Virulence of an Emerging Respiratory Pathogen, Genus Pandoraea, In Vivo and its Interactions with Lung Epithelial Cells

Gillian Herbert
Institute of Technology Tallaght

Anne Costello
Technological University Dublin, anne.costello@tudublin.ie

Lydia Fabunmi
Technological University Dublin, lydia.fabunmi@tudublin.ie

Kirsten Schaffer
St. Vincent’s University Hospital

Kevin Kavanagh
National University of Ireland, Maynooth

Follow this and additional works at: https://arrow.tudublin.ie/ittsciart

Part of the Biochemistry Commons, Cell Biology Commons, Microbiology Commons, and the Molecular Biology Commons

See next page for additional authors

Recommended Citation

This Article is brought to you for free and open access by the School of Science and Computing at ARROW@TU Dublin. It has been accepted for inclusion in Articles by an authorized administrator of ARROW@TU Dublin. For more information, please contact yvonne.desmond@tudublin.ie, arrow.admin@tudublin.ie, brian.widdis@tudublin.ie.

This work is licensed under a Creative Commons Attribution-Noncommercial-Share Alike 3.0 License
Authors
Gillian Herbert, Anne Costello, Lydia Fabunmi, Kirsten Schaffer, Kevin Kavanagh, Emma M. Caraher, Máire Callaghan, and Siobhan McClean

This article is available at ARROW@TU Dublin: https://arrow.tudublin.ie/ittsciart/26
Pathogenicity and Virulence

Virulence of emerging respiratory pathogen, genus Pandoraea, in vivo and its interactions with lung epithelial cells.

Running Title: Pandoraea and virulence

Anne Costello\textsuperscript{1,2}, Gillian Herbert\textsuperscript{1}, Lydia Fabunmi\textsuperscript{1}, Kirsten Schaffer\textsuperscript{3}, Kevin A. Kavanagh\textsuperscript{4}, Emma M. Caraher\textsuperscript{1,2}, Máire Callaghan\textsuperscript{1,2}, Siobhán McClean\textsuperscript{1,2*}

\textsuperscript{1}Centre of Microbial Host Interactions and \textsuperscript{2}Centre of Applied Science for Health, ITT Dublin, Tallaght, Dublin 24 Ireland.

\textsuperscript{3}Department of Microbiology, St. Vincent's University Hospital, Elm Park, Dublin, Ireland.

\textsuperscript{4}Dept of Biology, National University of Ireland, Maynooth, Co Kildare, Ireland.

* Corresponding author: Dr Siobhán McClean, Institute of Technology Tallaght Dublin, Old Blessington road, Tallaght, Dublin 24, Ireland.

\texttt{Siobhan.mcclean@ittdublin.ie}

Phone: +353-1-4042794

Fax: +353-1-4042404

Abbreviations: Bcc, \textit{Burkholderia cepacia} complex; CF, cystic fibrosis; transepithelial resistance (TER)
**Summary**

*Pandoraea* species have emerged as opportunistic pathogens among cystic fibrosis (CF) and non-CF patients. *P. pulmonicola* is the predominant *Pandoraea* species among Irish CF patients. The objective of this study was to investigate the pathogenicity and potential mechanisms of virulence of Irish *P. pulmonicola* isolates and strains from other *Pandoraea* species. Three patients from whom the *P. pulmonicola* isolates were isolated have died. In *vivo* virulence of these and other *Pandoraea* strains was examined by determining killing of *Galleria mellonella* larvae. *P. pulmonicola* strains generally were the most virulent tested, with three showing a comparable or greater level of virulence *in vivo* relative to another CF pathogen, *Burkholderia cenocepacia*, while strains from two other species, *P. apista* and *P. pnomenusa*, were considerably less virulent. For all *Pandoraea* species, whole cells were required for larval killing, as cell free supernatants had little effect on larval survival. Overall, invasive *Pandoraea* strains showed comparable invasion of two independent lung epithelial cell lines, irrespective of whether they had a CF phenotype. *Pandoraea* strains were also capable of translocation across polarised lung epithelial cell monolayers. Although protease secretion was a common characteristic across the genus, it is unlikely to be involved in pathogenesis. In conclusion, while multiple mechanisms of pathogenicity may exist across the *Pandoraea* genus, it appears that lung cell invasion and translocation contributes to the virulence of *P. pulmonicola* strains.
Introduction

Pandoraea species are characterized as non-spore forming, catalase, positive aerobic Gram negative rods with polar flagella, that are often mistakenly identified as *Burkholderia cepacia* complex (Bcc) or *Ralstonia* spp. (Schneider et al., 2006; Stryjewski et al., 2003). The genus *Pandoraea* contains five named species: *P. apista*, *P. pulmonicola*, *P. pnomenusa*, *P. sputorum*, *P. norimbergensis* (Stryjewski et al., 2003; Vandamme et al., 2000) and have been isolated from patients with respiratory tract infections as well as from soil, water and food (Schneider et al., 2006). Its closest phylogenetic relative is the genus *Burkholderia*, and like members of the genus *Burkholderia*, it has recently emerged as a pathogen in the cystic fibrosis (CF) population. CF is a genetically inherited disorder caused by mutations in the cystic fibrosis transmembrane regulator (CFTR), a chloride channel. The main source of morbidity and mortality for CF patients, however, is the bacterial pathogens that they encounter throughout their lives, including, *Pseudomonas aeruginosa* and to a lesser extent, Bcc. Although *Pandoraea* species have also been isolated from sputum samples of CF patients, there is still very little known about their mechanisms of pathogenicity or their roles in CF lung disease (LiPuma, 2003). In addition, *Pandoraea* has been isolated from both CF and non-CF patients from a variety of clinical samples including, blood, sputum, urine, the upper airways and lung tissue (Pimentel & MacLeod, 2008). The recovery of *Pandoraea* isolates from the blood of patients indicates that this organism is capable of invading tissue (Atkinson et al., 2006; Stryjewski et al., 2003). Antibiotic therapy for infection is difficult due to the limited number of antibiotics to which these species are susceptible: tetracycline, imipenem and trimethoprim-sulfamethoxazole.
A systematic review of 102 publications recommended that CF patients colonised with either *Pandoraea* or Bcc species should be segregated from other non-colonised patients (Vonberg & Gastmeier, 2005). However, the clinical significance of colonization with these organisms remains unclear (Atkinson *et al.*, 2006) and there are limited and conflicting data available on the clinical outcome of patients colonised with *Pandoraea*. Two CF patients chronically colonised with *P. apista* were culture negative post-bilateral transplants after antibiotic therapy (Atkinson *et al.*, 2006). However, apparent patient-to-patient transmission of *P. apista* has been demonstrated, and four of the patients exhibited a decrease in lung function post-acquisition (Jorgensen *et al.*, 2003). *P. pnomenusa* sepsis and subsequent death has been reported in a non-CF patient after a lung transplant (Stryjewski *et al.*, 2003). A recent case report highlighted nine previous reports of *Pandoraea* bacteraemia, only one of these was identified as being in a CF patient (Pimentel & MacLeod, 2008).

The predominant *Pandoraea* species among Irish CF patients is *P. pulmonicola*. We have previously shown that the Irish *P. pulmonicola* isolates were unusual within the genus as they all showed an ability to invade human lung epithelial A549 cells *in vitro* (Caraher *et al.*, 2008). The three patients from whom the *P. pulmonicola* isolates were obtained have since died. Therefore the virulence of these *P. pulmonicola* isolates required further study. We compared a selection of these isolates with other strains an *in vivo* virulence model, *Galleria mellonella* and also examined the interactions between some of these *P. pulmonicola* isolates and lung epithelial cells in more detail. The *G. mellonella* model was chosen as it has previously been used to examine virulence of a
number of CF pathogens including Bcc, *P. aeruginosa* and *Aspergillus fumigatus* (Kavanagh & Reeves, 2004; Reeves *et al.*, 2004; Seed & Dennis, 2008). The non-Irish strains were all sourced from a commercial bacterial culture collection and were all originally isolates from CF lung (Table 1).
Materials and Methods

Bacterial Strains and Cell lines. The origins of the Pandoraea strains examined in this study are listed in Table 1. The non-Irish isolates were purchased from BCCM/LMG, University of Ghent, Belgium. Non-invasive E. coli strain NCIB 9485 was used as a control in all experiments. All isolates were routinely grown on Tryptic soy agar (TSA), Tryptic soy broth (TSB), Luria–Bertani (LB) agar or LB broth at 37 °C. All cell lines were maintained in a humidified atmosphere at 37°C in 5% CO₂. Calu-3 cells (subbronchial epithelial cells) were obtained from the European Collection of Cell Cultures (ECACC) and maintained as previously described. (Shen et al., 1994). 16HBE14o⁻ (human bronchial epithelial cells with functional CFTR) and CFBE41o⁻ (CF bronchial epithelial cells) were kindly donated by Dr. Dieter Gruenert (University of California, San Francisco, UCSF) and maintained in fibronectin-vitrogen coated flasks containing MEM supplemented with 5 mM L-glutamine, 50 U ml⁻¹ penicillin, 50 µg ml⁻¹ streptomycin and 10% (v/v) FBS.

Determination of virulence in vivo using Galleria mellonella larvae. Sixth instar larvae of G. mellonella were obtained from Live Foods Direct (Sheffield, England) and stored in wood shavings in the dark at 15 °C. Ninety larvae (0.2 to 0.4g) were randomly chosen for each strain assayed, and all experiments were performed on three independent occasions. Bacterial cells were harvested by centrifugation at 4000 g for 10 minutes, washed in PBS, the O.D 600 was obtained and cultures diluted to a standard of 0.1 OD 600. Bacterial cultures were then serially diluted to 10⁻⁷. Bioburden was ascertained, post
injection, by plate counts of dilutions $10^{-4}$ to $10^{-7}$, plated in triplicate. Ten larvae were inoculated with 20 µl of each dilution, through the last pro-leg into the haemocoel using a 0.3 ml Myjector syringe (Terumo Europe, Leuven, Belgium) and incubated in Petri dishes, on filter paper, at 37 °C for 72 h in the dark. The control group consisted of 10 larvae injected with 20 µl sterile PBS. No more than one control larva died in any given trial. In instances where more than one larva died in the control group, the data from the entire experiment was discarded. Larval death was followed for 72h, at 24 h intervals, by visual inspection and by the lack of movement when stimulated. Three independent trials were conducted consisting of 10 larvae per bacterial concentration for each specified strain. Results are presented as LD$_{50}$ values, i.e. the inoculum concentrations which resulted in 50% killing of the larvae in 72 h. Survival over time was also monitored and plotted against the serially diluted bacteria (1 to 1 x $10^6$ CFU) and representative graphs of three experiments are shown. To assess the virulence of cell-free supernatant (CFS), bacterial cultures were pelleted by centrifugation at 4000rpm for 10 mins. Supernatants were then passed through 0.22 µm pore filters to remove all bacteria. An aliquot of 20 µl were injected into 10 larvae. Sterile broth was used as control.

**Invasion assay.** The ability of *Pandoraea* strains to invade epithelial cells was investigated as outlined previously (Caraher *et al.*, 2008). Lung epithelial cells (16HBE14o or CFBE41o) were seeded on 24 well plates (4×10$^5$ cells per well) in media containing 10% FBS, without antibiotics and cultured for 24 h at 37°C in 5% CO$_2$. All bacterial strains were grown to an optical density at 600 nm (O.D.$_{600}$) of 0.6 and
resuspended at the appropriate dilution, as determined by growth curves, in tissue culture media without antibiotics. These bacterial suspensions were then added to the epithelial cells at multiplicity of infection of 50:1, as used previously. Intracellular invasion was determined over 2 h following antibiotic killing of extracellular bacteria with 1mg amikacin ml$^{-1}$. The percentage invasion was determined as follows: c.f.u. recovered from lysed cells / c.f.u. applied to the cells) X 100.

**Measurement of trans-epithelial electrical resistance (TER).** Calu-3 cells were seeded onto Transwell® polycarbonate inserts (0.4 μm) and after one day, the cells were maintained at an air-liquid interface, in order to promote differentiation (Foster *et al.*, 2000). The trans-epithelial electrical resistance (TER) was monitored for 14 days using an EVOM voltohmmeter (World Precision Instruments) to ensure polarisation and formation of tight junctions and the cells were fed basolaterally on alternate days. Prior to infection of the epithelial cells, each strain was grown to an O.D of 0.6 at 600 nm and 0.5 ml of the appropriate dilution of bacterial suspension was added to the apical compartment and incubated at 37°C. *E. coli* strain, NCIB 9485, was used as a negative control. TER was measured at 0, 1, 2 and 4 h post-infection.

**Translocation studies.** In order to examine the translocation of bacteria through the epithelial monolayer, Calu-3 cells were seeded at a density of 7.5×10$^5$ cells per insert on 3.0 μM Transwell® polycarbonate inserts, a pore size which enables passage of the bacteria to the basolateral chamber (Duff *et al.*, 2006). The cells were cultured fed basolaterally on alternate days for a period of up to 21 days. After apical application of
bacteria (MOI = 50:1), TER was monitored up to 3 h and the basolateral media was sampled periodically and serial diluted in Ringer’s solution to determine the cfu ml$^{-1}$ that translocated through the cell monolayer (Duff et al., 2006).

Protease activity assay. For detection of general protease secretion, 100 µL of cell free supernatants (CFS) from an overnight culture of each of the Pandoraea strains, were placed into wells on skimmed milk agar plates (3 and 10% w/v) containing nutrient agar. This method provides a convenient and clear determination of protease detection on visual inspection and is suitable for evaluating a multiple bacterial strains simultaneously. Sterile broth was used as a negative control and E. coli strain, NCIB9485, and Burkholderia cenocepacia strain, BC7, were used as positive controls. The plates were incubated at 37°C for 24- to- 48 h and any zones of inhibition were measured (mm) using digital Vernier callipers (Lennox, Ireland).

Statistical analysis. Dunn’s multiple comparison method was used to compare invasiveness of individual strains compared to the negative control. A p-value of <0.05 was deemed to be significant in each case. Students’ t-test was used to compare the TER values of bacteria treated monolayers relative to control at individual time points, apparent permeability coefficients and also to compare % survival of G. mellonella at specific time points following exposure to cell free supernatants. In vivo virulence data for individual strains was compared with one-way ANOVA (Minitab).
Results

Patients and isolates. Patient 1 was an adult female patient that was colonised with *P. pulmonicola* at 36 years (RL7177). She was chronically colonised and a second isolate (RL8228) was obtained nine months after the first isolate. Her forced expiratory volume (FEV$_1$) fell from 54% at the time of first isolation of *P. pulmonicola* to 41% at the time of the second isolation and continued to decline down to 21% at 44 months after the first *P. pulmonicola* isolation. Her hospital admissions increased from an average of 0.6 per year prior to *Pandoraea* acquisition to 3 per year in the subsequent 3 years. She died 52 months after her first *Pandoraea* isolation, having spent the last 21 months as an in-patient.

Patient 2 was an adult female that was colonised with *P. pulmonicola* (RL0345) at 23 years. She was chronically colonised and a second *Pandoraea* isolate was obtained 8 months after the first isolation and identification. Her FEV$_1$ fell from 35% to 33% in the year prior to first isolation of *P. pulmonicola*, and dropped from 33% to 26% in the 12 months post-*P. pulmonicola* isolation. Although her hospital admissions did not change substantially post-acquisition of *P. pulmonicola* (2.5 admissions per year in the preceding years compared to four in the subsequent 12 months), she died 14 months after first isolation of *Pandoraea*.

Patient 3 was also an adult female patient colonised with *P. pulmonicola* (RL0308) at 19 years. Her hospital admissions increased from an average of two per year in the three
years prior to *Pandoraea* isolation to 4 per year following *Pandoraea* isolation. She had a lung transplant 5 months after first isolation of *Pandoraea*. Her FEV$_1$ was 64% at 7 months post-transplant and decreased to 39%, 11 months post-transplant. She subsequently died 2 years and 1 month after transplantation, *Pandoraea* was not identified in the patient post-transplant.

*P. pulmonicola* strains are more virulent in vivo than other *Pandoraea* species. We first examined the survival of *G. mellonella* larvae in response to the four Irish clinical *P. pulmonicola* isolates and to five other strains within the genus *Pandoraea* and compared them with values obtained for the *B. cenocepacia* strain, J2315. This Bcc strain was chosen as it has been previously tested in the *G. mellonella* model (Seed & Dennis, 2008). Although variability was observed across the genus, there were clear species-specific effects on *G. mellonella* survival. The four most virulent *Pandoraea* strains, with the lowest LD$_{50}$ at 72 h, were all *P. pulmonicola* strains (Table 1). Four out of six *P. pulmonicola* strains examined (RL7177, RL8228, LMG 18107 and LMG 18108) were quite virulent and showed comparable or greater virulence relative to that of *B. cenocepacia* strain, J2315 (Table 1, p = 0.147). The fact that the four most virulent strains included two patient isolates and two from a commercial source indicates that this effect is not biased towards Irish clinical isolates or commercially-sourced isolates. Both *P. apista* strains examined (LMG16407 and LMG 16408) exhibited high LD$_{50}$ values, indicating that they were less virulent. The only *P. pmomenusa* strain examined also had a high LD$_{50}$ value. Interestingly, the two sequential *P. pulmonicola* isolates (RL7177
and RL8228) obtained from Patient 1 showed a dramatic reduction in LD_{50} over time between isolates indicating that virulence had increased with time.

The kinetics of killing varied across the genus. Individual *P. pulmonicola* strains became more lethal with time following the injection (fig 1a to 1c), while *P. apista* and *P. pnomenusa* strains, all had their optimal effect within 24 h with no significant enhancement after that time (for example, *P. apista* LMG 16408, Fig. 1d). All other *P. pulmonicola* strains also showed this time-dependent effect (data not shown).

**Cellular invasion** *Pandoraea* spp is independent of cystic fibrosis phenotype. We have previously shown that only a subset of *Pandoraea* strains was capable of invading lung epithelial cells. Indeed, the Irish *P. pulmonicola* strains were unusual in that the majority of these were capable of invading A549 cells (Caraher et al., 2008). To investigate the invasive potential of the *P. pulmonicola* isolates further, we examined the potential of a selection of these strains to invade lung epithelial cells with, and without, a CF phenotype in order to examine whether cells with a CF phenotype (CFBE41o- cells) were more susceptible to invasion by members of the genus, relative to lung epithelial cells with functional CFTR (16HBE14o- cells). An MOI of 50:1 was used as previous studies had shown that invasion was optimal at this MOI. Three clinical *P. pulmonicola* isolates which were previously found to invade A549 cells (Caraher et al., 2008) were selected, including two sequential isolates (RL7177 and RL8228) from one patient, to examine their potential to invade these two distinct cell lines (16HBE14o- and CFBE41o-). These were compared with another strain previously shown to be moderately invasive,
P. pnomenusa strain, LMG18087. Three Pandoraea strains (P. pulmonicola RL0345 and RL8228, and P. pnomenusa LMG18087) were invasive in the 16HBE14o- cells, when compared to the negative control E. coli strain, NCIB9485, (p <0.05 by Dunn’s multiple comparison method in each case) (Fig 2). The remaining isolate, P. pulmonicola RL7177, was classified as non-invasive because the invasion values were not significantly different from that of the control E. coli strain, NCIB9485. Only two of the Pandoraea isolates, P. pulmonicola RL0345 and RL8228, invaded CFBE41o- cells (Fig 2) when compared to the negative control E. coli, NCIB9485 (P<0.05). The other two isolates, P. pnomenusa LMG18087 and P. pulmonicola RL7177, were not significantly more invasive of CFBE cells than E. coli strain, NCIB 9485. There was no statistically significant difference in invasion of any strain into CFBE41o- cells versus 16HBE14o- cells. Although these cell lines are distinct, this study suggests that cells with a CF phenotype are no more susceptible to invasion by Pandoraea strains than those with functional CFTR. Interestingly, a consistent increase in invasive potential in the two sequential isolates, RL7177 and RL8228, in both lung epithelial cell lines suggests that these strains become more invasive with time.

P. pulmonicola strains can translocate through polarised lung epithelia. Pandoraea has been isolated from the blood of patients (Atkinson et al., 2006; Daneshvar et al., 2001; Stryjewski et al., 2003) but the majority of strains previously examined were not invasive (Caraher et al., 2008). Therefore, we wanted to examine whether Pandoraea strains were able to invade through the lung epithelium from the apical side to the basolateral side, Calu-3 cells were grown as polarised monolayers on filters with pore
size sufficiently large (3 μm) to enable bacteria to pass through the filter to the basolateral chamber. Although development of a tight epithelium was slower than that observed for Calu-3 cells when cultured on the smaller pore-size (0.4 μm) filters (Duff et al., 2006), the TER peaked at 150 Ω/cm² over that of blank filters on day 21. All experiments were carried out on monolayers that had a TER of no less than 150 Ω/cm² above that of the control well. Bacterial counts from the basolateral chamber indicated that all four strains examined appeared to translocate within 3 h (Fig. 3a). The *P. apista* strain, LMG16408, (previously shown to be non-invasive) (Caraher et al., 2008), was the slowest to translocate, reaching levels in the basolateral chamber of 1% (2 x 10⁵) of bacteria applied over the 3 h. During this time, all the other strains appeared to translocate, in excess of 1x10⁶ cfu ml⁻¹. The strain which translocated most readily was *P. pnomenusa* strain, LMG 18087.

To evaluate if the observed translocation of *Pandoraea* strains was mediated by disruption of tight junctions, Calu-3 cells were grown on 0.4 μM Transwell® filters for 14 days, by which time the TER were routinely greater than 1000 Ω/cm². The sequential *P. pulmonicola* isolates, RL7177 and RL8228, both resulted a significant drop in TER over time when applied to Calu-3 monolayers, relative to *E. coli* strain, NCIB 9485, p<0.015 for both strains (Fig 3b). Three other strains which were shown to translocate were examined namely, *P. pulmonicola* RL0345, *P. pnomenusa* LMG18087 and *P. apista* LMG16408 did not cause a statistically significant reduction in TER. We have previously shown that the virulent CF pathogen *B. cenocepacia* strain, BC7 disrupted tight junction integrity in polarised Calu-3 cells (Duff et al., 2006); therefore this was
included as a positive control. The drop in TER following infection with the *B. cenocepacia* strain, BC7, was found to be comparable with that of both *P. pulmonicola* isolates, RL7177 and RL8228. The TER at 4 h fell to 64% ± 14 following infection with BC7 (excluded from Fig 3b for clarity), compared to 59% for RL7177 and 66% for RL8228.

**Pandoraea isolates secrete proteases but these are not involved in larval killing.**

Protease secretion by *Pandoraea* strains could play a role in disruption of tight junctions or general lung tissue damage; therefore we wanted to determine the levels of protease secretion in a selection of the clinical Irish isolates and other strains. The protease activities of a range of *Pandoraea* isolates were examined on 3% or 10% skimmed milk agar plates together with *E. coli* NCIB9485 and *B. cenocepacia* strain BC7 which were used as a positive controls in these studies (Gingues *et al.*, 2005; Swamy & Goldberg, 1982). All Irish *P. pulmonicola* isolates examined produced significant levels of protease activity. This level was comparable to all other *Pandoraea* strains examined and was also comparable to the levels of protease activity of both the *E. coli* strain, NCIB9485 and the *B. cenocepacia* strain, BC7 (Fig. 4).

The role of proteases in pathogenesis was examined by injecting CFS from a series of *Pandoraea* isolates into the *G. mellonella* model. In contrast to the killing of larvae by whole cells, when CFS were administered to the larvae, no killing was observed by *Pandoraea* strains examined up to 1 week post-injection. This clearly indicates that in general cellular components rather than secreted factors play a role in killing among the
Pandoraea genus. This contrasts with the effects of CFS from B. cenocepacia strain J2315 (Fig 5A) which resulted in significant killing of G. mellonella larvae within 72 h (70%). Interestingly, in spite of the lack of effect on survival, the CFS of certain strains, e.g. P. pulmonicola strains, LMG18107 and LMG18108, and P. pnomenusa strain, LMG 18087, had a reversible pathogenic effect on the larvae. The larvae underwent melanisation within 30 minutes (Fig 5C) and subsequently recovered without any effect on survival. This melanisation was never observed in the control larvae injected with LB broth only (Fig 5B), however, melanisation always preceded death of larvae that were injected with whole cells (Fig 5D).
Discussion

*Pandoraea* species are a relatively recently identified pathogen among CF and non-CF patients (LiPuma, 2003; Pimentel & MacLeod, 2008; Stryjewski *et al.*, 2003). Although *Pandoraea* has been isolated from the blood and sputum samples of patients, very little is known about its virulence or mechanisms of pathogenicity. *P. pulmonicola* are the most commonly identified *Pandoraea* species among Irish CF patients. However, there is very little known about this species in particular, in terms of the outcomes of infected patients or its pathogenicity, therefore we have focussed, although not exclusively, on this species. All three patients from whom *P. pulmonicola* was isolated were female. This is an unavoidable bias in this study, however, there were not enough patients to allow a more gender-balanced approach. In our experience, no gender bias has been observed in *Pandoraea* colonisation.

Firstly, in order to compare the potential virulence of *Pandoraea* species and strains, we evaluated nine strains in an *in vivo* virulence model. The larvae of *G. mellonella* has recently been used to evaluate a series of Bcc strains for their virulence and their responses to phage therapies (Seed & Dennis, 2008, 2009). They have also been used to study the virulence of other bacterial and fungal pathogens (Jander *et al.*, 2000; Reeves *et al.*, 2004). The host response of *G. mellonella* to infection shares many similarities with that of vertebrates (Kavanagh & Reeves, 2004), making it a cheaper alternative to other *in vivo* models. It has advantages over other *in vivo* models, such as *Caenorhabditis elegans*, as *Galleria* larvae can survive at 37°C. The pattern of survival of *G. mellonella* following infection with *Pandoraea* strains was species-specific. Although, the *in vivo* studies are limited in that they were performed in a single model, it
is clear that the *P. pulmonicola* strains were among the most virulent of the three species tested in this model, while other species, *P. apista* and *P. pnomenusa* were only weakly virulent (Table 1).

*P. pulmonicola* was also the species in which the majority of strains displayed a potential for epithelial cell uptake (Caraher *et al.*, 2008), therefore, in order to further investigate invasion as a mechanism of virulence within these *Pandoraea* strains, we compared invasion in cells with, and without, a CF phenotype. Although the magnitude of invasion into CFBE cells was less in general, the pattern of invasion (i.e. invasive or not) of *Pandoraea* isolates into both independent cell lines was comparable with invasion of A549 cells. In particular, two Irish *P. pulmonicola* isolates, RL0345 and RL8228, which showed considerable invasive capacity of A549 cells, were also found to be significantly invasive in both 16HBE14o⁻ and CFBE41o⁻ epithelial cell models. This demonstrates the ability of certain *Pandoraea* isolates to invade three independent lung epithelial cells and indicates that invasion is strain-specific rather than cell phenotype-dependent. The lack of enhanced susceptibility to invasion of cells with a CF phenotype over non-CF cells would agree with a recent summary of nine *Pandoraea* bacteraemia cases reported (Pimentel & MacLeod, 2008), at least three of which were identified as being in non-CF patients. The finding that *P. pulmonicola* isolate, RL8228, was both more invasive of human lung cells and more virulent to larvae than the earlier isolate (RL7177) from the same patient, suggests that invasion plays a role in pathogenesis for some members of the species. However, the finding that two other *P. pulmonicola* strains (LMG18107 and LMG18108) which were very virulent towards the larvae, but
were not previously shown to be invasive, indicates that other mechanisms of pathogenicity are also at play.

In order to explore mechanisms of lung invasion further, the ability of a selection of isolates to disrupt epithelial integrity prior to entering the blood stream was examined. Human lung epithelial cells, such as Calu-3, show permeability properties which are comparable to native lung epithelia and therefore are a useful model for studying the absorption barrier of the lung (Forbes & Ehrhardt, 2005). We and others have shown that Bcc strains can disrupt the tight junction integrity of, and penetrate through, lung epithelial monolayers (Duff et al., 2006; Kim et al., 2005). Although all four Pandoraea isolates tested had the ability to translocate through the monolayer, albeit at different rates, two of the strains examined translocated without having any effect on TER (Fig. 3). This suggests that these pathogens move through the monolayer via an alternative route to the paracellular pathway.

Despite the diversity across the genus, all Pandoraea strains tested secreted significant levels of protease and the three Pandoraea species examined showed comparable protease activities. Bacterial proteases have been implicated in a number of pathogenic processes, including disruption of epithelial integrity, inflammation and tissue damage. However, the lack of effect of CFS in the in vivo model suggests that the secreted proteases, in general, may not play any major role in pathogenesis. Furthermore, the lack of correlation between protease activity and tight junction disruption suggests that proteases are not likely to be responsible for this effect. In contrast, members of the closely related Bcc and also Ps. aeruginosa express metalloproteases which have been shown to be involved in virulence (Kooi et al., 2006;
McKevitt & Woods, 1984). In addition, serine proteases have also been identified in B. cenocepacia, and although one of these has been shown to play a role in iron acquisition, their roles in pathogenicity have not been confirmed (Flannagan et al., 2007; Whitby et al., 2006). The relatively high levels of general protease secretion across the genus are interesting, and the identity of these proteases is currently being investigated. It remains a possibility that specific proteases may play a role in virulence of Pandoraea species.

Overall, the lack of effect of CFS on G. mellonella survival for any species within the genus, indicates that the whole cells are the main mediator of virulence rather than secreted exoproducts strengthening the suggestion for direct interaction between the bacterial cells and the host cells as part of the virulence process.

This study shows that the genus Pandoraea is quite divergent in terms of their virulence characteristics, indeed, it was named for the potential Pandora’s box of genetic diversity associated with these organisms (Vandamme et al., 2000). In general, P. pulmonicola strains are the most invasive and also the majority of strains from this species showed the most virulence in the in vivo model, however, to the best of our knowledge, there have been no reported cases of P. pulmonicola related bacteraemia.

Given the small numbers involved, it is difficult to correlate in vitro behaviour with clinical outcomes in patients; however, it is significant that all P. pulmonicola colonised patients from which the isolates in this study were obtained, have died. Further studies would be required on much greater numbers to draw any definite conclusions.

P. apista strains examined showed the lowest virulence in G. mellonella, were non-invasive and P. apista strain LMG16408 was the slowest to translocate across the polarised lung epithelium, however, this species has been associated with at least two
cases of septicaemia (Daneshvar et al., 2001; Johnson et al., 2004). Only one *P. pnomenusa* strain, LMG18087, was included in this study and although invasive of lung cells expressing CFTR, was not invasive in cells with a CF phenotype. This strain showed the greatest level of trans-epithelial translocation among the four examined. Interestingly, four cases of *P. pnomenusa* associated bacteraemia have been reported (Daneshvar et al., 2001; Stryjewski et al., 2003). Indeed, all three isolates identified as *P. pnomenusa* by Daneshvar et al., were isolated from blood which led the authors to suggest that this species might have increased potential for invasive disease (Daneshvar et al., 2001). The mechanism of *P. pnomenusa* translocation is not linked to tight junction opening; therefore further analysis of this species is warranted.

The changes in sequential *P. pulmonicola* isolates, RL7177 and RL8228, are noteworthy. The later isolate is more invasive of lung epithelial cells and more virulent to larvae. Furthermore, in our previous study, we demonstrated that the early isolate, RL7177 was the only *Pandoraea* strain out of 19 examined which was capable of biofilm formation (Caraher et al., 2008), suggesting that this strain also undergoes a mucoid to non-mucoid change over time. This change from a mucoid to a non-mucoid state has also been observed in Bcc isolates from chronically infected patients (Zlosnik et al., 2008), indicating that the same phenotype change arises among *P. pulmonicola* isolates, however, more sequential isolates would need to be studied to confirm this. In addition, further study in the existing sequential isolates is being performed to elucidate the apparent alteration in virulence over time.

Overall, it is clear that *P. pulmonicola* strains show comparable *in vivo* virulence to *B. cenocepacia* strain J2315 in a *G. mellonella* model. A subset of *P. pulmonicola*
strains were shown to invade lung epithelial cells irrespective of CF status and four 
strains examined showed the potential to translocate across the lung epithelium. In 
addition, although protease secretion is a common feature among all strains examined, 
there is no clear evidence to date that this may be a virulence mechanism in this genus. 
It is likely that individual species within the *Pandoraea* genus have the potential to 
express very different mechanisms of pathogenesis, as demonstrated by differences in 
intracellular invasion, tight junction disruption and larval killing. This is comparable to 
the situation in Bcc strains which appear to use different virulence mechanisms to 
compete for survival in different environments and hosts (Uehlinger *et al.*, 2009). There 
are limited clinical data published on *Pandoraea* infections, and the numbers of isolates 
evaluated in this study are also limited, therefore it is difficult to draw clear conclusions 
regarding the mechanisms of pathogenicity and the effects on patients. In addition, 
major host-related factors are also at play and furthermore, given that many *Pandoraea* 
colonised patients are also co-colonised with other pathogens, the interplay between 
competing species must also be considered. However, it is clear that *P. pulmonicola* 
does contribute to the deterioration of colonised patients and has a direct interaction with 
lung epithelial cells.

**Acknowledgements:**

This study was supported by PRTLI Cycle 4 (supported by the European Union Regional 
Development Plan, the Irish Government National Development Plan 2007-2013 and
administered by the Higher Education Authority in Ireland) and by the Technology
Sector Research Post-graduate R&D Skills Programme (PRDSP), Strand I.
The authors would also like to thank Dr Dieter Gruenert for kindly providing us
with the 16HBE 14o- and CFBE41o- cells. We are grateful James Reilly, ITT Dublin for
advice on the statistical analysis of the data. In addition, we would like to thank the
Professor Philip Murphy and Jonathan Collins, National CF Reference Laboratory,
Adelaide, Meath and incorporating the National Children’s Hospital, Tallaght, Ireland for
providing the Irish P. pulmonicola isolates.
References:


Legends:

Fig. 1. Kinetics of larval killing by *Pandoraea* strains. Survival of *G. mellonella* at 24 h (●), 48 h (○) or 72 h (★) when infected with either *P. pulmonicola* strains, RL8228 (A), RL0345 (B), LMG18108 (C) or *P. apista* strain, LMG 16408 (D). Each data set is from a single trial (n=10) which was representative of three independent trials with the specified strain. The data are presented as % larval survival from 10 larva injected per trial. No more than one control larva died in any given trial.

Fig. 2. Comparison of invasion by *Pandoraea* strains in two lung epithelial cells, 16HBE14o− cells and CFBE41o−, compared to the negative control *E. coli* strain, NCIB9485. Bars represent % invasion (c.f.u. obtained after cell lysis as a percentage of c.f.u. applied) of at least five independent experiments for 16HBE14o− cells and at least three independent experiments for CFBE41o−. Error bars represent ± SEM; * denotes invasion significantly greater than *E. coli*, as determined by Dunn’s multiple comparison test (p<0.05).

Fig. 3. The translocation of *Pandoraea* strains through polarised Calu-3 epithelial monolayers. A) Cumulative bacterial counts taken from basolateral chamber of Calu-3 cell monolayers grown on 3 μm Transwell® filters at 1, 2 and 3 h post infection with *Pandoraea* strains expressed total CFU translocated /ml. The results are the average data from at least two independent experiments ± SEM. B) The effect of *Pandoraea* strains on TER at 0, 1, 2 and 4 h post-infection, presented as % of the control (1092 ± 225) and representing the mean of at least three independent experiments, error bars represent ± SEM.
Fig. 4. Protease secretion from *Pandoraea* species as determined on skimmed milk agar plates. Bars represent zones of clearance (± standard deviation) measured following 48 h incubation of *Pandoraea* cell free supernatants on 3% (black bars) or 10% (grey bars) (v/v) skimmed milk plates from three independent experiments.

Fig. 5. The effects of cell free supernatant on survival of *G. mellonella*. A) The bars represent % survival of 10 larvae at 24 h, 48 h, 72 h, respectively. Each data point represents the mean of three independent experiments ± SEM; * p<0.05, relative to LB injected control group. B-D) Effect of *P. pulmonicola* RL7177 CFS on *G. mellonella*, B) control larvae 30 min post-injection with TSB only; C) Larvae 30 min post-injection with CFS from LMG 18108; D) Larvae 48 h post-injection with whole cells, indicating killed larvae among melanised larvae.
TABLE 1. Summary of origins of *Pandoraea* strains and isolates used in this study and their relative LD50s values obtained in *G. mellonella* larvae 72 h post infection compared with *B. cenocepacia* strain, J2315.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Source</th>
<th>LD50 (72 h)</th>
<th>±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. pulmonicola</em></td>
<td>RL7177</td>
<td>CF Lung (Ireland, Pt 1)</td>
<td>$1 \times 10^4$</td>
<td>8.7$\times 10^3$</td>
</tr>
<tr>
<td></td>
<td>RL8228</td>
<td>CF Lung (Ireland, Pt 1)</td>
<td>171</td>
<td>159</td>
</tr>
<tr>
<td></td>
<td>RL0345</td>
<td>CF Lung (Ireland, Pt 2)</td>
<td>$3.85 \times 10^5$</td>
<td>$71.5 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>RL0308</td>
<td>CF Lung (Ireland, Pt 3)</td>
<td>$5.12 \times 10^5$</td>
<td>$3.8 \times 10^5$</td>
</tr>
<tr>
<td></td>
<td>LMG18107</td>
<td>CF lung (USA)</td>
<td>1.54</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>LMG18108</td>
<td>CF lung (USA)</td>
<td>31.3</td>
<td>30</td>
</tr>
<tr>
<td><em>P. apista</em></td>
<td>LMG16407</td>
<td>CF lung (Denmark)</td>
<td>$8.8 \times 10^6$</td>
<td>$2.0 \times 10^6$</td>
</tr>
<tr>
<td></td>
<td>LMG16408</td>
<td>CF lung (Denmark)</td>
<td>$1.27 \times 10^6$</td>
<td>$4.4 \times 10^5$</td>
</tr>
<tr>
<td><em>P. pnomenusa</em></td>
<td>LMG18087</td>
<td>CF lung (UK)</td>
<td>$5.2 \times 10^6$</td>
<td>$6.7 \times 10^5$</td>
</tr>
<tr>
<td><em>B. cenocepacia</em></td>
<td>J2315</td>
<td>CF lung (USA)</td>
<td>4100</td>
<td>3412</td>
</tr>
<tr>
<td></td>
<td>BC7</td>
<td>CF lung (USA)</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

LD<sub>50</sub> is defined as the CFU required to kill 50% of the *G. mellonella* larvae in 72 h. These data represent the mean of three independent trials with ten larvae per group. ND: not determined.
Figure 1.

A  RL8228

% Survival vs CFU/injection

B  RL0345

% Survival vs CFU/injection

C  LMG 18108

% Survival vs CFU/injection

D  LMG 16408

% Survival vs CFU/injection
Figure 2.
Figure 3.

A

![Bar chart showing basolateral CFU/ml for different species and strains over time.]

B

![Line graph showing TER (%) Control over time for different species and strains.]

Species/strain

- P. pulmonicola
- P. pnomenusa
- P. apista

Time (hours)

TER (% Control)

- E. coli, NCIB9485
- P. pulmonicola, RL7177
- P. pulmonicola, RL8228
- P. pnomenusa, LMG 18087
- P. apista, LMG 16408
Figure 4.

Table of Species / Strains:

- RL7177
- RL8228
- RL0345
- LMG18087
- LMG16408
- NCIB9485
- BC7

Species / Strains:

- B. cenocepacia
- E. coli
- P. aeruginosa
- P. promenans
- P. pulmonocola

Diameter of Zone (mm)

Graph showing the diameter of zone in mm for different species and strains.
Figure 5.

A

![Bar graph showing survival rates for different species and strains at 24, 48, and 72 hours.](image)

Species / strain

- P. pulmonica
- P. promenusa
- P. apista
- B. cenocepacia

% Survival

- 24 h
- 48 h
- 72 h

*Significant difference.