**Article highlights**

A review of the state of the art of the fast-moving field of *Pseudomonas aeruginosa* genomics

Insight into the species population structure that shows the genetic diversity underlying its evolutionary and environmental flexibility.

Examination of high-risk multidrug resistant clone evolution and penetration into healthcare systems.

Consideration of alternatives and possible adjuncts to antibiotic chemotherapy including environmental control of AMR isolates.

Review possible interventions with potential to control or reduce the ability for *P. aeruginosa* to gain virulence via HGT.

**Expert Opinion**

Analysis of the genome of *Pseudomonas aeruginosa* has greatly intensified in recent years following the publication of the type strain PAO1 genome sequence in 2000. Phenotypically diverse and a coloniser of a large variety of natural habitats, this species is able to infect both plants and humans using the same virulence armamentarium. The genomes of 4660 *P. aeruginosa* are in the public domain and these have started to reveal the reasons for the species evolutionary success. For a species with a relatively large genome of about 6.3 million nucleotides coding for an estimated 5600 genes the essential core genome appears surprisingly small – only 321 genes. The vast majority of *P. aeruginosa* genes are shared between a small number of isolates with approximately half found in only one isolate. This unusually high level of genetic diversity is driven by frequent horizontal gene transfer events between members of the species as well as extra-species associations that are evidenced by, for example the acquisition of antibiotic resistance genes on mobile genetic elements such as plasmids containing carbapenem resistance and even colistin resistance determinants. Perhaps thankfully in this species, although multiple drug resistance (MDR) is commonplace even in environmental isolates, extreme (XDR) and pan-drug resistances are much less common and largely restricted to a relatively small number of genetic backgrounds. These high-risk clones can be easily identified by genome sequencing and there is good potential for this to become routine so that rapid diagnostics can provide antibiotic sensitivity and even virulence potential data in a clinically useful timeframe that can minimise the untargeted, empirical use of that valuable resources - antibiotics. Genomic sequencing is now used to trace *P. aeruginosa* outbreaks retrospectively but technology is currently sufficiently advanced to allow real-time outbreak analyses in hospitals and other healthcare settings. Currently this is financially unviable in most settings but the near future promises declining costs for genomic sequencing and future innovations will reduce the human resource requirements of current epidemiological analyses.

*Pseudomonas aeruginosa* exists almost exclusively in biofilms which provide challenges for therapeutic intervention due to the low metabolic activity of some cells, the inability of some antibiotics to adequately penetrate the biofilm matrix and the strong attachment between biofilms and human tissue and indwelling device materials. Biofilms are also obviously also the place where intra- and inter-species gene exchange occurs allowing virulence genes and antibiotic resistance genes to move. With the current dearth in antibiotic drug discovery for gram-negative nosocomial pathogens in particular the future of antimicrobial chemotherapy will need to be supplemented by other

approaches to target pathogen biofilms in humans and also perhaps in environmental reservoirs of antibiotic resistance. Controlling the diverse biofilms associated with *P. aeruginosa* will be challenging due to the multispecies nature of many infective biofilms however targeting the species quorum-sensing network could be a possible anti-virulence strategy. Future therapies based on depleting biofilm directly using recombinant dispersins or phage-encoded polysaccharide depolymerases could also provide additional tools to limit the spread of *P. aeruginosa.* The removal or reduction of *P. aeruginosa* from water sources or wastewater processes through photocatalysis or other low-energy disinfection processes could also offer a promising future approach to reducing the reservoir of AMR *P. aeruginosa* **before** they impact on human health.

## 1. Introduction

*Pseudomonas aeruginosa* is ubiquitous in many natural environments such as soils and bodies of water, in addition to being a major cause of infections in humans, other animals and a cause of soft rot infections in plants. This is due to its ability to survive and even thrive under a wide range of temperatures, on different nutrient sources and the species inherent and acquired virulence and resistances to antibiotics, disinfectants and other antimicrobial compounds [1]. It has been classified as a major species associated with multiple antimicrobial resistance of urgent public health concern by the Infectious Diseases Society of America [2], Centers for Disease Control and Prevention and the World Health Organization. It is a leading cause of severe, life-threatening nosocomial human infections and the major pathogen associated with lung infections in patients with cystic fibrosis [3, 4] due to the pathogens various and successful virulence mechanisms.

### 1.1 Virulence

Damage to the bacterial host, and hence bacterial pathogenicity, is due to the action of virulence factors, a multifactorial process whereby bacterially-derived molecules enable entry, replication, and persistence that may lead to tissue damage [5]. A recent review by Diggle and Whiteley [6] described the major virulence systems of *P. aeruginosa* virulence and these are shown in Figure 2.

Understanding virulence in *P. aeruginosa* has been a key research field for decades, with many factors identified as common across infection of both humans and plants, for example toxA, plcS and gacA, as well as the phenazine pigment compound pyocyanin [7, 8]. A measure of the complexity and our lack of knowledge of the gene repertoire of the species is that we cannot discern human pathogenic genotypes from those infecting plants. This isillustrated in Figure 1 by the very high genetic similarity between clinical and non-clinical (environmental) isolates and the plant isolate PA14 . Quorum sensing (QS), the ability for bacteria to produce and detect signalling molecules (autoinducers) is known to play an important role in virulence (Figure 3), with mutations in QS genes or specific blocking/targeting of QS molecules able to reduce virulence of *P. aeruginosa* [9, 10, 11], although notably, such QS

mutants are not always avirulent [10]. Siderophores are small molecular weight molecules secreted by bacteria for iron-chelating, and are also responsible for regulation of virulence. Pyoverdine, a major siderophore in *P. aeruginosa*, is itself a virulence factor and regulates at least two others factors (endotoxin A and an endoprotease) [12, 13]. Virulence factors can also be activated by mechanochemical sensory systems (e.g. type-IV pili) once the *P. aeruginosa* cell recognises a surface (for example the chp system) [14] aiding in colonisation and biofilm formation. Virulence factors can also be secreted by *P. aeruginosa* and packaged into outer-membrane vesicles and delivered to a target host at relatively long distances [15].

Horizontal gene transfer (HGT), the process where genetic material is passed between different bacterial cells, is the most significant driver in shaping the genome of *P. aeruginosa* [16]. HGT can occur due to a number of intra- and even inter-species processes including conjugation, transduction and transformation. Transduction, the process by which genetic information is obtained as a consequence of viral (bacteriophage) infection has long been studied in *P. aeruginosa* [17], considered a process naturally occurring in the environment, and has even been demonstrated with 'large' jumbo-phage [18]. Similarly, conjugation, the process by which bacteria transfer genetic material between cells, is also well studied in *P. aeruginosa [19]*. Recent literature has examined the effect of conjugation on antibiotic resistance [20], and how biofilm phenotype may encourage this [21]. Natural transformation in *P. aeruginosa*, meaning the uptake of genetic material from the environment, is less studied/observed in the literature [22], with the ability to uptake and recombine naked DNA has not been proven conclusively to occur in this species [23] although numerous circumstantial pieces of evidence support this mechanism of HGT. Bacteria also have mechanisms to resist HGT. Restriction-modification (R-M) systems are a highly diverse group on enzymes that can be considered 'bacterial immunity' by defending bacterial cells from extra-cellular DNA. It is also hypothesised that R-Ms play a broader role in the bacterial cell, with a recent study demonstrating *P. aeruginosa* R-M systems potentially impacting virulence [24]. CRISPR-Cas is another cellular mechanism that can defend a bacterial cell from DNA uptake and integration by protecting against bacteriophage transduction [25]. One study of CRISPR-Cas systems within 672 genomes found reduced genome size to be associated with particular CRISPR-Cas types compared to those without such a system [26], however, the wider impact on HGT in this species is still unclear [27, 28].

Virulence in *Pseudomonas aeruginosa* is largely shaped by high rates of horizontal gene transfer (HGT) [29] that transfers regions of DNA between cells. Pathogenicity islands (PI) are horizontally acquired

genetic elements present in the accessory genome [16] that are frequently found in virulent clinical isolates of *P. aeruginosa*. The first such, called PAPI-1 and PAPI-2, contain homologues of genes found in other human and plant pathogens [30]. A recent study found 11 out of 21 identified GI in an ocular multi-drug resistant isolate of *P. aeruginosa* contained virulence genes. A recent analysis of 1,311 *P. aeruginosa* pangenomes Freschi, Vincent [29] concluded HGT has a non-negligible effect on the evolution of the bacteria, and are potentially implicated in the emergence of virulence. It has also been suggested that virulence traits in *P. aeruginosa* are selected for and maintained even when interaction with a human host is absent, potentially driven by interaction with other eukaryotic organisms in the wider environment, e.g. amoeba [22]. As greater numbers of genomes for *P. aeruginosa* become available, pangenome/comparative genome studies will continue shedding light on the genomic diversity of virulence.

However, there is little discussion in the literature on the control of HGT, and its impact on virulence and the wider issues of *P. aeruginosa* in healthcare settings. This review therefore seeks to discuss these considerations in the wider context of *P. aeruginosa* as a human pathogen with consideration of relevant original research on *Pseudomonas aeruginosa* pathogenicity and population structure with a strong emphasis on recent large genomic and pan-genomic studies on this very successful, diverse and adaptive bacterial species. All literature searches were performed in PubMed and Web of Science in the period May 2019 – March 2020.

### 1.2 Lessons from Genomics?

Since the first genome sequence of a *P. aeruginosa* isolate (PAO1) was published in 2000 [31] the species has been the subject of many genome sequencing studies - at the time of writing 4660 genomes are currently in the public domain making it one of the most studied pathogen species. PAO1 is the most commonly used *P. aeruginosa* laboratory strain and the subject of ongoing international efforts to ascribe functions to its 5570 open reading frames, in a genome of 6,264,403bp, through the Pseudomonas Genome DB project (accessible at http://www.pseudomonas.com) [32]. *P. aeruginosa* genomes are approximately twice the size of that of the average bacterial species with around 6.3 million base pairs [31, 33]. What is clearly apparent from genomic sequencing efforts to date is that the species has a very large pan-genome, the full complement of genes found in the species, with a very small number of core - genes found in every isolate and an extremely large accessory genome. A 2019 study [34] using genomics and analysis of gene knockouts from isolates grown under different conditions calculated that the number of core essential genes to be 321, which comprises only 6.6% of the *P. aeruginosa* genome. A study the previous year by Freschi and colleagues [29] estimated that

the core genome was only 1.2% of the pan-genome based upon genomes that they sequenced or were publicly available in 2016. Both of these studies and other large genome sequencing projects describe a species with a minimal essential genome (compared with other bacterial species such as *S. pneumoniae* and *C. jejuni,* which has approximately 851 and 866 core genes out of an estimated 1990 and 1623 genes respectively [35]). According to the Freschi study, 50.1% of unique genes are found in single isolates.

Early DNA sequencing studies suggested *P. aeruginosa* was divided into two main groups represented by PAO1 [31] - isolated from a chronic wound in the 1950s and PA14 [36]- an isolate from a plant soft-rot infection, however recent studies show a much more complex population structure comprising at least five major clades according to one genomic analysis [37] although data from a 2015 study of human infection and environmental isolates not included in this analysis suggests a more complex picture [26] (Figure 1). The particularly large accessory genome of *P. aeruginosa* is obviously a major contributor to the species versatility that allows it to exploit different environmental niches as a ubiquitous organism in soil and water and also as a commensal that can on occasion cause infections in plants and animals including humans. The *P. aeruginosa* pan-genome contains many genes that are unique to a particular isolate and provides the species with an extremely large variety of possible phenotypes [38].

### 1.3 Antibiotic Resistance

Although antibiotic resistance (ABR) is not considered to be a virulence factor, however, understanding ABR is essential due to the increasingly important role both play in the clinical setting [39]. In its 2013 publication "Antibiotic Resistant Threats in the United States, 2013", CDC reported 51, 000 cases of *P. aeruginosa* infections a year. 6700 (13.1%) of these were multidrug resistant (MDR), that is, untreatable with three or more antibiotic classes commonly used to treat such infections. These were found to have caused 440 deaths a year in the study period [40]. European CDC data for 2018 show a variable rate of MDR in Europe overall, with 32.1% of P. aeruginosa isolates resistant to at least one antibiotic, and 19.2% isolates resistant to two or more antibiotics [41]. This report suggests large inter-country variations for resistance across all antibiotics, with generally higher resistance percentages reported in southern and eastern Europe compared to northern Europe and resistance to carbapenems, the most commonly used antibiotics to treat nosocomial gram-negative infections, remains common in many EU/EEA countries. Resistance to polymyxin antibiotics (colistin and polymyxin B), often referred to as the antibiotic class of last resort for gram-negative infections still appears to be rare in Europe, the United States, Canada and Latin America with 0.5 – 1.1% of

isolates being resistant [42] although resistance rates were higher in China with 22.2% resistance among XDR (eXtremely Drug-Resistant, ie, resistant to four or more antibiotic classes) *P. aeruginosa* from bloodstream infections, according to a 2016 report [42, 43].

**1.4 Population Structure**

Recombination is the major mechanism driving sequence divergence in this species and this enables the frequent acquisition and dissemination of both virulence and antibiotic resistance genes. The very large pan-genome and large number of genes found in only one isolate indicate a very fluid species [26, 37]. Analysis of the core genome also shows a highly recombining population. Multilocus sequence typing (MLST), first described in 1998 for the characterization of *Neisseria meningitidis* [44], has been applied to the study of all common bacterial pathogens, as well as fungal species. As the method relies on DNA sequencing of specific regions of (typically) seven core metabolic / housekeeping gene loci and the online deposition of sequence- and meta- data, it can be used to study pathogen epidemiology at the local, national and international level. It has also been an invaluable tool for studying the evolution and global spread of antibiotic resistant and / or hyper-virulent lineages of species such as *Staphylococcus aureus* (including methicillin-resistant [MRSA] clones) [45], *Streptococcus pneumoniae* [46], *Escherichia coli* [47] and of course *Pseudomonas aeruginosa* [48]. MLST has also been used to examine and compare evolutionary parameters between species.

Comparison of the alleles for individual species allows an estimation to be made of the ratio of recombination (R) to point mutation (M) per MLST allele or per nucleotide. Two studies using this methodology estimated that MLST loci nucleotides were changed 8.4 - 9.5 times more often by recombination compared to point mutation which is much lower than the naturally transformable *S. pneumoniae* (50 times) and *N. meningitidis* (80 times), species that have epidemic population structures but are demonstrably naturally transformable [49]. However these latter two species have much smaller pan-genomes presumably due to their niche adaptation to almost exclusively colonise and infect mammals and have much lower numbers of prophage, plasmids and other mobile genetic elements compared to *P. aeruginosa*.

In an era in which rapid, inexpensive genome sequencing is now widely available traditional MLST is rarely used and most epidemiological studies nowadays extract core-genome (cg)MLST data from assembled or unassembled whole genome sequences [50, 51] for comparison with database sequences and isolate information held at, for example, the PubMLST website [50, 51, 52]. This allows the identification of multiple antibiotic resistant “high risk” clones (see below) and phylogenetic and phylogeographic comparisons of users isolates with the >3300 isolates present in the *P. aeruginosa* database.

### 1.5 High Risk Clones

In common with other pathogens with epidemic population structures, *P. aeruginosa* is notable for the presence of genotypes that are over-represented in samples from human disease and carriage. These so-called "high risk" clones are indicative of an epidemic population structure where genotypes increase in frequency through rapid transmission in hospital and other environments probably due to antibiotic selection and enhanced virulence in certain vulnerable patient populations. Clonal spread of *P. aeruginosa* genotypes belonging to clonal complex (CCs) 111, 175, 235 and 395 are associated with the global dissemination of multidrug resistant genes including metallo-beta lactamases $bla_{VIM,}$ IMP, SPM and NDM that confer carbapenem resistance [53, 54] (Figure 1). Carbapenemase genes are much more common in high-risk clones compared to other isolates and these may also have enhanced virulence – one study found isolates belonging to CC111 and 235 clones were highly virulent in comparison to ST175, a third, less significant clone in an invertebrate infection model [55].

### 1.6 Biofilm / Secretion Systems

In common with many bacterial pathogens *P. aeruginosa* cells exist within complex microbial communities known as biofilm; found at surface-liquid, surface-air and liquid-air interfaces. *P. aeruginosa* can thrive in biofilm due to the extracellular polymeric substances (EPS) it can produce, helping the bacteria resist desiccation, mechanical removal and the actions of antibiotics and disinfectants [56]. When *P. aeruginosa* forms biofilm in human tissue, cells are difficult to eradicate and represent infectious foci that can lead to severe systemic disease. Polymicrobial biofilms are considered a "melting pot" for horizontal gene transfer within and between bacterial species [57], recognized as important niches for MDR evolution. Isolates belonging to the two high-risk CC 111 and 235 described earlier have been shown to produce significantly more biofilm compared to those from non-high risk clones [58]. Numerous links between virulence and biofilm have been reported in the literature, with the presence of *P. aeruginosa* in polymicrobial increasing virulence of other bacterial species [59]. For example, it is suggested that the virulence factor phenazinethe, the enzyme polyphosphate kinase (ppk) and the HD-GYP domain proteins can modulate/regulate biofilm in addition to virulence [60, 61, 62]. Whilst there is still considerable work to be done to unpick the relationship between *P. aeruginosa*, virulence, HGT and biofilm production, it is clear that acknowledging this interplay will be an important aspect of future research.

### 1.7 Bacteriophage and HGT of virulence: friend or foe?

Bacteriophage (phage) are viruses that infect bacteria, and are considered of significant interest for their antibiofilm mechanism. Once a phage enters the cell, one of two life-cycles can occur. The lytic cycle results in the destruction (or lysis) of the infected cell, preceded by transcription of the phage DNA in order to synthesise new phage. The resultant destruction of the cell releases the newly formed phage into the environment to continue infection of the bacterial community. Therefore, there is significant interest in the ability for phage to infect and destroy bacterial cells (and particularly those in biofilm), with numerous review articles available describing the breadth of research in this area e.g. [63, 64].

However, some phage follow the second life cycle, known as the lysogenic cycle. Here, following infection, the phage DNA integrates with the bacterial DNA by a process known as transduction (a form of HGT), whilst the bacterial cell continues its usual lifestyle and remains able to reproduce. This DNA, known as a mobile genetic element (MGE), can also carry genes that may increase a bacterial cells ability to survive, including virulence factors and antimicrobial resistance mechanisms. MGE, from phage and other sources, are well documented as an important avenue of evolution in *P. aeruginosa* e.g. [65, 66, 67]. One large genomic study suggests that the presence in a *P. aeruginosa* genome of phage transduction - targeting CRISPR-Cas sytems is associated with reduced antibiotic resistance [26]. Therefore, understanding this process and targeting the mobile genetic elements of disease to stop the spread of virulence factors by HGT is of significant interest [68].

## 2. Control Strategies

### 2.1 Antibiofilm approaches

As described above, *P. aeruginosa* biofilm provides an ideal scenario to share virulence genes through HGT. Therefore, understanding how to remove or control biofilm, and therefore limit potential HGT of virulence genes, is an important and active research field. Various molecules have been identified as having both antibiofilm and anti-virulence effects. For example, the flavanol quercetin [69], ZnO nanoparticles [70], garlic [71] and cinnamon oil [72] all have been shown to have both antibiofilm and anti-virulence effects. The genetic links between antibiofilm and anti-virulence activity has also been explored, for example, in a *typA* (a GTPase) mutant that has demonstrably lower virulence and reduced biofilm [73].

A more targeted approach to biofilm control relies on the ability for bacterial cells to revert from biofilm to planktonic (unattached and free-floating), causing dispersal of cells. However, this process is not easy, due to the cementing of cells into complex 3D structures which form biofilm [74]. Dispersins are molecules that trigger cell dispersal. For example, the signalling molecules nitric oxide (NO) [75], succinate, glutamate, glucose [76] and the dendrimer FD2 [77] have all been shown to trigger enhanced dispersal of *P. aeruginosa* cells from biofilm. Innovations in electrochemical engineering may also provide one avenue for researchers to find new dispersins. In 2018, Robb demonstrated the use of pyrolytic graphite electrodes to assess potential dispersal-inducing properties of 2-aminoimidazole (2-AI) [78]. Such analytical technologies may enable microbiologists to better understand and measure the dispersal potential of novel antimicrobial and anti-biofilm molecules.

Another targeted approach for biofilm control is the use of depolymerase, enzymes which are able to breakdown polymeric substances. Depolymerase produced by *P. aeruginosa* has been a topic of research for decades [79], with much focus on bacteriophage-encoded depolymerases [80, 81]. It is believed that phage with depolymerase can degrade extracellular polymeric substances (EPS - the 'cement' of the biofilm), gaining easier access to host receptors. This ability to degrade the EPS also means the phage are likely able to infect cells within a biofilm more efficiently. For example, PT-6, a P. aeruginosa-infecting phage was able to reduce viscosity of four different alginate polymers by two-thirds within 15 minutes [82]. A recent review found 160 putative depolymerases from 143 phage, including sialidases, levanases, xylosidases, dextranases, hyaluronidases, peptidases as well as pectate/pectin lyases [83]. These represent potential anti-virulence strategies for the reduction of prevention of *P. aeruginosa* biofilm formation. Whilst there is a lack of literature describing the impact of antibiofilm measures on HGT, given the potential for HGT in biofilm (as discussed earlier), controlling biofilm should be considered a potential intervention strategy for the potential of *P. aeruginosa* to acquire virulence genes (and other genetic material). This is particularly important in environments where HGT of virulence genes could lead to particularly negative outcomes e.g. clinical biofilm.

### 2.2 Controlling Quorum Sensing

Quorum sensing is well known to be important in *P. aeruginosa* biofilm development [84], which as described previously, can be considered an important enabler for HGT. Therefore, strategies to control or reduce QS, and therefore reduce biofilm formation, may provide an indirect strategy for reducing HGT. *P. aeruginosa* has at least four quorum-sensing signalling networks, Las, Rhl, Pqs and Iqs [85].

The presence of QS molecules is associated with the production of extracellular DNA [86] and elevated rates of horizontal gene transfer (HGT) [87]. A study by Rossmann, Racek [88] found that high concentrations of the universal AI-2 compound in an already established biofilm promoted the release of phage capable of infecting other bacteria, increasing their virulence. In *E. coli*, transfer of antibiotic resistant plasmids can be enhanced by the presence of autoinducers, however, QS inhibitors were able to inhibit the transfer [89]. Various QS inhibitors have been demonstrated to block *P. aeruginosa* QS molecules. A synthetic derivative of a naturally occurring furanone has demonstrated antagonistic activity against *P. aeruginosa* QS, increasing susceptibility of biofilm to antibiotics and increased clearance in a mouse model [90]. The compound meta-bromo-thiolactone (mBTL), has also demonstrated inhibition of virulence factor production in *P. aeruginosa*, in addition to inhibiting biofilm formation by partially inhibiting LasR and RhlL [91]. Other compounds such as the essential oil 6-Ginerol, produced by fresh ginger, has similarly demonstrated QS inhibition [92]. Furthermore, presence of QS inhibitors has been shown to supress the CRISPR-Cas9 system in *P. aeruginosa*, making the bacterial cell more prone to death resulting from phage infection [93]. These are only an example of the wide range of research currently ongoing to understand the impact of QS in *P. aeruginosa* and many other microorganisms, a new and fast paced field [94]. Whilst specific literature describing the impact of QS control on HGT of virulence genes is lacking, it is clear that QS control has huge potential to indirectly limit HGT of virulence and the inevitable increase of isolate virulence.

### 2.3 Molecular Diagnostics

Rapid genomic or metagenomic sequencing from patient samples should soon deliver a future where key features such as species diversity antibiotic sensitivity and virulence gene complement can be determined in a clinically relevant timescale to guide appropriate and specific intervention strategies including patient isolation and specific antibiotic regimens. Rapid, real-time outbreak analysis and *in silico* resistome characterisation will lead to more targeted and thus more effective antibiotic administration that will also reduce the diversifying selection on microbial communities that is imposed by inappropriate antibiotic administration. The potential for Nextgen genomic sequencing to deliver epidemiologically useful information within a clinically useful timeframe was first demonstrated during an outbreak of *Staphylococcus aureus* in a paediatric setting in Cambridge, UK in 2012 [95]. The potential for *in silico* resistome analysis as a tool to determine the antibiotic sensitivity of *P. aeruginosa* has been successfully demonstrated in two separate studies [53, 96] and NGS is becoming a commonly used tool in retrospective analyses of *P. aeruginosa* outbreaks, for example in a 2014 study tracing an outbreak due to an XDR ST111 clone in the United Kingdom [97].

Therefore, there do not seem to be any significant barriers (except cost) to the routine use of genome sequencing in hospital laboratories for outbreak control and antibiotic prescribing purposes. With few therapeutic options for treating some *P. aeruginosa* infections, rapid information on antibiotic sensitivity of an individual isolate could be crucial and it could be used to guide and minimise inappropriate use of new antibiotics as they are developed.

The use of new agents such as therapeutic antibodies and antimicrobial peptides, perhaps co-administered with antibiotics, could also be effectively targeted for the control of "high-risk" *P. aeruginosa* genotypes based on rapid genomic profiling in hospital settings.

### 2.4 Controlling environmental conditions likely to encourage HGT - photocatalysis

AMR is increasingly recognised as a problem outside of the clinic with many organisms acquiring and transmitting resistance and virulence genes within and between environmental, agricultural and animal (including human) reservoirs. Thus, control of key human pathogens such as *P. aeruginosa* in the environment could play a major role in reducing HGT of resistance and virulence loci.

Wastewater is considered a hotspot for horizontal gene transfer [98]. Overuse and potential misuse of antibiotics in farming, industry and medicine (both human and veterinary) as well as irresponsible use of pollutants such as heavy metals and disinfectants have resulted in the inevitable discharge into waste water [99]. These conditions cause cellular stress, which alongside high bacterial numbers (and subsequent populations of bacteriophage) and biofilm formation [100] have led to a uniquely blurred boundary between the human and natural world where HGT is readily facilitated. Isolates of *P. aeruginosa*, is a common member of the wastewater community, have demonstrated an increase in QS production linked with potential virulence and biofilm formation [101]. More generally, transfer of antimicrobial resistance genes (ARG) in wastewater has become a dominant line of research [102], with suggestion that bacteria gaining ARGs via phage may also have an increased capacity for virulence [103]. Therefore, interventions that target conditions within aquatic environments that promote HGT should be a priority.

Advanced treatment technologies such as photocatalysis have demonstrated efficiency at removing contaminants from wastewater [102]. Over recent years, photocatalysis, a relatively simple, yet efficient technique of mineralizing organic contaminants to carbon dioxide and water without the production of secondary pollutants, has gained popularity as a safe, cheap and sustainable method for water decontamination [104, 105]. Titanium dioxide (Titania, $TiO_2$) is the most studied

photocatalytic material for decontamination, depollution and disinfection processes [105, 106], killing *P. aeruginosa* (and other bacteria) through the generation of radical oxygen species [107, 108, 109, 110], and can be favoured over other antimicrobial active substances due to its long-term antimicrobial action [111]. In addition to killing bacteria, photocatalysis has also been shown to break down viruses, and a wide variety of organic materials [112, 113, 114]. All of these properties target and reduce the factors that can increase HGT (large number of bacterial cells, pollutants and biofilm).

Despite the obvious benefits of such an antimicrobial material, fundamental questions relating to the effect of photocatalytic coatings on the microbiology of water have been raised in the literature. Critically, some advanced treatment technologies do not provide a rapid bacterial kill, and will create conditions likely to cause cellular stress and potentially leading to the cell SOS response, encouraging horizontal gene transfer.

## 3. Conclusion

*Pseudomonas aeruginosa* occupies a huge variety of environmental niches being a ubiquitous in many soils and watercourse habitats. These isolates are frequently indistinguishable from those colonising and infecting humans. The species diversity and genome plasticity reflects this – the species has a very small core genome of a few hundred genes representing 1% of the species genes discovered thus far. Approximately half of the genes found in *P. aeruginosa* have only been seen in one isolate. A significant proportion of this pan- genome especially that related to antibiotic resistance is mobile, present on elements such as plasmids, transposons and insertion sequences however the chromosome itself is also subject to high rates of recombinational change through HGT. The rapid detection and characterization of globally disseminated “High Risk” genotypes (clones) that are particularly associated with enhanced virulence and MDR, XDR and PDR resistance to antibiotics, through molecular diagnostics should enable more targeted treatments based on inferred antibiotic sensitivity from genome data or where no antibiotic is available, treatment with other agents in development such as therapeutic monoclonal antibodies or antimicrobial peptides. Anti-virulence molecules that interfere with inter-cell quorum sensing and signalling or approaches that destroy mixed strain and species biofilms where gene exchange occurs at high frequency represent future approaches for limiting the proliferation of *P. aeruginosa* genes for antibiotic resistance and virulence that represent an increasing threat to human health.

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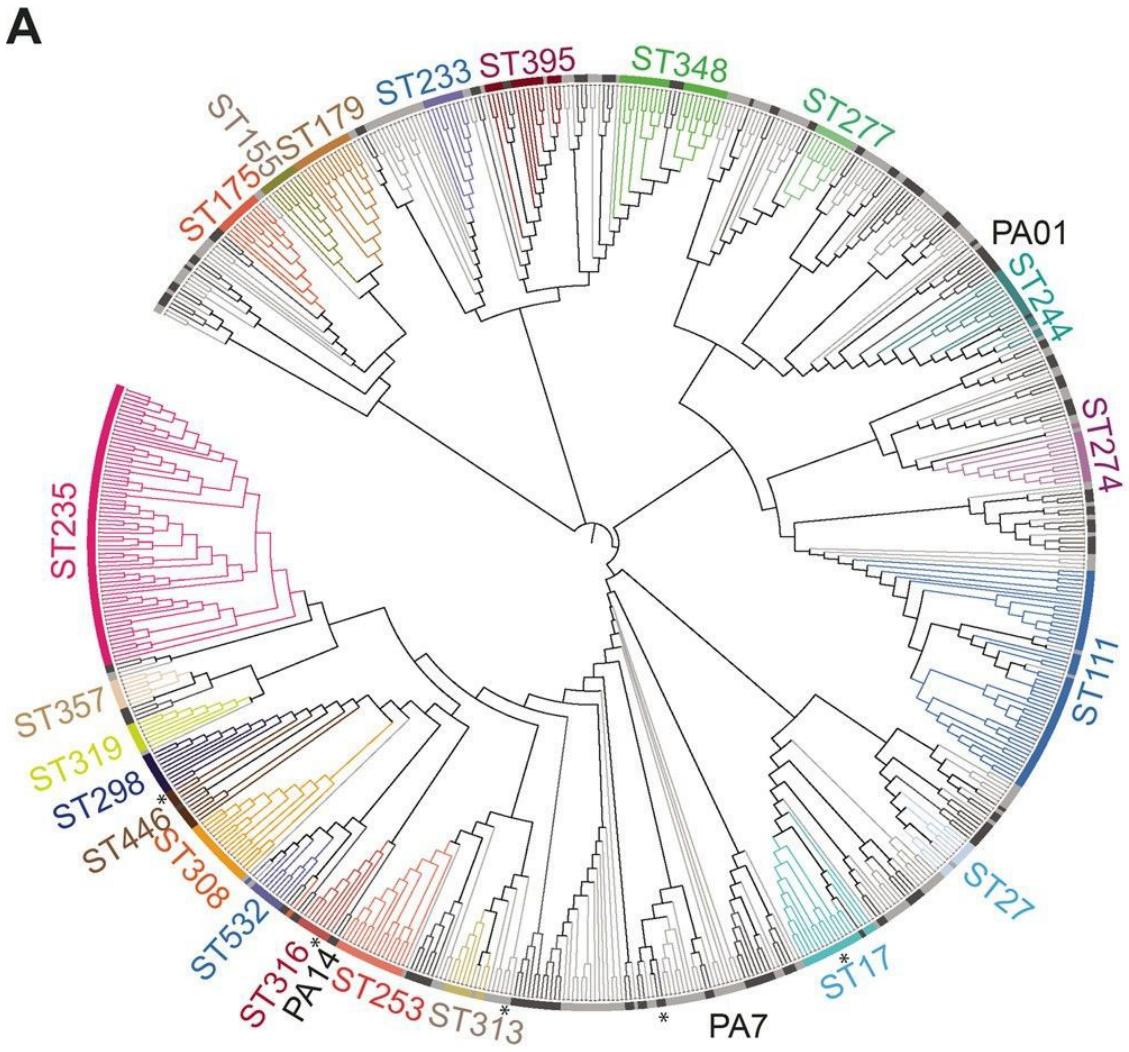
Figure 1. Genomic diversity of *P. aeruginosa* clinical isolates. (A) Phylogenetic tree of *P. aeruginosa* clinical isolates. Phylogenetically related ST groups with >7 members are shown in different colors. The prevalent lineages constitute 52% of all strains analyzed. ST groups that have only 1 member are delineated in grey, while black denotes phylogenetic lineages with 2 to 6 members. The phylogenetic locations of the common laboratory strains PAO1, PA14, and PA7 are marked in black for reference, while the 4 nonclinical isolates are labelled with small black asterisks. Figure reprinted from Van Belkum et al. mBio. 2015 [36].

Figure 2. Summary of key virulence determinants and factors important for biofilm formation produced by Pseudomonas aeruginosa. Reproduced from Diggle and Whiteley, 2019 [110].

Figure 3. The hierarchy quorum sensing network in *Pseudomonas aeruginosa*. Reproduced from Lee and Zhang, Protein and Cell, 2014 [80].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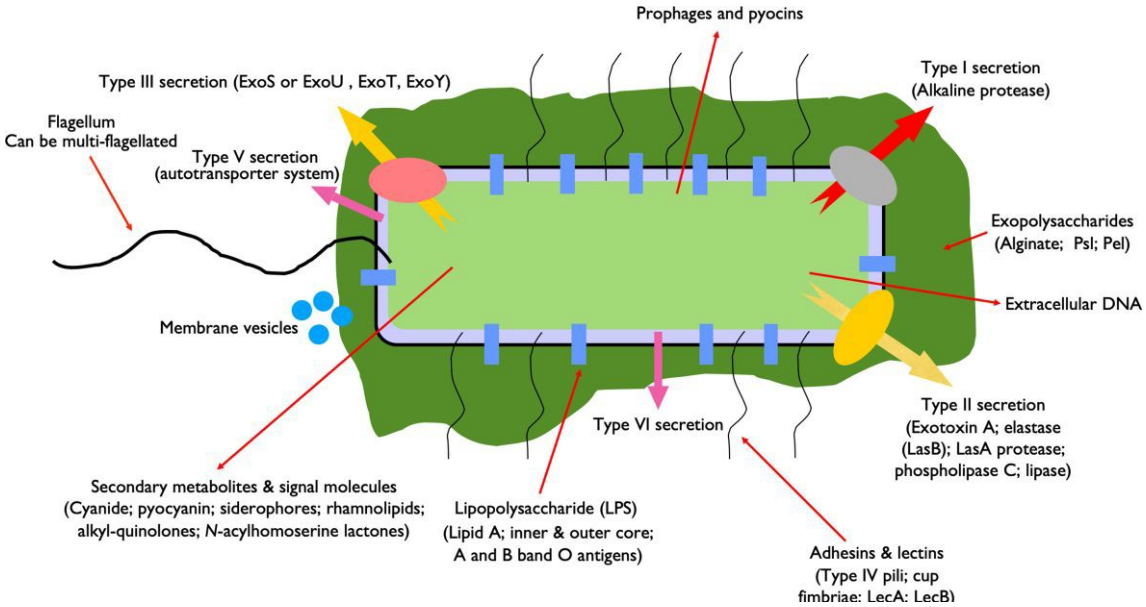
Prophages and pyocins
Type III secretion (ExoS or ExoU , ExoT, ExoY)
Type I secretion
(Alkaline protease)
Flagellum
Can be multi-flagellated
Type V secretion
(autotransporter system)
Exopolysaccharides
(Alginate; Psl; Pel)
Extracellular DNA
Membrane vesicles
Type VI secretion
Type II secretion
(Exotoxin A; elastase
(LasB); LasA protease;
phospholipase C; lipase)
Secondary metabolites & signal molecules
(Cyanide; pyocyanin; siderophores; rhamnolipids;
alkyl-quinolones; N-acylhomoserine lactones)
Lipopolysaccharide (LPS)
(Lipid A; inner & outer core;
A and B band O antigens)
Adhesins & lectins
(Type IV pili; cup
fimbriae; LecA; LecB)

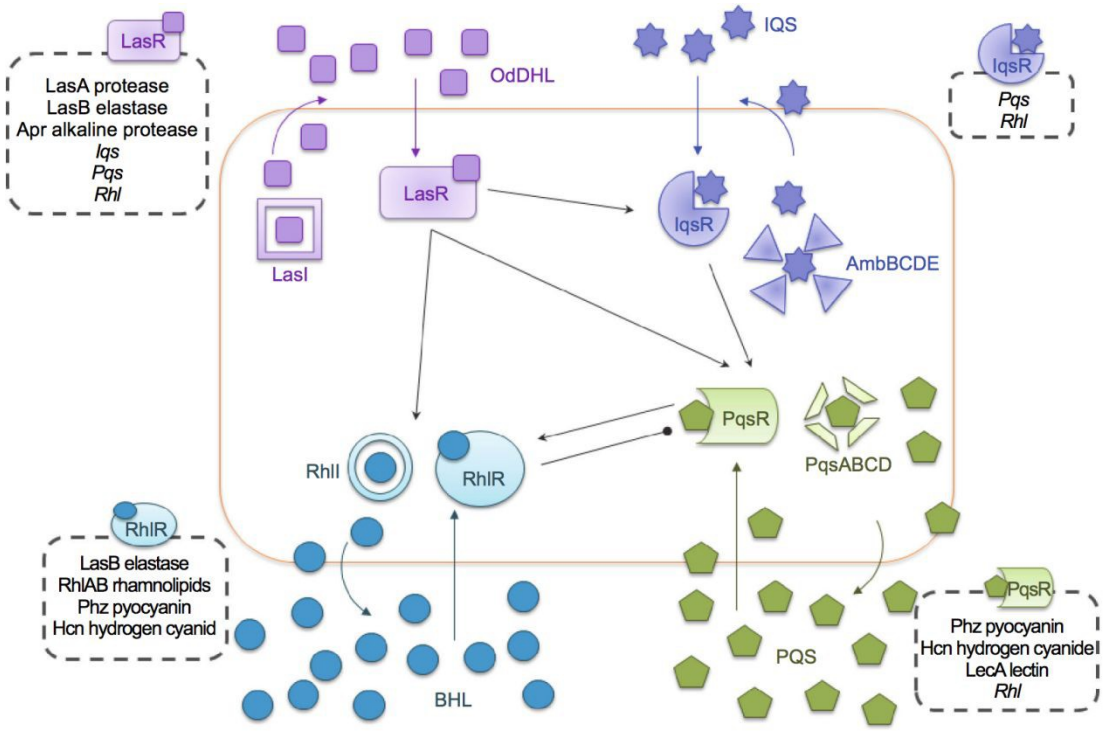


**Figure 3. Schematic representation of the four QS signaling networks in *P. aeruginosa* and their respective regulons.** Arrows indicate a stimulatory effect. Perpendicular lines indicate an inhibitory effect.