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Host lysolipid differentially modulates virulence factor expression and antimicrobial susceptibility in *Pseudomonas aeruginosa*

James C. McSorley[1], Alison C. MacFadyen[2], Leena Kerr[3] and Nicholas Peter Tucker[1,*]

Abstract

Lysophosphatidic acid (LPA) occurs naturally in inflammatory exudates and has previously been shown to increase the susceptibility of *Pseudomonas aeruginosa* to β-lactam antibiotics whilst concomitantly reducing accumulation of the virulence factors pyoverdine and elastase. Here it is demonstrated that LPA can also exert inhibitory effects upon pyocyanin production in *P. aeruginosa*, as well as influencing susceptibility to a wide range of chemically diverse non β-lactam antimicrobials. Most strikingly, LPA markedly antagonizes the effect of the polycationic antibiotics colistin and tobramycin at a concentration of 250 µg ml^{-1} whilst conversely enhancing their efficacy at the lower concentration of 8.65 µg ml^{-1}, approximating the maximal physiological concentrations found in inflammatory exudates. Transcriptomic responses of the virulent strain UCBPP-PA14 to LPA were analysed using RNA-sequencing along with BioLog phenoarrays and whole cell assays in attempts to delineate possible mechanisms underlying these effects. The results strongly suggest involvement of LPA-induced carbon catabolite repression together with outer-membrane (OM) stress responses whilst raising questions about the effect of LPA upon other *P. aeruginosa* virulence factors including type III secretion. This could have clinical relevance as it suggests that endogenous LPA may, at concentrations found *in vivo*, differentially modulate antibiotic susceptibility of *P. aeruginosa* whilst simultaneously regulating expression of virulence factors, thereby influencing host–pathogen interactions during infection. The possibility of applying exogenous LPA locally as an enhancer of select antibiotics merits further investigation.

AUTHOR SUMMARY

We show for the first time that lysophosphatidic acid (LPA) can reduce production of the exotoxin pyocyanin in *P. aeruginosa*, whilst variably affecting its susceptibility to a wide range of antibiotics. The transcriptomic response of the virulent strain UCBPP-PA14 as determined by RNA-sequencing (RNA-seq), taken together with the results of BioLog phenoarrays with the same strain and the results of growth kinetic assays, strongly suggest that *P. aeruginosa* utilizes LPA as a preferred carbon source leading to carbon catabolite repression of the pyocyanin biosynthesis pathway. PhoPQ and PmrAB are interlinked two-component regulatory systems, the *spe* and *arn* operons which they regulate, govern spermidine production and lipopolysaccharide modification, respectively; marked upregulation of all these genes in response to LPA, strongly suggests that this lysolipid provokes a membrane stress response.

INTRODUCTION

Pseudomonas aeruginosa is amongst the most versatile of opportunistic pathogens, capable of exploiting a wide range of virulence factors to cause an array of infective conditions in various hosts, including humans [1]. These include acute entities such as nosocomial pneumonia, urinary tract infections, neutropenic sepsis, burn wound infection, otitis externa and keratitis [1]. It may also be involved, often along with other pathogens, in chronic disease: airway infection in the context of cystic fibrosis or

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Author affiliations: [1]Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, UK; [2]Institute of Biodiversity, Animal Health & Comparative Medicine, Graham Kerr Building, University of Glasgow, Glasgow, G12 8QQ, UK; [3]Institute of Life and Earth Sciences, School of Energy, Geoscience, Infrastructure and Society, Heriot-Watt University, Riccarton, Edinburgh EH14 4AS, UK.

***Correspondence:** Nicholas Peter Tucker, nick.tucker@strath.ac.uk

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Abbreviations: LPA, Lysophosphatidic acid; LPS, lipopolysaccharide; MHB, Mueller–Hinton Broth; MIC, Minimum inhibitory concentration; OM, Outer-membrane.

Three supplementary tables and four supplementary figures are available with the online version of this article.

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other bronchiectatic conditions as well as infection of decubitus ulcers, especially amongst diabetics [1]. Regulation of pathogenic attributes in this organism is extremely complex and multifaceted but in general, the suite of virulence factors it employs will differ according to the acuity or chronicity of infection [1, 2]. Moreover, in chronic infections, *P. aeruginosa* generally adopts a biofilm lifestyle [1, 2]. Biofilms are dense bacterial colonies containing organisms of varying metabolic state encased within a matrix of polysaccharides and extracellular DNA [1, 2]. Organisms within biofilm are generally manifold more tolerant to antibiotics than are their planktonic counterparts [1, 2]. Furthermore, the biofilm matrix hinders phagocytosis of bacteria living within it. These features make chronic *P. aeruginosa* infections, such as those occurring in bronchiectatic airways, almost impossible to eradicate; antibiotic therapy may temporarily reduce organism load but seldom, if ever, achieves bacteriologic clearance as it typically does in acute infection [1, 2]. Treatment for such infections therefore tends to be palliative rather than curative [2]. Amongst *P. aeruginosa* virulence factors, elastase functions as a destructive protease, which histologically degrades the matrices of infected tissues, causing fibrosis and aiding progression of disease [2]. Additionally, elastase has been shown to cleave a range of host bactericidal defence proteins including complement fragments, serum IgG, secretory IgA, transferrin and pulmonary surfactant proteins [3, 4]. Pyocyanin, another secreted virulence factor, is a blue coloured, redox active, tricyclic phenazine known to induce airway fibrosis, upregulate futile hyperinflammatory host responses and cause destruction of secretory goblet cells in the airways [5]. Pyocyanin also promotes leucocyte apoptosis and retards ciliary beat at airway epithelial surfaces, hindering the host response to pathogens secreting it [6]. *P. aeruginosa* is inherently resistant to most available antibiotics and has the capacity to readily acquire additional resistances to the precious few agents, which are intrinsically active against it [7]. As a Gram-negative species, much of the innate antibiotic resistance of *P. aeruginosa* owes to the incorporation of an outer membrane (OM) in its diderm cell structure [7, 8]. The OM is an asymmetric lipid bilayer, which impedes permeability of toxic solutes on the basis of mass, charge and/or hydrophobicity; many antibiotics such as benzylpenicillin, macrolides and vancomycin, do not pass efficiently into the Gram-negative organisms, which possess it [7, 9]. Increasing resistance to antimicrobials mandates that new avenues be explored for control of *P. aeruginosa* infections. These may include the coformulation of antibiotics with potentiator molecules which nullify bacterial resistance mechanisms or, alternatively, the use of compounds, which disable bacterial virulence factors. It was previously shown that lysophosphatidic acid (LPA) enhances the susceptibility of several *P. aeruginosa* strains to the anionic β-lactams ampicillin, piperacillin and ceftazidime in lysogeny broth and suggested that this effect could be exploited therapeutically [10]. The antibacterial effect of LPA was especially marked against a mucoid subset of clinical isolates from cystic fibrosis airways and bacteriostatic effects were observed against these strains even without the addition of antibiotics [10]. It has been suggested that the unusually potent effect of LPA against mucoid strains may be due to the abbreviation of the O-antigen chains typically expressed on the surface of these strains. Lysophospholipids have previously been shown to exhibit bactericidal activity against Gram-positive bacteria, which lack the OM permeability barrier characteristic of Gram-negative species such as *P. aeruginosa* [10–13]. In the experience of those investigators, LPA did not affect the antibiotic activity of either nalidixic acid, a naphthyridone inhibitor of bacterial DNA gyrase, or the aminoglycoside gentamicin, an inhibitor of protein synthesis although these agents were tested only at fixed subinhibitory concentrations against strain PAO1 [10]. Laux and colleagues found that LPA was able to attenuate biofilm formation, alginate and pyoverdine production by *P. aeruginosa* strain PAO1 [14]. These investigators also demonstrated that these effects were reversible by divalent cation supplementation, indicating a mechanism of action related to the known chelating properties of LPA [14]. Moreover, they presented evidence from *lasB::gfp* fusion reporter experiments suggesting that the effect on elastase production was partially due to a direct transcriptional effect at the *lasB* promoter [14]. This provides only a partial explanation to the effects of LPA on elastase synthesis as Western-blot analysis revealed that treated cultures still produced some elastase. Albeit, this was at much lower levels and largely (93% of total) in the form of catalytically inert 53 and 51 kDa proenzymes rather than the 33 kDa active enzyme which was present exclusively in the control supernatants [14].

Therefore, LPA clearly induces aberrant processing of elastase at the post-transcriptional level by a thus far undefined mechanism. At least for strain PAO1, those investigators procured no evidence to suggest that LPA-induced loss of terminal O-antigen from the outer membrane (OM) lipopolysaccharide (LPS) [10, 14]. This led us to investigate whether LPA could also affect the expression of other virulence factors in *P. aeruginosa* or increase its susceptibility to classes of antibiotic other than β-lactams; we also aimed to ascertain the mechanisms underlying the effects of LPA upon *P. aeruginosa*. In order to do this, we utilized a range of phenotypic assays and attempted to reconcile their results with those of transcriptomic experiments performed on *P. aeruginosa* strain PA14 cultured in the presence of LPA.

METHODS

Bacterial strains

Bacterial strains used in this work are listed in Table S1. Strains were stored frozen at −80 °C in Microbank advanced microbial storage vials (Pro-Lab Diagnostics, Wirral, UK) and revived by inoculating 5 ml of LB (Melford, London, UK) with a single bead and growing at 37 °C with agitation at 250 r.p.m. until turbidity appeared. The resultant broth cultures were then subcultured onto LB agar plates (Melford, London, UK) and incubated for 24–48 h at 37 °C to obtain isolated colonies, which were then transferred to 3–5 ml volumes of Mueller–Hinton Broth (MHB) (Oxoid, Basingstoke, UK) and grown overnight at 37 °C with shaking at 250 r.p.m. to obtain stationary phase starter cultures for use in experiments.

Preparation of media containing LPA

In total, 12.5 mg of powdered LPA (Avanti Polar Lipids, Alabaster, AL, USA) was accurately weighed out and added to a glass round-bottomed flask that had previously been cleaned sequentially with boiling water and 70% ethanol before being dried in an oven. Then, 5 ml of chloroform was added and swirled to suspend the lipid and kill microbial contaminants. The chloroform was then removed by rotary evaporation at 250 r.p.m. in a water bath set to 60 °C to obtain a dry, uniform lipid film. Any excess traces of chloroform were then removed briefly by applying a nitrogen stream. The mouth of the round-bottomed flask was then sealed with cling film and it was transferred to a UV cross-linker oven set to full power for 45 min to further ensure sterility. A 10 ml aliquot of sterile PBS in a 50 ml Falcon tube was then heated in a water bath set to 60 °C – above the phase transition temperature of LPA. This preheated PBS was immediately added to the round-bottomed flask containing the sterilized lipid film upon removal from the water bath, the flask was then swirled to dissolve the LPA and obtain a clear solution of micellar LPA. This micellar LPA solution was then added to a 50 ml Falcon tube containing 40 ml of MHB with cation concentrations of 78 µM Ca 2+and 225 µM Mg2+ (Oxoid, Basingstoke, UK). This MHB contained 250 µg ml^{-1} and was used for the growth curve measurements, assays of virulence factors and assays of biofilm formation reported in this chapter. Control MHB lacking LPA was prepared using preheated PBS vehicle in place of the micellar LPA solution. For MIC assays, twice the initial concentration of LPA was used to account for the twofold dilution of the media in these tests when topped up with inoculum. For agar motility assays, half the volume of PBS was used to solubilize LPA so as not to reduce the agar viscosity excessively.

Growth curves

MHB with and without the addition of 250 µg ml^{-1} LPA was prepared as described and decanted into 10 ml aliquots in sterile universal tubes in matched LPA and control pairs for each strain. These were then each inoculated with 25 µl of overnight MHB culture and shaken to mix. A multi-channel pipette was then used to add 200 µl volumes of each culture to the wells of a 96-well plate in such an array as to produce triplicate results for each strain in both the absence and presence of LPA, in other words, 3 wells per test condition.

Uninoculated controls of MHB with and without LPA were also included. 0.03% resazurin solution was prepared by adding 15 mg of resazurin sodium (Sigma Aldrich, UK) to 50 ml of sterile PBS, shaking to dissolve and then filter sterilizing. Then, 20 µl of 0.03% resazurin solution was added to all wells of the aforementioned plate before it was sealed with a breathe easy membrane (Sigma Aldrich, Poole, UK) and transferred to a Biotek kinetic plate reader with temperature preset to 37 °C and platform set to shake continuously at full speed.

Absorbances at 570 and 600 nm wavelengths were then recorded by the instrument every 15 min for 24 h and saved as a Microsoft Excel spreadsheet. The experiment was repeated in triplicate and the mean results were used to calculate the percentage reduction of resazurin using the equation given below [15].

$$\frac{(E_{\mathrm{OXI}}600\times A_{570})-(E_{\mathrm{OXI}}570\times A_{600})}{(E_{\mathrm{RED}}570\times C_{600})-(E_{\mathrm{RED}}600\times C_{570})}\times 100$$

where

$E_{\mathrm{OXI}}570$=molar extinction coefficient (E) of oxidized resazurin at 570 nm=80586

$E_{\mathrm{OXI}}600$=molar extinction coefficient (E) of oxidized resazurin at 600 nm=117216

$E_{\mathrm{RED}}570$=molar extinction coefficient (E) of reduced resazurin at 570 nm=155677

$E_{\mathrm{RED}}600$=molar extinction coefficient (E) of reduced resazurin at 600 nm=14652

A_{570}=absorbance of test wells at 570 nm

A_{600}=absorbance of test wells at 600 nm

C_{570}=absorbance of negative control well (media, resazurin, no bacteria) at 570 nm

C_{600}=absorbance of negative control well (media, resazurin, no bacteria) at 600 nm

MIC determinations

Antibiotics used were supplied in the form of powders as follows: ceftazidime (as hydrate), chloramphenicol, ciprofloxacin, colistin, erythromycin, nalidixic acid (as sodium salt), tetracycline, tobramycin and trimethoprim (Sigma Aldrich, Poole, UK). Stock solutions of ceftazidime, colistin, nalidixic acid, tetracycline and tobramycin were prepared at a concentration of 1.024 mg ml^{-1} in distilled water and filter sterilized. Stock solutions of chloramphenicol and erythromycin were prepared at concentrations of 25 mg ml^{-1} in 100% ethanol. Ciprofloxacin stock solution was prepared at a concentration of 1 mg ml^{-1} in 0.05 N hydrochloric acid. Trimethoprim stock solution was prepared at a final concentration of 20 mg ml^{-1} in a 15% solution of acetic acid. The wells in three inner rows of a 96 plate were filled with 100 µl of MHB prepared with or without 500 µg ml^{-1} LPA as described – three rows with LPA and three without per plate. Antibiotics were then diluted across the rows from left to right to obtain a series of

nine doubling dilutions in columns 2 to 10 of the microtitre plate containing twice the desired concentration of antibiotic and drug-free controls with and without LPA in column 11. Inocula of each isolate were then diluted with fresh MHB to an A_{600} value of 0.1±0.02, then further diluted in a 1:150 ratio with more MHB to obtain an inoculum containing approximately1×10^6 c.f.u. ml^{-1}, 100 µl of inoculum were then used to complete the volumes of the microtitre plate wells to 200 µl~5×10^5 c.f.u. ml^{-1}. One plate was used per test, perimeter wells were avoided due to the possibility of discrepant 'edge effects' caused by evaporation; these were filled with an equal volume of sterile MHB to minimize evaporation, then plate lids were attached and they were sealed with parafilm. Plates were then stacked and secured with autoclave tape before being secured in a shaking incubator set to 37 °C and 250 r.p.m. with the aid of oblong metal clamps. Plates were removed from incubation after 18 h and opened before 20 µl of 0.03% resazurin solution was added to each well using a multichannel pipette. Plates were resealed and incubated as before for a further 2 h. They were then removed from incubation and fluorescent emissions at a wavelength of 590 nm were read after excitation at a wavelength of 540 nm in a plate reader. Results were analysed for significance at the α=0.05 level using one-way ANOVA. The MIC was defined as the lowest antibiotic concentration at which fluorescence was less than 50% of that in the drug-free control, this had previously been found to correspond with turbidity-based MIC values recorded in the absence of LPA and a separate uninoculated plate filled in the same format was read to ensure that neither LPA nor the test drugs themselves had substantially reduced the metabolic indicator dye, thus confounding the results.

Quantifying pyocyanin in agitated cultures

Inocula were diluted with fresh MHB to an A_{600} value of 0.2±0.02 before being further diluted in a 1:30 ratio with MHB with or without the addition of 250 µg ml^{-1} LPA, prepared as previously described to give a starting inoculum of ~1 x 10^7 cfu ml^{-1}. Triplicate wells of a 96-well plate for each isolate and test condition were then filled with 180 µl of inoculum. Perimeter wells were avoided to avoid the possibility of discrepant 'edge effects' occurring due to evaporation, these and any other unused wells were filled with an equal volume of sterile MHB and sealed with parafilm. Plates were then stacked and secured with autoclave tape before being secured in a shaking incubator set to 37 °C and 250 r.p.m. with the aid of oblong metal clamps. Plates were then removed from incubation after 24 h and immediately assessed for production of pyocyanin by recording the A_{695} value of each well [16]. Plates were then opened before 20 µl of 0.03% resazurin metabolic indicator solution was added to each well using a multichannel pipette. Plates were resealed and returned to incubation under the previously stated conditions for a further hour after which F_{590} emissions were measured following excitation with light at a wavelength of 540 nm to quantify resazurin reduction as a surrogate marker for growth in each well. Percentage growth in triplicate LPA treated wells versus matched control wells was determined by dividing the F_{590} reading of the LPA-treated well by that of its matched control and multiplying by 100 [16]. Pyocyanin levels quantified in each well were then normalised for growth in the same well. Assays were repeated three times and normalized results were processed as bar charts displaying the mean of nine observations plus standard error of the mean. Results were analysed for significance at the α = 0.05 level via one-way ANOVA.

Quantifying virulence-related exoproducts in biofilm cultures

Inocula were diluted with fresh cation supplemented MHB to an A_{600} value of 0.2±0.02 before being further diluted in a 1:30 ratio with cation supplemented MHB to obtain a starting inoculum of ~1 x 10^7 cfu ml^{-1}. This culture was then added to the eight inner wells of a 24-well plate in 1.8 ml aliquots and outer wells were filled with an equal volume of sterile water to minimize evaporation. Lids were then attached to plates and they were sealed with parafilm before being incubated statically at 37 °C for 24 h to allow bacterial attachment and biofilm formation on the polystyrene well surfaces. After 24 h, plates were removed from incubation and all liquid culture was removed by pipetting, leaving only preformed biofilms on the wells. Quadruplicate wells were then refilled with 2 ml volumes of cation supplemented MHB prepared with or without the addition of 250 µg ml^{-1} LPA as described. Three plates were set up per experiment to give a total of 12 wells per test condition. Plates were resealed with parafilm and incubated statically at 37 °C for a further 48 h to enable production of virulence-associated exoproducts. After incubation, plates were opened and well contents were mixed by pipetting and scraping the well surfaces with the pipette tip to resuspend the biofilm as far as possible, visible biofilm was disrupted by repeated shearing using a sterile 1 ml disposable syringe. Control and LPA treated samples were then pooled in triplicate and transferred to 50 ml Falcon tubes to give a total of four tubes containing ~6 ml of the LPA-treated sample and four tubes containing ~6 ml of control sample. A 200 µl aliquot of each pooled, homogenized culture was then taken from each of the tubes and transferred to the wells of a 96-well plate, 20 µl of 0.03% resazurin solution was then added to each well of the 96-well plate before it was incubated at 37 °C with shaking at 250 r.p.m. for an hour. F_{590} emissions were read from each well after excitation with light at a wavelength of 540 nm. Percentage growth in quadruplicate samples from LPA-treated tubes versus matched control wells was determined by dividing the F_{590} reading of the LPA-treated sample by that of its matched control and multiplying by 100. The remaining contents in each tube (~5.8 ml) were centrifuged for 15 min at 4200 r.p.m. to remove the cells and the resulting supernatant was filter sterilized and retained for assays of pyocyanin, elastase and total exoprotease described below, results were analysed for significance at the α = 0.05 level via one-way ANOVA in all cases. Pyocyanin was extracted and assayed based on a method previously reported in the literature [17]. Overall, 5 ml samples of each quadruplicate supernatant

were transferred to 50 ml Falcon tubes to which 3 ml of chloroform had previously been added. The tubes were then capped and vortexed on full power for 5 s before being centrifuged at 4200 r.p.m. for 10 min to allow formation of separate aqueous and organic phases. Then, 2 ml of the organic phase (containing oxidized blue pyocyanin) was siphoned off by pipetting and transferred to a separate 15 ml Falcon tube. In total, 1 ml of 0.2 N hydrochloric acid was then added to the chloroform extract, the tube was capped and vortexed for 5 s on full power. The tube was then centrifuged at 4200 r.p.m. for 10 min to obtain distinct organic and acidified aqueous phases. Overall, 150 µl of the acidic aqueous phase (containing reduced red pyocyanin) was then transferred to a 96-well plate and A_{520} was measured. The A_{520} value was then multiplied by 17.072 (the molar extinction coefficient of pyocyanin) to reveal the extracted pyocyanin concentration in µg ml^{-1}, this value was then multiplied by 0.66 to correct for the fact that only 2/3 of the organic phase had been sampled [17]. Altogether, 0.2 N hydrochloric acid was used as a negative control. Elastase activity was measured using a method previously reported in the literature that uses a granular elastin-dye conjugate, elastin congo-red [16, 18]. Briefly, 25.7mg samples of elastin congo-red granules (Sigma Aldrich, Poole, UK) were weighed out and dispensed into the conical base of a 20ml universal tube. One tube was used per test supernatant. The granules were then suspended by adding 1.8 ml of elastase buffer and 200 µl of test supernatant. Tubes were then capped and transferred to a shaking incubator set at 37 °C and 250 r.p.m. for 4 h. Thereafter, 1 ml aliquots were transferred into 1.5 ml microcentrifuge tubes and spun down at 4 ˚C/13300 r.p.m. to remove granules. After, 150 µl volumes of the resulting supernatant were then transferred to a 96-well plate and read at $A_{495.}$ A negative control was made by using a tube treated with MHB in place of culture supernatant. Total exoprotease activity in supernatants was quantified using a method previously described in the literature that uses a casein conjugated to an azo-dye as a promiscuous protease substrate [16]. In total, 250 µl of 2% filter sterilized azocasein solution in total exoprotease assay buffer was added to 2 ml microcentrifuge tubes containing 150 µl of filtered culture supernatant and vortexed for 5 s before being incubated statically at 37 °C for 45 min to allow substrate digestion. Reactions were then stopped by precipitating out any undigested substrate by adding 1.2 ml of 10% trichloroacetic acid per tube prior to incubating for 15 min at room temperature and then centrifuging at 13300 r.p.m. for a further 10 min. Then, 900 µl volumes of the resulting supernatant were transferred to fresh 2 ml microcentrifuge tubes containing 750 µl of 1M NaOH to neutralize the trichloroacetic acid. Next, 150 µl volumes of the centrifuged supernatant were then transferred to 96-well plates and A_{440} was read. A negative control was made by using a tube treated with MHB in place of culture supernatant. All results were normalized to account for any differences in growth, as measured by fluorescence of reduced resazurin, that had been observed in the cultures prior to centrifugation and filtration.

Biofilm formation assays

To assess whether LPA affected biofilm formation, a simple microtitre plate assay was used, based on modifications of that proposed previously [19]. Each test isolate was inoculated into MHB prepared with and without 250 µg ml^{-1} LPA at a cell density of ~1.0×10^{7} cfu ml^{-1}. Then, 180 µl of this culture was pipetted into the wells of a 96-well plate in such a manner as to produce six replicate wells for each strain and test condition. The plate lid was then attached and sealed with parafilm to minimize evaporation before the plate was incubated statically at 37 °C for 24 h. After incubation, culture was siphoned out of the wells using a multichannel pipette and the wells were rinsed twice with 200 µl volumes of sterile PBS so that only biofilm would remain on the well surfaces. Thereafter, 180 µl volumes of filtered 0.1% crystal-violet solution were added to each well and left in place for 5 min to stain the biofilms. PBS rinsing was then repeated once after the crystal violet had been pipetted off and 200 µl of 30% acetic acid was added to each well and left for 15 min to allow leaching of biofilm-absorbed crystal violet. After this time, the acid was transferred to a fresh 96-well plate and absorbance of leached crystal violet, which is proportional to biofilm formation, was read at 570 nm in a plate reader. The assay was repeated on two occasions to give 12 replicates per condition. Results were analysed for significance at the α= 0.05 level via one-way ANOVA.

Field emission SEM of adherent organisms

The protocol used to grow and fix biofilms and prepare them for scanning electron microscopy was an adaptation of that previously described by Harrison and colleagues, differing in that glass coverslips were used instead of the Calgary biofilm device [20]. Overnight LB cultures were diluted in fresh MHB to an A_{600} value of 0.2±0.02. These adjusted cultures were then further diluted 1:30 with fresh MHB, prepared with or without the addition of 250 µg ml^{-1} LPA, to obtain starter inocula of each strain containing approximately 1×10^{7} c.f.u. ml^{-1}. Round 13 mm glass coverslips were placed into the inner wells of a 24-well plate and the lid was attached. Coverslips were then sterilized by placing the 24-well plate into a UV crosslinker oven and running on full power for 30 min. Next, 1 ml of turbidity adjusted inoculum was then pipetted into each well containing coverslips and the remaining outer wells were filled with an equal volume of sterile water to minimise evaporation. The 24-well plates were then sealed around the perimeter with parafilm and incubated statically at 37 °C for 24 h to allow biofilm formation. After incubation, coverslips were transferred using forceps to fresh 24-well plates with wells containing 1 ml of fixative :- 2.5% electron microscopy grade glutaraldehyde in 0.1 M cacodylate buffer. Fixation was continued for 2 h at room temperature before coverslips were sequentially rinsed for 10 min in cacodylate buffer and 18.2 MOh water and then air dried for 120 h. Coverslips were then mounted on stalks, sputter-coated in gold and imaged under FE-SEM.

Swarming motility assays

Swarming motility was assayed using adaptations of a method previously reported in the literature [21]. Molten MH agar (0.5% agar content) was decanted in 45 ml volumes into 4 separate 50 ml polypropylene tubes half of which were then topped up with 5 ml of micellar LPA solution and the other half with preheated PBS, capped tightly and swirled vigorously to mix. Three petri dishes each of agar (30 ml) with or without the addition of 250 µg ml^{-1} LPA were then poured and allowed to air dry for 2 h prior to use. Three separate overnight cultures of PA14 in MHB were diluted with fresh MHB to an A_{600} value of 0.4. Thereafter, 1 µl volumes of diluted culture were then pipetted up and stab inoculated into the centre of the agar medium in each plate in such a way as to have an LPA containing and matched control plate prepared from each of the three cultures. Upright plates were incubated statically at 37 °C for 20 h. After this time, tendril diameters were measured with a ruler and the triplicate means for each group were calculated and graphed with standard error values. Statistical significance was analysed by one-way ANOVA at α=0.05.

BioLog phenotype microarrays

BioLog PM-1 plates were set up as per the manufacturer's instructions with one modification; LPA dissolved in PBS was added to the inoculating fluids to obtain a final concentration of 250 µg ml^{-1}, not exceeding 10% of the total volume (BioLog Phenotype MicroArrays, Manual, Part No. 00A 037, Rev A 06/2011, www.biolog.com). Controls were prepared using an equal volume of sterile PBS without LPA. Growth in plates was recorded over 48 h in an Omnilog plate reader with dedicated software. Triplicate results were then averaged and plotted to allow observance of growth differences between LPA-treated and matched control wells.

RNA sequencing

Growth conditions were optimized during a trial run prior to RNA extraction, using pyocyanin production as a surrogate marker of LPA activity. In total, 15 mg of LPA was weighed and added to a small round-bottomed flask that had been pre-cleaned with 70% ethanol and dried in an oven. Then, 5 ml of chloroform was added to the flask and swirled to fully suspend the LPA. The chloroform was then evaporated to dryness using a Buchi rotary evaporator with rotor set to 200 r.p.m. and water bath at 55 °C, residual chloroform was then removed under a nitrogen stream. The flask was then sealed with parafilm to prevent entry of contaminants before being placed in a UV crosslinker oven set to full power for 30 min to kill remaining microbes. Next, 10 ml volumes of sterile PBS were decanted into each of two 50 ml Falcon tubes, which were then capped and heated in a water bath set to 70 °C in to raise the temperature of the PBS to levels exceeding the transition temperature of LPA (~50 °C). The round-bottomed flask containing dried LPA lipid film was then opened in a laminar flow cabinet and 10 ml of the pre-heated PBS was aseptically added and swirled immediately after retrieval from the water bath. This enabled the lipid film to be solubilized to clarity. The resulting 10 ml aliquot of LPA solution was then pipetted into a Falcon tube containing 40 ml of MHB to obtain a final concentration of 300 mg l^{-1} LPA, whilst 10 ml of warmed PBS was added to another Falcon tube containing 40 ml of MHB as a control. Thereafter, 15 ml volumes of MHB containing 300 mg l^{-1} were transferred to each of three sterile 250 ml conical flasks, matched controls were prepared using the LPA-free MHB. Triplicate PA14 cultures each grown in 5 ml MHB for 16 h at 250 r.p.m./37 °C from separate isolated colonies on a fresh LB streak plate, were then used to inoculate the conical flasks in three LPA/control pairs to obtain three biological replicates for each condition – 100 µl of overnight culture was added to each flask. The flasks were then sealed with sterile aluminium foil attached with autoclave tape and incubated at 37 °C with 250 r.p.m. agitation. A_{695} values of 100 µl aliquots withdrawn from each conical flask at 20 min intervals were then recorded for 8 h to obtain approximate measures of pyocyanin concentration over time and triplicate cfu ml^{-1} spread plate counts were also recorded at these intervals to normalize for growth. The time point at which there was a maximal mean difference in A_{695} values between LPA-treated and matched control cultures after correction for differences in growth was taken as the time point at which LPA was exhibiting maximal effect on the log phase cultures – this corresponded to 4 h and 40 min. Cultures for RNA extraction were thus prepared exactly as described for the trial run and then harvested after 4 h and 40 min of growth by adding 1 ml of culture (~1×10^9 c.f.u.) to a 50 ml Falcon tube containing 2 ml of Qiagen RNA protect bacteria reagent (Qiagen, Manchester, UK). The tubes were then capped and shaken before being left at room temperature for 5 min and centrifuged at 4200 r.p.m. for 10 min. Supernatants were then siphoned off and the resulting cell pellets, three LPA treated and three matched controls, were immediately stored at −80 °C. RNA was extracted from cell pellets using an Ambion RiboPure Bacteria Kit and depleted using the Ambion Microbe Express Kit (Life Technologies, Inchinnan, UK). rRNA depleted samples were reverse transcribed to cDNA libraries and amplified using the Ion Total RNA-Seq Kit (version 2) prior to being loaded on an Ion 316 chip for sequencing using the Ion Torrent Personal Genome Machine (Life Technologies, Inchinnan, UK). The sequenced reads were mapped to the UCBPP-PA14 reference genome using EDGE-pro software [22], to generate gene-expression levels, prior to filtering and primary statistical analysis being undertaken with the DESeq2 package (Bioconductor.org). To enable systematic gene classification and detection of functional enrichment, differentially regulated genes were mapped to the PAO1 reference genome and assigned to 1 of 18 functional categories based on COG (cluster of orthologous groups of proteins) annotations from the *Pseudomonas* genome project [23]. Only 23 genes not mapping to any of the 18 categories were identified, these genes were assigned to 1 of 2 arbitrarily created categories; W for genes not otherwise classified despite having PAO1 orthologues or X for genes not otherwise assigned due to the lack of a PAO1 orthologue. Enrichment for upregulated and downregulated genes in each functional category was determined using Fisher's exact test. Overrepresentation, or underrepresentation, respectively, were considered evident where LPA regulated genes in a

given functional category were proportionately over or underrepresented relative to their abundance in the PAO1 genome, with statistical significance at the α=0.1 level. Functions and pathway involvement of genes were also determined using annotations from the *Pseudomonas* genome project database [23], KEGG database [24, 25] and searches of the open literature.

RESULTS AND DISCUSSION

LPA variably affects growth rates of different *P. aeruginosa* strains

Since LPA has previously been demonstrated to slow the growth rates of PAO1 and several mucoid CF sputum isolates, it was deemed of interest to test whether LPA would impede growth of other *P. aeruginosa* strains. This hypothesis was tested by plotting growth curves for several *P. aeruginosa* strains cultured in broth media, representing both mucoid and non-mucoid CF sputum isolates. Strains tested included two previously sequenced isogenic non-mucoid/mucoid pairs isolated from the same patients, as well as isolates previously found to belong to the highly virulent Liverpool epidemic strain (LES).

Fourteen *P. aeruginosa* strains were cultured in Mueller–Hinton Broth (MHB) with or without the addition of 250 µg ml^{-1} LPA for 24 h at 37 °C with agitation, growth being monitored at intervals of every 0.25 h. Owing to the turbidity potentially imparted by LPA precipitates, reduction of resazurin, a metabolic indicator dye, was monitored rather than turbidity; blue resazurin is metabolically reduced by growing cells to a pink intermediate product, resorufin, whose increasing concentration is directly proportional to growth rate.

Eventually, resorufin is further reduced to a colourless product, dihydroresorufin and as a result, maximum peaks in the growth curves presented in this section do not correspond to the beginning of stationary phase but rather to the time point at which resorufin concentration peaked and began to decline in late exponential phase. Stationary phase therefore corresponds, approximately, with the levelling of the curve after an initial period of decline.

Generation times were extrapolated, to the nearest 0.25 h, from the upward sloping regions of the curve, corresponding with mid-exponential phase, time taken to exit lag phase was also derived from the curves. For 8 of 14 strains tested, J1532, C1426, J1385, S2, S38, A36, LES400 and LES431, LPA reduced generation times two–fourfold (Fig. S1). With the sole exception of strains S2 and S38, for which LPA extended lag phase by approximately 4.5 and 5.0 h, respectively, no effect on the time taken to exit lag phase was evident for any of these eight strains. In the case of another four strains: PA14, PAO1, mucoid isolates 28 and C1433no effect of LPA upon growth rate was apparent (Fig. S1). No data could be recorded for another strain, A38, as LPA entirely abolished its growth at the tested concentration. These findings were highly unexpected given that a much lower micellar LPA concentration (85 ±7.8 µg ml^{-1}) had previously been shown elsewhere to reduce the growth of strain PAO1 in shaken LB cultures by 24% over an 18 h incubation period and that even more profound reductions in growth rate (~2–5 log reduction in final viable counts after 18 h growth) were also recorded for six mucoid CF isolates tested by this group [1]. The possibility that the differences observed were due to use of a different growth medium and/or to the nature of the test isolates themselves cannot be ruled out as MHB was used in place of LB in the current work and PAO1, which exhibited the least discrepancy, was the only strain common to both experiments. The MHB used in the current experiments had a lower net content of Ca^{2+} and Mg^{2+} ions, at 78 and 225 µM, respectively, relative to the LB used in the original study, which had 263 µM Ca^{2+} and 225 µM Mg^{2+} [10]. LPA had been previously shown to exert its effects via cation chelation and these were reversible where the action of LPA was overwhelmed by sufficiently high cation concentrations [10]. In order to delineate the role of cation concentration in the observed discrepancies, pilot experiments on a subset of strains (PAO1, PA14, J1385, J1532, C1426 and C1433) were carried out in the same Mueller–Hinton broth, supplemented with Ca^{2+} and Mg^{2+} to net concentrations of 500 µM and 412 µM, respectively (Fig. S2). These endpoint experiments measured growth over 18 h in both LPA treated and control cultures, indicating that LPA did limit growth of these strains under such conditions (Fig. S2). It can thus be inferred that *P. aeruginosa* responds differently to this lysolipid under cation replete conditions.

Based on the growth curves observed, we hypothesized that *P. aeruginosa* utilizes LPA, or its breakdown products (glycerol and fatty acids) as carbon sources (Fig. S1). This would provide an explanation for the increased growth rates we observed in response to LPA especially given the fact that a higher concentration of micellar lipid (250 µg ml^{-1}) was used in our experiment relative to that used by the other investigators [10]. Curiously, all strains exhibiting enhanced growth in response to LPA originated as CF sputum isolates. This may have significance as LPA occurs naturally in inflammatory exudates of the airway [26] and strains adapted to CF airways have shown enhanced capacity to use glycerol as a carbon source [27]. It has previously been found that mucoid strains are generally more susceptible to LPA, possibly due to stunting of O-antigen chains on the outer membrane LPS [10]. Of the three mucoid isolates studied, only J1532, the least mucoid as previously judged by colonial morphology, increased growth in response to LPA. It is thus possible that the growth stimulating effects of LPA were counterbalanced by increased membrane damage in mucoid strains C1433 and MUC28 meaning that no net change in growth rate was detected. LPA was shown to slow the growth of a single isolate, S1, extending its lag phase by approximately 4 h and increasing the generation time thereafter from approximately 1.75 to 2.25 h (Fig. S1). For another isolate, A38, growth was completely inhibited by addition of 250 µg ml^{-1} LPA and therefore no curve was plotted.

Table 1. MICs of antimicrobials against 13 *P. aeruginosa* strains+/-LPA

DRUG	CAZ		CHL		CIP		COL		ERY		NAL		TET		TMP		TOB	
LPA [250 μG ml^{-1}]	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
A36	2	0.5	16	4	0.06	0.03	0.5	32	128	64	64	32	4	0.25	64	16	0.06	2
C1426	2	0.5	32	8	0.06	0.03	0.5	32	128	128	256	128	32	4	32	4	0.5	16
C1433*	8	1	32	4	0.03	0.03	0.5	32	128	32	64	32	32	8	32	16	0.25	8
J1385	2	0.5	16	8	0.06	0.03	0.5	32	64	64	64	64	16	4	32	16	0.5	8
J1532*	2	1	8	4	0.06	0.125	0.5	32	16	16	128	128	8	8	16	16	0.5	8
LES400	64	8	4	0.5	0.125	0.125	0.5	32	32	64	128	128	2	0.5	64	16	4	32
LES431	256	64	1	0.25	1	0.25	0.5	32	128	32	1024	256	1	0.25	4	1	0.5	8
MUC28*	32	1	0.5	0.5	0.5	0.125	0.25	32	16	16	256	128	0.5	0.5	64	64	0.25	8
PAO1	2	0.5	8	4	0.06	0.03	0.5	32	64	16	64	64	8	2	16	8	0.25	2
PA14	0.5	0.25	8	4	0.06	0.03	0.5	32	64	32	64	64	16	4	32	8	0.25	1
S1	128	16	8	2	4	0.5	0.5	32	1024	128	256	128	4	0.5	64	16	64	512
S2	128	16	8	2	2	2	0.5	32	1024	1024	1024	1024	16	4	32	8	64	512
S38	256	16	8	4	4	1	0.5	32	512	64	512	128	4	0.5	64	32	64	512
LPA [8.65 μG ml^{-1}]	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
PA14	NT	NT	NT	NT	NT	NT	0.5	0.25	NT	NT	NT	NT	NT	NT	NT	NT	0.25	0.125

Ceftazidime (CAZ), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (COL), erythromycin (ERY), nalidixic acid (NAL), tetracycline (TET), trimethoprim (TMP) and tobramycin (TOB) *mucoid strains. Colour coding: green=synergistic/additive; yellow=indifferent; red=antagonistic. (*n*=3).

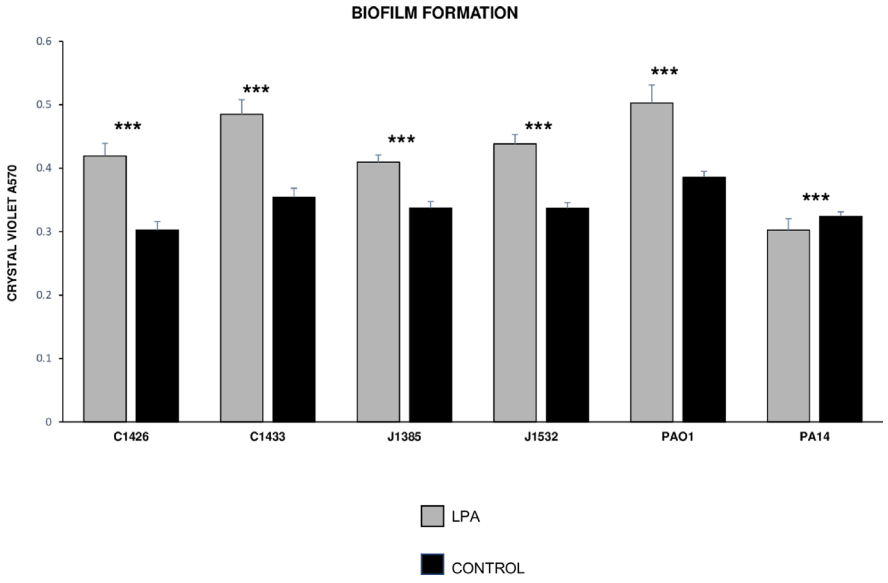

Fig. 1. Mean biofilm formation quantified using crystal-violet assays with absorbance at 570 nm (n = 12). Significance denoted *** (P< 0.05).

PA14 can utilize LPA as a sole carbon source

In order to assess the potential effects of LPA upon carbon catabolism, strain PA14 was cultured in triplicate PM-1 BioLog phenotype microarray plates containing various lone carbon sources, with and without the addition of 250 μg ml^{-1} LPA. In negative control wells containing no carbon source (A1) and in wells containing other lone carbon sources on which PA14 did not grow, growth could only be detected in the LPA supplemented plates and not the matched controls (Fig. S3). This confirms that LPA, or breakdown products thereof, are being utilized as a sole carbon source by the organism.

LPA may be either synergistic or antagonistic with different antimicrobial classes against *P. aeruginosa*

In order to determine whether LPA influenced the actions of antimicrobials upon *P. aeruginosa* isolates, antibiotic MICs were measured by broth microdilution for 14 strains with and without the addition of 250 μg ml^{-1} LPA (Table 1). Chemically diverse agents with differing modes of action were assayed including the following representatives: *β*-lactams (ceftazidime), fluoroquinolones (ciprofloxacin), naphthyridones (nalidixic acid), tetracyclines (tetracycline), phenylpropanoids (chloramphenicol), macrolides (erythromycin), diaminopyrimidines (trimethoprim), aminoglycosides (tobramycin) and polymyxins (colistin). An LPA concentration of 8.65 μg ml^{-1}, approximating the maximum concentrations reported in serum, was also tested for tobramycin and colistin (Table 1). As would have been expected based on previously reported findings [10], LPA consistently potentiated the activity of ceftazidime, a third-generation cephalosporin widely used in the treatment of pseudomonal infections. Synergy was evident as a 2- to 32-fold drop in MIC in the presence of LPA for 13 of 14 strains. Profoundly antagonistic interactions were evident between 250 μg ml^{-1} LPA and colistin for all test isolates and were essentially uniform in degree, bringing about a 64- to 128-fold rise in MIC values to 32 μg ml^{-1} for all test strains (Table 1). Three explanations could be put forward for this phenomenon and they are not mutually exclusive. The first and most obvious is that LPA may be interacting physicochemically with colistin in such a way as to render it inactive. This hypothesis seems feasible given that polymyxins have been demonstrated to bind with bilayers of phosphatidic acid, which differs from LPA only by an extra acyl chain [28]. The second explanation is that sequestration of Mg^{2+} by LPA might lead to the transcriptional activation of the interlinked *phoPQ-oprH*, *pmrAB* and *arn* operons, which coordinate adaptive resistance to polycationic antibiotics such as polymyxins, aminoglycosides and mammalian defence peptides in Gram-negative bacteria [29–31].

Finally, OM binding sites for LPA and colistin may overlap, leading to competitive exclusion of colistin by LPA. As with colistin, marked antagonism between 250 μg ml^{-1} LPA and tobramycin was evident for all strains. This was highly unexpected as conditions of divalent cation limitation, as would be induced by LPA, have long been known to enhance the activity of aminoglycosides [32]. Further to this point, MIC values reported here may be artificially low as it was decided not to supplement the MHB batch used with additional cations out of concern that this may have obscured weak effects of LPA. Potential reasons for the observed antagonism between LPA and tobramycin are essentially the same as those already proposed for colistin. Aminoglycosides have been shown to bind to phosphatidic acid and other negatively charged phospholipids [33]. However, in the case of tobramycin, unlike that of colistin, considerable variations in the magnitude of MIC increase were apparent even between strains with comparable MIC values in the absence of LPA (Table 1). As the presence of different bacterial strains would not be anticipated to interfere with

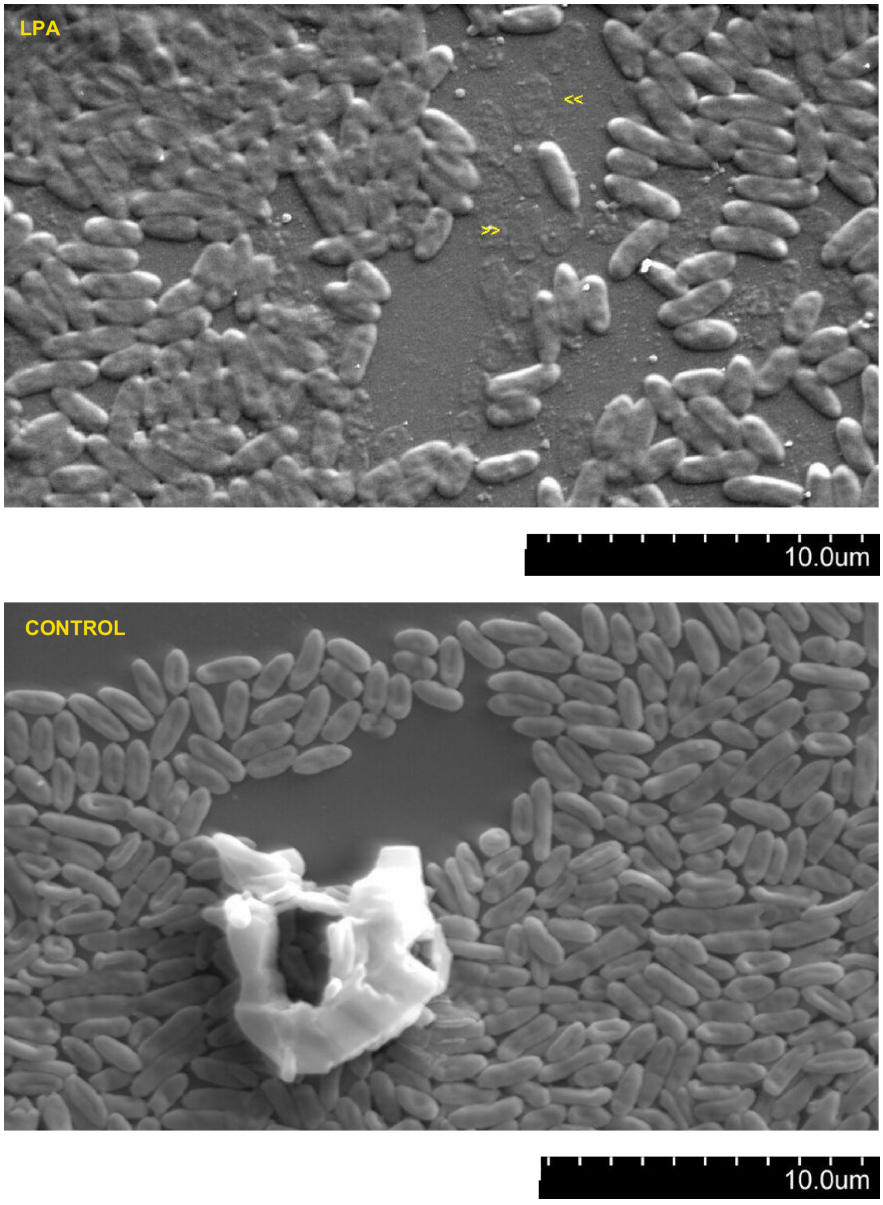

Fig. 2. Ghost cell formation (yellow arrows) are evident in LPA treated vs control PA14 cells adhered on glass coverslips. Note the gross appearance of cells, consistent with OM blebbing. FE-SEM micrograph.

the stoichiometry of any physicochemical interactions between LPA and tobramycin, it seems highly improbable that this is the sole factor underpinning the antagonistic interactions observed in these MIC determinations. Interestingly, in a trial experiment with strain PA14 using a far lower LPA concentration (~8.7 μg ml^{-1} or 20 μM) akin to the maximum recorded to occur *in vivo* [26], it was found that LPA reduced the MICs of both colistin and tobramycin twofold (Table 1). This may indicate that there is a window in which LPA concentrations are sufficiently high to permeabilize the OM or exert other antibiotic potentiating effects but are not high enough to either inactivate polycationic antimicrobials or sufficiently deplete Mg^{2+}ions and trigger expression of adaptive resistance genes. This may imply that LPA could be an effective antibiotic synergist at very low, non-toxic, concentrations, not much greater than those that occur naturally [26]. Moreover, since human defence peptides found in the airway, such as LL-37, promote apoptosis like LPA and have a mechanism of antibacterial action similar to that of colistin, it seems likely that LPA may play a role in the innate immunological response of the airway to infection by pathogens such as *P. aeruginosa* [26, 34]. It might be of interest to note that isolate A38 and all other isolates (S1, S2 and S38) exhibiting slowed or inhibited growth, either in the form of extended lag phases, longer generation times or both, in response to LPA, were all LES isolates displaying high-level tobramycin resistance and were in fact the only tobramycin-resistant isolates included in this experiment (Fig. S1). Conversely,

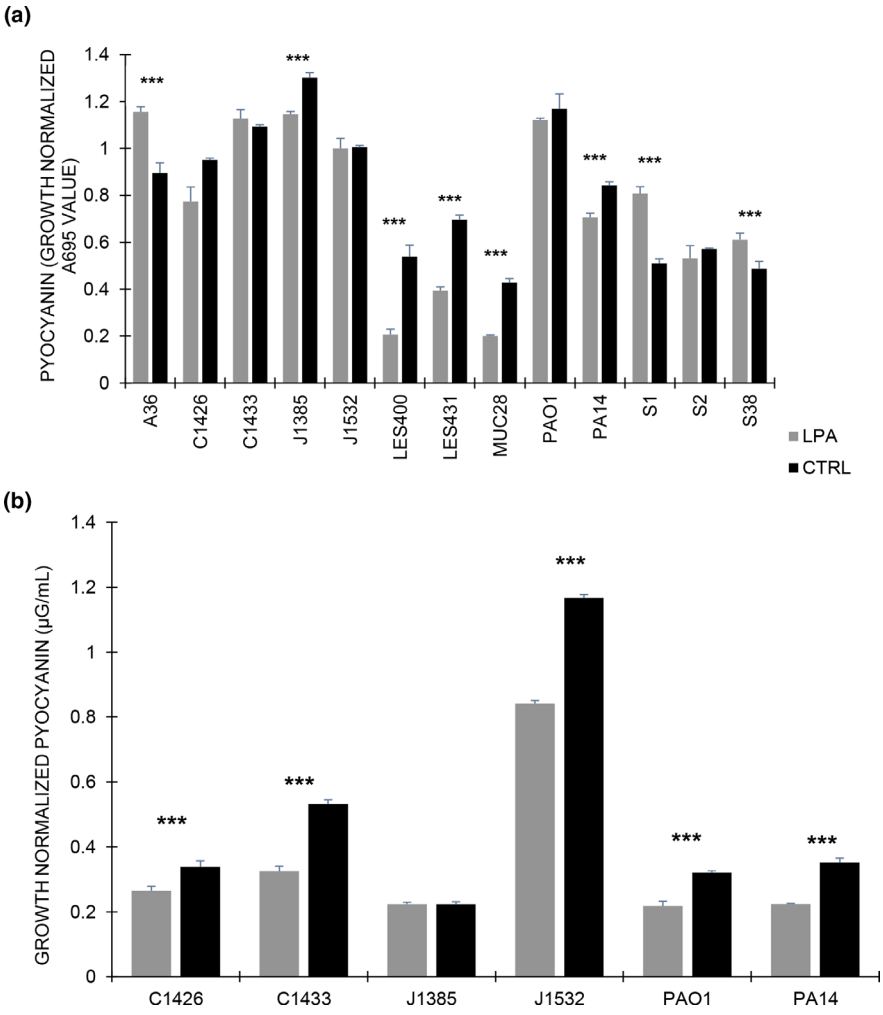

Fig. 3. LPA effects on pyocyanin production. (a) Mean absorbances of pyocyanin at 695 nm+SEM, normalized for growth differences in treated vs control samples of planktonic culture (n = 9). Significance denoted *** (P < 0.05). (b) Mean pyocyanin concentrations accumulated over 48 h in biofilm cultures, normalized to growth in treated vs control samples+SEM (n = 4). Significance denoted *** (P< 0.05).

tobramycin-sensitive LES isolates A36, LES400 and LES431 did not show any decrease in growth rate in response to LPA and were actually amongst the isolates whose growth was most stimulated by this lipid, the generation time of LES400 being quartered by LPA (Fig. S1). This finding may indicate an inverse relationship between sensitivity to tobramycin and LPA in these isolates, this hypothesis is further reinforced by the finding that LPA antagonizes the activity of tobramycin under identical conditions (Table 1); this may suggest competition between these two molecules for overlapping binding sites on the OM.

Although interactions of LPA with ceftazidime, colistin and tobramycin were qualitatively consistent across strains, interactions with chloramphenicol, erythromycin, quinolones, tetracycline and trimethoprim varied according to the specific strain. This suggests that synergistic interactions of LPA with these agents might be affected by other factors in addition to the OM permeabilizing effects of LPA. It is of interest that whilst mucoid strain J1532 was considerably more susceptible than its non-mucoid isogenic counterpart J1385 to chloramphenicol, erythromycin, tetracycline and trimethoprim, synergy was absent with tetracycline and trimethoprim for J1532, a *mexB* and *lpxO2* mutant [35].

LPA increases biofilm formation in static cultures

LPA has previously been shown to decrease biofilm formation by *P. aeruginosa* strain PAO1 [14], therefore, we utilized crystal-violet binding assays to quantify biofilm formation in LPA-treated and matched control cultures. Supplementation with 250 µg ml^{-1} LPA increased biofilm formation over 24 h by six of seven strains tested in static MHB cultures: A36, C1426, C1433, J1385, J1532 and PAO1, this was statistically significant for all strains but A36, LPA did not promote biofilm formation by strain PA14 under these conditions (Fig. 1). The finding that LPA enhanced biofilm formation by six of seven tested isolates, including reference

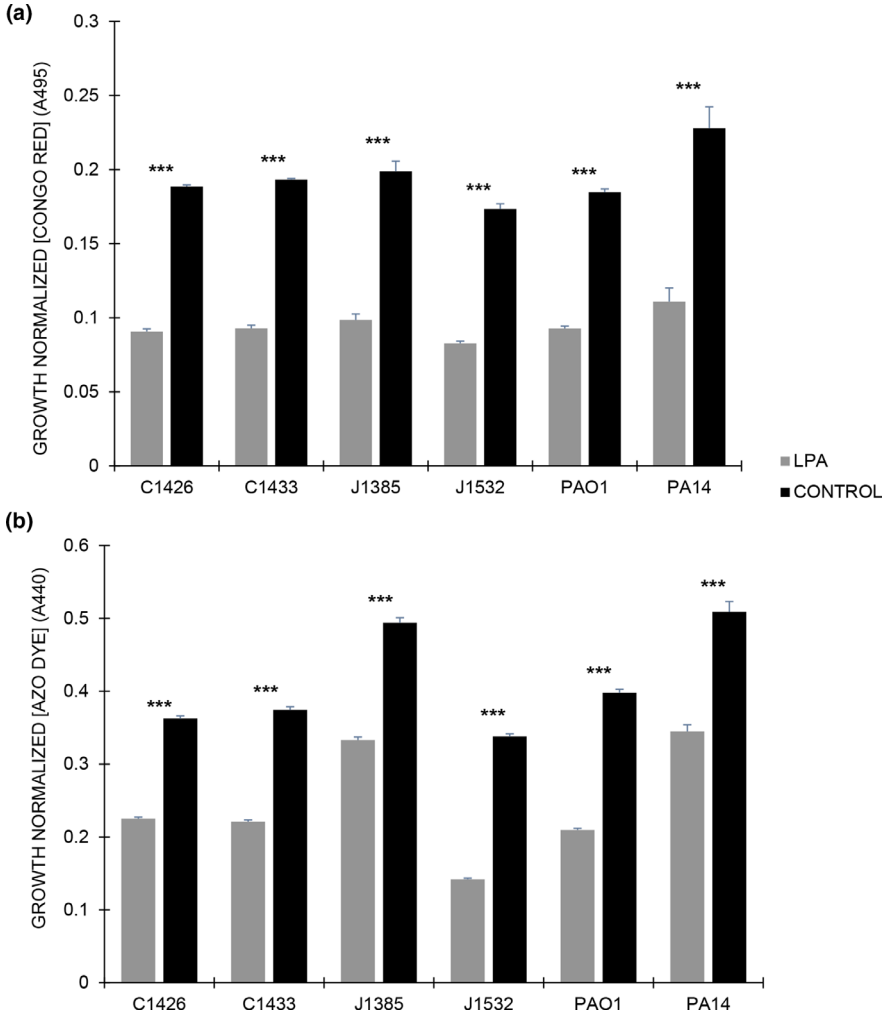

Fig. 4. (a) Mean elastase activity detected after 48 h by elastin congo-red assay in biofilm cultures+SEM, normalized for growth differences in LPA treated versus control cultures (n = 4). Significance denoted *** (P < 0.05). (b) Mean net protease activity detected after 48 h by azocasein digestion assay in biofilm cultures+SEM, normalized for growth differences in LPA treated versus control cultures (n = 4). Significance denoted *** (P< 0.05).

strain PAO1, was highly unexpected and difficult to explain given that chelators have previously been shown to reduce biofilm formation [14]. Laux and colleagues had previously reported that 80 µM LPA markedly reduced biofilm formation on the surfaces of a 24-well plate inoculated with PAO1 in LB cultures as determined via crystal-violet staining and appeared to also reduce formation of readily visible pellicle biofilm under these conditions [14]. They attributed these findings to a marked reduction in alginate production by the organism as confirmed by a carbazole-based colorimetric assay for uronic acids [14]. This study was published at a time when the prevailing view was that alginate was the primary exopolymer involved in biofilm formation and it has subsequently become accepted that other exopolysaccharides such as Pel and Psl, as well as other factors such as eDNA, are more important for biofilm formation by non-mucoid strains, and at least for PAO1, the contribution of alginate is probably negligible [36, 37]. In principle, carbazole assays detect the uronic acid monomers liberated upon acid hydrolysis of alginate but are sensitive to interference from neutral sugars and it has been suggested that the positive results obtained when these assays are applied to the exopolymeric substance of PAO1 biofilms, and possibly also biofilms of other non-mucoid strains, are due to the presence of eDNA and not alginate, as was once widely believed to be the case [36, 37]. The relative contributions of Pel and Psl polysaccharides to the biofilm matrix is strain dependent, some strains produce both in a redundant fashion, such that loss of either single polysaccharide does not substantially alter biofilm formation [38]. PAO1, utilises primarily Psl but can produce smaller quantities of Pel under certain circumstances whereas a minority of tested strains, as typified by PA14, are wholly reliant on Pel for biofilm formation and lack the psl gene cluster [38, 39]. Curiously, PA14 was the only tested isolate that did not exhibit enhanced biofilm formation in the presence of LPA in this experiment (Fig. 1). This may be of significance given that all other test isolates are genetically capable of Psl production [27].

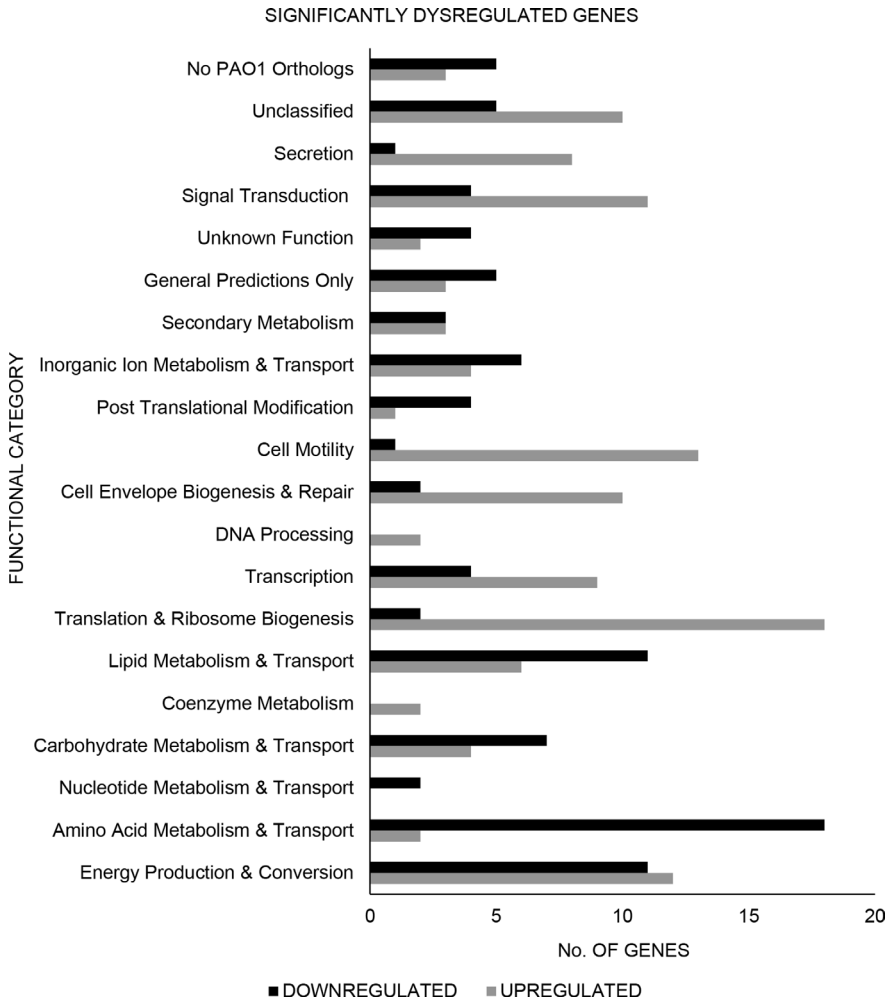

Fig. 5. Number of LPA regulated genes in PA14 mapping to each COG as curated in the *Pseudomonas* genome project [56].

LPA increases swarming motility in PA14

Swarming motility and biofilm formation are generally considered to be opposing behaviours in *P. aeruginosa* [40, 41]. LPA has previously been shown to decrease biofilm formation by strain PAO1 in crystal-violet adherence assays [14]. Moreover, other phospholipids have been shown to stimulate swarming motility in various bacterial species [19]. Therefore, we predicted that *P. aeruginosa* swarming would be stimulated by LPA exposure. This was tested using modifications of a simple assay previously described in the literature [21]. Briefly, the organism was stab inoculated on the middle of agar plates with and without the addition of LPA. Swarming motility was then quantified by measuring the diameter over which visible tendrils had spread from a central stab inoculation site in semisolid MHA medium during a 20 h incubation with and without the addition of 250 µg ml^{-1} LPA . In the presence of LPA, strain PA14 mean swarm distance was, significantly, increased by 66% (Fig. S4). Other phospholipids have previously been shown to act as chemoattractants, which stimulate swarming, swimming and twitching motility [19, 42].

Moreover, both swimming and twitching motility have previously been shown to play roles in biofilm formation [40]. Type IV pili and flagellae may both act as surface adhesins and structural biofilm components whilst twitching motility aids horizontal biofilm expansion and swimming motility enables vertical biofilm expansion [40, 41, 43].

LPA induces OM blebbing and ghost cell formation in surface adhered PA14

As LPA is suspected of having a membrane active mechanism, we endeavoured to visualize, in some detail, organisms exposed to it. Surface adhered cells of PA14 displayed evidence of ghost cell formation when grown in MHB with the addition of 250 µg ml^{-1} LPA – these effects were not observed in matched controls grown in MHB without LPA (Fig. 2). This supports the hypothesis that LPA induces loss of LPS via cation chelating activity.

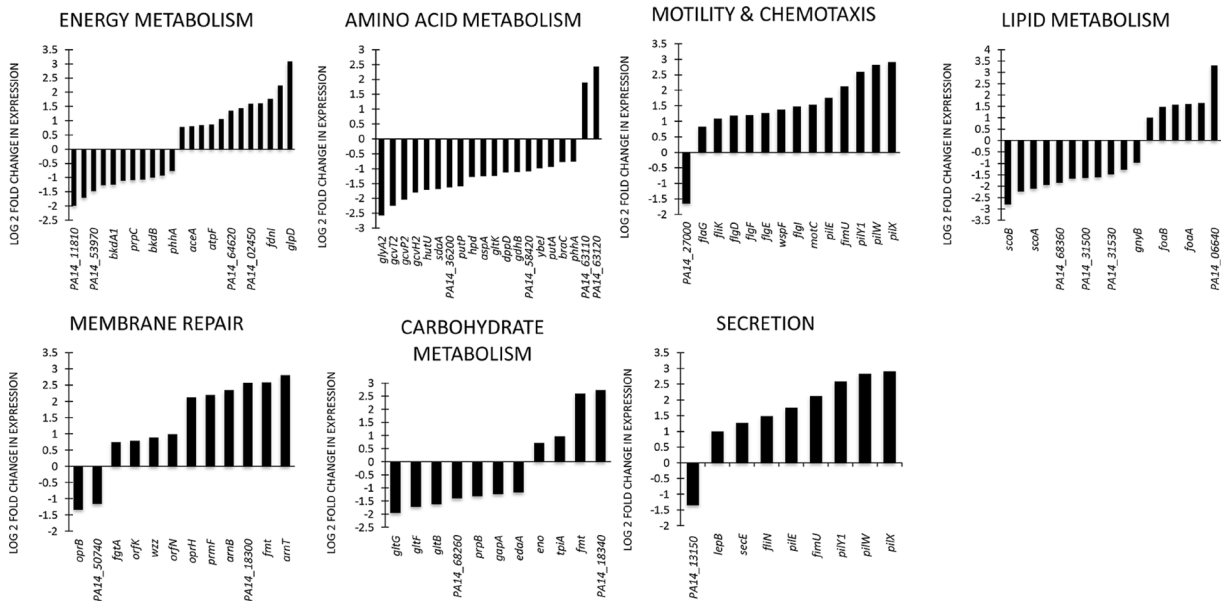

Fig. 6. Differential regulation of individual genes in COG functional categories showing statistical overrepresentation or underrepresentation ($P< 0.1$) ($n = 3$). Categories displayed include energy metabolism (C), amino acid metabolism (E), carbohydrate metabolism (G), lipid metabolism (I), membrane biogenesis and repair (M), motility and chemotaxis (N) and secretion (U).

LPA decreases pyocyanin production by *P. aeruginosa* in planktonic and biofilm states

LPA is known to inhibit production of several virulence factors including exoproteases and pyoverdine [14]. Given that production of one of these virulence factors, elastase, has been previously shown to be regulated via *lasR* expression, we sought to determine whether LPA also decreases production of pyocyanin, another *lasR* regulated virulence factor [44]. This was tested in both planktonic and biofilm cultures, using assays previously reported elsewhere [16, 17]. In agitated MHB cultures, addition of 250 µg ml^{-1} LPA reduced pyocyanin levels produced by 9 of 13 strains over 24 h: C1426, J1385, J1532, LES400, LES431, mucoid isolate 28, PAO1, PA14 and S2 (Fig. 3). Conversely, under the same conditions, LPA increased pyocyanin production by 4 of 13 strains: A36, C1433, S1 and S38 (Fig. 3).

In statically grown biofilm MHBII cultures, addition of 250 µg ml^{-1} LPA decreased pyocyanin production over 48 h by five of six strains tested : C1426, C1433, J1532, PAO1 and PA14 with statistical significance in all cases (Fig. 3). For a single strain, J1385, LPA had no effect on pyocyanin accumulation under these conditions (Fig. 3). Cation supplementation and extended incubation were used in these experiments to optimize biofilm formation.

In planktonic cultures, there appears to be an inverse relationship between growth rate and pyocyanin production with LPA increasing pyocyanin production in isolates whose growth it simultaneously decreases and vice versa. A36 appears to be an exception to this trend, however, it should be noted that this strain appeared to enter stationary growth phase at approximately the same time in LPA treated and control cultures even though LPA effectively halved its doubling time during exponential phase (Fig. S1). The reasons underlying these differential effects of LPA on pyocyanin production are not immediately apparent. Nonetheless, it has long been known that pyocyanin production does generally have an inverse correlation with growth rates and levels of oxidative respiration in the producing strain [45]. Pyocyanin production is also known to be elevated in the presence of high calcium concentrations [46], implying that the observed reductions in pyocyanin production by this subset of isolates may have been partly due to the Ca^{2+} chelating effects of LPA. The results of phenotypic microarray studies conducted on PA14 in the absence and presence of LPA using BioLog PM-1 plates strongly suggest that *P. aeruginosa* is capable of metabolizing LPA as a carbon source (Fig. S3). LPA breakdown would be expected to yield fatty acids and glycerol, the former of which can be readily oxidized to succinate, a preferred carbon source in pseudomonads [47, 48]. Production of pyocyanin is known to be increased during growth on certain amino acids whilst being subject to carbon catabolite repression by fatty acids [48, 49]. This may also provide a partial explanation for the observed reduction in pyocyanin accumulation that occurred in these nine isolates in the presence of LPA. Potential explanations for reduced pyocyanin production in biofilm cultures are broadly the same as those discussed for planktonic cultures and include increased metabolic rate and carbon catabolite repression.

Table 2. Manually curated genes. (A) Membrane stress associated genes that are differentially regulated in response to LPA but do not map to COG M. (B) Genes involved in motility and/or secretion that either do not map to COGs N and U or exhibit overlap. COGs are as follows: energy metabolism (C), amino acid metabolism (E), carbohydrate metabolism (G), transcription (K), membrane biogenesis and repair (M), motility and chemotaxis (N), post-translational modifications, turnover and chaperoning (O), inorganic ion metabolism (P), secondary metabolism (Q), unknown function (S), signal transduction (T) and secretion (U). Data based on a cut-off value of ≥1.5-fold difference in gene expression at α level of 0.1 (*n*=3)

(A) Membrane stress regulon members not mapping to COG M	**Log 2-fold change in expression**	**COGs**	**Xref for KEGG**	***Padj***
opdH *cis*-aconitate uptake porin	−3.123	unclassified		8.56E-07
ccoN cytochrome C oxidase subunit (cbb-3 type)	−1.865	O	pau02020 pau00190 pau01100	6.07E-10
oprC outer-membrane copper receptor	−1.591	P		0.00133
oprE anaerobic porin	−0.905	unclassified		0.03108
PA14_25840 putative electron transfer flavoprotein ubiquinone oxidoreductase	0.780	C		0.03501
mucA antisigma factor	0.888	T		0.00416
algU sigma factor	0.927	K		0.01743
algP alginate regulator	0.944	unclassified		0.04932
sigX ECF sigma factor	1.005	K		0.00103
PA14_57880 putative toluene ABC efflux protein	1.182	Q	pau02010	0.04054
phoP two-component regulator	1.360	TK	pau02020 pau01503	5.94E-06
algZ/fimS alginate biosynthesis protein	1.362	T	pau02020	0.01342
phoQ two-component sensor	1.927	T	pau02020 pau01503	9.94E-11
arnF 4-amino-4-deoxy-L-arabinose-phosphoundecaprenol flippase	2.329	unclassified	pae01503	2.93E-07
PA14_63120 putative spermidine synthase	2.424	E	pau01100 pau00480 pau00270 pau00410 pau00330	6.88E-13
pmrB two-component sensor	2.514	T	pau02020 pau02024	6.94E-09
PA14_63130 conserved hypothetical protein	2.603	unclassified		4.22E-14
arnD polysaccharide deacetylase	2.728	G	pau01503 pau00520	5.39E-09
pmrA two-component regulator	3.100	TK	pau02020 pau02024	9.80E-16
(B) Genes involved in secretion and /or motility	**Log 2-fold change in expression**	**COGs**	**Xref for KEGG**	***padj***
PA14_27000 putative chemotaxis protein	−1.648	N		5.32E-05
flaG hypothetical protein	0.826	N		0.04403
popB translocator protein	0.927	S		0.02943
lepB signal peptidase	1.000	U	pau02024 pau03060	0.03141
popD translocator protein	1.073	unclassified		0.03843
PA14_45830 putative flagellar hook protein	1.087	N	pau02040	0.04403

Continued

Table 2. Continued

(A) Membrane stress regulon members not mapping to COG M	Log 2-fold change in expression	COGs	Xref for KEGG	*Padj*
cheY two-component regulator	1.088	T	pau02020 pau02030	0.0094
flgD flagellar rod protein	1.181	N	pau02040	0.00523
flgF flagellar rod protein	1.200	N	pau02040	0.03506
flgE flagellar rod protein	1.266	N	pau02040	0.00066
spcU chaperone	1.269	unclassified		0.04924
secE secretion protein	1.272	U	pau02024 pau03060 pau03070	0.00075
exoT exotoxin	1.362	T		0.00953
algZ/fimS alginate biosynthesis protein	1.362	T	pau02020	0.01342
wspF putative cheB like methylesterase	1.383	N	pau02020 pau02025	0.03046
flgI flagellar P ring protein	1.472	N	pau02040	0.0024
fliN flagellar motor switch protein	1.491	U	pau02040 pau02030	0.02655
motC chemotaxis protein	1.536	N	pau02020 pau02030 pau02040	0.00138
pilE type IV fimbrial biogenesis protein	1.756	NU		0.0002
fimU type IV fimbrial biogenesis protein	2.124	NU		0.00081
pilY1 type IV fimbrial biogenesis protein	2.591	NU		3.59E-14
pilW type IV fimbrial biogenesis protein	2.830	NU		1.59E-09
pilX type IV fimbrial biogenesis protein	2.904	NU		1.71E-10

LPA reduces elastase and total protease production by *P. aeruginosa* in biofilm states

Given that LPA was previously shown to decrease elastase and total exoprotease secretion in PAO1 (14), we endeavoured to confirm these findings using a wider range of strains. Assays employed have been reported elsewhere [16, 50]. LPA at 250 µg ml^{-1} significantly decreased accumulation of active elastase over 48 h in statically grown biofilm MHBII cultures for all six strains tested (Fig. 4). Under the same conditions, this LPA concentration also significantly reduced total exoprotease production by these strains (Fig. 4).

LPA effects upon the PA14 transcriptome

Since lysolipids are thought to induce envelope stress, we determined the transcriptomic response to LPA-treated planktonic cultures of PA14 using RNA-Seq. Genes that were differentially regulated in planktonic MHB cultures of PA14 exposed to 250 µg ml^{-1} LPA were grouped into functional categories, mapped to the PAO1 reference genome and assigned to 1 of 18 COG (cluster of orthologous groups of proteins) annotations from the *Pseudomonas* genome project database [23]. Differential gene regulation was determined for each COG, normalized to their relative abundances in the PAO1 genome (Fig. 5). Expression differences of ≥1.5-fold were deemed to be biologically significant and were displayed individually (Fig. 6). Overrepresentation was evident for the following COGs: energy metabolism (downregulated), amino acid metabolism (downregulated), carbohydrate metabolism (downregulated), lipid metabolism (downregulated), cell envelope biogenesis and repair (upregulated), motility and chemotaxis (upregulated) and secretion (upregulated). Underrepresentation was evident for only a single category; amino acid metabolism genes (upregulated); note that downregulated genes in this category were simultaneously overrepresented, suggesting an overall repression of genes involved in amino acid metabolism. These transcriptomic data correlate with the phenotypic data obtained for LPA-treated cultures and are consistent with the hypotheses proposing that this lysolipid disrupts OM architecture, induces swarming motility and reduces pyocyanin production by repressing catabolism of aromatic amino acids and inducing the glyoxylate pathway. Some differentially regulated genes of interest

mapped to more than one COG or did not map to anticipated COGs based on function, for instance, the COG mappings for *phoPQ* and *pmrAB* genes, indicate only their involvement in transcriptional regulation (categories T and K) but do not reveal their clear involvement in membrane stress as they do not map to the corresponding category for that function (M). These genes were curated manually based upon KEGG pathways [23–25] and publications in the literature, then tabulated separately (Table 2). Several genes associated uniquely with type III secretion are also highly upregulated in response to LPA challenge including *exoU*, *spcU*, *popB* and *popD* (Table 2).

It would therefore be of interest to confirm whether LPA can increase type III secretion as it does swarming motility. It is possible that raised production of type III secretion apparatus, flagellae and/or pili by LPA may increase the antigenicity of *P. aeruginosa* and lead to heightened immunogenicity via Toll-like receptors *in vivo*. A similar phenomenon has already been reported for other Gram-negative pathogens, including *Salmonella enterica* and enhanced type III secretion has also been linked with glyoxylate flux [47, 51]. Individual differentially regulated genes are tabulated (Table S2, available in the online version of this article) and these were also annotated from the *Pseudomonas* genome project and KEGG databases [23–25]. Extreme upregulation of many genes involved in OM biogenesis and repair in response to LPA, including the interlinked *phoPQ* and *pmrAB* two-component regulators and genes governed by them including *oprH* as well as members of the *arn* and *spe* operons [30, 52], strongly suggest LPA induced OM stress accounts for the antibiotic potentiating effects of this lipid (Table 1, Fig. 6).

Although many genes encoding Mex system components were found to be downregulated in response to LPA, none passed the significance threshold when filtered by *padj* value (Table S2). Some of these missed the threshold by only a narrow margin including *PA14_18780* (*mexQ*) and *PA14_56890* (*mexW*), which are components of the respective MexPQ-OpmE and MexVW-OprM efflux systems, both able to extrude many drugs, which are potentiated by LPA and the former of which is not constitutively expressed [53, 54]. It may therefore be possible that downregulation of efflux plays a role in LPA mediated antibiotic potentiation, reinforcing OM permeabilizing effects. The possibility that LPA directly affects the configuration or energy supply of efflux systems cannot be excluded either and amphipathic molecules of varying charge (including lysolipids) have previously been shown to affect the configuration of membrane channels [55]. Indeed, some amphipaths already clinically employed as drugs for non-infective conditions, such as the phenothiazines are well-known efflux pump inhibitors [53, 56].

Many of the sugar nucleotide monomers used to produce Psl polysaccharide are also involved in the biosynthesis of A-band LPS and functional redundancy has been noted between genes involved in production of LPS (*wbpW*) and Psl (*pslB*) polysaccharide [57]. In RNA-seq analyses with strain PA14, *wbpW* (*PA14_71970*) was found to be upregulated (Table S1), as were several other genes involved in the synthesis and export of LPS components such as *orfK*, *orfN*, *wzz*, *wzt* and *eno* [57–59], implying that LPS is overexpressed in response to LPA (Fig. 6). Upregulation of LPS linked genes in response to LPA makes sense given that LPA has been shown to chelate cations required in LPS bridging, presumably inducing its loss from the OM [10]. It is possible that similar upregulation of LPS genes in strains other than PA14 occurs, increasing the cellular pool of sugar nucleotides available for LPS biosynthesis, and potentially also increasing biosynthesis of Psl polysaccharide.

Equally, effects of LPA on the structure of LPS itself may affect biofilm formation; absence of the O-polysaccharide, specifically the B-band, has previously been demonstrated to facilitate adhesion of *P. aeruginosa* to polystyrene and biofilm formation ranked by A- and B-band O-polysaccharide expression was found to be as follows: A+B−>A−B−>A+B+>A−B for PAO1 and its mutants [58]. Abbreviation of core polysaccharide has also been found to enhance both adherence and cohesion of cells [58]. It seems feasible that removal of LPS components (B-band LPS) by LPA might increase adhesion or alternatively, that enhanced expression of A-band LPS in response to this lipid may increase adhesion. In this regard, it is of interest to note that strain PA14, the only tested strain found not to increase biofilm formation in response to LPA, asides from lacking Psl, is also unusual in that it lacks A-band LPS due to the presence of SNPs in the *wbpX* and *migA* genes [59]. Several other possible explanations also exist for the increased biofilm formation that was observed to occur in the presence of LPA. Whilst biofilm formation and some forms of active motility are often considered to be opposing and mutually exclusive behaviours, flagellae and type IV pili have been implicated in certain stages of biofilm formation, in which they dually function as adhesins and structural components, they are also important for horizontal expansion of the biofilm upon the underlying substratum as well as in vertical differentiation and dispersal, respectively [40, 41, 43]. Therefore, upregulation of genes encoding type IV pilus and/or flagellum biogenesis in response to LPA (Fig. 5) may contribute to enhanced biofilm formation. The diguanylate cyclase GcbA (*PA14_64050*) is significantly downregulated in response to LPA (Table S2). GcbA has been shown to be essential for the post-transcriptional activation of BdlA, which is in turn essential for biofilm dispersal [60]. Downregulation of this gene in response to LPA may thus have increased biofilm development in these experiments. The *PA14_33530* locus is significantly upregulated in the presence of LPA and encodes an adhesion protein that may be involved in biofilm formation (Table S2). It should be noted that cells growing in the biofilm mode exhibit differential gene regulation that may differ spatiotemporally from that of cells grown in planktonic cultures but also from that of cells growing in different regions of the same biofilm. Therefore, the fact that the transcriptomic analyses in the present work were carried out using RNA extracted from planktonic cultures at a set time point may place considerable limitations on their predictive powers with regards to biofilm phenotypes.

Increased swarmingby PA14 in response to LPA is consistent with the transcriptomic data, which reveal significant upregulation of genes involved in flagellum and type IV pilus biogenesis, motility and chemotaxis (Fig. 6). Many genes involved in motility, chemotaxis and the biosynthesis of type IV pili and flagellae are highly upregulated upon LPA exposure in transcriptomic analyses (Fig. 6.).

Polyamines, which induce LPS cross bridging under Ca^{2+}/Mg^{2+} deplete conditions, inducing resistance to polycationic antimicrobials whilst increasing susceptibility to other molecules, including various β-lactams and chloramphenicol, are trafficked to the OM [30, 52]. The *speD2* (*PA14_63110*) and *speE2* (*PA14_63120*) genes for spermidine production are highly upregulated in response to LPA (Fig. 6) suggesting that surface localized spermidine may play a role in the modulation of antimicrobial sensitivity that occurs in response to LPA. The fact that LPA represses many genes from COGs C and E, which are involved in metabolism of branched chain amino acids strongly suggests that LPA, or its breakdown products, engage in carbon catabolite repression under these experimental conditions. In transcriptomic analyses, downregulation of *lasB* was detected in the presence of LPA whilst *lasA* transcription was unaltered (Table S2). This may have been due to the early timepoint, during exponential growth phase, at which RNA was extracted from cells – 4.66 h after first exposure to LPA. The possibility that significant differences in transcription would have ensued given a longer incubation time cannot be discounted. The transcriptomic analysis did however reveal significant downregulation of the *dsbA2* gene in response to LPA. The *dsb* genes have been shown to encode disulphide bond isomerases, which are essential for normal folding and catalytic activity of exoproteases including elastase [61, 62].

It is therefore possible that some exoproteases, such as elastase, even if transcribed normally, appear deficient in LPA-treated cultures owing to inactivity imposed by defective folding or other post-translational modifications, this would be in fitting with the previously reported finding of LPA causing elastase to be secreted as an inactive proenzyme.

Conclusions

In summary, LPA appears to influence the growth rate and antibiotic susceptibility of *P. aeruginosa* in a cation-dependent manner. Evidence from electron micrographs and transcriptomic data strongly suggest that this results from a membrane stress response. Moreover, LPA reduces production of pyocyanin in diverse clinical isolates of *P. aeruginosa,* a phenomenon which may result from catabolite repression of aromatic amino acids. Unexpectedly, it was discovered that LPA generally enhances biofilm formation whilst markedly increasing swarming motility. The clinical ramifications of this remain unclear at present but these results strongly suggest that the role of LPA in host–pathogen interactions merits further investigation as does its potential utility as an antibiotic 'enhancer' agent.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Behzadi P, Baráth Z, Gajdács M. It's not easy being green: a narrative review on the microbiology, virulence and therapeutic prospects of multidrug-resistant *Pseudomonas aeruginosa Antibiotics (Basel)* 2021;10:42.
2. Hauser AR, Jain M, Bar-Meir M, McColley SA. Clinical significance of microbial infection and adaptation in cystic fibrosis. *Clin Microbiol Rev* 2011;24:29–70.
3. Schultz DR, Miller KD. Elastase of Pseudomonas aeruginosa: inactivation of complement components and complement-derived chemotactic and phagocytic factors. *Infect Immun* 1974;10:128–135.
4. Döring G, Obernesser HJ, Botzenhart K, Flehmig B, Høiby N, *et al*. Proteases of *Pseudomonas aeruginosa* in patients with cystic fibrosis. *J Infect Dis* 1983;147:744–750.
5. Caldwell CC, Chen Y, Goetzmann HS, Hao Y, Borchers MT, *et al*. *Pseudomonas aeruginosa* exotoxin pyocyanin causes cystic fibrosis airway pathogenesis. *Am J Pathol* 2009;175:2473–2488.
6. Hall S, McDermott C, Anoopkumar-Dukie S, McFarland AJ, Forbes A, *et al*. Cellular effects of pyocyanin, a secreted virulence factor of *Pseudomonas aeruginosa*. *Toxins (Basel)* 2016;8:E236.
7. Nikaido H, Nakae T. The outer membrane of gram-negative bacteria. *Adv Microb Physiol* 1979;20:394591.
8. Angus BL, Carey AM, Caron DA, Kropinski AM, Hancock RE. Outer membrane permeability in *Pseudomonas aeruginosa*: comparison of a wild-type with an antibiotic-supersusceptible mutant. *Antimicrob Agents Chemother* 1982;21:299–309.
9. Yoshimura F, Nikaido H. Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. *J Bacteriol* 1982;152:636–642.
10. Krogfelt KA, Utley M, Krivan HC, Laux DC, Cohen PS. Specific phospholipids enhance the activity of beta-lactam antibiotics against *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 2000;46:377–384.
11. Van Rensburg CE, Jooné GK, O'Sullivan JF, Anderson R. Antimicrobial activities of clofazimine and B669 are mediated by lysophospholipids. *Antimicrob Agents Chemother* 1992;36:2729–2735.
12. Skindersoe ME, Krogfelt KA, Blom A, Zhang J, Jiang G, *et al*. Dual action of lysophosphatidate-functionalised titanium: interactions with human (MG63) osteoblasts and methicillin resistant *Staphylococcus aureus*. *PLoS One* 2015;10:e0143509.

13. Kabara JJ, Swieczkowski DM, Conley AJ, Truant JP. Fatty acids and derivatives as antimicrobial agents. *Antimicrob Agents Chemother* 1972;2:23–28.
14. Laux DC, Corson JM, Givskov M, Hentzer M, Møller A, *et al*. Lysophosphatidic acid inhibition of the accumulation of *Pseudomonas aeruginosa* PAO1 alginate, pyoverdin, elastase and LasA. *Microbiology (Reading)* 2002;148:1709–1723.
15. O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem* 2000;267:5421–5426.
16. Jiricny N, Molin S, Foster K, Diggle SP, Scanlan PD, *et al*. Loss of social behaviours in populations of *Pseudomonas aeruginosa* infecting lungs of patients with cystic fibrosis. *PLoS ONE* 2014;9:e83124.
17. Essar DW, Eberly L, Hadero A, Crawford IP. Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. *J Bacteriol* 1990;172:884–900.
18. Huang J, Sonnleitner E, Ren B, Xu Y, Haas D. Catabolite repression control of pyocyanin biosynthesis at an intersection of primary and secondary metabolism in *Pseudomonas aeruginosa*. *Appl Environ Microbiol* 2012;78:5016–5020.
19. Bonner PJ, Shimkets LJ. Phospholipid directed motility of surface-motile bacteria. *Mol Microbiol* 2006;61:1101–1109.
20. Harrison JJ, Turner RJ, Ceri H. High-throughput metal susceptibility testing of microbial biofilms. *BMC Microbiol* 2005;5:53.
21. Chow S, Gu K, Jiang L, Nassour A. Salicylic acid affects swimming, twitching and swarming motility in *Pseudomonas aeruginosa*, resulting in decreased biofilm formation. *JEMI* 2011;15:22–29.
22. Magoc T, Wood D, Salzberg SL. EDGE-pro: estimated degree of gene expression in prokaryotic genomes. *Evol Bioinform Online* 2013;9:127–136.
23. Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, *et al*. Enhanced annotations and features for comparing thousands of *Pseudomonas* genomes in the *Pseudomonas* genome database. *Nucleic Acids Res* 2016;44:D646-53.
24. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 2000;28:27–30.
25. Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M. KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res* 2016;44:D457-62.
26. Ediger TL, Toews ML. Dual effects of lysophosphatidic acid on human airway smooth muscle cell proliferation and survival. *Biochim Biophys Acta* 2001;1531:59–67.
27. Daniels JB, Scoffield J, Woolnough JL, Silo-Suh L, *et al*. Impact of glycerol-3-phosphate dehydrogenase on virulence factor production by *Pseudomonas aeruginosa*. *Can J Microbiol* 2014;60:857–863.
28. Beurer G, Warncke F, Galla H-J. Interaction of polymyxin B1 and polymyxin B1 nonapeptide with phosphatidic acid monolayer and bilayer membranes. *Chem Phys Lipids* 1988;47:155–163.
29. Mulcahy H, Charron-Mazenod L, Lewenza S, *et al*. Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *PLoS Pathog* 2008;4:e1000213.
30. Johnson L, Mulcahy H, Kanevets U, Shi Y, Lewenza S. Surface-localized spermidine protects the *Pseudomonas aeruginosa* outer membrane from antibiotic treatment and oxidative stress. *J Bacteriol* 2012;194:813–826.
31. Nicas TI, Hancock RE. Outer membrane protein H1 of *Pseudomonas aeruginosa*: involvement in adaptive and mutational resistance to ethylenediaminetetraacetate, polymyxin B, and gentamicin. *J Bacteriol* 1980;143:872–878.
32. Washington JA, Snyder RJ, Kohner PC, Wiltse CG, Ilstrup DM, *et al*. Effect of cation content of agar on the activity of gentamicin, tobramycin, and amikacin against *Pseudomonas aeruginosa*. *J Infect Dis* 1978;137:103–111.
33. Alexander AM, Gonda I, Harpur ES, Kayes JB. Interaction of aminoglycoside antibiotics with phospholipid liposomes studied by microelectrophoresis. *J Antibiot* 1979;32:504–510.
34. Barlow PG, Beaumont PE, Cosseau C, Mackellar A, Wilkinson TS, *et al*. The human cathelicidin LL-37 preferentially promotes apoptosis of infected airway epithelium. *Am J Respir Cell Mol Biol* 2010;43:692–702.
35. Stewart L, Ford A, Sangal V, Jeukens J, Boyle B, *et al*. Draft genomes of 12 host-adapted and environmental isolates of *Pseudomonas aeruginosa* and their positions in the core genome phylogeny. *Pathog Dis* 2014;71:20–25.
36. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS, *et al*. Extracellular DNA required for bacterial biofilm formation. *Science* 2002;295:1487.
37. Wozniak DJ, Wyckoff TJO, Starkey M, Keyser R, Azadi P, *et al*. Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. *Proc Natl Acad Sci U S A* 2003;100:7907–7912.
38. Colvin KM, Irie Y, Tart CS, Urbano R, Whitney JC, *et al*. The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. *Environ Microbiol* 2012;14:1913–1928.
39. Mann EE, Wozniak DJ. Pseudomonas biofilm matrix composition and niche biology. *FEMS Microbiol Rev* 2012;36:893–916.
40. O'Toole GA, Kolter R. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* 1998;30:295–304.
41. Klausen M, Heydorn A, Ragas P, Lambertsen L, Aaes-Jørgensen A, *et al*. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol Microbiol* 2003;48:1511–1524.
42. Miller RM, Tomaras AP, Barker AP, Voelker DR, Chan ED, *et al*. *Pseudomonas aeruginosa* twitching motility-mediated chemotaxis towards phospholipids and fatty acids: specificity and metabolic requirements. *J Bacteriol* 2008;190:4038–4049.
43. Murray TS, Ledizet M, Kazmierczak BI. Swarming motility, secretion of type 3 effectors and biofilm formation phenotypes exhibited within a large cohort of *Pseudomonas aeruginosa* clinical isolates. *J Med Microbiol* 2010;59:511–520.
44. Juhas M, Eberl L, Tümmler B. Quorum sensing: the power of cooperation in the world of *Pseudomonas*. *Environ Microbiol* 2005;7:459–471.
45. Whooley MA, McLoughlin AJ. The regulation of pyocyanin production in *Pseudomonas aeruginosa*. *European J Appl Microbiol Biotechnol* 1982;15:161–166.
46. Sarkisova S, Patrauchan MA, Berglund D, Nivens DE, Franklin MJ. Calcium-induced virulence factors associated with the extracellular matrix of mucoid *Pseudomonas aeruginosa* biofilms. *J Bacteriol* 2005;187:4327–4337.
47. Rojo F. Carbon catabolite repression in *Pseudomonas* : optimizing metabolic versatility and interactions with the environment. *FEMS Microbiol Rev* 2010;34:658–684.
48. Chung JCS, Rzhepishevska O, Ramstedt M, Welch M. Type III secretion system expression in oxygen-limited Pseudomonas aeruginosa cultures is stimulated by isocitrate lyase activity. *Open Biol* 2013;3:120131.
49. Palmer GC, Palmer KL, Jorth PA, Whiteley M. Characterization of the *Pseudomonas aeruginosa* transcriptional response to phenylalanine and tyrosine. *J Bacteriol* 2010;192:2722–2728.
50. Ohman DE, Cryz SJ, Iglewski BH. Isolation and characterization of *Pseudomonas aeruginosa* PAO mutant that produces altered elastase. *J Bacteriol* 1980;142:836–842.
51. Subramanian N, Qadri A. Lysophospholipid sensing triggers secretion of flagellin from pathogenic salmonella. *Nat Immunol* 2006;7:583–589.
52. Kwon DH, Lu CD. Polyamine effects on antibiotic susceptibility in bacteria. *Antimicrob Agents Chemother* 2007;51:2070–2077.

53. Mima T, Sekiya H, Mizushima T, Kuroda T, Tsuchiya T. Gene cloning and properties of the RND-type multidrug efflux pumps MexPQ-OpmE and MexMN-OprM from *Pseudomonas aeruginosa*. *Microbiol Immunol* 2005;49:999–1002.
54. Li Y, Mima T, Komori Y, Morita Y, Kuroda T, *et al*. A new member of the tripartite multidrug efflux pumps, MexVW-OprM, in *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 2003;52:572–575.
55. Martinac B, Adler J, Kung C. Mechanosensitive ion channels of *E. coli* activated by amphipaths. *Nature* 1990;348:261–263.
56. Ughachukwu P, Unekwe P. Efflux pump-mediated resistance in chemotherapy. *Ann Med Health Sci Res* 2012;2:191–198.
57. Byrd MS, Sadovskaya I, Vinogradov E, Lu H, Sprinkle AB, *et al*. Genetic and biochemical analyses of the *Pseudomonas aeruginosa* Psl exopolysaccharide reveal overlapping roles for polysaccharide synthesis enzymes in Psl and LPS production. *Mol Microbiol* 2009;73:622–638.
58. Lau PCY, Lindhout T, Beveridge TJ, Dutcher JR, Lam JS. Differential lipopolysaccharide core capping leads to quantitative and correlated modifications of mechanical and structural properties in *Pseudomonas aeruginosa* biofilms. *J Bacteriol* 2009;191:6618–6631.
59. Hao Y, Murphy K, Lo RY, Khursigara CM, Lam JS, *et al*. Single-nucleotide *Polymorphisms* found in the migA and wbpX glycosyltransferase genes account for the intrinsic lipopolysaccharide defects exhibited by *Pseudomonas aeruginosa* PA14. *J Bacteriol* 2015;197:2780–2791.
60. Petrova OE, Cherny KE, Sauer K, ed. The diguanylate cyclase GcbA facilitates *Pseudomonas aeruginosa* biofilm dispersion by activating BdlA. *J Bacteriol* 2015;197:174–187.
61. Malhotra S, Silo-Suh LA, Mathee K, Ohman DE. Proteome analysis of the effect of mucoid conversion on global protein expression in *Pseudomonas aeruginosa* strain PAO1 shows induction of the disulfide bond isomerase, dsbA. *J Bacteriol* 2000;182:6999–7006.
62. Braun P, Ockhuijsen C, Eppens E, Koster M, Bitter W, *et al*. Maturation of *Pseudomonas aeruginosa* Elastase. *J Biol Chem* 2001;276:26030–26035.

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