Accessory genome of the multi-drug resistant ocular isolate of *Pseudomonas aeruginosa* PA34

Dinesh Subedi1*, Gurjeet Singh Kohli2, Ajay Kumar Vijay1, Mark Willcox1, Scott A. Rice2,3,4

1 School of Optometry and Vision Science, University of New South Wales, Sydney, Australia, 2 The Singapore Centre for Environmental Life Sciences Engineering, Nanyang Technological University, Singapore, Singapore, 3 The School of Biological Sciences, Nanyang Technological University, Singapore, Singapore, 4 The ithree Institute, The University of Technology Sydney, Sydney, New South Wales, Australia

*d.subedi@unsw.edu.au*

Abstract

Bacteria can acquire an accessory genome through the horizontal transfer of genetic elements from non-parental lineages. This leads to rapid genetic evolution allowing traits such as antibiotic resistance and virulence to spread through bacterial communities. The study of complete genomes of bacterial strains helps to understand the genomic traits associated with virulence and antibiotic resistance. We aimed to investigate the complete accessory genome of an ocular isolate of *Pseudomonas aeruginosa* strain PA34. We obtained the complete genome of PA34 utilising genome sequence reads from Illumina and Oxford Nanopore Technology followed by PCR to close any identified gaps. In-depth genomic analysis was performed using various bioinformatics tools. The susceptibility to heavy metals and cytotoxicity was determined to confirm expression of certain traits. The complete genome of PA34 includes a chromosome of 6.8 Mbp and two plasmids of 95.4 Kbp (pMKPA34-1) and 26.8 Kbp (pMKPA34-2). PA34 had a large accessory genome of 1,213 genes and had 543 unique genes not present in other strains. These exclusive genes encoded features related to metal and antibiotic resistance, phage integrase and transposons. At least 24 genomic islands (GIs) were predicted in the complete chromosome, of which two were integrated into novel sites. Eleven GIs carried virulence factors or replaced pathogenic genes. A bacteriophage carried the aminoglycoside resistance gene *(AAC(3)-IId)*. The two plasmids carried other six antibiotic resistance genes. The large accessory genome of this ocular isolate plays a large role in shaping its virulence and antibiotic resistance.

Introduction

*Pseudomonas aeruginosa* is associated with many opportunistic and nosocomial human infections such as pneumonia, septicemia, corneal ulcers (microbial keratitis) and chronic infections in cystic fibrotic lungs [1, 2]. Antibiotic resistance in this bacterium is alarmingly on the
Pseudomonas aeruginosa has been included in one of the top three priority pathogens urgently requiring new antimicrobial therapies for treatment by the World Health Organization [3]. Resistance to different antimicrobials in P. aeruginosa is due to its inherent capacity to oppose the action of antibiotics and its capacity to acquire genetic elements that often carry antibiotic resistance genes [4].

The study of the mechanisms used by different strains of P. aeruginosa to become resistant to antibiotics or acquire virulence have benefited from genome sequencing and these have identified a number of mobile genetic elements (MGEs) [5–7]. However, many of the genomes investigated are not completely closed and, in this context, accessory genes associated with resistance or virulence in addition to mutations may be missed. This may limit the understanding whether resistance and virulence genes are present on MGEs or “standard” chromosomal regions as well as their prevalence in general [8]. For example, less than 5% of P. aeruginosa draft genomes are complete; furthermore, only 37 plasmids of the total (3003 as of 15/10/2018) in the NCBI P. aeruginosa database are complete. Additionally, many of the isolates sequenced are derived from cystic fibrosis infections, while relatively few ocular and other isolates are represented. This is an important source of genetic information for P. aeruginosa as specific subpopulations are thought to be associated with microbial keratitis [9]. Notably, those subpopulations are predominantly characterised by possession of genes associated with type IV pili twitching motility, cytotoxicity (exoU), and certain genomic islands [9]. Indeed, to date, the complete genome of only one P. aeruginosa keratitis isolate has been published [10]. Very little information is available about the accessory genomes of ocular isolates.

P. aeruginosa strain PA34 (referred to as PA34 hereafter) is a multi-drug resistant microbial keratitis isolate which is resistant to gentamicin, imipenem, ciprofloxacin and moxifloxacin. Analysis of its draft genome revealed that PA34 belongs to sequence type 1284 based on multilocus sequence typing and carried at least 12 acquired resistance genes and possessed the exoU gene [11], an important effector gene in the pathogenesis of microbial keratitis [12]. A class I integron (I1427) that carries two antibiotic resistance genes has been shown to be integrated into a Tn3-like transposon carried by PA34 [13]. However, the genetic context of these resistance and pathogenic genes has not been completely elucidated. Hence, in this study we sought to analyse the complete genome of PA34, to examine the genetic structure of accessory genome and to compare the complete genome of PA34 with other genomes of P. aeruginosa from the public database.

Materials and methods

Bacterial strain and microbiology

The strain Pseudomonas aeruginosa PA34 was isolated in 1997 from the cornea of a microbial keratitis patient in a tertiary eye care centre L.V. Prasad Eye Institute, Hyderabad, India. The cause of microbial keratitis was recorded as trauma induced by a stone in the right eye of a 21-year old male. Three complete genomes of P. aeruginosa were used for comparison: I) P. aeruginosa PAO1, which has the most complete and curated annotations [14, 15], II) P. aeruginosa PA14, an exoU positive strain [16] and III) P. aeruginosa VRFP04, an Indian microbial keratitis isolate [10].

The minimum inhibitory concentration (MIC) to three heavy metals (mercury, copper and cobalt) was determined by broth dilution methods as described elsewhere with minor modification [17, 18]. PA34 and PAO1 (as the control strain) were examined against Hg²⁺, Cu²⁺ and Co²⁺ by using twofold serial dilutions from 16mM– 0.31mM. The metal salt solutions (HgCl₂, CuCl₂ and CoCl₂) were prepared in deionised water, sterilized by membrane filtration and added to Mueller-Hinton broth (Oxoid Ltd, Hampshire, UK) to obtain the
required concentrations. The experiments were performed in triplicate and repeated three times. Wilcoxon matched-pairs signed rank test was used to confirm significant difference between metal tolerance and the strain. A p-value of <0.05 was taken as significant.

Cytotoxicity was examined by the trypan blue dye exclusion assay [19]. Briefly, human corneal epithelial cells (HCEC) were grown in 96 well plates in the presence of SHEM (DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 1.05mM CaCl$_2$, 0.5% DMSO, 2ng/mL epidermal growth factor (Gibco), 1% ITS-X (Gibco)). 200µL of 5x10$^5$ CFU/mL $P$. aeruginosa in SHEM was used to expose confluent HCEC. Following 3 h incubation at 37˚C, the cells were stained with 0.4% trypan blue. The stained cells were observed by microscopy and photographs were taken for the quantitative determination of cytotoxicity. $P$. aeruginosa PAO1 (a non-cytotoxic strain) were used as a control. The experiment was performed in triplicate and repeated three times.

MinION sequencing and complete genome assembly
Genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) as per the manufacturer’s protocol. The amount of extracted DNA was determined using a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). The library was prepared using the Ligation Sequencing Kit 1D R9 Version as per the manufacturer’s instructions. The sequencing library was loaded into the Flow Cell Mk 1 Spot-ON of the MinION system using Library Loading Bead Kit R9 Version according to the manufacturer’s instructions. The raw reads were obtained using the MinKNOW v1.7.14 in a 24-h run experiment and base calling was performed using Albacore v2.0.2. A total of 50,831 reads ranging from 174bp to 58,887bp (mean = 3,292bp) were obtained. The sequence reads from Illumina (which was obtained using MiSeq (Illumina, San Diego, CA) generating 300bp paired-end reads [11]) and MinION data were assembled using three hybrid assembly pipe-lines; Unicycler v0.4.3 [20], Japsa v1.8.0 [21] and Spades v3.12.0 [22]. Spades resulted in the best assembly in terms of contig numbers (Table 1). The genome coverage was calculated using BBmap v35.82 [23].

All contigs were reordered with reference to the three complete genomes of $P$. aeruginosa. (PAO1, PA14, and VRFPA04) using MUMmer v3.0 [24]. Gaps between the contigs were determined by ABACAS v1.3.1 [25] using the “circular reference genomes” flag. Pseudogenomes of different sizes were observed after comparison with different reference strains and in all comparisons, contigs 6 and 7 were observed as unused contigs indicating they were not part of the chromosome. This was further verified by performing a BLAST of unused contigs against reference genomes. The fewest gaps between contigs in the PA34 genome (99 bp) were observed when PA14 was used as the scaffold compared to the other strains. Further, the size of the PA34 pseudogenome was slightly less than the size of the draft genome (~6.8 Mbp) [11]

| Contig number | Length (bp) | Mean coverage (fold) | G+C content (%) |
|---------------|-------------|----------------------|-----------------|
| 1             | 3254829     | 54.8                 | 66.3            |
| 2             | 1907753     | 57.9                 | 65.4            |
| 3             | 996852      | 48.0                 | 66.4            |
| 4             | 466064      | 47.7                 | 65.7            |
| 5             | 184581      | 63.3                 | 66.9            |
| 6             | 95404       | 49.8                 | 57.2            |
| 7             | 26862       | 108.9                | 61.0            |

Table 1. Assembly result from Spades.

https://doi.org/10.1371/journal.pone.0215038.t001
when using PA14 as the scaffold genome. A gap of 99 bp is an indication of a joining point between two contigs [25]. Therefore, this comparison was considered as the best and was further used to complete the genome of PA34. Primers were designed for each gap (S1 and S2 Tables) using Primer3web v4.1.0 [26] and the gap size was verified by PCR. Using this approach, we were able to close the gaps between contigs to generate a single contiguous chromosome based on contigs 1–5. Contigs 6 and 7 had a lower G+C content than rest of the contigs and showed similarity with different plasmids based BLASTn searches against NCBI database. Contigs 6 and 7 were therefore estimated to represent two different plasmids and were both circularised, referred to here as pMKPA34-1 and pMKPA34-2 (Plasmid 1 and 2 of Microbial Keratitis strain PA34).

Bioinformatics analysis

The complete chromosomal genome was annotated using NCBI Prokaryotic Annotation Pipeline [27]. Plasmids were annotated by Prokka v1.7 [28] followed by manual examination and curation using information from ISsaga [29], the Rapid Annotations using Subsystems Technology (RAST v2.0) [30] and NCBI BLASTn searches. Genomic islands were predicted on the basis of MAUVE [31] whole genome alignment against *P. aeruginosa* strains PAO1, PA14 and VRFPA04. Through this comparison, DNA blocks of four contiguous open reading frames were predicted as genomic islands of PA34 if these blocks were present in PA34 but absent in anyone of the three reference genomes used for comparison [5]. Pangenome analysis was conducted using Roary v3.12.0 [32], and all four genomes (PAO1, PA14, VRFPA04 and PA34) were annotated using Prokka to avoid annotation biases. Other software used for analysis were CRISPReFinder database [33] CGview [34], Easyfig [35], SnapGene Viewer v4.3.2 [36], VENNY v2.1.0 [37] and Resfinder V3.0 [38]. The nucleotide sequence of the complete chromosome and two plasmids were made available in the NCBI database under GenBank accession numbers CP032552, MH547560 and MH547561.

Results and discussion

General features of *P. aeruginosa* PA34 genome

The statistics of the complete genome of *P. aeruginosa* PA34 (Table 2) were broadly matched with other published complete genomes of *P. aeruginosa* [39]. A total of 6,462 coding sequences (CDS) were predicted in the 6.8 Mbp genome of PA34 using NCBI Prokaryotic Genome Annotation Pipeline (PGAP) [40]. Of the predicted CDS, 6,314 were predicted to form functional proteins and 148 were pseudogenes.

Table 2. Genomic features of *P. aeruginosa* PA34.

| Characteristics                     | Value         |
|-------------------------------------|--------------|
| Genome size in base pairs           | 6,810,079    |
| G+C content %                       | 66.1         |
| Number of genes                     | 6,544        |
| Number of coding sequences (CDS)    | 6,462        |
| Number of protein coding genes      | 6,314        |
| Number of pseudogenes               | 148          |
| Total RNA genes                     | 82           |
| complete 5S, 16S, 23S rRNAs         | 4, 4, 4      |
| tRNAs                               | 65           |
| ncRNAs                              | 5            |

https://doi.org/10.1371/journal.pone.0215038.t002
In the genomic comparison with three reference genomes (PAO1, PA14 and VRFPA04) and the genome of PA34, it was estimated there were 7,643 orthologs in the pangenome. Of those orthologs, 5078 were common amongst all four strains and are suggested to represent the size of the core genome. PA34 had the largest accessory genome compared to these reference strains (having 1,213 genes [~20% of total genes]) that had 543 genes unique to PA34. These exclusive genes were determined to encode features related to metal and antibiotic resistance, phage integrase and transposons. Furthermore, in the context of individual reference genomes, PA34 has 886, 737, 946 genes with no orthologs in PAO1, PA14, and VRFPA04, respectively (Fig 1) (See S1 File for full annotations of exclusive orthologs). A high proportion of strain-specific accessory genomic elements are common in P. aeruginosa strains and this represents the diversity of its genome [41, 42]. However, in this study, we observed a lower range (3–10%) of exclusive orthologues than previously reported range (10–20%) [41]. The difference observed may be related to the use of the complete genome for the analysis over the use of draft genomes in previous studies. Since draft genomes are likely to be mis-assembled due to the presence of a high content of repetitive sequences in bacteria [43], analysis based on the complete genome is expected to be more relevant. PA34 shared 124 orthologs with VRFPA04, an eye isolate, and those genes were associated with chloramphenicol resistance, pathogenesis (type IV secretion system) and phages.

The diversity of core and accessory genomes observed in this study contradicts findings on environmental species P. fluorescens, where comparative genomics of three isolates demonstrated...
that they shared a core of 61% genes (vs 66% in this study) and 22–27% of unique genes to each isolate [44]. Furthermore, comparative genomics of three strains of the plant pathogen P. syringae showed a smaller core genome of 56% [45] than that of P. aeruginosa observed in this and another study [46]. The overall low diversity amongst P. aeruginosa strains compared to other Pseudomonas spp. may result from the selection of closely related strains (i.e. those containing exoU) in the present study.

No significant CRISPR and associated Cas genes were observed in PA34 based on comparison with the CRISPycasFinder database [33]. CRISPR-Cas system may be negatively correlated with the size of the accessory genome and acquired antibiotic resistance genes because this system restricts the invasion and incorporation of MGEs. Thus, strains without a CRISPR-Cas system are expected to have a larger genome size [47].

Genomic islands

Genomic islands (GIs) refer to blocks of horizontally acquired DNA that integrate into certain loci in the core genome [48]. Such loci are known as Regions of Genomic Plasticity (RGPs). GIs should have a minimum of four contiguous open reading frames, which are not present in any of the set of genomes to which they are compared [5]. More than 80 RGPs have been identified in the genome of P. aeruginosa [42]. The position of RGPs in the genome are indicated by homologous flanking loci in PAO1 [5]. RGPs constitute a major portion of the accessory genome and are essential for the adaption of P. aeruginosa in diverse habitats. In this study, 24 RGPs were observed in the complete genome of PA34 (Table 3 and Fig 2) when compared with genomes of P. aeruginosa strains PAO1, PA14 and VRPA04. A study has reported 27 to 37 RGPs in individual genomes of five P. aeruginosa strains [5]. The difference in number of observed RGPs may be due to a difference in the number of genomes used for comparison. Nevertheless, out of 24 GIs in the current study, two GIs were integrated into loci which have not been reported previously [42] and were deemed as GIs MKPA34-GI1 and MKPA34-GI2 in this study (Table 3). The GI MKPA34-GI1 was 68.9 Kbp, was integrated between PA2858 and PA2859 homologues of PAO1 and carried genes for mercury and chromate resistance. The GI MKPA34-GI2 had a size of 39.9 Kbp, encodes genes associated with phages and was integrated into loci flanking 4856/4857 of PAO1. In addition, three GIs (RGP2, RGP50 and RGP57) were associated with the type IV secretion system.

Integrative conjugative elements (ICEs) are chromosomally integrated self-transmissible MGEs that can exist as circular extrachromosomal elements and are antecedents of various GIs [48]. Our analysis revealed three ICEs in the complete genome of PA34. The ICEs observed in RGP5 and RGP29 were related to clc-like ICE elements (Genbank Accession number AJ617740). The parental clc element contains genes for chlorocatechol (clc) degradation [50]. However, these genes were lost from both RGP5 and RGP29 of PA34. Nevertheless, genes required for integration and conjugation were identified in both of the ICEs in RGP5 and RGP29 (Fig 3A) indicating that these elements may have the capacity to transfer within and between species. This may be the reason for observing clc-like GIs in two different loci in this strain. In addition, RGP5 carried mercury resistance genes, which are not present in the parental clc elements. This finding suggests that the clc-like elements may incorporate antibiotic resistance genes during genetic recombination.

The third ICE observed in RGP41 of PA34 was related to the pKLC102 family (GenBank Accession number AY257538) and was similar to P. aeruginosa pathogenicity island PAPI-1.
(GenBank Accession number AY273869) first identified in *P. aeruginosa* clone C (Fig 3B). PAPI-1 is self-transmissible and carries virulence factors such as genes for type IV pilus biogenesis and the *cupD* gene clusters. The *cupD* genes are essential for the formation of cell surface fimbriae that are involved in biofilm formation [51]. Interestingly, the *cupD* gene cluster appears to have been lost from the RGP41 of PA34, although the genes for type IV pilus biogenesis are still present.

In addition, various members of pKCL102 ICE family are found to be associated with the carriage of *exoU/spcU* genes [48]. These GIs have frequently been referred as *exoU* islands such as *exoU* island A, *exoU* island B, *exoU* island C and PAPI-2 [52, 53]. Possession of the *exoU* gene, that encodes the type III secretion system effector cytotoxin ExoU, markedly enhances the virulence of *P. aeruginosa* [54]. An *exoU*-island of 7.5 Kbp carrying five open reading frames was observed in PA34 (RGP7). In contrast to other *exoU*-islands that contain genes associated with integration, transposition, conjugations and sometimes antibiotic resistance [55], the *exoU* island observed here contains only three genes that have homology to a site-specific integrase (Fig 4A). The presence of both PAPI-1 and PAPI-2 like elements that act synergistically in virulence [56] may indicate an enhanced virulence of PA34.

To determine whether the *exoU* gene was functional, cytotoxicity of PA34 was examined in a human corneal epithelium cell line and compared with *P. aeruginosa* PAO1 which is a non-cytotoxic invasive strain [57]. Microscopic examination after staining with trypan blue indicated that PA34 was highly cytotoxic (Fig 5) showing a large number of dead cells. Based on

---

**Table 3. Regions of genomic plasticity (RGP) [5, 42] with associated features in PA34.**

| RGP ID   | Flanking homologous loci in PAO1 | Start   | End     | Size (bp) | G+C (%) | CDS | Key features                                                                 |
|----------|----------------------------------|---------|---------|-----------|---------|-----|-----------------------------------------------------------------------------|
| RGP46    | 0041/0042                        | 51,800  | 63,858  | 12,059    | 53      | 14  | Filamentous hemagglutinin/transposase                                        |
| RGP2     | 0256/0264                        | 294,565 | 300,887 | 6,323     | 59      | 5   | Type IV secretion system protein                                            |
| RGP58    | 3366/3368                        | 1,706,074 | 1,724,960 | 18,887 | 54      | 9   | IS3 family transposase                                                      |
| RGP32    | 3222/3223                        | 1,892,296 | 1,897,940 | 5,645    | 63      | 5   |                                                                            |
| RGP31    | 3141/3160                        | 1,961,341 | 1,970,838 | 9,498    | 46      | 9   | LPS O-antigen chain length regulator—replacement island                     |
| MKP3A4-GI1 | 2858/2859                     | 2,284,401 | 2,353,046 | 68,646   | 58      | 86  | Chromate and mercury resistance operons                                     |
| RGP29    | 2817/2820                        | 2,391,918 | 2,475,873 | 83,956   | 64      | 97  | Integrative conjugative element                                             |
| RGP56    | 2793/2795                        | 2,495,327 | 2,539,745 | 44,419   | 61      | 65  | Phage elements                                                              |
| RGP28    | 2727/2737                        | 2,588,565 | 2,602,472 | 13,908   | 55      | 11  | ISpa37 elements                                                             |
| RGP84    | 2603/2604                        | 2,744,268 | 2,753,449 | 9,182    | 62      | 13  | Phage regulatory proteins                                                   |
| RGP25    | 2455/2464                        | 2,940,859 | 2,950,847 | 9,989    | 52      | 9   |                                                                            |
| RGP73    | 2397/2403                        | 3,022,522 | 3,055,524 | 33,003   | 68      | 5   | Pyoverdin (*pvdE*) gene—replacement island                                  |
| RGP23    | 2217/2235                        | 3,231,884 | 3,557,062 | 125,179  | 59      | 116 | AAC(3)-IId, tunicamycin and copper resistance protein and phage (gp37)     |
| RGP50    | 1655/1656                        | 3,976,745 | 3,985,520 | 8,776    | 63      | 5   | Type IV secretion system protein                                            |
| RGP77    | 1397/1398                        | 4,269,893 | 4,277,302 | 7,410    | 60      | 6   | Type IV secretion system protein                                            |
| RGP9     | 1087/1092                        | 4,605,383 | 4,612,609 | 7,227    | 64      | 9   | Flagellar (*fliC*) gene replacement island                                 |
| RGP7     | 0976/0988                        | 4,719,909 | 4,727,427 | 7,519    | 58      | 5   | Effector protein (*exoU*) island                                            |
| RGP86    | 0831/0832                        | 4,880,992 | 4,906,589 | 25,598   | 59      | 23  | IS3 family transposase                                                      |
| RGP5     | 0714/0730                        | 5,010,479 | 5,090,313 | 79,835   | 63      | 91  | Integrative conjugative element/murcury resistance system                   |
| RGP60    | 4524/4526                        | 5,429,650 | 5,444,149 | 14,500   | 64      | 14  | Pilus (*pilA*) gene—replacement island                                      |
| RGP41    | 4541/4542                        | 5,459,741 | 5,545,278 | 85,538   | 60      | 105 | Integrative conjugative element/twitching motility protein                  |
| RGP42    | 4673/4674                        | 5,699,240 | 5,733,605 | 34,366   | 60      | 34  | IS66 family transposase                                                     |
| MKP3A4-GI2 | 4856/4857                    | 5,945,432 | 5,981,336 | 35,905   | 65      | 49  | Phage proteins (*Pseudomonas* phage MP38)                                   |
| RGP62    | 5149/5150                        | 6,328,034 | 6,341,398 | 13,365   | 55      | 10  | IS5 family transposase                                                      |

https://doi.org/10.1371/journal.pone.0215038.t003
the percentage of dead cells, maximum cytotoxicity (of score 4) was observed in PA34 [19]. Previous studies have shown that possession of exoU is associated with in vitro and in vivo cytotoxicity, for example by using knockout mutants of P. aeruginosa strains such as PA103 and PAO1 [58, 59]. The results in the current study suggest that exoU in RGP7 of PA34 was transcribed and translated into a functional protein that retained cytotoxicity. The finding also demonstrates that the size of the exoU island was not associated with activity. However, direct confirmation of this result by producing exoU knockout of P. aeruginosa PA34 is required to confirm this result.

A mercury resistance operon was also observed in the MKPA34-GI1 RGP that also carried chromate resistance genes and features related to transposition and conjugation. A BLASTn
search against the plasmid database in NCBI did not show significant sequence similarity with MKPA34-G1. However, 51% of the MKPA34-G1 sequence was loosely identical (205 matches with an average identity of 97%) to the complete genome of *Pseudomonas stutzeri* strain 273 (GenBank accession number CP015641.1). *Pseudomonas stutzeri* is an environmental
organism and has the capacity to degrade pollutants such as toxic metals [60]. These observations indicate that the MKPA34-GI1 may have been acquired by horizontal transfer from organisms in mercury polluted environments. The ability of MKPA34-GI1 to transfer between strains needs to be confirmed by further experiments that will also help to confirm the MKPA34-GI1 RGP loci is mobile.

Fig 4. Graphical representation of BLASTn comparison of various genomic islands of PA34. Protein-coding regions are represented by the orange arrows and key features/associated genes are shown. The gradient grey shading represents regions of nucleotide sequence identity (100% to 79%) determined by BLASTn analysis. Figures are drawn to scale using Easyfig [35]. (A) A comparison of exoU-island (RGP7) of PA34 with four exoU-islands of different strains (exoU island A, exoU island B, exoU island C and PAPI2) (xerC = site specific integrase, exoU = type III secretion system effector protein ExoU, spcU = ExoU chaperon protein, PA0975/PA0989 = franking loci in PAO1) (B) A comparison of a genomic island (MKPA34-GI2) of PA34 with Pseudomonas phage MP38 (GenBank Accession number NC_011611) (traA = transposase A, hni = host nuclease inhibitor, vip = virion protein, tcp = tail component protein).

https://doi.org/10.1371/journal.pone.0215038.g004
In order to compare the phenotypic susceptibility to heavy metals, MIC to Hg$^{++}$, Cu$^{++}$ and Co$^{++}$ were examined. High mercury tolerance was observed in PA34 ($p < 0.05$) in comparison to PAO1 (Fig 6), possibly due to the presence of mercury resistance operon in two different GIs (RGP5 and MKPA34-GI1) in PA34. Copper tolerance was slightly higher in PA34 than PAO1 ($p > 0.05$). Although bacteria require copper as a cofactor for many metabolic processes and can tolerate low concentration of Cu$^{++}$, decreasing sensitivity to copper can be associated with acquisition of copper resistance genes [61]. The presence of a copper resistance operon in RGP23 in PA34 may be associated with higher MIC to Cu$^{++}$. On the other hand, cobalt tolerance, for which no acquired genes were observed in this study was low in both PA34 and PAO1 ($p < 0.05$). 

*P. aeruginosa* can tolerate Cu$^{++}$ and other heavy metals including Co$^{++}$ mainly due to P-type ATPase transporter and resistance nodulation cell division efflux pump [62]. In PAO1, the P-type ATPase encoded by PA3920 was found to be important for copper tolerance [63]. In addition, bacterial plasmids also carry metal resistance determinants [64, 65]. However, plasmid associated metal resistance determinates were not detected in PA34. 

The *Pseudomonas* phage MP38 was identified in the MKPA34-GI2 (flanking loci homologous to 4856/4857 of PAO1) (Fig 4B). MP38 is D3112-like phage which is a transposable and has been isolated from many clinical isolates of *P. aeruginosa* [66]. We examined the presence of MP38 in genomes of different *P. aeruginosa* and found that a similar phage was integrated into VRFP404 near the PA4204 homologue of PAO1. This confirms the result of another study [67] that has shown that transposable phages may have variable integration sites, indicating that the phage MP38 does not have any specific integration site in the genome of *P. aeruginosa*. Therefore, the MKPA34-GI2 integration site may not be a constant RGP for *P. aeruginosa*. 

Four RGPs (RGP31, RGP73, RGP9 and RGP60) of PA34 were associated with replacement of pathogenic genes and were related to lipo-polysaccharide O-antigen, pyoverdine (*pvdE*), flagella (*fliC*) and pilus (*pilA*) synthesis. Despite the replacement islands contains the same genes and being integrated into the same loci in the core genome, they highly diverse between strains [68]. These components are important for interaction of a bacterium with external environments including other species or hosts and hence are under continuous selection pressure [68–71]. The *pvdE* and *fliC* orthologs have been shown to vary greatly between different strains of *P. aeruginosa* [72]. The replacement islands may contribute to higher virulence in PA34 although this needs to be tested for swimming or twitching motility and for expression of *pvdE* under low iron condition.
The largest GI (RGP23) observed in PA34 was 125.1 Kbp and carried resistance genes for aminoglycosides (AAC(3)-IId) and tunicamycin. An insertion sequence of the family IS1182 was observed in this GI, which may be responsible for carriage of these resistance genes. A BLASTn search revealed that the antibiotic resistance genes were best matched with those of Acinetobacter sp. WCHA45 plasmid pNDM1_010045 (GenBank accession number NZ_CP028560.1) and were similar to many from other members of the Enterobacteriaceae where IS1182 is also present. In addition, a phage tail protein gp37, which was first identified in Enterobacter phage T4 [73] was found inserted into this island. This suggests that this resistance island may be derived from phages.

**Plasmid features**

The Illumina and ONT reads of the whole genome sequence of PA34 were assembled into seven contigs (> 500 bp) using hybrid strategies in SPAdes. Out of seven contigs, contigs 6 and 7 had a G+C content that less than that of other contigs and showed a significant match with plasmids in the NCBI database. This suggests that contigs 6 and 7 are likely to represent two different plasmids carried by PA34 and were named here as pMKPA34-1 and pMKPA34-2. pMKPA34-1 is 95.4 kbp with 57% G+C content and pMKPA34-2 is 26.8 kbp with 61% G+C content. Automatic annotation followed by manual confirmation with BLASTn revealed 98...
CDS in pMKPA34-1 and 33 CDS in pMKPA34-2. Out of 98 predicted genes in pMKPA34-1, 46 were predicted to encode proteins with unknown functions whilst all 33 CDS in pMKPA34-2 were predicted to encode known proteins.

**General features of pMKPA34-1.** The putative plasmid pMKPA34-1 contains a replication gene (*repE*), a chromosome partition gene (*smc*), a plasmid stabilization gene (*parB*) and a plasmid conjugal transfer mating pair stabilization protein (*traN*). These genes may help replication, maintenance and transfer of the plasmid [74, 75] (Fig 7). Based on BLASTn searches against the NCBI plasmid database, pMKPA34-1 had the best match (31% query cover with 94% identity) with plasmid pLIB119 of *P. stutzeri* strain 19SMN4 (GenBank accession number CP007510), followed by *Citrobacter freundii* strain 18–1 plasmid pBKPC18-1 (23% query cover with 99% identity) (Fig 7). The *parA, smc* and *repE* genes display high similarity to the corresponding genes of pLIB119. The presence of the *traN* gene, which encodes an outer...
membrane protein and is essential for F-mediated bacterial conjugation [75], indicates the potential exchangeability of pMKPA34-1. This transfer system is common amongst bacterial plasmids notably in Enterobacteriaceae [76] and its presence in pMKPA34-1 may indicate that the plasmid could be exchanged with members of the Enterobacteriaceae.

Transposase, integrase and recombinase genes formed a major portion of the mobile genetic elements in pMKPA34-1. ISSage analysis identified two putative prophage integrases, and site-specific recombinases (xerC and xerD) of the tyrosine family. The XerC-XerD system, which is found on both the chromosome and on plasmids helps separation and segregation of newly replicated bacterial dimeric chromosome by resolving it to monomers [77]. This system is essential in the segregation of multicity plasmids and contributes to the stable inheritance of multicity plasmids [78, 79].

Plasmid pMKPA34-1 carries at least six antibiotic resistance genes. Five resistance genes (trimethoprim; dfrA15, chloramphenicol; cmlA1, aminoglycosides; APH(3”)-Ib/APH(6)-Id, and beta-lactam; blaNPS,1) are located in the Tn3-like transposon which also carries a class I integron (In1427) possessing dfrA15 and cmlA1 [13]. Additionally, pMKPA34-1 carries a multi-drug efflux gene (acrB). The acrB gene has high similarity to the corresponding gene of pBKPC18-1 (GenBank accession number CP022275), a resistance plasmid isolated from Citrobacter freundii strain 18–1. This efflux pump is similar to acrA and acrB of E. coli and responsible for resistance to hydrophilic compounds that include disinfectants [80]. The results suggest these movable resistance genes may be related to enteric bacteria.

**General features of pMKPA34-2.** The plasmid pMKPA34-2 lacks identifiable replication genes. However, the genome coverage statistics (109x vs. 50x) (Table 1) suggests it may have a higher copy number than the pMKPA34-1. pMKPA34-2 may use alternative mechanisms for replication. In the BLASTn search against the NCBI plasmid database, pMKPA34-2 was best matched (29% query cover with 88% identity) with plasmid pSSE-ATCC-43845 of Salmonella enterica subsp. enterica serovar Senftenberg strain 775WP (GenBank accession number CP016838), followed by plasmid tig00000727 of Klebsiella pneumoniae strain AR_0158 (GenBank accession number CP021699) (14% query cover with 99% identity) (Fig 8). Moreover, pMKPA34-2 carried the series of genes (tnsA, tnsB, tnsC, tnsD and tnsE) that are associated with transposition and are related to Tn7 transposons, a phage integrase and a putative resolvase. Upstream of these mobile genetic elements, a multi drug export protein gene (mepA) was identified. MepA is multi drug efflux transporter of MATE (Multidrug And Toxic Compound Extrusion) family whose role in P. aeruginosa is not well understood.[81]. However, MepA has been associated with resistance to many antibiotics and microbicidal dyes (crystal violet and ethidium bromide) in Staphylococcus aureus [82]. Indeed, the presence of the mepA gene in this plasmid and its association with mobile genetic elements suggest that this gene may have been acquired through horizontal gene transfer.

**Conclusions**

The large accessory genome of P. aeruginosa strain PA34 indicates that this strain has a diverse genomic structure. The strain harbours twenty-four genomic islands and two plasmids that carry various metal and antibiotic resistance genes as well as several genes associated with virulence. This may be associated with the observance of higher antibiotic resistance, mercury tolerance and in-vitro cytotoxicity of PA34. The *in-silico* analysis showed that six antibiotic resistance genes were present in two different plasmids and one antibiotic resistance gene plus various mercury resistance genes were integrated into different GIs and these resistance genes have sequence similarities with that of either other environmental or enteric bacteria. Furthermore, the genome of the PA34 has been integrated by phage element (gp37) that has its origin in enteric bacteria.
and carried aminoglycoside resistance gene (AAC(3)-IId). These findings suggest that resistance and virulence in PA34 may have evolved due to environmental selection pressure where organisms acquire traits to survive predation by other inhabitants. Thus acquired traits enhance the pathogenesis process in human. Given the eye is susceptible to being infected by environmental strains of *P. aeruginosa*, examination of a larger number of eye isolates may be necessary to uncover any additional acquired genetic features associated with microbial keratitis. This will help our understanding of different aspects of *Pseudomonas* keratitis.

**Supporting information**

S1 Table. Gaps in pseudogenome of PA34 with comparison with PA14. (DOCX)
S2 Table. Primer proposed for the gap validation.
(DOCX)

S1 File. Annotation of exclusive orthologs of strains.
(XLS)

Acknowledgments
The authors would like to acknowledge Associate Professor Federico Lauro of the Asian School of the Environment and the Singapore Centre for Environmental Life Sciences Engineering (SCELSE) for providing Oxford Nanopore Technology facility for DNA sequencing. We are also thankful to UNSW high performance computing facility KATANA for providing us cluster time for data analysis. Thanks to Sidra Sarwat UNSW for her help with cytotoxicity assay.

Author Contributions

Conceptualization: Dinesh Subedi, Ajay Kumar Vijay, Mark Willcox, Scott A. Rice.

Data curation: Dinesh Subedi, Gurjeet Singh Kohli.

Formal analysis: Dinesh Subedi, Gurjeet Singh Kohli.

Funding acquisition: Mark Willcox.

Investigation: Dinesh Subedi, Gurjeet Singh Kohli.

Methodology: Dinesh Subedi.

Project administration: Mark Willcox.

Resources: Mark Willcox, Scott A. Rice.

Supervision: Ajay Kumar Vijay, Mark Willcox, Scott A. Rice.

Validation: Mark Willcox.

Writing – original draft: Dinesh Subedi, Ajay Kumar Vijay, Mark Willcox.

Writing – review & editing: Dinesh Subedi, Ajay Kumar Vijay, Mark Willcox, Scott A. Rice.

References

1. Lyczak JB, Cannon CL, Pier GB. Establishment of Pseudomonas aeruginosa infection: lessons from a versatile opportunist. Microbes Infect. 2000; 2(9):1051–60. PMID: 10967285.
2. Stapleton F, Dart JK, Seal DV, Matheson M. Epidemiology of Pseudomonas aeruginosa keratitis in contact lens wearers. Epidemiol Infect. 1995; 114(3):395–402. Epub 1995/06/01. PMID: 7781727.
3. WHO priority pathogens list for R&D of new antibiotics [Internet]. 2017.
4. Livermore DM. Multiple mechanisms of antimicrobial resistance in Pseudomonas aeruginosa: our worst nightmare? Clin Infect Dis. 2002; 34(5):634–40. https://doi.org/10.1086/338782 PMID: 11823954.
5. Mathee K, Narasimhan G, Valdes C, Qiu X, Matewish JM, Koehrsen M, et al. Dynamics of Pseudomonas aeruginosa genome evolution. Proc Natl Acad Sci U S A. 2008; 105(8):3100–5. https://doi.org/10.1073/pnas.0711982105 PMID: 18287045.
6. Jeukens J, Boyle B, Kukavica-Ibrulj I, Ouellet MM, Aaron SD, Charette SJ, et al. Comparative genomics of isolates of a Pseudomonas aeruginosa epidemic strain associated with chronic lung infections of cystic fibrosis patients. PLoS One. 2014; 9(2):e87611. Epub 2014/02/08. https://doi.org/10.1371/journal.pone.0087611 PMID: 24505294.
7. Stewart L, Ford A, Sangal V, Jeukens J, Boyle B, Kukavica-Ibrulj I, et al. Draft genomes of 12 host-adapted and environmental isolates of Pseudomonas aeruginosa and their positions in the core
8. Beatson SA, Walker MJ. Microbiology. Tracking antibiotic resistance. Science. 2014; 345(6203):1454–5. https://doi.org/10.1126/science.1260471 PMID: 25237090.

9. Stewart RM, Wiehmann L, Ashelford KE, Preston SJ, Frimmersdorf E, Campbell BJ, et al. Genetic characterization indicates that a specific subpopulation of *Pseudomonas aeruginosa* is associated with keratitis infections. J Clin Microbiol. 2011; 49(3):993–1003. https://doi.org/10.1128/JCM.02036-10 PMID: 21227987.

10. Murugan N, Malathi J, Umashankar V, Madhavan HN. Unraveling genomic and phenotypic nature of multidrug-resistant (MDR) *Pseudomonas aeruginosa* VRPA04 isolated from keratitis patient. Microbiol Res. 2016; 193:140–9. Epub 2016/11/09. https://doi.org/10.1016/j.micres.2016.10.002 PMID: 27825482.

11. Subedi D, Vijay AK, Kohli GS, Rice SA, Willcox M. Comparative genomics of clinical strains of *Pseudomonas aeruginosa* strains isolated from different geographic sites. Sci Rep. 2014; 3(1):6203. https://doi.org/10.1038/srep04159 PMID: 25237090.

12. Fleischig SM, Lee EJ, Wu C, Andika RC, Vallas V, Portoles M, et al. Cytotoxic strains of *Pseudomonas aeruginosa* can damage the intact corneal surface in vitro. J Antimicrob Chemother. 2018; 73(6):1417–7. PMID: 29474453

13. Subedi D, Vijay AK, Kohli GS, Rice SA, Willcox M. Nucleotide sequence analysis of NPS-1 beta-lactamase and a novel integron (In1427)-carrying transposon in an MDR *Pseudomonas aeruginosa* keratitis strain. J Antimicrob Chemother. 2018. Epub 2018/03/17. https://doi.org/10.1093/jac/dky073 PMID: 29547943.

14. Klockgether J, Munder A, Neugebauer J, Davenport CF, Staneke F, Larbig KD, et al. Genome diversity of *Pseudomonas aeruginosa* PAO1 laboratory strains. J Bacteriol. 2010; 192(4):1113–21. https://doi.org/10.1128/JB.01515-09 PMID: 20023018.

15. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warren P, Hickey MJ, et al. Complete genome sequence of *Pseudomonas aeruginosa* PA01 laboratory strains. J Bacteriol. 2010; 192(4):1113–21. https://doi.org/10.1128/JB.01515-09 PMID: 20023018.

16. Lee DG, Urbach JM, Wu G, Liberati NT, Feinbaum RL, Miyata S, et al. Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial. Genome Biol. 2006; 7(10):R90. Epub 2006/10/14. https://doi.org/10.1186/gb-2006-7-10-r90 PMID: 17038190.

17. Rapid methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically—Eleventh Edition. CLSI. 2012; 23(M07-A11).

18. Teitzel GM, Parsek MR. Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. Appl Environ Microb. 2003; 69(4):2313–20. https://doi.org/10.1128/AEM.69.4.2313-2320.2003 PMID: 12676715.

19. Fleischig SM, Zaidi TS, Preston MJ, Grout M, Evans DJ, Pier GB. Relationship between cytotoxicity and corneal epithelial cell invasion by clinical isolates of *Pseudomonas aeruginosa*. Infect Immun. 1996; 64(6):2288–94. Epub 1996/06/01. PMID: 8675339.

20. Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS computational biology. 2017; 13(6):e1005595. https://doi.org/10.1371/journal.pcbi.1005595 PMID: 28594827.

21. Nguyen SH, Duarte TP, Coin LJ, Cao MD. Real-time demultiplexing Nanopore barcode nanosequencing data with npBarcode. Bioinformatics. 2017; 33(24):3988–90. https://doi.org/10.1093/bioinformatics/btx537 PMID: 28961965.

22. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 2012; 19(5):455–77. https://doi.org/10.1089/cmb.2012.0021 PMID: 22506599.

23. Bushnell B. BBMap short read aligner. University of California, Berkeley, California URL http://sourceforge.net/projects/bbmap. 2016.

24. Kurtz S, Phillippy A, Delcher AL, Smoot M, Shumway M, Antonescu C, et al. Versatile and open software for comparing large genomes. Genome Biology. 2004; 5(2):R12. https://doi.org/10.1186/gb-2004-5-2-r12 PMID: 14759262.

25. Assael S, Keane TM, Otto TD, Newbold C, Berriman M. ABACAS: algorithm-based automatic contiguation of assembled sequences. Bioinformatics. 2009; 25(15):1968–9. https://doi.org/10.1093/bioinformatics/btp347 PMID: 19497936.

26. Unterlass A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3—new capabilities and interfaces. Nucleic Acids Res. 2012; 40(15):e115–e. https://doi.org/10.1093/nar/gks596 PMID: 22730293.
27. Tatusova T, DiCuccio M, Badretdin A, Chetvernin V, Nawrocki EP, Zaslavsky L, et al. NCBI prokaryotic genome annotation pipeline. Nucleic Acids Research. 2016; 44(14):6614–24. https://doi.org/10.1093/nar/gkw569 PMID: 27342282

28. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 2014; 30(14):2068–9. https://doi.org/10.1093/bioinformatics/btu153 PMID: 24642063.

29. Varani AM, Siguier P, Gourbeyre E, Charneau V, Chandler M. IISsaga is an ensemble of web-based methods for high throughput identification and semi-automatic annotation of insertion sequences in prokaryotic genomes. Genome Biol. 2011; 12(3):R30. https://doi.org/10.1186/gb-2011-12-3-r30 PMID: 21443786

30. Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, et al. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res. 2014; 42(D1):D206–D14. https://doi.org/10.1093/nar/gkt1226 PMID: 24293654

31. Darling AE, Mau B, Perna NT. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS One. 2010; 5(6):e11147. https://doi.org/10.1371/journal.pone.0011147 PMID: 20593022.

32. Page AJ, Cummings CA, Hunt M, Wong VK, Reuter S, Holden MTG, et al. Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics. 2015; 31(22):3691–3. https://doi.org/10.1093/bioinformatics/btv421 PMID: 26198102

33. Couvin D, Bernheim A, Toffano-Nioche C, Touchon M, Michalik J, Néron B, et al. CRISPRCas Finder, an update of CRISPRFinder, includes a portable version, enhanced performance and integrates search for Cas proteins. Nucleic Acids Res. 2018; 46(W1):W246–W51. https://doi.org/10.1093/nar/gky425 PMID: 29790974

34. Grant JR, Arantes AS, Stothard P. Comparing thousands of circular genomes using the CGView Comparison Tool. BMC Genomics. 2012; 13:202. https://doi.org/10.1186/1471-2164-13-202 PMID: 22621371.

35. Sullivan MJ, Petty NK, Beaton SA. Easyfig: a genome comparison visualizer. Bioinformatics. 2011; 27(7):1009–10. Epub 2011/02/01. https://doi.org/10.1093/bioinformatics/btr039 PMID: 21278367.

36. Biotech G. SnapGene Viewer. Glick B, editor. 3(3).

37. Venny. An interactive tool for comparing lists with Venn’s diagrams [Internet]. 2007–2015. http://bioinfogp.cnb.csic.es/tools/venny/index.html.

38. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother. 2012; 67(11):2640–4. https://doi.org/10.1093/jac/dks261 PMID: 22782487.

39. Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman FS. Enhanced annotations and features for comparing thousands of Pseudomonas genomes in the Pseudomonas genome database. Nucleic Acids Res. 2016; 44(D1):D646–53. Epub 2015/11/19. https://doi.org/10.1093/nar/gkv1227 PMID: 26578582.

40. Tatusova T, DiCuccio M, Badretdin A, Chetvernin V, Nawrocki EP, Zaslavsky L, et al. NCBI prokaryotic genome annotation pipeline. Nucleic Acids Res. 2016; 44(14):6614–24. Epub 2016/06/28. https://doi.org/10.1093/nar/gkw569 PMID: 27342282.

41. Pohl S, Klockgether J, Eckweiler D, Khaledi A, Schniederjans M, Chouvarine P, et al. The extensive set of accessory Pseudomonas aeruginosa genomic components. FEMS Microbiol Lett. 2014; 356(2):235–41. https://doi.org/10.1111/1574-6968.12445 PMID: 24763999.

42. Klockgether J, Cramer N, Wiehlmann L, Davenport CF, Tummel B. Pseudomonas aeruginosa genomic structure and diversity. Front Microbiol. 2011; 2:150. https://doi.org/10.3389/fmicb.2011.00150 PMID: 21808635.

43. Ricker N, Qian H, Fulthorpe RR. The limitations of draft assemblies for understanding prokaryotic adaptation and evolution. Genomics. 2012; 100(3):167–75. https://doi.org/10.1016/j.ygeno.2012.06.009. PMID: 22750566.

44. Silby MW, Cerdeno-Tarraga AM, Vernikos GS, Giddens SR, Jackson RW, Preston GM, et al. Genomic and genetic analyses of diversity and plant interactions of Pseudomonas fluorescens. Genome Biol. 2009; 10(5):R51. https://doi.org/10.1186/gb-2009-10-5-r51 PMID: 19432983.

45. Almeida NF, Yan S, Lindeberg M, Studholme DJ, Schneider DJ, Condon B, et al. A draft genome sequence of Pseudomonas syringae pv. tomato T1 reveals a type III effector repertoire significantly divergent from that of Pseudomonas syringae pv. tomato DC3000. Mol Plant Microbe Interact. 2009; 22(1):52–62. Epub 2008/12/09. https://doi.org/10.1094/MPMI-22-1-0052 PMID: 19061402.

46. Ozer EA, Allen JP, Hauser AR. Characterization of the core and accessory genomes of Pseudomonas aeruginosa using bioinformatic tools Spine and AGEnt. BMC Genomics. 2014; 15(1):737. https://doi.org/10.1186/1471-2164-15-737 PMID: 25168460.
van Belkum A, Soriaga LB, LaFave MC, Akella S, Veyrieras JB, Barbu EM, et al. Phylogenetic Distribution of CRISPR-Cas Systems in Antibiotic-Resistant Pseudomonas aeruginosa. MBio. 2015; 6(6): e01796–15. Epub 2015/11/26. https://doi.org/10.1128/mBio.01796-15 PMID: 26604259.

Kung VL, Ozer EA, Hauser AR. The accessory genome of Pseudomonas aeruginosa. Microbiol Mol Biol Rev. 2010; 74(4):621–41. https://doi.org/10.1128/MMBR.00027-10 PMID: 21119020.

Winstanley C, Langille MG, Fothergill JL, Kukavica-Ibrulj I, Paradis-Blcau C, Sanschagrin F, et al. Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the Liverpool Epidemic Strain of Pseudomonas aeruginosa. Genome Res. 2009; 19(1):12–23. Epub 2008/12/03. https://doi.org/10.1101/gr.086882.108 PMID: 19047519.

Ravatn R, Studer S, Springael D, Zehnder AJ, van der Meer JR. Chromosomal integration, tandem amplification, and deamplification in Pseudomonas putida F1 of a 105-kilobase genetic element containing the chlorocatechol degradative genes from Pseudomonas sp. Strain B13. J Bacteriol. 1998; 180(17):4360–9. PMID: 9721270.

Mikkelsen H, Ball G, Giraud C, Filloux A. Expression of Pseudomonas aeruginosa CupD fimbral genes is antagonistically controlled by RcsB and the EAL-containing PvrR response regulators. PLoS One. 2009; 4(6):e6018. Epub 2009/06/24. https://doi.org/10.1371/journal.pone.0006018 PMID: 19547710.

Kulasekara BR, Kulasekara HD, Wolfgang MC, Stevens L, Frank DW, Lory S. Acquisition and evolution of the exoU locus in Pseudomonas aeruginosa. J Bacteriol. 2006; 188(11):4037–50. https://doi.org/10.1128/JB.02000-05 PMID: 16707695.

He J, Baldini RL, Deziel E, Saucier M, Zhang Q, Liberati NT, et al. The broad host range pathogen Pseudomonas aeruginosa strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. Proc Natl Acad Sci U S A. 2004; 101(8):2530–5. https://doi.org/10.1073/pnas.0304622101 PMID: 14983043.

Hauser AR, Cobb E, Bodl M, Mariscal D, Valles J, Engel JN, et al. Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by Pseudomonas aeruginosa. Crit Care Med. 2002; 30(3):521–8. PMID: 11990909.

Roy Chowdhury P, Scott M, Worden P, Huntington P, Hudson B, Karagiannis T, et al. Genomic islands 1 and 2 play key roles in the evolution of extensively drug-resistant ST235 isolates of Pseudomonas aeruginosa. Open Biol. 2016; 6(3). https://doi.org/10.1098/rsob.150175 PMID: 26962050.

Harrison EM, Carter ME, Luck S, Ou HY, He X, Deng Z, et al. Pathogenicity islands PAPI-1 and PAPI-2 contribute individually and synergistically to the virulence of Pseudomonas aeruginosa strain PA14. Infect Immun. 2010; 78(4):1437–46. Epub 2010/02/04. https://doi.org/10.1128/IAI.00621-09 PMID: 20123716.

Evans DJ, Frank DW, Finck-Barbancon V, Wu C, Fleischg SM. Pseudomonas aeruginosa invasion and cytotoxicity are independent events, both of which involve protein tyrosine kinase activity. Infect Immun. 1998; 66(4):1453–9. PMID: 9529067.

Finck-Barbancon V, Goranson J, Zhu L, Sawa T, Wiener-Kronish JP, Fleischg SM, et al. ExoU expression by Pseudomonas aeruginosa correlates with acute cytotoxicity and epithelial injury. Mol Microbiol. 1997; 25(3):547–57. PMID: 9302017.

Allerwelt M, Coleman FT, Grout M, Pribe GP, Pier GB. Acquisition of expression of the Pseudomonas aeruginosa ExoU cytotoxin leads to increased bacterial virulence in a murine model of acute pneumonia and systemic spread. Infect Immun. 2000; 68(7):3998. https://doi.org/10.1128/IAI.68.7.3998-4004.2000 PMID: 10858214.

Lalucat J, Bennasar A, Bosch R, García-Valdés E, Palleroni NJ. Biology of Pseudomonas stutzeri. Microbiol Mol Biol Rev. 2006; 70(2):510–47. https://doi.org/10.1128/MMBR.00047-05 PMID: 16760312.

Elguindi J, Wagner J, Rensing C. Genes involved in copper resistance influence survival of Pseudomonas aeruginosa on copper surfaces. J Appl Microbiol. 2009; 106(5):1448–55. Epub 02/23. https://doi.org/10.1111/j.1365-2672.2009.04148.x PMID: 19239551.

Nies DH. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol Rev. 2003; 27(2–3):313–39. https://doi.org/10.1016/S0168-6445(03)00048-2 PMID: 12829273.

Teitzel GM, Geddie A, De Long SK, Kirisits MJ, Whiteley M, Parsek MR. Survival and growth in the presence of elevated copper: transcriptional profiling of copper-stressed Pseudomonas aeruginosa. J Bacteriol. 2006; 188(20):7242–56. Epub 2006/10/04. https://doi.org/10.1128/JB.00837-06 PMID: 17015663.

Ohtake H, Cervantes C, Silver S. Decreased chromate uptake in Pseudomonas fluorescens carrying a chromate resistance plasmid. J Bacteriol. 1987; 169(6):3853–6. Epub 1987/08/01. PMID: 3112130.

Brown NL, Misra TK, Winnie JN, Schmidt A, Seiff M, Silver S. The nucleotide sequence of the mercuric resistance operons of plasmid R100 and transposon Tn501: further evidence for mer genes which
enhance the activity of the mercuric ion detoxification system. Mol Gen Genet. 1986; 202(1):143–51. Epub 1986/01/01. PMID: 3007931.

66. Wang PW, Chu L, Guttman DS. Complete sequence and evolutionary genomic analysis of the Pseudomonas aeruginosa transposable bacteriophage d3112. J Bacteriol. 2004; 186(2):400–10. https://doi.org/10.1128/JB.186.2.400-410.2004 PMID: 14702309.

67. Vergnaud G, Midoux C, Blouin Y, Bourkaltseva M, Krylov V, Pourcel C. Transposition behavior revealed by high-resolution description of Pseudomonas aeruginosa Saltovirus integration sites. Viruses. 2018; 10(5). https://doi.org/10.3390/v10050245 PMID: 29735891.

68. Smith EE, Sims EH, Spencer DH, Kaul R, Olson MV. Evidence for diversifying selection at the poydine locus of Pseudomonas aeruginosa. J Bacteriol. 2005; 187(6):2138–47. Epub 2005/03/04. https://doi.org/10.1128/JB.187.6.2138-2147.2005 PMID: 15743962.

69. Chibeau A, Ceyssens P-J, Hertveldt K, Volckaert G, Matthijs S, et al. The adsorption of Pseudomonas aeruginosa bacteriophage φKMV is dependent on expression regulation of type IV pili genes. FEMS Microbiol Lett. 2009; 296(2):210–8. https://doi.org/10.1111/j.1574-6968.2009.01640.x PMID: 19459952.

70. Kuzio J, Kropinski AM. O-antigen conversion in Pseudomonas aeruginosa PAO1 by bacteriophage D3. J Bacteriol. 1983; 155(1):203–12. PMID: 6190794.

71. Pier GB. Pseudomonas aeruginosa lipopolysaccharide: a major virulence factor, initiator of inflammation and target for effective immunity. Int J Med Microbiol. 2007; 297(5):277–95. https://doi.org/10.1016/j.ijmm.2007.03.012 PMID: 17466590.

72. Subedi D, Vijay AK, Kohli GS, Rice SA, Wilcox M. Comparative genomics of clinical strains of Pseudomonas aeruginosa strains isolated from different geographic sites. Sci Rep. 2018; 8(1):15668. https://doi.org/10.1038/s41598-018-34020-7 PMID: 30353070.

73. Cerritelli ME, Wall JS, Simon MN, Conway JF, Steven AC. Stoichiometry and domainal organization of the long tail-fiber of bacteriophage T4: a hinged viral adhesin. J Mol Biol. 1996; 260(5):767–80. Epub 1996/08/02. https://doi.org/10.1006/jmbi.1996.0436 PMID: 8709154.

74. del Solar G, Giraldo R, Ruiz-Echevarría MJ, Espinosa M, Díaz-Orejas R. Replication and Control of Circular Bacterial Plasmids. Microbiol Mol Biol Rev. 1998; 62(2):434–64. PMID: 9618448.

75. Klimke WA, Rypien CD, Klinger B, Kennedy RA, Rodriguez-Maillard JM, Frost LS. The mating pair stabilization protein, TraN, of the F plasmid is an outer-membrane protein with two regions that are important for its function in conjugation. Microbiology. 2005; 151(Pt 11):3527–40. https://doi.org/10.1099/mic.0.28025-0 PMID: 16272376.

76. Lawley TD, Klimke WA, Gubbins MJ, Frost LS. F factor conjugation is a true type IV secretion system. FEMS Microbiol Lett. 2003; 224(1):1–15. https://doi.org/10.1016/S0378-1097(03)00430-0 PMID: 12855161.

77. Castillo F, Benmohamed A, Szatmari G. Xer site specific recombination: Double and single recombinase systems. Front Microbiol. 2017; 8:453. Epub 2017/04/05. https://doi.org/10.3389/fmictb.2017.00453 PMID: 28373867.

78. Arciszewska LK, Sherratt DJ. Xer site-specific recombination in vitro. EMBO J. 1995; 14(9):2112–20. PMID: 7744017.

79. Blakely GW, Davidson AO, Sherratt DJ. Sequential strand exchange by XerC and XerD during site-specific recombination at dif. J Biol Chem. 2000; 275(14):9930–6. Epub 2000/04/01. PMID: 10744667.

80. Ma D, Cook DN, Alberto M, Pon NG, Nikaido H, Hearst JE. Genes acrA and acrB encode a stress-induced efflux system of Escherichia coli. Mol Microbiol. 1995; 16(1):45–55. PMID: 7651136.

81. Kuroda T, Tsuchiya T. Multidrug efflux transporters in the MATE family. Biochimica et Biophysica Acta (BBA)—Proteins and Proteomics. 2009; 1794(5):763–8. https://doi.org/10.1016/j.bbabap.2008.11.012.

82. Kaatz GW, McAleese F, Seo SM. Multidrug resistance in Staphylococcus aureus due to overexpression of a novel Multidrug and Toxin Extrusion (MATE) transport protein. Antimicrob Agents Chemother. 2005; 49(5):1857–64. https://doi.org/10.1128/AAC.49.5.1857-1864.2005 PMID: 15855507.