UC Berkeley

UC Berkeley Previously Published Works

Title

Human tear fluid modulates the Pseudomonas aeruginosa transcriptome to alter antibiotic susceptibility

Permalink

https://escholarship.org/uc/item/58g550z5

Authors

Tabor, Lauren M
Grosser, Melinda R
Metruccio, Matteo MME
et al.

Publication Date

2021-10-01

DOI

10.1016/j.jtos.2021.07.004

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

Ocul Surf. Author manuscript; available in PMC 2023 April 27.

Published in final edited form as:

Ocul Surf. 2021 October ; 22: 94–102. doi:10.1016/j.jtos.2021.07.004.

Human tear fluid modulates the *Pseudomonas aeruginosa* transcriptome to alter antibiotic susceptibility

Lauren M. Tabor[1,*], **Melinda R. Grosser**[1,*,+], **Matteo M. M. E. Metruccio**[1], **Naren G. Kumar**[1], **Yvonne T. Wu**[1], **Vincent Nieto**[1], **David J. Evans**[1,2], **Suzanne M.J. Fleiszig**[1,3,#]

[1]School of Optometry, University of California, Berkeley, CA USA

[2]College of Pharmacy, Touro University California, Vallejo, CA USA

[3]Graduate Groups in Vision Science, Microbiology, and Infectious Diseases & Immunity, University of California, Berkeley, CA USA

Abstract

Purpose—Previously, we showed that tear fluid protects corneal epithelial cells against *Pseudomonas aeruginosa* without suppressing bacterial viability. Here, we studied how tear fluid affects bacterial gene expression.

Methods—RNA-sequencing was used to study the *P. aeruginosa* transcriptome after tear fluid exposure (5 h, 37 C). Outcomes were further investigated by biochemical and physiological perturbations to tear fluid and tear-like fluid (TLF) and assessment of bacterial viability following tear/TLF pretreatment and antibiotic exposure.

Results—Tear fluid deregulated ~180 *P. aeruginosa* genes ≥ 8 fold versus PBS including downregulating *lasI, rhlI, qscR* (quorum sensing/virulence), *oprH, phoP, phoQ* (antimicrobial resistance) and *arnBCADTEF* (polymyxin B resistance). Upregulated genes included *algF* (biofilm formation) and *hemO* (iron acquisition). qPCR confirmed tear down-regulation of *oprH, phoP* and *phoQ*. Tear fluid pre-treatment increased *P. aeruginosa* resistance to meropenem ~5-fold (4 μg/ml), but enhanced polymyxin B susceptibility ~180-fold (1 μg/ml), the latter activity reduced by dilution in PBS. Media containing a subset of tear components (TLF) also sensitized bacteria to polymyxin B, but only ~22.5-fold, correlating with TLF/tear fluid Ca^{2+} and Mg^{2+} concentrations. Accordingly, *phoQ* mutants were not sensitized by TLF or tear fluid. Superior activity of tear fluid versus TLF against wild-type *P. aeruginosa* was heat resistant but proteinase K sensitive.

Conclusion—*P. aeruginosa* responds to human tear fluid by upregulating genes associated with bacterial survival and adaptation. Meanwhile, tear fluid down-regulates multiple virulence-associated genes. Tears also utilize divalent cations and heat resistant/proteinase K sensitive component(s) to enhance *P. aeruginosa* sensitivity to polymyxin B.

[#]Correspondence: Dr. Suzanne M. J. Fleiszig, School of Optometry, University of California, Berkeley, CA 94720 USA. Tel. 1 (510) 643-0990, fleiszig@berkeley.edu.

[*]Contributed equally to first authorship

[+]Present addresses: University of North Carolina, Asheville, NC USA

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Keywords

Tear fluid; *Pseudomonas aeruginosa* ; RNA-sequencing; Antimicrobial susceptibility; *oprH-phoP*/*phoQ*; *arn* operon; Polymyxin B; Tear-like fluid

1. Introduction

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen able to cause both acute and chronic life threatening infections at multiple body sites. It is also the leading cause of contact lens-related microbial keratitis, a sight-threatening disease of the cornea [1–3]. Due to emerging *P. aeruginosa* clinical isolates that exhibit resistance to commonly used antibiotics, the World Health Organization (WHO) recently promoted *P. aeruginosa* to "critical status" on the Global Priority Pathogen List [4,5]. Therefore, there is an urgent need to elucidate the molecular mechanisms driving *P. aeruginosa* pathogenesis and antibiotic susceptibility [6,7].

Despite a wide array of genes devoted to antimicrobial resistance and virulence regulation, *P. aeruginosa* is unable to infect the healthy cornea in the absence of contact lens wear [1,8,9]. Nevertheless, corneal epithelial cells raised *in vitro* are highly sensitive to *P. aeruginosa*, even if inoculated with 5-log fewer bacteria [1,10,11]. Contributing to the vastly reduced susceptibility *in vivo*, is the presence of mucosal fluid (tear fluid) [3,12]. Blinking distributes tear fluid across the corneal surface, physically removing microbes that might otherwise cause infection. In addition, multiple tear fluid components have direct antimicrobial activity and/or can counter bacterial adhesion. These include secretory IgA, lysozyme, lactoferrin, antimicrobial peptides, surfactant proteins, glycoproteins and mucins [1,3,9,13–15].

Mechanisms by which tear fluid protects the corneal epithelium against bacteria extend beyond reducing bacterial viability or bacterial adhesion [1,3,16]. We previously reported that tear fluid could act directly on corneal epithelial cells to enhance their resistance to bacterial invasion and cytotoxic effects. This was associated with a multitude of changes to gene expression in tear fluid treated epithelial cells, including upregulation of host stress response factors NF-κB and AP-1 [12]. In another study, we found that fluid could enhance the transepithelial resistance (TER) of corneal epithelial cell multilayers associated with reduced susceptibility to traversal by bacteria [13]. Increased TER can enhance epithelial cell polarity, another cellular defense against bacteria [3,17,18]. We further showed that tear fluid mediated effects on epithelial cells were in part driven by alterations to microRNA expression, including upregulation of microRNA-762, which negatively regulates two factors (RNase7 and ST2) that play critical roles in host cell immunity [1,12,19].

We have also shown that tear fluid can act on bacteria without reducing their viability. Using *P. aeruginosa*, we have found that tears interfere with biofilm formation, dispersal of existing biofilms [20,21], and the suppression of both twitching [22,23] and swimming motility [16]. These properties have all been connected to virulence *in vivo* during both chronic and acute infections, and they each support the lifestyle of this adaptable microbe in *in vitro* settings [12]. While we identified the glycoprotein DMBT1 (Deleted in Malignant Brain Tumors 1) as the tear fluid component responsible for inhibiting twitching motility [22,23], tear

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

ingredients that compromise biofilm formation and suppress swimming remain unknown. The contribution of changes to bacterial gene expression is also yet to be determined.

To help fill these knowledge gaps, here we performed an unbiased analysis of the impact of human tear fluid on the transcriptome of *P. aeruginosa* strain PAO1. The results showed that several genes associated with virulence and antibiotic resistance were impacted, the pattern suggesting tear-mediated defenses and also countering bacterial adaptations. Follow-up experiments showed that tear fluid impacts antibiotic susceptibility differentially depending on the agent tested, including increasing resistance to meropenem and enhancing susceptibility to polymyxin B. Sensitization to polymyxin B was correlated with downregulation of the *phoPQ*, *pmrAB*, and *arnBCADTEF* operons known associated with polymyxin B resistance. An investigation of tear fluid components responsible for polymyxin B sensitization revealed roles for divalent cations Ca^{2+} and Mg^{2+} and additionally heat resistant/proteinase K susceptible ingredients. These results have potential for development of novel therapeutic strategies. They also highlight the importance of considering the *in vivo* environment when studying microbial pathogenesis or evaluating the potential efficacy of antimicrobial treatments.

2. Methods

2.1 Bacterial culture and generation of mutants

P. aeruginosa (strain mPAO1) obtained from the laboratory of Dr. Manoil (University of Washington, Seattle, WA) was used for all experiments. Bacteria were grown on tryptic soy agar (TSA) plates (16 h, 37 °C) to obtain “lawn” colony densities, and in some experiments then grown in tryptic soy broth (TSB) (16 h, 37 °C). A *phoQ* clean deletion mutant was generated *via* allelic exchange using the phage λ-Red recombination and CRISPR/Cas9 negative selection system as described in Chen *et al.* 2018 [24]. To design the programmable portion of a single guide RNA (sgRNA) that would direct Cas9 to the wild-type *phoQ* gene, a 20 bp sequence from *phoQ* with adjacent PAM motif was selected and ordered as two oligos. These were annealed and cloned via Golden Gate assembly into pACRISPR (Addgene), transformed into *E. coli* Dh5α, and verified by sequencing. Next, a repair template was generated by PCR-amplifying 500-bp regions flanking the *phoQ* gene. These fragments were cloned simultaneously via Gibson Assembly into the XhoI/XbaI sites of pACRISPR+sgRNA, transformed into *E. coli* Dh5α, and verified by PCR. The complete the pACRISPR-*phoQ* plasmid was transformed *via* electroporation into mPAO1 already containing the plasmid pCasPA (Addgene), with arabinose added to induce Cas9 expression from pCasPA; transformants were selected on LB agar with 150 μg/ml carbenicillin and 100 μg/ml tetracycline. Both plasmids were cured by negative counter-selection on 5 % sucrose LB agar. Mutants were confirmed by PCR and gel electrophoresis. PAO1Δ*pho*Q was cultured as for wild-type PAO1.

2.2 Tear fluid collection

Tear fluid was collected from healthy volunteers (male and female) under an approved protocol (Committee for the Protection of Human Subjects, University of California, Berkeley). This research followed the tenets of the Declaration of Helsinki. Informed

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

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. Tear fluid was pooled as needed to obtain sufficient volume for each experiment.

2.3 RNA-sequencing and analysis

Bacteria were grown on TSA overnight, then colonies inoculated into TSB and cultured in a shaking incubator (16 h, 37 °C). The following day, the TSB culture was sub-cultured in fresh TSB and incubated for 2 h at 37 °C until reaching an absorbance of ~0.4 at 650 nm. Bacteria were centrifuged at ~ 5,000 × g for 5 min and the bacterial pellet reconstituted in 2 ml of tear fluid or PBS control and incubated for 5 h at 37 °C with agitation. The 5 h time point for was chosen for initial analysis to allow sufficient time for *P. aeruginosa* to express tear-associated changes in gene expression. The samples were then centrifuged at 5,000 × g for 5 min to obtain a bacterial pellet for RNA extraction. RNA was extracted from samples in each condition according to the protocol in RNeasy kit (Qiagen) including on-column DNase steps to remove any contaminating genomic DNA. The RNA was first checked with a nanodrop to assess concentration, and the quality evaluated using the Agilent Model 2100 bioanalyzer (RIN > 8) before proceeding with RNA-sequencing. The rRNA was then depleted using Ribozero kit from Epicenter (rRNA content < 10 %), this rRNA-depleted RNA was then used to generate the libraries according to the RNA-Sequencing Library Construction Kit (Ambion). Sequencing of the libraries was performed using a HiSeq 2000 (Illumina). RNA sequencing involved one replicate per condition, and sequencing quality, significance and differential gene expression determined using Rockhopper [25,26].

Gene set enrichment analysis on differentially-expressed genes was performed using ADAGE [27,28]. Enriched networks were combined with RNA-sequencing fold-change information *via* Cytoscape (https://cytoscape.org), then plotted within an untargeted interactome. ADAGE is a neural network model which identifies gene signatures associated with various biological processes. Each gene holds a weight value associated with each node, represented by edge strength. Gene signatures are determined from gene sets that contribute the highest positive and negative weights to a given node. Thus, both a positive and negative gene signature are produced from a single node [27,28].

2.4 Tear-like fluid (TLF) and subcomponents

The formula for the TLF used has been previously published [29]. TLF buffer solution was made in a 500 ml volume of Dulbecco PBS (PBS containing magnesium and calcium salts). Sodium bicarbonate ($NaHCO_3$) and D-glucose were dissolved into solution for a final concentration of 1.37 and 0.1 mg/ml, respectively. TLF lipid solution was prepared as follows: concentrated lipid stock solution (1000x) was prepared in 50/50 chloroform/methanol. The following reagents were added to 10 ml of chloroform/methanol solution: cholesteryl linoleate (24 mg/ml), linalyl acetate (18 mg/ml), triolein (14.5 mg/ml), oleic acid (10.4 mg/ml), undecylenic acid (2.6 mg/ml) and cholesterol (1.6 mg/ml). To prepare a TLF mucin-lipid solution: 0.075 g of mucins were added to 0.5 ml of lipid stock solution within

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

a 1 L flask. 10 ml of TLF buffer solution was added to the mucin-lipid mixture before the solution was heated and stirred for 1 h. Additional TLF buffer solution was then added to a total volume of 500 ml. To prepare complete TLF, the following proteins were dissolved one at a time into the TLF mucin-lipid solution: Acid alpha 1 glycoprotein (0.05 mg/ml), bovine serum (0.1%), gammaglobulins (0.3 mg/ml), B lactoglobulin (lipocalin) (1.3 mg/ml), lysozyme (2 mg/ml) and lactoferrin (2 mg/ml). Lactoferrin and Lysozyme were the final components added. The final solution was kept overnight at 4 C, then allowed to warm to room temperature before incubation with bacteria. All reagents were obtained from Sigma (St. Louis, MO) except D-glucose obtained from Fisher Scientific (Pittsburgh, PA).

2.5 Tear fluid and TLF pretreatment and antibiotic viability assays

P. aeruginosa was cultured on TSA (as a “lawn” of bacteria) and diluted in Sigma-Aldrich PBS to an absorbance of ~15 at a 650 nm. Then, 1–2 μl of bacterial suspension was added to ~18 μl of experimental incubation media (Tear fluid, TLF, PBS or M63) to a total volume of 20 μl and final absorbance of ~1.0. M63 minimal medium [30] was used as an additional control to account for differences in bacterial growth during pretreatment that could potentially influence antibiotic efficacy. In controls, *P. aeruginosa* mPAO1 used for this study showed a growth rate in tear fluid between that of M63 medium and PBS (data not shown). The solutions were incubated within 1.5 ml microcentrifuge tubes (4 h, 37°C) without shaking, then pelleted *via* centrifugation (5,000 × g, 5 min). Each solution was gently resuspended in 2 ml of TSB to form the various pretreated bacterial inocula. For each pretreated inoculum, 100 μl was added to each of 4 wells of a 96-well microplate and antibiotic stock solutions added to 2 of those 4 wells, with 2 wells left as untreated controls. Antibiotic and control mixtures were incubated while shaking (2 h or 4 h, 37 C, 180 rpm). The following concentrations of antibiotics were used: polymyxin B (1 and 0.5 μg/ml), meropenem (4 μg/ml), ceftazidime (4 μg/ml), tobramycin (2 μg/ml), tetracycline (64 μg/ml), rifampicin (64 μg/ml). For polymyxin B, anhydrous polymyxin B was reconstituted in 1 ml of deionized water, then diluted to make a stock solution of 0.05 mg/ml that was used to achieve final concentrations of 1 or 0.5 μg/ml. The remainder of the 2 ml pretreated inoculum was serially diluted. Each dilution was plated onto TSA plates in quadruplicate and incubated (16 h, 37 C) for enumeration of bacterial colonies (CFU) in the pretreated inoculum. The 96-well plate was removed after incubation and serial dilutions plated in duplicate onto TSA plates and incubated (16 h, 37 C) for enumeration of bacterial colonies (CFU) post-antibiotic exposure. Percent survival was determined versus the pretreated inoculum.

2.6 Statistical analysis

Data were expressed as the mean ± standard error of the mean (SEM) unless otherwise stated. Significance of differences between groups was determined by one-way or two-way ANOVA followed by Tukey’s test for post-hoc multiple comparisons or Student’s t-Test as appropriate. Microsoft Prism software was used. P values of < 0.05 were considered significant, and all experiments were performed at least three times or as otherwise indicated.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

3. Results

3.1 *P. aeruginosa* responds to human tear fluid with genome-wide transcriptome changes

RNA-sequencing analysis was used to study the impact of human tear fluid exposure on the transcriptome of *P. aeruginosa* strain PAO1 after 5 h at 37 C. Results were compared to incubation in PBS under the same conditions. Exposure of *P. aeruginosa* to tear fluid resulted in a significant alteration of bacterial gene expression; ~180 genes with ≥ 8-fold dysregulation (up or down-regulated) were differentially expressed relative to bacteria exposed to PBS. Genes that experienced ≥ 4-fold changes were identified and included in a reduced gene network (Fig 1A, Supplemental Fig S1). Genes upregulated included *algF* associated with biofilm formation (11-fold) and both *phuS* and *hemO* associated with iron acquisition (7-fold, and 17-fold, respectively) (Table 1). Downregulated genes included *lasI*, *rhlI* and *qscR*, involved in quorum sensing and virulence (20-fold, 10-fold, and 10-fold, respectively), in addition to *oprH, phoP, phoQ*, and *pmrB* involved in two-component regulated resistance (55-fold, 15-fold, 9-fold, and 4-fold, respectively) (Table 1).

A subsequent analysis was performed to determine if differentially expressed genes included those associated with virulence and antimicrobial response pathways. Gene set enrichment analysis was performed using ADAGE [27,28], which identifies common gene groups in public databases and assigns them to established gene signatures. The analysis showed that bacterial expression of gene clusters involving antibiotic resistance and quorum sensing were significantly altered in *P. aeruginosa* exposed to tear fluid (Fig 1A, Supplemental Table S1). The *pqq* genes included in Sig089pos (Supplemental Table S1) are known to induce aerobic ethanol oxidation and were found upregulated in tear fluid. Additionally, there were several downregulated gene signatures associated with antibiotic resistance, including: Sig098pos which included a majority of *arn* genes, and Sig137pos which included *phoP, phoQ* and *oprH* genes (Fig. 1A, Supplemental Table S1). Tear fluid down-regulation of *oprH*, *phoP* and *phoQ* was confirmed *via* qPCR (Fig 1B).

3.2 Prior exposure to human tear fluid impacts *P. aeruginosa* antibiotic susceptibility

Since tear fluid exposure impacted a significant number of genes involved in environmental sensing, stress responses, and antibiotic resistance, we next asked if tear fluid impacted bacterial survival in a panel of antibiotics representing some different classes of clinically used agents on the eye or at other tissue sites. Thus, 4 h of prior exposure to tear fluid was compared to 4 h prior exposure to PBS and M63 medium controls (see Methods). After the 4 h exposure to tear fluid or control media, bacterial susceptibility to treatment with antibiotics was then determined over a second incubation period of either 2 h (Fig. 2A) or 4 h (Fig. 2B). Antibiotic concentrations chosen were based upon typical reported sensitivity data for *P. aeruginosa* [e.g. see 28] and our experience with the mPAO1 strain used for this study. Susceptibility to most antibiotics (2 h survival assay) was not significantly affected by prior incubation in tear fluid relative to pretreatment with M63 or PBS. Those included tobramycin (2 μg/ml), ceftazidime (4 μg/ml), tetracycline (64 μg/ml) and rifampin (64 μg/ml) (Fig 2A). Tear fluid exposure prior to meropenem (4 μg/ml) resulted in increased bacterial survival (3-fold difference) relative to bacteria pre-incubated in M63 medium ($p < 0.05$, two-way ANOVA). In contrast, tear fluid exposure prior to polymyxin B (1 μg/ml)

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

resulted in enhanced bacterial susceptibility ($p < 0.01$, two-way ANOVA). A 4 h survival assay showed similar results (Fig. 2B) with tear fluid-associated survival of meropenem greater than both M63 and PBS controls. Since polymyxin B (1μg/ml) combined with a 4 h survival assay after 4 h tear fluid pre-exposure resulted in no survivors (Fig 2B), a reduced concentration (0.5 μg/ml) and survival assay time (2 h) was subsequently used to better resolve *P. aeruginosa* survival.

3.3 Tear fluid concentration influences its sensitization of *P. aeruginosa* to polymyxin B

Having shown that human tear fluid sensitizes *P. aeruginosa* to killing by the antimicrobial peptide polymyxin B, we next explored the mechanisms involved. Previously, we showed that when corneal epithelial cells were inoculated with *P. aeruginosa in vitro*, the protective effects of tear fluid were susceptible to dilution, with activity lost after a 3-fold dilution in PBS [16]. Multiple cell types at the ocular surface express various antimicrobial peptides, some with mechanisms of action similar to the pore-forming antibiotic polymyxin B [32–35]. Thus, we examined if tear fluid sensitization of *P. aeruginosa* to polymyxin B was also susceptible to dilution.

Tear fluid was used whole or diluted into PBS v/v at 1:2 (50%), 1:4 (25%), and 1:8 (12.5%), then used to pretreat bacteria for 4 h prior to polymyxin B treatment (0.5 μg/ml, 2 h) (Fig 3). Results showed a dilution-dependent loss of tear sensitizing activity approaching the level of PBS controls ($p < 0.001$, one-way ANOVA). Pretreatment with 12.5 % tear fluid resulted in a significant increase in bacterial survival of polymyxin B exposure compared to pretreatment with undiluted (100 %) tear fluid ($p < 0.05$, one-way ANOVA) and confirmed the loss of sensitizing activity at that dilution.

3.4 Tear-like fluid (TLF) sensitizes *P. aeruginosa* to polymyxin B with reduced efficacy

Tear-like fluid (TLF) is a defined medium containing some key mucins, proteins, and lipids found in human tear fluid [36]. Thus, TLF was used to explore the role of a subset of tear fluid components in sensitizing bacteria to polymyxin B. Results showed that TLF pretreatment (4 h) could sensitize *P. aeruginosa* to polymyxin B versus PBS (0.5 μg/ml, 2 h, $p < 0.001$, one-way ANOVA) (Fig 4). While TLF had significant activity (~22.5-fold sensitization) it was relatively less effective than whole human tear fluid which sensitized ~180-fold ($p < 0.001$).

3.5 Ca^{2+} and Mg^{2+} contribute to *P. aeruginosa* polymyxin B sensitization

Having found that the subset of tear components in TLF contained polymyxin B sensitization activity, we next examined which components contributed. TLF was separated into; 1) its salt buffer and 2) its salt buffer with lipid components. Surprisingly, there was no significant difference between either fraction and TLF in ability to sensitize bacteria to polymyxin B (Supplemental Fig S2). This suggested that buffer components were responsible. TLF and human tear fluid both contain biologically relevant concentrations of Ca^{2+} and Mg^{2+}, previously shown to modulate expression of polymyxin resistance associated genes in *P. aeruginosa* [37,38]. Thus, we explored their role in TLF induced sensitization of *P. aeruginosa*. Results confirmed that *P. aeruginosa* pretreated with PBS supplemented with tear fluid/TLF relevant concentrations of Ca^{2+} and Mg^{2+} (~1.5 mM

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

and ~1 mM, respectively) became sensitized to polymyxin B (0.5 μg/ml, 2 h) compared to pretreatment with unsupplemented PBS (Fig 5) ($p < 0.05$, one-way ANOVA). PBS supplementation with other candidate components in both tears and TLF (bicarbonate and glucose, lysozyme and lactoferrin) did not result in sensitization. These finding implicated Ca^{2+} and Mg^{2+} concentrations within tear fluid in the sensitization of *P. aeruginosa* to polymyxin B.

3.6 *P. aeruginosa phoQ* is involved with tear fluid-induced sensitization to polymyxin B

The *arnBCADTEF* operon, regulated by the cation-sensitive *phoPQ* operon [38], can induce polymyxin B resistance in Gram-negative bacteria [33,37,39]. Thus, we next tested the hypothesis that tear fluid cation components (Ca^{2+}/Mg^{2+}) induce *P. aeruginosa* polymyxin B sensitivity through transcriptional suppression of *arnBCADTEF*. A clean deletion of *phoQ* was constructed in *P. aeruginosa* (PCR-verified prior to experimentation). This deletion results in constitutive expression of the *phoPQ* regulon and thus OprH and the *arnBCADTEF* protein product (L-Ara4N) that mediate resistance [37–42]. Results showed that the PAO1Δ*phoQ* mutant was not sensitized to killing by polymyxin B (0.5 μg/ml, 2 h treatment) (Fig 6). Indeed, it showed 10–100-fold higher average survival rates in these assays relative to wild-type PAO1 (e.g. compare Fig 6 with Fig 5). These results support the hypothesis that *phoQ* and the regulon it controls are important for tear fluid sensitization of *P. aeruginosa* to polymyxin B *via* Ca^{2+} and Mg^{2+}.

3.7 Tear fluid contains heat resistant and proteinase K sensitive factors that can sensitize *P. aeruginosa* to polymyxin B

The results presented above in Fig 5 confirmed that undiluted human tear fluid was much more potent than either TLF or PBS supplemented with Ca^{2+} and Mg^{2+} in sensitizing *P. aeruginosa* to polymyxin B. This suggested that human tear fluid contained components beyond Ca^{2+} and Mg^{2+} that also contribute to this activity.

To determine if a heat sensitive protein was involved, tear fluid was boiled for 10 min at 95 ^{0}C prior to treating bacteria. Boiled tears retained full sensitizing activity compared to untreated tear fluid (Fig 7). Since small peptides can be heat stable yet susceptible to protease enzyme digestion, tear fluid was next treated with the broad-spectrum serine proteinase, proteinase K (50 μg/mL, 2 h, 37 C). Proteinase K-digested tear fluid (for 2 h) had significantly reduced ability to sensitize *P. aeruginosa* to polymyxin B compared to untreated tear fluid ($p < 0.05$, one-way ANOVA). As expected, tear fluid retained low level activity similar to TLF (Fig 7).

Taken together, these results suggested that human tear fluid sensitization of *P. aeruginosa* to killing by polymyxin B involves two superimposed mechanisms, one involving Ca^{2+} and Mg^{2+} ion driven alterations to the bacterial transcriptome, the other a heat-resistant peptide.

4. Discussion

Our previously published studies showed that human tear fluid can protect corneal epithelial cells against *P. aeruginosa in vivo* and *in vitro* without suppressing bacterial viability through protective actions on both the corneal epithelial cell and the bacteria. We

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

subsequently showed that fluid bolsters the resistance of corneal epithelial cells by triggering alterations to the epithelial cell transcriptome involve microRNA regulation. Less is known about how tear fluid exerts non-lethal effects on *P. aeruginosa* which include suppression of known virulence determinants.

Here, we used RNA-sequencing to gain a global understanding of how human tear fluid impacts gene expression in *P. aeruginosa*, and we found ~180 genes impacted ≥ 8 fold. These included genes involved in adaptation to stress and virulence, as well as several key clusters associated with antibiotic resistance. We chose to focus further analysis on antibiotic resistance, because related gene clusters were profoundly altered. These included downregulation of the *oprH-phoP-phoQ, pmrAB*, and *arnBCADTEF* operons connected to polymyxin B resistance, which we explored in more detail. Results showed that tear fluid profoundly sensitized *P aeruginosa* to polymyxin B, and the divalent cations Ca^{2+} and Mg^{2+} were identified as the driver. The much greater potency of human tear fluid compared to TLF containing similar Ca^{2+} and Mg^{2+} concentrations (~1.5 mM Ca^{2+}, ~1 mM Mg^{2+}) pointed to a second mechanism driving polymyxin B sensitization that is absent from TLF and acts independently from or synergistically with divalent cations. The additional activity associated with tear fluid, which was heat-stable but proteinase K degradable, is likely to be peptide(s)-associated.

PhoPQ and PmrAB are two-component regulatory systems that modulate expression of *oprH* and the *arnBCADTEF* operon induced in environmental conditions of low divalent cation concentrations (Ca^{2+} and Mg^{2+}) [32–34, 37]. Mutations in these two-component regulatory systems (e.g. *phoQ*) are commonly found in naturally occurring polymyxin-resistant strains of *P. aeruginosa* and result in constitutive expression of *oprH* the *arnBCADTEF* operon [7,37,40]. Data obtained using a *phoQ* mutant was consistent with the hypothesized involvement of *arn*-mediated polymyxin resistance. A clean deletion of the *phoQ* gene, which causes constitutive expression of the *arnBCADTEF* operon, elicited polymyxin tolerance across all pre-treatment conditions, including tears and TLF (Fig 6). Indeed, the *phoQ* mutant incubated in control medium continued to grow after polymyxin B exposure, unlike wild-type bacteria under those conditions.

The mechanism by which *arnBCADTEF* expression confers resistance to polymyxin B involves formation of an aminoarabinose protein product, 4-amino-4-deoxy-L-arabinose (L-Ara4N) [7,39,43], thought to competitively inhibit polymyxin-LPS binding, thus preventing bacterial death [7,44]. Our results showing that polymyxin B sensitization induced by Ca^{2+} and Mg^{2+} levels in tear fluid and TLF (0.3–2.0 mM and 0.3–1.1 mM, respectively) depends on *phoQ* (which modulates *arnBCADTEF* expression) suggests that the tear fluid and TLF sensitization mechanism involves reduced production of the protective L-Ara4N protein.

Pinpointing the identity of other active ingredients and mechanisms by which they sensitize *P. aeruginosa* to polymyxin B will require further study. Our results suggested involvement of a heat-stable peptide(s). Mechanistic possibilities include a factor(s) that alters bacterial gene expression (same or different genes), or a factor that acts directly on bacteria to exert additive or synergistic activity with polymyxin B in targeting the bacterial cell wall/membrane [34,35]. Indeed, the findings of this study may relate to the presence

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

of endogenous ocular antimicrobial peptides (AMPs), which include defensins, LL-37 and fragments of keratin 6A [14,35,45–47]. AMPs are present in tear fluid, secreted by various ocular surface cells, and a subset share a similar mode of interaction with bacterial membranes as polymyxin B [8,35,38]. However, the relationship, if any, is likely to be complex given that AMPs can activate (upregulate) the *pmrAB* two-component regulatory system encoding polymyxin B resistance [38], essentially the opposite phenomenon.

The ability of tear fluid to sensitize *P. aeruginosa* to polymyxin B was gradually reduced with serial dilution in PBS. Notwithstanding, bacteria pre-treated with only 12.5 % tear fluid remained significantly more sensitive to polymyxin B than PBS treated bacteria, showing a robust defense mechanism(s) that could potentially tolerate the wide range of salt concentrations naturally present at the ocular surface in the healthy human population (0.3–2.0 mM Ca^{2+}, 0.3–1.1 mM Mg^{2+}) [48]. That said, potential clinical impacts (positive or negative) might still be evident under ocular or systemic disease conditions that affect tear fluid cation concentrations and/or composition, such as hyper/hypocalcemia [49], dry eye disease, application of eye drops or contact lens wear.

While tear fluid downregulating some antibiotic resistance genes (e.g. polymyxin B), it upregulated others. This likely reflects deliberate adaptation/survival responses in this highly adaptable opportunistic pathogen. An example was upregulation of the *pqq* operon, a complex regulatory network known to mediate chloramphenicol resistance in *Pseudomonas putida* [50]. While we screened an entire panel of antibiotics for efficacy after *P. aeruginosa* was exposed to tear fluid, the only significant result (aside from increased susceptibility to polymyxin B) was increased resistance to meropenem, a carbapenem antibiotic. Previous literature has established outer membrane porin and efflux pump overexpression as the primary mechanisms facilitating meropenem resistance [51–53], however, these changes were not found in our RNA sequencing analysis. It is possible that some of the differences in meropenem sensitivity observed reflect differences in bacterial growth rate or stress responses between pre-exposure conditions that could affect bacterial physiology when resuspended in TSB for antibiotic sensitivity assays, e.g. differences in adaptation to nutrient rich TSB and subsequent growth. That noted, 4 h tear pre-exposure significantly increased meropenem survival over both M63 medium and PBS controls suggesting a tear-mediated effect that may be independent of those factors. Thus, the mechanism(s) by which tear fluid mediates meropenem exposure resistance is/are unclear and is another finding warranting further investigation. In a separate (unpublished) study, we have observed the *in vivo* evolution of several *P. aeruginosa* clones with elevated meropenem resistance versus wild-type inoculum. Whether this relates to the presence of tear fluid remains to be determined.

Beyond impacting antibiotic resistance genes, the results revealed several other adaptive changes in *P. aeruginosa* gene expression in response to tear fluid. They included deregulation of multiple genes associated with biofilm formation, iron acquisition, and quorum sensing all known to be associated with virulence. Since the healthy ocular surface readily clears even high inocula of *P. aeruginosa*, logic dictates that these adaptive responses to tear fluid are not sufficient to mediate infection under normal circumstances [12,13]. However, further studies to determine their significance in the context of corneal injury, contact lens wear, and infection might be of value.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

5. Conclusion

The results of this investigation show that tear fluid exposure exerts a significant impact on *P. aeruginosa* gene expression, including modulating susceptibility to antibiotics. Potentially benefitting the host, tear fluid utilizes divalent cations to downregulate multiple bacterial genes associated with resistance to polymyxin B. This is augmented by other tear fluid components, likely peptide in nature, to greatly enhance susceptibility to this cationic antibiotic with a mechanism of action similar to endogenous antimicrobial peptides. Other changes to the transcriptome induced by tear fluid potentially benefit the bacteria. These include inducing the expression of multiple genes that encode known virulence determinants and that support survival under conditions of stress. We also noted enhanced resistance to meropenem, an antibiotic commonly utilized to treat *P. aeruginosa* infection.

Taken together, these results provide a mere glimpse of the complexities involved in the balancing act of interactions between pathogen and host that usually results in the *status quo* of infection resistance. Understanding these complexities and how the scales “tip” when the cornea becomes susceptible to infection and/or when therapeutics or contact lenses are introduced, is key to developing eyecare strategies that are effective without surprising (unwanted) consequences.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Thanks to Dr. Manoil, University of Washington, for providing *P. aeruginosa* mPAO1.

Funding and disclosures

This work was supported by the National Institutes of Health EY024060 (SMJF), American Heart Association (MG), American Optometric Foundation (Vistakon Research Grant) (YW/SMJF) and the Australian National Health and Medical Research Council (CJ Martin Fellowship) (YW). The funding sources were not involved in study design, data collection or analysis, decision to publish or article preparation. The authors have no commercial or proprietary interest in any concept or product described in this article.

References

[1]. Fleiszig SMJ, Kroken AR, Nieto V, Grosser MR, Wan SJ, Metruccio MME, et al. Contact lens-related corneal infection: Intrinsic resistance and its compromise. Prog Retin Eye Res 2019:76;100804. 10.1016/J.PRETEYERES.2019.100804. [PubMed: 31756497]

[2]. Hilliam Y, Kaye S, Winstanley C. *Pseudomonas aeruginosa* and microbial keratitis. J Med Microbiol 2020;69:3–13. 10.1099/jmm.0.001110. [PubMed: 31750813]

[3]. Kwong MSF, Evans DJ, Ni M, Cowell BA, Fleiszig SMJ. Human tear fluid protects against *Pseudomonas aeruginosa* keratitis in a murine experimental model. Infect Immun 2007;75:2325–32. 10.1128/IAI.01404-06. [PubMed: 17325054]

[4]. Asokan GV, Ramadhan T, Ahmed E, Sanad H. WHO global priority pathogens list: A bibliometric analysis of medline-pubmed for knowledge mobilization to infection prevention and control practices in Bahrain. Oman Med J 2019;34:184–93. 10.5001/omj.2019.37. [PubMed: 31110624]

[5]. De Freitas LC. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. WHO 2013;43:348–65. 10.1590/S0100-15742013000100018.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

[6]. Ferrer-Espada R, Shahrour H, Pitts B, Stewart PS, Sánchez-Gómez S, Martínez-de-Tejada G. A permeability-increasing drug synergizes with bacterial efflux pump inhibitors and restores susceptibility to antibiotics in multi-drug resistant *Pseudomonas aeruginosa* strains. Sci Rep 2019;9:1–12. 10.1038/s41598-019-39659-4. [PubMed: 30626917]

[7]. Han M-L, Zhu Y, Creek DJ, Lin Y-W, Gutu AD, Hertzog P, et al. Comparative metabolomics and transcriptomics reveal multiple pathways associated with polymyxin killing in *Pseudomonas aeruginosa*. MSystems 2019;4.e00149–18. 10.1128/msystems.00149-18.

[8]. Evans DJ, Fleiszig SMJ. Why does the healthy cornea resist *Pseudomonas aeruginosa* infection? Am J Ophthalmol 2013;155:961–970 e2. 10.1016/j.ajo.2013.03.001. [PubMed: 23601656]

[9]. Wan SJ, Ma S, Evans DJ, Fleiszig SMJ. Resistance of the murine cornea to bacterial colonization during experimental dry eye. PLoS One 2020;15.e0234013. 10.1371/journal.pone.0234013.

[10]. Fleiszig SM, Zaidi TS, Pier GB. *Pseudomonas aeruginosa* invasion of and multiplication within corneal epithelial cells *in vitro*. Infect Immun 1995;63:4072–7. [PubMed: 7558321]

[11]. Fleiszig SMJ, Zaidi TS, Preston MJ, Grout M, Evans DJ, Pier GB. Relationship between cytotoxicity and corneal epithelial cell invasion by clinical isolates of *Pseudomonas aeruginosa*. Infect Immun 1996;64:2288–94. 10.1128/iai.64.6.2288-2294.1996. [PubMed: 8675339]

[12]. Mun JJ, Tam C, Evans DJ, Fleiszig SMJ. Modulation of epithelial immunity by mucosal fluid. Sci Rep 2011;1:8. 10.1038/srep00008. [PubMed: 22355527]

[13]. Augustin DK, Heimer SR, Tam C, Li WY, Le Due JM, Evans DJ, et al. Role of defensins in corneal epithelial barrier function against *Pseudomonas aeruginosa* traversal. Infect Immun 2011;79:595–605. 10.1128/IAI.00854-10. [PubMed: 21115716]

[14]. Tam C, Mun JJ, Evans DJ, Fleiszig SMJ. Cytokeratins mediate epithelial innate defense through their antimicrobial properties. J Clin Invest 2012;122:3665–77. 10.1172/JCI64416. [PubMed: 23006328]

[15]. Fleiszig SM, Zaidi TS, Ramphal R, Pier GB. Modulation of *Pseudomonas aeruginosa* adherence to the corneal surface by mucus. Infect Immun 1994;62:1799–804. [PubMed: 8168942]

[16]. Fleiszig SMJ, Kwong MSF, Evans DJ. Modification of *Pseudomonas aeruginosa* interactions with corneal epithelial cells by human tear fluid. Infect Immun 2003;71:3866–74. 10.1128/IAI.71.7.3866-3874.2003. [PubMed: 12819071]

[17]. Fleiszig SMJ, Vallas V, Jun CH, Mok L, Balkovetz DF, Roth MG, et al. Susceptibility of epithelial cells to *Pseudomonas aeruginosa* invasion and cytotoxicity is upregulated by hepatocyte growth factor. Infect Immun 1998;66:3443–6. [PubMed: 9632620]

[18]. Fleiszig SMJ, Evans DJ, Do N, Vallas V, Shin S, Mostov KE. Epithelial cell polarity affects susceptibility to *Pseudomonas aeruginosa* invasion and cytotoxicity. Infect Immun 1997;65;2861–7. [PubMed: 9199460]

[19]. Mun J, Tam C, Chan G, Kim JH, Evans D, Fleiszig S. MicroRNA-762 is upregulated in human corneal epithelial cells in response to tear fluid and *Pseudomonas aeruginosa* antigens and negatively regulates the expression of host defense genes encoding RNase7 and ST2. PLoS One 2013;8:e57850. 10.1371/journal.pone.0057850. [PubMed: 23469087]

[20]. Singh PK, Parsek MR, Greenberg EP, Welsh MJ. A component of innate immunity prevents bacterial biofilm development. Nature 2002;417:552–5. 10.1038/417552a. [PubMed: 12037568]

[21]. Wu YT, Tam C, Zhu LS, Evans DJ, Fleiszig SMJ. Human tear fluid reduces culturability of contact lens-associated *Pseudomonas aeruginosa* biofilms but induces expression of the virulence-associated type III secretion system. Ocul Surf 2017;15:88–96 10.1016/j.jtos.2016.09.001. [PubMed: 27670247]

[22]. Li J, Wan SJ, Metruccio MME, Ma S, Nazmi K, Bikker FJ, et al. DMBT1 inhibition of *Pseudomonas aeruginosa* twitching motility involves its N-glycosylation and cannot be conferred by the Scavenger Receptor Cysteine-Rich bacteria-binding peptide domain. Sci Rep 2019;9:13146. 10.1038/s41598-019-49543-w. [PubMed: 31511582]

[23]. Li J, Metruccio MME, Smith BE, Evans DJ, Fleiszig SMJ. Mucosal fluid glycoprotein DMBT1 suppresses twitching motility and virulence of the opportunistic pathogen *Pseudomonas aeruginosa*. PLoS Pathog 2017;13:e1006392. 10.1371/journal.ppat.1006392. [PubMed: 28489917]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

[24]. Chen W, Zhang Y, Zhang Y, Pi Y, Gu T, Song L, et al. CRISPR/Cas9-based genome editing in *Pseudomonas aeruginosa* and cytidine deaminase-mediated base editing in *Pseudomonas* species. IScience 2018;6:222–31. 10.1016/j.isci.2018.07.024. [PubMed: 30240613]

[25]. McClure R, Balasubramanian D, Sun Y, Bobrovskyy M, Sumby P, Genco CA, et al. Computational analysis of bacterial RNA-Seq data. Nucleic Acids Res 2013;41:e140. 10.1093/nar/gkt444. [PubMed: 23716638]

[26]. Tjaden B. A computational system for identifying operons based on RNA-seq data. Methods 2020;176:62–70. 10.1016/j.ymeth.2019.03.026. [PubMed: 30953757]

[27]. Tan J, Hammond JH, Hogan DA, Greene CS. ADAGE-based integration of publicly available *Pseudomonas aeruginosa* gene expression data with denoising autoencoders illuminates microbe-host interactions. MSystems 2016;1:e00025–15. 10.1128/msystems.00025-15. [PubMed: 27822512]

[28]. Tan J, Huyck M, Hu D, Zelaya RA, Hogan DA, Greene CS. ADAGE signature analysis: Differential expression analysis with data-defined gene sets. BMC Bioinformatics 2017;18:512. 10.1186/s12859-017-1905-4. [PubMed: 29166858]

[29]. Mirejovsky D, Patel AS, Rodriguez DD, Hunt TJ. Lipid adsorption onto hydrogel contact lens materials. Advantages of Nile red over oil red O in visualization of lipids. Optom Vis Sci 1991;68:858–64. 10.1097/00006324-199111000-00005. [PubMed: 1766647]

[30]. Elbing KL, Brent R. Media preparation and bacteriological tools. Curr. Protoc. Protein Sci 1998;13: Hoboken, NJ, USA: John Wiley & Sons, Inc., p. A.4A.1–A.4A.5. 10.1002/0471140864.psa04as13.

[31]. Sader HS, Huband MD, Castanheira M, Flamm RK. *Pseudomonas aeruginosa* antimicrobial susceptibility results from four years (2012 to 2015) of the International Network for Optimal Resistance Monitoring Program in the United States. Antimicrob Agents Chemother 2017;61. 10.1128/AAC.02252-16.

[32]. Haynes RJ, Tighe PJ, Dua HS. Antimicrobial defensin peptides of the human ocular surface. Br J Ophthalmol 1999;83:737–41. 10.1136/bjo.83.6.737. [PubMed: 10340986]

[33]. Trimble MJ, Mlynárčik P, Kolář M, Hancock REW. Polymyxin: Alternative mechanisms of action and resistance. Cold Spring Harb Perspect Med 2016;6:a025288. 10.1101/cshperspect.a025288. [PubMed: 27503996]

[34]. Andersson DI, Hughes D, Kubicek-Sutherland JZ. Mechanisms and consequences of bacterial resistance to antimicrobial peptides. Drug Resist Updat 2016;26:43–57. 10.1016/J.DRUP.2016.04.002. [PubMed: 27180309]

[35]. McDermott AM. The role of antimicrobial peptides at the ocular surface. Ophthalmic Res 2009;41:60–75. 10.1159/000187622. [PubMed: 19122467]

[36]. Sterner O, Aeschlimann R, Zürcher S, Osborn Lorenz K, Kakkassery J, Spencer ND, et al. Friction measurements on contact lenses in a physiologically relevant environment: Effect of testing conditions on friction. Investig Ophthalmol Vis Sci 2016;57:5383–92. 10.1167/iovs.16-19713. [PubMed: 27737459]

[37]. Barrow K, Kwon DH. Alterations in two-component regulatory systems of phoPQ and pmrAB are associated with polymyxin B resistance in clinical isolates of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2009;53:5150–4. 10.1128/AAC.00893-09. [PubMed: 19752280]

[38]. McPhee JB, Lewenza S, Hancock REW. Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. Mol Microbiol 2003;50:205–17. 10.1046/j.1365-2958.2003.03673.x. [PubMed: 14507375]

[39]. Schurek KN, Sampaio JLM, Kiffer CRV, Sinto S, Mendes CMF, Hancock REW. Involvement of pmrAB and phoPQ in polymyxin B adaptation and inducible resistance in non-cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2009;53:4345–51. 10.1128/AAC.01267-08. [PubMed: 19635950]

[40]. Moskowitz SM, Brannon MK, Dasgupta N, Pier M, Sgambati N, Miller AK, et al. PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. Antimicrob Agents Chemother 2012;56:1019–30. 10.1128/AAC.05829-11. [PubMed: 22106224]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

[41]. Soncini FC, García E, García G, Scovi VÉ, Groisman EA. Transcriptional autoregulation of the *Salmonella typhimurium* phoPQ operon. J Bacteriol 1995; 177:4364–71. [PubMed: 7543474]

[42]. Macfarlane ELA, Kwasnicka A, Ochs MM, Hancock REW. PhoP-PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. Mol Microbiol 1999;34:305–16. 10.1046/j.1365-2958.1999.01600.x. [PubMed: 10564474]

[43]. Poole K, Lau CHF, Gilmour C, Hao Y, Lam JS. Polymyxin susceptibility in *Pseudomonas aeruginosa* linked to the MexXY-OprM multidrug efflux system. Antimicrob Agents Chemother 2015;59:7276–89. 10.1128/AAC.01785-15. [PubMed: 26369970]

[44]. Lo Sciuto A, Imperi F. Aminoarabinosylation of lipid A is critical for the development of colistin resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2018;62:e01820–17. 10.1128/AAC.01820-17. [PubMed: 29263076]

[45]. Jiang YY, Xiao W, Zhu MX, Yang ZH, Pan XJ, Zhang Y, et al. The effect of human antibacterial peptide LL-37 in the pathogenesis of chronic obstructive pulmonary disease. Respir Med 2012;106:1680–9. 10.1016/j.rmed.2012.08.018. [PubMed: 22981321]

[46]. Davidopoulou S, Theodoridis H, Nazer K, Kessopoulou E, Menexes G, Kalfas S. Salivary concentration of the antimicrobial peptide LL-37 in patients with oral lichen planus. J Oral Microbiol 2014;6:1–6. 10.3402/jom.v6.26156.

[47]. Yanagi S, Ashitani JI, Ishimoto H, Date Y, Mukae H, Chino NN, et al. Isolation of human β-defensin-4 in lung tissue and its increase in lower respiratory tract infection. Respir Res 2005;6:130. 10.1186/1465-9921-6-130. [PubMed: 16269090]

[48]. Woodward AM, Senchyna M, Argüeso P. Differential contribution of hypertonic electrolytes to corneal epithelial dysfunction. Exp Eye Res 2012;100:98–100. 10.1016/j.exer.2012.04.014. [PubMed: 22564970]

[49]. Nagai N, Ogata F, Kawasaki N, Ito Y, Funakami Y, Okamoto N, et al. Hypercalcemia leads to delayed corneal wound healing in ovariectomized rats. Biol Pharm Bull 2015;38:1063–9. 10.1248/bpb.b15-00227. [PubMed: 26133716]

[50]. Gliese N, Khodaverdi V, Görisch H. The PQQ biosynthetic operons and their transcriptional regulation in *Pseudomonas aeruginosa*. Arch Microbiol 2010;192:1–14. 10.1007/s00203-009-0523-6. [PubMed: 19902179]

[51]. Masuda N, Ohya S. Cross-Resistance to meropenem, cephems, and quinolones in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 1992;36:1847–51.

[52]. Meletis G, Exindari M, Vavatsi N, Sofianou D, Diza E. Mechanisms responsible for the emergence of carbapenem resistance in *Pseudomonas aeruginosa*. Hippokratia 2012;16:303–7. [PubMed: 23935307]

[53]. Li H, Luo Y-F, Williams BJ, Blackwell TS, Xie C-M. Structure and function of OprD protein in *Pseudomonas aeruginosa*: From antibiotic resistance to novel therapies. Int J Med Microbiol 2011;302:63–8. 10.1016/j.ijmm.2011.10.001.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

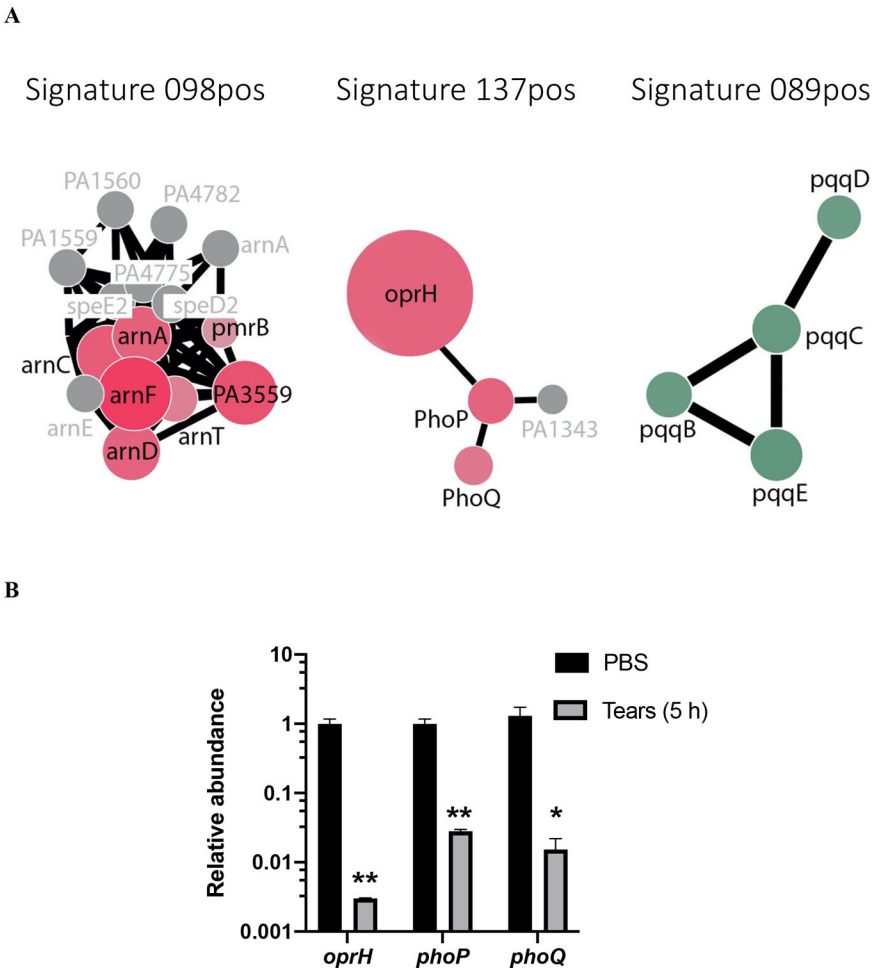

Fig 1.

Clusters of functionally related genes differentially expressed in response to 5 h tear fluid exposure relative to PBS control. The three most significant gene clusters are shown (see full dataset analysis in Supplemental Fig S1). Correlation networks were obtained from ADAGE [27] and annotated with the RNA sequencing experimental data to incorporate differentially expressed genes. Upregulated genes (green) and down-regulated genes (red) in response to tear fluid treatment are indicated, and sizes scaled to represent absolute fold changes. Genes not represented in the RNA-sequencing dataset, but included in the ADAGE gene signature analysis, are also shown (gray). Correlation networks were named using pre-established gene signatures from ADAGE, e.g. Signature 098pos (see Methods, Supplemental Table S1). (B) qPCR validation of *oprH, phoP, phoQ* (Signature 137pos) after 5 h tear fluid pretreatment relative to PBS. Combined results of two experiments are shown. Data were expressed as the mean ± standard deviation, * $p < 0.05$, ** $p < 0.01$ (Student's t Test).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

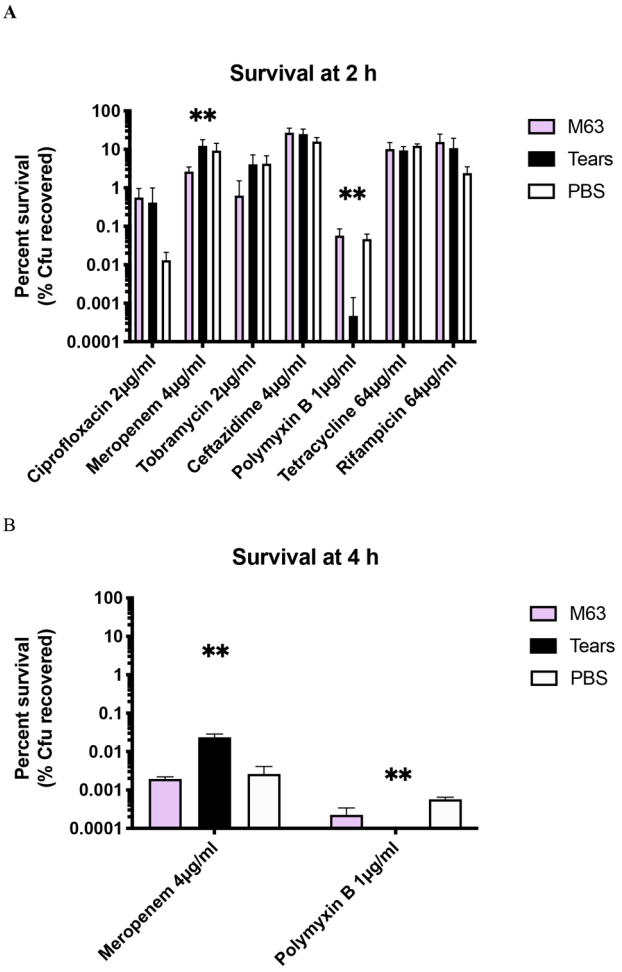

Fig 2.

(A) Tear fluid pretreatment enhances *P. aeruginosa* sensitivity to polymyxin B. Percent recovery of PAO1 to determine susceptibility to various antibiotics after 4 h pretreatment with tear fluid. After tear fluid, M63 (minimal medium) or PBS incubation, PAO1 was treated for 2 h with the following antibiotics: meropenem (4 µg/ml), tobramycin (2 µg/ml), ceftazidime (4 µg/ml), polymyxin (1 µg/ml), tetracycline (64 µg/ml), rifampicin (64 µg/ml). Tear fluid pre-exposure significantly enhanced sensitivity to polymyxin B. (B) Percent recovery of PAO1 to determine susceptibility to meropenem (4 µg/ml) or polymyxin B (1 µg/ml) after 4 h pretreatment with tear fluid and 4 h antibiotic exposure. Further antibiotic exposure showed a significant difference in meropenem survival between tear fluid-pretreated bacteria and M63 and PBS controls, and no recovery of bacteria pretreated with tear fluid then exposed to polymyxin B. ** $p < 0.01$ (two-way ANOVA with Tukey's post-hoc analysis). The combined results of three to four experiments (A) and two experiments (B) are shown.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

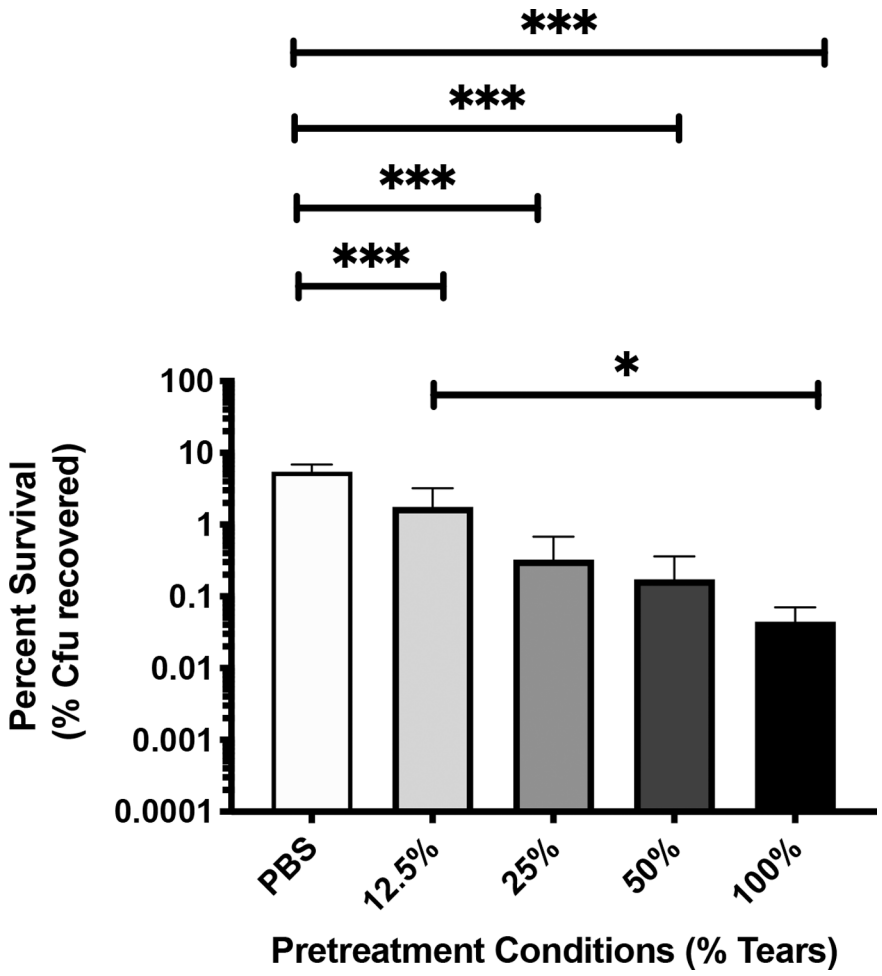

Fig 3.

Tear fluid dilution mitigates *P. aeruginosa* PAO1 sensitivity to polymyxin B. Bacteria were exposed to tear fluid at various dilutions in PBS for 4 h. Subsequent sensitivity to polymyxin B (0.5 μg/ml, 2 h survival assay) followed a linear decrease with greater tear dilution. Significance is shown relative to the PBS control. All comparisons were also significant relative to 12.5 % tears. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (one-way ANOVA with Tukey's post-hoc analysis). Combined results of five experiments are shown.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

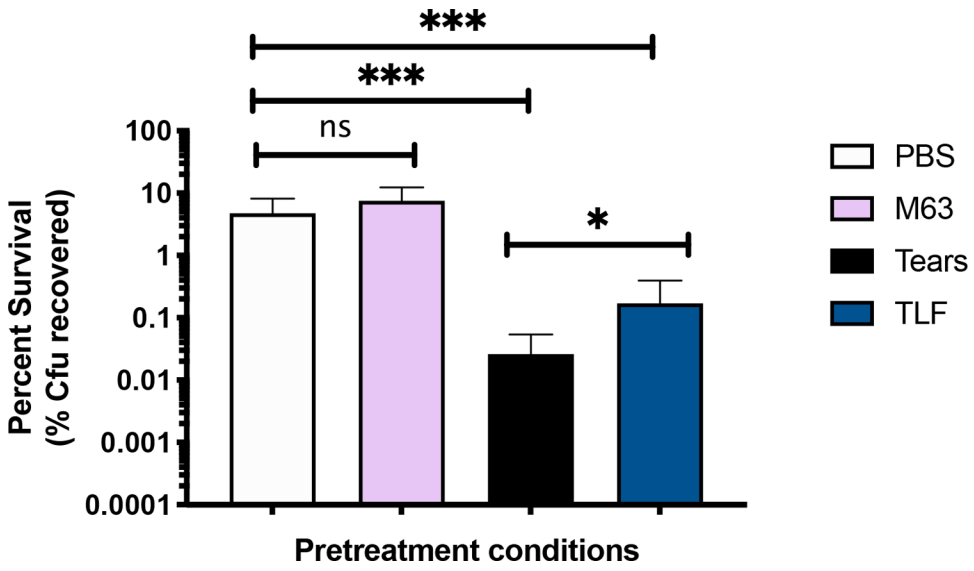

Fig 4.

(A) Tear-like fluid (TLF) also sensitizes *P. aeruginosa* to polymyxin B mediated killing. Pretreatment of *P. aeruginosa* PAO1 with tear like fluid (4 h) reduced the recovery of PAO1 after polymyxin B treatment (0.5 μg/ml, 2 h), * $p < 0.05$ Tears versus TLF pretreatment, *** $p < 0.001$ Tears or TLF pretreatment versus PBS pretreated controls (one-way ANOVA with Tukey's post-hoc analysis). Combined results of six to nine experiments are shown.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

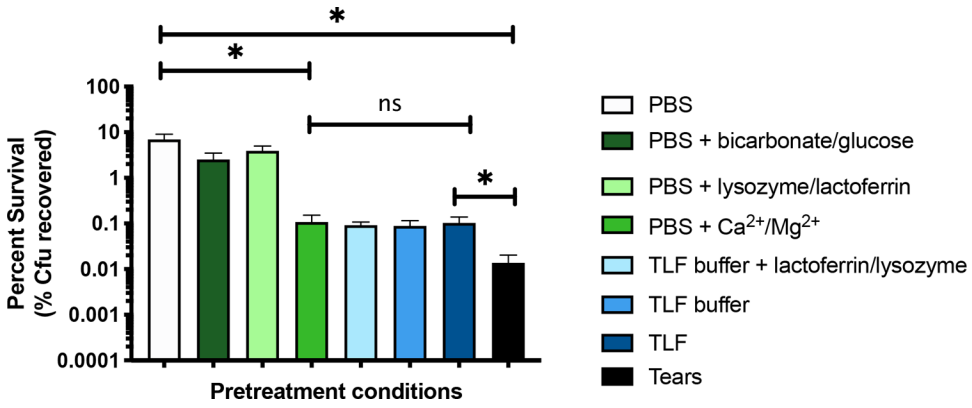

Fig 5.

Calcium and magnesium cation supplementation increased *P. aeruginosa* susceptibility to polymyxin B (0.5 μg/ml, 2 h). A 4 h pretreatment with media containing Ca^{2+} (~1.5 mM) and Mg^{2+} (~1.0 mM) [PBS supplemented with Ca^{2+} and Mg^{2+}, TLF supplemented with lactoferrin and lysozyme, TLF buffer, TLF] each resulted in lower survival of *P. aeruginosa* PAO1 rates relative to pretreatments lacking Ca^{2+} and Mg^{2+} [PBS, PBS with added lactoferrin and lysozyme, and PBS with added bicarbonate and glucose]. Tear fluid caused additional sensitization versus TLF. * $p < 0.05$ (one-way ANOVA with Tukey's post-hoc analysis). Combined results of three experiments are shown.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

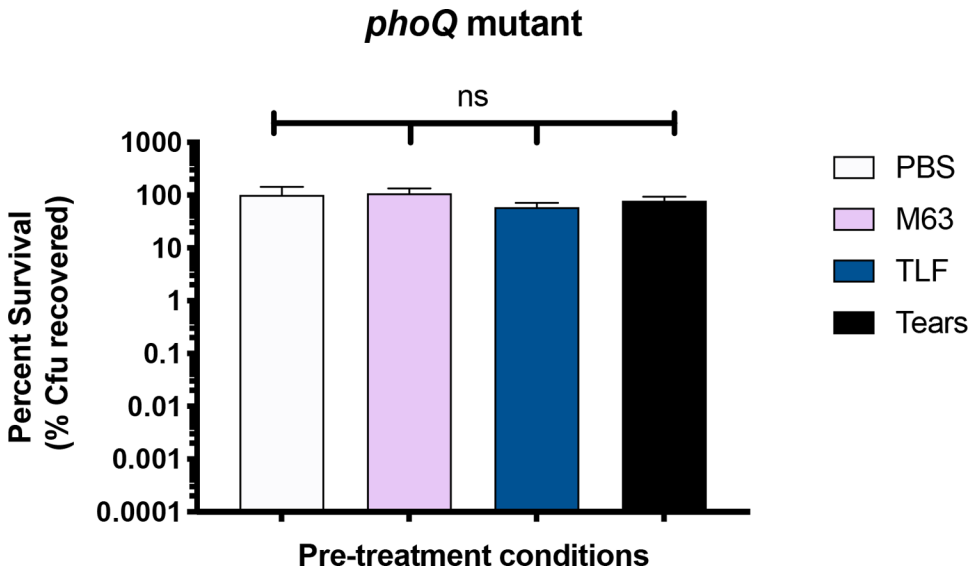

Fig 6.

A *P. aeruginosa phoQ* clean deletion mutant loses sensitization to polymyxin B by tear fluid or TLF. PAO1Δ*phoQ* was pretreated with tear fluid, TLF, PBS or M63, TLF for 4 h prior to polymyxin B exposure (0.5 μg/ml, 2 h). There was no significant difference in *P. aeruginosa* survival between pretreatment conditions, all of which showed bacterial recovery consistent with polymyxin B resistance (ns = no significance, one-way ANOVA with Tukey's post-hoc analysis). Combined results of two experiments are shown.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

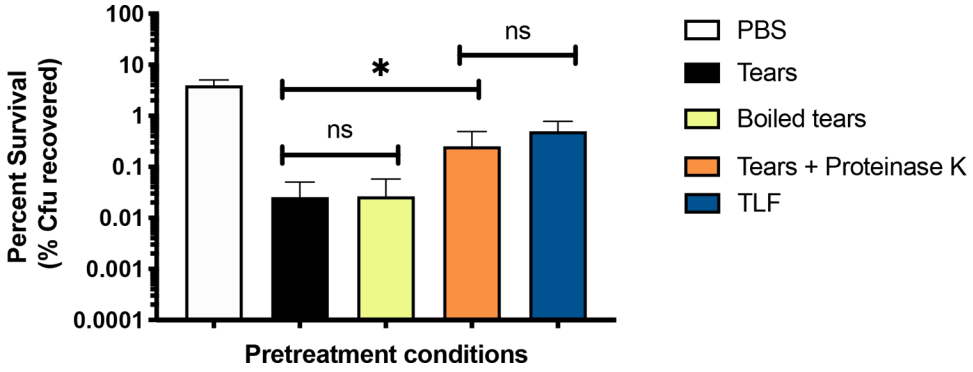

Fig. 7.

A heat-resistant, proteinase K-sensitive tear factor(s) is involved with sensitizing *P. aeruginosa* to polymyxin B (0.5 μg/ml, 2 h). Prior tear fluid treatment with the broad-spectrum serine proteinase (proteinase K, 50 μg/ml, 2 h) reduced tear-mediated sensitization of *P. aeruginosa* PAO1 to the level of TLF. However, boiling tear fluid (10 min, 95°C) prior to PAO1 pretreatment did not affect tear-mediated sensitization to polymyxin B. * $p < 0.05$, ns = no significance (one-way ANOVA with Tukey's post-hoc analysis). Combined results of six experiments are shown.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 1.

P. aeruginosa genes deregulated after exposure to human tear fluid (5 h, 37 C)

Gene Name	PAO1 ID	Fold Change (Tears/PBS)	Gene Function
ANTIBIOTIC/STRESS RESPONSE			
phoP	PA2512	−15	Two-component response regulator (polymyxin B resistance)
phoQ	PA1180	−9	Two-component response sensor (polymyxin B resistance)
oprH	PA1178	−55	Low Mg^{2+} inducible outer membrane protein precursor
arnD	PA3555	−15	Polymyxin resistance protein
pmrB	PA4777	−4	Two-component regulator system signal sensor (polymyxin B resistance)
QUORUM SENSING/BIOFILM			
lasI	PA1432	−20	Quorum sensing and virulence response
rhlI	PA3476	−10	Autoinducer synthesis protein (Quorum sensing activator)
rhlR	PA3477	−7	Autoinducer synthesis protein (Quorum sensing activator)
qscR	PA1898	−10	Quorum-sensing control repressor
algF	PA3550	+11	Alginate biosynthesis protein
IRON METABOLISM			
hemO	PA0672	+17	Heme oxygenase
phuS	PA4709	+7	Heme uptake