Advances in understanding Pseudomonas
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F1000Prime Reports 2014, 6:9 (doi:10.12703/P6-9)

Abstract

*Pseudomonas aeruginosa*, the type species of pseudomonads, is an opportunistic pathogen that colonizes a wide range of niches. Current genome sequencing projects are producing previously inconceivable detail about the population biology and evolution of *P. aeruginosa*. Its pan-genome has a larger genetic repertoire than the human genome, which explains the broad metabolic capabilities of *P. aeruginosa* and its ubiquitous distribution in aquatic habitats. *P. aeruginosa* may persist in the airways of individuals with cystic fibrosis for decades. The ongoing whole-genome analyses of serial isolates from cystic fibrosis patients provide the so far singular opportunity to monitor the microevolution of a bacterial pathogen during chronic infection over thousands of generations. Although the evolution in cystic fibrosis lungs is neutral overall, some pathoadaptive mutations are selected during the within-host evolutionary process. Even a single mutation may be sufficient to generate novel complex traits provided that predisposing mutational events have previously occurred in the clonal lineage.

Advances in understanding Pseudomonas by genomics

Pseudomonads are renowned for their metabolic versatility and widespread spatiotemporal distribution [1]. The type species *Pseudomonas aeruginosa* has become a major opportunistic pathogen for humans and this is probably the underlying incentive for most research that is currently performed on *P. aeruginosa*. Hence if we want to talk about advances in understanding Pseudomonas we can confine ourselves to *P. aeruginosa*. Thanks to the revolution of next generation sequencing, most new knowledge – at least in quantitative terms – has been generated during the last few months and years in Pseudomonas genomics. We are now starting to understand the evolution and population biology of *P. aeruginosa* and its interaction with the environment at the whole genome scale, which can provide us with some general insight into the diversity, ecology and evolution of the microbial world.

Population genomics

*P. aeruginosa* is a fascinating ubiquitous microorganism that can thrive at low densities within the range of 4°C to 42°C in inanimate aquatic habitats and can colonize the surface of animate hosts ranging from worms and flies to plants and mammals [1].

The *P. aeruginosa* population has an epidemic structure [2]. With the exception of a genomic region around the origin of replication, DNA segments can freely recombine between clonal complexes, although the individual clonal frames remain remarkably stable over time (our own unpublished genome sequencing data). By genotyping a large collection of strains from environmental and disease habitats with a custom-made multi-marker array [3] we have identified more than a thousand different clonal complexes, the majority of which are rare [3-4]. Just 20 clones make up about 40% of the contemporary population. Members of the two major clones C [5] and PA14 [6] were sampled from salt and fresh water, secluded national reserves, anthropogenically polluted sites, plants, wild and domestic animals and acute and chronic human infections. In other words, these two global clones are everywhere. However, the 20 next frequent clones predominate in particular
geographic areas and/or habitats. Numerous clones still have no representative among the subset of human infections and, conversely, clones that had previously caused outbreaks of nosocomial infection still lack an environmental isolate in our strain collection. These data suggest that the *P. aeruginosa* population consists of global and local generalists on the one hand and niche specialists on the other.

To verify this statement, genome data mining may provide the first evidence. During the last decade most information about the genetic repertoire of *P. aeruginosa* has been gathered from the genomes of the reference strains PAO1 [7] and PA14 [8]. Matching phenotypes were seen for orthologs of metabolic enzymes, whereas complex phenotypes (such as virulence traits) were combinatorial in nature, that is, the encoded function of a gene was modified by epistatic interactions [8]. Even a rather simple trait such as colony morphology was induced by mutagenesis of a peculiar gene in one strain, but not in another [9]. These pilot studies provide evidence that phenotypic diversity in the *P. aeruginosa* population cannot solely be ascribed to sequence variation and/or the presence or absence of genes, but rather results from an interplay between trait-shaping key genes, genetic modifiers and clonal genomic background. However, prior to addressing this complex issue, we need an inventory of genes and sequence variants to describe the genomic diversity of *P. aeruginosa*.

Ongoing genome sequencing projects in our own and other laboratories indicate that the core genome common to all *P. aeruginosa* consists of somewhat more than 4,000 genes. The so-called regions of genome plasticity [10,11] make up the variable accessory genome of about 10,000 genes. Variable subsets of this accessory gene pool are present in individual strains and clonal complexes [11]. On top of that, between dozens and hundreds of unique genes previously unknown in *P. aeruginosa* have regularly been observed whenever a strain of a yet uncharacterized clonal complex was subjected to genome sequencing [12-16]. Since more than 1,000 clonal complexes have already been identified in *P. aeruginosa*, we can estimate a pool of 100,000 further “private” genes that are rare or very rare in the *P. aeruginosa* population. The publication of the *P. aeruginosa* PAO1 genome sequence in the year 2000 [7] classified 2,500 PAO1 open reading frames as hypotheticals of unknown function. Since then, 300 of this number have been functionally characterized by the Pseudomonas community [17], so one can imagine the challenge remaining to achieve a comprehensive insight into the encoded genetic repertoire of the *P. aeruginosa* pan-genome.

Another subset of genes that had been underappreciated prior to the advent of RNA-sequencing are small regulatory RNAs (sRNAs) that, for example, shape the adaptive response to environmental cues by titrating regulatory proteins or base-pairing with target mRNAs [18]. RNA-sequencing of PAO1 [19] or PA14 [20] bacteria grown in planktonic culture in Luria-Bertani (LB) broth has already identified over 500 novel small RNAs or cis-antisense RNAs in addition to the previously known 44 sRNAs. Further sRNAs will probably show up under other experimental conditions and in other clonal complexes, because non-coding RNAs are preferentially localized in the accessory genome [20].

The closest homologs of genes of the accessory genome are typically found in β- and γ-proteobacteria, mostly in other pseudomonads, enterobacteriaceae, and species of the genera Burkholderia [21,22], Ralstonia [23] and Cupriavidus [24] that had been classified as pseudomonads prior to the introduction of 16S rDNA sequence-based molecular taxonomy [25]. Burkholderia, Ralstonia and Cupriavidus bacteria are still called honorary pseudomonads because, like the true pseudomonads, they are metabolically versatile and can utilize a broad range of compounds as carbon and nitrogen sources and even degrade halogenated xenobiotics. The operons that encode these extraordinary metabolic capabilities are typically located in genomic islands, which are shuffled across genus and species barriers by horizontal gene transfer [26,27]. An intriguing example is a PAGI-2 island that was detected with 100% sequence identity in a *Cupriavidus metallidurans* isolate from heavy-metal polluted soil and some 1,000 miles apart in a *P. aeruginosa* isolate from the chronically infected airways of a patient with cystic fibrosis [28]. In other words, the GC-rich *P. aeruginosa* apparently exchanges its gene pool of the accessory genome with other GC-rich proteobacteria that enjoy a similar lifestyle and metabolic versatility.

The intraspecies transfer of genomic islands among *P. aeruginosa* strains has been determined to occur at a frequency of $10^{-6}$ to $10^{-2}$ [28-30], meaning that only a minority of cells within the *P. aeruginosa* community is capable of horizontal transfer of the island [31]. The activation occurs at random and is associated with a loss of fitness to grow, which apparently restricts the number of transfer-competent donors to a few cells.

Genome mobility in *P. aeruginosa* is typically accomplished by horizontal gene transfer of plasmids, transposons, genomic islands and bacteriophages. Recent work has made *P. aeruginosa* into a model organism for the study of bacterial genome integrity in the context of
the co-evolution of phage and bacteria [32,33]. Bacterial CRISPR/Cas systems mediate phage resistance. These systems consist of clustered regularly interspaced short palindromic repeats (CRISPR) coupled with cas (CRISPR-associated) genes. Similar to RNA interference in eukaryotes, these CRISPR/Cas systems use small RNAs for sequence-specific detection and neutralization of invading genomes. In P. aeruginosa PA14, the type I-F CRISPR/Cas system was shown to prevent the replication of phages [32] and, conversely, the genomes of phages infecting strain PA14 harbour the first known “anti-CRISPR” genes that mediate the inhibition of a CRISPR/Cas system [33].

**Genome evolution in chronic cystic fibrosis lung infection**

P. aeruginosa causes a wide range of infections in humans that can vary from local to systemic, subacute to chronic, and superficial and self-limiting to life-threatening [1]. Chronic airway infections with P. aeruginosa are regularly seen in patients with advanced stages of chronic obstructive pulmonary disease [34,35] and individuals suffering from the autosomal recessive trait cystic fibrosis [36]. The basic defect of a perturbed ion, water and pH homeostasis predisposes the cystic fibrosis airways to colonization with opportunistic pathogens [37], predominantly Staphylococcus aureus and P. aeruginosa. Once P. aeruginosa has taken up residence in the cystic fibrosis lungs for more than a year, the organism is notoriously resistant to eradication by chemotherapy. Unless the patients become co-infected by a transmissible strain, more than 50% of patients will still be harbouring the initially acquired clone after 20 years of colonization [38]. The chronic infections of the stomach with Helicobacter pylori [39] and of the cystic fibrosis airways with P. aeruginosa [38] are the only cases of infections with extracellular bacteria in humans where the causative agent persists in its niche for decades and does not disseminate into other organs. These two scenarios provide a unique opportunity to monitor the genome evolution of a human pathogen in a disease habitat over many years.

Maynard Olson’s group has investigated the genetic adaptation of P. aeruginosa to cystic fibrosis airways in the single child of a cystic fibrosis newborn cohort who became chronically colonized with P. aeruginosa during infancy [40]. Numerous loci were hit by mutation in the child’s serial airway isolates collected over an 8-year observation period. Of these, the gene loci encoding multidrug efflux pumps and regulators of quorum sensing and alginate biosynthesis were subsequently confirmed by genotyping of a large strain collection to be hot-spots of mutation of P. aeruginosa isolates in cystic fibrosis lungs [40].

The cystic fibrosis clinics in Copenhagen and Hanover have regularly collected P. aeruginosa from the airways of all their patients who became chronically colonized in the 1970s and 1980s [38,41]. The analysis of these unique strain collections taken from numerous patients should provide a more comprehensive insight into the micro-evolution of P. aeruginosa during the chronic infection of cystic fibrosis airways than the single case of the infant from the US and hence whole genome sequencing of serial isolates has been performed at both sites.

The molecular epidemiology was different at the two clinics. Patients from the Hanover clinic became colonized with clones that are found at a similar frequency in the environment and other cystic fibrosis clinics [38]. In contrast, the Copenhagen patient cohort became chronically colonized with two highly related clones (DK1 and DK2) by patient-to-patient spread [38,41].

At the Hanover clinic, no turnover of clones was caused by nosocomial acquisition from other patients. Hence the microevolution of the initially acquired clone in patients’ airways could be followed over 30 years (currently ongoing). We started with the investigation of microevolution of the globally most abundant clones C and PA14 in two cystic fibrosis patients’ airways [42]. Both clones underwent phenotypic conversion that is typical for the cystic fibrosis lung habitat [43], that is, they became less motile, secreted less siderophores and virulence effectors and became deficient in the lipopolysaccharide (LPS) O-antigen, but they remained non-mucoid [42]. Late isolates were only slightly compromised in competitive growth in the presence of first isolates which, did not confirm the common belief in the literature that adaptation in the cystic fibrosis lungs is accompanied by a loss of global fitness of P. aeruginosa. For clone C, a loss-of-function mutation in the DNA repair gene mutL generated a bushy structure of multiple hypermutable clades [44-46] that accumulated close to 1,000 de novo mutations, but interestingly the mutation rate returned to normal in the mutL-deficient lineages after 10 years of colonization. No hypermutators arose in the PA14 lineage. The initial PA14 clade diverged into three clades during the first years of colonization, but after 15 years only one clade persisted in the cystic fibrosis lungs indicating that only this clade was endowed with the features to cope with the on-going remodelling and dedifferentiation of the chronically inflamed airways.

We are currently investigating the genome evolution of P. aeruginosa in contrasting patient cohorts with a very mild or very severe course of their infection (our own unpublished data). Exopolysaccharide biosynthesis,
antimicrobial resistance and global regulators of lifestyle and metabolism were found to be common functional categories whose genes were hit by mutations in all *P. aeruginosa* clones, irrespective of the severity of infection in the cystic fibrosis host. Microevolution, however, was not uniform in the patients’ lungs. For example, the *P. aeruginosa* clone inhabiting the most severely affected lungs generated progeny with stop mutations or drastic amino acid changes in key genes of lifestyle, whereas the *P. aeruginosa* clone that has persisted in a patient with normal lung function had diverged into co-existing clades which, accumulated benign, probably modifying amino acid substitutions, but no stop mutations.

These two cases demonstrate that the acquisition of a few mutations can make a major impact on bacterial phenotype. The fact that a single loss-of-function mutation can have dramatic consequences is also the “take home lesson” of intraclonal genome comparisons [47]. Clones CHA and TB were chosen for analysis because the clone members CHA and TBCF10839 confer unusual and severe traits of virulence. Both strains undermine man’s major host defence against *P. aeruginosa*, that is, killing by macrophages or neutrophils. By virtue of its type III secretion system, strain CHA destroys host defence cells by a mechanism called “pack swarming” whereby dozens of bacteria simultaneously attack and destroy the mammalian cell [48]. For genome comparison, a clone CHA isolate from the river Ruhr and the first *P. aeruginosa* isolate from a cystic fibrosis patient from another clinic were chosen [47]. These two other strains are not capable of pack swarming. The genomes of the three strains were more than 99.9 % identical. Each strain was carrying a few specific elements in its accessory genome and some dozen strain-specific single nucleotide polymorphisms (SNPs) reflecting the diverse spatiotemporal origin of the strains. However, the exceptional pathogenicity of strain CHA was attributed to a combined deletion of the lactate dehydrogenase and gacS genes, the latter being the major switch between the sessile biofilm-forming and the planktonic exoprotein-secreting lifestyles. Disruption of gacS created a strain that is both mucoid and proficient in the secretion of virulence effectors.

The second example of clone TB is equally illuminating [49]. The two cystic fibrosis strains TBCF121838 and TBCF10839 just differed in one gene deletion, less than 10 SNPs and one insertion of a genomic island from each other, but their phenotype was dramatically different in transcriptome, proteome, metabolome and adherence [46]. TBCF121838 was efficiently killed by neutrophils, whereas TBCF10839 could grow and multiply in polymorphonuclear cells (PMNs). The atypical virulence of strain TBCF10839 was caused by the pleiotropic consequences of a deletion within the pilQ gene that is essential for pilus biogenesis. The other pili were still synthesized [50], secreted by alternative routes and facilitated the intracellular survival of TBCF10839 in PMNs. In conclusion, microevolution in *P. aeruginosa* can generate novel complex traits by a few or even single mutations, provided that predisposing mutational events had occurred before in the clonal lineage [49].

Complementary to our study on the genome evolution of distinct clones in a cohort of patients seen at the Hanover clinic, a retrospective study has been performed on the genome evolution of the transmissible DK2 clone in a cohort of patients seen at the Copenhagen cystic fibrosis clinic [51-54]. Genome sequencing of 55 bacterial isolates collected from 21 cystic fibrosis patients over 38 years uncovered 8,530 mutations [54]. Overall, no evidence was found for either intragenic bias of mutations or for positive selection within coding regions. The 65 most frequently mutated genes, however, showed clear signs of positive selection. A large part of these genes should be involved in niche adaptation because they are associated with the composition of the cell envelope, antibiotic resistance and evasion of the host response.

Besides these investigations on the DK1 and DK2 clones from Denmark, genomic and phenotypic analyses are being pursued on further transmissible *P. aeruginosa* strains that have caused patient-to-patient spread in cystic fibrosis clinics, namely in Australia, Canada and the United Kingdom [55-64]. A consortium from these three countries headed by Fiona Brinkman (Vancouver), Roger Levesque (Quebec City) and Craig Winstanley (Liverpool) will sequence a further 1000 *P. aeruginosa* genomes in the context of cystic fibrosis lung infections. It is anticipated that we will be able to link the data to the Pseudomonas.com website, integrate the information with patient data registries in cystic fibrosis and develop a user-friendly pipeline for researchers and clinicians.

**Further current topics of Pseudomonas research**

This report focuses on our progress in understanding Pseudomonas in the context of genomics and its application to evolution, epidemiology and clinical microbiology. Besides these global genome-wide approaches, more thematically-oriented research is prospering in the
Pseudomonas field. Several major topics of interest were discussed at the Pseudomonas 2013 conference:

(a) the world of non-coding RNAs [18, 65, 66];
(b) the dissection of sigma factor regulons [67];
(c) structure and function of the type II, type III and type VI secretion systems (T6SSs) [68-80];
(d) the physiological functions of secondary metabolites such as phenazines [81-85];
(e) quorum sensing inhibitors [86, 87];
(f) the sessile biofilm-forming lifestyle [88-94];
(g) and the interaction of \( \text{P. aeruginosa} \) with its competitors in the microbial community [73, 77, 78, 82-85, 95].

Of these topics, the ongoing progress in the understanding of the T6SS [96] is particularly impressive. T6SSs are molecular nanomachines allowing Gram-negative bacteria to transport and inject proteins into a wide variety of target cells [76, 78]. The T6SS is composed of 13 core components and displays structural similarities with the tail-tube of bacteriophages [76, 97]. The phage uses a tube and a puncturing device to penetrate the cell envelope of target bacteria and inject DNA. T6SS is considered to be an inverted bacteriophage device creating a specific path in the bacterial cell envelope to drive effectors and toxins to the surface [76]. The \( \text{P. aeruginosa} \) PAO1 genome encodes three T6SSs, namely H1-, H2-, and H3-T6SS.

\( \text{P. aeruginosa} \) H1-T6SS targets at least three effector proteins (Tse1-3) to recipient Gram-negative cells [73, 74, 98]. The Tse2 protein is a cytoplasmic effector that induces the quiescence of target cell proliferation, thus providing a pronounced fitness advantage for \( \text{P. aeruginosa} \) in interspecies competition under T6S-conducive conditions [77].

The role of H3-T6SS is still unknown, but the function and regulation of H2-T6SS are starting to be elucidated [77]. H2-T6SS secretes a phospholipase D that releases choline from phosphatidylcholine and thereby leads to a severely perturbed membrane phospholipid composition in the recipient cell. Thus the H2-T6SS delivers an enzyme that is active against both prokaryotic [77] and eukaryotic membranes [75, 99]. This strategy of membrane targeting may contribute to the fitness of \( \text{P. aeruginosa} \) in interspecies competition under T6S-conducive conditions [77].

**Abbreviations**

Cas, CRISPR-associated; CRISPR, clustered regularly interspaced short palindromic repeat; LB, Luria-Bertani; LPS, lipopolysaccharide; PMNs, polymorphonuclear cells; SNPs, single nucleotide polymorphisms; sRNA, small regulatory RNA; T6SS, type VI secretion system.

**Disclosures**

The authors declare that they have no disclosures.

**Acknowledgments**

Work in the authors’ laboratory is supported by the Deutsche Forschungsgemeinschaft (SFB 900, project A2), the Bundesministerium für Bildung und Forschung (Medizinische Infektionsgenomik, Az 0315827A) and the Christiane Herzog Stiftung.

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