Virulence adaptations of *Pseudomonas aeruginosa* isolated from patients with non-cystic fibrosis bronchiectasis

Taylor E. Woo,1 Jessica Duong,1 Nicole M. Jervis,1 Harvey R. Rabin,2,3 Michael D. Parkins2,3 and Douglas G. Storey1,2

1Department of Biological Sciences, University of Calgary, 2500 University Drive NW, Calgary, Alberta T2N 1N4, Canada
2Department of Microbiology, Immunology and Infectious Diseases, University of Calgary, 2500 University Drive NW, Calgary, Alberta T2N 1N4, Canada
3Department of Medicine, University of Calgary, 2500 University Drive NW, Calgary, Alberta T2N 1N4, Canada

*Pseudomonas aeruginosa* is a major pathogen in chronic lung diseases such as cystic fibrosis (CF) and non-cystic fibrosis bronchiectasis (nCFB). Much of our understanding regarding infections in nCFB patients is extrapolated from findings in CF with little direct investigation on the adaptation of *P. aeruginosa* in nCFB patients. As such, we investigated whether the adaptation of *P. aeruginosa* was indeed similar between nCFB and CF. From our prospectively collected biobank, we identified 40 nCFB patients who had repeated *P. aeruginosa* isolates separated by ≥6 months and compared these to a control population of 28 CF patients. A total of 84 nCFB isolates [40 early (defined as the earliest isolate in the biobank) and 41 late (defined as the last available isolate in the biobank)] were compared to 83 CF isolates (39 early and 44 late). We assessed the isolates for protease, lipase and elastase production; mucoid phenotype; swarm and swim motility; biofilm production; and the presence of the *lasR* mutant phenotype. Overall, we observed phenotypic heterogeneity in both nCFB and CF isolates and found that *P. aeruginosa* adapted to the nCFB lung environment similarly to the way observed in CF isolates in terms of protease and elastase expression, motility and biofilm formation. However, significant differences between nCFB and CF isolates were observed in lipase expression, which may allude to distinct characteristics found in the lung environment of nCFB patients. We also sought to determine virulence potential over time in nCFB *P. aeruginosa* isolates and found that virulence decreased over time, similar to CF.

INTRODUCTION

Bronchiectasis is a pathologic diagnosis defined by permanent dilation and widening of the respiratory airways and is common in chronic suppurative lung diseases like cystic fibrosis (CF) and non-cystic fibrosis bronchiectasis (nCFB) (Weycker et al., 2005). Patients with bronchiectasis may present with common symptoms including sputum production, recurrent respiratory infections and airway obstruction manifesting in thickened bronchial walls, establishment of chronic infections and increased levels of inflammatory markers (Mhanna et al., 2001; Seitz et al., 2010; Bergin et al., 2013; Gupta et al., 2015).

Abbreviations: CF, cystic fibrosis; nCFB, non-cystic fibrosis bronchiectasis.

Bronchiectasis arises as a consequence of complications induced by mutations in the CF transmembrane conductance regulator CFTR. However, multiple other non-CFTR mechanisms exist that culminate in bronchiectasis, termed nCFB. Typically considered an ‘orphan disease’, the incidence of nCFB has risen by 8.7% annually between 2000 and 2007 (Barker & Bardana, 1988; Seitz et al., 2012) and is estimated to cost over $630 million annually in the US healthcare system. Causes of bronchiectasis include immune dysregulation (including autoimmune disorders), obstruction of the airways and complications from infections or injuries (Al-Shirawi et al., 2006; McShane et al., 2012).

In patients with CF and nCFB, accumulation of thick mucus in the lungs and impaired mucociliary clearance allow respiratory infections (Martens et al., 2011). While a
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myriad of infecting organisms have been identified, the archetypal pathogen of suppurative lung diseases is *Pseudomonas aeruginosa* (King et al., 2007; Goeminne et al., 2012). During the initial phase of infection, *P. aeruginosa* is postulated to utilize various virulence factors, including proteases, lipases and elastases, which help to facilitate initial infection (Gillham et al., 2009; Chalmers & Hill, 2013). Following the acute phase, initiation of biofilm formation, increased alginate production, down-regulation of motility genes and attenuation of virulence contribute to the establishment of chronic infections (Harmer et al., 2013; Hogardt & Heesemann, 2013). Chronic *P. aeruginosa* infections in both nCFB and CF patients have been associated with a decline in respiratory function and an increased risk of morbidity and mortality (Kerem et al., 1996; Ho et al., 1998; Kosorok et al., 2001; Emerson et al., 2002; Li et al., 2005). However, these trends may not account for the pathogenesis of all isolates of *P. aeruginosa*. Evidence for phenotypic and genotypic variation within *P. aeruginosa* isolates suggests that variable adaptation may occur in response to different niches found within the lung environment (Sousa & Pereira, 2014; Foweraker et al., 2005). This variability may extend to virulence factor production and, as a result, may influence the rate of disease progression in individuals with either disease.

Research investigating the pathogenesis of infection within the CF lung has often been extrapolated to nCFB but there is little supporting evidence to enable correlation between the two diseases. Critically, treatments that demonstrate a clinical benefit in CF do not necessarily confer the same benefit in nCFB (O’Donnell et al., 1998; Drobnic et al., 2005; Barker et al., 2014), which suggests that direct extrapolation may not be appropriate. In order to develop strategies to optimally manage chronic *P. aeruginosa* infections in patients with nCFB, understanding the pathogenesis of chronic *P. aeruginosa* derived specifically from this patient population is required. We hypothesized that *P. aeruginosa* would adapt similarly in nCFB patients and CF patients. In this study, we used two longitudinal sets of *P. aeruginosa* isolates, one from nCFB patients and the other one from CF patients, to examine virulence factor production of isolates from the two cohorts. In addition, we set out to observe whether the attenuation of virulence traits occurs over time, akin to that observed in CF (Lorè et al., 2012).

**METHODS**

**Bacterial strains and culture conditions.** *P. aeruginosa* isolates were collected from patients attending the Calgary Adult Cystic Fibrosis Clinic and the Calgary Bronchiectasis Clinic from 1986 to 2013. The Conjoint Health Research Ethics Board E-23087 granted permission for the collection and analysis of these strains. *P. aeruginosa* was identified from routine sputum samples submitted for semi-quantitative sputum evaluation during regular clinical visits and was prospectively inventoried and stored at −80°C in the Calgary Adult CF Clinic Biobank (Parkins et al., 2014). We retrospectively audited the biobank to identify adults with nCFB who were repeatedly positive for *P. aeruginosa* for more than 6 months. This biobank included every isolate from every adult with CF who had chronic infection spanning >6 months.

Each isolate was cultured in tryptic soy broth and incubated overnight at 37°C prior to use in each assay, which was conducted in three replicate replicates (n=9) unless otherwise stated. For the protease, lipase and elastase assays, each culture was normalized to an OD$_{600}$ of 0.3 and spotted in triplicate on each plate.

**Protease.** Protease production was assayed as previously described using dialysed BHI agar plates (Sokol et al., 1979). Protease activity was quantified by measurement of the zone of clearance after incubation for 24 h at 37°C.

**Lipase.** Lipase plates were prepared as described by Lonon et al. (1988), containing 1% (w/v) of bactopeptone, 0.5% (w/v) of sodium chloride, 0.01% (w/v) of calcium chloride, 1.5% (w/v) of noble agar and 1% (w/v) of Tween 80. Lipase activity was measured by the cloudiness around the cells after 48 h incubation at 37°C.

**Reverse elastase.** Elastase expression was measured through the breakdown of elastin as described by Rust et al. (1994). Agar containing 0.8% (w/v) nutrient broth and 2% (w/v) noble agar was dissolved in distilled water and was pH-adjusted to 7.5. This composed the bottom layer of the plate. An elastin preparation containing 0.8% (w/v) nutrient broth, 2% (w/v) noble agar and 0.5% (w/v) elastin was then overlaid onto the medium. The resulting zone of clearance was measured after 24 h incubation at 37°C followed by 48 h at 4°C.

**Swim and swarm motility.** Swim motility was assessed by using 0.3% LB agar plates as performed by Murray & Karmazin (2006) and was measured by the distance travelled from the point of inoculation after an incubation period of 72 h at 22°C with extra humidity.

Swarm plates were prepared according to Kohler et al. (2000) using M8 medium supplemented with 0.2% (w/v) glucose, 0.05% (w/v) glutamate, 0.5% (w/v) noble agar and 0.024% (w/v) MgSO$_4$. The radial distance of swarming for each isolate was then recorded after incubation for 48 h at 37°C.

**Biofilm assay.** Each culture was normalized to an OD$_{600}$ of 0.001 in tryptic soy broth and inoculated in triplicate into two Calgary Biofilm Devices (CBDs) and incubated at 37°C for 24 h and assessed with two complementary assays: the biofilm biomass assay and viable cell count assay adapted from a protocol by Ceri et al. (1999).

**Biofilm biomass assay.** Biomass production was quantified using the crystal violet assay. After incubation, the CBD lid, which contained 96 pegs, was washed with sterile water, allowed to dry and then stained with 1% crystal violet followed by three consecutive washes with PBS. The lid was then decolorized in another 96-well plate containing methanol and the absorbance was recorded at an optical density of 540 nm in a Perkin Elmer Victor X4 plate reader.

**Viable cell count assay.** After incubation for 24 h, the CBD lid was placed into a 96-well plate containing 0.85% saline and sonicated (5510 Branson) for 10 min. Tenfold serial dilutions were then carried out and 20 µl of each dilution for each isolate was spotted on a tryptic soy agar plate and incubated for 24 h at 37°C. The numbers of colonies were counted as a measure of viable cells in the biofilm.
**Mucoidy.** The mucoid phenotype was qualitatively assessed by a slime appearance on Pseudomonas Isolation Agar after incubation for 48 h at 37°C.

**Phenotypic identification of LasR mutants.** LasR mutants were identified by growth at 37°C after incubation for 48 h on M9 medium supplemented with 0.1% (w/v) adenosine and 1.5% (w/v) noble agar as done previously (Sandoz et al., 2007).

**Mutation frequency determination.** Mutation frequency was determined using a rifampicin screen described by Oliver et al. (2000). Strains were considered hypermutators if their mutation frequency was found to be 20-fold greater than that of our control strain PAO1.

**Statistical analysis.** Statistical analysis was conducted using Prism 5.0 (GraphPad). Column statistics allowed the calculation of the mean value of the three triplicate trials (n=9) of each isolate for the protease, lipase, elastase, swarm and slime motility, biofilm, mucoidy and LasR assays. Using a D value <0.05 for significance, we used the nonparametric, two-tailed Mann–Whitney U test to compare the phenotypic assays between our nCFB and CF cohorts. Similarly, we also used the Mann–Whitney U test to analyse the phenotypic assays between the two reference points (early and late) in our nCFB cohort. For dichotomous variables, we used a combination of Fisher’s exact test and chi-squared test (α<0.05) to determine whether significant differences existed in our cohorts with respect to the LasR, mucoidy and hypermutator assays.

**Hierarchal clustering analysis.** Phenotypic clustering of virulence factors was done through the use of CLUSTER 3.0 (Stanford University) using the unweighted pair group method. The dendrogram was generated based on criteria set by Duong et al. (2015) and visualized using Java TreeView (Saldanha, 2004).

### RESULTS

Forty patients with nCFB, meeting inclusion criteria of repeated *P. aeruginosa* isolation spanning >6 months were identified. From these patients, we selected 81 isolates to study: 40 early isolates and 41 late isolates. The median time between early and late isolate collection for patients was 3.16 years (range: 0.5–21.4 years). Our CF control cohort was derived from 28 patients chosen randomly from a collection of patients known to be infected with both unique and epidemic strains and included 39 early and 44 late isolates (Duong et al., 2015). The median time between early and late CF isolates collected was 14 years (range: 2–24 years).

**Comparison of phenotypic traits in *P. aeruginosa* isolated from patients with CF and nCFB**

We first wanted to determine how *P. aeruginosa* phenotypically adapts to the environment of the bronchiectatic lung environment. In order to do so, we compared the variation in expression of a number of phenotypic traits between CF and nCFB isolates. Overall, we observed a diverse range of expression for each phenotype assessed in *P. aeruginosa* isolates from both nCFB and CF (Fig. 1). When the virulence factor production was compared between our nCFB and CF isolates, we found that the overall distribution was heterogeneous. Furthermore, no significant differences were found between nCFB and CF isolates based on the medians of protease and elastase production, swim and swarm motility, mucoidy or biofilm growth (Fig. 1a, b, d–i).

On the other hand, isolates derived from individuals with nCFB were found to exhibit significantly lower levels of lipase activity (P=0.0003) compared to CF isolates (Fig. 1c). Furthermore, in terms of isolates that do not produce lipase, we found that the nCFB cohort had significantly more lipase-null isolates [53/84 (63%)] as compared to CF isolates [29/83 (35%)] (P=0.0003). Likewise, we observed a significantly higher proportion of non-elastase-producing isolates [51/84 (61%)] in the nCFB isolates as compared to 27/83 (33%) isolated from patients with CF (P=0.0003) (Fig. 1c), suggesting that differences in the elastase- and lipase-null isolates exist between the two patient cohorts. Furthermore, as lipase and elastase production are both regulated by the Las/Rhl quorum sensing systems (Rust et al., 1996; Reimmann et al., 1997), we sought to examine the proportion of LasR mutant phenotype isolates that also were null for elastase and lipase activity. As LasR mutants are deficient in nucleoside hydrolase (Nuh) activity, they are unable to grow on adenosine as their sole carbon source (Heurlier et al., 2005). In total, we found 34/51 (67%) of non-elastase-producing nCFB isolates and 26/53 (49%) of lipase-null isolates were positive for the LasR mutant phenotype.

### Hierarchical clustering of phenotypic traits from nCFB and CF isolates

Fig. 1 demonstrates that the distribution and means of individual traits were similar with only lipase being significantly different. However, to enable a more comprehensive assessment of phenotypes, we examined the entire set of traits for each isolate by clustering the isolates from both nCFB and CF isolates. The isolates clustered into two large clades, referred to as clade I and clade II (Fig. 2). Clade I had a total number of 82 isolates, with 50 (61%) nCFB and 32 (39%) CF isolates. Conversely, a total of 85 isolates in clade II consisted of 34 (40%) nCFB and 51 (60%) CF isolates (Fig. 2). A comparison between the two clades for the distribution of nCFB and CF isolates within each clade was found to be significantly different (P=0.0067) suggesting that nCFB isolates dominated clade I and CF isolates dominated clade II.

Knowing this distribution, we then assessed whether virulence factor production was different in each clade. Overall, we found that the clades separated by relative levels of virulence factor production (Fig. 2). *P. aeruginosa* isolates found in clade I (consisting mainly of nCFB-derived isolates) showed lower levels of virulence expression (protease, elastase and lipase), swim and swarm motility and biofilm formation relative to isolates found in clade II (consisting mainly of CF-derived isolates). In addition to the relatively low levels of virulence expression, we also found a larger abundance of elastase-null and lipase-null isolates within clade I. Conversely, we found that the majority of clade II isolates had above average elastase and lipase production.
Early and late expression of virulence factors in nCFB isolates

We compared P. aeruginosa in the nCFB subset taken at different time points to investigate whether attenuation of virulence occurred over time as has been observed in CF isolates (Lorè et al., 2012). Accordingly, we observed a significant decrease in protease, elastase, swim motility, biomass and viable cell count between early and late in our CF cohort (Fig. 3). However, we did not observe significant differences with respect to the production of protease, lipase or mucoidy assays in our nCFB cohort. Furthermore, when we evaluated the LasR mutant phenotype, we found that the 18/40 (45%) of early isolates and 25/41 (61%) of our late isolates were positive for mutant phenotype (data not shown). However, we did observe a significant decline in elastase expression (P=0.0435) between

Fig. 1. Comparison of P. aeruginosa isolates between nCFB (n=84) and CF (n=83) isolates. Isolates were characterized for protease (a), elastase (b) and lipase (c) production; swim (d) and swarm (e) motility; mucoidy (f); planktonic growth (g); biomass production (h); and viable cell count in biofilm (i). Black horizontal bars represent the mean of each group. Statistical analysis of median was determined to be significant if P<0.05.
early and late isolates (Fig. 3b). Similarly, while swarm motility did not differ, we observed a reduction in swim motility between both reference points (Fig. 3d). With respect to biofilm growth, we found that the biofilm capabilities remained similar between early and cohorts. However, planktonic growth was observed to be reduced ($P=0.0385$) in late nCFB isolates (Fig. 3g). Furthermore, we sought to investigate the proportion of hypermutator strains in a small subset of 20 nCFB isolates and observed no significant differences between early (3/10) and late (5/10) isolates.

**DISCUSSION**

*P. aeruginosa* isolated from nCFB patients has both similarities and distinct differences in virulence factor production when compared to CF isolates

While the aetiology of nCFB and CF are markedly different, we found that *P. aeruginosa* derived from nCFB patients display similar adaptation strategies compared to those isolates from CF. Critically, we observed a substantial degree of phenotypic heterogeneity among all the *P. aeruginosa* isolates examined in this study. As the...
assumption of phenotypic diversity of *P. aeruginosa* has previously been demonstrated in CF (Workentine et al., 2013; Clark et al., 2015), it appears that phenotypic diversity is also evident within our cohort of nCFB isolates. The broad range of virulence potential in our cohort is likely due to the extensive genome plasticity found within *P. aeruginosa*, which influences the adaptation strategies of *P. aeruginosa* to these unique lung microenvironments (Shen et al., 2006). The capability of these isolates to colonize these unique niches may play a role in influencing the levels of virulence and survival of *P. aeruginosa* (Mathee et al., 2008).

Furthermore, we observed a high degree of similarity of virulence factor production, motility and biofilm formation. The expression of these virulence traits is likely influenced by the lung environment and has been suggested to be important in the colonization by *P. aeruginosa* (Cowell et al., 2003; Tingpej et al., 2007; Gellatly et al., 2012). The similar levels of protease and elastase expression, swim and swarm motility and biofilm formation suggest that these virulence traits play a similar role in the adaptation strategy of *P. aeruginosa* during the initial colonization of individuals with nCFB to that in CF (Drake & Montie, 1988; O'Toole & Kolter, 1998). In particular, biofilms have been

**Fig. 3.** Comparison of *P. aeruginosa* isolates from nCFB at early (*n*=40) and late (*n*=41) reference points to early (*n*=39) and late (*n*=44) CF isolates. Isolates were characterized for protease (a), elastase (b) and lipase (c) production; swim (d) and swarm (e) motility; mucoidy (f); planktonic growth (g); biomass production (h); and viable cell count in biofilm (i). Black horizontal bars represent the mean of each group. The median distribution was considered significant at *P*<0.05.
documented within the lungs of individuals with CF (Singh et al., 2000) and adaption to the biofilm mode of growth is thought to be a critical factor in the persistence and resistance to both the immune system and exogenous antibiotics (Drenkard & Ausubel, 2002; Mah et al., 2003). In concordance with findings by Perez et al. (2013), similar levels of biofilm characteristics between our patient cohorts in our study support the important role of biofilm formation in both nCFB and CF.

While we found many phenotypic similarities between P. aeruginosa isolates from both groups, we also observed distinct differences between both diseases. For instance, we observed a higher proportion of elastase-null nCFB isolates relative to our CF isolates. Elastase plays a role in host tissue invasion, immune evasion and tight junction disruption and is under the control of the LasIR quorum sensing system (Cowell et al., 2003; Kuang et al., 2011; Nomura et al., 2014). As LasR is a positive regulator of lasB (gene encoding elastase), mutations in the lasR would result in a non-elastase-producing isolate, which has been shown to provide a selective growth advantage on carbon and nitrogen sources, including amino acids (Gambello & Igleswki, 1991; Luján et al., 2007; D’Argenio et al., 2007). As we found a high proportion or elastase-null isolates with the LasR mutant phenotype, we suspect that mutations in lasR may also be an important factor in the adaptation of P. aeruginosa to the nCFB lung environment.

One notable distinction observed in our study is the markedly lower lipase expression found in our nCFB cohort. Bacterial lipases, including those secreted by P. aeruginosa, contribute to pathogenesis by breaking down the lipid component of lung surfactants to provide free fatty acids for growth and allow direct targeting of the host membrane (Lonon et al., 1988; Barth & Pitt, 1996; Gellatly et al., 2012). The breakdown of fatty acids required for beta-oxidation likely serves as an important source of carbon and nitrogen for growth (Barth & Pitt, 1996; Kang et al., 2008). The lungs of individuals with CF have been observed to have higher levels of total phospholipids relative to those with chronic bronchiectasis (Girod et al., 1992; Puchelle et al., 2002) which we suspect contributes to the differential levels of lipase expression observed between our patient cohorts.

**Expression of virulence factors by P. aeruginosa generally decreases over time in nCFB**

The dogma in CF research is that, over time, there is a reduction in virulence in P. aeruginosa (Loré et al., 2012). This has not previously been reported in nCFB – but presumed. Within our study, we observed a trend towards decreased virulence factor production over time and, in particular, observed a significant decrease in elastase production and swim motility. We suspect that our findings are likely due to environmental pressures which select against elastase expression over the course of infection in individuals with suppurative lung diseases (Smith et al., 2006). The decrease in swim motility was likely due to the transcriptional down-regulation of motility-related genes and the establishment of biofilms, which are considered a hallmark of chronic infections in both CF and nCFB (Déziel et al., 2001; Hogardt & Heesemann, 2013; Varga et al., 2015).

Interestingly, we also found that the proportion of mucoid and lasR mutant phenotypes of P. aeruginosa isolates did not change between the early and late nCFB isolates. The acquisition of lasR and mucoid phenotypes has been shown to be an adaptation strategy to the lung environment and may be an indicator of disproportionate disease progression in CF (Li et al., 2005; Smith et al., 2006; Hoffman et al., 2009; LaFayette et al., 2015). As such, our findings suggest that this may not be as common in nCFB and provide an interesting distinction between the two diseases. Taken altogether, it is possible that the different inflammatory environment of nCFB may place a smaller pressure on P. aeruginosa to acquire a mucoid phenotype and lasR mutations over time (Bergin et al., 2013).

An important limitation in this work is the retrospective nature of our study. As the isolates tested were obtained for the biobank upon clinic visit, it is uncertain how long or at what stage the patients presented with the disease prior to collection of the sputum. Rather than ‘early’ and ‘late’, we might be comparing ‘late’ and ‘later’ isolates – isolates that may have previously already undergone reduction in expression. Indeed, the presentation of nCFB is insidious, with 30–40 years passing between the onset of symptoms and a clinical diagnosis, time in which infections can develop and evolve (Pasteur et al., 2000; King et al., 2006). Accordingly, any longitudinal cohort study following nCFB patients for infections and changes in virulence potential is going to be subject to the same age-related limitations as our own, something not observed in CF cohorts where historically 75% of patients were diagnosed before age 2 years (Cystic Fibrosis Foundation, 2015). Furthermore, heterogeneity within chronically infecting populations of P. aeruginosa has now been established in both nCFB and CF (Gillham et al., 2009; Workentine et al., 2013). While we sampled a large number of isolates, our sampling included only a small number from each individual patient and therefore might not have adequately represented the phenotypic diversity. Lastly, differences in antibiotic therapy between patients with nCFB and CF may influence the differences in the phenotypic adaptation observed within our study.

In conclusion, the importance of P. aeruginosa in employing various strategies of colonizing the CF lung has been extensively investigated (Foweraker et al., 2005; Hogardt & Heesemann, 2013). However, this relationship has been largely unexplored in the case of nCFB with many conclusions inferred from pre-existing CF research. As such, the aim of our study was to address this current gap of knowledge with respect to the adaptation of P. aeruginosa in nCFB. Indeed, we found similar adaptation strategies
between isolates of *P. aeruginosa* from nCFB patients – which, due to the broad range of environmental niches, display a wide spectrum of virulence and motility (Pujana *et al.*, 1999; Penesyan *et al.*, 2015). While differences in airway characteristics exist between CF and nCFB, we have found that *P. aeruginosa* largely adapts in a similar manner, extending from virulence factor production to motility and biofilm formation, and that virulence tends to decrease over time. These adaptations may be influenced by differences in mucus viscosity, ciliary clearance and anoxic environments, as well as host responses. We have provided evidence suggesting that differential levels of lipase expression exist between nCFB and CF and may further influence the adaptation of *P. aeruginosa* in response to the unique characteristics of each lung environment. Overall, this study has established that, with a few exceptions owing to fundamental differences between the disease processes, *P. aeruginosa* causing chronic infection in patients with nCFB undergo phenotypic adaption similar to that observed for isolates derived from individuals with CF.

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**REFERENCES**


