The exopolysaccharide Psl contributes to biofilm structure and antibiotic tolerance and may play a role in the failure to eradicate *Pseudomonas aeruginosa* biofilms in children with cystic fibrosis. The study objective was to determine whether there were any differences in Psl in *P. aeruginosa* isolates that were successfully eradicated compared to those that persisted, despite inhaled tobramycin treatment, in children with CF. Initial *P. aeruginosa* isolates were collected from children with CF undergoing eradication treatment, grown as biofilms and labeled with 3 anti-Psl monoclonal antibodies (Cam003/Psl0096, WapR001, WapR016) before confocal microscopy visualization. When grown as biofilms, *P. aeruginosa* isolates from children who failed antibiotic eradication therapy, had significantly increased Psl0096 binding compared to isolates from those who cleared *P. aeruginosa*. This was confirmed in *P. aeruginosa* isolates from the SickKids Eradication Cohort as well as the Early Pseudomonas Infection Control (EPIC) trial. Increased anti-Psl antibody binding was associated with bacterial aggregation and tobramycin tolerance. The biofilm matrix represents a potential therapeutic target to improve *P. aeruginosa* eradication treatment.

**ARTICLE**

**OPEN**

**The role of Psl in the failure to eradicate *Pseudomonas aeruginosa* biofilms in children with cystic fibrosis**

Amanda J. Morris1, Lindsay Jackson1, Yvonne CW Yau2, Courtney Reichhardt3, Trevor Beaudoin1, Stephanie Uwumarenogie1, Kevin M. Guttman1, P. Lynne Howell4,5, Matthew R. Parsek3, Lucas R. Hoffman6, Dao Nguyen7, Antonio DiGiandomenico8, David S. Guttman9, Daniel J. Wozniak10 and Valerie J. Waters1,11,12,13

The exopolysaccharide Psl contributes to biofilm structure and antibiotic tolerance and may play a role in the failure to eradicate *Pseudomonas aeruginosa* from cystic fibrosis (CF) airways. The study objective was to determine whether there were any differences in Psl in *P. aeruginosa* isolates that were successfully eradicated compared to those that persisted, despite inhaled tobramycin treatment, in children with CF. Initial *P. aeruginosa* isolates were collected from children with CF undergoing eradication treatment, grown as biofilms and labeled with 3 anti-Psl monoclonal antibodies (Cam003/Psl0096, WapR001, WapR016) before confocal microscopy visualization. When grown as biofilms, *P. aeruginosa* isolates from children who failed antibiotic eradication therapy, had significantly increased Psl0096 binding compared to isolates from those who cleared *P. aeruginosa*. This was confirmed in *P. aeruginosa* isolates from the SickKids Eradication Cohort as well as the Early Pseudomonas Infection Control (EPIC) trial. Increased anti-Psl antibody binding was associated with bacterial aggregation and tobramycin tolerance. The biofilm matrix represents a potential therapeutic target to improve *P. aeruginosa* eradication treatment.

**INTRODUCTION**

Cystic fibrosis (CF) is a genetic disease characterized by *Pseudomonas aeruginosa* pulmonary infection1. To prevent the detrimental outcomes associated with chronic infection, antimicrobial treatment is used to eradicate initial *P. aeruginosa* infection2–5. However, in 10–40% of cases, eradication therapy fails, with no clear superiority of one antibiotic regimen over another, and the reasons for this are not entirely understood6. In addition to antibiotic treatment, clearance of the organism from the airways depends on mucociliary action and immune-mediated mechanisms such as phagocytosis by neutrophils7,8. Studies that have examined outcomes of eradication therapy have not identified any host factors, such as gender or lung function, that are associated with failure to clear *P. aeruginosa*9,10. *P. aeruginosa* phenotypes characteristic of chronic pulmonary infection, such as mucoidy status, decreased motility and wrinkly colony morphology, have occasionally been identified as risk factors for failure of antibiotic eradication therapy11,12.

Using a collection of new onset *P. aeruginosa* isolates from children with CF undergoing antibiotic eradication treatment, we showed that Staphylococcal protein A (SpA) bound to the exopolysaccharide Psl in *P. aeruginosa* isolates that failed eradication therapy but bound much less in isolates successfully cleared13. This Psl-SpA interaction led to *P. aeruginosa* aggregation within biofilms and tolerance to high concentrations of tobramycin. These data suggest that, although the reasons for the failure of eradication therapy are likely multifactorial, Psl may be playing a role. Psl is a neutral repeating pentasaccharide that contributes to cell–cell and cell–substrate attachment adhesion, aggregation and biofilm formation in vitro14–19. Patients with invasive *P. aeruginosa* infections have serum antibodies against Psl, however, we do not know whether this occurs in CF patients20. Psl also protects *P. aeruginosa* from antimicrobials, including tobramycin and ciprofloxacin21, by forming a barrier matrix, and from activities of the innate immune system such as phagocytosis by neutrophils22–24. However, its contribution to the persistence of *P. aeruginosa* in the CF airways following inhaled antibiotic treatment is not known.

Therefore, the goals of this study were to examine Psl production and function in *P. aeruginosa* isolates that were successfully eradicated compared to those that persisted, despite inhaled tobramycin treatment, in the airways of children with CF. To do so, we used two sets of *P. aeruginosa* isolates, from the SickKids Eradication Cohort and the Early Pseudomonas Infection Control (EPIC) trial11,25. In addition, we used three separate anti-Psl antibodies (Cam003/Psl0096, WapR001 and WapR016), which recognize distinct epitopes and vary in their characteristics for promoting opsonization and phagocytosis and preventing epithelial cell binding20,26. We identified differences in Psl0096 binding between persistent and eradicated *P. aeruginosa* isolates with corresponding differences in bacterial aggregation and tobramycin tolerance.
RESULTS
Quantification of Psl production in eradicated and persistent P. aeruginosa isolates

Initial investigations focused on determining whether there were any differences in the quantity of secreted and cell-associated Psl produced by eradicated and persistent isolates from the complete SickKids collection (29 persistent, 63 eradicated isolates). Biofilms were grown and sonicated to disrupt cell aggregation and lyse the bacteria. The supernatant and lysate were then incubated with three separate anti-Psl monoclonal antibodies recognizing distinct epitopes (Cam003/Psl0096, WapR001 and WapR016). Figure 1 illustrates that there was no difference in the amount of Psl detected via densitometric analysis of signal intensity between the eradicated (N = 63 isolates) and persistent (N = 29 isolates) isolates, either in the supernatant or lysate components.

Comparative anti-Psl antibody binding to in vitro grown biofilms of eradicated and persistent isolates

In order to visualize Psl within intact biofilms (without sonication), P. aeruginosa were grown as biofilms and then stained with fluorescently labeled anti-Psl antibodies. For these detailed experiments, seven eradicated and seven persistent isolates from the SickKids collection were used. These isolates were chosen to represent 1 isolate per patient and to have similarities in other phenotypic characteristics which may influence eradication success, such as motility, mucoid status, and planktonic tobramycin minimum inhibitory concentrations (MICs) between the eradicated and persistent groups of isolates, as previously published.

Representative images of an eradicated and a persistent isolate are shown in Fig. 2, demonstrating increased anti-Psl antibody binding in the persistent P. aeruginosa biofilm. Figure 3 depicts the volume of anti-Psl antibody staining (per 100,000 µm² of biofilm) in all persistent versus eradicated isolates. In the SickKids collection, there was significantly more anti-Psl antibody binding of Psl0096 (Fig. 3A) and WapR001 (Fig. 3B) antibodies in persistent compared to eradicated P. aeruginosa.

To validate these findings in a second dataset, we repeated the same experiments using seven persistent and seven eradicated isolates (from separate patients) from the EPIC cohort. Although there were no differences between groups using the WapR001 and WapR016 antibodies, Psl0096 bound significantly more in the persistent P. aeruginosa isolates compared to the eradicated ones, as seen in the SickKids cohort (Fig. 3).

Effects of anti-Psl antibody binding in the presence of tobramycin in P. aeruginosa biofilms

We previously noted that Spa-Psl binding led to bacterial aggregation and tobramycin tolerance in persistent isolates. To determine whether this finding was a Psl specific phenomenon and would occur with the increased binding of Psl0096 to persistent isolates, we investigated the effects of anti-Psl antibody binding on tobramycin resistance in P. aeruginosa biofilms. Biofilms of the SickKids seven eradicated and seven persistent isolates were grown for 24 h and then exposed to Psl0096 followed by tobramycin for an additional 24 h. Tobramycin significantly reduced the amount of biofilm volume for the eradicated isolates in media alone as well as in the presence of IgG and Psl0096 (Fig. 4A). However, for the persistent isolates, tobramycin significantly reduced biofilm volume only in the media and IgG control conditions; in the presence of Psl0096, there was no significant reduction in P. aeruginosa biovolume. Furthermore, in comparison to the IgG controls (to account for the non-specific effects of antibody binding), persistent isolates also had significant reductions in biofilm surface to volume ratio in the presence of Psl0096, indicating increased aggregation; increased aggregation was not observed in the eradicated group (Fig. 4B).

Figure 4C shows representative images of a persistent and an eradicated isolate.

We then used an ATP assay to investigate the metabolic activity of the remaining P. aeruginosa in the presence of tobramycin. For the eradicated isolates, tobramycin again significantly reduced ATP production under all conditions (Fig. 5). In the persistent
group, tobramycin significantly reduced ATP production only in the control conditions and not in the presence of Psl0096. When colony-forming units (CFUs) were measured under the same conditions, however, tobramycin was able to significantly reduce the colony count in both the persistent and eradicated groups (Supplemental Fig. 1).

Psl polymorphism among eradicated and persistent P. aeruginosa isolates
To determine whether there were any underlying genetic differences among the seven persistent and seven eradicated SickKids isolates, we examined the 14 locus Psl region from pslA – pslN and identified all non-synonymous (missense) mutations. These genes total to 17,657 bp and span the region from 2,453,667 to 2,472,076 on the PAO1 genome (note that some psl genes overlap). There was an average of 0.81 ± 0.549 (stddev) non-synonymous polymorphisms per strain across all 14 loci, with no variation found in pslD, and only one polymorphism found in both pslA and pslG. The most polymorphic locus was pslB with an average of 1.57 non-synonymous polymorphisms per strain. There were no statistically significant differences in the number of polymorphisms per gene (or when all genes were considered together) between eradicated and persistent strains (Supplemental Fig. 2).

CdrA expression in eradicated and persistent P. aeruginosa isolates
Given that CdrA is a Psl associated matrix protein that plays a significant role in aggregation, we investigated the expression of CdrA in the 21 persistent and 25 eradicated SickKids isolates grown as biofilms. Supplemental Fig. 3 shows that CdrA expression was detected (particularly in the supernatant fraction) in a similar number of persistent compared to eradicated isolates. Size differences in CdrA were observed between isolates in the western blot analysis. This is consistent with past observations of P. aeruginosa isolates.

DISCUSSION
This study demonstrated that there was increased binding of anti-Psl antibodies in P. aeruginosa isolates (when grown as biofilms) from CF patients, from both the SickKids and EPIC cohort, who failed antibiotic eradication therapy compared to those who did not; anti-Psl antibody binding led to the aggregate formation in biofilms and tobramycin tolerance.

To our knowledge, this is the largest study to examine the specific contribution of Psl in the failure to eradicate P. aeruginosa with antibiotic therapy in children with CF. Psl is involved in early bacterial surface attachment and is an important structural component of the extracellular matrix of biofilms. Psl production can also confer resistance to antibiotics such as colistin, tobramycin, and ciprofloxacin, likely through a protective barrier effect. Furthermore, Psl prevents complement deposition and opsonization, inhibiting bacterial killing by phagocytes. In vitro studies of Psl have included P. aeruginosa strains from CF patients with chronic infection but not from those with early infection. It is important to understand the significance of Psl in initial P. aeruginosa colonization which is more amenable to antibiotic treatment and clearance than chronic infection.

In our study, we noted differential binding of anti-Psl antibodies to biofilm cells of eradicated versus persistent isolates. Monoclonal antibodies to the Class I epitope (Cam003 or its affinity optimized derivative, Psl0096) were previously found to be the most active in promoting opsonophagocytic killing, in preventing P. aeruginosa from binding to epithelial cells and preferentially bind to the surface of in vitro grown biofilms. We also noted that mAb Psl0096 was the most discriminatory mAb in terms of binding between persistent and eradicated isolates, both for the SickKids and EPIC cohort. There were no significant differences in the number of polymorphisms in Psl genes between the groups to explain our finding. Furthermore, the amount of Psl as measured by dot blot analysis (using Psl0096) was similar between both groups. However, when P. aeruginosa was allowed to form a mature biofilm, undisturbed by sonication or processing, the increased affinity to Psl0096 was demonstrated. It is possible that persistent isolates grown as biofilms produce more surface Psl with a conformation that allows Psl0096 to bind more avidly than do eradicated isolates. In a screening study using synthetic oligosaccharides, Class I mAbs (Cam003/Psl0096) did not bind any of the oligosaccharides, suggesting the presence of a Psl-associated functional group or modification that is sensitive to experimental conditions such as alkaline exposure.
with anti-Psl antibodies was simply an effect of adding antibodies as the aggregation seen in our study was Psl specific. Antibody depletion experiments demonstrated by our collaborators in a study using immunohistochemical techniques to visualize CF sputum. It is not known what the specific trigger for this aggregation may be in vivo; simply growing the persistent isolates as biofilms did not reveal persistence in the presence of tobramycin, suggesting that the ability to form biofilms alone may not fully explain persistence in the CF airways. We previously showed that Staphylococcal protein A (SpA) can bind to Psl in our persistent P. aeruginosa strains leading to aggregation and tobramycin tolerance. However, this aggregative phenotype is not dependent on SpA. The presence of P. aeruginosa aggregates expressing Psl in the airways of a CF patient with chronic infection has been demonstrated by our collaborators in a study using immunohistochemical techniques to visualize CF sputum. It is not known whether anti-Psl antibodies can be found in the sputum of CF patients colonized with Psl. In conclusion, we demonstrated that Psl is important in the persistence of P. aeruginosa infection in most children with CF. In addition, we do not have the data in either cohort to confirm that failure of eradication treatment was due to the persistence of the same strain of P. aeruginosa, as these were clinical isolates and genotyping is not done routinely in the clinical microbiology laboratory. We focused our study on the role of Psl solely within P. aeruginosa biofilms but have not included potential contribution of host factors such as neutrophils, which clearly interact with Psl. Finally, we visualized host-derived polymers at the site of chronic infections may physically connect bacterial cells via a bridging mechanism leading to Psl mediated aggregation.

This study had several limitations. The SickKids Eradication cohort was limited to children with CF who could produce sputum and the study defined eradication based on an early time point (1 week post treatment). To make our findings more generalizable, we validated our results using a second isolate collection, from the EPIC cohort, that was obtained from oropharyngeal swabs, and for which eradication was defined at the 3 month post treatment follow up visit. Thus, our data suggest that differences in Psl are important in the persistence of P. aeruginosa infection in most children with CF. In addition, we do not have the data in either cohort to confirm that failure of eradication treatment was due to the persistence of the same strain of P. aeruginosa, as these were clinical isolates and genotyping is not done routinely in the clinical microbiology laboratory. We focused our study on the role of Psl solely within P. aeruginosa biofilms but have not included the potential contribution of host factors such as neutrophils, which clearly interact with Psl. Finally, we visualized P. aeruginosa biofilms in vitro but have not yet confirmed our study findings in vivo, directly in the sputum of children with CF; this is the focus of a currently ongoing observational study.

In conclusion, we demonstrated that P. aeruginosa isolates that fail to be cleared from the airways of children with CF following inhaled tobramycin therapy, have increased binding to anti-Psl antibodies when grown as biofilms, leading to aggregation and tobramycin tolerance. These components of the biofilm matrix represent possible therapeutic targets to improve the success of early P. aeruginosa eradication treatment.
METHODS

P. aeruginosa isolate collections

The SickKids cohort consisted of clinical P. aeruginosa isolates (N = 92) from sputum samples obtained from 67 pediatric CF patients undergoing eradication treatment with inhaled tobramycin for new-onset P. aeruginosa infection at SickKids (Toronto, Canada) from 2011 to 2018 (ongoing). New-onset infection was defined as a sputum culture positive for P. aeruginosa with at least 3 P. aeruginosa negative respiratory tract cultures in the prior 12 months. Eradication was defined based on a P. aeruginosa negative culture of the first respiratory tract specimen taken after the end of inhaled tobramycin therapy (1 week post end of
A. The mean biofilm volume (measured in µm³) for each isolate (done in triplicate) was calculated and the mean of all isolates was plotted with standard error of the mean (SEM). Comparisons with and without tobramycin treatment were performed for each condition using non-parametric Mann–Whitney test \(*p < 0.001, \ast \ast p < 0.01, \ast \ast \ast p < 0.05, \) ns not significant. B In the presence of tobramycin, aggregation was measured using a live-cell stain to visualize the biofilm. The mean biofilm surface to volume ratio (µm²/µm³) was calculated for each isolate (done in triplicate) and the mean of all isolates was plotted with standard error of the mean (SEM). Comparisons to IgG control were performed for each condition using non-parametric Mann–Whitney test \(*p < 0.001, \ast \ast p < 0.01, \ast \ast \ast p < 0.05, \) ns not significant. C Representative images are shown for a persistent (Pa375) and eradicated (Pa558) isolate.

**Fig. 4** SickKids *P. aeruginosa* isolates (N = 7 persistent; N = 7 eradicated) were grown as biofilms for 24 h after which antibodies (LB control, IgG or anti-Psl 0096) and tobramycin 1000 µg/ml (or LB alone) were added for the following 24 h before imaging was performed.

The mean biofilm volume (measured in µm³) for each isolate (done in triplicate) was calculated and the mean of all isolates was plotted with standard error of the mean (SEM). Comparisons with and without tobramycin treatment were performed for each condition using non-parametric Mann–Whitney test \(*p < 0.001, \ast \ast p < 0.01, \ast \ast \ast p < 0.05, \) ns not significant. B In the presence of tobramycin, aggregation was measured using a live-cell stain to visualize the biofilm. The mean biofilm surface to volume ratio (µm²/µm³) was calculated for each isolate (done in triplicate) and the mean of all isolates was plotted with standard error of the mean (SEM). Comparisons to IgG control were performed for each condition using non-parametric Mann–Whitney test \(*p < 0.001, \ast \ast p < 0.01, \ast \ast \ast p < 0.05, \) ns not significant. C Representative images are shown for a persistent (Pa375) and eradicated (Pa558) isolate.

**Fig. 5** SickKids *P. aeruginosa* isolates (N = 7 persistent; N = 7 eradicated) were grown as biofilms for 24 h after which antibodies (LB control, IgG or anti-Psl 0096) and tobramycin (or LB alone) were added for the following 24 h before ATP was measured. The mean ATP activity (measured in Relative Light Units (RLU) \(\times 10^7\) for each isolate (done in triplicate) was calculated and the mean of all isolates was plotted with standard error of the mean (SEM). Comparisons with and without tobramycin treatment were performed for each condition using non-parametric Mann–Whitney test \(*p < 0.001, \ast \ast p < 0.01, \ast \ast \ast p < 0.05, \) ns not significant.

In addition, we also used the larger *P. aeruginosa* isolate collection \(N = 194\) from the EPIC trial of early eradication (2004–2009) to compare isolate density and biofilm properties.

### Psl dot blot analysis

*P. aeruginosa* isolates, PAO1 and Δpsl (Psl deletion strain [PAO1Δpsl]) were grown overnight in lysogeny broth (LB) with shaking (225 rpm). The overnight culture was diluted 1:100 and grown for 3 h at 37 °C to obtain an OD of 0.1 at 600 nm (early log phase). Of this culture, 1 mL was transferred to a Sarstedt 96-well Tissue Culture plate (Thermo Fisher Scientific, Mississauga, ON) and grown for 48 h at 37 °C with LB replacement after the first 24 h. The wells were washed 1x with phosphate-buffered saline (PBS) and 200 µL of PBS was added back into the wells. The cell-attached fraction of the cells was disrupted and the optical density (OD) of the cell suspension in PBS was normalized to 0.2 at 600 nm, followed by centrifugation (3000 x g, 4 °C, 15 min).

The centrifuged product consisted of a cell pellet and supernatant. The cell pellet was re-suspended in 500 µL of 0.9 % NaCl and lysed by bath sonication (VWR Ultrasonic S0D, 5 x 2 min) with 2 min cooling on ice after each interval. The supernatant (pre-lysis) and lysate (post-lysis) were loaded into the Minifold II Slot-Blot manifold system (50 µL each) and spotted on a nitrocellulose membrane. The blots were air-dried and blocked with 2% bovine serum albumin (BSA), followed by 24 h incubation at 4 °C. The blots were rinsed 3x with 1x tris buffered saline with Tween 20 (TBST) and incubated with 1:5000 primary anti-Psl antibody in 1x TBST for 1 h at room temperature (RT). The primary antibody was decanted and the wells were rinsed 3x with 1x TBST, then incubated with 1:3000 secondary goat anti-rabbit IgG antibody (Bio-Rad, Mississauga, ON) for 1 h at RT. The secondary antibody was decanted and the blots were rinsed 3x with 1x TBST, after which 50 µL of 1x TBST was added back onto the blots to prevent drying. Bound antibody was detected using a SuperSignal West Atto chemiluminescent substrate (Thermo Fisher Scientific, Mississauga, ON). Blots were imaged using the ChemiDoc Imaging System and variations in signal intensities were quantified with signal accumulation mode. The mean intensity value for each isolate (done in triplicate) was calculated as arbitrary units (a.u.).

### Confocal imaging of *P. aeruginosa* biofilms

*P. aeruginosa* isolates were grown in Nunc Lab-Tek II, 8-chambered cover glass slides (Thermo Fisher Scientific, Mississauga, ON) as previously described. *P. aeruginosa* was grown in LB overnight at 37 °C on a shaker (225 rpm). The overnight culture was diluted 1:100 and grown to an OD of 0.1 at 600 nm (early log phase), after which 220 µL of the culture was seeded into an 8-chambered cover glass slide (Thermo Fisher Scientific, Mississauga, ON) and incubated at 37°C.

To measure antibody binding alone, after 48 h of growth, media was removed and fluorescently labeled anti-Psl antibody (56 µg/mL) was added for 90 min. Media was then removed from the wells and 200 µL of Syto-9™ live-cell fluorescent stain (Thermo Fisher Scientific) was added and incubated for 45 min at RT. The wells were then gently washed 2x with LB and 200 µL of LB was added back into the wells prior to confocal laser scanning microscopy (CLSM). This procedure was repeated in triplicates for each *P. aeruginosa* isolate.

To measure antibody binding in the presence of tobramycin, after 24 h of growth, media was removed and replaced with fluorescently labeled anti-Psl antibody (56 µg/mL) for 1 h at RT. Then, either 100 µL of LB alone or LB with tobramycin (final concentration 100 µg/mL) was added back into the wells and 200 µL of Syto-9™ live-cell fluorescent stain (Thermo Fisher Scientific) was added and incubated for 45 min at RT. The wells were then gently washed 2x with LB and 200 µL of LB was added back into the wells prior to CLSM. This procedure was repeated in triplicates for each *P. aeruginosa* isolate.

Images were acquired using a Quorum Wave FX Borealis Spinning Disk Confocal Microscope, based on a Yokohama CSU-10 scan head. Fluorescently labeled (red) anti-Psl antibody and live cell (green) stains were excited using 561 and 491 nm excitation lines, respectively, and visualized with a 25x/0.80 Zeiss water immersion lens, coupled to a 1.6x magnification coupler. Image acquisition was performed using the Quorum Volocity 6.3 software. A total of 6 z-stack images were acquired per well in increments of 0.3 µm. Anti-Psl binding within *P. aeruginosa* biofilms was calculated using Volocity software to quantify total volume of voxels in the green and red channels. Surface-to-biovolume ratio, as a measure of *P. aeruginosa* aggregation, was quantified as previously described, using Comstat2 software as a plugin to ImageJ. All images are included in the Supplemental materials as Supplemental Fig. 4.
Bacterial counts of *P. aeruginosa* biofilms

Colony-forming units (CFU) counts were determined by growing *P. aeruginosa* biofilms in cation-adjusted Mueller Hinton broth (CAMHB) in 8-chambered cover glass slides (Thermo Fisher Scientific, Mississauga, ON), under the same conditions previously described herein for confocal imaging. After 24 h growth with subsequent 24 h growth with exposure to antibodies and tobramycin, media was removed, washed 2x with PBS, and 500 µL of fresh PBS was added into the wells. The cell-attached fraction of the wells were disrupted and cell suspensions were serial diluted, then plated on blood agar. Plates were grown for 24 h at 37 °C and CFUs were counted. This procedure was repeated in triplicates for each *P. aeruginosa* isolate, reporting mean CFU counts (log transformed) as previously described.

**ATP assays of *P. aeruginosa* biofilms**

Cell metabolic activity was assessed using a modified ATP assay method for biofilms.[36,37] In brief, *P. aeruginosa* was grown overnight in CAMHB (Sigma-Aldrich, Oakville, ON) at 37 °C with shaking (225 rpm). The overnight culture was diluted 1:100 and grown to an OD of 0.1 at 600 nm. Of this log phase culture, 220 µL was added to 8 wells of a white Greiner Medium Binding 96-well plate (Sigma-Aldrich, Oakville, ON) and incubated for 24 h at 37 °C. After 24 h, media was removed and the wells were washed 2x with CAMHB. Subsequently, 100 µL of anti-Psl antibody (56 µg/mL) was added to designated wells and incubated for 1 h at RT. Either 100 µL of CAMHB alone or CAMHB with tobramycin (final concentration 1000 µg/mL) was added to designated wells and incubated for 24 h at 37 °C. Media was then removed and the wells were washed 3x with CAMHB. Finally, to measure ATP in the cell-attached fraction of the wells, 100 µL of Bac Titer-Glo™ reagent (Promega, Madison, WI) was added. The cell-attached fraction of the wells was disrupted and the plate was gently mixed on an orbital shaker for 10 min prior to luminescence reading as per the manufacturer’s protocol. This procedure was repeated in triplicates for each *P. aeruginosa* isolate.

**Psl gene polymorphisms**

To determine whether there were mutational differences in the Psl genes of persistent versus eradicated isolates, we analyzed the gene region from 2453667-2472076 spanned by the 14 psl genes, pslA, pslB, pslC, pslD, pslE, pslF, pslG, pslH, pslI, pslJ, pslK, pslM, pslN for polymorphisms in each of the seven persistent and seven eradicated SickKids *P. aeruginosa* strains. Whole genome sequence data for each of the 14 Psl genes were previously collected via DNA extraction and sequencing[13]. The data for each strain was then mapped to PAO1 as a reference, and single nucleotide polymorphisms called non-synonymous (missense) mutations found in each psl locus and compared polymorphism levels between persistent and eradicated strains on a per gene (and across all genes) basis and for the entire Psl region. All data are available through Bioproject PRJNAS56419 with the following Genbank accessions: JAGHM W000000000 (Pa50), JAGHMV000000000 (Pa263), JAGHMU000000000 (Pa342), JAGHM R000000000 (Pa375), JAGHMQ000000000 (Pa380), JAGHMP000000000 (Pa404), JAGHMO000000000 (Pa505), JAGHMN000000000 (Pa531), JAGHM L000000000 (Pa558), JAGHML000000000 (Pa565), JAGHMK000000000 (Pa580), JAGHMN000000000 (Pa549).

**Anti-CdrA western blot analysis of static biofilms**

Sample preparation for western blot analysis was performed as previously described.[38] Briefly, static biofilms were cultured in 6-well plates using TSA for 24 h at 30 °C. Biofilms were then harvested by passage through an 18-gauge syringe. Biofilms were normalized by OD 600 nm. Biofilms were separated by centrifugation into supernatant and cellular fractions. Samples were then analyzed by western blot analysis as previously described[39] using an anti-CdrA antibody raised against CADGKTRKVYG-DADPS (Genscript). This amino acid sequence is a conserved stretch of amino acids in the C-terminal region of the protein.

**Statistical analysis and institutional approvals**

All statistical analyses were done using GraphPad 5.0. Continuous variables were compared using non-parametric Mann-Whitney test; p-value of <0.05 was considered significant. This study was approved by the SickKids Research Ethics Board (REB#1000065841) with waiver of consent due to de-identified nature of bacterial isolates.

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**DATA AVAILABILITY**

The data that support the findings of this study are available from the corresponding author upon reasonable request. All data are available through Bioproject PRJNAS56419 for the above mentioned Genbank accessions.

Received: 4 February 2021; Accepted: 1 July 2021; Published online: 04 August 2021

**REFERENCES**

1. Cohen, T. S. & Prince, A. Cystic fibrosis: a mucosal immunodeficiency syndrome. *Nat. Med.* 18, 509–519 (2012).
2. Emerson, J., Rosenfeld, M., McMamara, S., Ramsey, B. & Gibson, R. L. *Pseudomonas aeruginosa* and other predictors of mortality and morbidity in young children with cystic fibrosis. *Pediatr. Pulmonol.* 34, 91–100 (2002).
3. Henry, R. L., Mellis, C. M. & Petrovic, L. Mucoid *Pseudomonas aeruginosa* is a marker of poor survival in cystic fibrosis. *Pediatr. Pulmonol.* 12, 158–161 (1992).
4. Konstan, M. W. et al. Risk factors for rate of decline in forced expiratory volume in one second in children and adolescents with cystic fibrosis. *J. Pediatr.* 151, 134–139, 139 e131 (2007).
5. Paten, F., Doring, G. & Nikolazik, W. H. Effect of inhaled tobramycin on early *Pseudomonas aeruginosa* colonisation in patients with cystic fibrosis. *Lancet* 358, 983–984 (2001).
6. Schelschlaeete, P., Haerynck, F., Van daele, S., Deseyne, S. & De Baets, F. Eradication therapy for *Pseudomonas aeruginosa* colonization episodes in cystic fibrosis patients not chronically colonized by *P. aeruginosa*. *J. Cyst. Fibros.* 1, 8–13 (2013).
7. Knowles, M. R. & Boucher, R. C. Mucus clearance as a primary innate defense mechanism for mammalian airways. *J. Clin. Invest.* 109, 571–577 (2002).
8. Williams, B. J., Dehnboest, J. & Blackwell, T. S. *Pseudomonas aeruginosa*: host defence in lung diseases. *Respirology* 15, 1037–1056 (2010).
9. Stanosevic, S., Waters, V., Mathew, J. L., Taylor, L. & Ratjen, F. Effectiveness of inhaled tobramycin in eradicating *Pseudomonas aeruginosa* in children with cystic fibrosis. *J. Cyst. Fibros.* 13, 172–178 (2014).
10. Mayer-Hamblett, N. et al. Initial *Pseudomonas aeruginosa* treatment failure is associated with exacerbations in cystic fibrosis. *Pediatr. Pulmonol.* 47, 125–134 (2012).
11. Vidya, P. et al. Chronic infection phenotypes of *Pseudomonas aeruginosa* are associated with failure of eradication in children with cystic fibrosis. *Eur. J. Clin. Microbiol. Infect. Dis.* 35, 67–74 (2016).
12. Mayer-Hamblett, N. et al. *Pseudomonas aeruginosa* phenotypes associated with eradication failure in children with cystic fibrosis. *Clin. Infect. Dis.* 59, 624–631 (2014).
13. Beaudoin, T. et al. *Staphylococcus aureus* interaction with *Pseudomonas aeruginosa* biofilm enhances tobramycin resistance. *NPJ Biofilms Microbiomes* 3, 25 (2017).
14. Jackson, K. D., Starkey, M., Kremmer, S., Parsen, M. R. & Wozniak, D. J. Identification of psl, a locus encoding a potential exopolysaccharide that is essential for antibiotic defence in *Pseudomonas aeruginosa*. *Mucosal Immunol.* 5, 158–167 (2012).
15. Ma, L., Lu, H., Sprinkle, A., Parsen, M. R. & Wozniak, D. J. *Pseudomonas aeruginosa* Psl is a galactose- and mannose-rich exopolysaccharide. *J. Bacteriol.* 189, 8353–8356 (2007).
16. Byrd, M. S. 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.* 73, 622–638 (2009).
17. Yang, L. et al. Distinct roles of extracellular polymeric substances in *Pseudomonas aeruginosa* biofilm development. *Environ. Microbiol.* 13, 1705–1717 (2011).
18. Chew, S. C. et al. Dynamic remodeling of microbial biofilms by functionally distinct exopolysaccharides. *MBio* 5, e01536–01514 (2014).
19. Ma, L. et al. Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. *PLoS Pathog.* 5, e1000354 (2009).
20. DiGiandomenico, A. et al. Identification of broadly protective human antibodies to *Pseudomonas aeruginosa* exopolysaccharide Psl by phenotypic screening. *J. Exp. Med.* 209, 1273–1287 (2012).
21. Billings, N. et al. The extracellular matrix Component Psl provides fast-acting antibiotic defense in *Pseudomonas aeruginosa* biofilms. *PLoS Pathog.* 9, e100326 (2013).
22. Thanabalasuriar, A. et al. Bispecific antibody targets multiple Pseudomonas aeruginosa evasion mechanisms in the lung vasculature. J. Clin. Investig. 127, 2249–2261 (2017).

23. Mishra, M. et al. Pseudomonas aeruginosa Psl polysaccharide reduces neutrophil phagocytosis and the oxidative response by limiting complement-mediated opsonization. Cell Microbiol. 14, 95–106 (2012).

24. Rybtke, M., Jensen, P. O., Nielsen, C. H. & Toller-Nielsen, T. The extracellular polysaccharide matrix of Pseudomonas aeruginosa biofilms is a determinant of polymorphonuclear leukocyte responses. Infect. Immun. https://doi.org/10.1128/IAI.00631-20 (2020).

25. Treggiari, M. et al. Comparative efficacy and safety of 4 randomized regimens to treat early Pseudomonas aeruginosa infection in children with cystic fibrosis. Arch. Pediatr. Adolesc. Med. 165, 847–856 (2011).

26. Ray, V. A. et al. Anti-Psl targeting of Pseudomonas aeruginosa biofilms for neutrophil-mediated disruption. Sci. Rep. 7, 16065 (2017).

27. Reichhardt, C. et al. The versatile Pseudomonas aeruginosa biofilm matrix protein CdrA promotes aggregation through different extracellular exopolysaccharide interactions. J. Bacteriol. https://doi.org/10.1128/JB.00216-20 (2020).

28. Byrd, M. S., Pang, B., Mishra, M., Swords, W. E. & Wozniak, D. J. The Pseudomonas aeruginosa exopolysaccharide Psl facilitates surface adherence and NF-kappaB activation in A549 cells. mbio https://doi.org/10.1128/mBio.00140-10 (2010).

29. Zhao, K. et al. Psl trails guide exploration and microcolony formation in Pseudomonas aeruginosa biofilms. Nature 497, 388–391 (2013).

30. Jones, C. J. & Wozniak, D. J. Psl produced by mucoid Pseudomonas aeruginosa contributes to the establishment of biofilms and immune evasion. MBio https://doi.org/10.1128/mBio.00864-17 (2017).

31. Colvin, K. M. et al. The Pel and Psl polysaccharides provide Pseudomonas aeruginosa structural redundancy within the biofilm matrix. Environ. Microbiol. 14, 1913–1928 (2012).

32. Huse, H. K. et al. Pseudomonas aeruginosa enhances production of a non-alginate exopolysaccharide during long-term colonization of the cystic fibrosis lung. PLoS ONE 8, e62621 (2013).

33. Li, H. et al. Epitope mapping of monoclonal antibodies using synthetic oligosaccharides uncovers novel aspects of immune recognition of the Psl exopolysaccharide of Pseudomonas aeruginosa. Chemistry 19, 17425–17431 (2013).

34. Ray, V. A. et al. Anti-Psl targeting of Pseudomonas aeruginosa biofilms for neutrophil-mediated disruption. Sci. Rep. 8, 9637 (2018).

35. Borlee, B. R. et al. Pseudomonas aeruginosa uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix. Mol. Microbiol. 75, 827–842 (2010).

36. Reichhardt, C., Wong, C., Passos da Silva, D., Wozniak, D. J. & Parsek, M. R. CdrA Interactions within the Pseudomonas aeruginosa biofilm matrix safeguard it from proteolysis and promote cellular packaging. MBio https://doi.org/10.1128/mBio.01376-18 (2018).

37. Staudinger, B. J. et al. Conditions associated with the cystic fibrosis defect promote chronic Pseudomonas aeruginosa infection. Am. J. Respir. Crit. Care Med. 189, 812–824 (2014).

38. Chmiel, J. F. et al. Antibiotic management of lung infections in cystic fibrosis. I. The microbiome, methicillin-resistant Staphylococcus aureus, gram-negative bacteria, and multiple infections. Ann. Am. Thorac. Soc. 11, 1120–1129 (2014).

39. Jennings, L. K. et al. Pseudomonas aeruginosa aggregates in cystic-fibrosis sputum produce exopolysaccharides that likely impede current therapies. Cell Rep. 34, 108782 (2021).

40. Lee, B. et al. Heterogeneity of biofilms formed by nonmucoid Pseudomonas aeruginosa isolates from patients with cystic fibrosis. J. Clin. Microbiol. 43, 5247–5255 (2005).

41. Caceres, S. M. et al. Enhanced in vitro formation and antibiotic resistance of nonattached Pseudomonas aeruginosa aggregates through incorporation of neutral polymer products. Antimicrob. Agents Chemother. 58, 6851–6860 (2014).

42. Phan, J., Gallagher, T., Oliver, A., England, W. E. & Whiteson, K. Fermentation products in the cystic fibrosis airways increase inflammation and dormant-associated expression profiles in a CF clinical isolate of Pseudomonas aeruginosa. FEMS Microbiol. Lett. https://doi.org/10.1093/femsle/fny082 (2018).

43. Secor, P. R., Michaels, L. A., Ratjen, A., Jennings, L. K. & Singh, P. K. Entropically driven aggregation of bacteria by host polymers promotes antibiotic tolerance in Pseudomonas aeruginosa. Proc. Natl Acad. Sci. USA 115, 10780–10785 (2018).

44. Strand, S. P., Várum, K. M. & Østgaard, K. Interactions between chitosans and bacterial suspensions: adsorption and flocculation. Colloids Surf. B Biointerfaces 27, 71–81 (2003).

45. Schwarz-Linek, J. et al. Polymer-induced phase separation in suspensions of bacteria. Europhys. Lett. 89, 68003 (2010).

46. Blanchard, A. C. et al. Effectiveness of a stepwise Pseudomonas aeruginosa eradication protocol in children with cystic fibrosis. J. Cyst. Fibros. 16, 395–400 (2017).

47. Kelly, K. A., Benedetti, Y., Yau, V., Waters, D. N. The Journal of Infectious Diseases. https://doi.org/10.1093/infdis/jia102 (2021).

48. Morris, A. J., Li, A., Jackson, L., Yau, Y. C. W. & Waters, V. Quantifying the effects of antimicrobials on in vitro biofilm architecture using COMSTAT Software. JoVE, e61759, https://doi.org/10.3791/61759 (2020).

49. Yau, Y. C. et al. Randomized controlled trial of biofilm antimicrobial susceptibility testing in cystic fibrosis patients. J. Cyst. Fibros. 14, 262–266 (2015).

50. Beaudoin, T. et al. Activity of a novel antimicrobial peptide against Pseudomonas aeruginosa biofilms. Sci. Rep. 8, 14728 (2018).

51. Stiefel, P. et al. Is biofilm removal properly assessed? Comparison of different quantification methods in a 96-well plate system. Appl. Microbiol. Biotechnol. 100, 4135–4145 (2016).

52. Seemann, T. Snippy. https://github.com/tseemann/snippy (2015).

ACKNOWLEDGEMENTS

Funded by a grant from the US Cystic Fibrosis Foundation (WATERS17G0). C.R. was supported by a K99 “Pathway to Independence” Award (5K99GM134121-02) and a Postdoc-to-Faculty Transition Award from the Cystic Fibrosis Foundation (REICH19H5).

AUTHOR CONTRIBUTIONS

A.J.M., L.J., C.R., T.B., S.J.U. and K.M.G. performed the experiments. Y.C.W.Y., P.L.H., M.R.P., L.R.H., D.N., D.S.G., D.J.W. and V.W. contributed to the study design, data interpretation and analysis, and writing of the manuscript. A.D. contributed experimental materials and contributed to the writing of the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41522-021-00234-3.

Correspondence and requests for materials should be addressed to V.J.W.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021