2010-11

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

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Recommended Citation

Herbert, G., Costello, A., Fabunmi, L., Schaffer, K., Caraher, E., Callaghan, M. and McClean, M. Virulence of an emerging respiratory pathogen, genus Pandoraea, in vivo and its interactions with lung epithelial cells. Letters in Applied Microbiology 02/2010; 50(5):500- DOI 10.1099/jmm.0.022657-0

Funder: PRTLI Cycle 4 & PRDSP1

Pathogenicity and Virulence

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

Running Title: *Pandoraea* and virulence

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Abbreviations: Bcc, *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

67 (Schneider *et al.*, 2006).

68 A systematic review of 102 publications recommended that CF patients colonised
69 with either *Pandoraea* or Bcc species should be segregated from other non-colonised
70 patients (Vonberg & Gastmeier, 2005). However, the clinical significance of
71 colonization with these organisms remains unclear (Atkinson *et al.*, 2006) and there are
72 limited and conflicting data available on the clinical outcome of patients colonised with
73 *Pandoraea.* Two CF patients chronically colonised with *P. apista* were culture negative
74 post-bilateral transplants after antibiotic therapy (Atkinson *et al.*, 2006). However,
75 apparent patient- to- patient transmission of *P. apista* has been demonstrated, and four of
76 the patients exhibited a decrease in lung function post-acquisition (Jorgensen *et al.*,
77 2003). *P. pnomenusa* sepsis and subsequent death has been reported in a non-CF patient
78 after a lung transplant (Stryjewski *et al.*, 2003). A recent case report highlighted nine
79 previous reports of *Pandoraea* bacteraemia, only one of these was identified as being in
80 a CF patient (Pimentel & MacLeod, 2008).

81 The predominant *Pandoraea* species among Irish CF patients is *P. pulmonicola.*
82 We have previously shown that the Irish *P. pulmonicola* isolates were unusual within the
83 genus as they all showed an ability to invade human lung epithelial A549 cells *in vitro*
84 (Caraher *et al.*, 2008). The three patients from whom the *P. pulmonicola* isolates were
85 obtained have since died. Therefore the virulence of these *P. pulmonicola* isolates
86 required further study. We compared a selection of these isolates with other strains an *in*
87 *vivo* virulence model, *Galleria mellonella* and also examined the interactions between
88 some of these *P. pulmonicola* isolates and lung epithelial cells in more detail. The *G.*
89 *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 ^{o}C. All cell lines were maintained in a humidified atmosphere at 37^{o}C in 5% CO_2. Calu-3 cells (sub-bronchial 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^{o}C in 5% CO_2. All bacterial strains were grown to an optical density at 600 nm ($O.D._{600}$) of 0.6 and

140 resuspended at the appropriate dilution, as determined by growth curves, in tissue culture
141 media without antibiotics. These bacterial suspensions were then added to the epithelial
142 cells at multiplicity of infection of 50:1, as used previously. Intracellular invasion was
143 determined over 2 h following antibiotic killing of extracellular bacteria with 1mg
144 amikacin ml^{-1}. The percentage invasion was determined as follows: c.f.u. recovered
145 from lysed cells / c.f.u. applied to the cells) X 100.
146
147 **Measurement of trans-epithelial electrical resistance (TER).** Calu-3 cells were seeded
148 onto Transwell® polycarbonate inserts (0.4 μm) and after one day, the cells were
149 maintained at an air-liquid interface, in order to promote differentiation (Foster *et al.*,
150 2000). The trans-epithelial electrical resistance (TER) was monitored for 14 days using
151 an EVOM voltohmmeter (World Precision Instruments) to ensure polarisation and
152 formation of tight junctions and the cells were fed basolaterally on alternate days. Prior
153 to infection of the epithelial cells, each strain was grown to an O.D of 0.6 at 600 nm and
154 0.5 ml of the appropriate dilution of bacterial suspension was added to the apical
155 compartment and incubated at 37°C. *E. coli* strain, NCIB 9485, was used as a negative
156 control. TER was measured at 0, 1, 2 and 4 h post-infection.
157
158 **Translocation studies.** In order to examine the translocation of bacteria through the
159 epithelial monolayer, Calu-3 cells were seeded at a density of 7.5×10^5 cells per insert on
160 3.0 μM Transwell® polycarbonate inserts, a pore size which enables passage of the
161 bacteria to the basolateral chamber (Duff *et al.*, 2006). The cells were cultured fed
162 basolaterally on alternate days for a period of up to 21 days. After apical application of

163 bacteria (MOI = 50:1), TER was monitored up to 3 h and the basolateral media was
164 sampled periodically and serial diluted in Ringer's solution to determine the cfu ml^{-1} that
165 translocated through the cell monolayer (Duff *et al.*, 2006).
166
167
168 **Protease activity assay.** For detection of general protease secretion, 100 µL of cell free
169 supernatants (CFS) from an overnight culture of each of the *Pandoraea* strains, were
170 placed into wells on skimmed milk agar plates (3 and 10% w/v) containing nutrient agar.
171 This method provides a convenient and clear determination of protease detection on
172 visual inspection and is suitable for evaluating a multiple bacterial strains simultaneously.
173 Sterile broth was used as a negative control and *E. coli* strain, NCIB9485, and
174 *Burkholderia cenocepacia* strain, BC7, were used as positive controls. The plates were
175 incubated at 37^{o}C for 24- to- 48 h and any zones of inhibition were measured (mm) using
176 digital Vernier callipers (Lennox, Ireland).
177
178 **Statistical analysis.** Dunn's multiple comparison method was used to compare
179 invasiveness of individual strains compared to the negative control. A p-value of <0.05
180 was deemed to be significant in each case. Students' t-test was used to compare the TER
181 values of bacteria treated monolayers relative to control at individual time points,
182 apparent permeability coefficients and also to compare % survival of *G. mellonella* at
183 specific time points following exposure to cell free supernatants. *In vivo* virulence data
184 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

208 years prior to *Pandoraea* isolation to 4 per year following *Pandoraea* isolation. She had
209 a lung transplant 5 months after first isolation of *Pandoraea*. Her FEV_1 was 64% at 7
210 months post-transplant and decreased to 39%, 11 months post-transplant. She
211 subsequently died 2 years and 1 month after transplantation, *Pandoraea* was not
212 identified in the patient post-transplant.
213
214 ***P. pulmonicola* strains are more virulent *in vivo* than other *Pandoraea* species.** We
215 first examined the survival of *G. mellonella* larvae in response to the four Irish clinical *P.*
216 *pulmonicola* isolates and to five other strains within the genus *Pandoraea* and compared
217 them with values obtained for the *B. cenocepacia* strain, J2315. This Bcc strain was
218 chosen as it has been previously tested in the *G. mellonella* model (Seed & Dennis,
219 2008). Although variability was observed across the genus, there were clear species-
220 specific effects on *G. mellonella* survival. The four most virulent *Pandoraea* strains,
221 with the lowest LD_{50} at 72 h, were all *P. pulmonicola* strains (Table 1). Four out of six *P.*
222 *pulmonicola* strains examined (RL7177, RL8228, LMG 18107 and LMG 18108) were
223 quite virulent and showed comparable or greater virulence relative to that of *B.*
224 *cenocepacia* strain, J2315 (Table 1, $p = 0.147$). The fact that the four most virulent
225 strains included two patient isolates and two from a commercial source indicates that this
226 effect is not biased towards Irish clinical isolates or commercially-sourced isolates. Both
227 *P. apista* strains examined (LMG16407 and LMG 16408) exhibited high LD_{50} values,
228 indicating that they were less virulent. The only *P. pnomenusa* strain examined also had
229 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,

253 *P. pnomenusa* strain, LMG18087. Three *Pandoraea* strains (*P. pulmonicola* RL0345 and
254 RL8228, and *P. pnomenusa* LMG18087) were invasive in the 16HBE14o$^-$ cells, when
255 compared to the negative control *E. coli* strain, NCIB9485, ($p < 0.05$ by Dunn's multiple
256 comparison method in each case) (Fig 2). The remaining isolate, *P. pulmonicola*
257 RL7177, was classified as non-invasive because the invasion values were not
258 significantly different from that of the control *E. coli* strain, NCIB9485. Only two of the
259 *Pandoraea* isolates, *P. pulmonicola* RL0345 and RL8228, invaded CFBE41o$^-$ cells (Fig
260 2) when compared to the negative control *E. coli,* NCIB9485 ($P<0.05$). The other two
261 isolates, P. *pnomenusa* LMG18087 and *P. pulmonicola* RL7177, were not significantly
262 more invasive of CFBE cells than *E. coli* strain, NCIB 9485. There was no statistically
263 significant difference in invasion of any strain into CFBE41o- cells versus 16HBE14o-
264 cells. Although these cell lines are distinct, this study suggests that cells with a CF
265 phenotype are no more susceptible to invasion by *Pandoraea* strains than those with
266 functional CFTR. Interestingly, a consistent increase in invasive potential in the two
267 sequential isolates, RL7177 and RL8228, in both lung epithelial cell lines suggests that
268 these strains become more invasive with time.
269
270 ***P. pulmonicola* strains can translocate through polarised lung epithelia.** Pandoraea
271 has been isolated from the blood of patients (Atkinson *et al.*, 2006; Daneshvar *et al.*,
272 2001; Stryjewski *et al.*, 2003) but the majority of strains previously examined were not
273 invasive (Caraher *et al.*, 2008). Therefore, we wanted to examine whether *Pandoraea*
274 strains were able to invade through the lung epithelium from the apical side to the
275 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 /cm^2 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 /cm^2 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 10^5) of bacteria applied over the 3 h. During this time, all the other strains appeared to translocate, in excess of 1x10^6 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 uM Transwell® filters for 14 days, by which time the TER were routinely greater than 1000 Ω /cm^2. 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

299 included as a positive control. The drop in TER following infection with the *B.*
300 *cenocepacia* strain, BC7, was found to be comparable with that of both *P. pulmonicola*
301 isolates, RL7177 and RL8228. The TER at 4 h fell to 64% $\pm$ 14 following infection with
302 BC7 (excluded from Fig 3b for clarity), compared to 59 % for RL7177 and 66 % for
303 RL8228.
304
305 ***Pandoraea* isolates secrete proteases but these are not involved in larval killing.**
306 Protease secretion by *Pandoraea* strains could play a role in disruption of tight junctions
307 or general lung tissue damage; therefore we wanted to determine the levels of protease
308 secretion in a selection of the clinical Irish isolates and other strains. The protease
309 activities of a range of *Pandoraea* isolates were examined on 3% or 10% skimmed milk
310 agar plates together with *E. coli* NCIB9485 and *B. cenocepacia* strain BC7 which were
311 used as a positive controls in these studies (Gingues *et al.*, 2005; Swamy & Goldberg,
312 1982). All Irish *P. pulmonicola* isolates examined produced significant levels of protease
313 activity. This level was comparable to all other *Pandoraea* strains examined and was
314 also comparable to the levels of protease activity of both the *E. coli* strain, NCIB9485
315 and the *B. cenocepacia* strain, BC7 (Fig. 4).
316
317 The role of proteases in pathogenesis was examined by injecting CFS from a series of
318 *Pandoraea* isolates into the *G. mellonella* model. In contrast to the killing of larvae by
319 whole cells, when CFS were administered to the larvae, no killing was observed by
320 *Pandoraea* strains examined up to 1 week post-injection. This clearly indicates that in
321 general cellular components rather than secreted factors play a role in killing among the

322 *Pandoraea* genus. This contrasts with the effects of CFS from *B. cenocepacia* strain
323 J2315 (Fig 5A) which resulted in significant killing of *G. mellonella* larvae within 72 h
324 (70%). Interestingly, in spite of the lack of effect on survival, the CFS of certain strains,
325 e.g. *P. pulmonicola* strains, LMG18107 and LMG18108, and *P. pnomenusa* strain, LMG
326 18087, had a reversible pathogenic effect on the larvae. The larvae underwent
327 melanisation within 30 minutes (Fig 5C) and subsequently recovered without any effect
328 on survival. This melanisation was never observed in the control larvae injected with LB
329 broth only (Fig 5B), however, melanisation always preceded death of larvae that were
330 injected with whole cells (Fig 5D).
331
332

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^{o}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

356 is clear that the *P. pulmonicola* strains were among the most virulent of the three species
357 tested in this model, while other species, *P. apista* and *P. pnomenusa* were only weakly
358 virulent (Table 1).

359 *P. pulmonicola* was also the species in which the majority of strains displayed a
360 potential for epithelial cell uptake (Caraher *et al.*, 2008), therefore, in order to further
361 investigate invasion as a mechanism of virulence within these *Pandoraea* strains, we
362 compared invasion in cells with, and without, a CF phenotype. Although the magnitude
363 of invasion into CFBE cells was less in general, the pattern of invasion (i.e. invasive or
364 not) of *Pandoraea* isolates into both independent cell lines was comparable with invasion
365 of A549 cells. In particular, two Irish *P. pulmonicola* isolates, RL0345 and RL8228,
366 which showed considerable invasive capacity of A549 cells, were also found to be
367 significantly invasive in both 16HBE14o$^-$ and CFBE41o$^-$ epithelial cell models. This
368 demonstrates the ability of certain *Pandoraea* isolates to invade three independent lung
369 epithelial cells and indicates that invasion is strain-specific rather than cell phenotype-
370 dependent. The lack of enhanced susceptibility to invasion of cells with a CF phenotype
371 over non-CF cells would agree with a recent summary of nine *Pandoraea* bacteraemia
372 cases reported (Pimentel & MacLeod, 2008), at least three of which were identified as
373 being in non-CF patients. The finding that *P. pulmonicola* isolate, RL8228, was both
374 more invasive of human lung cells and more virulent to larvae than the earlier isolate
375 (RL7177) from the same patient, suggests that invasion plays a role in pathogenesis for
376 some members of the species. However, the finding that two other *P. pulmonicola*
377 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;

401 McKevitt & Woods, 1984). In addition, serine proteases have also been identified in *B.*
402 *cenocepacia*, and although one of these has been shown to play a role in iron acquisition,
403 their roles in pathogenicity have not been confirmed (Flannagan *et al.*, 2007; Whitby *et*
404 *al.*, 2006). The relatively high levels of general protease secretion across the genus are
405 interesting, and the identity of these proteases is currently being investigated. It remains
406 a possibility that specific proteases may play a role in virulence of *Pandoraea* species.
407 Overall, the lack of effect of CFS on *G. mellonella* survival for any species within the
408 genus, indicates that the whole cells are the main mediator of virulence rather than
409 secreted exoproducts strengthening the suggestion for direct interaction between the
410 bacterial cells and the host cells as part of the virulence process.

411 This study shows that the genus *Pandoraea* is quite divergent in terms of their
412 virulence characteristics, indeed, it was named for the potential Pandora's box of genetic
413 diversity associated with these organisms (Vandamme *et al.*, 2000). In general, *P.*
414 *pulmonicola* strains are the most invasive and also the majority of strains from this
415 species showed the most virulence in the *in vivo* model, however, to the best of our
416 knowledge, there have been no reported cases of *P. pulmonicola* related bacteraemia.
417 Given the small numbers involved, it is difficult to correlate *in vitro* behaviour with
418 clinical outcomes in patients; however, it is significant that all *P. pulmonicola* colonised
419 patients from which the isolates in this study were obtained, have died. Further studies
420 would be required on much greater numbers to draw any definite conclusions.

421 *P. apista* strains examined showed the lowest virulence in *G. mellonella,* were
422 non-invasive and *P. apista* strain LMG16408 was the slowest to translocate across the
423 polarised lung epithelium, however, this species has been associated with at least two

424 cases of septicaemia (Daneshvar *et al.*, 2001; Johnson *et al.*, 2004). Only one *P.*
425 *pnomenusa* strain, LMG18087, was included in this study and although invasive of lung
426 cells expressing CFTR, was not invasive in cells with a CF phenotype. This strain
427 showed the greatest level of trans-epithelial translocation among the four examined.
428 Interestingly, four cases of *P. pnomenusa* associated bacteraemia have been reported
429 (Daneshvar *et al.*, 2001; Stryjewski *et al.*, 2003). Indeed, all three isolates identified as *P.*
430 *pnomenusa* by Daneshvar *et al.*, were isolated from blood which led the authors to
431 suggest that this species might have increased potential for invasive disease (Daneshvar
432 *et al.*, 2001). The mechanism of *P. pnomenusa* translocation is not linked to tight
433 junction opening; therefore further analysis of this species is warranted.

434 The changes in sequential *P. pulmonicola* isolates, RL7177 and RL8228, are
435 noteworthy. The later isolate is more invasive of lung epithelial cells and more virulent
436 to larvae. Furthermore, in our previous study, we demonstrated that the early isolate,
437 RL7177 was the only *Pandoraea* strain out of 19 examined which was capable of biofilm
438 formation (Caraher *et al.*, 2008), suggesting that this strain also undergoes a mucoid to
439 non-mucoid change over time. This change from a mucoid to a non-mucoid state has
440 also been observed in Bcc isolates from chronically infected patients (Zlosnik *et al.*,
441 2008), indicating that the same phenotype change arises among *P. pulmonicola* isolates,
442 however, more sequential isolates would need to be studied to confirm this. In addition,
443 further study in the existing sequential isolates is being performed to elucidate the
444 apparent alteration in virulence over time.

445 Overall, it is clear that *P. pulmonicola* strains show comparable *in vivo* virulence
446 to *B. cenocepacia* strain J2315 in a *G. mellonella* model. A subset of *P. pulmonicola*

447 strains were shown to invade lung epithelial cells irrespective of CF status and four
448 strains examined showed the potential to translocate across the lung epithelium. In
449 addition, although protease secretion is a common feature among all strains examined,
450 there is no clear evidence to date that this may be a virulence mechanism in this genus.
451 It is likely that individual species within the *Pandoraea* genus have the potential to
452 express very different mechanisms of pathogenesis, as demonstrated by differences in
453 intracellular invasion, tight junction disruption and larval killing. This is comparable to
454 the situation in Bcc strains which appear to use different virulence mechanisms to
455 compete for survival in different environments and hosts (Uehlinger *et al.*, 2009). There
456 are limited clinical data published on *Pandoraea* infections, and the numbers of isolates
457 evaluated in this study are also limited, therefore it is difficult to draw clear conclusions
458 regarding the mechanisms of pathogenicity and the effects on patients. In addition,
459 major host-related factors are also at play and furthermore, given that many *Pandoraea*
460 colonised patients are also co-colonised with other pathogens, the interplay between
461 competing species must also be considered. However, it is clear that *P. pulmonicola*
462 does contribute to the deterioration of colonised patients and has a direct interaction with
463 lung epithelial cells.
464
465
466 **Acknowledgements:**
467 This study was supported by PRTLI Cycle 4 (supported by the European Union Regional
468 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.

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566
567

Legends:

568 Fig.1. Kinetics of larval killing by *Pandoraea* strains. Survival of *G. mellonella* at 24 h
569 (•), 48 h (o) or 72 h (▾) when infected with either *P. pulmonicola* strains, RL8228 (A),
570 RL0345 (B), LMG18108 (C) or *P. apista* strain, LMG 16408 (D). Each data set is from a
571 single trial (n=10) which was representative of three independent trials with the specified
572 strain. The data are presented as % larval survival from 10 larva injected per trial. No
573 more than one control larva died in any given trial.
574
575 Fig**. 2**. Comparison of invasion by *Pandoraea* strains in two lung epithelial cells,
576 16HBE14o$^-$ cells and CFBE41o$^-$, compared to the negative control *E. coli* strain,
577 NCIB9485. Bars represent % invasion (c.f.u. obtained after cell lysis as a percentage of
578 c.f.u. applied) of at least five independent experiments for 16HBE14o$^-$ cells and at least
579 three independent experiments for CFBE41o$^-$. Error bars represent ± SEM; * denotes
580 invasion significantly greater than *E. coli,* as determined by Dunn's multiple comparison
581 test ($p<0.05$).
582
583 Fig. 3. The translocation of *Pandoraea* strains through polarised Calu-3 epithelial
584 monolayers. A) Cumulative bacterial counts taken from basolateral chamber of Calu-3
585 cell monolayers grown on 3 μm Transwell® filters at 1, 2 and 3 h post infection with
586 *Pandoraea* strains expressed total CFU translocated /ml. The results are the average data
587 from at least two independent experiments ± SEM. B) The effect of *Pandoraea* strains
588 on TER at 0, 1, 2 and 4 h post-infection, presented as % of the control (1092 $\pm$ 225) and
589 representing the mean of at least three independent experiments, error bars represent ±
590 SEM.

591

592 Fig. 4. Protease secretion from *Pandoraea* species as determined on skimmed milk agar
593 plates. Bars represent zones of clearance (± standard deviation) measured following 48 h
594 incubation of *Pandoraea* cell free supernatants on 3% (black bars) or 10% (grey bars)
595 (v/v) skimmed milk plates from three independent experiments.

596

597 Fig. 5. The effects of cell free supernatant on survival of *G. mellonella*. A) The bars
598 represent % survival of 10 larvae at 24 h, 48 h, 72 h, respectively. Each data point
599 represents the mean of three independent experiments ± SEM; * $p<0.05$, relative to LB
600 injected control group. B-D) Effect of *P. pulmonicola* RL7177 CFS on *G. mellonella*, B)
601 control larvae 30 min post-injection with TSB only; C) Larvae 30 min post-injection
602 with CFS from LMG 18108; D); Larvae 48 h post-injection with whole cells, indicating
603 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.

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

LD_{50} 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.

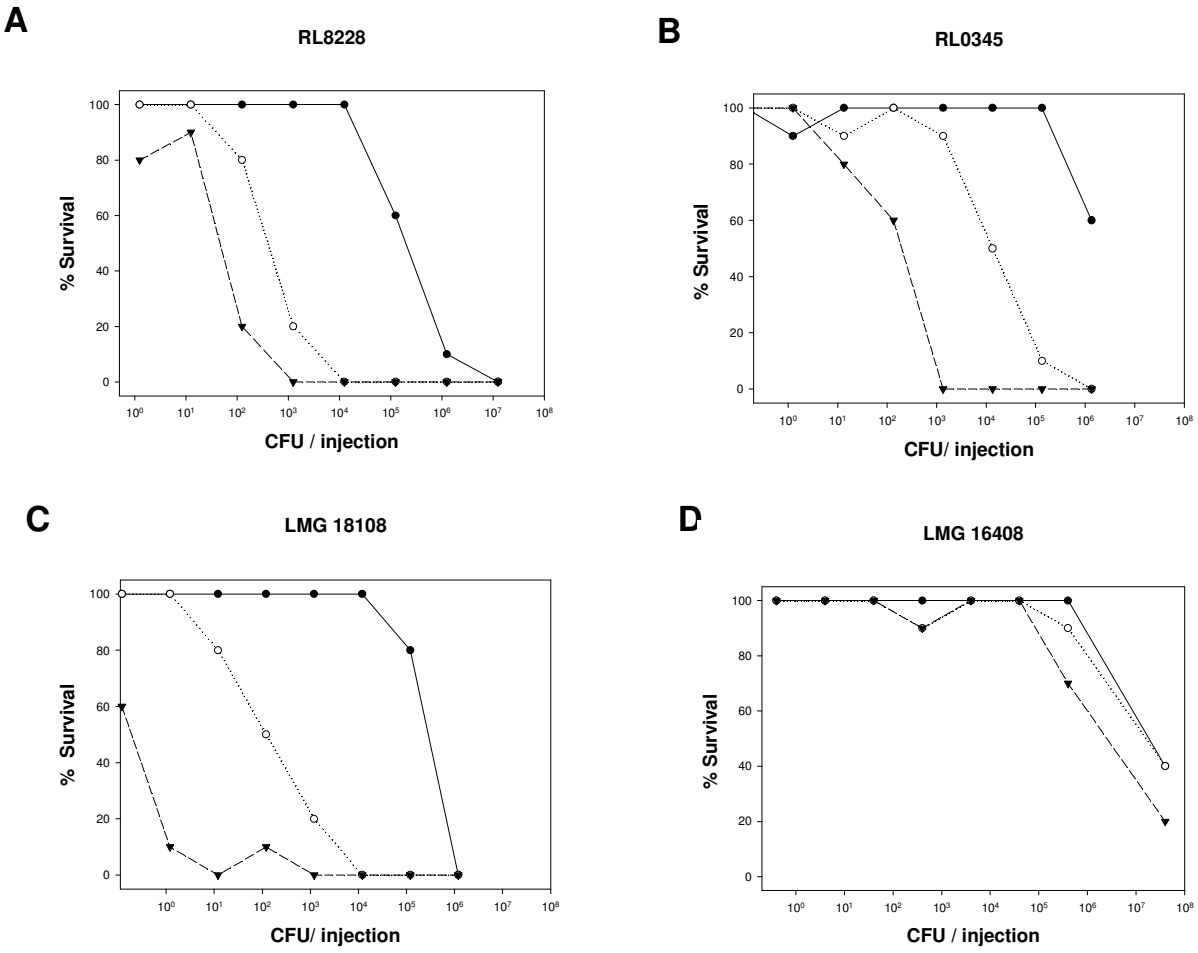

A
RL8228
% Survival
CFU / injection
B
RL0345
% Survival
CFU/ injection
C
LMG 18108
% Survival
CFU/ injection
D
LMG 16408
% Survival
CFU / injection

Figure 2.

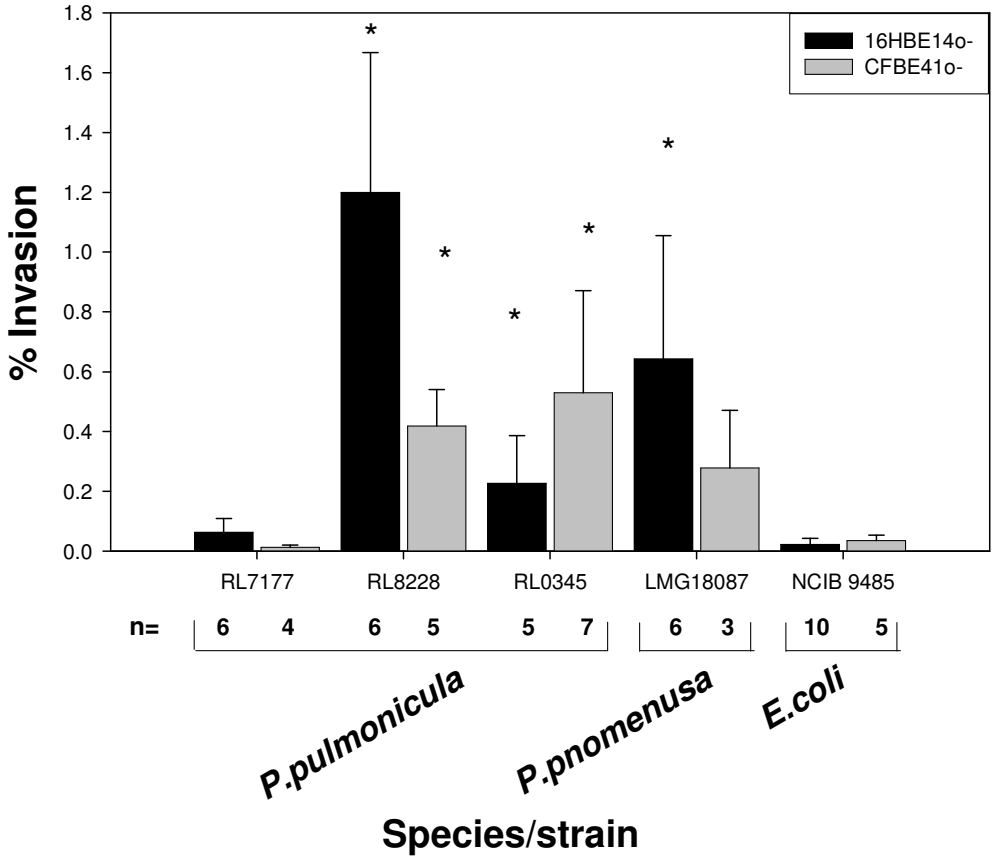
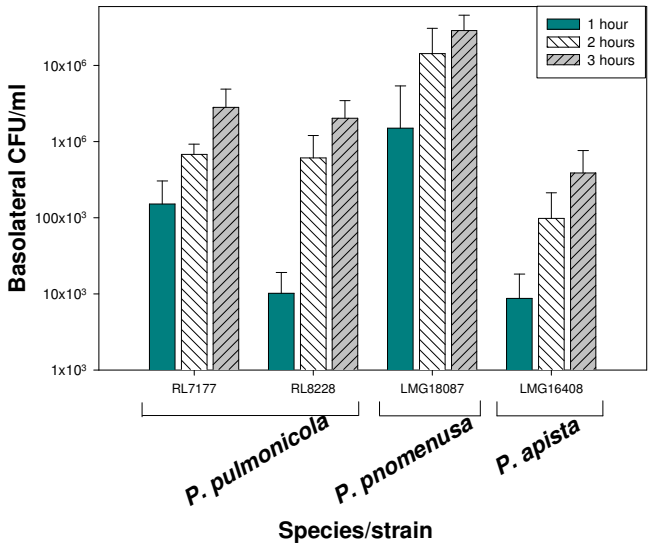

16HBE14o-
CFBE41o-
% Invasion
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
RL7177
RL8228
RL0345
LMG18087
NCIB 9485
n=
6 4 6 5 5 7 6 3 10 5
P.pulmonicula
P.pnomenusa
E.coli
Species/strain

Figure 3.

A


1 hour
2 hours
3 hours
Basolateral CFU/ml
10x10⁶
1x10⁶
100x10³
10x10³
1x10³
RL7177
RL8228
LMG18087
LMG16408
P. pulmonicola
P. pnomenusa
P. apista
Species/strain

B

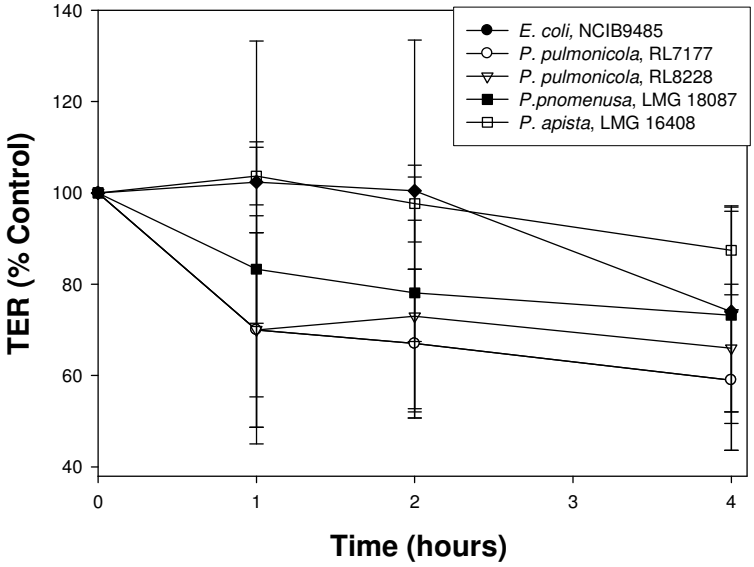

E. coli, NCIB9485
P. pulmonicola, RL7177
P. pulmonicola, RL8228
P.pnomenusa, LMG 18087
P. apista, LMG 16408
TER (% Control)
140
120
100
80
60
40
0
1
2
3
4
Time (hours)

Figure 4.

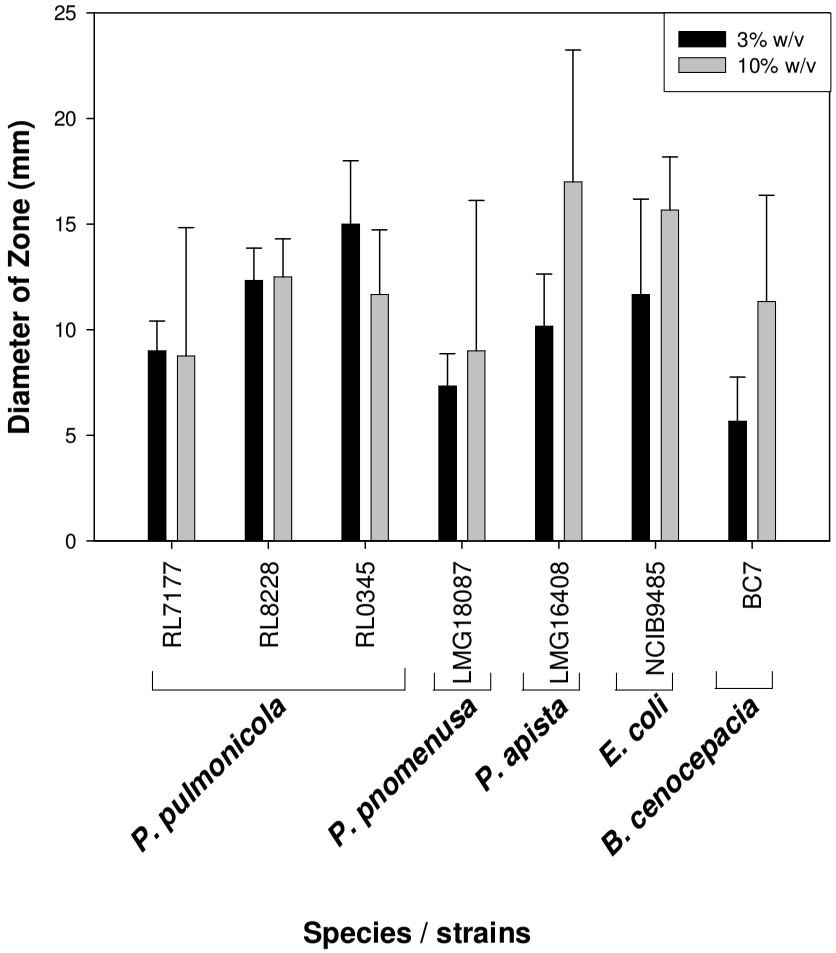
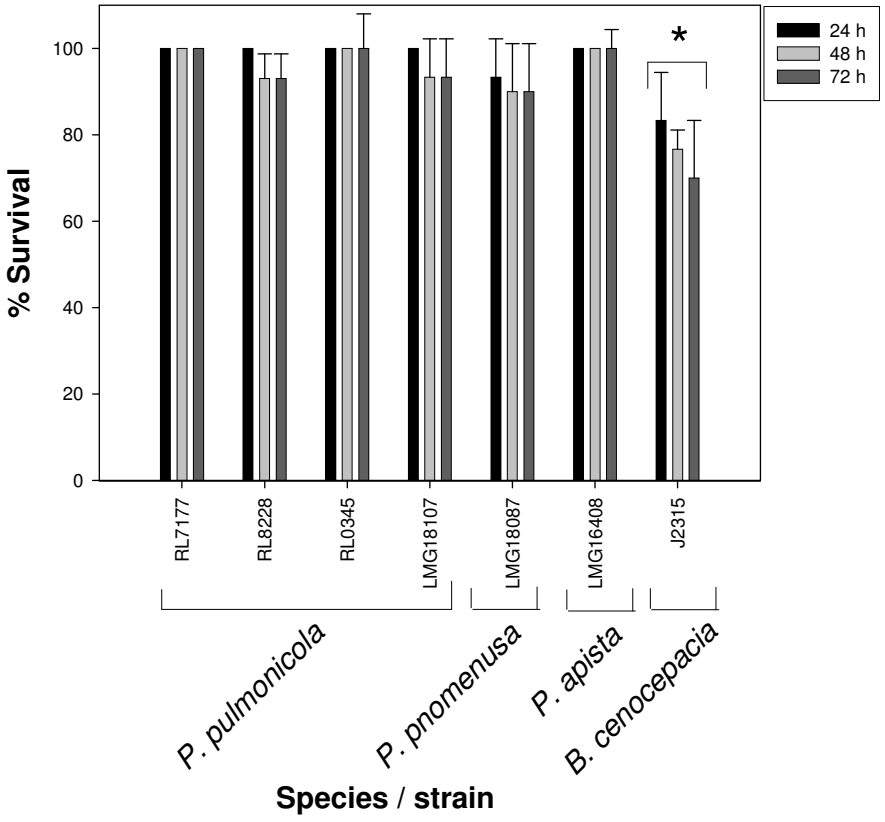
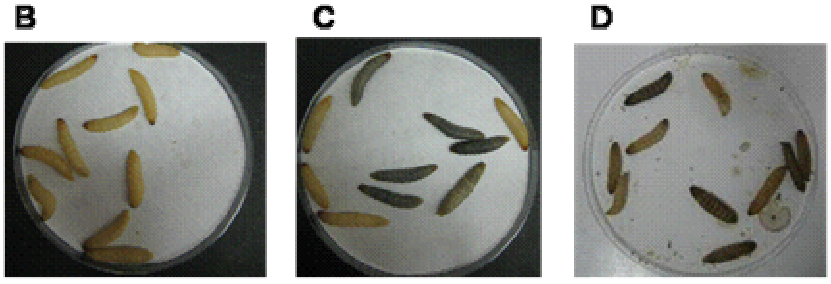

25
20
15
10
5
0
Diameter of Zone (mm)
3% w/v
10% w/v
RL7177
RL8228
RL0345
LMG18087
LMG16408
NCIB9485
BC7
P. pulmonicola
P. pnomenusa
P. apista
E. coli
B. cenocepacia
Species / strains

Figure 5.

A


24 h
48 h
72 h
*
100
80
60
40
20
0
% Survival
RL7177
RL8228
RL0345
LMG18107
LMG18087
LMG16408
J2315
P. pulmonicola
P. pnomenusa
P. apista
B. cenocepacia
Species / strain


B
C
D