Protease IV production in *Pseudomonas aeruginosa* from the lungs of adults with cystic fibrosis

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Protease IV is important in the pathogenesis of *Pseudomonas aeruginosa*-induced microbial keratitis, but little is known of its role in cystic fibrosis (CF) lung infection. In this study protease IV production was examined in 43 *P. aeruginosa* isolates (24 non-clonal and 19 clonal) from the lungs of chronically infected adult patients attending the Royal Prince Alfred Hospital CF Clinic, Sydney, Australia. Overall, 32/43 (74 %) isolates were positive for protease IV protein by Western blotting and 22/43 (51 %) had evidence of active protease IV on gelatin zymography. Clonal strains were 1.6 times more likely than non-clonal strains to produce protease IV [18/19 (95 %) versus 14/24 (58 %), RR = 1.6, CI 1.1–2.3, $P = 0.007$] and 3 times more likely to secrete the protein [16/19 (84 %) versus 6/24 (25 %), RR = 3.4, CI 1.6–6.9, $P < 0.001$]. Nine of the ten strains negative by both Western blotting and zymography were non-clonal, and all but one of these was positive for the protease IV gene. There was a marked strain-to-strain variation in the amount of protease IV produced. Secretion of protease IV by clonal strains may enhance their infectivity and ability to adapt to the changing CF lung environment. Overall the findings suggest that protease IV plays an important role in the pathogenesis of *P. aeruginosa* infection in the CF lung.

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INTRODUCTION

Pseudomonas aeruginosa lung infection is a major cause of morbidity and mortality in patients with cystic fibrosis (CF) (Mellins *et al.*, 1968). The pathogenesis of *P. aeruginosa* infection depends on a broad spectrum of virulence factors, including proteases. Proteases such as elastase (LasB), alkaline protease (AprA) and staphylolysin (LasA) have been characterized in many clinical settings, including the CF lung (Goodman & Lory, 2004). Much less is known of

the distribution and properties of the recently described protease IV (PrpL) (O'Callaghan *et al.*, 1996).

Protease IV, a 26 kDa serine endoprotease (O'Callaghan *et al.*, 1996; Engel *et al.*, 1998a), is secreted by most *P. aeruginosa* isolates causing microbial keratitis and in association with other proteases has a major role in corneal virulence (O'Callaghan *et al.*, 1996; Engel *et al.*, 1997, 1998b; Wilderman *et al.*, 2001). The virulence of protease IV in ocular infection has been attributed to the destruction of host proteins, including fibrinogen and components of the immune system (Engel *et al.*, 1998a). Protease IV also degrades structural proteins such as elastin, facilitating bacterial adhesion and infection. Limited *in vitro* data

Abbreviations: CF, cystic fibrosis; CI, confidence interval; RR, relative risk.

suggest that protease IV may contribute to acute lung injury induced by *P. aeruginosa* through loss of surfactant function (Malloy *et al.*, 2005). There is little information on protease IV secretion by clinical lung isolates.

P. aeruginosa has a unique relationship with the CF lung. Infections in early childhood are often acute and transient, and virulence factors, including the proteases, are expressed at high levels (Jain *et al.*, 2004). However, over time the organism undergoes changes that enable it to persist indefinitely in the face of aggressive antibiotic therapy (Jain *et al.*, 2004). These changes include down-regulation of virulence factors required for acute infection, such as the proteases, and an up-regulation of genes required for biofilm development.

It is widely accepted that most CF patients acquire unique (non-clonal) *P. aeruginosa* strains from the environment, although clonal *P. aeruginosa* strains have been reported with increasing frequency in recent years (Jones *et al.*, 2001; McCallum *et al.*, 2001; Scott & Pitt, 2004). Two clonal strains, the Australian epidemic strain (AES)-1 (formerly known as the Melbourne epidemic strain, MES, m16 or PI) and AES-2 (PII) currently infect from 10 to 40 % of patients in six CF clinics in eastern Australia (Armstrong *et al.*, 2003; O'Carroll *et al.*, 2004; H. Service, C. Lee, B. Rose, M. Elkins, P. Bye & C. Harbour, unpublished data). The properties conferring the enhanced colonization of clonal strains have not been defined. The major aim of this study was to examine protease IV production in a series of clonal and non-clonal *P. aeruginosa* isolates from the chronically infected CF lung.

METHODS

Investigations were carried out on 43 *P. aeruginosa* isolates from 43 patients attending the adult CF clinic at the Royal Prince Alfred Hospital, Sydney, Australia between 2001 and 2004. The study was approved by the Institutional Ethics Committee (approval X02-0320). Subjects gave informed consent to the work. The male to female ratio was 21:22; the mean age was 27 years and the age range 18–58 years. All patients had been chronically infected with *P. aeruginosa* for a minimum of 4 years. Eighteen of the forty-three (42 %) isolates were derived from sputa collected at the time of exacerbation of symptoms and the remaining twenty-five (58 %) at routine clinic visits. The proportions within the clonal and non-clonal groups were similar. Genotyping by PFGE carried out using our published methods (Anthony *et al.*, 2002) showed that 24 were non-clonal strains and that 19 were clonal (14 AES-1 and 5 AES-2). The proportion of clonal versus non-clonal strains was consistent with the distribution of strains in this clinic.

Bacteria were grown in tryptone soya broth to late exponential phase ($OD_{600} \approx 1.6$) at 37 °C with rapid agitation. The wound-derived *P. aeruginosa* reference strain PAO1 (Holloway *et al.*, 1979) was used as a control. Supernatants were collected and filtered through a 0.22 µm filter to remove any remaining cells. Cell suspensions were used for the detection of the *prpL* gene.

Western blotting. Western blotting to detect protein was carried out on 21 µl supernatant using standard techniques. Blots were incubated overnight with rabbit polyclonal anti-protease IV antibody

(1:5000) (kindly donated by Brett Thibodeaux, Department of Microbiology, Immunology and Parasitology, Louisiana State University, Medical Center, New Orleans, LA, USA) and bands then imaged using a calibrated densitometer and QuantityOne software (Bio-Rad). All isolates were examined twice on different occasions.

Gelatin zymography. Gelatin zymography to assess protease IV activity was carried out on 21 µl supernatant using 7.5 % polyacrylamide gels containing 0.1 % (w/v) gelatin (Zhu *et al.*, 2002). Zymograms were imaged using a calibrated densitometer and QuantityOne software (Bio-Rad). All isolates were tested twice on different occasions.

PCR for the protease IV gene. DNA was extracted from cell suspensions using the *microLYSIS* kit (Microzone). PCR was carried out using combinations of two sets of primers covering different parts of the protease IV gene: those described by Cabellero and colleagues (Caballero *et al.*, 2004) (designated here as CabF and CabR) and the in-house designed primers 5'-TATTTCCGCCGACTCC-CTGTA-3' (TCF) and 5'-GAATAGACGCCGCTGAAATC-3' (TCR). The CabF/TCR combination yielded a 353 bp product, the TCF/CabR combination a 780 bp product and the TCF/TCR combination a 752 bp product. PCRs were performed in 50 µl mixes containing 25 µl Green GoTaq master mix (Promega), 1.25 µM forward and reverse primers, 2.5 µl 4 % (v/v) glycerol and 2.5 µl template. An annealing temperature of 60 °C was used for each of the PCRs.

Dot hybridization. A DIG-labelled DNA probe was generated from the PAO1 strain using the CabF/TCR primer combination (above) according to the manufacturer's instructions (Roche Diagnostics). Bacterial DNA extractions were dot blotted onto Hybond-N+ membrane (General Electric Healthcare). Blots were then hybridized using DIG EasyHyb buffer (Roche Diagnostics). Following a standard high-stringency wash, hybrids were visualized by standard alkaline phosphatase colorimetric detection (Bio-Rad). *Pseudomonas putida* cells were used as a negative control.

Statistics. Tests of association between the production and secretion of protease IV and genotype were carried out using the Mantel-Haenszel method and Pearson's chi-square tests. *P* values <0.05 were considered significant. Relative risk (RR) and 95 % confidence intervals (CIs) were used to determine the strength of the associations.

RESULTS AND DISCUSSION

Western blotting

Overall 32 of the 43 (74 %) isolates and the PAO1 reference strain produced a band of 26 kDa equivalent to that of the protease IV control (Fig. 1, Table 1). Although most positive samples produced a single band of the predicted size, a small proportion of samples with a strong band at 26 kDa showed additional faint bands, including those at approximately 17 and 31 kDa. Other studies have also demonstrated these extraneous bands, with or without strong bands at 26 kDa, and have attributed them to the presence of precursor protease IV protein or to aggregation of the protein (Engel *et al.*, 1998a; Traidej *et al.*, 2003; Caballero *et al.*, 2004). The 17 kDa band has been reported to represent an enzymically active breakdown product of protease IV formed by autodigestion. Precursor protein may interfere with transport of the protease to the exterior of the cell. However, there was no association between the

Fig. 1. Composite figure of Western blots and corresponding isolates on 1% gelatin zymograms indicating the presence of protease IV protein at 26 kDa and evidence of protease IV activity at around 350 kDa. Lane 1, PAO1; lanes 2–5, AES-1; lanes 6–7, AES-2; lanes 8–10, non-clonal isolates.

presence of extraneous bands on the Western blots and protease IV activity in the zymograms.

Zymography

Although the protease IV enzyme has a molecular mass of 26 kDa, it aggregates under mild SDS denaturing conditions, and is resolved at a molecular mass of 350 kDa (Caballero *et al.*, 2001). Overall, 22 of the 43 (51%) isolates showed a diffuse region of activity at a molecular mass consistent with protease IV (Table 1). Ten of the eleven (91%) zymography-negative isolates were also negative on the Western blot. As shown in Fig. 1, there was no relationship between the intensity of the band on the Western blot and protein activity on the zymogram.

PCR for the *prpL* gene

PCR was carried out on the ten isolates producing negative results by Western blotting and zymography. Nine of the ten were positive for at least one of the PCR assays confirming that the *prpL* gene is conserved among CF isolates; seven yielded a product using the CabF/TCR primer combination and two using the TCF/CabR and TCF/TCR combinations. The remaining sample (non-clonal) was negative for all PCRs. All amplicons were of the size predicted from the primer design. The isolate with negative results on all PCRs was also negative by dot hybridization, suggesting that the *prpL* gene was lost or mutated.

Protease IV expression: non-clonal versus clonal isolates

The expression profile for protease IV in the non-clonal strains was markedly different from that in the clonal strains (Table 1). Collectively, clonal isolates were 1.6 times more likely to produce protease IV than non-clonal isolates ($P=0.007$), and were 3 times more likely to have active

protein ($P<0.001$). Sixteen of eighteen (89%) Western blot-positive clonal isolates had protease activity on the zymogram compared with only five of fourteen (36%) Western blot-positive non-clonal isolates. All but one of the isolates testing negative by Western blotting and zymography fell into the non-clonal group. Recent studies have indicated that *P. aeruginosa* adapts to the hostile environment of the CF lung by progressively undergoing phenotypic or genotypic changes that down-regulate virulence factors required for acute infection and up-regulate factors required for biofilm formation (Smith *et al.*, 2006). Our finding that only about half of the non-clonal isolates produced protease IV and less than one quarter secreted the protein suggests that protease IV is among the virulence factors usually down-regulated with chronic infection in the CF lung. Nonetheless, there was considerable strain-to-strain variation in the amount of protease IV protein produced by non-clonal strains, and a few produced higher levels of the protein than seen in any of the clonal isolates or PAO1. It has been reported that the expression of virulence factors is increased on exacerbation of symptoms (Grimwood *et al.*, 1993). However, there was no relationship between protease IV production and the exacerbation of symptoms at the time of collection of the sputum.

Our finding that most isolates of both clonal strains secreted protease IV was of particular interest. The amount of protease IV produced by isolates of clonal strains was relatively uniform between and across the two groups, and in general was slightly less than that of PAO1. There is evidence in support of person-to-person spread of clonal strains, but acquisition from a common source has not been excluded. It is likely that the enhanced infectivity of clonal strains is multifactorial. However, we hypothesize that clonal strains, in contrast to non-clonal strains, have the ability to adapt for long-term survival in the CF lung by resisting modifications to the expression of virulence factors

Table 1. Production of protease IV by *P. aeruginosa* isolates from the CF lung

	Non-clonal (<i>n</i> =24)	Clonal			Total (<i>n</i> =43)	RR (95% CI) clonal vs non-clonal
		AES-1 (<i>n</i> =14)	AES-2 (<i>n</i> =5)	AES-1 + AES-2 (<i>n</i> =19)		
Western blotting	14 (58%)	13 (93%)	5 (100%)	18 (95%)	32 (74%)	RR=1.6 CI 1.1–2.3
Zymography	6 (25%)	12 (85%)	4 (80%)	16 (84%)	22 (51%)	RR=3.4 CI 1.6–6.9

such as protease IV, and that this promotes infectivity and/or facilitates person-to-person spread.

CF lung versus corneal keratitis isolates

Comparisons of data from non-clonal CF isolates with our findings from a study of microbial keratitis (Zhu *et al.*, 2002) showed that the proportion of isolates with active protease IV was much higher among the keratitis group; 82 % (14/17) of microbial keratitis isolates compared with 25 % of non-clonal CF isolates. This discrepancy is likely to reflect both the different anatomical site and the nature of infection (acute in the case of keratitis versus chronic in the case of CF).

In summary, this is the first study of the protease IV protein in a substantial number of *P. aeruginosa* isolates from the CF lung. This study has shown that the protease IV gene is highly conserved among CF lung isolates, which suggests that protease IV may have an important role in the pathogenesis of *P. aeruginosa* at this site. However, further studies will be needed to establish the contribution of protease IV to acute lung infection in young CF patients.

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