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Lipotoxin F of *Pseudomonas aeruginosa* is an AlgU-dependent and alginate-independent outer membrane protein involved in resistance to oxidative stress and adhesion to A549 human lung epithelia

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Chronic lung infection with *P. aeruginosa* and excessive neutrophil-associated inflammation are major causes of morbidity and mortality in patients with cystic fibrosis (CF). Overproduction of an exopolysaccharide known as alginate leads to the formation of mucoid biofilms that are resistant to antibiotics and host defences. Alginate overproduction or mucoidy is controlled by a stress-related ECF sigma factor AlgU/T. Mutation in the anti-sigma factor MucA is a known mechanism for conversion to mucoidy. Recently, we showed that inactivation of a kinase (KinB) in nonmucoid strain PAO1 results in overproduction of alginate. Here, we report the initial characterization of lipotoxin F (LptF, PA3692), an OmpA-like outer membrane protein that exhibited increased expression in the mucoid PAO1*kinB* mutant. The lipotoxin family of proteins has been previously shown to induce inflammation in lung epithelia, which may play a role in CF disease progression. Expression of LptF was observed to be AlgU-dependent and upregulated in CF isolates. Deletion of *lptF* from the *kinB* mutant had no effect on alginate production. Deletion of *lptF* from PAO1 caused a differential susceptibility to oxidants that can be generated by phagocytes. The *lptF* and *algU* mutants were more sensitive to hypochlorite than PAO1. However, the *lptF* mutant displayed increased resistance to hydrogen peroxide. LptF also contributed to adhesion to A549 human lung epithelial cells. Our data suggest that LptF is an outer membrane protein that may be important for *P. aeruginosa* survival in harsh environments, including lung colonization in CF.

INTRODUCTION

Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) cause cystic fibrosis (CF), which affects the normal respiratory, gastrointestinal and nutritional functions of the body (Lyczak *et al.*, 2002). In CF patients, the respiratory tract system is impaired not only by defective CFTR but also by microbial infections with a variety of pathogens, such as *Pseudomonas aeruginosa*, due to decreased mucociliary clearance (Govan & Deretic, 1996). Chronic lung infection with *P. aeruginosa* leads to increased morbidity and mortality in CF (Lyczak *et al.*, 2002). Biofilm formation in CF lungs by *P. aeruginosa* facilitates survival through resistance to host immune responses and increased antibiotic resistance (Govan & Deretic, 1996). Biofilm formation in CF lungs is also dependent upon bacterial communication or quorum sensing (QS) (Singh *et al.*, 2000).

Conversion of *P. aeruginosa* to mucoid phenotype or overproduction of exopolysaccharide alginate has clearly been shown to be protective for survival (Govan & Deretic,

Abbreviations: CF, cystic fibrosis; LC, liquid chromatography; MudPIT, multidimensional protein identification technology; QS, quorum sensing; TLR, Toll-like receptor.

The GenBank/EMBL/DDBJ accession number for the *algU mucA* sequence of *P. aeruginosa* CF149 is FJ649224.

A supplementary table, listing oligonucleotides used in this study, is available with the online version of this paper.

1996). MucA is a negative regulator of alginate production that sequesters the alginate master regulator, ECF sigma factor AlgU (Schurr *et al.*, 1996), to the inner membrane (Rowen & Deretic, 2000). Mutations in *mucA* cause constitutive production of alginate (Martin *et al.*, 1993) due to loss of MucA repression of AlgU. AlgU activates transcription of the *algD* biosynthetic operon (Deretic *et al.*, 1987), which then leads to alginate production (Wozniak & Ohman, 1994). Alginate production can also occur independently of *mucA* mutations through proteolytic derepression of MucA by the protease AlgW (Qiu *et al.*, 2007).

The two-component response regulator AlgB (PA5483) controls alginate production at the *algD* promoter (Wozniak & Ohman, 1994). AlgB and KinB (PA5484) are encoded on the chromosome in an operon, and KinB has been shown to phosphorylate AlgB (Ma *et al.*, 1997). However, phosphorylation of AlgB is not required for alginate production (Ma *et al.*, 1998; Damron *et al.*, 2009). AlgB is required for mucoidy (Goldberg & Ohman, 1984) and transcriptional activation of the *algD* biosynthetic operon (Leech *et al.*, 2008). Recently, we have observed that inactivation of *kinB* causes strain PAO1 to produce copious amounts of alginate (Fig. 1) (Damron *et al.*, 2009). Inactivation of *kinB* causes loss of AlgU repression by MucA and alginate production that is dependent upon AlgW, AlgB and the alternative sigma factor RpoN (σ^{54}) (Damron *et al.*, 2009). Alginate production provides protection for *P. aeruginosa*; however, alginate-independent, AlgU-dependent gene products are responsible for the detrimental inflammation (Firoved *et al.*, 2004). Of the 5567 proteins encoded in the PAO1 genome there are 113–186 predicted lipoproteins (Babu *et al.*, 2006). In mucoid strains, 70 % of genes with a >30-fold increase in expression encode lipoproteins (Firoved *et al.*, 2004). AlgU-dependent lipoproteins or lipotoxins cause activation of NF-κB in human lung epithelial cells through Toll-like receptor (TLR)2 (Firoved *et al.*, 2004). Lipotoxins have been shown to stimulate inflammatory responses (Firoved *et al.*, 2002, 2004). However, the physiological roles of these lipotoxins have not been characterized.

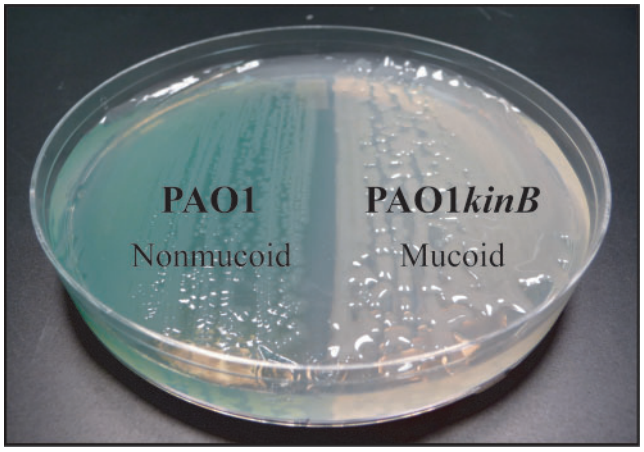

Fig. 1. Colony morphologies of *P. aeruginosa* PAO1 and isogenic mucoid variant PAO1*kinB*::*aacC1*. Inactivation of *kinB* in PAO1 causes alginate overproduction (Damron *et al.*, 2009).

Here we report that inactivation of *kinB* causes upregulation of an outer membrane protein known as lipotoxin F or LptF (PA3692). The *lptEF* promoter is highly upregulated in mucoid laboratory strains and CF isolates, and is controlled by AlgU. However, *lptF* has no effect on alginate production in the *kinB* mutant. We deleted *lptF* from PAO1 and observed increased resistance to hydrogen peroxide but increased susceptibility to killing by hypochlorite. Both nonmucoid and mucoid isolates from CF have increased *lptF* promoter expression. Also, PAO1Δ*lptF* exhibits decreased adherence to A549 human lung epithelial cells. The studies presented here suggest that LptF in *P. aeruginosa* is an important survival factor.

METHODS

Bacterial strains, growth conditions, sequencing and oligonucleotides. Bacterial strains and plasmids used in this study are shown in Table 1. *Escherichia coli* strains were grown at 37 °C in Lennox broth (LB) or LB agar supplemented, when necessary, with carbenicillin or tetracycline at concentrations of 100 and 20 μg ml^{-1}, respectively. *P. aeruginosa* strains were grown at 37 °C in LB or on *Pseudomonas* isolation agar (PIA) plates (Difco). When necessary, the PIA plates were supplemented with carbenicillin or tetracycline at concentrations of 300 and 200 μg ml^{-1}, respectively. Amplicon sequencing of plasmids and gene deletions were performed by the Marshall University Genomics Core Facility. The sequences of the oligonucleotides utilized this study are listed in Supplementary Table S1.

Multidimensional protein identification technology (MudPIT) analysis. To identify the proteins present in *P. aeruginosa* total cell lysates, MudPIT with tandem MALDI-TOF/TOF MS was employed. Strains were streaked on PIA and cultured for 24 h at 37 °C. Cells were harvested and total protein samples were prepared by processing cell lysates with Epicentre ReadyPreps. Total protein samples (50 μg) were desalted using C_4 ProteaTip SpinTips (Protea Biosciences) according to the manufacturer's protocol. The collected samples were lyophilized and dissolved in 100 μl 50 mM ammonium bicarbonate in 20 % acetonitrile for tryptic digestion. The samples were then reduced and alkylated with 10 μl 250 mM DTT (60 min at 55 °C), and 10 μl 625 mM iodoacetamide (60 min at room temperature in the dark). Proteolytic digestion was performed in 50 mM ammonium bicarbonate buffer using a trypsin : protein ratio of 1 : 100. The digestion was carried out overnight at 37 °C. The digests were cleaned by repeated lyophilization and reconstitution in 0.1 M acetic acid. After final lyophilization, the digests were reconstituted in a strong cation-exchange loading buffer (5 mM ammonium formate in 20 % acetonitrile, pH 3.0) to be fractionated with ProteaTip spin tips according to the manufacturer's protocol. The SpinTip was transferred to a fresh centrifuge tube to collect the sample during elution with 200 μl elution solution. Eight different elution solutions were used to fractionate the peptides (20, 60, 100, 150, 200, 250, 400 or 500 mM ammonium formate in 20 % acetonitrile) in a stepwise manner. The collected fractions were cleaned by repeated lyophilization and reconstitution in 0.1 M acetic acid. After the final lyophilization, the digests were reconstituted in liquid chromatography (LC) run buffer. The fractions were then submitted to LC MALDI spotting and MALDI-TOF/TOF spectral analysis to survey

Table 1. Bacterial strains and plasmids used in this study

The *P. aeruginosa* isolates used in this study were obtained from the following individuals: P. Phibbs, East Carolina University Genetic Stock Center, Greenville, NC; J. Goldberg, University of Virginia, Charlottesville, VA; G. Pier, Harvard Medical School, Boston, MA; D. Speert, University of British Columbia, BC, Canada; J. Govan, University of Edinburgh, Edinburgh, UK.

Strain or plasmid	Phenotype, genotype and description*	Source
***P. aeruginosa* strains**		
PAO1	Alg^- prototroph	P. Phibbs
PAO1Δ*algU*	Alg^-, PAO1 in-frame deletion of *algU* (PA0762)	Damron *et al.* (2009)
PAO1Δ*lptF*	Alg^-, PAO1 in-frame deletion of *lptF* (PA3692)	This study
PAO1Δ*rpoN*	Alg^-, PAO1 in-frame deletion of *rpoN* (PA4462); non-motile	Damron *et al.* (2009)
PAO1*kinB*::*aacC1*	Alg^+, PAO1 *kinB*::Gm^R	Damron *et al.* (2009)
PAO1*kinB*::*aacC1*Δ*algW*	Alg^-, PAO1*kinB*::*aacC1* in-frame deletion of *algW* (PA4446)	Damron *et al.* (2009)
PAO1*kinB*::*aacC1*Δ*algU*	Alg^-, PAO1*kinB*::*aacC1* in-frame deletion of *algU* (PA0762)	Damron *et al.* (2009)
PAO1*kinB*::*aacC1*Δ*lptF*	Alg^+, PAO1*kinB*::*aacC1* in-frame deletion of *lptF* (PA3692)	This study
PAO1*kinB*::*aacC1*Δ*rpoN*	Alg^-, PAO1*kinB*::*aacC1* in-frame deletion of *rpoN* (PA4462); non-motile	Damron *et al.* (2009)
383	Nonmucoid CF isolate from the sputum of a patient at Children's Hospital, Boston, MA, USA, on 16 June 1980	J. Goldberg (Hanna *et al.*, 2000)
2192	Mucoid CF isolate from the sputum of a patient at Children's Hospital, Boston, MA, USA, on 18 June 1980, isogenic to 383	J. Goldberg (Hanna *et al.*, 2000)
CF29	Alg^+ *mucA* mutant	J. Govan (Head & Yu, 2004)
CF149	Alg^+ *mucA* mutant, *algU* suppressor mutant	G. Pier (Head & Yu, 2004)
CFO42	Alg^+ mucoid isolate (1978)	D. Speert (Head & Yu, 2004)
CFO23o	Alg^+ mucoid isolate from same sputum sample (1998) as CFO23s and CFO23s	D. Speert (Head & Yu, 2004)
CFO23s	Alg^+ mucoid isolate from same sputum sample as CFO23o and CFO23w	D. Speert (Head & Yu, 2004)
CFO23w	Alg^+ mucoid isolate from same sputum sample as CFO23o and CFO23s	D. Speert (Head & Yu, 2004)
***E. coli* strains**		
DH5α	F^-, ϕ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(r_k^-, m_k^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1	Laboratory strain
TOP10	DH5α derivative	Invitrogen
Plasmids		
pRK2013	*Tra Mob ColE1* Km^R	Figurski & Helinski (1979)
pCR4-TOPO	TA cloning vector; 3.9 kb; Ap^R Km^R	Invitrogen
pHERD20T	pUCP20T P_{lac} replaced by 1.3 kb AflII–*Eco*RI fragment of *araC*–P_{BAD} cassette	Qiu *et al.* (2008)
pHERD20T-*lptF*	*lptF* (PA3692) from PAO1 in pHERD20T *Eco*RI/*Hin*dIII	This study
pEX100T	*Pseudomonas* suicide vector, *sacB*, *oriT*, Cb^R	Schweizer & Hoang (1995)
pEX100T-*Not*I	*Pseudomonas* suicide vector with *Not*I restriction site fused into *Sma*I of pEX100T, *sacB*, *oriT*, Cb^R	Qiu *et al.* (2007)
pEX100T-Δ*lptF*	A 1.8 kb fragment flanking *lptF* fused with pEX100T-*Not*I with in-frame deletion of *lptF*	This study
miniCTX-*lacZ*	Gene delivery vector for inserting genes at the CTX phage *att* site on the *P. aeruginosa* chromosome; Tc^R	Hoang *et al.* (2000)
miniCTX-P_{lptEF}-*lacZ*	Complete P_{lptEF} promoter (949 bp upstream of ATG of *lptF*) fused *Hin*dIII/*Eco*RI with *lacZ* for integration at the CTX phage *att* site on the *P. aeruginosa* chromosome; Tc^R	This study
pMRPQ-1	GFP expression plasmid; Cb^R	Davies *et al.* (1998)

*Alg^-, nonmucoid phenotype; Alg^+, mucoid phenotype; Ap^R, ampicillin resistant; Cb^R, carbenicillin resistant; Km^R, kanamycin resistant; Tc^R, tetracycline resistant.

the proteome of the *P. aeruginosa* strains. The relative quantity of the protein within the sample was calculated by a comparison of the number of amino acids identified by MS with those corresponding to the full-length peptide. The ratio of the identified peptides within the sample was also normalized against the various expected lengths of the peptides and displayed as the relative percentage of peptides identified within the sample.

SDS-PAGE, total protein preparation and peptide mass spectrometric sequencing. Total protein preparations were obtained by processing cell lysates with Epicentre ReadyPreps. Protein concentrations were determined using the Bio-Rad D_C Protein Assay. SDS-PAGE (14 % polyacrylamide) was performed to separate total cell lysates for staining with R250 Coomassie stain. Selected upregulated protein bands were excised from the gel for

direct mass spectrometric sequencing. Gel pieces were destained with Protea Silver destaining solution (Protea Biosciences). The pieces were dehydrated and then rehydrated with acetonitrile and 50 mM ammonium bicarbonate, respectively. Proteins in the gel pieces were reduced and alkylated with 250 mM DTT (60 min, 55 °C) and 650 mM iodoacetamide (60 min at room temperature in the dark), respectively. Digestion was performed with 625 ng trypsin in 50 mM ammonium bicarbonate buffer overnight. Extraction of peptides was performed using 5 % formic acid in 50 % acetonitrile and with 50 mM ammonium bicarbonate. Three cycles of dehydration, rehydration and supernatant collection were performed, and the recovered peptides were dried down in a lyophilizer to be purified with an acetic acid rinse in addition to a final lyophilization.

The LC-MALDI MS system used was an ABI Tempo LC MALDI spotter with Tempo LC MALDI v.2.00.09 data acquisition and processing software. Lyophilized-digested samples were reconstituted, and 5 µl was injected onto a Chromolith CapRod monolith column 150 × 0.1 mm (Merck). The peptides were eluted from the column using an acetonitrile/trifluoroacetic acid gradient (2–72 % acetonitrile in 25 min) and spotted directly onto a MALDI plate. The MALDI spots were analysed using an ABI 4800 MALDI-TOF/TOF analyser operated with 4000 Series Explorer software. The MS acquisition was in reflector mode positive ion mode with 400 laser shots per spectrum performed. The 15 strongest precursors were chosen for MS-MS, and the MALDI spot was interrogated until at least four peaks in the MS-MS spectra achieved a signal : noise ratio ⩾70. The resulting MS/MS spectra were analysed using ABI Protein ProteinPilot software 2.0. The spectral data were compared with the *Pseudomonas* Genome Project version 2 database for identification of the peptides and corresponding proteins.

Analysis of outer membrane proteins. *P. aeruginosa* strains were streaked on PIA and cultured for 24 h at 37 °C. The cells were scraped from the plates and suspended in PBS (pH 7.4, Sigma-Aldrich). The cells were harvested by centrifugation at 7000 ***g***. The cell pellet was suspended in 2 % sarkosyl with 2 mM PMSF protease inhibitor in PBS. The cells were lysed by sonication for 1 min on ice. The lysate was clarified by low-speed centrifugation. The supernatant was taken and centrifuged at 40 000 ***g*** for 1 h. The resulting pellet, containing outer membrane proteins, was resuspended in Tris-buffered saline (TBS; Protea Biosciences). The protein concentration was determined. The preparations were separated by SDS-PAGE and visualized by silver staining with Bio-Rad Silver Stain Plus.

β-Galactosidase activity assay of P_{lpteEF}–*lacZ* promoter fusion. The MiniCTX-*lacZ* (Hoang *et al.*, 2000) integration gene delivery vector was used for inserting promoter fusions into the CTX phage *attB* site on the *P. aeruginosa* chromosome. A 949 bp length upstream of the *lptF* start site was cloned into the *Hin*dIII/*Eco*RI sites of MiniCTX-*lacZ*. The construct was sequenced to show that no mutations had occurred during the cloning. MiniCTX-P_{lptEF}–*lacZ* was transferred to recipient strains by pRK2013 conjugation. Strains with integration into the *attB* site were selected on PIA supplemented with tetracycline (200 µg ml^{-1}) and were passed through three isolations. The β-galactosidase activity assay was based on the method originally described by Miller (1972), with the following modification. The cells were grown on PIA with antibiotics for selection in triplicate for 24 h at 37 °C and harvested in PBS. Cell density was measured by OD_{600}. The β-galactosidase activity was assayed after toluene permeabilization of the cells. The reported values represent the means of samples in triplicate from three independent experiments with standard error indicated.

Mutant strain construction. For in-frame deletion of *lptF*, the upstream and downstream sequence fragments (1 kb) flanking *lptF* were PCR-amplified and fused using the crossover PCR method. The PCR products with the in-frame deletion of the target gene were then cloned into pCR4-TOPO. The subcloned in-frame deletion fragment was then digested and ligated into the pEX100T-*Not*I vector. The resulting vectors were sequenced to show that no mutations had occurred apart from the intended specific gene deletion. A two-step allelic exchange procedure was employed with the pEX100T constructs for gene disruption or in-frame deletion. The single-crossover merodiploid exconjugants were selected based on carbenicillin resistance and sensitivity on 10 % sucrose (*sacB*). After incubation of the merodiploids in LB, the double-crossover recombinants were isolated from the PIA plates supplemented with 10 % (w/v) sucrose. The disruption or in-frame deletion of the target gene was confirmed by antibiotic-resistance assays, PCR amplification of the flanking region of the target gene with multiple sets of primers, and amplicon sequencing.

Analysis of alginate production. *P. aeruginosa* strains were grown at 37 °C on PIA plates in triplicate for 24 h. The resulting bacterial growth was removed from plates and suspended in PBS. The OD_{600} of the suspension in PBS was measured. The suspensions were assayed for the amount of uronic acid in comparison with a standard curve made with D-mannuronic acid lactone (Sigma-Aldrich), as previously described (Damron *et al.*, 2009).

Susceptibility to killing by hydrogen peroxide and hypochlorite. Sensitivity to hydrogen peroxide and hypochlorite was determined by measuring the radius of the growth inhibition zone surrounding filter disks (6 mm diameter, BBL). A 25 ml volume of LB agar was poured into 100 × 15 mm plates. Overnight cultures were diluted with LB, and 100 µl OD_{600} 0.1 culture was added to 3 ml molten 0.6 % soft agar and gently mixed. The culture–soft agar suspension was then overlaid on the 25 ml of LB agar. Disks were soaked with 10 µl fresh stock solutions of 10 % hydrogen peroxide or 6 % hypochlorite. The disks were then applied to the soft agar-containing plate. The zone of inhibition was scored after 24 h incubation at 37 °C by measuring the radius.

Cell culture methods. A549 lung epithelial cells (ATCC catalogue no. CCL-185) were purchased from ATCC. The cells were cultivated in F-12K medium supplemented with 10 % fetal bovine serum (ATCC) and antibiotics (pen-strep, MP Biomedicals) in 100 × 20 mm tissue culture treated dishes (Greiner Bio-One) and subcultured every 2–3 days. One day prior to experimental use, they were grown to 80–90 % confluence and split at a ratio of 1 : 1.

A549 epithelial cell adherence assay. Adherence was measured by incubation of A549 cells with GFP-tagged *P. aeruginosa* harbouring pMRPQ-1 (Davies *et al.*, 1998). A549 cells were harvested by treatment with 1 ml trypsin (0.25 %, Hyclone) for 10 min followed by gentle pipetting to remove any adherent cells. Live harvested cells were quantified by using erythrosin B (10 % in PBS, Fisher) exclusion dye and counted on a haemocytometer. A549 cells (1.5×10^5) were resuspended in 300 µl F-12K medium plus 10 % fetal bovine serum. GFP-tagged *P. aeruginosa* was added to the cells at a ratio of 100 : 1, and the mixture was rotated end over end at room temperature in a 1.5 ml microcentrifuge tube for 15 min. The cells were washed twice with 500 µl FACS buffer (3 % BSA, 0.02 % sodium Azide, 1 mM EDTA in PBS) and analysed for GFP fluorescence using a Becton Dickinson FACSAria cell sorter. Ten thousand cells were counted in each sample. Data were analysed using Flowjo software 8.8.2. Threshold gates were drawn based on a no-bacteria control. Results were reported as a percentage of PAO1-treated cells. All experiments were conducted in triplicate with three independent trials. Within each trial, data were normalized to the average adherence percentage of PAO1. The average normalized percentage for each trial was then calculated and used in statistical analysis. Student's *t* tests were performed to determine reported *P* values.

RESULTS

MudPIT detection of peptides in PAO1 *kinB* mutant and PAO1 *kinB/rpoN* double mutant

KinB is a histidine kinase of a two-component signal transduction system with the alginate response regulator AlgB (Ma *et al.*, 1997). In our previous studies, we observed that inactivation of alginate regulator *kinB* in PAO1 caused alginate overproduction (Fig. 1) (Damron *et al.*, 2009). This suggested that KinB is a negative regulator of alginate production in the wild-type *mucA* strain PAO1. Since inactivation of *kinB* causes mucoidy (PAO1*kinB*::*aacC1*), we hypothesized that AlgU-dependent gene products, as well as genes controlled by KinB–AlgB, would be upregulated. We sought to identify proteins upregulated in the *kinB* mutant to discover members of the KinB regulon. To do so, we subjected total protein extracts to MudPIT analysis. MudPIT analysis utilizes two liquid-column chromatographic separations and tandem MALDI-TOF MS peptide fingerprinting to identify peptides in a complex sample. The conditions of these experiments allowed the identification of the peptides present in the highest concentrations in the proteomes analysed.

Alginate production by the *kinB* mutant requires *rpoN* (Damron *et al.*, 2009). Therefore, we compared the proteomes of the *kinB* mutant and the nonmucoid *kinB/rpoN* double mutant. In mucoid PAO1*kinB*::*aacC1*, AlgD was present, but it was absent in the *kinB/rpoN* double mutant (Table 2). AlgD, or GDP-mannose 6-dehydrogenase, is responsible for the initial enzymic steps leading to alginate production in *P. aeruginosa*. Another differentially expressed peptide observed between the proteomes of the *kinB* mutant and the *kinB/rpoN* double mutant was azurin (PA4922) (Table 2). Azurin is a QS-regulated redox protein that is located in the periplasm (Nouwens *et al.*, 2003; Sriramulu *et al.*, 2005). Azurin is secreted by *P. aeruginosa* in response to eukaryotic proteins and induces apoptosis of macrophages (Zaborina *et al.*, 2000). In the *kinB* mutant, azurin formed 1.5 % of the peptides identified; however, in the *kinB/rpoN* mutant, azurin represented 15.4 % of peptides identified.

Five of the 12 peptides identified in the *kinB* mutant have been suggested to be controlled by QS (Table 2). QS-regulated proteins were observed in the *kinB* mutant, but only one (azurin) in the *kinB/rpoN* double mutant. PA4739 is a small periplasmic hypothetical protein that has been shown to be upregulated in response to QS signals (Schuster *et al.*, 2003) and hydrogen peroxide (Salunkhe *et al.*, 2005). PA0041 is similar to *Bordetella pertussis* haemagglutinin exoprotein (Jacob-Dubuisson *et al.*, 2001). PA0041 was detected in the *kinB* mutant but not the *kinB/rpoN* double mutant (Table 2). Since PA0041 is a secreted protein, it may be a component of the exopolysaccharide matrix of the *kinB* mutant (Fig. 1).

Identification of mucoidy-coupled lipotoxin F

The periplasmic chaperone SurA was identified in the *kinB* mutant (Table 2). SurA has been shown to assist in folding of outer membrane proteins OmpA, OmpF and LamB in *E. coli* (Lazar & Kolter, 1996). In our analysis, only one potential outer membrane protein was observed, PA3692 or LptF (Firoved *et al.*, 2004). Many lipoproteins or lipotoxins have been shown to be upregulated in mucoid *mucA* mutants (Firoved *et al.*, 2004) and in the presence of the cell wall inhibitor D-cycloserine (Wood *et al.*, 2006). According to the Pseudomonas Genome Database version 2 (http://www.pseudomonas.com), LptF (PA3692) is a conserved OmpA-like lipoprotein. The C-terminal 110 residues are 49 % identical to *P. aeruginosa* major porin OprF. We observed that LptF was upregulated in the *kinB* mutant; however, it was absent from the *kinB/rpoN* double mutant (Table 2). To validate the observations from the MudPIT analysis, total protein extracts of PAO1 and PAO1*kinB*::*aacC1* were separated by SDS-PAGE and visualized by Coomassie staining (data not shown). A significantly upregulated protein was observed in PAO1*kinB*::*aacC1* total protein extracts with an apparent mass of 27 kDa (Fig. 2, lane 2). The protein was identified as LptF (PA3692) by direct peptide fingerprint analysis.

LptF is an outer membrane protein

Computational analysis of the *P. aeruginosa* genome showed that LptF has a predicted type II signal peptide for export (Lewenza *et al.*, 2005). To confirm that LptF is in fact an outer membrane protein, outer membrane proteins from PAO1 and PAO1*kinB*::*aacC1* were prepared by the sarkosyl method. Total protein extracts and sarkosyl-insoluble proteins were separated and visualized by silver staining (Fig. 2). LptF is upregulated in the outer membrane protein fraction of PAO1*kinB*::*aacC1*; however, it is also present in PAO1 (Fig. 2, lanes 3 and 4). Lipotoxins have been shown to activate the host inflammatory response (Firoved *et al.*, 2004); however, their physiological functions have not been investigated, and therefore we further characterized lipotoxin F.

Expression of P_{lptEF} is AlgU-dependent and upregulated in CF isolates

We reasoned that since LptF was upregulated in *mucA* mutants (Firoved *et al.*, 2004) and in the mucoid *kinB* mutant, it was likely to be AlgU-dependent. LptE and LptF are encoded in the genome as an operon (Firoved *et al.*, 2004). Interestingly, the *lptEF* promoter does not contain an AlgU consensus sequence (Firoved *et al.*, 2004). A *lacZ* fusion with the *lptEF* promoter was constructed and integrated into the *P. aeruginosa* chromosome to compare expression of P_{lptEF} in various strains. P_{lptEF} was active in nonmucoid strains PAO1 and PA14 (Fig. 3). P_{lptEF} expression was observed to be AlgU-dependent and could be restored upon expression of AlgU *in trans* (Fig. 3). Also deletion of *algU* from PAO1*kinB*::*aacC1* caused complete loss of detectable P_{lptEF} (Fig. 3).

Since it was clear that *lptEF* expression was AlgU-dependent from previous research and data obtained in

Table 2. Proteins identified in total protein lysates of mucoid strain PAO1*kinB*::*aacC1* and nonmucoid strain PAO1*kinB*::*aacC1*Δ*rpoN* as determined by LC-MALDI TOF/TOF mass spectrometry

Strain	Locus (protein name)*	Relative percentage of identified peptides within sample†	Description (functional class)
PAO1*kinB*::*aacC1*	PA4739‡	16.6	Hypothetical protein (unknown)
	PA0594 (SurA)	14.1	Peptidyl-prolyl *cis-trans* isomerase (chaperones and heat-shock proteins)
	PA2518 (XylX)	10.7	Toluate 1,2-dioxygenase alpha subunit (carbon compound catabolism)
	PA0041‡	8.8	Probable haemagglutinin (toxins, enzymes, alginate)
	PA3540 (AlgD)	8.6	GDP-mannose 6-dehydrogenase (toxins, enzymes, alginate)
	PA2412	8.3	Hypothetical protein (unknown)
	PA2687 (PfeS)	7.8	Two-component sensor histidine kinase (regulatory systems)
	PA4385 (GroEL)	7.2	GroEL protein (chaperones and heat-shock proteins)
	PA2169‡	5.5	Hypothetical protein (unknown)
	PA3692 (LptF)‡	**5.5**	**Outer membrane protein (membrane proteins)**
	PA4277 (TufB)	5.5	Elongation factor Tu (post-translational modification)
	PA4922 (Azu)‡	1.5	Azurin precursor (energy metabolism)
PAO1*kinB*::*aacC1*Δ*rpoN*	PA4922 (Azu)‡	15.4	Azurin precursor (energy metabolism)
	PA1754 (CysB)	14.0	Transcriptional regulator (amino acid biosynthesis and metabolism)
	PA1337 (AnsB)	13.2	Glutaminase–asparaginase (amino acid biosynthesis and metabolism)
	PA5339	9.5	Hypothetical protein (unknown)
	PA5242 (Ppk)	7.4	Polyphosphate kinase (nucleotide biosynthesis and metabolism)
	PA4336	7.3	Hypothetical protein (unknown)
	PA4385 (GroEL)	6.3	GroEL protein (chaperones and heat-shock proteins)
	PA2952 (EtfB)	5.5	Electron transfer flavoprotein beta-subunit (energy metabolism)
	PA4244 (RplO)	3.8	Ribosomal protein L15 (post-translational modification)
	PA3686 (Adk)	3.3	Adenylate kinase (nucleotide biosynthesis and metabolism)
	PA0962	2.8	Probable DNA-binding stress protein (adaptation)
	PA3611	2.3	Hypothetical protein (unknown)
	PA0888 (AotJ)	2.0	Arginine/ornithine binding protein (transport of small molecules)
	PA3021	1.4	Hypothetical protein (unknown)
	PA0329	0.8	Hypothetical protein (unknown)
	PA2743 (InfC)	0.8	Translation initiation factor IF-3 (post-translational modification)
	PA0456	0.6	Probable cold-shock protein (adaptation)
	PA0981	0.6	Hypothetical protein (unknown)
	PA1804 (HupB)	0.5	DNA-binding protein HU (replication, recombination, modification and repair)
	PA1852	0.2	Hypothetical protein (unknown)
	PA2622 (CspD)	0.6	Cold-shock protein (adaptation)
	PA2966 (AcpP)	0.6	Acyl carrier protein (fatty acid and phospholipid metabolism)
	PA3031	0.5	Hypothetical protein (unknown)
	PA3745 (RpsP)	0.6	Ribosomal protein S16 (replication, recombination, modification and repair)

*PA loci number designations are according to the Pseudomonas Genome Project; http://www.pseudomonas.com.
†This value refers to the relative quantity of the protein within the sample. The value is calculated by comparison of the number of amino acids identified corresponding to the full-length peptide. The ratio of the identified peptides within the sample was also normalized against the various expected lengths of the peptides and displayed as the percentage of peptides identified within the sample.
‡Indicates protein has been shown to be regulated by quorum sensing.

this study, we hypothesized that mucoid CF isolates would have increased expression of the *lptEF* promoter. Strain 383 is a nonmucoid CF isolate (Hanna *et al.*, 2000) and strain 2192 is a strain isogenic to 383 but with a *mucA* mutation (Hanna *et al.*, 2000). Interestingly, P_{lptEF} was upregulated in both nonmucoid CF isolate 383 and mucoid CF isolate 2192 (Fig. 3) compared with lab strain PAO1 (Fig. 3). However, no P_{lptEF} expression was detected in nonmucoid CF149. This indicates that CF149 harbours an *algU* mutation. When we sequenced *algU* and *mucA* in CF149, we found that this strain carries both *algU* and *mucA* mutations. The *algU* gene of CF149 has a missense

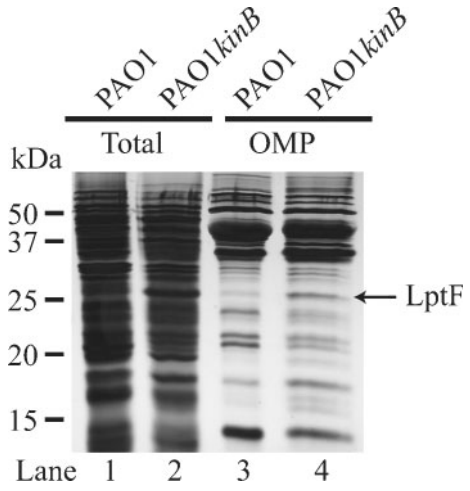

Fig. 2. SDS-PAGE of total and outer membrane proteins from *P. aeruginosa* strains PAO1 and PAO1*kinB*::*aacC1* reveals that LptF is an outer membrane protein. Outer membrane proteins (OMP) were isolated from total protein lysates by precipitation in 2% sarkosyl. A 60 μg sample of the protein preparation was separated and submitted to silver staining. The apparent molecular masses are indicated based on comparison with protein ladder standards (10–250 kDa). The arrow indicates the position of LptF in the separations.

mutation (C_{182} to T_{182}), resulting in an amino acid change from Ala_{61} to Val_{61}. The *mucA* mutation is a deletion of a C at 374, which causes a frameshift with the formation of a premature stop at TGA_{386} (GenBank accession number FJ649224). This further suggests that P_{lptEF} expression is AlgU-dependent. Several other CF isolates showed high *lptEF* expression (Fig. 3). Even within one CF sputum sample, several morphologies were observed, and each exhibited a different level of *lptEF* expression (Fig. 3, strains CFO23o, s and w). These results show that the *lptEF* promoter is AlgU-dependent and upregulated in CF isolates.

LptF is not required for alginate production

Envelope proteins such as MucE can activate alginate overproduction in *P. aeruginosa* through regulated proteolysis of MucA by the serine protease AlgW (Qiu *et al.*, 2007). Since LptF was highly upregulated in the mucoid *kinB* mutant, we examined whether LptF expression plays a role in the signal transduction which leads to AlgW-dependent alginate production of this strain (Damron *et al.*, 2009). To test this, *lptF* was deleted from PAO1*kinB*::*aacC1*. However, both the *kinB* mutant and the *kinB/lptF* double mutant produced approximately 100 micrograms of alginate per millilitre per OD_{600} unit. Furthermore, overexpression of *lptF* in PAO1 from the P_{BAD} promoter of pHERD20T did not stimulate alginate production above the normal nonmucoid level (30 micrograms alginate per millilitre per OD_{600} unit). These results suggested that LptF does not activate alginate production. Therefore, we concluded that LptF is likely to be co-expressed with alginate and is not involved in the signalling pathway that leads to alginate production.

Deletion of *lptF* causes increased resistance to hydrogen peroxide in PAO1, but increased susceptibility to hypochlorite

In the CF lung, *P. aeruginosa* produces alginate for protection (Govan & Deretic, 1996). Since LptF is upregulated along with alginate production, we hypothesized that LptF serves as a protective factor. We first generated a PAO1 *lptF* deletion mutant and observed no

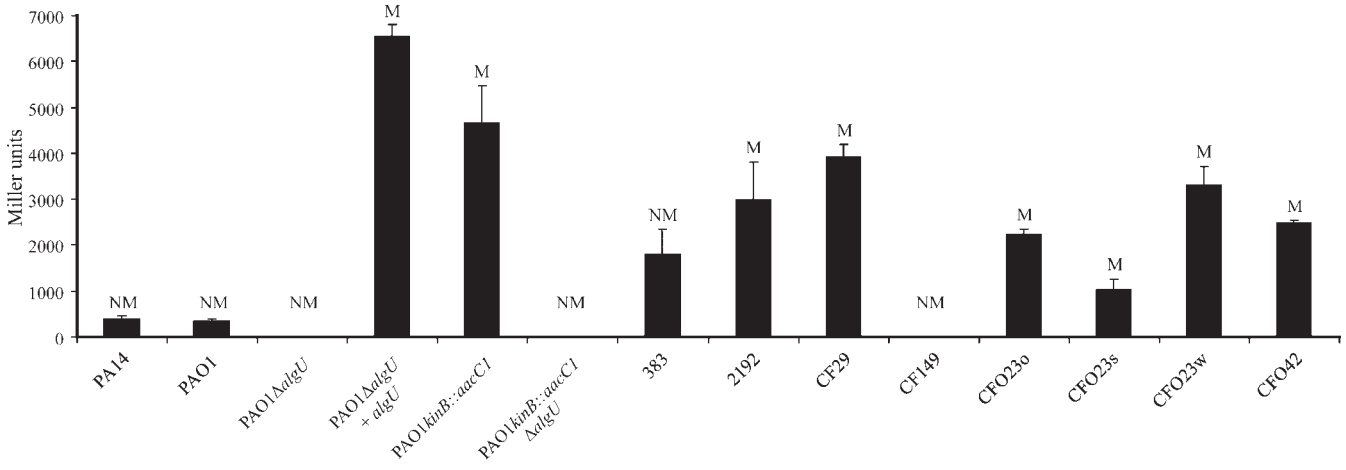

Fig. 3. β-Galactosidase activity from P_{lptEF}–*lacZ* reporter constructs integrated into the chromosome at the *attB* site (Hoang *et al.*, 2000) in laboratory and CF isolates. Note that expression of the *lptEF* promoter requires AlgU and that *lptEF* expression is upregulated in CF isolates. NM, nonmucoid strain; M, mucoid strain.

changes in growth rate compared with PAO1, showing that *lptF* is not an essential gene (data not shown). We next examined whether LptF has a protective role against hydrogen peroxide and hypochlorite. To test the role of *lptF* regarding cell membrane integrity, susceptibility assays were performed with hydrogen peroxide and hypochlorite (Table 3). Interestingly, PAO1Δ*lptF* was more resistant to hydrogen peroxide than PAO1 (Table 3). However, deletion of *algU* did not result in the same level of resistance to hydrogen peroxide. Deletion of *lptF* caused significantly increased susceptibility to hypochlorite (Table 3). Neutrophils utilize the generation of oxidants to kill microbes, and mucoid mutants are more resistant to hypochlorite killing (Learn *et al.*, 1987). Deletion of *algU* and *lptF* caused increased susceptibility to hypochlorite (Table 3). These data suggest that the AlgU-dependent proteins such as LptF protect *P. aeruginosa* from hypochlorite killing.

Deletion of *rpoN* or *lptF* decreases adhesion to A549 lung epithelial cells

Most lipotoxins are small lipoproteins that are likely housed in the inner leaflet or the periplasm. However, LptF is an outer membrane protein (Fig. 2). *E. coli* OmpA can participate in adhesion to surfaces and interactions with cells (Smith *et al.*, 2007); therefore, we were interested to see if LptF also has a role in adhesion. To test this hypothesis, we performed adherence assays with A549 lung epithelial cells. A constitutively GFP-expressing plasmid pMRPQ-1 (Davies *et al.*, 1998) was conjugated into PAO1, PAO1Δ*rpoN*, PAO1Δ*lptF* and mucoid strain PAO1*kinB*::*aacC1*. Pili and flagella expression are controlled by *rpoN* (Ishimoto & Lory, 1989; Totten *et al.*, 1990). TLR5, which is expressed on A549 cells, recognizes flagellin and promotes adherence of bacteria to cell surfaces (Hayashi *et al.*, 2001). Thus, PAO1Δ*rpoN* serves as a negative control for adhesion for our experiments. Epithelial cells were incubated with the indicated bacteria strains for 15 min at room temperature. The cells were then washed twice and analysed immediately by flow cytometry. Threshold gating was used to determine the percentage of GFP-positive cells (Fig. 4a), which is indicative of the adherence of the bacteria to the A549 cells. PAO1 readily adhered to A549 cells, and as expected, deletion of *rpoN* substantially decreased adhesion (Fig. 4b, c). In the absence of *lptF*, adhesion to A549 cells decreased to 71.5 % ± 7.9 compared with PAO1 (Fig. 4b). These data suggest that *lptF* is required in PAO1 for maximal adhesion to A549 cells. LptF is highly upregulated in PAO1*kinB*::*aacC1*, which produces copious amounts of alginate (Fig. 1). However, PAO1*kinB*::*aacC1* adherence is reduced compared with PAO1 (Fig. 4b).

Table 3. Altered sensitivity of *P. aeruginosa* strains to hydrogen peroxide and hypochlorite

Strain*	Growth inhibition zone [mean radius ± SEM (mm)]†	
	10 % Hydrogen peroxide	6 % Hypochlorite
PAO1	9.7 ± 0.3	8.2 ± 0.4
PAO1Δ*lptF*	7.8 ± 0.2‡	10.7 ± 0.3§
PAO1Δ*algU*	12.0 ± 0.8	12.2 ± 0.2§

*PAO1 is a wild-type nonmucoid *P. aeruginosa* strain. For statistical analysis, isogenic strains were compared with PAO1.

†Sensitivity to killing by hydrogen peroxide and hypochlorite is expressed as zones of inhibited growth around filter disks impregnated with 10 µl of the solution indicated. Zones were measured after 24 h at 37 °C and are the means of three experiments.

‡Indicates that the strain was more resistant to killing than PAO1. Student's unpaired *t* test; two tailed ($P<0.05$).

§Indicates that the strain was more susceptible to killing than PAO1. Student's unpaired *t* test; two tailed ($P<0.05$).

DISCUSSION

P. aeruginosa pulmonary infections cause detrimental and irreversible damage to the CF patient. Alginate overproduction by *P. aeruginosa* occurs in response to the conditions of the CF lung. Lipotoxins are co-expressed with alginate genes. We observed that LptF was the only lipotoxin identified in MudPIT proteome analysis of the mucoid *kinB* strain. We also confirmed that LptF is an outer membrane protein (Fig. 2). LptF upregulation in mucoid cells (Firoved *et al.*, 2002, 2004; Firoved & Deretic, 2003; Wood *et al.*, 2006) suggests that LptF has roles in establishment of mucoid biofilms. Collectively, these data warranted further investigation.

We first examined *lptF* expression and confirmed that it is controlled by AlgU (Fig. 3). P_{lptEF} expression is upregulated in both nonmucoid and mucoid CF isolates (Fig. 3). Since *lptF* expression is dependent upon AlgU, and the *lptEF* promoter does not have an AlgU consensus sequence, there are two possible mechanisms for AlgU-dependent expression of *lptF*. Either AlgU drives transcription of LptF directly or it does so indirectly through expression of another transcription factor. Ultimately, LptF expression depends on the alginate master regulator AlgU.

To further characterize LptF, we generated an unmarked deletion mutant for downstream analysis. PAO1Δ*lptF* was assayed for survival against killing by hydrogen peroxide and hypochlorite. Our data suggest that LptF has a role in resistance against hypochlorite; however, the deletion of *lptF* causes increased resistance to hydrogen peroxide. This difference suggests that LptF protection is specific for certain niches or environments. In the CF lung, *P. aeruginosa* forms biofilms (Singh *et al.*, 2000), and colonization of the CF lung occurs first by nonmucoid strains (Burns *et al.*, 2001). These early colonizing strains then establish an immunostimulatory phase of infection

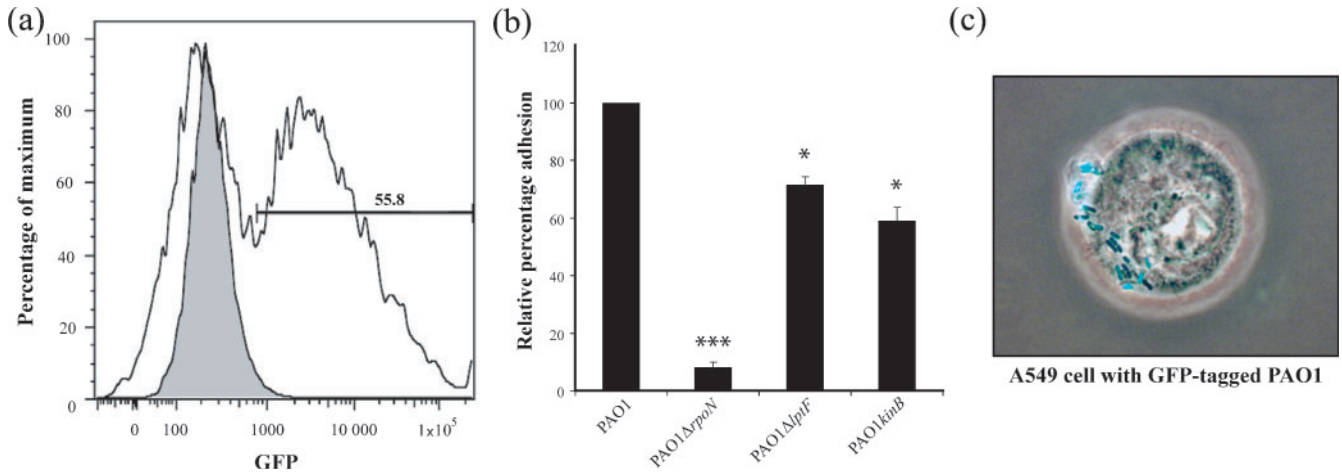

Fig. 4. Adherence of *P. aeruginosa* to A549 lung epithelial cells. (a) Flow cytometry threshold gating of GFP-positive A549 cells. GFP-positive A549 cells were determined by threshold gating based on A549 cells without GFP-tagged *P. aeruginosa* cells. Of A549 cells, 55.8 % were positive for GFP-tagged PAO1. (b) Relative percentage adhesion of GFP-tagged *P. aeruginosa* strains to A549 cells. Values were normalized to the observed amount of GFP-tagged PAO1 adhesion to A549 cells. Experiments were performed in triplicate with three independent studies. Student's *t* test was performed for comparison of each of the isogenic mutants with PAO1. Asterisks indicate significant differences (***$P<0.00001$; *$P<0.001$). (c) A549 lung epithelial cell with adherent GFP-tagged (pMRPQ-1) PAO1 cells.

(Feldman *et al.*, 1998), resulting in increased inflammation. Mucoid biofilm conversion occurs due to mutations in the anti-sigma factor *mucA* (Martin *et al.*, 1993). Furthermore, with conversion to mucoidy comes upregulation of the stimulatory lipotoxins. Lipotoxins such as LptF stimulate inflammatory responses through TLR2 (Firoved *et al.*, 2004). Motile strains with flagella activate TLR5 recognition (Zhang *et al.*, 2005). Therefore, immune responses due to the presence of *P. aeruginosa* occur starting with the initial infection and continue through the rest of the CF patient's life due to inability to eradicate *P. aeruginosa* from the CF lung (Costerton, 2001).

Our data show that deletion of *rpoN*, which controls expression of flagella and pili (Ishimoto & Lory, 1989; Totten *et al.*, 1990), severely attenuated adhesion to A549 epithelial cells. Flagella and pili are both required for early biofilm formation (O'Toole & Kolter, 1998). PAO1Δ*lptF*, like PAO1, is motile (data not shown), and adheres to A549 cells to a lesser extent than PAO1. This suggests that LptF is likely to be recognized independently by epithelial cells, which may allow *P. aeruginosa* to attach to the tissue surface. Alternatively, the loss of LptF could result in blockage of transport of extracellular factors necessary to adhere to epithelial cells. PAO1*kinB*::*aacC1* adhered to A549 cells less than PAO1. Although PAO1*kinB*::*aacC1* produces alginate, there are other factors, such as repression of motility factors by AlgU (Baynham *et al.*, 2006; Tart *et al.*, 2006), that could affect adherence.

Lipotoxins such as LptF likely not only cause the inflammatory response and detrimental tissue damage in the CF lung, but also protect *P. aeruginosa* and preserve the biofilm. MudPIT proteomic analysis of the mucoid *kinB* mutant suggests that AlgU-dependent LptF is the major lipotoxin expressed in the mucoid strain proteome (Table 2). Unlike most of the other lipotoxins, LptF is an outer membrane protein (Fig. 2). We also observed that *lptF* expression was upregulated in CF isolates (Fig. 3), and LptF may have roles in protection (Table 3) and adhesion to lung epithelia (Fig. 4). Since LptF is highly expressed in mucoid strains that cause chronic infection, it will be interesting to use synthetic peptides to further analyse the activation of the specific inflammatory response to LptF. Recently, azithromycin has been shown to downregulate expression of lipotoxins LptF, LptE, LptD, SlyB, OsmE and PA1323 (Skindersoe *et al.*, 2008). Also, other macrolides have been shown to alter biofilms (Wozniak & Keyser, 2004). Therefore, therapeutic treatments with azithromycin may be able to lessen the potential respiratory tract damage caused by *P. aeruginosa* lipotoxins such as LptF.

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