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Evaluation of *in vitro* virulence characteristics of the genus *Pandoraea* in lung epithelial cells

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Pandoraea species are emerging opportunistic pathogens capable of causing chronic lung infections in cystic fibrosis patients. This study examined the interactions of 17 *Pandoraea* isolates from the five identified species (*Pandoraea apista*, *Pandoraea norimbergensis*, *Pandoraea pulmonicula*, *Pandoraea sputorum* and *Pandoraea pnomenusa*) plus two *Pandoraea* genomospecies isolates with lung epithelial cells and their ability to form biofilms *in vitro*. Only three isolates showed an ability to invade A549 lung epithelial cells, and only one isolate was able to form biofilms. In contrast, all isolates triggered a pronounced pro-inflammatory response, with elevation of both interleukin (IL)-6 (two- to 19-fold) and IL-8 (10- to 50-fold) above that observed for a control strain of *Escherichia coli*. This property is likely to be a major factor in the pathogenesis of the genus.

INTRODUCTION

Cystic fibrosis (CF) is the one of the most common genetically inherited diseases in Caucasians, resulting in lung disease characterized by thickened mucus secretions in the lung, leading to recurrent and persistent respiratory infections. A hierarchy of opportunistic pathogens colonize the CF lung throughout life, often culminating in chronic infection with *Pseudomonas aeruginosa* and, less frequently, *Burkholderia cepacia* complex (Bcc) organisms. On occasion, other opportunistic Gram-negative bacteria are encountered, including various *Enterobacteriaceae*, *Achromobacter xylosoxidans* and *Stenotrophomonas maltophilia* (Gibson *et al.*, 2003). Although uncommon, members of the genus *Pandoraea* have been isolated from the lungs of CF patients. The genus comprises five named species, *Pandoraea apista*, *Pandoraea norimbergensis*, *Pandoraea pulmonicula*, *Pandoraea sputorum* and *Pandoraea pnomenusa* (Coenye *et al.*, 2000), and at least three other, as yet unnamed, genomospecies (Daneshvar *et al.*, 2001).

Pandoraea are motile, aerobic, non-fermentative, Gram-negative rods, but, as they are able to grow on Bcc-selective agar, they may be misidentified as Bcc organisms or *Ralstonia* spp., although they are taxonomically distinct from these (Coenye *et al.*, 2000). *Pandoraea* spp. have been isolated from the lungs and from blood cultures of CF patients, supporting an invasive role for this pathogen (Atkinson *et al.*, 2006; Daneshvar *et al.*, 2001), and from other patients (Daneshvar *et al.*, 2001; Stryjewski *et al.*, 2003), and some strains may be transmissible between patients (Jorgensen *et al.*, 2003). Colonization with *P. apista* has been associated with a decline in lung function in certain patients (Atkinson *et al.*, 2006; Jorgensen *et al.*, 2003) and in the formation of high antibody titres (Jorgensen *et al.*, 2003), but the impact and clinical significance of colonization with these organisms remains ambiguous.

A number of virulence factors have been associated with clinical virulence of *Ps. aeruginosa* and Bcc, including stimulation of pro-inflammatory cytokine secretion, biofilm formation and, in the case of Bcc, the ability to invade lung epithelial cells, which may contribute to the persistence of the strains in the CF lung (Burns *et al.*, 1996; Cieri *et al.*, 2002; Duff *et al.*, 2006). Indeed, biofilm formation by *Ps. aeruginosa* and Bcc has been specifically associated with increased resistance to antibiotics and maintenance of the bacteria in the lung (Caraher *et al.*, 2007a; Chernish & Aaron, 2003; Hoiby *et al.*, 2001; Yu & Head, 2002). Here, we report the results of studies on the interaction of a series of *Pandoraea* strains with lung epithelial cells and their ability to form biofilms *in vitro*, in

Abbreviations: Bcc, *Burkholderia cepacia* complex; CF, cystic fibrosis; IL, interleukin; MBC, minimum bactericidal concentration.

order to gain insight into their virulence potential in CF lung infections.

METHODS

Bacterial strains and growth. The origins of the *Pandoraea* strains are listed in Table 1. The non-Irish isolates were purchased from BCCM/LMG. *E. coli* strain NCIB 9485 was used as a negative control in all experiments. All isolates were routinely grown on Luria–Bertani (LB) agar or in LB broth at 37 °C, and growth curves were determined by incubating bacteria in LB broth (pH 7.0) at 37 °C with agitation at 200 r.p.m. Aliquots were removed at specific time intervals and OD_{600} was measured. Serial dilutions were made and aliquots (50 µl) were plated on LB agar plates, which were incubated at 37 °C for 48 h to determine viable counts, expressed as c.f.u. ml^{-1}.

Cell lines. A549 cells (passages 89 to 109) were purchased from the European Collection of Cell Cultures (ECACC) and maintained in RPMI 1640 supplemented with 5 mM L-glutamine and 5 mM HEPES with 10 % fetal bovine serum, without antibiotics.

Measurement of interleukin (IL)-6 and IL-8. Cells were seeded on 24-well plates (4×10^5 cells per well), cultured for 24 h at 37 °C in 5 % CO_2 and incubated with test strains at an m.o.i. of 50 bacteria per cell for 24 h. Cells were washed, the medium was replaced and plates were incubated for a further 24 h. The supernatants were removed, filtered through a 0.45 µm pore-size membrane to remove bacteria, and frozen prior to ELISA. The levels of IL-6 and IL-8 in supernatants (diluted 1 : 10) were determined by ELISA (R&D Systems) according to the manufacturer's instructions.

Table 1. Origins of the *Pandoraea* strains used in this study

Species/isolate	Source	Doubling time (h)
P. apista		
LMG 16407^T	CF lung (Denmark)	1.23
LMG 16408	CF lung (Denmark)	0.88
LMG 16411	CF lung (Denmark)	1.44
LMG 16409	CF lung (Denmark)	1.12
9963/95	CF lung (Denmark)	1.26
9967B/97	CF lung (Denmark)	1.15
P. pulmonicula		
LMG 18107	CF lung (USA)	0.80
LMG 18108	CF lung (USA)	1.63
RL0345	CF lung (Ireland)	0.72
RL0308	CF lung (Ireland)	2.20
S8228	CF lung (Ireland)	1.48
S7633	CF lung (Ireland)	0.86
S7177	CF lung (Ireland)	1.02
P. pnomenusa		
LMG 18087^T	CF lung (UK)	1.00
LMG 18817	CF lung (USA)	0.82
P. norimbergensis		
LMG 13019	Blood culture (Belgium)	1.10
P. sputorum		
LMG 18819^T	CF lung (USA)	1.12
Pandoraea genomospecies		
S0293	CF lung (Ireland)	0.88
S8166	CF lung (Ireland)	1.58

Antibiotic susceptibility tests. In order to establish the appropriate antibiotic concentrations required to kill extracellular bacteria in cell invasion assays, the MIC and minimum bactericidal concentration (MBC) were determined following exposure of strains to antibiotic for 2 h. An inoculum of 10^6 c.f.u. ml^{-1} of each strain was added to a 96-well plate containing serial dilutions of antibiotic in LB broth. The plate was incubated at 37 °C for 24 h and the OD_{600} was read to give the MIC. In addition, 50 µl of the cultures that had been exposed to antibiotic for 2 h was spread on LB agar and incubated for 48 h to establish the MBC of the antibiotic.

Invasion of epithelial cells. Invasion studies were carried out on A549 epithelial cells using an adaptation of the method of Martin & Mohr (2000). Cells were seeded on 24-well plates (4×10^5 cells per well), cultured for 24 h at 37 °C in 5 % CO_2 and incubated with bacterial suspensions at an m.o.i. of 50 for 2 h. The cells were washed three times with PBS and treated with 1 mg amikacin ml^{-1} for 2 h to kill any remaining extracellular bacteria. Intracellular bacteria were released by lysis with 0.5 % Triton X-100/50 mM EDTA and invasion was quantified by viable counts on LB agar. The percentage invasion was determined as follows: (c.f.u. ml^{-1} recovered from lysed cells/ c.f.u. applied to cells) × 100. Prior to lysis, the medium was removed and cultured in LB to ensure that no extracellular bacteria survived the antibiotic treatment.

Biofilm formation. Biofilm formation was examined by determining the ability of *Pandoraea* strains to form biofilms on microtitre plates (Caraher *et al.*, 2007a). Briefly, LB broth (100 µl per well) was inoculated with mid-exponential phase cultures (OD_{600} ~0.6) of individual strains at 1×10^6 or 1×10^7 c.f.u. ml^{-1} in quadruplicate. The plates were incubated at 37 °C for the specified times, rinsed thoroughly with water to remove non-adherent bacteria and air-dried. Crystal violet (1 %, w/v) was added to each of the wells at ambient temperature for 30 min, the plates were rinsed thoroughly and bound dye was dissolved with 95 % ethanol containing 0.05 % Triton X-100. The solubilized dye (100 µl) was transferred to a new polystyrene microtitre plate and A_{590} was determined in a Tecan plate reader. Biofilm formation was defined as those wells that had $A_{590}>0.05$.

Statistical analysis. Comparison of the invasiveness of the strains against the negative control was carried out using the Holm–Sidak method for multiple comparisons. Values of $P<0.05$ were deemed to be significant in all comparisons.

RESULTS AND DISCUSSION

Pandoraea strains elicit a potent pro-inflammatory response

Chronic lung inflammation is largely responsible for the morbidity in CF. In particular, accumulation of pro-inflammatory cytokines such as IL-8 and IL-6 results in lung tissue damage (Gibson *et al.*, 2003). We examined whether *Pandoraea* strains were able to trigger such a response, which could contribute to their pathogenesis in the lung. All 19 *Pandoraea* strains examined stimulated pro-inflammatory cytokines, in particular IL-8. Secretion of IL-8 exceeded 10 ng ml^{-1} following exposure of A549 cells to all strains, with the exception of *P. norimbergensis* strain LMG 13019 and *Pandoraea* genomospecies S8166 (Fig. 1a). These latter strains, however, also elicited a significant host response, with secretion of IL-8 over nine

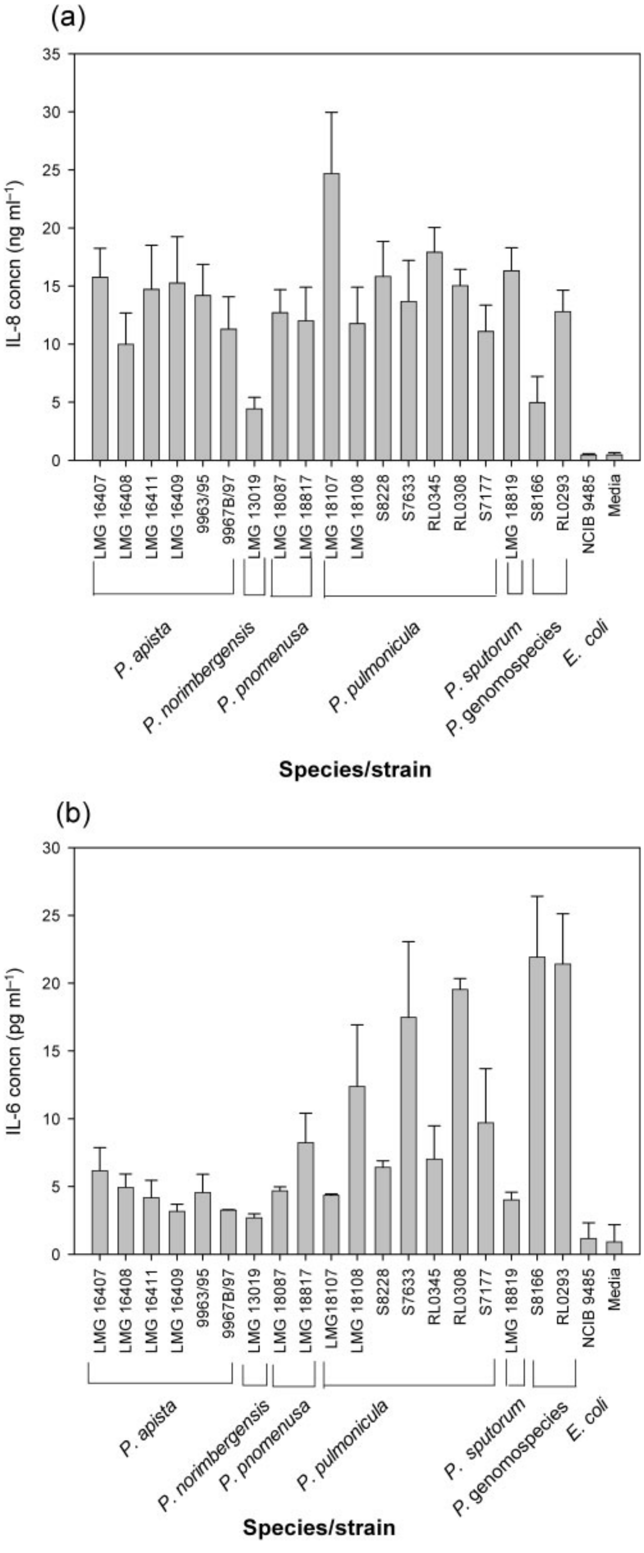

Fig. 1. Secretion of proinflammatory cytokines IL-8 (a) and Il-6 (b) from lung epithelial cells following stimulation with *Pandoraea* strains or an *E. coli* strain, or with medium only. Cytokine levels were measured by ELISA and results are expressed as mean ± SD of three independent assays carried out in duplicate.

times greater than that observed for *E. coli*. All *Pandoraea* strains also stimulated IL-6 secretion (Fig. 1b), although levels of secretion of this cytokine were less dramatic than those of IL-8. Maximal levels were secreted by *P. pulmonicula* strain RL0308 and by *Pandoraea* genomospecies isolates S8166 and RL0293 (17- to 19-fold greater than the levels secreted by *E. coli*) and were lowest in *P. norimbergensis* isolate LMG 13019 (twofold greater than *E. coli*). This finding suggests that this is a potential mechanism of pathogenesis for these organisms. Comparable increases in IL-8 and IL-6 production have been observed following colonization with the CF pathogens *Ps. aeruginosa* and Bcc (Bonfield *et al.*, 1995; Palfreyman *et al.*, 1997) and further amplify the inflammatory response in the lungs of CF patients. Indeed, the IL-8 secretion elicited by Bcc isolates is of the same order as that observed for *Pandoraea* in this study (Palfreyman *et al.*, 1997). However, whilst there was much more variability in IL-8 secretion among different species within the Bcc, the levels induced by the *Pandoraea* strains in this study were quite comparable.

IL-6 and IL-8 are constitutively upregulated in the CF airway and both are found early in nasal and bronchoalveolar lavage fluids of infants and children with CF compared with those without the defect (Khan *et al.*, 1995; Muhlebach & Noah, 2002; Noah *et al.*, 1997). Upregulation of these cytokines in CF patients has been shown to lead to increased neutrophil recruitment and further enhancement of inflammation in the lung (De Rose, 2002), whilst IL-6 is also important for the augmentation of antibody production and the acute-phase response (Khair *et al.*, 1996) The dramatic expression of these two interleukins by all *Pandoraea* strains examined indicates that this is an important mechanism of virulence for this genus and is likely to contribute to the pathogenesis of colonized CF patients. Many patients colonized with *Pandoraea* are co-colonized with other CF pathogens, in particular *Ps. aeruginosa* (Jorgensen *et al.*, 2003), and the stimulation of inflammatory effects by *Pandoraea* would most likely aggravate the chronic inflammation that results from infection with other pathogens.

Amikacin is a suitable antibiotic for invasion assays

The integrity of the *in vitro* invasion assay depends on the killing of all extracellular bacteria prior to cell lysis. We previously examined invasion of Bcc strains with a combination of ceftazidime and amikacin (Caraher *et al.*, 2007a; Duff *et al.*, 2006), and therefore in this study we tested all strains for susceptibility to these agents (1 mg ml^{-1} each). All strains except for one *P. apista* strain (9967B/97) were killed by concentrations of 512 μg amikacin ml^{-1} or less following a 2 h exposure, but the majority of strains survived exposure to ceftazidime (not shown). As a result, only amikacin (1 mg ml^{-1}) was used in the invasion assays to kill extracellular *Pandoraea* strains.

The amikacin-resistant *P. apista* strain was therefore excluded from the invasion experiments.

Invasion of A549 cells by *Pandoraea* strains

Invasion of epithelial cells can confer advantages on the pathogen, including the avoidance of host defence processes and protection from antimicrobial agents. Bcc organisms have the ability to invade lung epithelial cells (Cieri *et al.*, 2002; Duff *et al.*, 2006), and the levels of invasion of A549 cells were found to correlate with the virulence of strains in an *in vivo* mouse infection model (Cieri *et al.*, 2002). We found that only three of the 18 *Pandoraea* strains were able to invade A549 lung epithelial cells (Fig. 2), i.e. gave percentage invasion values significantly different from that obtained for the negative-control *E. coli* strain ($P<0.05$ for RL0308, RL0345 and S8228). All three of these strains were *P. pulmonicola*. Two other strains appeared to be moderately invasive (*P. pulmonicola* S7177 and *P. pnomenusa* LMG 18087), but values were not significantly different from the control ($P=0.05$). The remainder were classified as non-invasive.

The level of invasiveness of the three strains described above for A549 cells was comparable to that observed for a *Burkholderia multivorans* strain (LMG 13010) and a piliated *Burkholderia cenocepacia* strain (C5424) (Duff *et al.*, 2006). Overall, the ability to invade cells is not common among *Pandoraea* and therefore is unlikely to be a major contributor to strain virulence, in contrast to Bcc strains. The small number of invasive isolates and the fact that the patients were co-colonized with other pathogens make it difficult to correlate invasiveness with clinical outcome in patients. However, one of the invasive isolates, RL0345, was acquired from a pre-transplant patient who is doing well post-transplant. This patient was co-colonized with *Ps. aeruginosa* but experienced significant decline in the first 12 months following first identification with *Pandorae*a (forced expiratory volume in 1 s declined from 75 to 55 %, body mass dropped from 45 to 38 kg). The other invasive *P. pulmonicula* isolate, RL0308, was isolated from a patient who was also co-colonized with *Ps. aeruginosa* and who subsequently died, aged 19 years (J. Collins, unpublished data). The third invasive isolate, S8228, was identified in an Irish patient who experienced no clinical deterioration following *Pandoraea* colonization.

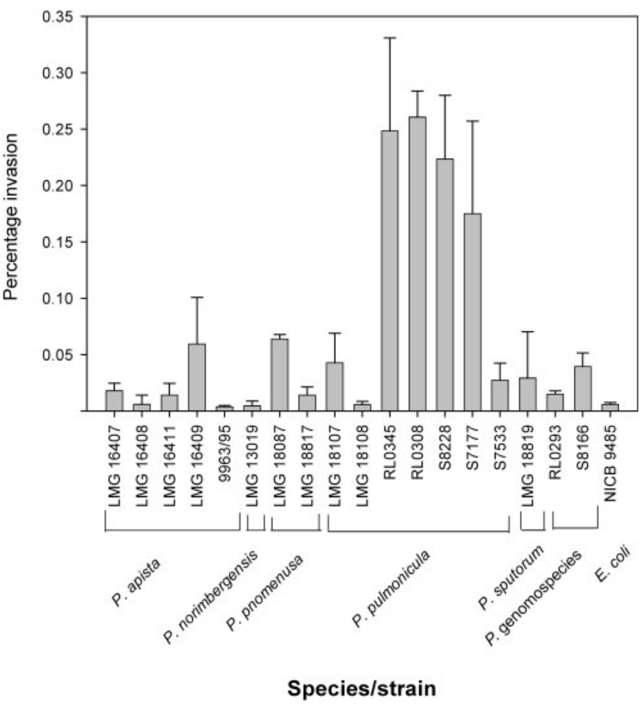

Fig. 2. Invasion of A549 lung epithelial cells by *Pandoraea* strains compared to that of *E. coli* strain NCIB 9485. Strains were applied to the cells for 2 h, extracellular bacteria were killed with 1 mg amikacin ml^{-1}, and the c.f.u. of internalized bacteria was determined and expressed as a percentage of the bacteria applied (2×10^7 cells). Results are shown as mean percentage invasion ± SEM for three independent assays.

Pandoraea does not readily form biofilms

Both Bcc and *Ps. aeruginosa* form strong biofilms, which have been associated with increased antibiotic resistance (Caraher *et al.*, 2007b; Desai *et al.*, 1998; Johnson *et al.*, 2004; Moskowitz *et al.*, 2004). We found only one *Pandoraea* strain, *P. pulmonicula* S7177, that was capable of forming biofilms (Fig. 3). This property was not related to the growth rate of the strain, as its doubling time of 61 min was comparable to that of other isolates that did not form biofilms (Table 1). Another *P. pulmonicula* strain, RL0345, formed a weak biofilm, with A_{590} values just above the threshold of 0.05 at all time points examined. The OD values of all other strains were <0.05 at 72 h (not shown). These findings suggest that biofilm formation is not a major virulence factor or a likely source of antimicrobial resistance *in vivo*.

Despite its emergence in CF patients, little has been published on the disease burden and pathogenicity of *Pandoraea* spp. Chronic lung infection of six patients with a single clone of *P. apista* was associated with decreased lung function (Jorgensen *et al.*, 2003) and two different *P. apista* strains were implicated in the deterioration of lung function in two patients, but it was unclear whether this was due directly to the presence of these organisms (Atkinson *et al.*, 2006). *P. apista* was also identified in blood cultures and sputum of a 16-year-old CF patient (Johnson *et al.*, 2004).

P. pnomenusa, however, was identified as the causative agent of sepsis and subsequent multiple organ failure in a post-transplant pulmonary sarcoidosis patient (Stryjewski *et al.*, 2003). Furthermore, cross-infection with *Pandoraea* spp. has been reported in CF centres (Coenye *et al.*, 2001; Jorgensen *et al.*, 2003). *Pandoraea* has also emerged recently in the Irish CF population, with *Pandoraea pulmonicula* and *Pandoraea*

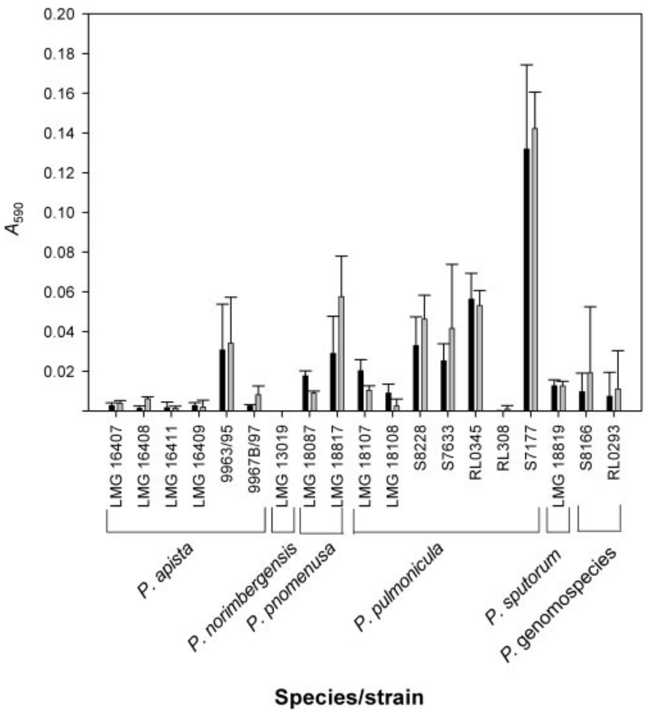

Fig. 3. Biofilm formation by members of the genus *Pandoraea* at 24 and 48 h. Strains were inoculated at a concentration of 10^7 c.f.u. ml^{-1} in 96-well plates, and the biofilms were stained at 24 h (filled bars) and 48 h (shaded bars) with crystal violet. Bars represent the mean ± SEM of three independent experiments.

genomospecies being the most predominantly identified (J. Collins and others, unpublished data). However, consistent with experience elsewhere, all *Pandoraea*-colonized Irish patients were co-colonized with other CF pathogens, making it difficult to draw correlations between clinical outcome and the *in vitro* analysis.

In conclusion, whilst a minority of *Pandoraea* strains examined were able to invade lung epithelial cells, the most dramatic and universal effect was the stimulated production of proinflammatory cytokines. Further studies are necessary to establish the significance of this property in lung infection in CF patients.

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