and other environmental stresses, and from which they can be resuscitated to reacquire virulence.[37–42] Despite the fact that tear fluid reduced the number of culturable bacteria in biofilms compared to PBS, it enhanced expression of the bacterial T3SS, a major virulence determinant. This was over and above the increased expression caused by biofilm growth compared to growth in suspension. Our published data show that the T3SS is involved in *P. aeruginosa* traversal of superificially-injured corneal epithelium in mouse models.[25,43] The net sum result on virulence of increasing T3SS expression while also reducing the number of culturable bacteria via tear fluid exposure will require further investigation. How virulence factors other than the T3SS are impacted by tear fluid also remains to be determined. Whatever the case, the data presented here support the notion that studying culturability alone may not provide enough information to understand the significance of bacterial contamination during lens wear.

Following discovery of the *P. aeruginosa* master regulator RetS by three independent laboratories, including our group,[44–46] a model evolved that *P. aeruginosa* utilizes it and other factors to reciprocally regulate T3SS genes and biofilm formation for the purposes of directing bacterial pathogenicity towards either acute infection and virulence or chronic infection and persistence.[45,47] Indeed, enhanced biofilm formation on abiotic surfaces has been observed using both T3SS and RetS mutants.[45,48] Further, RNA-sequencing has shown a distinct difference in the transcriptome of planktonic versus static-biofilm *P. aeruginosa* with the latter favoring T3SS suppression, production of biofilm matrix materials (e.g. polysaccharide), and adaptation to microaerophilic conditions.[5] However, others have published data that suggest the two phenotypes can occur simultaneously in the same population.[49] Our data showing greater T3SS gene expression in biofilm on contact lenses versus planktonically grown *P. aeruginosa* also challenges the idea that there is a neat division of *P. aeruginosa* into two states depending on how it is grown. Indeed, the *P.*

aeruginosa T3SS has actually been implicated in promoting adhesion to contact lenses, as has lens exposure to artificial tear fluid.[50] Thus, the reciprocal regulation model appears to be conditional on the experimental set-up. It is unclear why there would be more robust T3SS expression in biofilms grown on contact lenses when its expression is suppressed during biofilm growth in some other circumstances, but this could relate to properties of the material, or some other variable. For example, *P. aeruginosa* biofilms exposed to *Acanthamoeba castellanii* show induction of T3SS genes to kill the Amoebae.[51]

V. CONCLUSION

The results of this study contribute another piece to the puzzle of how contact lens wear promotes infection with *P. aeruginosa*. Due to its inherent resistance to killing, its capacity for adaptation, and its ubiquitousness in our environment, *P. aeruginosa* commonly contaminates contact lenses and lens care products.[52–54] On the surface of these materials, it can form robust biofilms,[30,33,55] including on contact lenses worn in vivo.[18,19] Biofilm formation,[56] and tear fluid,[57] can each promote outer membrane vesicle (OMV) release by *P. aeruginosa*, and OMVs can prime the cornea for subsequent bacteria adhesion.[57]

The data presented here show that biofilm growth on a lens and exposure to human tear fluid can also upregulate expression of the T3SS, which can enable *P. aeruginosa* to traverse susceptible corneal epithelium,[25,43] an essential event in the pathogenesis of corneal infection. In the absence of lens wear, *P. aeruginosa* (and other microbes) cannot infect a healthy corneal surface, which has anti-adhesive properties.[58–60] Since there is nothing for them to establish a foothold, any inoculated bacteria are rapidly removed by highly effective clearance mechanisms,[58] giving them insufficient time or opportunity to prime the surface for adhesion or to trigger expression of virulence factors required for tissue invasion.

Infection of the cornea can lead to rapid vision loss. Studies of the sequence of events involved in pathogenesis of infection versus those maintaining health at the ocular surface and elsewhere could lead to strategies for preventing infection. That would improve upon the current approach of treating infection after pathology is evident, and potential for loss of vision or life is already apparent.

Acknowledgments

This research was supported by the National Institutes of Health, EY011221 (SMJF), an American Optometric Foundation Vistakon Research Grant (YW/SMJF), and by the Australian National Health and Medical Research Council CJ Martin Fellowship (YW).

References

1. Bowden T, Harknett T. Contact lens wearer profile 2004. Contact Lens Anterior Eye. 2005; 28:37–45. [PubMed: 16318833]
2. Bourcier T, Thomas F, Borderie V, et al. Bacterial keratitis: predisposing factors, clinical and microbiological review of 300 cases. Br J Ophthalmol. 2003; 87:834–39. [PubMed: 12812878]
3. Willcox MD, Zhu H, Conibear TC, et al. Role of quorum sensing by Pseudomonas aeruginosa in microbial keratitis and cystic fibrosis. Microbiology. 2008; 154:2184–94. [PubMed: 18667552]
4. Stover CK, Pham XQ, Erwin AL, et al. Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. Nature. 2000; 406:959–64. [PubMed: 10984043]

5. Dotsch A, Eckweiler D, Schniederjans M, et al. The Pseudomonas aeruginosa transcriptome in planktonic cultures and static biofilms using RNA sequencing. PloS one. 2012; 7:e31092. [PubMed: 22319605]
6. Winstanley C, O'Brien S, Brockhurst MA. Pseudomonas aeruginosa. evolutionary adaptation and diversification in cystic fibrosis chronic lung infections. Trends Microbiol. 2016; 24:327–37. [PubMed: 26946977]
7. Valentini M, Filloux A. Biofilms and cyclic di-GMP (c-di-GMP) signaling: lessons from Pseudomonas aeruginosa and other bacteria. J Biol Chem. 2016; 291:12547–55. [PubMed: 27129226]
8. O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. Annu Rev Microbiol. 2000; 54:49–79. [PubMed: 11018124]
9. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science. 1999; 284:1318–22. [PubMed: 10334980]
10. Friedman L, Kolter R. Genes involved in matrix formation in Pseudomonas aeruginosa PA14 biofilms. Mol Microbiol. 2004; 51:675–90. [PubMed: 14731271]
11. Szczotka-Flynn LB, Imamura Y, Chandra J, et al. Increased resistance of contact lens-related bacterial biofilms to antimicrobial activity of soft contact lens care solutions. Cornea. 2009; 28:918–26. [PubMed: 19654521]
12. Drenkard E. Antimicrobial resistance of Pseudomonas aeruginosa biofilms. Microbes Infect. 2003; 5:1213–19. [PubMed: 14623017]
13. Evans DJ, Allison DG, Brown MR, et al. Effect of growth-rate on resistance of gram-negative biofilms to cetrimide. J Antimicrob Chemother. 1990; 26:473–8. [PubMed: 2254220]
14. Leid JG, Kerr M, Selgado C, et al. Flagellum-mediated biofilm defense mechanisms of Pseudomonas aeruginosa against host-derived lactoferrin. InfectI Immun. 2009; 77:4559–66.
15. Leid JG, Willson CJ, Shirtliff ME, et al. The exopolysaccharide alginate protects Pseudomonas aeruginosa biofilm bacteria from IFN-gamma-mediated macrophage killing. J Immunol. 2005; 175:7512–8. [PubMed: 16301659]
16. Meluleni GJ, Grout M, Evans DJ, et al. Mucoid Pseudomonas aeruginosa growing in a biofilm in vitro are killed by opsonic antibodies to the mucoid exopolysaccharide capsule but not by antibodies produced during chronic lung infection in cystic fibrosis patients. J Immunol. 1995; 155:2029–38. [PubMed: 7636254]
17. Breidenstein EB, de la Fuente-Nunez C, Hancock RE. Pseudomonas aeruginosa: all roads lead to resistance. Trends Microbiol. 2011; 19:419–26. [PubMed: 21664819]
18. McLaughlin-Borlace L, Stapleton F, Matheson M, et al. Bacterial biofilm on contact lenses and lens storage cases in wearers with microbial keratitis. J Appl Microbiol. 1998; 84:827–38. [PubMed: 9674137]
19. Tam C, Mun JJ, Evans DJ, et al. The impact of inoculation parameters on the pathogenesis of contact lens-related infectious keratitis. Invest Ophthalmol Vis Sci. 2010; 51:3100–6. [PubMed: 20130275]
20. Diaz MR, King JM, Yahr TL. Intrinsic and extrinsic regulation of type III secretion gene expression in Pseudomonas aeruginosa. Front Microbiol. 2011; 2:89. [PubMed: 21833328]
21. Fleiszig SM, Wiener-Kronish JP, Miyazaki H, et al. Pseudomonas aeruginosa-mediated cytotoxicity and invasion correlate with distinct genotypes at the loci encoding exoenzyme S. InfectI Immun. 1997; 65:579–86.
22. Vance RE, Rietsch A, Mekalanos JJ. Role of the type III secreted exoenzymes S, T, and Y in systemic spread of Pseudomonas aeruginosa PAO1 in vivo. Infect Immun. 2005; 73:1706–13. [PubMed: 15731071]
23. Anantharajah A, Mingeot-Leclercq MP, Van Bambeke F. Targeting the Type Three Secretion System in Pseudomonas aeruginosa. Trends Pharmacol Sci. 2016; 37:734–49. [PubMed: 27344210]
24. Lee EJ, Cowell BA, Evans DJ, et al. Contribution of ExsA-regulated factors to corneal infection by cytotoxic and invasive Pseudomonas aeruginosa in a murine scarification model. Invest Ophthalmol Vis Sci. 2003; 44:3892–8. [PubMed: 12939306]

25. Sullivan AB, Tam KP, Metruccio MM, et al. The importance of the Pseudomonas aeruginosa type III secretion system in epithelium traversal depends upon conditions of host susceptibility. Infect Immun. 2015; 83:1629–40. [PubMed: 25667266]
26. Fleiszig SM, Kwong MS, Evans DJ. Modification of Pseudomonas aeruginosa interactions with corneal epithelial cells by human tear fluid. Infect Immun. 2003; 71:3866–74. [PubMed: 12819071]
27. Bloemberg GV, O'Toole GA, Lugtenberg BJ, et al. Green fluorescent protein as a marker for Pseudomonas spp. Appl Environ Microbiol. 1997; 63:4543–51. [PubMed: 9361441]
28. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25:402–8. [PubMed: 11846609]
29. Wiley L, Bridge DR, Wiley LA, et al. Bacterial biofilm diversity in contact lens-related disease: emerging role of Achromobacter, Stenotrophomonas, and Delftia. Invest Ophthalmol Vis Sci. 2012; 53:3896–905. [PubMed: 22589441]
30. Willcox MD, Harmis N, Cowell, et al. Bacterial interactions with contact lenses; effects of lens material, lens wear and microbial physiology. Biomaterials. 2001; 22:3235–47. [PubMed: 11700795]
31. Furuhata K, Ishizaki N, Kawakami Y, et al. Bacterial contamination of stock solutions in storage cases for contact lens, and the disinfectant-resistance of isolates. Biocontrol Sci. 2010; 15:81–5. [PubMed: 20938091]
32. Wilson LA, Sawant AD, Ahearn DG. Comparative efficacies of soft contact lens disinfectant solutions against microbial films in lens cases. Arch Ophthalmol. 1991; 109:1155–7. [PubMed: 1867561]
33. Robertson DM, Parks QM, Young RL, et al. Disruption of contact lens-associated Pseudomonas aeruginosa biofilms formed in the presence of neutrophils. Invest Ophthalmol Vis Sci. 2011; 52:2844–50. [PubMed: 21245396]
34. Miller MJ, Wilson LA, Ahearn DG. Effects of protein,and human tears on adherence of Pseudomonas aeruginosa to hydrophilic contact lenses. J Clin Microbiol. 1988; 26:513–17. [PubMed: 3128579]
35. Oliver JD. The viable but nonculturable state in bacteria. J Microbiol. 2005; 43(Spec No):93–100. [PubMed: 15765062]
36. Ayrapetyan M, Williams TC, Oliver JD. Bridging the gap between viable but non-culturable and antibiotic persistent bacteria. Trends Microbiol. 2015; 23:7–13. [PubMed: 25449050]
37. Orman MA, Henry TC, DeCoste CJ, et al. Analyzing persister physiology with fluorescence-activated cell sorting. Methods Mol Biol. 2016; 1333:83–100. [PubMed: 26468102]
38. Nowakowska J, Oliver JD. Resistance to environmental stresses by Vibrio vulnificus in the viable but nonculturable state. FEMS Microbiol Ecol. 2013; 84:213–22. [PubMed: 23228034]
39. Weichart D, Kjelleberg S. Stress resistance and recovery potential of culturable and viable but nonculturable cells of Vibrio vulnificus. Microbiology. 1996; 142(Pt 4):845–53. [PubMed: 8936311]
40. Aurass P, Prager R, Flieger A. EHEC/EAEC O104:H4 strain linked with the 2011 German outbreak of haemolytic uremic syndrome enters into the viable but non-culturable state in response to various stresses and resuscitates upon stress relief. Environ Microbiol. 2011; 13:3139–48. [PubMed: 21951606]
41. Klancnik A, Guzej B, Jamnik P, et al. Stress response and pathogenic potential of Campylobacter jejuni cells exposed to starvation. Res Microbiol. 2009; 160:345–52. [PubMed: 19477271]
42. Dwidjosiswojo Z, Richard J, Moritz MM, et al. Influence of copper ions on the viability and cytotoxicity of Pseudomonas aeruginosa under conditions relevant to drinking water environments. Int J Hyg Environ Health. 2011; 214:485–92. [PubMed: 21742552]
43. Lee EJ, Evans DJ, Fleiszig SM. Role of Pseudomonas aeruginosa ExsA in penetration through corneal epithelium in a novel in vivo model. Invest Ophthalmol Vis Sci. 2003; 44:5220–7. [PubMed: 14638720]
44. Zolfaghar I, Angus AA, Kang PJ, et al. Mutation of retS, encoding a putative hybrid two-component regulatory protein in Pseudomonas aeruginosa, attenuates multiple virulence mechanisms. Microbes Infect. 2005; 7:1305–16. [PubMed: 16027020]

45. Goodman AL, Kulasekara B, Rietsch A, et al. A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in Pseudomonas aeruginosa. Dev Cell. 2004; 7:745–54. [PubMed: 15525535]
46. Laskowski MA, Osborn E, Kazmierczak BI. A novel sensor kinase-response regulator hybrid regulates type III secretion and is required for virulence in Pseudomonas aeruginosa. Mol Microbiol. 2004; 54:1090–103. [PubMed: 15522089]
47. Ventre I, Goodman AL, Vallet-Gely I, et al. Multiple sensors control reciprocal expression of Pseudomonas aeruginosa regulatory RNA and virulence genes. Proc Natl Acad Sci U S A. 2006; 103:171–6. [PubMed: 16373506]
48. Kuchma SL, Connolly JP, O'Toole GA. A three-component regulatory system regulates biofilm maturation and type III secretion in Pseudomonas aeruginosa. J Bacteriol. 2005; 187:1441–54. [PubMed: 15687209]
49. Mikkelsen H, Bond NJ, Skindersoe ME, et al. Biofilms and type III secretion are not mutually exclusive in Pseudomonas aeruginosa. Microbiology. 2009; 155:687–98. [PubMed: 19246740]
50. Shen EP, Tsay RY, Chia JS, et al. The role of type III secretion system and lens material on the adhesion of Pseudomonas aeruginosa to contact lenses. Invest Ophthalmol Vis Sci. 2012; 53:6416–26. [PubMed: 22918630]
51. Matz C, Moreno AM, Alhede M, et al. Pseudomonas aeruginosa uses type III secretion system to kill biofilm-associated amoebae. ISME J. 2008; 2:843–52. [PubMed: 18480848]
52. Wu YT, Willcox M, Zhu H, et al. Contact lens hygiene compliance and lens case contamination: A review. Cont Lens Anterior Eye. 2015; 38:307–16. [PubMed: 25980811]
53. Konda N, Motukupally SR, Garg P, et al. Microbial analyses of contact lens-associated microbial keratitis. Optom Vis Sci. 2014; 91:47–53. [PubMed: 24212183]
54. Stapleton F, Dart JK, Seal DV, et al. Epidemiology of Pseudomonas aeruginosa keratitis in contact lens wearers. Epidemiol Infect. 1995; 114:395–402. [PubMed: 7781727]
55. Zegans ME, Becker HI, Budzik J, et al. The role of bacterial biofilms in ocular infections. DNA Cell Biol. 2002; 21:415–20. [PubMed: 12167244]
56. Toyofuku M, Roschitzki B, Riedel K, et al. Identification of proteins associated with the Pseudomonas aeruginosa biofilm extracellular matrix. J Proteome Res. 2012; 11:4906–15. [PubMed: 22909304]
57. Metruccio MM, Evans DJ, Gabriel MM, et al. Pseudomonas aeruginosa outer membrane vesicles triggered by human mucosal fluid and lysozyme can prime host tissue surfaces for bacterial adhesion. Front Microbiol. 2016; 7:871. [PubMed: 27375592]
58. Mun JJ, Tam C, Kowbel D, et al. Clearance of Pseudomonas aeruginosa from a healthy ocular surface involves surfactant protein D and is compromised by bacterial elastase in a murine null-infection model. Infect Immun. 2009; 77:2392–8. [PubMed: 19349424]
59. Alarcon I, Tam C, Mun JJ, et al. Factors impacting corneal epithelial barrier function against Pseudomonas aeruginosa traversal. Invest Ophthalmol Vis Sci. 2011; 52:1368–77. [PubMed: 21051692]
60. Gipson IK, Spurr-Michaud S, Tisdale A, et al. Comparison of the transmembrane mucins MUC1 and MUC16 in epithelial barrier function. PloS one. 2014; 9:e100393. [PubMed: 24968021]

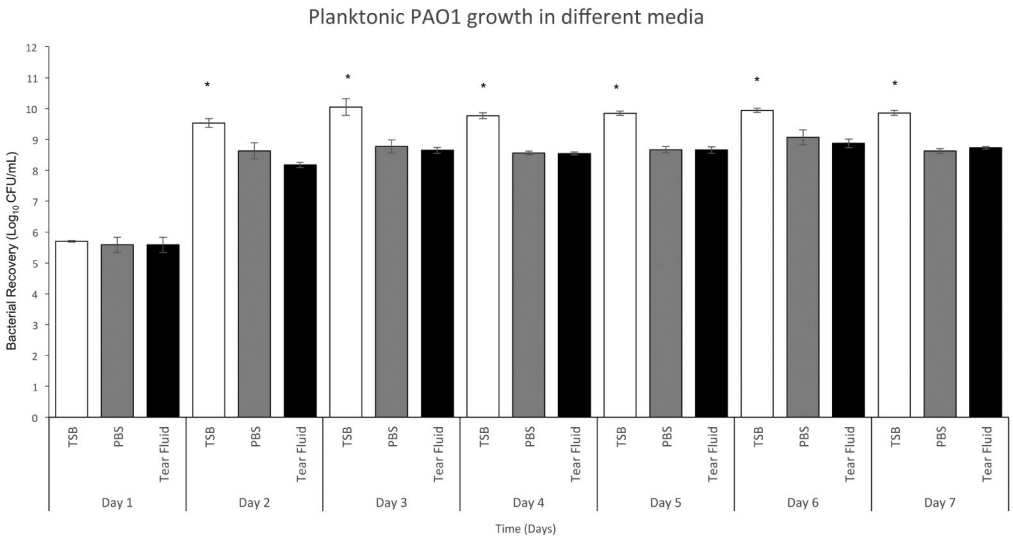

Figure 1.

Planktonic growth of *P. aeruginosa* strain PAO1 in TSB, PBS and tear fluid.* Growth in TSB was significantly higher than in PBS or tear fluid on each day after day 1 ($p<.01$, one-way ANOVA with Dunn's Multiple post-hoc analysis), but growth in PBS and tear fluid was not significantly different.

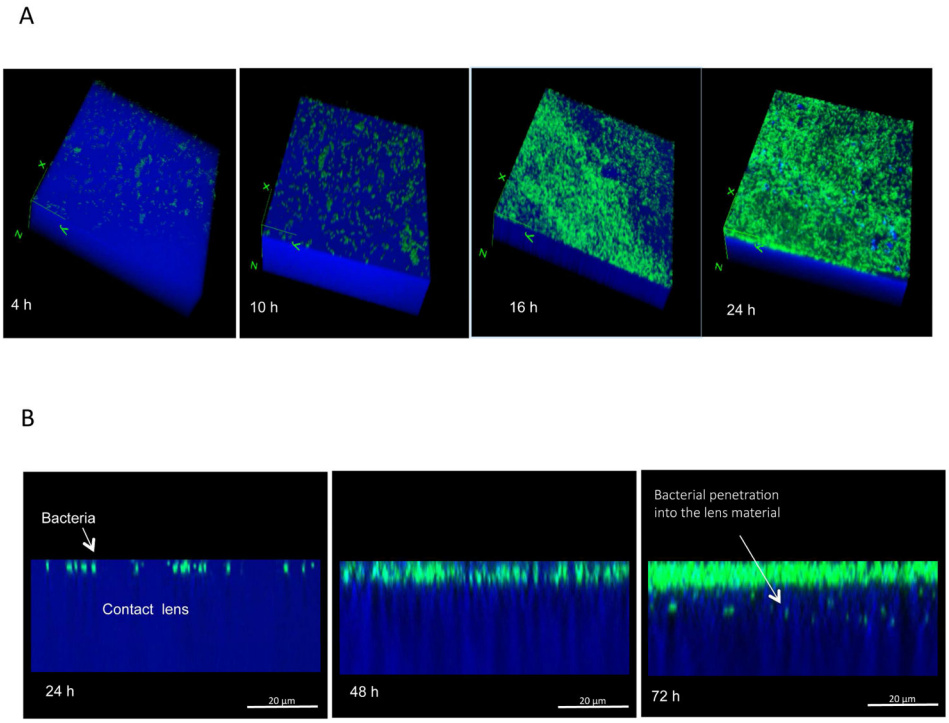

Figure 2.

Confocal microscopy images of *P. aeruginosa* biofilms (PAO1-GFP, green) on contact lenses (blue). A. 3-D images of biofilm development over 24 h. B. Orthoslice images of *P. aeruginosa* biofilm penetration of the contact lens over 72 h.

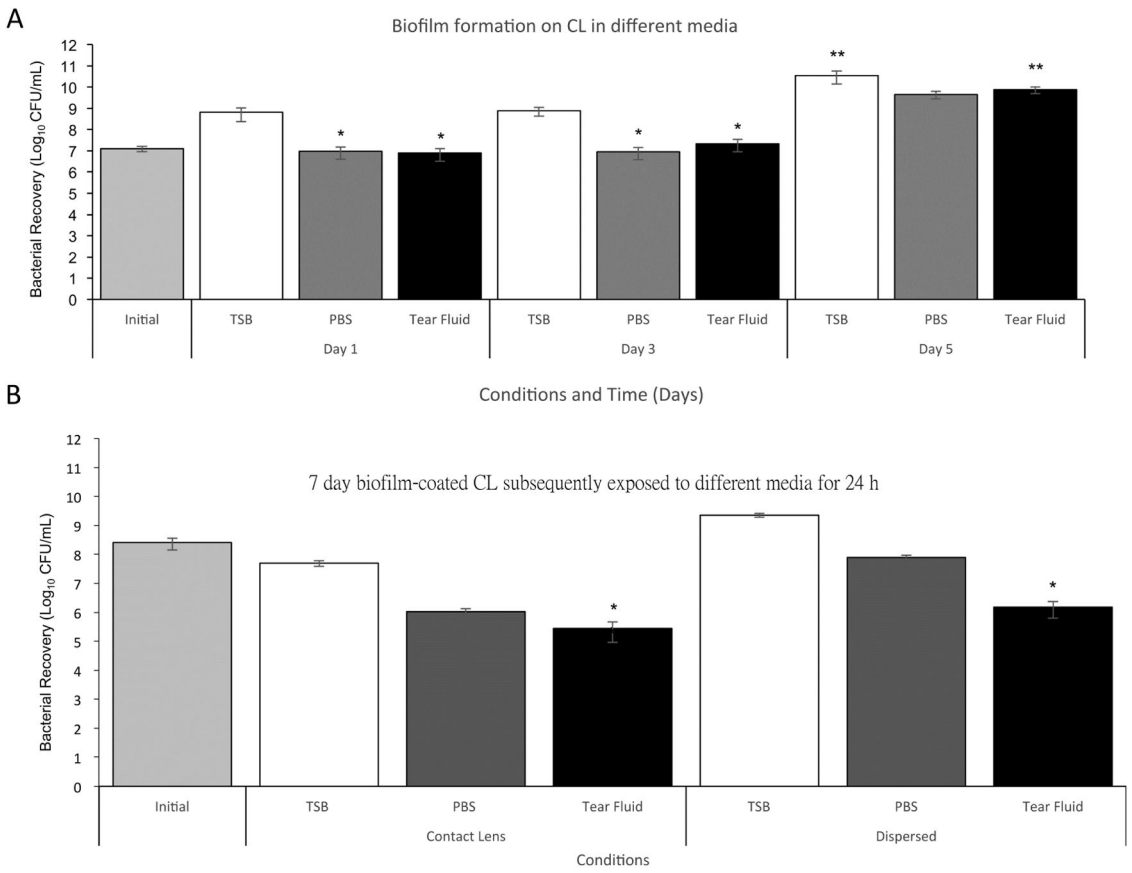

Figure 3.

A. Effect of human tear fluid on the development of *P. aeruginosa* PAO1 biofilms on contact lenses compared to PBS and TSB controls. Initial bacterial adhesion is indicated (see Methods). Biofilms were allowed to form over 5 days during exposure to the different media indicated at time zero.* 1 day and 3 day tear fluid- or PBS-grown biofilms showed significantly reduced bacterial culturability compared to TSB-grown biofilms at each time point ($p<.05$, one-way ANOVA with Dunn's Multiple post-hoc analysis), but did not differ from each other.** After 5 days, bacteria numbers were significantly higher in tear fluid or TSB than in the same media on days 1 and 3 ($p<.05$, one-way ANOVA with Dunn's Multiple post-hoc analysis), but not significantly different between media. B. Effect of human tear fluid on preformed biofilms of *P. aeruginosa* PAO1 grown on contact lenses for 7 days in TSB ("initial" refers to viability of 7 day bacterial biofilm).* Tear fluid exposure (24 h) significantly reduced both the number of bacteria that could be cultured from the biofilm, and from incubation media around the biofilm (dispersed), compared to both the PBS and TSB controls ($p<.001$ for each group, one-way ANOVA with Dunn's Multiple post-hoc analysis).

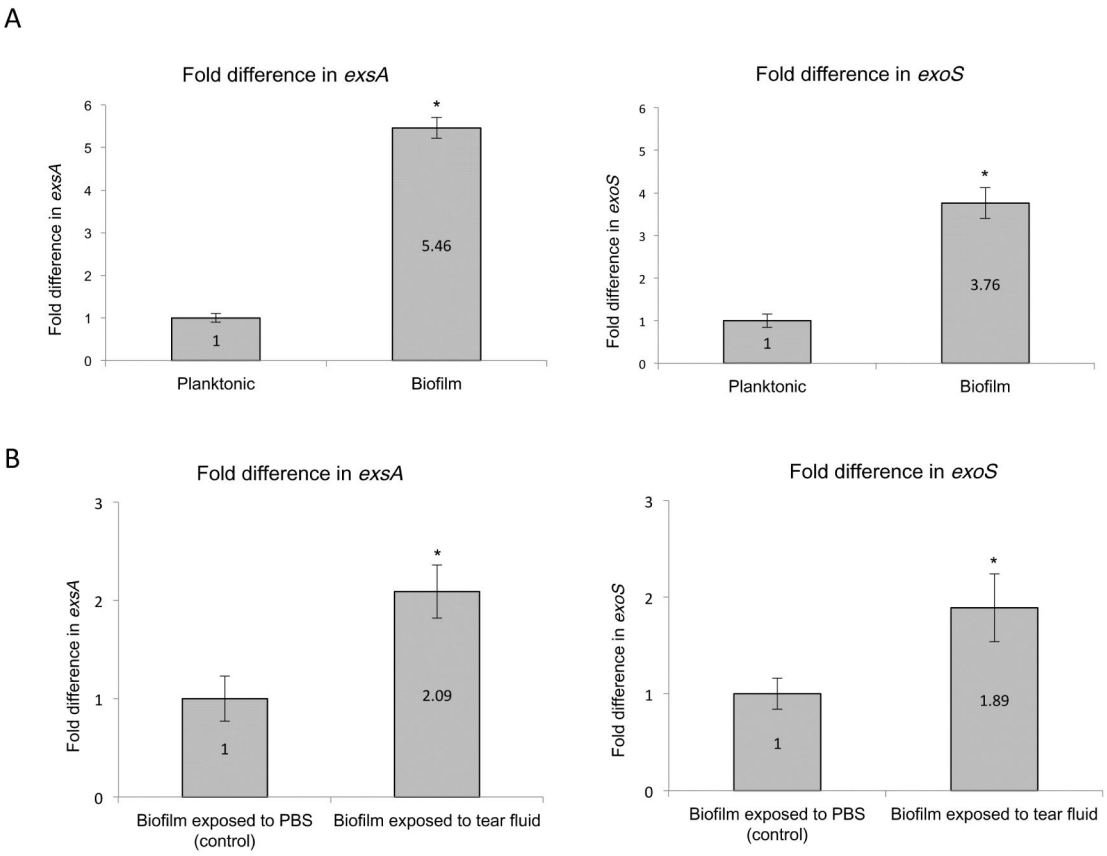

Figure 4.

P. aeruginosa T3SS gene expression in contact lens-associated biofilms by q-PCR. A. Expression of the T3SS transcriptional activator *exsA,* and effector toxin gene *exoS,* was significantly increased in contact lens-grown biofilms compared to bacteria grown in suspension (planktonic[* p=.02 for *exsA*, p=.01 for *exoS*, t-Test]). B. Expression of *exsA* and *exoS* was further upregulated in *P. aeruginosa* contact lens-grown biofilms exposed to human tear fluid for 5 h compared to biofilms without tear exposure (*p=.04 for *exsA*, p<. 001 for *exoS*, t-Test).