Title: *Aspergillus fumigatus* enhances elastase production in *Pseudomonas aeruginosa* co-cultures

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Running Title: The presence of *Aspergillus fumigatus* influences the production of *Pseudomonas* elastase

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Abstract

In the cystic fibrosis (CF) lung the presence of bacteria and fungi in the airways promotes an inflammatory response causing progressive lung damage, ultimately leading to high rates of morbidity and mortality. We hypothesised that polymicrobial interactions play an important role in promoting airway pathogenesis. We therefore examined the interplay between the most commonly isolated bacterial CF pathogen *Pseudomonas aeruginosa,* and the most prevalent filamentous fungi, *Aspergillus fumigatus,* to test this. Co-culture experiments showed that in the presence of *A. fumigatus* the production of *P. aeruginosa* elastase was enhanced. This was confirmed by the presence of zones of clearance on Elastin-Congo Red (ECR) agar, which was identified as elastase by mass spectrometry. When *P. aeruginosa* were grown in a co-culture model with mature *A. fumigatus* biofilms, 60% of isolates produced significantly more elastase in the presence of the filamentous fungi than in its absence ($p<0.05$). The expression of *lasB* also increased when *P. aeruginosa* isolates PA01 and PA14 were grown in co-culture with *A. fumigatus*. Supernatants from co-culture experiments were also significantly toxic to a human lung epithelial cell line (19-38% cell cytotoxicity) in comparison to supernatants from *P. aeruginosa* only cultures ($p<0.0001$). Here we report that *P. aeruginosa* cytotoxic elastase is enhanced in the presence of the filamentous fungi *A. fumigatus,* suggesting that this may have a role to play in the damaging pathology associated with the lung tissue in this disease. This indicates that patients who have a co-colonisation with these two organisms may have a poorer prognosis.

Introduction

There is a growing awareness of the importance of polymicrobial infections to human health.[1] An inclusive view that infectious agents do not act independently, but influence disease pathology as a result of interactions with other organisms and components of the host immune response, is contributing to the better understanding of microbial disease.[2] In the case of patients with cystic fibrosis (CF), it has been widely accepted that the altered ion transport associated with this disease gives rise to a viscous pulmonary mucous layer and impaired ciliary clearance, which compromises the host immune response and permits polymicrobial colonisation of the lungs.[3] Common opportunistic pathogens that infect the CF lungs, as identified by traditional culture methods from sputum samples, include *Staphylococcus aureus*, *Haemophilus influenza*, *Pseudomonas aeruginosa* and *Burkholderia* species.[4,5] The most commonly isolated pathogen is *P. aeruginosa*, which has been reported to colonise the airways of up to 75% of adult patients with CF.[6] However, recent advances in culture-independent, deep-sequencing technologies have revealed that the microbiome of the CF lung is much richer than previously appreciated comprising of a diverse range of bacterial and fungal pathogens,[7-9] of which *Aspergillus fumigatus* is the most prevalent filamentous fungi.[10] The propensity for chronic infection is aided further by polymicrobial biofilm formation, which protects the encased cells from antimicrobial therapy and the host immune response.[11] In addition, direct physical contact between organisms or indirect molecular signalling within the biofilm may influence microbial pathogenicity, which in turn could affect the disease outcome.[12,13]

CF airway disease is characterised by periods of stability punctuated by acute exacerbations. These are typified by decreased lung function, changes in cough,

sputum production, decreased energy levels and weight loss.[14] The ensuing inflammatory response causes progressive lung damage and is the primary cause of CF morbidity and mortality.[15] The microbes present in the lower airways promote this disease pathology, and a major contributing factor to *P. aeruginosa* virulence is attributed to the production of secreted virulence factors. These include exotoxin A, exoenzyme S, phospholipase C, alkaline protease, LasA, and elastase.[16] *Pseudomonas* elastase is a metalloprotease, which is thought to damage host tissues through hydrolysis of the proteins of the extracellular matrix (elastin and collagen) and by attacking intercellular tight junction proteins and breaching epithelial barriers.[17] Elastase also has the ability to degrade numerous components of the innate and adaptive immune response, including lysozyme and proteins involved in the opsonisation of pathogens.[18-21]

The interplay between the pathogens residing in the lung may be responsible for the acute exacerbations associated with CF, where the balance is tipped towards an environment with excess inflammatory, oxidative and proteolytic activity.[22] In fact, it has been reported that the lung function in patients co-infected with *A. fumigatus* and *P. aeruginosa* has been shown to decline,[12] a phenomenon that has also been shown with *Candida spp* and *P. aeruginosa* in the CF lung.[14] Therefore, we hypothesised that the interactions between these two organisms positively influences virulence factor production by *P. aeruginosa*. In this study we examined the relationship between the presence of *A. fumigatus* and the production of the key virulence factor, elastase, by *P. aeruginosa*. Determining the interactions between these two important organisms may result in new therapeutic strategies and improve the prognosis for patients with CF.

Materials and Methods

Microbial isolates and growth conditions

The laboratory strains of *P. aeruginosa* PA01 and PA14 were used along with two mucoid and non-mucoid clinical isolate pairs (a mucoid and a non-mucoid variant of 72707 and a mucoid and a non-mucoid variant of 64531) collected from children with CF (Royal Hospital for Sick Children, Glasgow), and two environmental strains of *P. aeruginosa* (DSM-6279 and DSM-8924), isolated from sewage and water respectively (DSMZ, Braunschweig, Germany). All strains were stored in Microbank vials (Pro-Lab Diagnostics, Cheshire, UK) at -80°C until required. *P. aeruginosa* isolates were sub-cultured from frozen stocks on Luria broth (LB) agar plates and grown in liquid culture in L-broth. The *A. fumigatus* isolate Af293 (MYA-4609, ATCC, Teddington, UK) was selected for this study and was cultured from frozen stocks on Sabouraud's dextrose (SAB) agar (Sigma-Aldrich, Dorset, UK) at 37°C for 72 h at which point the SAB agar was washed with 5 ml of sterile PBS containing 0.025% (v/v) Tween 20 to harvest the conidia. A stock solution of 1 x 10^8 conidia/ml was prepared in PBS and stored at 4°C for use in experiments.

P. aeruginosa and *A. fumigatus* agar plate interaction assay

To investigate the interactions between *P. aeruginosa* and *A. fumigatus*, two sterile 6 mm Whatman filter discs (Thermo Fisher Scientific, Loughborough, UK) were added at three different spacing distances (5, 10 and 20mm apart) to SAB agar plates. A 5µl volume of an overnight *P. aeruginosa* culture*,* adjusted in PBS to 1 x 10^5 cfu/ml, was added to one of the filter paper discs and a 5µl volume of *A. fumigatus,* standardised in PBS to 1 x 10^5 conidia/ml, was added to the other disc on each plate. The plates were incubated at 37°C and examined after 24 and 48 h to assess

whether growth of either isolate was inhibited. This experiment was performed with *A. fumigatus* Af293 and all *P. aeruginosa* isolates included in this study. Assays were performed in triplicate and repeated on at least one further occasion.

Elastase-Congo Red agar plate assay

To assess if the *P. aeruginosa* virulence factor elastase had a role to play in the inhibition of the growth of *A. fumigatus*, a stock solution of 20 mg/ml Elastin-Congo Red (ECR) (Sigma-Aldrich, UK) solution was prepared in 0.1mM Tris-HCl (pH 8.0) and this was added to molten LB agar to give a final concentration per plate of 2mg/ml (modified from a previously published method).[23,24] Two 6mm Whatman filter discs at spacing configurations of 5, 10 and 20mm apart were inoculated with 5µl of *P. aeruginosa* (1 x 10^5 cfu/ml) and 5µl *A. fumigatus* (1 x 10^5 conidia/ml), as described previously. The plates were incubated at 37°C for 24 h, following which they were examined for zones of clearing around the filter paper discs. This assay was performed with *A. fumigatus* Af293 and all *P. aeruginosa* isolates included in this study. Controls of *P. aeruginosa* only with 5µl of PBS instead of *A. fumigatus* on the adjacent disc were included in every experiment. Assays were performed in triplicate.

Protein isolation and identification

Total excreted protein was isolated from *P. aeruginosa* following a method adapted from a previously published method.[25] Briefly, isolate PA01 was grown at 37°C on twenty filter paper discs on ECR agar (LB agar/10mg/ml ECR) for 48 h, as described above. Following incubation, the agar was excised from the zones of clearing and suspended in 10ml of sterile deionised water for 72 h at 4°C to allow the protein to

diffuse into solution. This was then filtered (0.2µm filter) to remove bacterial cells, an equal volume of 100% trichloroacetic acid was added and incubated for 2 h at 4°C. The protein solution was centrifuged at 13,000 rpm for 1 h at room temperature, the pellet was then washed with acetone, centrifuged again for 10 min and re-suspended in 100µL of deionized water. To determine whether the isolated protein sample contained elastase, 5µl was spotted in triplicate onto an LB-ECR agar plate and incubated at 37°C for 24 h.

Peptides in the sample were separated by polyacrylamide gel electrophoresis on 1 mm NuPAGE 10% Bis-Tris gels using a BenchMark™ protein ladder (molecular weight (MW) 10-222kDa) (both Life Technologies, Paisley, UK) according to manufacturers' instructions. Proteins were silver stained to allow them to be identified using the SilverQuest™ Silver Staining Kit (Life Technologies). A protein band corresponding to the MW of elastase was identified and excised from the gel. The protein was then identified by the Glasgow Polyomics Facility (University of Glasgow). Briefly, peptides were solubilised in 0.5% formic acid and fractionated on a nanoflow uHPLC system (UltiMate™ RSLCnano, Thermo Scientific) before online analysis by electrospray ionisation (ESI) mass spectrometry on an Amazon ion trap MS/MS (Bruker Daltonics, Coventry, UK). Peptide separation was performed on a Pepmap C18 reversed phase column (Dionex/LC Packings, Thermo Scientific), using a 5 - 85% v/v acetonitrile gradient (in 0.5% v/v formic acid) run over 45 min at a flow rate of 0.2 µl/min. Mass spectrometric (MS) analysis was performed using a continuous duty cycle of survey MS scan followed by up to ten MS/MS analyses of the most abundant peptides, choosing the most intense multiply charged ions with dynamic exclusion for 120 s. The Matrix Science Mascot Daemon server (v2.1.06)

was used to search for matches to peptide sequences on the NCBI Genbank database.

P. aeruginosa and *A. fumigatus* co-culture model

A suspension of *A. fumigatus* Af293 conidia was prepared from refrigerated stocks to give a final concentration of 1 x 10^5 per ml in RPMI-1640 media (Sigma-Aldrich, UK). A 1ml volume of this suspension was added to the wells of a 12-well flat bottom tissue culture plate and incubated for 24 h at 37°C to form mature fungal biofilms.[26] Following incubation, the biofilms were rinsed twice with PBS to remove loosely attached cells. *A. fumigatus* has the ability to produce low levels of elastase therefore, for the purpose of this assay to detect only elastase produced by *P. aeruginosa*, the *A. fumigatus* biofilms were treated with 1ml of 100% methanol for 30 min to kill the biofilm-associated cells. Overnight cultures of all *P. aeruginosa* isolates were grown in 10ml of LB at 37°C and 180 rpm and adjusted to 1 x 10^5 cfu/ml with LB. The bacterial suspensions were added to the *A. fumigatus* biofilms and incubated at 37°C for 24 h. Control cultures of *P. aeruginosa* only and *A. fumigatus* biofilms only were included in each assay. All co-culture conditions were performed in triplicate and repeated on two further occasions. Following incubation the supernatants were collected from each well and filtered using a 0.2µm filter unit (Millipore, UK) to remove bacterial or fungal cells. The filtered supernatants were then stored at 4°C until use in the elastase activity assay.

Elastase activity assay

A 100µl volume of ECR stock (20 mg/ml in 0.1 mM Tris-HCl buffer [pH 8.0]) was added to each well of a round bottom 96-well plate. To this, 100µl of *P. aeruginosa*

supernatant was added to give a final well concentration of 10mg/ml ECR and the plate was incubated for 6 h at 37°C and 250 rpm.[24] A dilution series of porcine elastase (Sigma-Aldrich), from 64µg/ml to 0.125µg/ml, was prepared in sterile water and added to each plate, to construct a standard curve. Following incubation, the sample in each well was re-suspended by pipetting and the plate was centrifuged for 3 min at 500 rpm to pellet the undigested ECR and allow 100µl of each supernatant to be transferred to a 96-well flat bottom plate. The absorbance of each sample was read at 495nm on a FLUOstar Omega plate reader (BMG Labtech). The standard curve was plotted to show the concentration of elastase versus activity and this allowed the concentration of elastase present in each *P. aeruginosa* supernatant sample to be calculated.

Detection of *lasB* expression under co-culture conditions by RT-PCR

RNA was extracted from *P. aeruginosa* isolates PA14 and PA01 grown on filter paper discs from the agar plate co-culture proximity assay and from the biofilm co-culture experiments. To extract RNA from the cells grown on the filter paper discs, each disc was placed in 1ml of RNA Protect (Qiagen, Crawley, UK) and sonicated for 5 min in a water bath then vortexed for 10 s to remove bacterial cells from the surface. The cells were pelleted by centrifugation at 10,000 rpm for 5 min and the RNA Protect solution was removed. Cells were lysed using 200µl lysozyme at 1mg/ml in TE buffer and RNA was extracted from cells using the RNeasy mini kit (Qiagen) following the manufacturers' instructions. For *P. aeruginosa* cells grown in liquid culture with *A. fumigatus* biofilms, following incubation the *P. aeruginosa* cells were centrifuged at 10,000 rpm for 5 min and the cell pellet was resuspended in 1ml of RNA Protect for 5 min. Cells were re-centrifuged and the RNA Protect was

removed. The cell pellet was then treated with lysozyme solution and RNA was extracted using the RNeasy mini kit as above. RNA quantity and integrity was assessed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific).

From each sample, 300ng of RNA was converted to cDNA using the High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, Paisley, UK) following the manufacturers' instructions. Real time quantitative PCR reactions were set up in a MicroAmp™ Fast Optical 96-Well Reaction Plate (Applied Biosystems) and contained 10µl of Fast SYBR® Green Master Mix (Applied Biosystems), 1µl of forward and 1µl of reverse primer at a concentration of 10 µM (primers for target gene *lasB* (this study); lasB-F' TGATCGGCTACGACATCAAG, lasB-R' ATTGGCCAACAGGTAGAACG or the internal control *rpoD,*[27] rpoD-F' GGGCGAAGAAGGAAATGGTC, rpoD-R' CAGGTGGCGTAGGTGGAGAA), 2µl of cDNA for each sample (final concentration of 100ng) and 6µl of molecular grade water, to give a total reaction volume of 20µl. The reactions were performed on an One Step Plus instrument (Applied Biosystems) using the conditions; on step of 95°C for 20 sec, then 40 cycles of 95°C for 3 sec and 60°C for 30 sec. Melting curve analysis displayed a single specific product was observed for each primer pair. Reactions were performed in triplicate and repeated on one further occasion. Gene expression was calculated using the ΔΔCt method where the expression of *lasB* was normalised to the housekeeping gene *rpoD*.

The effect of co-culture supernatants on human lung epithelial cells

The human lung adenocarcinoma epithelial cell line A549 was used to examine the toxicity of the products of *A. fumigatus* and *P. aeruginosa* co-culture on the human host. From stocks stored in liquid nitrogen, A549 epithelial cells were cultured in

Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich) and 1% penicillin-streptomycin solution (x100) (Sigma-Aldrich) in a 75cm^2 tissue culture flask (Cellstar, Greiner Bio-One, UK) at 37°C and 5% CO_2 to achieve 70-80% confluency and then seeded at a density of 1 x 10^5 cells per well of a 24-well tissue culture plate. Cells were cultured in DMEM + 10% FBS for 24 h and then rinsed twice with sterile PBS. The *P. aeruginosa* filtered supernatants from the 24 h co-culture experiment were diluted 10-fold in cell culture medium and 1ml was added to each well of the tissue culture plate. Each sample was tested in triplicate and on each plate porcine elastase (10µg/ml and 1µg/ml), media only and 1% Triton X-100 (Sigma-Aldrich) were added to triplicate wells as controls (positive elastase control, negative control (live epithelial cells), 100% dead epithelial cells, respectively). Cells were incubated for 4 h at 37°C and 5% CO_2. The experiment was repeated on one further occasion. Following incubation the A549 cell supernatant from each well was collected and cell cytotoxicity was assessed using the Lactate Dehydrogenase (LDH) Activity Assay Kit (BioVision, Cambridge Biosciences, UK), following the manufacturers' instructions.

Statistical Analysis

Graph production and statistical analysis were performed using GraphPad Prism (version 4; La Jolla, CA, USA). Un-paired, two-tailed student t-tests were used to measure statistical differences between two independent groups in co-culture experiments, the LDH assay and in gene expression studies. Statistical significance was achieved if $p < 0.05$.

Results

A. fumigatus growth is inhibited by *P. aeruginosa* on solid agar

To investigate the interactions between *P. aeruginosa* and *A. fumigatus* a known concentration of *P. aeruginosa* PA14 and *A. fumigatus* Af293 was used to inoculate filter paper discs in decreasing proximity to each other (5, 10 and 20mm spacing). Following the incubation period, the radial growth pattern of *A. fumigatus* was inhibited in close proximity to the *P. aeruginosa* cells on the discs that were placed at 5 and 10mm apart, (Figure 1Ai, ii). With increasing distance between the filter paper discs (20mm spacing) *A. fumigatus* growth returned to normal (Figure 1Aiii). This phenomenon was observed for *A. fumigatus* using all isolates of *P. aeruginosa* included in this study (PA01, non-mucoid and mucoid clinical isolates (72707 and 64531), and environmental isolates (DSM-6279 and DSM-8924). Interestingly, at the 5mm spacing arrangement the growth of all *P. aeruginosa* isolates also appeared to be slightly reduced by the presence of *A. fumigatus* in comparison to growth of the pseudomonad at 10 and 20mm spacing, suggesting the complex interplay between these two species.

P. aeruginosa releases an inhibitory protease on solid media

Next, to determine whether the inhibition of radial growth was related to a secreted *P. aeruginosa* virulence factor within the SAB agar, we focussed on the metalloprotease elastase, which enables this bacterium to degrade host proteins and those of competing organisms, using an ECR agar plate assay. In the presence of elastase the red-pigmented ECR compound is degraded producing a zone of clearance of approximately 20mm (Figure 1B). Following incubation zones of clearance were observed by all strains of *P. aeruginosa* in the presence of *A.*

fumigatus at 5, 10 and 20mm apart. This would suggest that elastase, or a similar metalloprotease, is in fact produced by *P. aeruginosa* in the presence of *A. fumigatus.* The zones of clearance were measured for the 5, 10 and 20mm spacing intervals for all isolates and there was no significant difference in the size of zones produced at each of the distances, for example for isolate PA14 clearance zones measured 20.30, 20.33, 20.87mm, respectively (Figure 1C). The size of zones of clearance produced by all other isolates was similar to PA14 and this data is not shown. Moreover, the *P. aeruginosa*-only control produced a similar sized zone of clearance (20.90mm), suggesting that elastase is produced constitutively and not as a direct result of stimulus from this fungal pathogen. However, this may be due to the diffusion limitations of elastase through the agar and highlights a possible disadvantage of this assay.

P. aeruginosa releases elastase in solid agar media

To confirm the hypothesis that elastase production was responsible for the zone of clearing in the ECR-LB agar assay, the total protein was extracted from the zone of inhibition. A 5µl volume of the protein extract was spotted in triplicate onto an LB-ECR agar plate to determine if it retained activity and zones of clearing were produced. The protein extract was separated by polyacrylamide gel electrophoresis to identify the dominant proteins present. Silver staining revealed that there was principally a large dense negatively stained band on the gel with a molecular weight of approximately 30kDa, which corresponds to the molecular weight of extracellular *P. aeruginosa* elastase (33kDa) [28]. This band was excised from the gel and identified by electrospray ionisation (ESI) mass spectrometry on an Amazon ion trap MS. The Matrix Sciences Mascot report produced following a NCBI Genbank database search

showed that the top match for peptide sequences in the preparation excised from the zone of clearing on ECR agar, with 34 matching peptide sequences and a score of 522 (significance threshold of $p<0.05$), was elastase LasB (*P. aeruginosa* UCBPP-PA14, gi|116051721). This protein has a mass of 53.79-kDa on the database and contains a 20-kDa signal sequence and propeptide which precede the mature 33-kDa extracellular elastase protein [28]. This suggests that *P. aeruginosa* elastase was involved in producing the zones of clearance on ECR agar and further investigation of the expression of this protease in liquid co-culture conditions with *A. fumigatus* is warranted.

A. fumigatus biofilms enhance *P. aeruginosa* elastase production in co-culture

All *P. aeruginosa* isolates included in this study were incubated for 24 h in the presence of mature, methanol-killed *A. fumigatus* biofilms. The relative concentration of elastase in each of the filtered *P. aeruginosa* supernatants was detected by ECR assay with a standard curve created using a dilution series of porcine elastase. To establish a baseline for elastase production the supernatant from each isolate grown in the absence of *A. fumigatus* was tested (Table 1). The constitutive production of elastase varied between each of the isolates. There did not appear to be a correlation between the production of elastase and phenotype (mucoid or non-mucoid) or source of the isolate (clinical or environmental). In the presence of methanol-killed *A. fumigatus*, there was an overall trend towards the increased production of elastase in comparison to the *Pseudomonas*-only control. For isolates PA14, PA01, non-mucoid clinical isolate 72707, non-mucoid isolate 64531 and environmental isolate 8924 the increase in elastase production in the presence *of A. fumigatus* biofilms was statistically significant ($p=0.0089$, $p<0.0001$, $p=0.04$, $p=0.033$

and p<0.0001, respectively) (Figure 2). There was no significant change in elastase production in the other three *P. aeruginosa* isolates (mucoid isolate 72707, mucoid isolate 64531 and environmental isolate 6279) included in this study (data not shown).

A. fumigatus **biofilms differentially induce expression of** ***lasB*** **in** ***P. aeruginosa***

To examine the influence of *A. fumigatus* on the transcription of *lasB*, which encodes the elastase protein, fluorescent RT-PCR was performed using primers specific for the *lasB* gene and an internal reference gene *rpoD*. The specificity of these primers was confirmed by performing melt-curve analysis, for each gene of interest, and observing a single peak. The expression of *lasB* was determined in the well-characterised laboratory strains of *P. aeruginosa*, PA01 and PA14. The isolates were grown on filter paper discs opposite *A. fumigatus* at spacing intervals of 5, 10 and 20mm, as described above. Following incubation, RNA was isolated from the cells on the filter paper disc and RT-PCR was performed. *P. aeruginosa* grown on a filter paper disc in the absence of *A. fumigatus* was included in each experiment as a control. The relative expression of *lasB* was compared to the constitutively expressed internal control gene *rpoD* (Figure 3). For isolate PA14, the expression of *lasB* increased by 1.7-fold (SD +/-0.38) in cells incubated in closest proximity to *A. fumigatus* (5mm) in comparison to the PA14-only control. At 10mm and 20mm spacing distances, *lasB* expression was down-regulated by 0.76-fold (SD +/-0.28) and 0.62-fold (SD +/-0.11), respectively which was significantly less than the expression at 5mm (p=0.03 and p=0.03, unpaired, two-tailed t-test). For isolate PA01, the expression of *lasB* increased by 1.57-fold (SD +/-0.49) and 1.05-fold (SD +/-0.16) at 5mm and 10mm intervals from *A. fumigatus* and expression of *lasB* was

down-regulated at the 20mm spacing interval by 0.355-fold (SD +/-0.2). The difference in expression of *lasB* in PA01 at 5mm and 20mm spacing was significant (p=0.036, unpaired, two-tailed t-test).

When the expression of *lasB* was examined in PA14 and PA01 cells grown in the *A. fumigatus* biofilm co-culture model a trend of increased expression when the two species were in close contact was also observed. PA14 cells incubated in the presence of methanol-killed Af293 24 h biofilms increased the expression of *lasB* by 7.43-fold (SD +/-1.87) and the PA01 cells expressed 8.73-fold (SD +/-2.17) more *lasB* than *Pseudomonas* only control cultures. This data correlates with the results of the ECR assay (Figure 2) which showed that in the presence of *A. fumigatus* there was an increased concentration of elastase detected in *P. aeruginosa* supernatants, and suggests that the presence of the fungi stimulates the production of this important virulence factor in *P. aeruginosa*.

The effect of co-culture supernatants on a lung epithelial cell line

The human lung adenocarcinoma epithelial cell line A549 was cultured to a density of 1×10^5 cells/per well in a 24 well plate and exposed for four hours to supernatants from the co-culture experiment. These included supernatants from PA01 only cultures, PA01 + methanol-killed *A. fumigatus* (Af293) co-culture, PA14 only culture, PA14 + methanol-killed Af293 co-culture, and Af293 methanol-killed biofilms only. Porcine elastase at a concentration of 10µg/ml (E10) and 1µg/ml (E1) were added to the plate as elastase controls, and A549 cells with media only and A549 cells treated with 1% Triton-X were added as negative (untreated cells) and positive (100% killed cells) controls, respectively. Following exposure to the co-culture samples, the A549

cell supernatants were removed from the culture plates and used in the LDH cytotoxicity kit (BioVision). Cell cytotoxicity (%) was calculated by comparing the presence of LDH in supernatants from A549 cells treated with co-culture supernatants to those of the positive and negative control (Figure 4). A549 cell viability was significantly reduced in cells treated with PA14 and PA01 grown in co-culture with methanol-killed *A. fumigatus* biofilms (killing 37.46% of cells (SD +/- 7.495) and 19.36% of cells (SD +/-0.6215), respectively) in comparison to *P. aeruginosa*-only controls ($p<0.0001$ for both). Elastase at 10µg/ml and 1µg/ml was significantly cytotoxic to A549 cells (37.96% killed (+/- SD 5.275) and 13.67% killed (+/- SD 4.271), ($p=0.0007$ and $p=0.015$, respectively)) and the presence of PA14, PA01 and Af293 alone was not significantly cytotoxic to this human lung adenocarcinoma epithelial cell line.

Discussion

The morbidity and mortality associated with CF is linked to the irreversible decline in lung function caused by microbial colonisation of the airways and the resulting overactive neutrophilic immunological response.[15] Infection of the lower airways by the important opportunistic bacterial pathogen *P. aeruginosa* correlates with a more rapid decline in pulmonary function, worsening nutritional status, frequent hospital admissions and a shorter life expectancy.[29,30] *P. aeruginosa* is the most commonly isolated organism in CF,[6] however patients are rarely colonised by one single pathogen and it is now widely recognised that the CF lung has a rich polymicrobial microbiome,[31-33] which influences the stability of the condition of the patient and can cause an inflammatory exacerbation.[8] It is therefore imperative that the interplay between *P. aeruginosa* and other common CF-related pathogens is studied to better understand the impact co-culture has on the production of inflammatory mediators and virulence factors.

Several studies have previously examined lung function in CF patients colonised by *Aspergillus* alone and found that although patient age and antibiotic use are risk factors for airway colonisation by this fungi, its presence did not have a significant impact on the decline of lung function.[34,35] However, Amin *et al* discovered in a retrospective study of CF patients that there was a statistically significant relationship between *A. fumigatus* and *P. aeruginosa* co-colonisation and lower pulmonary function ($p < 0.0006$).[36] Within the CF lower airway polymicrobial infections can persist in the form of mixed species biofilms. Both *P. aeruginosa* and *A. fumigatus* possess the ability to form multi-cellular biofilm consortia, making it inherently difficult to eradicate the infection, but additionally the direct physical contact between the organisms or indirect molecular interactions may influence

439 microbial pathogenicity.[12,36] One study previously carried out in our laboratory
440 examined direct and indirect interactions between these two important pathogens
441 and discovered that *P. aeruginosa* could significantly impede filamentous growth and
442 biofilm formation in *A. fumigatus*, possibly through the release of small diffusible
443 molecules involved in inter-cellular communication.[13]

444 Although there are limited reports investigating the interaction between *P.*
445 *aeruginosa* and *A. fumigatus,* significant work has been carried out with *P.*
446 *aeruginosa* and the polymorphic fungus *Candida albicans*. One important study by
447 Hogan *et al.* showed that *P. aeruginosa* can interfere with germination of *C. albicans*
448 by secretion of the molecule 3-oxo-C12 homoserine lactone.[37,38] *Pseudomonas* can
449 also attach to localised areas of the hyphal surface and induce cell lysis, but are
450 unable to attach to or kill the yeast form of *C. albicans*. Trejo-Hernandez and co-
451 workers also recently compared the proteome of these two organisms in mixed
452 culture and found that there was differential expression of virulence proteins,
453 multidrug resistance-associated proteins, and proteases in both pathogens when
454 cultured together.[39] This highlights the importance of microbial interactions in relation
455 to the progression of disease within the host and suggests that co-infection with
456 *Pseudomonas* spp. and *Aspergillus* spp. may have a negative impact on the health
457 of the patient and merits further investigation.

458 One important virulence factor produced by *P. aeruginosa* which exhibits
459 toxicity towards host cells is the metalloprotease elastase. This protease is thought
460 to hydrolyse proteins of the extracellular matrix and degrade components of the host
461 immune response, including lysozyme and proteins involved in bacterial
462 opsonisation.[17-21] Elastase has also recently been implicated in the pathology of
463 chronic rhinosinusitis through the disruption of tight junctions and increased

permeability of human nasal epithelial cells.[40] This virulence factor may also have a role to play in another chronic inflammatory condition – microbial infection of the CF lung. In our initial experiments, when *P. aeruginosa* was grown adjacent to *A. fumigatus* at increasing spacing intervals it was clearly observed that *P. aeruginosa* had an inhibitory effect on the growth of *A. fumigatus* at the closest spacing intervals and this effect was reduced in a distance dependant manner (Figure 1A). The same inhibitory effect was observed for all isolates of *P. aeruginosa* included in this study. The presence of elastase in the secreted products of *P. aeruginosa* in this assay was confirmed by growing the two organisms on ECR-LB agar and observing zones of clearance and degradation of the red ECR compound (Figure 1B). The protein was extracted from zones of clearing and the presence of *P. aeruginosa* elastase was confirmed by electrospray ionisation (ESI) mass spectrometry (MS) (Figure 1D).

The production of elastase by *P. aeruginosa* in overnight cultures was then examined using the liquid ECR assay to monitor degradation of the ECR compound over a 4 h period. The activity of the culture supernatants was compared to a standard curve of known concentrations of this protease to estimate the concentration of elastase produced by each *P. aeruginosa* isolate. A range of isolates including laboratory strains, clinical isolates and those found in environmental sources were used to examine whether expression of this protease is specific to strains from a particular source or phenotype. One early study by Nicas and Iglewski (1986) reported that >85% of clinical and environmental strains in their collection of 113 isolates had the ability to produce elastase.[41] In our study, the concentration of elastase produced in overnight cultures varied between each isolate. Clinical isolate 72707 and environmental isolate 8924 constitutively produced significant levels of elastase, similar to PA01 and PA14, while clinical isolate 64531

and environmental isolate 6279 produced very low amounts of elastase. Interestingly, when the concentration of elastase was compared between non-mucoid and mucoid paired clinical isolates, the non-mucoid phenotype of 72707 produced significantly more elastase than the paired mucoid phenotype ($p=0.04$), but for clinical isolate 64531 the non-mucoid phenotype produced significantly less elastase than the mucoid phenotype ($p<0.05$), however the concentration of elastase produced by 64531 in general was extremely low. In CF pathology, patients initially become colonised by *P. aeruginosa* isolates with a non-mucoid phenotype which appears to switch over time to a mucoid phenotype as a result of environmental cues within the CF lung.[42] The fact that non-mucoid isolate 72707 produced significantly more elastase than its mucoid counterpart may indicate that elastase plays a role in early colonisation of the airways, and merits further study with a larger collection of non-mucoid/mucoid paired isolates.

To better understand the influence of a competing organism on the production of elastase by *P. aeruginosa* in the CF lung, a biofilm co-culture model with *A. fumigatus* was employed. *A. fumigatus* biofilms were formed for 24 h and then killed with methanol to eliminate the possibility of fungal proteases interfering with the results of the assay. Supernatants were tested with the liquid ECR assay as before and the concentration of elastase produced was compared to *P. aeruginosa* only controls. Five isolates including PA14, PA01, non-mucoid clinical isolate 72707, non-mucoid clinical isolate 64531 and environmental isolate 8924, produced a significantly higher concentration of elastase in the presence of methanol-killed *A. fumigatus* biofilms, than the *P. aeruginosa* isolates alone. There was no statistically significant change in the production of elastase by the other three isolates. When the expression of the *lasB* gene, which encodes elastase, was analysed in isolates PA14

and PA01 it increased significantly in close proximity to *A. fumigatus* in the disc spacing and co-culture assays (1.7-fold and 1.57-fold [5mm disc spacing], 7.43-fold and 8.73-fold [co-culture], respectively). The gene expression data corresponds with the results of the ECR assay and shows that the presence of *A. fumigatus* has the ability to alter the transcriptome of *P. aeruginosa* with a possible impact on the virulence of this microbe within the CF lung.

To examine the possibility that polymicrobial infections enhanced virulence, the human lung adenocarcinoma epithelial cell line A549 was used to examine the potential consequence of a bacterial/fungal co-infection with *P. aeruginosa* and *A. fumigatus* in the CF lung. The A549 cells were exposed to supernatants from the co-culture experiments and an LDH assay was used to measure cell cytotoxicity. The supernatants produced by PA14 and PA01 co-cultured with methanol-killed *A. fumigatus* biofilms were significantly cytotoxic to the A549 cell line, killing 37.46% of cells and 19.36% of cells, respectively. Cell cytotoxicity was similar to that observed by adding elastase at 10µg/ml and 1µg/ml to A549 cells (killing 37.96% of cells and 13.67% of cells, respectively). The concentration of elastase was chosen to represent the clinically-relevant concentrations produced by these isolates previously in the ECR assay. Supernatants from PA01, PA14 and *A. fumigatus* Af293 only cultures were not significantly cytotoxic to A549 cells ($p<0.05$). Though, cellular toxicity could be caused by another agent co-produced with elastase in the co-culture model. Further studies are needed to investigate the *Pseudomonas* elastase specific cytotoxicity in the co-infection model.

Conclusion

In this study we have shown that the metalloprotease elastase is secreted constitutively by a range of *P. aeruginosa* isolates and that this production is enhanced in the majority of isolates tested in this study in the presence of the common CF fungal pathogen *A. fumigatus.* Although *P. aeruginosa* is known to utilise a myriad of virulence factors in the course of infection, and we accept that multiple secreted factors may play a part in pathogenicity, the experiments with co-culture and the human lung adenocarcinoma epithelial cell in this study show that *P. aeruginosa* elastase could be an important contributor to lung epithelial cell cytotoxicity. Increased knowledge of the interplay between microbes commonly present in the CF airway microbiome will help guide future treatment options and improve the prognosis for patients with this disease.

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Declaration of Interests

The authors have no competing interests in publishing this work.

List of Abbreviations

CF: cystic fibrosis, CFU: colony forming unit, ECR: Elastin-Congo Red, LB: Luria broth, PBS: phosphate buffered saline, MS: Mass Spectrometry, SD: standard deviation, PA: *Pseudomonas aeruginosa*.

Author's contributions

All authors listed contributed sufficiently to this project. KS was responsible for the project design, experimental work, data analysis and writing the manuscript. SK contributed to the experimental work and RR was involved in the experimental work and helped write the manuscript. WGM and CW assisted in interpretation of the results and correction of the manuscript. GR was responsible for the study as a whole and was involved in project design, interpretation of data and developing of the manuscript.

References

1. Peters BM, Jabra-Rizk MA, O'May GA, Costerton JW, Shirtliff ME. Polymicrobial interactions: impact on pathogenesis and human disease. *Clin. Microbiol. Rev.* Jan 2012;25(1):193-213.

2. Singer M. Pathogen-pathogen interaction: a syndemic model of complex biosocial processes in disease. *Virulence.* Jan-Feb 2010;1(1):10-18.

3. Sheppard MN, Nicholson AG. The pathology of cystic fibrosis. *Curr. Diagn. Pathol.* 2010;8:50-59.

4. Gibson RL, Burns JL, Ramsey BW. Pathophysiology and management of pulmonary infections in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* Oct 15 2003;168(8):918-951.

5. Harrison F. Microbial ecology of the cystic fibrosis lung. *Microbiology.* Apr 2007;153(Pt 4):917-923.

6. Valenza G, Tappe D, Turnwald D, et al. Prevalence and antimicrobial susceptibility of microorganisms isolated from sputa of patients with cystic fibrosis. *J Cyst Fibros.* Mar 2008;7(2):123-127.

7. Huang YJ, Lynch SV. The emerging relationship between the airway microbiota and chronic respiratory disease: clinical implications. *Expert Rev. Respir. Med.* Dec 2011;5(6):809-821.

8. Price KE, Hampton TH, Gifford AH, et al. Unique microbial communities persist in individual cystic fibrosis patients throughout a clinical exacerbation. *Microbiome.* 2013;1(1):27.

9. Stressmann FA, Rogers GB, van der Gast CJ, et al. Long-term cultivation-independent microbial diversity analysis demonstrates that bacterial communities infecting the adult cystic fibrosis lung show stability and resilience. *Thorax.* Oct 2012;67(10):867-873.

10. Bakare N, Rickerts V, Bargon J, Just-Nubling G. Prevalence of *Aspergillus fumigatus* and other fungal species in the sputum of adult patients with cystic fibrosis. *Mycoses.* Feb 2003;46(1-2):19-23.

11. Yang L, Liu Y, Wu H, Hoiby N, Molin S, Song ZJ. Current understanding of multi-species biofilms. *Int J Oral Sci.* Apr 2011;3(2):74-81.

12. Duan K, Dammel C, Stein J, Rabin H, Surette MG. Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. *Mol. Microbiol.* Dec 2003;50(5):1477-1491.

13. Mowat E, Rajendran R, Williams C, et al. *Pseudomonas aeruginosa* and their small diffusible extracellular molecules inhibit *Aspergillus fumigatus* biofilm formation. *FEMS Microbiol. Lett.* Dec 2010;313(2):96-102.

14. Goss CH, Burns JL. Exacerbations in cystic fibrosis. 1: Epidemiology and pathogenesis. *Thorax.* Apr 2007;62(4):360-367.

15. Lyczak JB, Cannon CL, Pier GB. Lung infections associated with cystic fibrosis. *Clin. Microbiol. Rev.* Apr 2002;15(2):194-222.

16. Lee DG, Urbach JM, Wu G, et al. Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial. *Genome Biol.* 2006;7(10):R90.

17. Azghani AO. *Pseudomonas aeruginosa* and epithelial permeability: role of virulence factors elastase and exotoxin A. *Am. J. Respir. Cell Mol. Biol.* Jul 1996;15(1):132-140.

18. Alcorn JF, Wright JR. Degradation of pulmonary surfactant protein D by *Pseudomonas aeruginosa* elastase abrogates innate immune function. *J. Biol. Chem.* Jul 16 2004;279(29):30871-30879.

19. Jacquot J, Tournier JM, Puchelle E. In vitro evidence that human airway lysozyme is cleaved and inactivated by *Pseudomonas aeruginosa* elastase and not by human leukocyte elastase. *Infect. Immun.* Feb 1985;47(2):555-560.

20. Kuang Z, Hao Y, Walling BE, Jeffries JL, Ohman DE, Lau GW. *Pseudomonas aeruginosa* elastase provides an escape from phagocytosis by degrading the pulmonary surfactant protein-A. *PLoS One.* 2011;6(11):e27091.

21. Theander TG, Kharazmi A, Pedersen BK, et al. Inhibition of human lymphocyte proliferation and cleavage of interleukin-2 by *Pseudomonas aeruginosa* proteases. *Infect. Immun.* Jul 1988;56(7):1673-1677.

22. Cathcart GR, Quinn D, Greer B, et al. Novel inhibitors of the *Pseudomonas aeruginosa* virulence factor LasB: a potential therapeutic approach for the attenuation of virulence mechanisms in pseudomonal infection. *Antimicrob. Agents Chemother.* Jun 2011;55(6):2670-2678.

23. Hamood AN, Griswold J, Colmer J. Characterization of elastase-deficient clinical isolates of *Pseudomonas aeruginosa*. *Infect. Immun.* Aug 1996;64(8):3154-3160.

24. Schad PA, Bever RA, Nicas TI, Leduc F, Hanne LF, Iglewski BH. Cloning and characterization of elastase genes from *Pseudomonas aeruginosa*. *J. Bacteriol.* Jun 1987;169(6):2691-2696.

25. Be'er A, Ariel G, Kalisman O, et al. Lethal protein produced in response to competition between sibling bacterial colonies. *Proc. Natl. Acad. Sci. U. S. A.* Apr 6 2010;107(14):6258-6263.

26. Mowat E, Butcher J, Lang S, Williams C, Ramage G. Development of a simple model for studying the effects of antifungal agents on multicellular communities of *Aspergillus fumigatus*. *J. Med. Microbiol.* Sep 2007;56(Pt 9):1205-1212.

27. Savli H, Karadenizli A, Kolayli F, Gundes S, Ozbek U, Vahaboglu H. Expression stability of six housekeeping genes: A proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. *J. Med. Microbiol.* May 2003;52(Pt 5):403-408.

28. Bever RA, Iglewski BH. Molecular characterization and nucleotide sequence of the *Pseudomonas aeruginosa* elastase structural gene. *J. Bacteriol.* Sep 1988;170(9):4309-4314.

29. Emerson J, Rosenfeld M, McNamara S, Ramsey B, Gibson RL. *Pseudomonas aeruginosa* and other predictors of mortality and morbidity in young children with cystic fibrosis. *Pediatr. Pulmonol.* Aug 2002;34(2):91-100.

30. Nixon GM, Armstrong DS, Carzino R, et al. Clinical outcome after early *Pseudomonas aeruginosa* infection in cystic fibrosis. *J. Pediatr.* May 2001;138(5):699-704.

31. Delhaes L, Monchy S, Frealle E, et al. The airway microbiota in cystic fibrosis: a complex fungal and bacterial community--implications for therapeutic management. *PLoS One.* 2012;7(4):e36313.

32. Sibley CD, Parkins MD, Rabin HR, Duan K, Norgaard JC, Surette MG. A polymicrobial perspective of pulmonary infections exposes an enigmatic pathogen in cystic fibrosis patients. *Proc. Natl. Acad. Sci. U. S. A.* Sep 30 2008;105(39):15070-15075.

33. Sibley CD, Surette MG. The polymicrobial nature of airway infections in cystic fibrosis: Cangene Gold Medal Lecture. *Can. J. Microbiol.* Feb 2011;57(2):69-77.

34. Bargon J, Dauletbaev N, Kohler B, Wolf M, Posselt HG, Wagner TO. Prophylactic antibiotic therapy is associated with an increased prevalence of *Aspergillus* colonization in adult cystic fibrosis patients. *Respir. Med.* Nov 1999;93(11):835-838.

35. de Vrankrijker AM, van der Ent CK, van Berkhout FT, et al. *Aspergillus fumigatus* colonization in cystic fibrosis: implications for lung function? *Clin. Microbiol. Infect.* Sep 2011;17(9):1381-1386.

36. Amin R, Dupuis A, Aaron SD, Ratjen F. The effect of chronic infection with *Aspergillus fumigatus* on lung function and hospitalization in patients with cystic fibrosis. *Chest.* Jan 2010;137(1):171-176.

37. Antunes LC, Ferreira RB, Buckner MM, Finlay BB. Quorum sensing in bacterial virulence. *Microbiology.* Aug 2010;156(Pt 8):2271-2282.

38. Hogan DA, Vik A, Kolter R. A *Pseudomonas aeruginosa* quorum-sensing molecule influences *Candida albicans* morphology. *Mol. Microbiol.* Dec 2004;54(5):1212-1223.

39. Trejo-Hernandez A, Andrade-Dominguez A, Hernandez M, Encarnacion S. Interspecies competition triggers virulence and mutability in *Candida albicans-Pseudomonas aeruginosa* mixed biofilms. *ISME J.* Oct 2014;8(10):1974-1988.

40. Nomura K, Obata K, Keira T, et al. *Pseudomonas aeruginosa* elastase causes transient disruption of tight junctions and downregulation of PAR-2 in human nasal epithelial cells. *Respir. Res.* 2014;15:21.

41. Nicas TI, Iglewski BH. Production of elastase and other exoproducts by environmental isolates of *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* May 1986;23(5):967-969.

42. Fegan M, Francis P, Hayward AC, Davis GH, Fuerst JA. Phenotypic conversion of *Pseudomonas aeruginosa* in cystic fibrosis. *J. Clin. Microbiol.* Jun 1990;28(6):1143-1146.

Figure 1. *P. aeruginosa* and *A. fumigatus* interactions

A) *P. aeruginosa* PA14 and *A. fumigatus* Af293 agar plate interaction assay. *P. aeruginosa* was grown on the disc on the left of each image and *A. fumigatus* was grown on the disc on the right. The growth of *A. fumigatus*, on the disc on the right of the image for 5mm and 10mm spacing (i and ii), is inhibited in close proximity to *P. aeruginosa*. Growth recovered when the discs were placed 20mm apart. B) PA14 grown on ECR-LB agar for 24 h at 37°C. The zone of clearing indicates the release of elastase and degradation of the ECR complex. C) Zones of clearing of ECR-LB agar were compared for PA14 and all other *P. aeruginosa* isolates grown in the presence and absence of *A. fumigatus* at 5mm, 10mm and 20mm spacing intervals and there was no significant difference in the size of the zones of clearing in relation to the distance between the two organisms. Graph displays the mean diameter of zones of clearing for isolate PA14 and error bars indicate the standard deviation between results (One-way ANOVA, no post-test, PA14 p=0.134). D) Silver stained gel with BenchMark™ protein ladder of total protein isolated from the zone of clearing produced by isolate PA01 on ECR agar. Dominant negatively stained protein band approximately 30kDa M.W. (highlighted), excised and identified by nano-electrospray tandem mass spectrometry.

Figure 2. *P. aeruginosa* elastase production in the presence of *A. fumigatus* biofilms. *A. fumigatus* biofilms were grown for 24 h, killed with methanol and then incubated in co-culture with a range of *P. aeruginosa* isolates. The supernatants from the co-culture experiments were used in the ECR assay and the concentration of elastase (µg/ml) in each sample was determined by comparing the change in absorbance (degradation of the ECR compound) to a standard curve. Isolates PA14, PA01, non-mucoid clinical isolate 72707, non-mucoid clinical isolate 64531 and environmental isolate 8924 all produced significantly more elastase in the presence of methanol-killed *A. fumigatus* 24 h biofilms than in *P. aeruginosa*-only control cultures ($p=0.008$, $p<0.0001$, $p=0.04$, $p=0.033$ and $p<0.0001$, respectively). The mean value is plotted for each of the conditions and error bars represent the standard deviation between replicate samples. *=$p<0.05$, **=$p<0.001$, ***=$p<0.0001$.

Figure 3. The relative expression of *lasB* in *P. aeruginosa* PA01 and PA14 grown in close proximity to *A. fumigatus* Af293. The relative expression (fold-

change) of *lasB* was determined for *P. aeruginosa* isolates PA01 and PA14 grown on filter paper discs 5mm, 10mm and 20mm distance apart from *A. fumigatus* Af293, using the constitutively expressed *rpoD* as an internal control and comparing the expression of *lasB* to *P. aeruginosa* grown on a filter paper disc in the absence of *Aspergillus*. The expression of *lasB* was down-regulated in PA01 and PA14 increasing distance from *A. fumigatus*. This was statistically significant by student un-paired, two tailed t test for PA01 at 20mm spacing and PA14 at 10mm and 20mm spacing (*=p<0.05). The mean expression is plotted and error bars represent the standard deviation between replicate samples.

Figure 4. LDH cytotoxicity assay evaluation of the effect of co-culture supernatants on the human lung adenocarcinoma epithelial cell line A549. Lung adenocarcinoma epithelial cells (A549) were exposed for four hours to supernatants from the co-culture experiments and cell cytotoxicity was measured using the LDH cytotoxicity kit (BioVision). A549 epithelial cells were exposed to supernatants from PA01 only, PA01 + methanol-killed *A. fumigatus* (Af293) co-culture, PA14 only, PA14 + methanol-killed Af293 co-culture, Af293 methanol-killed biofilm only, porcine elastase at a concentration of 10µg/ml (E10), porcine elastase at a concentration of 1µg/ml (E1), A549 cells with media only (negative control) and A549 cells treated with 1% Triton-X (100% dead positive control). Cell cytotoxicity (%) was calculated by comparing the presence of LDH in supernatants from A549 cells treated with co-culture supernatants to those of the positive and negative control. A549 cell viability was significantly reduced in cells treated with PA14 and PA01 grown in co-culture with methanol-killed *A. fumigatus* biofilms in comparison to *P. aeruginosa*-only controls (unpaired, two-tailed t-test, p<0.0001 for both). Elastase at 10µg/ml (E10) and 1µg/ml (E1) was also significantly cytotoxic to A549 cells (p=0.0007 and p=0.015, respectively). The presence of supernatants from PA14, PA01 and Af293 cultured alone was not significantly cytotoxic to this human lung adenocarcinoma epithelial cell line. The mean cell cytotoxicity (%) is plotted and error bars represent the standard deviation between replicate samples. *=p<0.05, ***=p<0.0001.