Characterization of protease IV expression in *Pseudomonas aeruginosa* clinical isolates

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Expression of protease IV by *Pseudomonas aeruginosa* during ocular infections contributes significantly to tissue damage. However, several *P. aeruginosa* strains isolated from ocular infections or inflammatory events produce very low levels of protease IV. The aim of the present study was to characterize, genetically and phenotypically, the presence and expression of the protease IV gene in a group of clinical isolates that cause adverse ocular events of varying degrees, and to elucidate the possible control mechanisms of expression associated with this virulence factor. Protease IV gene sequences from seven clinical isolates of *P. aeruginosa* were determined and compared to *P. aeruginosa* strains PAO1 and PA103-29. Production and enzyme activity of protease IV were measured in test strains and compared to that of quorum-sensing gene (*lasR*) mutants and the expression of other virulence factors. Protease IV gene sequence similarities between the isolates were 97.5–99.5 %. The strains were classified into two distinct phylogenetic groups that correlated with the presence of exo-enzymes from type three secretion systems (TTSS). Protease IV concentrations produced by PAO1/*lasR* mutants and the two clinical isolates with a *lasR* gene deficiency were restored to levels comparable to strain PAO1 following complementation of the quorum-sensing gene deficiencies. The protease IV gene is highly conserved in *P. aeruginosa* clinical isolates that cause a range of adverse ocular events. Observed variations within the gene sequence appear to correlate with presence of specific TTSS genes. Protease IV expression was shown to be regulated by the Las quorum-sensing system.

Received 23 May 2011

Accepted 14 September 2011

INTRODUCTION

Inflammation and infection in the cornea (keratitis) arising from *Pseudomonas aeruginosa* infection can lead rapidly to scarring and loss of sight unless prompt and targeted treatment is initiated (Sankaridurg *et al.*, 2000). *P. aeruginosa* virulence is attributed, in part, to the production of several destructive proteins and stimulation of host immune response in the ocular tissues (Hazlett, 2002). The virulence factors most commonly associated with *P. aeruginosa*-induced ocular damage are exo-enzymes S (*exoS*) and U (*exoU*) (Fleiszig *et al.*, 1997), elastase (*lasB*) (Kessler & Blumberg, 1987), alkaline protease (*aprA*) (Twining *et al.*, 1993) and protease IV (*piv*) (O'Callaghan *et al.*, 1996). Another protease, *P. aeruginosa* small protease (PASP), can also cause rapid corneal damage (Marquart

et al., 2005). Other important pathogenic mechanisms include the genes involved in translocation and activation of virulence proteins via the type II (*xcp*) and type III secretory pathways (Sandkvist, 2001; Yahr *et al.*, 1997).

Protease IV is a lysine-specific endoprotease and a significant virulence factor for pathogenesis of *P. aeruginosa* in the eye and lung (Caballero *et al.*, 2004; Engel *et al.*, 1997; Wilderman *et al.*, 2001). Mature protease IV degrades important host immunological proteins such as complement and IgG (Engel *et al.*, 1998). Protease IV also compromises the integrity of structural proteins such as elastin (Wilderman *et al.*, 2001), causing tissue damage and facilitating bacterial infection. Protease IV degrades the iron-binding proteins lactoferrin and transferrin (Wilderman *et al.*, 2001) enabling *P. aeruginosa* to scavenge iron from the host via the siderophore, pyoverdine. Pyoverdine has a high affinity for iron and sequesters it from the surrounding environment, facilitating iron uptake by the bacterium (Ochsner *et al.*, 1995). Moreover, pyoverdine has been shown to act as a signal molecule for the synthesis of protease IV (Wilderman *et al.*, 2001).

Abbreviations: AHL, acylhomoserine lactones; MK, microbial keratitis; PASP, *P. aeruginosa* small protease; TLCK, N- α -*p*-tosyl-L-lysine chloromethyl ketone; TTSS, type three secretion systems.

The GenBank/EMBL/DDBJ accession numbers for the consensus sequences of the ocular strains of *P. aeruginosa* determined in this study are DQ351518–DQ351524.

Controlled expression of several key *P. aeruginosa* virulence factors, including elastase (*lasB*) (Pearson *et al.*, 1997), exotoxin A (Gambello *et al.*, 1993), type II secretory pathway proteins (*xcp*) (Chapon-Hervé *et al.*, 1997) and pyoverdine (Kim *et al.*, 2005; Stintzi *et al.*, 1998) are mediated by population density bio-sensor mechanisms, a process known as quorum sensing. *P. aeruginosa* has two quorum-sensing systems, the Las and Rhl systems, each influencing the expression of a range of virulence factors (Passador *et al.*, 1993; Pearson *et al.*, 1994). The systems are organized in a hierarchical manner such that the Rhl system is partially controlled by Las activation (Latifi *et al.*, 1996). The Las and Rhl quorum-sensing molecules are acylhomoserine lactones (AHL). Once the bacterial population reaches an appropriate size, activation of the quorum-sensing-controlled virulence genes is initiated.

Protease IV has been reported as a key virulence factor in the pathogenesis of *P. aeruginosa* in the eye (Engel *et al.*, 1997). However, a number of 'low protease'-producing strains of *P. aeruginosa* have been isolated from microbial keratitis and corneal inflammation events (Zhu *et al.*, 2002, 2004). In the current study, the protease IV gene sequence was determined for clinical isolates causing a range of adverse ocular events. Sequence similarities were correlated with the presence or absence of exo-enzymes of the type three secretion system (TTSS). The presence and activity of protease IV was determined for each of the low-protease-producing isolates and regulation of protease IV production by the Las quorum-sensing system was demonstrated.

METHODS

Bacterial strains, plasmids, and growth conditions. The six strains of *P. aeruginosa* used in this study (Table 1) were isolated from the eyes or contact-lens storage cases of subjects during asymptomatic routine contact-lens wear or following corneal infection or inflammation events. *P. aeruginosa* PAO1 was included as a reference strain. The isogenic quorum-sensing mutant of strain PAO1 (PAO1Δ*lasRI*::*Gm*) and the *P. aeruginosa* shuttle vector pUCPSK were kindly supplied by Dr Scott Beatson (Institute for Molecular Bioscience, University of Queensland, Australia). *Escherichia coli* strain DH5α, from our laboratory collection, was used during the construction of the *lasRI* plasmid. Bacteria were stored at -80 °C in tryptic soy broth (TSB, Oxoid) containing 30 % (v/v) glycerol and were cultured at 37 °C on tryptic soy agar (TSA, Oxoid). For general laboratory work, colonies of *P. aeruginosa* were inoculated into 5 ml TSB and incubated at 37 °C with vigorous shaking for 18 h to stationary phase, achieving an OD₆₆₀ of 2.0. Bacterial cells were recovered by centrifugation at 16 000 g at 4 °C for 10 min and the supernatant was passed through a 0.22 μm filter to remove any remaining cells. For growth of bacteria containing vector DNA, media were prepared as above with the following antibiotics as required: 100 μg ampicillin ml⁻¹ for *E. coli* and 400 μg carbenicillin ml⁻¹ and/or 100 μg gentamicin ml⁻¹ for *P. aeruginosa*. For genetic transformation experiments, *E. coli* cells were rendered chemically competent. An overnight cell culture was grown as above, washed once in sterile PBS (pH 7.4) and resuspended in ice-cold 0.15 M MgCl₂ for 30 min (Mercer & Loutit, 1979). For *P. aeruginosa* cell competency, the same process was employed but with the cultures grown overnight at 43 °C (Holloway, 1965).

PCR amplification of the protease IV gene. PCRs were performed to amplify the entire protease IV gene and promoter region. Based on the published sequence of strain PAO1 (<http://www.pseudomonas.com>), novel primers were designed using the GCG software within BioManager (<http://biomanager.info/>) to produce an amplicon of 1876 bp, identical to non-coding regions surrounding the *piv* gene. Primers were synthesized by Invitrogen. The primer sequences were as follows: PIVflk-for, 5'-AGAGCCACTCCAGACCAAAC-3'; PIVflk-rev, 5'-GGATAAACGGCGGATAACAC-3'.

Genomic DNA for each PCR was prepared by incubating 1 μl fresh overnight bacterial broth culture with 19 μl microLYSIS buffer (Microzone) and heating in a thermal cycler (Corbett) using the following conditions: 65 °C for 5 min, 96 °C for 2 min, 65 °C for 4 min, 96 °C for 1 min, 65 °C for 1 min, 96 °C for 30 s and then cooled to 25 °C.

PCR amplification was performed using 3 units of the high-fidelity proofreading polymerase *Pfu* (Promega) in a 50 μl reaction containing buffer (20 mM Tris/HCl, pH 8.8; 10 mM KCl; 10 mM (NH₄)₂SO₄; 2 mM MgSO₄; 0.1 % Triton X-100 and 0.1 mg BSA ml⁻¹), 200 μM each dNTP (Promega), 0.2 μM each primer and 2 μl template DNA/microLYSIS mixture. Reactions were performed for an initial denaturation period of 95 °C for 7 min followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 90 s, and a final extension step at 72 °C for 10 min. PCR products were separated by electrophoresis through a 1.5 % agarose gel in TBE buffer (45 mM Tris, pH 8.0; 45 mM boric acid; 1 mM ethylenediaminetetraacetic acid), stained with SybrSafe (Invitrogen) and visualized under UV illumination.

Protease IV gene sequencing. Amplicons from each ocular strain were purified using Wizard SV Gel and PCR Clean Up System kits (Promega), blunt-end cloned into pCR4-TOPO according to the manufacturer's instructions and sequenced using a nested deletion method on the ABI Prism 3700 Automated DNA platform (Supamac). The nested deletion sequences for each clone were compiled and aligned using BioEdit version 7.0.5 (<http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html>; Hall, 1999) to produce a consensus sequence for each strain. Each new consensus sequence was made available in the NCBI GenBank database, under accession numbers DQ351518–DQ351524. Each sequence was aligned with the published gene sequence of strains PAO1 (Stover *et al.*, 2000) and PA103-29 (Caballero *et al.*, 2004) using BioEdit. A phylogenetic tree was generated using fastDNAMl (Olsen *et al.*, 1994) software within the BioManager service.

Zymography analysis. Gelatin zymography techniques based on those of Twining *et al.* (1993) were employed to determine protease IV activity in the isolates. Cell-free culture supernatants were pre-incubated with or without the protease IV specific inhibitor N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK, Sigma-Aldrich) (Engel *et al.*, 1998). TLCK was prepared as a stock solution of 10 mM in 5 mM HCl. Aliquots of each supernatant were incubated for 30 min at 25 °C with 0.2 volumes of TLCK stock solution or 0.2 volumes of 5 mM HCl as a control. Treated supernatants were combined with SDS sample buffer (final concentration: 50 mM Tris/HCl, pH 6.8; 10 % v/v, glycerol; 0.2 % w/v, bromophenol blue; 0.5 % w/v, SDS), and 15 μl of the mixture was loaded into a 7.5 % SDS-polyacrylamide gel containing 0.1 % gelatin (Type A from porcine skin, Sigma-Aldrich) with a 4 % stacker. A pre-stained molecular mass marker was included (Kaleidoscope Precision Plus, Bio-Rad). Electrophoresis was performed at 4 °C under non-reducing conditions and bands were resolved at 25 mA per gel until the 50 kDa pre-stained marker band was approximately 10 mm from the edge of the gel. To allow protein renaturation, SDS was removed by gentle agitation with 2.5 % Triton X-100 (Sigma-Aldrich) in distilled water for 1 h. Gels were rinsed

Table 1. Strains of *P. aeruginosa* and plasmids used in this study

Strain or plasmid	TTSS gene	Characteristics and/or genotype*	Source
Strains			
Paer1	<i>exoS</i>	CLARE isolate, low protease producer, <i>lasRI</i> ⁻	Zhu <i>et al.</i> (2002)
Paer2	<i>exoU</i>	Asymptomatic ocular isolate, low protease producer, <i>rhlR</i> ⁻ <i>rhlAB</i> ⁻	Zhu <i>et al.</i> (2002)
Paer3	<i>exoS</i>	Asymptomatic ocular isolate, low protease producer, <i>lasRI</i> ⁻	Zhu <i>et al.</i> (2002)
Paer17	<i>exoU</i>	MK isolate CL related, low protease producer	Zhu <i>et al.</i> (2002)
Paer26	<i>exoU</i>	MK isolate CL related, low protease producer	Zhu <i>et al.</i> (2002)
6206	<i>exoU</i>	MK isolate, low protease producer	Fleiszig <i>et al.</i> (1996)
6294	<i>exoS</i>	MK isolate, high protease producer	Fleiszig <i>et al.</i> (1996)
PAO1 (ATCC 15692)	<i>exoS</i>	Wild-type, wound isolate	Holloway <i>et al.</i> (1979)
PAO1Δ <i>lasRI</i> :: <i>Gm</i>		PAO1 with a <i>Gm</i> ^r cassette in <i>lasRI</i>	Beatson <i>et al.</i> (2002)
PAO1Δ <i>lasRI</i> + <i>lasRI</i>		PAO1Δ <i>lasRI</i> :: <i>Gm</i> with pUC <i>lasRI</i>	This study
Paer1+ <i>lasRI</i>		Paer1 with pUC <i>lasRI</i>	This study
Paer3+ <i>lasRI</i>		Paer3 with pUC <i>lasRI</i>	This study
<i>E. coli</i> DH5α		<i>hsdR</i> , <i>recA</i> , <i>lacZYA</i> ϕ80 <i>lacZ</i> ΔM15	Lab collection
Plasmids			
pUCPSK		Cloning vector	Watson <i>et al.</i> (1996)
pUC <i>lasRI</i>		pUCPSK containing <i>lasRI</i>	This study

*CLARE, contact-lens-induced acute red eye; CL, contact lens; *Gm*^r, gentamicin resistance gene.

twice in distilled water and incubated in development buffer (50 mM Tris/HCl, pH 8.0; 10 mM CaCl₂; 1 mM ZnCl₂; 150 mM NaCl) at 37 °C for 24 h. Gels were stained and destained simultaneously with 0.01 % Coomassie blue R-250 in glacial acetic/methanol/distilled water (1:3:6) for 1 h, according to the method of Leber & Balkwill (1997). Zymogram experiments were repeated twice, beginning from frozen bacterial stock cultures.

Chromogenic substrate assay. The activity of protease IV in cell-free culture supernatants was measured by the breakdown of the chromogenic substrate Chromozym PL (Roche) which reacts specifically with protease IV in *P. aeruginosa* culture supernatants (Caballero *et al.*, 2004). Briefly, 20 µl pre-treated sample (with or without TLCK as for zymography) and 40 µg of Chromozym PL were combined in reaction buffer (50 mM Tris/HCl, pH 8.0; 150 mM NaCl) to a total volume of 100 µl. *A*₄₀₅ was measured at 5 min intervals for a total of 3 h. Protease IV activity ml⁻¹ in supernatant was calculated as follows: $V/(v \times E \times d) \times \Delta A \text{ min}^{-1}$; where *V*=total assay volume; *v*=sample volume; *E*=extinction coefficient (at 405 nm=10.4); *d*=path length (100 µl volume in microtitre plate≈0.53 cm); and $\Delta A \text{ min}^{-1}$ =the maximum change in absorbance min⁻¹. To determine maximal rate of change in absorbance, the data for each strain were first plotted as a function of time (data not shown). The protease activity was then normalized to account for variation in bacterial cell growth as observed at OD₆₆₀; total protease activity was divided by optical density to result in µM/OD₆₆₀.

Samples were analysed in neat and ten-fold concentrated states. To concentrate supernatants, 500 µl each sample was applied to Amicon Ultrafree-MC 5 kDa molecular mass cut off centrifugal filters (Millipore) and centrifuged at 16 000 *g* at 20 °C until the volume of the retentate was reduced to 50 µl.

Protein immunoblot analysis. The presence of protease IV protein from *P. aeruginosa* isolates was examined using western blotting. Culture supernatants were analysed in neat or concentrated forms, as in the chromogenic substrate experiments, but were not pre-treated with TLCK. Volumes (30 µl) of each supernatant and a purified

sample of protease IV (kindly supplied by Professor R. O'Callaghan and Dr B. Thibodeaux, Louisiana State University, USA), were prepared in NuPAGE lithium dodecyl sulphate sample buffer (Invitrogen) and heat denatured for 10 min in a water bath at 80 °C. Fifteen microlitres of each was loaded onto a pre-cast 4–12 % NuPAGE Novex Bistris gel in conjunction with the XCell Sure Lock system (Invitrogen) together with 5 µl pre-stained molecular mass marker (Kaleidoscope Precision Plus Broad Range, Bio-Rad) as was used for the zymogram experiments. After resolving in PAGE, protein bands were transferred to polyvinylidene fluoride membranes (Bio-Rad). The membranes were blocked for 1 h in 3 % BSA (Bovostar, Bovogen) and incubated overnight with 15 ml rabbit polyclonal anti-protease IV antibody (1:2000 in blocking solution; kindly supplied by Professor R. O'Callaghan and Dr B. Thibodeaux, Louisiana State University, USA). Unbound primary antibody was removed by three PBS washes. The membranes were then incubated with 15 ml of secondary antibody solution, 1:2000 anti-rabbit IgG conjugated to alkaline phosphatase (Sigma-Aldrich) in blocking solution, for 1 h. The targeted bands were visualized after adding 5-bromo-4-chloro-3-indolylphosphate-nitro blue tetrazolium (Roche).

Genetic manipulations and complementation. The *lasRI* region of the PAO1 genome was amplified by PCR with primers supplied by Dr Scott Beatson (Beatson *et al.*, 2002) using the same conditions as in the amplification of the protease IV gene, except with an annealing temperature of 58 °C. The amplified DNA was blunt-end cloned into the *EcoRV* restriction enzyme site of *P. aeruginosa* shuttle vector pUCPSK. The resulting construct, pUC*lasRI*, was verified for sequence integrity and orientation by a vector (T7)- and insert (*lasRI*-r)-specific PCR using conditions as above. The pUC*lasRI* plasmid was transformed into competent *E. coli* DH5α cells. After culturing in TSB containing ampicillin, replicated plasmids were purified using the Aurum Plasmid Mini kit (Bio-Rad). Purified pUC*lasRI* (100 ng) was then used to transform chemically competent *P. aeruginosa* strains PAO1Δ*lasRI*::*Gm*, Paer1 and Paer3. Transformants were plated onto solid media containing ampicillin and carbenicillin and incubated at 37 °C overnight. A

single colony of each complemented *P. aeruginosa* strain was cultured in TSB containing ampicillin and carbenicillin and then subcultured into TSB containing no antibiotics. Supernatants were prepared and analysed as before by Western blotting and Chromozym PL assay for the presence and activity of the mature protease IV protein.

Pyoverdine production. Pyoverdine concentrations in cell-free bacterial supernatants were determined using the method of Wilderman *et al.* (2001). Briefly, OD₆₆₀ of overnight cultures was monitored, cell-free culture supernatant was diluted 1:10 with PBS, and A₄₀₅ was determined using a spectrophotometer. The production of pyoverdine (μM) was normalized by bacterial growth; total protease activity was divided by optical density to result in μM/OD₆₆₀.

Nomenclature. Protease IV has also been referred to in the literature as endoprotease IV and the gene referred to as *piv*.

RESULTS

DNA sequencing of protease IV genes from clinical isolates

Sequences were made available via the GenBank Database under accession numbers DQ351518–DQ351524. Similarities between protease IV gene sequences ranged from 97.5 to 99.5 %. There were size variations between the PCR products from each isolate, indicating that the regions flanking the coding protease IV region were not genetically identical. The similarity of each strain was calculated relative to all strains and this was displayed as a nearest-neighbour phylogenetic tree (Fig. 1). The strains were divided into two groups in the tree: group A comprising strains PA103-29, 6206, Paer2, Paer17 and Paer26; group B comprising PAO1, 6294, Paer1 and Paer3 (Fig. 1). DNA sequences of the protease IV genes of the test strains are aligned in Fig. 2. In comparison with the published gene sequence of PAO1, there were 24 occurrences of nucleotide variations that were conserved in all group A strains. Seven of these variations were within the promoter region and 17 were within the coding sequence. There were seven instances in which strains Paer1, Paer3 and 6294 of group B showed conserved nucleotide variations that differed from sequences of strain PAO1 and the group A strains. The differences occurred within the ORF of the protease IV coding sequence.

When the coding sequences were translated, four conserved amino acid variations occurred at residues 23, 57, 102 and 137 within the signal sequence and pro-peptide (Fig. 3). These conserved changes were seen in all group A strains (Leu-23 to Ser, Ile-57 to Thr, Thr-102 to Ala [or Val for strain 6206], and His-137 to Arg) and in strains Paer1 and Paer3 from group B at residues 102 and 137 (Thr-102 to Ala and His-137 to Arg). Non-conserved amino acid changes in the signal or pro-peptide were Leu-7 to Pro in strain Paer1, Asp111 to Glu in strain Paer26, Pro-19 to Leu in strain 6294, Ser-127 to Arg in strain 6206 and Ser-165 to Pro in strain Paer2. Four non-conserved amino acid substitutions were noted within the mature protease. Three of these occurred in strain Paer1 (Cys-224 to Arg, Gln-314

Fig. 1. Nearest-neighbour phylogenetic tree based on analysis of protease IV gene sequences of clinical isolates tested in this study and published reference sequences from the NCBI database. Proximity of isolates indicates those that are most genetically similar based on *piv* gene sequences. Square brackets indicate that Paer17 was most genetically similar in the protease IV gene sequence to strains containing exotoxin gene *exoU*, despite being *exoS*⁺/*exoU*[−].

to Arg and Lys-331 to Asn) and the other occurred in strain Paer2 (Ser-280 to Trp). There were no amino acid changes in the putative transmembrane domains (Caballero *et al.*, 2004) Arg-275–Ala-292, Arg-341–Gly-360 or Tyr-376–Ala-393 and there were no changes to the catalytic triad (Traidej *et al.*, 2003) His-283, Asp-333 and Ser-409.

Detection of protease IV in clinical isolates

The activities of protease IV in *P. aeruginosa* isolates were visualized by gelatin zymography. A gelatinolytic band at an apparent molecular mass of ~350 kDa was seen in all samples (Fig. 4). This band has previously been suggested to be protease IV (Caballero *et al.*, 2001); although the enzyme has a molecular mass of 26 kDa, it aggregates under SDS reducing conditions and is resolved at a high molecular mass within the gel (Caballero *et al.*, 2001). The protease IV band size and intensity were maximal in strain 6294. The bands of protease IV activity in the low-protease-producing strains Paer1, Paer 2, Paer 3, Paer 17, Paer 26 and 6206 were not as large or dense as those of strains 6294 or PAO1. Pre-treatment of the supernatant with the specific inhibitor TLCK, appeared to inhibit protease IV activity on the zymogram. Other protease bands were either weak or not visible in the low-protease-producing strains and the gelatinolytic band in lane 5 (Fig. 4) with an apparent molecular mass of ~80 kDa was most likely PASP (Marquart *et al.*, 2005). Protease IV activities in *P. aeruginosa* culture supernatants were determined (Table 2). In neat bacterial supernatants, only strains 6294 and PAO1 produced detectable amounts of protease IV, at 28.01 and 12.05 mU ml^{−1}/OD₆₆₀, respectively. Protease IV activity in strains Paer1, Paer 2, Paer 3, Paer 17, Paer 26 and 6206 was only detected after ten-fold concentration of supernatants and ranged from 0.10–0.18 mU ml^{−1}/OD₆₆₀. In all samples pre-treated with TLCK, no protease IV activity was detected, even in the ten-fold concentrated samples.

Fig. 2. Sequence analysis of the protease IV (*piv*) genes of the test strains and the previously published *piv* gene sequences of strains PAO1 and PA103-29. Full stops indicate nucleotides matching PAO1. Nucleotide variations from the *piv* gene sequence of strain PAO1 are indicated. Underlined sequences represent putative trans-membrane domains (Caballero *et al.*, 2004). Highlighted bases represent nucleotide variations leading to changes in amino acid residues. Hyphens indicate nucleotides not determined.

Western blotting was performed to determine the relative level of the protease IV protein in each supernatant. In neat supernatants, only strains 6294 and PAO1 produced a band corresponding to the protease IV standard (results not shown). An extension of the development stage produced a number of additional higher molecular mass bands in

samples other than 6294 and PAO1 as seen in previous work by Caballero *et al.* (2004). For ten-fold concentrated supernatants, a band of the same molecular mass as the protease IV control (25 kDa) was seen in all strains (Fig. 5). In addition, a band at an apparent molecular mass of 23 kDa was seen in all concentrated samples.

Fig. 3. Translated protein coding sequences of the protease IV genes of the test strains and strain PA103-29, aligned to reference strain PAO1. Full stops represent amino acid residues matching those of strain PAO1. Amino-acid variations from the protein sequence of strain PAO1 are indicated with the appropriate letter. Underlined sequences represent putative transmembrane domains (Caballero *et al.*, 2004). Highlighted residues represent catalytic triad (Traidej *et al.*, 2003). Asterisks represent stop codons.

Fig. 4. Protease activity of *P. aeruginosa* strains. Analysed by 7.5% gelatin zymogram, using 15 µl aliquots of *P. aeruginosa* bacterial culture supernatants mixed with SDS loading buffer. (a) Supernatants run in absence of the protease IV inhibitor TLCK. (b) Supernatants run in the presence of protease IV inhibitor TLCK. Lanes: 1, Paer1; 2, Paer2; 3, Paer3; 4, Paer17; 5, Paer26; 6, 6294; 7, 6206; 8, PAO1; M, molecular mass marker.

Complementation of *lasRI*-deficient isogenic mutant and clinical isolates

The quorum-sensing mutant PAO1Δ*lasRI*::*Gm* and the previously reported *lasRI*-deficient clinical isolates Paer1 and Paer3 exhibited little or no protease IV production when examined via chromogenic substrate assay and Western blotting. These strains were successfully complemented with pUC*PlasRI* to form PAO1Δ*lasRI*+*lasRI*, Paer1+*lasRI* and Paer3+*lasRI*, as indicated by the restoration of LasB elastase activity (data not shown). The use of the chromogenic substrate indicated protease IV production was restored in the complemented *lasRI* mutant PAO1Δ*lasRI* and clinical isolates of Paer1 and Paer3 to similar levels to that exhibited by wild-type strain PAO1 (Fig. 6a). Immunoblot analysis of culture supernatants also confirmed that protease IV protein production in these strains was increased to levels similar to that of wild-type strain PAO1 (Fig. 6b).

Table 2. Protease IV activity in cell-free supernatants of strains of *P. aeruginosa*

The supernatant of each *P. aeruginosa* strain was allowed to react with the specific protease IV substrate Chromozym PL to determine the number of enzyme units ml⁻¹. All bacterial cultures had OD₆₆₀ 2.0 after overnight growth and final results were normalized according to estimated cell number. Values represent the mean of three repeated tests with SD values shown in parentheses. Concentrated supernatants were ten-fold concentrated. ND, Not determined.

Strain	Activity (mU ml ⁻¹ /OD ₆₆₀) (SD)	
	Neat supernatant	Concentrated supernatant
Paer1	0	0.18 (0.06)
Paer2	0	0.10 (0.03)
Paer3	0	0.10 (0.05)
Paer17	0	0.10 (0.08)
Paer26	0	0.18 (0.07)
6206	0	0.16 (0.03)
6294	28.01 (9.65)	ND
PAO1	12.05 (2.74)	ND

Pyoverdine concentrations in cell-free supernatants

Pyoverdine concentrations in cell-free supernatants of wild-type strains PAO1 and 6294 were 2421 and 2273 µM/OD₆₆₀, respectively. Pyoverdine production levels in 'low-protease'-producing strains Paer1, Paer2, Paer3, Paer17, Paer26, 6206 and PAO1Δ*lasRI*::*Gm* were all below 1050 µM/OD₆₆₀ and were between 30 and 43 % of the concentration produced by strain PAO1. In complemented strains Paer1+*lasRI*, Paer3+*lasRI* and PAO1Δ*lasRI*+*lasRI*, levels of pyoverdine production were restored to ≥98 % of that produced by PAO1 (Table 3).

DISCUSSION

Protease IV gene sequences were characterized in seven *P. aeruginosa* isolates. This work extends that of Caballero *et al.* (2004) who compared the protease IV sequence of strain PA103-29 to that of PAO1. Up to 2.5 % (43/1706 nt) nucleotide variation was observed between all strains in the current study. Caballero *et al.* (2004) previously reported the coding sequence of protease IV genes in strains PAO1 and PAO103-29 exhibiting only 1.5 % variation (26/1706 nt). This is the first time, to our knowledge, that multiple sequences for whole protease IV virulence genes have been determined and further demonstrates that protease IV genes of both virulent and avirulent *P. aeruginosa* strains show negligible DNA variation. However, following DNA sequence alignment, it was possible to distinguish two divergent phylogenetic groups: group A, containing strains PAO103-29, Paer2, Paer17, Paer26 and 6206; and group B, containing strains PAO1, Paer1, Paer3 and 6294. It is of interest that these similarities correlate with the presence of the genes that confer an invasive or cytotoxic phenotype (Fleiszig *et al.*, 1996, 1997; Zhu *et al.*, 2006). Strains containing *exoU* (group A) or *exoS* (group B) were found to be most similar with regards to their *piv* gene sequences. Strain Paer17 was the only exception to these findings, containing *exoS* but grouping in group A (with the *exoU*-containing isolates) based on *piv* gene sequence similarity data.

There were a number of conserved gene mutations leading to amino acid substitution in the protein sequence,

Fig. 5. Western blot analysis of protease IV expression in strains of *P. aeruginosa*. Bacterial supernatants were concentrated ten-fold. Lanes: 1, Paer1; 2, Paer2; 3, Paer3; 4, Paer17; 5, Paer26; 6, 6294; 7, 6206; 8, PAO1; M, Molecular mass marker; PIV, purified protease IV control.

however there was no correlation between any of these mutations and a low-protease-producing phenotype. There were no amino acid changes that consistently created or removed potential auto-cleavage sites (lysine residues), premature stop codons or alterations to the catalytic triad (Traidej *et al.*, 2003). The conserved amino acid changes detected in our clinical isolates agreed with those detected by Caballero *et al.* (2004) while the non-conserved amino acid substitutions are reported here for the first time, to our knowledge. These results indicate that the potential to secrete protease IV may be determined by upregulation of protease IV gene expression rather than variations in gene structure causing a structurally altered protein. The role and function of the non-conserved amino acid substitutions are not known and warrant further investigation.

Within the isolates examined, Paer2, Paer17, Paer26 and 6206, despite being low-protease-producing strains, were virulent in a murine scratch keratitis model (Zhu *et al.*, 2004). Of these strains, Paer17 is the only *exoS*-containing isolate in group A; it causes microbial keratitis (MK) and is a low-protease-producing strain. All other MK isolates in the present study either possess *exoU* or are high protease producers, an observation consistent with the findings of others (Winstanley *et al.*, 2005). Thus, it is of interest that strain Paer17 is more similar to *exoU*-containing isolates in phylogenetic analysis of *piv* gene sequences. Lee *et al.* (2006) have reported pathogenicity-related genes, the presence or absence of which do not correlate directly with degree of virulence, suggesting virulence is more complex than simple autonomous functioning of virulence genes. Strain-variable virulence-associated genes have been reported within genomic islands of *P. aeruginosa* (Battle *et al.*, 2009). These mobile genetic elements can contribute to virulence in a combinatorial manner with other accessory genome 'modules', resulting in strain-specific virulence (Lee *et al.*, 2006). There are 45 specific genome

Fig. 6. Protease IV production in the quorum-sensing mutants after complementation of strains with the plasmid containing *lasRI*. (a) Units of protease IV enzyme per millilitre of *P. aeruginosa* culture supernatant as determined by chromogenic substrate assay. *x* axis: 1, PAO1; 2, Paer1; 3, Paer3; 4, PAO1Δ*lasRI*::*Gm*; 5, Paer1+*lasRI*; 6, Paer3+*lasRI*; 7, PAO1Δ*lasRI*+*lasRI*; 8, PAO1+pUCPSK. Data represent the mean of three experiments. Bars represent SEM. (b) Western blot analysis of bacterial culture supernatants. Lanes: 1–7, as for panel (a); PIV, purified protease IV control; M, pre-stained Kaleidoscope Precision Plus broad-range molecular mass marker.

modules that tend to be associated in MK strains (Stewart *et al.*, 2011). The similarity of the *piv* gene sequence of strain Paer17 to those of strains possessing the *exoU* gene rather than the *exoS* gene, corresponding to increased virulence of this low-protease-containing strain, suggests that the *piv* gene might be part of a genomic island containing other virulence factors that combine with genome modules within this strain to increase virulence. Singh *et al.* (2010) recently suggested a reciprocal regulation of the TTSS and *Pseudomonas* Quinolone Signal (PQS), both regulated by the *las* and *rhl* quorum-sensing systems (Blevins *et al.*, 2005). Furthermore, host defences can disrupt quorum sensing of invading *P. aeruginosa* (Chun *et al.*, 2004), suggesting a complex interplay of virulence

Table 3. Pyoverdine production in strains of *P. aeruginosa*

All results represent the means of three repeated experiments $\pm$ SD values. Figures in parentheses show percentage of pyoverdine in comparison to the wild-type strain PAO1.

Strain	Concentration of pyoverdine ($\mu\text{M}/\text{OD}_{660}$)
Paer1	800 $\pm$ 11 (33 %)
Paer2	834 $\pm$ 17 (34 %)
Paer3	933 $\pm$ 108 (39 %)
Paer17	1035 $\pm$ 43 (43 %)
Paer26	922 $\pm$ 44 (38 %)
6206	737 $\pm$ 40 (30 %)
6294	2273 $\pm$ 155 (94 %)
PAO1	2421 $\pm$ 141 (100 %)
PAO1 Δ lasRI::Gm	864 $\pm$ 208 (36 %)
Paer1 + lasRI	2768 $\pm$ 16 (114 %)
Paer3 + lasRI	2369 $\pm$ 458 (98 %)
PAO1 Δ lasRI + lasRI	2485 $\pm$ 242 (103 %)

factors and an orchestration of host and pathogen responses during infection, all of which requires further elucidation.

From our previous studies it has been found that all the low-protease-producing strains used in this study (Paer1, Paer2, Paer3, Paer17, Paer26 and 6206) also produced low extracellular concentrations of *N*-acylhomoserine lactones (AHLs), suggesting the quorum-sensing circuits in these isolates are not fully active (Zhu *et al.*, 2002). These isolates had no detectable elastase activity *in vitro*, a virulence factor that is directly regulated by the *las* quorum-sensing system. Paer1 and Paer3 were found to be deficient in the *lasRI* gene, and Paer2 was deficient in the *rhlR* and *rhlAB* genes (Zhu *et al.*, 2004). We also confirmed that PAO1 Δ lasRI had decreased protease IV production. By replacing the defective *lasRI* genes, we demonstrated that protease IV secretion can be restored to the normal levels. Restoration of protease IV secretion was also observed in clinical isolates that were known to be defective in *lasRI* genes (Paer1 and Paer3), suggesting that protease IV production is also controlled, at least in part, through quorum-sensing regulation; these findings are in agreement with the work of Arevalo-Ferro *et al.* (2003). However, control may not be direct as there is no evidence of a *lux* box (Blevess *et al.*, 2005) within any of the gene sequences. Traidej *et al.* (2003) suggested that protease IV can utilize the type II secretion pathway to travel to the cell exterior. The *las* quorum-sensing system directly controls the expression of the type II *xcp* gene cluster, producing channels that allow direct transportation of proteins to the cell exterior (Chapon-Hervé *et al.*, 1997). When the *las* quorum-sensing system is disrupted, it is likely that protease IV, and other virulence-associated proteins are unable to be effectively transported and activated.

Protease IV production is influenced by the availability of iron in the *P. aeruginosa* culture, which also controls the

alternative sigma factor PvdS (Wilderman *et al.*, 2001). PvdS regulates expression of genes required for pyoverdine production, and is therefore upregulated when *P. aeruginosa* is in an iron-deficient environment. Pyoverdine upregulates production of itself as well as protease IV (Lamont *et al.*, 2002) and its synthesis is also controlled by *lasRI* (Stintzi *et al.*, 1998). In the current study, the low-protease-producing isolates also demonstrated low pyoverdine production. In each of the genetically restored quorum-sensing mutants, PAO1 Δ lasRI, Paer1 and Paer3, the increase in protease IV secretion to normal levels correlated with an increase in pyoverdine production, confirming both pyoverdine and protease IV productions are under control of the *lasRI* gene. The direct influence of pyoverdine on the production of protease IV warrants further investigation.

In summary, we have identified that all the ocular isolates tested have a functional protease IV gene, which is highly conserved yet can be classified into two distinct phylogenetic groups. The expression and secretion of protease IV in clinical isolates was determined to be under the control of the *las* quorum-sensing system, a global regulator of pathogenesis. Further *in vivo* analysis of pathogenic clinical isolates is needed to elucidate the complex interrelationship between quorum sensing, TTSS and protease IV production of *P. aeruginosa*.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Scott Beatson for supplying the isogenic PAO1 mutants, pUCPSK plasmid and *lasRI* primer sequences. Much appreciation is also given to Professor O'Callaghan and Dr B. Thibodeaux for providing the rabbit polyclonal anti-protease IV antibody and purified protease IV protein standard. This work was supported by the Australian Federal Government through the Cooperative Research Centre program.

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