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# A Pilot Study of the Genetic Differences between *P. aeruginosa* Ocular Isolates Using Suppression Subtractive Hybridization

**TCR Conibear, H Zhu, MDP Willcox***

School of Optometry and Vision Science, University of New South Wales, New South Wales, Australia

***Corresponding author:** Mark Willcox, Professor, School of Optometry and Vision Science, University of New South Wales, Sydney, New South Wales 2052, Australia

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## Abstract

*Pseudomonas aeruginosa* is associated with microbial keratitis during contact lens wear. Strains isolated from contact lenses may be virulent or avirulent in mouse models, but the differences between such strains are not fully understood. This study examines genetic differences between such strains. Well characterized strains (n=8; 5 strains isolated from patients with corneal infections, 2 from patients with non-infectious inflammatory conditions and 1 from the contact lens of an asymptomatic contact lens wearer) of *P. aeruginosa* were examined. Polymerase chain reaction was used to evaluate their possession of the genes vfr and algR that can regulate virulence factor production. Suppression subtractive hybridization (SSH) was used to determine genetic differences between isolates. All strains possessed vfr and algR. SSH demonstrated that strain 6294 isolated from microbial keratitis and virulent in mouse models possessed seven genes in common with other virulent strains that were absent from avirulent strains, including lasI an important quorum sensing gene and an integrase. One gene of 6294 appeared to be unique to that isolate. Several genes were found to be associated with virulent but not avirulent isolates of *P. aeruginosa*. Next steps should be to analyze what role, if any, the products of these genes have in the virulence of strains and the pathology of keratitis.

**Keywords:** Keratitis; Regulatory genes; *Pseudomonas aeruginosa*; Virulence; Virulence genes

## Introduction

*P. aeruginosa* is able to survive in and colonise a wide variety of environments including fresh and salt water, soil, plants, insects, nematodes and mammals [1-3]. The adaptability of *P. aeruginosa* is clearly demonstrated by survival in these environments, and this allows interaction with a plethora of other microbial species. These species, at a basic level, can be seen as genomic libraries of potential benefit to *P. aeruginosa*. *P. aeruginosa* may utilize horizontal gene transfer mechanisms to accumulate and express random, or perhaps even targeted, regions of foreign DNA. Any beneficial genomic additions are likely to pass through successive generations, and potentially create novel isolated epidemic strains. Indeed, epidemic strains of *P. aeruginosa* are known to occur in isolates from cystic fibrosis [4-6] and these can be differentiated on their genetic make-up.

Inflammation and infection of the cornea caused by *P. aeruginosa* can rapidly lead to scarring and loss of sight unless treated quickly and effectively [7]. In the eye, a combination of several *P. aeruginosa* virulence factors are associated with cellular damage and induction of the host immune response [8]. These include exoenzymes S (exoS), and U (exoU), [9] elastase (lasB), [10] alkaline protease (aprA) [11] and protease IV (prpL) [12]. *P. aeruginosa* also possess many transcriptional regulators such as Vfr, [13,14] AlgR [15] and quorum sensing systems [16] which control the expression of many keratitis-related virulence genes. Vfr controls the production of elastase, exotoxin A and twitching motility via type IV pili partly by controlling the las quorum sensing system in *P. aeruginosa* [13,14,17]. AlgR also regulates twitching motility, the production of hydrogen cyanide, pyocyanin and pyoverdin and quorum sensing via the rhl system [15,18,19].

However, there are both virulent and avirulent phenotypes of *P. aeruginosa*, [20,21] with the avirulent types causing the non-infectious inflammatory condition termed contact lens-induced acute red eye [22]. Even within virulent types there are those that cause acute cytotoxicity of corneal epithelial cells (cytotoxic strains) and those that can invade corneal epithelial cells (Invasive Strains). These two phenotypes are distinguished genotypically by possession of two type III secretion genes, exoS or exoU [23]. Indeed, the possession of exoU, along with a small group of other genes, has been shown to identify a specific sub-population or clone that is associated predominantly with infectious keratitis [23,24]. ExoU possessing strains are also associated with increased resistance to fluoroquinolones and beta-lactam antibiotics [25]. Using multilocus sequence typing, two other clonal types have been associated with keratitis, ST308 in Indian strains [26] and

ST235 in UK strains [27].

The technique known as suppression subtractive hybridization (SSH) can be used to identify genetic differences between related strains. This technique essentially hybridizes the cDNA of two strains, with pieces of cDNA that differ between the strains remaining unhybridized. Polymerase chain reaction is then used to amplify this remaining cDNA which represents differentially expressed sequences or different genomic sequences. SSH has been used to study differences in many bacteria [28]. This technique was used to identify a unique genetic locus that can be used to detect the presence of clonal cystic fibrosis isolates from Australia [6] and the UK, [4] to identify the accessory genome of cystic fibrosis isolates, [5] or virulence genes (using the similar technique of representational difference analysis) in a highly pathogenic strain of *P. aeruginosa* [29]. SSH has not been used with ocular isolates of *P. aeruginosa*. Furthermore, most previous studies have used isolates from infections to study genetic relatedness. However, as strains can be isolated from asymptomatic people and from non-infectious events that can occur during contact lens wear, we were interested to study the relationship between these types of strains and those isolated from microbial keratitis.

## Methods and Materials

### Bacterial Strains, Growth Conditions

The *P. aeruginosa* strains used in this study are given in (Table 1). Strains were isolated from contact lens wearing subjects following a corneal inflammatory event (Paer1), routine microbiology analyses of asymptomatic patients (Paer2 and -3) or after corneal infection (Paer17, -26, 6294 and 6206) [28,30]. Previously, these strains had been characterized in terms of their serotype, [31,32] production of proteases (elastase, alkaline protease and protease IV), [30,32,33] pyoverdine and rhamnolipids production, [33,34] production of quorum sensing acylated homoserine lactones, [30] possession of genes for protease IV, [33] type IV secretion, [32,33] and quorum sensing, [34] as well as resistance to antibiotics, [32] phenotype of invasion or cytotoxicity to epithelial cells, [30] and virulence in a mouse model of corneal infection [20,21,34]. All bacteria were stored at -80˚C in Trypticase Soya Broth (TSB; Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC, Australia) containing 30% glycerol, and were cultured at 37˚C on Trypticase Soya agar (Thermo Fisher Scientific Australia). For general laboratory work, colonies of *P. aeruginosa* were inoculated into 5 ml TSB and incubated at 37˚C with vigorous shaking for 18 hours to reach stationary phase.

| **Strains** | **Source of strain[32] and year of isolation** | **Sero-type [31, 32]** | **Production of:** | | | | **Possession of genes:** | | | **Resis-tance to antibiot-ics[32]** | **Virulence in mouse kerati-tis model[20, 21, 34]** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | **Proteases PIV/ LasB/ AprA/[30, 33] protease type[30, 32]** | **Py-over-dine pro-duc-tion [33]** | **Rham-nolipid produc-tion [34]** | **AHLs BHL/ HHL/ OHHL/ OdDHL [30]** | **Protease piv (group) [33]/ aprA,/ lasB [34]** | **Type IV secre-tion genes exoU/ exoS[32, 33]** | **Quorum-sensing genes lasI/ lasR/ rhlAB/ rhlR[34]** | | |
| Paer1 | Contact lens from CLARE event; Australia, 1993 | I (O:1) | +/-/-/III | + | + | -/+/-/+ | +(B)/+/+ | -/+ | -/-/+/+ | S | AV |
| Paer2 | Contact lens of as-ymptomatic wearer; Australia, 1994 | E (O:11) | +/-/-/IIb | + | - | -/-/-/+ | +(A)/+/+ | +/- | +/+/-/- | S | V |
| Paer3 | Contact lens disin-fection case of as-ymptomatic wearer; Australia, 1994 | I (O:1) | +/-/-/III | + | + | -/+/-/+ | +(B)/+/+ | -/+ | -/-/+/+ | S | AV |
| Paer17 | Cornea during microbial keratitis; Australia, 1994 | D (O:9) | +/+/+/III | + | + | -/+/-/+ | +(A)/+/+ | -/+ | +/+/+/+ | TIC, ATM, OFX, MXF | V |
| Paer26 | Contact lens disinfecting case of a person with microbial keratitis; Australia, 1998 | C (O:7/8) | ±/-/-/IIb | + | + | -/+/-/+ | +(A)/+/+ | +/- | +/+/+/+ | S | V |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 6206 | Cornea of a person with microbial keratitis; USA, 1992 | E (O:11) | ±/+/-/IIb | + | + | -/-/-/+ | +(A)/+/+ | +/- | +/+/+/+ | S | V |
| 6294 | Cornea of a person with microbial keratitis; USA, 1992 | G (O:6) | +++/++ +/++/I | ++ | ND | +/+/+/+ | +(B)/+/+ | -/+ | +/+/+/+ | S | V |
| PAO1 | Standard strain; Wound isolate; Australia, 1954 | (O:5) | ++/++ /++/I | ++ | ND | +/+/+/+ | +(B)/+/+ | -/+ | +/+/+/+ | ND | V |
| CLARE = contact lens induced acute red eye; S = susceptible; TIC = ticarcillin; ATM, aztreonam; OFX, ofloxacin; MXF, moxifloxacin; AV = avirulent in mouse model of keratitis; V = virulent in mouse model of keratitis. | | | | | | | | | | | |

**Table 1:** Characteristics of the *P. aeruginosa* strains used in the study.

**Polymerase Chain Reactions for Detection of Virulence Genes**

Oligonucleotide primers used in this study and their appropriate annealing temperatures are shown in (Table 2). All primers were synthesized by Invitrogen (Mulgrave, Vic, Australia). Vfr primers were designed using Prime (GCG) on the Biomanager suite (Australian National Genomic Information Services website: www.angis.org.au), using the PAO1 sequence from the Pseudomonas database. For template DNA in each polymerase chain reaction (PCR), bacterial DNA was released by incubating 1 ml of fresh overnight bacterial broth culture with 19 ml of microLYSIS™ buffer (Microzone Ltd., Sussex, UK) which was heated in a thermal cycler using the following conditions: 65˚C, 5 minutes; 96˚C, 2 minutes; 65˚C, 4 minutes; 96˚C, 1 minute; 65˚C, 1 minute; 96˚C, for 30 seconds and then cooled to 25˚C. DNA prepared by this method was used immediately or stored in small aliquoted amounts below -70˚C. Template DNA was not exposed to repeat freeze thaw cycles. PCR was carried out in a reaction mixture containing 12.5 ml of BioMix Red (32 mM $(NH_4)_2SO_4$, 125 mM Tris-HCl, pH 8.8, 0.02% Tween 20, 2 mM dNTP's, 2.5 mM $MgCl_2$, DNA polymerase 0.05 units/µl; BioLine GmbH, Luckenwalde, Germany), 100 pmol of each primer, 1 µl of Microlysis/bacterial DNA template, and brought to a reaction volume of 25 ml with nuclease free water. Parameters for the amplification cycles were: denaturation for 5 minutes at 94˚C, then 30 cycles of 94˚C for 30 seconds, annealing at 52-62˚C for 30 seconds, and extension at 72˚C for 90 seconds, followed by a final extension period of 72˚C for 7 minutes. The expected amplification product size for each reaction is listed in (Table 2). PCR reactions were analyzed by electrophoresis though 1.5% agarose (in 45 mM Tris, pH 8.0, 45 mM boric acid and 1mM ethylenediaminetetraacetic acid buffer) gel, stained with SybrSafe (*In vitro* gen) and viewed under ultra-violet illumination. PCRs were performed at least twice for each primer pair and used freshly prepared template DNA on each separate occasion.

| **Primers** | **Sequence** | **Annealing Temp (˚C)** | **Product Size (base pairs)** |
|---|---|---|---|
| *vfr*-f | 5'-TGTTCTTCCAGGAGCGTGG-3' | 58 | 946 |
| *vfr*-r | 5'-TCGCAAAATCACATCGAC-3' | | |
| *algR*-f[14] | 5'-CGTGAGGATCCCGGACGGCACTAC-3' | 55 | 921 |
| *algR*-r[14] | 5'-ACGAAGCTTACATGGGATATTCCG-3' | | |
| **Oligonucleotides** | **Sequence** | | |
| Adaptor 1[35] | 5-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3 | | |
| | and 3-GGCCCGTCCA-5 | | |
| Adaptor 2[35] | 5-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3 | | |
| | and 3-GCCGGCTCCA-5 | | |
| P1[35] | 5-CTAATACGACTCACTATAGGGC-3 | | |
| NP1[35] | 5-TCGAGCGGCCGCCCGGGCAGGT-3 | | |
| NP2[35] | 5-AGCGTGGTCGCGGCCGAGGT-3 | | |

**Table 2:** Primers and oligonucleotides used in this study.

**Suppression Subtractive Hybridization (SSH)**

The oligonucleotides used for subtractive hybridizations are shown in (Table 2) [35]. SSH was used to compare the genomes of *P. aeruginosa* Paer3 and 6294. The SSH protocol used in this study was adapted from the methods originally described for Helicobacter pylori DNA, [35] initially using 6294 DNA as the tester and Paer3 as the driver DNA. The following minor changes to the original protocol [35] were made: RsaI restriction enzyme was used to digest the DNA; in the first PCR reaction, the final 72°C extension was increased to 7 minutes; the products of the second PCR were inserted into the pCR®4-TOPO® TA cloning kit (ThermoFisher Scientific, North Ryde, Australia) according to the manufacturer's instructions. During sequencing, any vector DNA was removed in silico from each sequence using VecScreen at http://www.ncbi.nlm.nih.gov/VecScreen. Sequences were then submitted to the Genbank Database

(National Centre for Biotechnology Information, Bethesda, MD) under accession numbers DQ436444-DQ436454 and screened for similarity to known protein sequences (BLASTx; http://www.ncbi.nlm.nih.gov/BLAST). The SSH protocol was repeated twice using identical techniques but reversing the 6294 and Paer3 DNA pools to determine whether Paer3 contained any additional DNA sequences to 6294.

**Dot Blot Hybridizations**

To determine the possession of the potential unique sequences in clones and other *P. aeruginosa* strains, 5mL of PCR product from each subtracted library clone or 10ml genomic DNA from other *P. aeruginosa* strains to be probed was heat denatured (95˚C for 10 minutes, then 0˚C for 10 minutes) and applied to Hybond N+ membrane and fixed by baking at 80˚C for 2 hours. Labelled 6294 and Paer3 DNA was prepared by incubating 1 mg of heat denatured RsaI digested genomic DNA with 0.25 mM random primer (Promega Australia, Sydney, Australia), 1 mM ATP, CTP, GTP (each), 0.65 mM UTP, 0.35 mM DIG-11-UTP (Roche Applied Science, Mannheim, Germany), reaction buffer (50 mM Tris-HCl, pH 7.2, 10 mM MgSO4, 0.1 mM DTT) and 10 units of DNA polymerase I (Promega Australia) in a total volume of 50 µl at 37˚C for 18 hours. A positive control used prpL primers (forward 5'-AGAGCCACTCCAGACCAAAC-3'; reverse 5'-GGATAAACGGCGGATAACAC-3') and PAO1 DNA extract as template for the PCR reaction. The probes were purified using the Wizard SV Gel and PCR Cleanup Kit (Promega Australia), and the concentration estimated by spectrophometric absorbance readings at 260/280 nm. Pre-hybridization of the membrane occurred in nuclease free glass tubes (Hybaid) containing 0.125 µl/cm2 DIG Easy Hyb solution (Roche Applied Science) for 2 hours at 42˚C in a rotisserie-style hybridizing oven. The appropriate amount of probe was denatured by incubation for 5 minutes in a boiling water bath and then held on ice for 5 minutes. A small portion of the pre-hybridization solution was removed and mixed with the appropriate volume of denatured DIG labelled probe (final concentration 5 ng/ml). The probe solution was added back to the tube and allowed to hybridize at 42˚C overnight. Unbound probe was removed by performing two successive low stringency washes for 5 minutes each in 5 X SSC buffer (0.75 M NaCl, 0.075 M Na citrate, pH 7.0), 0.1% (w/v) SDS (sodium dodecyl sulfate) at 25˚C. This was followed by a high stringency wash in 1 X SSC, 0.1% (w/v) SDS at 42˚C; and finally in 0.1 X SSC, 0.1% (w/v) SDS at 42˚C. Membranes were then incubated for 5 minutes in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) containing 3% (v/v) Tween 20 and blocked for 2 hours in maleic acid buffer containing 1% (w/v) blocking reagent (Roche Applied Science). The membranes were then incubated with a 1:5000 dilution of alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Science) in the blocking solution for 30 minutes. Membranes were washed twice for 15 minutes each in maleic acid buffer containing 3% (v/v) Tween 20, and DNA spots that bound the labelled probe were detected with Nitroblue Tetrazolium-5-Bromo-4-Chloro-3-Indolylphosphate (NBT-BCIP) in alkaline phosphatase detection buffer (100 mM Tris HCl, 100 mM NaCl, pH 9.5) until suitable development had occurred. Image analysis was performed by using Quantity One software and GS-800 calibrated densitometer (Bio-Rad Australia, Gladesville, NSW, and Australia) to determine the percentage of probe bound to the membrane, in comparison to the wildtype strain PAO1.

**Gel Analysis and Sequencing**

A 5 µl aliquot of each 6294-specific amplified fragment was analyzed by electrophoresis though a 1.5% (w/v) agarose gel in TBE buffer (45 mM Tris, pH 8.0, 45 mM boric acid and 1 mM EDTA), stained with SybrSafe (Invitrogen) and viewed under ultra-violet illumination. A DNA size comparison was included to allow size estimation of each clone fragment (BenchTop 1 kb Ladder, Promega Australia). Clones that produced a PCR product with a clear single band of a unique size to all other fragments were selected for subsequent analysis. Fragments were purified from the original PCR reaction using a Wizard SV Gel and PCR Cleanup Kit (Promega Australia), following the manufacturer's instructions. The DNA sequence of each fragment was analyzed using an ABI Prism 3700 Automated DNA platform. Matches to each sequence were then found using the BLASTx search tool on the NCBI database website.

**Results**

All isolates were positive for vfr and algR by PCR. After SSH, a total of 144 clones were picked at random over both experiments. After dot blot screening (Figure 1), 31 of the DNA fragment inserts were identified to be present in strain 6294 and not in Paer3. Clones were then screened using PCR amplification and gel electrophoresis. Eleven clones were deemed suitable for further analysis and sent to be DNA sequenced (Lanes 1, 4, 5, 11, 13, 16, 18, 22, 23, 28, 30) (Figure 1).

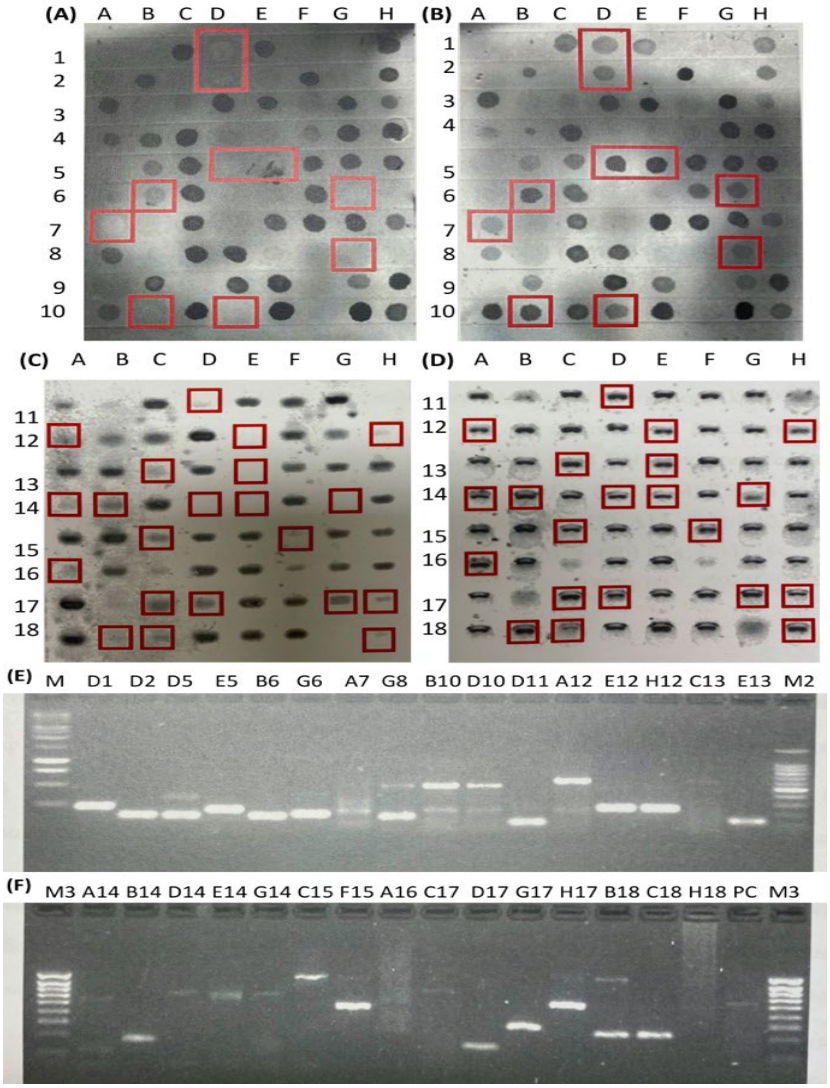


**Figure 1:** Amplified DNA dotted onto nitrocellulose membranes and probed with labelled Paer 3 (A and C) or 6294 (B and D) genomic DNA. Positions of the areas where there were differences between the hybridization results are shown in red quadrilaterals and the corresponding positions (e.g. E5) were then assigned as the clone identifications. The lower part of the figure shows the results of PCR amplification of DNA sequences that showed homology to the 6294 probe but not the Paer 3 probe. M = 1Kb DNA ladder (Promega Australia); M2 = 100bp DNA ladder (Promega Australia); M3 = Hyperladder IV (Bioline); PC = positive control prpL gene. Other lanes correspond to positions in A-D above.

Seven of the 11 sequences had significant similarity (>96%) to published *P. aeruginosa* genes. One sequence was too short to produce significant similarity to the database and was therefore excluded from further analysis. The analysis of each sequenced library fragment is shown in (Table 3). Two fragments (E5, B6) were of a higher G+C content, two (C18, D18) of lower G+C content and three (E12, E13 and F15) had approximately the average G+C content of PAO1. All fragments were submitted to Genbank and their accession numbers are given in (Table 3).

| Fragment No. | Clone ID | Insert Size (bp) | G+C Content (%) | GenBank Accession No.* | Genus, Species | Protein Similarities BLASTx [E Value, Residue identity] |
|---|---|---|---|---|---|---|
| DNA fragments missing from Paer3 genome compared to PAO1 | | | | | | |
| 1 | E5 | 166 | 72 | DQ436445 | *P. aeruginosa* | xanthine dehydrogenase family protein molybdopterin-binding subunit, partial [5e-15, 34/34] Sequence ID: RCI69845.1 |
| 2 | E12 | 97 | 67 | DQ436448 | *P. aeruginosa* | hypothetical protein P797_32705 [1e-07, 24/25] Sequence ID: AIL00036.1<br><br>benzoylformate decarboxylase, partial [2e-07, 24/25] Sequence ID: PBD22408.1 |
| 3 | E13 | 92 | 61 | DQ436449 | *P. aeruginosa* | tyrosine-type recombinase/integrase [6e-07, 23/23] Sequence ID: WP_079387396.1 |
| 4 | F15 | 414 | 66 | DQ436452 | *P. aeruginosa* | Autoinducer synthetase *lasI* [5e-65, 70/70] Sequence ID: VFT21013.1 |
| 5 | B6 | 116 | 72 | DQ436446 | *P. aeruginosa* | hypothetical protein CVT20_25495, partial [3e-09, 26/27] Sequence ID: PKG09998.1 |
| 6 | C18 | 185 | 44 | DQ436454 | *P. aeruginosa* | DUF2235 domain-containing protein, partial [1e-28, 53/53] Sequence ID: WP_144205624.1 |
| 6# | D18 | 186 | 42 | DQ436444 | *P. aeruginosa* | DUF2235 domain-containing protein, partial [4e-31, 56/56] Sequence ID: WP_144205624.1 |
| DNA fragments present in 6294 but not PAO1 | | | | | | |
| 7 | H17 | 454 | 54 | DQ436453 | *P. aeruginosa* | AAA family ATPase [3e-95, 150/150] Sequence ID: WP_134225397.1 |
| 8 | B14 | 137 | 57 | DQ436450 | No significant similarity | |
| 9 | C15 | 750 | 57 | DQ436451 | *P. aeruginosa* | hypothetical protein [1e-178, 240/241] Sequence ID: WP_105750495.1 |
| *, The DNA fragment assigned to accession number DQ436447 was too short to give significant homology to any sequence after vector sequences were removed and was therefore disregarded. #, Clone D18 was mapped to different loci of the same gene as C18. | | | | | | |

**Table 3:** Protein similarities as identified by BLASTx searches.

Each clone identified from the library screening was used to probe each of the strains used in this study (Table 4). The majority of the subtracted library clones (D1, E5, B6, E12, E13, F15, C18) were found to be present in all of the screened *P. aeruginosa* strains tested except Paer1 and Paer3. The sequences of clones B14, C15 and H17 were absent in all strains other than 6294. There was no significant hybridization between any of the probes and the negative control strains of *P. putida* and *E. coli*. All strains hybridized with DNA to prpL, except the negative controls.

| Clone Probe ID | Fragment Size (bp) | 6294 / Paer3 | | Isolates screened for presence of subtracted sequences9 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 6294 | Paer3 | Paer1 | Paer2 | Paer17 | Paer26 | 6206 | PAO1 | *P. putida* | *E. coli* |
| D18 | 186 | + | - | - | + | + | + | + | + | - | - |
| E5 | 166 | + | - | - | + | + | + | + | + | - | - |
| B6 | 116 | + | - | - | + | + | + | + | + | - | - |
| E12 | 97 | + | - | - | + | + | + | + | + | - | - |
| E13 | 92 | + | - | - | + | + | + | + | + | - | - |
| F15 | 414 | + | - | - | + | + | + | + | + | - | - |
| C18 | 185 | + | - | - | + | + | + | + | + | - | - |
| B14 | 137 | + | - | - | - | - | - | - | - | - | - |
| C15 | 750 | + | - | - | - | - | - | - | - | - | - |
| H17 | 454 | + | - | - | - | - | - | - | - | - | - |
| *prpL* | 1047 | + | + | + | + | + | + | + | + | - | - |

**Table 4:** Dot blot hybridization of the subtracted sequences with strains used in the study.

After reversing the 6294 and Paer3 DNA pools, no DNA fragments were identified that were unique to the Paer3 genome once homologous 6294 DNA sequences were removed. This suggests that there were no additional genes or significant differences within the Paer3 genome.

## Discussion

The *P. aeruginosa* strains used in the current study were chosen based upon the diversity of the ocular conditions they were isolated from and genotypic and phenotypic traits. All of these strains possessed the *vrf* and *algR* genes that encode for their transcription factors. Vrf is a global regulator of virulence factor expression, controlling the expression of exotoxin A, protease, type IV pili, a type III secretion system as well as another transcription regulatory system, the las quorum-sensing, which controls the expression of hundreds of additional genes, including multiple virulence factors [13,14,17,36,37]. The type III secretion effectors ExoS, ExoT, and ExoU have been shown to have important roles in the pathogenesis of *P. aeruginosa* keratitis, [38,39] as has exotoxin [40] Type IV pili that mediate twitching motility, [41] AlgR activates alginate production and twitching motility but represses the *P. aeruginosa* quorum-sensing system Rhl which is responsible for rhamnolipid production [19] and hydrogen cyanide, [15] pyocyanin and pyoverdine production [18]. The Rhl system appears to be less important than the Las system during keratitis, [34] whereas loss of the ability to produce pyoverdine reduces the virulence of *P. aeruginosa* during keratitis [42]. These data suggest that *vfr* would be important for strains to induce keratitis, but *algR* may be less important. However, the fact that strains, isolated as far apart in years as 1954 (PAO1) to 1998 (Paer26), have retained these genes suggests that they are important in survival of the organism.

In this study we identified genetic elements of pathogenic *P. aeruginosa* strain 6294 that were not present or not transcribed or significantly mutated in the non-pathogenic strain Paer3. By selecting strain 6294 for this study, instead of using the type strain PAO1, we were not only able to identify genes absent from the Paer3 genome, but also novel genes added to the 6294 strain that could potentially contribute to virulence during keratitis.

Six of the nine genes identified from the 6294 genome were not present in the cDNA library of Paer3. These six gene fragments were also absent from the cDNA library of strain Paer1, another avirulent isolate. The identification that the gene for the quorum-sensing signal generating enzyme LasI (clone F15) was missing from the cDNA library of Paer1 and Paer3 was a significant finding from this study and confirms previous findings [34] thus validating the SSH technique used in the current study. This enzyme produces an acylated homoserine molecule involved in the regulation of the Las quorum-sensing system [43-46]. A previous study demonstrated that the lasI gene controls virulence of *P. aeruginosa* in the eye [34]. This confirms previous evidence that shows strains Paer1 and -3 were deficient in this gene and that this was at least in part responsible for the attenuated virulence seen in the scratched mouse cornea model [34].

The probable tyrosine-type recombinase/integrase (Clone E13) of *P. aeruginosa* is of interest. Whilst this particular gene has not been implicated in *P. aeruginosa* virulence, these types of integrases catalyze recombination between DNA sequences that share limited identity and so may facilitate genomic rearrangements and integrations of mobile genetic elements within the chromosome of *P. aeruginosa*. The absence of this integrase in Paer3 and Paer1 may be an indication that, at some point in the evolution of these strains, the loss of this gene, and possibly surrounding genetic information, was favorable to survival. Conversely, the integrase may have been damaged or lost, preventing horizontal transfer of

important virulence factors, and perhaps contributing to the more limited genetic difference of Paer3 compared to 6294. The role of these genes and their proteins in ocular virulence of *P. aeruginosa* requires further investigation with site-specific mutants.

Three of the nine genes identified by this study were unique to the cDNA of 6294 when compared to all the other strains used in this study, but two had homologues with genes from other *P. aeruginosa* strains. These two DNA fragments contained an AAA family ATPase or a hypothetical protein. AAA family ATPases couple energy generation from ATP hydrolysis to mechanical force and have been associated with flagella expression and rapid translation from a motile state to the sessile biofilm state [47], and also several other cellular functions. Perhaps possession or transcription of these genes gives certain strains of *P. aeruginosa* a competitive advantage. The ATPase is identical to that of several *Pseudomonas aeruginosa* strains (192S190811BSL_PA1, 192S190811BSL_PA2 192S190811BSL_PA3) isolated from sputa of a cystic fibrosis patients in Canada, and the hypothetical protein was identical to a gene from strains from China (such as WCHPA075045, WCHPA075022) and from throat swabs of cystic fibrosis patients in Germany (strains OY3, BJ2) and a microbial keratitis isolate (PA40) from India. The hypothetical protein may be involved in pathogenesis or other important functions of the virulent strains.

The unique DNA fragment B14 might be used to create a specific probe to identify 6294 and very closely related strains. Alternatively, lack of DNA fragments D1, E5, B6, E12, E13, F15 or C18 has the possibility of identifying avirulent strains such as Paer1 or Paer3. This might be important when people present with Contact Lens-Induced Acute Red Eye (CLARE). Having the ability to clearly identify avirulent strains that might be cultured from contact lenses at the time of a CLARE event may reduce the use of antibiotics, which are not needed to treat CLARE (Simply removing the contact lens is an appropriate treatment), [48] and so reduce the possibility of creating antibiotic resistance in strains. There is clear evidence that topical use of fluoroquinolones is associated with increased isolation of resistant strains from eyes [49,50].

## Conclusion

In conclusion, this study identified several genes in the pathogenic microbial keratitis isolate that were absent in two strains that had been isolated from either a non-infectious inflammatory condition associated with bacterial colonisation of contact lenses, CLARE, or a strain isolated from the contact lens disinfecting storage case of an asymptomatic contact lens wearer, both of which had previously been shown to be avirulent in a mouse keratitis model. These genes might confer virulence phenotypes to *P. aeruginosa* and this should be followed up using strains deleted in those genes in the mouse model of keratitis.

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