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Evolutionary steps in the conversion of environmental bacteria into opportunistic hospital pathogens

Origin and Evolution of Nosocomial *Pseudomonas aeruginosa*

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Evolutionary steps in the conversion of environmental bacteria into opportunistic hospital pathogens: Origin and Evolution of Nosocomial Pseudomonas aeruginosa

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DEDICATION

To Sara.

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RESUMEN

El origen de nuevos agentes patógenos es una de las mayores preocupaciones para la humanidad por motivos de una posible diseminación rápida y con consecuencias graves tales como incremento en la mortalidad y la morbilidad humanas y de otros animales. En este manuscrito, describo una serie de experimentos cuyo objetivo es investigar el origen de *Pseudomonas aeruginosa*, un organismo patógeno oportunista y una bacteria ambiental.

En el Capítulo 2 se expone el estado del arte sobre los estudios evolutivos que describen las fuerzas de presión selectiva en la evolución de *P. aeruginosa* y su impacto en su potencial virulento.

En el Capítulo 3, describimos un estudio cuyo objetivo fue realizar una comparación de *P. aeruginosa* presente en la microbiota intestinal y en sitios de infección de pacientes inmunocomprometidos y críticamente enfermos. Nuestros hallazgos revelan que *P. aeruginosa* pudo haber estado presente en el intestino de los pacientes antes de la presencia de la infección sistémica, demostrando que la bacteria coloniza el intestino antes de infectar otros órganos.

En el Capítulo 4 se ponen a consideración los resultados de experimentos diseñados para investigar diferencias fisiológicas encontradas entre las cepas de *P. aeruginosa* encontradas en objetos inanimados en un hospital y las cepas obtenidas de infecciones de pacientes en el mismo periodo de tiempo y en el mismo hospital.

En el Capítulo 5, hacemos una recopilación de las conclusiones derivadas de los resultados presentados en esta tesis y se exponen ideas para futuros estudios relacionados.

ABSTRACT

The origin of pathogens is a major concern for humanity due to their potential to spread rapidly and cause significant morbidity and mortality. In this manuscript, I describe a series of experiments designed to investigate the origin of *Pseudomonas aeruginosa*, an opportunistic pathogen and environmental bacterium.

Chapter II presents a literature review of current ideas about the forces driving the evolution of *P. aeruginosa* and their impact on its virulence potential.

In Chapter III, we conducted a study to analyze the genetic comparison of *P. aeruginosa* present in the intestinal microbiota and infection sites of immunocompromised and critically ill patients. Our findings reveal that *P. aeruginosa* can be present in the intestines even before the onset of a systemic infection, demonstrating that this bacterium colonizes the intestine before infecting other organs.

Chapter IV investigates the physiological differences between *P. aeruginosa* strains found on hospital inanimate objects and those isolated from infections.

The Chapter 5, conclusions of this manuscript summarize the findings and suggest ideas for future studies.

TABLE OF CONTENTS

LIST OF TABLES 11

LIST OF FIGURES 12

CHAPTER I

GENERAL INTRODUCTION 14

CHAPTER II

Pseudomonas aeruginosa transition from environmental generalist to human pathogen 19

CHAPTER III

Endogenous origin of *Pseudomonas aeruginosa* infecting hospitalized patients in Ecuador 38

CHAPTER IV

Insights into Physiological Adaptations of *Pseudomonas aeruginosa*: Contrasting Clinical and Environmental Isolates in a Hospital Setting 61

CHAPTER V

GENERAL CONCLUSION 94

LIST OF TABLES

Table 1. Information on patients, origin and type of infection, and discrepancies between clinical and rectal samples. 49

Table S1. Bioinformatic tools employed. 50

Table S2. Metadata of the clinical history of the included patients. 51

Table S3. Number of isolates carrying resistant genes detected by at least two out of three databases Comprehensive Antibiotic Resistance Database (CARD) Resistance Gene Identifier (RGI), National Center for Biotechnology Information NCBI AMRFinder, and the Center for Genomic Epidemiology Resfinder. 52

Table S4. Phenotypic and genotypic antibiotic resistance analysis of relatedness by Fisher´s exact test and OR-Fisher with 95% confidence interval. Bold numbers represent a statistically significant relation between the phenotype and the genotype. 53

Not published Annex Table 1. Resistance genotypes and phenotypes discrepancies between bacteria from the same clade (Highlighted cells). ResFinder database profiles were confirmed by AMRFinder database profiles. 1=gene presence, 0=gene absence, R=resistant phenotype, S=sensitive phenotype. 59

Not published Annex Table 2. Patients not included in the phylogenetic analysis. Resistance phenotypes and origin of samples. R=resistant phenotype, S=sensitive phenotype. 60

Table 1. Description of the environmental samples taken during the surveillance and the number of *Pseudomonas aeruginosa* isolates. 86

Supplementary Table 1. Two tails T-test *t* values and correspondent *p* values from the difference between the zone diameter area of inhibition due to antibiotic resistance Kirby-Bauer technique for nine antibiotics of environmental versus clinical isolates. 87

LIST OF FIGURES

Figure 1. Diverse *Pseudomonas aeruginosa* strains (blue bacteria) thrive in the environment degrading organic matter. Some strains have improved aptitude to grow in human tissue (brown bacteria); a subset of them have evolved additional adaptations and can be transmitted from human to human. 37

Figure 1. Phylogenetic tree using IQTREE software (maximum likelihood) of 5.29 Mbps length alignment of 53 *P. aeruginosa* isolates (including the reference sequence). Blue branches represent isolates from Hospital_1C. Red branches represent isolates from Hospital_2P. Shaded boxes represent each patient's isolates. The yellow shade shows the ST-253 epidemic clade. The numbers in nodes indicate bootstrap values using 1000 pseudo-replicates; the bootstrap number is shown as a percentage (%). Resistance phenotypes are shown on the right. Resistant strains with at least one genetic marker statistically significantly related to its phenotype are drawn in filled circles, and the ones with the resistant phenotype but not the genetic feature are in empty circles. Susceptible isolates are not illustrated. A single asterisk denotes the recurrence isolates. Double asterisks denote rectal isolates collected on the admission date. 54

Figure S1. Whole genome core SNPs phylogenetic tree obtained by kSNPs software using maximum likelihood GTR model. Number of core SNPs from 53 sequences (including the reference sequence): 49188. The clades resemble the clustering from Figure 1. The bootstrap is a percentage. 55

Figure S2. Average Nucleotide Identity phylogenetic tree and matrix obtained by ANIclustermap software, skani. The matrix denotes the closely related strains in darker squares, resembling the phylogenetic tree from Figure 1. 56

Figure S3. Timeline of the ST strains isolated from Hospital_1C (blue) and Hospital_2P (red). Horizontal bars correspond to the stay length at the hospital from each patient. The circle's position shows the date when the clinical sample was taken. 57

Figure S4. Partial Pearson's r heatmap, conditioned on the variable of the number of SNPs per million base pairs between the genomes of the isolates and the reference sequence *P. aeruginosa* AE004091.2. *p < 0.05 **p < 0.01 ***p <0.001 58

Figure S1. UPGMA phylogenetic tree of the *aroE* allele's sequences from pathogenic and environmental isolates. Seventy-seven sequences: 9 from PubMLST allele reference sequences and 68 from this study were included in the tree, and 499 nucleotide sites were used. The clinical isolates are drawn as orange-filled squares (Type). The bootstraps are annotated as percentages; branch lengths are annotated upside the bootstrap value. 88

Figure 1. Water survival bacterial counts, CFU/ml transformed to LN of clinical and environmental strains at 12 time points. Error bars represented 95% confidence intervals. 89

Figure 2. Survival differences among clinical and environmental *P. aeruginosa* strains with the bacteria grazer *T. pyriformis* host at different time points. A. Box plots showing the count on bacterial density without ciliates CFU/ml (control). B. Box plots showing the count on bacterial density with the ciliate *T. pyriformis* CFU/ml (test). C. Box plots showing the ciliate density of the tests in cells/ml. D. Box plots showing the comparison of the counts of bacteria density in CFU/ml between the controls and the tests. E. Box

plots showing the MOI changes in time intervals (bacteria:ciliates). Error bars 95% confidence intervals. Counts display as the natural logarithm LN. 90

Figure 3. Antibiotic resistance boxplots of the zone diameters differences among clinical and environmental strains under antibiotic resistance Kirby-Bauer test for nine antibiotics. Error bars 95% confidence interval. 91

Figure 4. *In-vitro* competitions. A. Photo improved by artificial intelligence of the left=lysis, right-up corner=halo, right-down=no inhibition. B. Bar Plots of the mean halo or lysis activity times per bacteria under nutrient and minimal growth conditions. 92

Figure 5. Swarming, swimming, and twitching motility diameter in clinical versus environmental strains (mm). Error bars 95% confidence interval. Photos under the boxplots represent the motility differences on agar tests. 93

CHAPTER I

GENERAL INTRODUCTION

During the mid-19th century, silk production in Europe suffered significant economic losses due to the spread of disease among silk-producing worms. In 1865, Louis Pasteur, who was already renowned for his studies in chemistry, fermentation, and the pasteurization method, was assigned to find the cause of the *Bombyx mori* disease. After five years of research, he discovered that at least two microorganisms were responsible for the disease. Even though many microorganisms had been described, this was the first time one was recognized as the cause of an infectious disease. Following this discovery, Pasteur, Robert Koch, and other scientists worked tirelessly to describe methods to identify animal disease etiological agents, including human infectious diseases. At the same time, scientists began to develop methods for eradicating microorganisms from the sick. In 1867, Joseph Lister published the methods of antisepsis to avoid infections in surgical procedures with tremendous success in reducing mortality. These findings began a bacteriological era of discoveries. These findings and even the rising industry of antibiotics contributed to the idea that bacteria are mainly pathogenic, and for instance, the idea that hygiene methods must include antibacterial practices for bacteria eradication. These anti-bacterial ideas impacted the explanations of the origin of the pathogenic bacterial species. Bacterial virulence was understood as the result of bacterial specifical adaptation to animal immunological responses.

In contrast, Bass Becking, one of the introducers to environmental ecology studies, stated, “Everything is everywhere, but the environment selects” in the 1934 publication “Geobiology or Introduction to the Science of Environment.” Bass Becking proposed that the genes and, for instance, the species that carry them have relative ease of moving between the earth. Every gene can be found in every environment; however, the selective forces in each niche select the gene and species diversification and abundance.

Studies on the interaction of soil bacteria have found that there is an important antagonistic activity among kin bacteria, especially between bacteria living in the same niche. This antagonism triggers the adaptation of survival strategies, specifically, resistance to toxin activity and nutritional specialization [1].

It is well known that T6SS antagonistic mediated activity and toxin-mediated antagonism are critical in each niche [2]. The animal intestinal microbiota has long been considered a stable niche due to the commensal (and possibly mutualistic) relationships between the host immune system and the diet. However, T6SS antagonistic activity in the intestinal microbiota may provide colonization resistance from other bacteria [1,2].

In the bacterial microbiota, functional redundancy is more critical in selecting bacterial species than maintaining an actual taxonomy composition [3]. Thus, competition with new colonizers may select for more antagonistic activity and resistance to competition

among pathogenic strains and may deploy microbiota in the presence of a strong antagonistic strain [2].

Environmental bacteria that evolve outside of an animal host live under a wide range of niches and gradients that act as selective pressures, enriching the phenotypes and genotypes of a given species [3]. For instance, the environment is a source of bacteria adapted to antagonistic interaction, which may enable them to colonize new niches, which can show similar or less harsh antagonistic forces than the environment where they evolved.

Opportunistic human pathogens can originate from two main sources: the host's microbiota and the environment [4]. However, these pathogens can only infect humans when the immune system is compromised due to severe illness, microbiota translocation to another organ outside the intestines or mucosa, or immunocompromised states.

There is a growing public interest in investigating the origins of the emergence of human pathogens [5]. This interest is triggered by the continuous changes in the environment and the identification of increasing risk factors among humans, such as aging, antibiotic resistance, and the rapid spread potential of new pathotypes [5].

We have selected *Pseudomonas aeruginosa*, an environmental bacterium, as a model over other opportunistic human pathogens because of its morbidity importance and frequent isolation (against other environmental opportunistic bacteria) in Ecuadorian hospitalized patients [6–9].

References

[1] Peterson SB, Bertolli SK, Mougous JD. The Central Role of Interbacterial Antagonism in Bacterial Life. Current Biology 2020;30:R1203–14. https://doi.org/10.1016/j.cub.2020.06.103.

[2] Zhao W, Caro F, Robins W, Mekalanos JJ. Antagonism toward the intestinal microbiota and its effect on Vibrio cholerae virulence. Science (1979) 2018;359:210–3. https://doi.org/10.1126/science.aap8775.

[3] Baquero F, Coque TM, Galán JC, Martinez JL. The Origin of Niches and Species in the Bacterial World. Front Microbiol 2021;12. https://doi.org/10.3389/fmicb.2021.657986.

[4] Huang M, Wu Y, He P. Advances in humans and animals opportunistic pathogens from environment infecting plants by crossing kingdoms. Wei Sheng Wu Xue Bao 2016;56:188–97.

[5] World Health Organization. 2021 Antibacterial agents in clinical and preclinical development: an overview and analysis. Geneva: World Health Organization; 2022.

[6] Barbecho Coraisaca DV. Susceptibilidad antimicrobiana en *Pseudomonas* spp., en el Hospital General Docente Cuenca-Ecuador. Revista Vive 2021;4:484–99. https://doi.org/10.33996/revistavive.v4i12.108.

[7] Salgado Yepez E, Bovera MM, Rosenthal VD, González Flores HA, Pazmiño L, Valencia F, et al. Device-associated infection rates, mortality, length of stay and bacterial resistance in intensive care units in Ecuador: International Nosocomial Infection Control Consortium's findings. World J Biol Chem 2017;8:95. https://doi.org/10.4331/wjbc.v8.i1.95.

[8] Pachay Solórzano JW, Pachay Parrales VE. *Pseudomonas aeruginosa* y su evolución de resistencia a los antibióticos en un hospital de segundo nivel en Portoviejo, Ecuador. QhaliKay Revista de Ciencias de La Salud ISSN: 2588-0608 2021;5:50. https://doi.org/10.33936/qkrcs.v5i2.3002.

[9] Lascano-Vaca Y, Ortiz-Prado E, Gomez-Barreno L, Simbaña-Rivera K, Vasconez E, Lister A, et al. Clinical, genetic and microbiological characterization of pediatric patients with cystic fibrosis in a public Hospital in Ecuador. BMC Pediatr 2020;20:111. https://doi.org/10.1186/s12887-020-2013-6.

CHAPTER II

Pseudomonas aeruginosa transition from environmental generalist to human pathogen

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Pseudomonas aeruginosa transición de generalista ambiental a patógeno humano

Abstract

Opportunistic bacteria *Pseudomonas aeruginosa* is a major concern as an etiological agent of nosocomial infections in humans. Many virulence factors used to colonize the human body are the same as those used by *P. aeruginosa* to thrive in the environment, such as membrane transport, biofilm formation, oxidation/reduction reaction, and others. The origin of *P. aeruginosa* is mainly from the environment; the adaptation to mammalian tissues may follow a source-sink evolution model. The environment is the source of many lineages, some of them capable of adaptation to the human body. Some lineages may adapt to humans and go through reductive evolution in which some genes are lost. The understanding of this process may be critical in order to implement better methods of controlling outbreaks in hospitals.

Keywords: bacteria, host adaptation, evolution, opportunistic,

Resumen

La bacteria oportunista *Pseudomonas aeruginosa* es una de las principales preocupaciones por su rol como agente etiológico de infecciones nosocomiales humanas. En muchos casos, los mismos factores de virulencia que le permiten a *P. aeruginosa* causar infecciones en humanos pueden ser empleados para prosperar en el ambiente, como los sistemas de transporte de membranas, la formación de biopelículas, las reacciones de oxidación / reducción y otros. El origen de *P. aeruginosa* es principalmente el ambiente; mas, su adaptación a los tejidos de los mamíferos puede seguir un modelo de evolución del tipo fuente-sumidero. El ambiente es la fuente de muchos linajes de esta bacteria, donde algunos de ellos son capaces de adaptarse al cuerpo humano. Algunos linajes pueden adaptarse a los humanos y pasar por una evolución reductiva en la que se pierden algunos genes. La comprensión de este proceso puede ser fundamental para implementar mejores métodos de control de brotes en los hospitales.

Palabras clave: bacteria, adaptación al hospedador, evolución, oportunista

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative aerobic rod bacterium, ubiquitous in the environment, and an opportunistic human pathogen [1]. It accounts for 9-19% prevalence of bacterial nosocomial infections and 7% of community-acquired pneumonia cases [2, 3]. *Pseudomonas aeruginosa* infections occur in the respiratory tract [4,5], eyes [6], ears, skin wounds [7], bloodstream [1], or surgical site infections [8]. *P. aeruginosa* can colonize human intestines and skin, and it can take advantage of

any host's immunodeficiency to produce acute or chronic systemic infections [4, 9]. Moreover, it is argued that *P. aeruginosa* infections are acquired from the bacterial population that colonizes the proximal environment of the host [10].

Pseudomonas aeruginosa prospers in soil, tap water, plants, intestinal contents, food, puddles, and swimming pools; it has some remarkable abilities to survive in the presence of heavy metals and chlorine [11]. *P. aeruginosa* lives surrounded by predatory amoeba, which may have selected bacterial cells with the ability to survive phagocytosis [12]. Living outside the host, *P. aeruginosa* adapts to changes (physical and chemical) and competes with other environmental microorganisms. This bacterium is a generalist and heterotrophic, possessing an arsenal of enzymes to oxidize many organic carbon sources, mostly decaying organic matter (and even xenobiotic compounds), to obtain energy [13–19].

One of the main problems associated with *P. aeruginosa* is the occurrence of nosocomial outbreaks where the source of the bacteria is unknown; many times, hospitals have resorted to extreme measures such as plumbing system replacements to stop *P. aeruginosa* dissemination [20, 21]. Also, multidrug resistance *P. aeruginosa* is one of the most critical concerns of hospital-acquired infections [8].

In this review, we describe the complexity of the evolutionary processes involved in the adaptation of environmental *P. aeruginosa* to human tissues. The sporadic infections by environmental strains and infections by strains adapted to humans are examined. We

will not address antibiotic resistance which is comprehensively covered by other manuscripts [22–24].

SPORADIC INFECTIONS BY ENVIRONMENTAL STRAINS

Pseudomonas aeruginosa infections with environmental strains involve the migration of bacteria from a heterogeneous ecosystem (environment) to a more homogeneous and restrictive ecosystem (human tissues). This movement is also known as a source- sink dynamic. The source is the environment to which the bacteria are adapted, and the sink is the human tissue that is often a harsher milieu. The bacterial growth rate in the sink may not compensate for the death rate; therefore, the bacterial population in the sink is maintained by a constant introduction of bacteria from the source (environment outside the host) [25].

The adaptation of *P. aeruginosa* to different environments implies multiple mechanisms of DNA modifications such as inheritance of mutations, homologous recombination, horizontal gene transfer (acquisition of accessory genome), and gene deletion [26, 27]. Environmental strains of *Pseudomonas* obtain nutrients from human tissues and neutralize immune responses using the same genes that are useful for dealing with environmental challenges [14, 28, 29]. For example, a type 3 secretion system (T3SS) is a translocation apparatus enabling the bacteria to export effector proteins from the bacterial cell to a eukaryotic cell without an extracellular step. Effector proteins can cause different consequences in the eukaryotic cell; exoenzyme (Exo) U is a phospholipase that induces cell death of predatory amoebas [28, 30] whereas ExoS, another T3SS-exported effector protein, is involved in anti-predatory responses against

free-living amoeba [31]. The same T3SS and its ExoU phospholipase also kill macrophages [32]; the effector protein, ExoS, has two domains that act in ADP-ribosylation and GTPase activities in host cell proteins [32] and also activates Toll-like receptors in phagocytes [33]. In the environment outside the host, stress caused by toxic levels of metal ions like copper, iron, and zinc induces the synthesis of pyoverdine, superoxide dismutase, fumarate hydratase, metal cation efflux transporter CzrA, ATP-binding cassette transporters (ABC transporters), copper resistance oxidase proteins, and others [34]. These molecules protect *P. aeruginosa* from oxidative insults and increase the availability of iron. In the host, the same molecules protect bacteria against oxidative reactions (from neutrophils or macrophages) and help to capture iron [35], as iron is sequestered in the host's proteins [36]. Iron associates and inactivates Fur-like transcriptional repressors, which downregulate many genes required to scavenge iron from animal tissues [37]. The range of niches in different environments outside of humans is reflected in vast *P. aeruginosa* diversity [38]; this diversity makes it challenging to identify the environmental source of clinical strains, and some researchers indicate that all environmental *P. aeruginosa* could cause human disease [39]. In the last years, whole-genome sequencing (WGS) provided irrefutable evidence of the environmental origin of clinical *P. aeruginosa* [26, 27, 40, 41]. Some environmental clones are genetically indistinguishable from clinical isolates [39]; recently, whole-genome sequence comparison of geographically related strains (belonging to ST-1146, Mallorca, Spain) obtained from the environment and a clinical case showed no genetic differences, although the clinical isolate had a mutation in the *oprD* gene (causing carbapenem resistance) [42]. Nevertheless, recent evidence suggests also that not all environmental *P. aeruginosa* strains may be able to cause

human infections; apparently some phenotypes are required to become human pathogens [43, 44]. Some evolutionary steps enabling *P. aeruginosa* strains to colonize human tissues may have occurred in the environment outside the host [27]; selective forces in niches outside the host may simulate some conditions in host tissues. For example, the abundance of environmental amoeba may select bacterial lineages able to survive macrophage attack. Nucleotide polymorphisms along the genome of clinical isolates (including epidemic and non-epidemic CF strains) have signs of positive selection (high rates of non-synonymous mutations, dN/ dS > 1) [26, 45]; among the genes under positive selection are those involved in LPS biosynthesis, flagella, secretion systems, iron scavenging, and iron uptake [26]. Analysis of *P. aeruginosa* genomes also shows that strains causing clinical infections have suffered higher levels of homologous recombination in genes similar to those showing signs of positive selection: membrane transport structures, biofilm formation, oxide/reduction, and other cell wall functions [27]. The recombination in the genome regions where positive selection is observed and the coincidence of recombinations in the same genes in many clinical isolates suggest that natural selection is affecting these strains.

INFECTIONS BY STRAINS ADAPTED TO HUMANS

Recent evidence suggests that human-to-human transmission of *P. aeruginosa* may be associated with the ability of these strains to colonize intestines; patients suffering from *P. aeruginosa* infections were colonized by the same clones [46]. However, some *P. aeruginosa* may become adapted to humans and transmitted from person to person as happens among pulmonary strains of cystic fibrosis patients [47]. Human-adapted *P. aeruginosa* strains have suffered similar evolutionary pressures as strains causing

sporadic infections; however, they have acquired additional adaptations during chronic infections, such as cystic fibrosis (CF) (Fig. 1). *Pseudomonas* strains, causing CF infections, tend to form a different group of *P. aeruginosa* populations from strains causing other types of infections in hospitals [27]. CF lung may select strains with additional aptitudes [26, 27] that may have been acquired in inhospitable niches outside the host before adaptation to human tissues [26, 27, 48, 49]. Some of these CF strains become epidemic strains (found in different continents and transferred from person to person) and show positive selection in core genome genes involved in oxidation/ reduction, membrane transport, and type III secretion systems, among others [26]. Going back to source-sink dynamics, if the sink conditions are not too harsh, the adaptation may occur especially if there is a constant migration rate [50] or in the presence of temporal less restrictive conditions [51]; chronic infections in CF patients may represent this. When an environmental generalist (such as *P. aeruginosa*) with a large genome (many genes required to thrive in an environment outside the host) [52–55] enters human tissue, a reductive evolution is prone to occur [18, 52–55]. In this scenario, evolution from environmental to pathogen involves gene loss due to small effective population size and lack of purifying selection; this gene loss is especially intense if these genes are unnecessary in the new niche (Fig. 1) [53, 56].

Longitudinal studies of cystic fibrosis *P. aeruginosa* strains have improved our understanding of evolutionary changes during chronic infections. Strains from these chronic infections show more profound evolutionary changes which probably occurred during infection of CF patients; paradoxically, some of these changes involve the loss of some apparent virulence factors. Evidence of reductive evolution may be apparent by the selection of amino acid auxotrophs in some strains [57, 58], loss of twitching

motility [44], or reduction of type IV pili [59]; chronic infections of cystic fibrosis lungs may select patho-adaptive phenotypes [60]. Lungs from cystic fibrosis patients go through oxidative stress by the accumulation of hemoglobin, ferrous iron, and transferrin [61]. Pyoverdine (a siderophore involved in iron scavenging in human tissue) no longer seems a critical factor to obtain iron which may select pyoverdine mutants in genes such as *pvdS* (a sigma factor involved in pyoverdine transcription) and the pyoverdine gene and PrrF (an RNA that reduces pyoverdine expression and increases expression of HemO) [60]. An alternative iron-acquisition mechanism may take over in these mutants, allowing iron to be taken from the heme molecules; these strains may require heme oxygenase (HemO), which breaks the heme and releases biliverdin, carbon dioxide, and iron [62].

Also, the long-term colonization of the lungs selects variants of unusual biofilm formation. In *P. aeruginosa*, biofilm-mucoid phenotypes show increased expression of the exopolysaccharide PsI and alginate. PsI induction occurs by at least six different mutations in operons which occur when the infection lingers for a long time [63]. These strains also show mutations in the *lasR* gene, a transcriptional regulator of biofilm formation and other virulence genes [64]. Other *P. aeruginosa* adaptations to human hosts include higher mutation rates [65, 66], increased synthesis of multidrug-efflux pumps, higher antibiotic resistance, mucoid phenotypes, increased biofilm formation, higher tendency to form micro-colonies in tissue [67, 68], upregulation of metalloproteinases, lipid A modifications, reduced fucosyltransferase 2 expression, and better adhesion to tissues [27]. These host- adapted phenotypes have not been found outside the human host even in the same environment where infected patients reside

[43, 44]. Some genotypes (LESA and ESB) could be found in CF patients on different continents [26, 27], suggesting human-to-human transmission. Evidence of human-to-human transmission has also been observed in some non-CF lineages (ST-111, ST-235, and ST-175) that caused infections in five hospitals in France during four years [47]—some of these were described in other countries [69]—and in the strain O12, a clone which caused a different type of non-CF infection in different countries during different years [39]. These observations may indicate that non-CF *P. aeruginosa* strains could also adapt to human tissues.

Additionally, adaptive evolution of bacteria to a new niche may involve antagonistic pleiotropy [70]; a mutation that is adaptive in the new environment could be detrimental in the original environment [71] and may account for many additional variations found in these strains. In vitro studies indicate that *P. aeruginosa* derived from CF patients are outcompeted by environmental *P. aeruginosa* [56, 72], which means that selective pressures during the host colonization make *P. aeruginosa* less capable of competing in the environment outside the host and that these strains are possibly transmitted from person to person [27].

CONCLUSIONS

The environment outside the host may contain a variety of ecosystems with characteristics that allow the selection of bacterial lineages to colonize human tissue and evade the immune attack. The environment outside the host is also an endless source of opportunistic pathogens, which can infect immunocompromised patients. Controlling and preventing *P. aeruginosa* infections in hospitals are very complex

endeavors that may require an understanding of *P. aeruginosa* population genetics of the strain causing the outbreak and its relationship with the environment. It is critical to recognize the physiological diversity of the different strains of *P. aeruginosa* causing infections. Current information suggests that the routes of transmission of the opportunistic pathogen *P. aeruginosa* are diverse: inanimate objects (such as plumbing and fixtures), human carriers (intestinal or respiratory tract), plants, animals, rivers, etc. It is imperative to use molecular tools to establish whether isolates obtained from different patients or during different timeframes are clonal; genomic information may allow exploring the presence of this pathogen in human carriers, inanimate objects, water sources, etc. Control and preventive measures should be diverse and in agreement with the strain's origin. Sometimes these measures may require the use of disinfectants or antibiotic treatment of carriers; in other cases, they may require the removal of plumbing or fixtures or other sources of organic matter or water sources.

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AUTHOR CONTRIBUTIONS

Both authors wrote and reviewed the manuscript.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

[1] Juan, C., Peña, C., & Oliver, A. (2017). Host and Pathogen Biomarkers for Severe *Pseudomonas aeruginosa* Infections. The Journal of Infectious Diseases, 215(suppl_1), S44-S51. doi: https://doi.org/10.1093/infdis/jiw299

[2] Ding, C., Yang, Z., Wang, J., Liu, X., Cao, Y., Pan, Y., Han, L., & Zhan, S. (2016). Prevalence of *Pseudomonas aeruginosa* and antimicrobial-resistant *Pseudomonas aeruginosa* in patients with pneumonia in mainland China: A systematic review and meta-analysis. International Journal of Infectious Diseases, 49, 119-128. doi: https://doi.org/10.1016/j. ijid.2016.06.014

[3] Ribeiro, Á. C. da S., Crozatti, M. T. L., Silva, A. A. da, Macedo, R. S., Machado, A. M. de O., & Silva, A. T. de A. (2020). *Pseudomonas aeruginosa* in the ICU: Prevalence, resistance profile, and antimicrobial consumption. Revista da Sociedade Brasileira de Medicina Tropical, 53, e20180498. doi: https://doi.org/10.1590/0037-8682-0498-2018

[4] Monsó, E. (2017). Microbiome in chronic obstructive pulmonary disease. Annals of Translational Medicine, 5(12), 251- 251. doi: https://doi.org/10.21037/atm.2017.04.20

[5] Rodrigo-Troyano, A., & Sibila, O. (2017). The respiratory threat posed by multidrug resistant Gram-negative bacteria: MDR-GNB in respiratory infections. Respirology, 22(7), 1288-1299. doi: https://doi.org/10.1111/resp.13115

[6] Teweldemedhin, M., Gebreyesus, H., Atsbaha, A. H., Asgedom, S. W., & Saravanan, M. (2017). Bacterial profile of ocular infections: A systematic review. BMC Ophthalmology, 17(1). doi: https://doi.org/10.1186/s12886-017-0612-2

[7] Roser, D. J., Van Den Akker, B., Boase, S., Haas, C. N., Ashbolt, N. J., & Rice, S. A. (2014). *Pseudomonas aeruginosa* dose response and bathing water infection. Epidemiology and Infection, 142(03), 449-462. doi: https://doi.org/10.1017/ S0950268813002690

[8] Moremi, N., Claus, H., Vogel, U., & Mshana, S. E. (2017). Surveillance of surgical site infections by *Pseudomonas aeruginosa* and strain characterization in Tanzanian hospitals does not provide proof for a role of hospital water plumbing systems in transmission. Antimicrobial Resistance & Infection Control, 6(1), 6-56. doi: https://doi.org/10.1186/ s13756-017-0216-x

[9] De Soyza, A., & Winstanley, C. (2018). *Pseudomonas aeruginosa* and Bronchiectasis. In J. Chalmers, E. Polverino, & S. Aliberti (Eds.), Bronchiectasis (pp. 157-180). Springer International Publishing. Cham. doi: https://doi. org/10.1007/978-3-319-61452-6_12

[10] Engelhart, S., Krizek, L., Glasmacher, A., Fischnaller, E., Marklein, G., & Exner, M. (2002). *Pseudomonas aeruginosa* outbreak in a haematology–oncology unit associated with contaminated surface cleaning equipment. Journal of Hospital Infection, 52(2), 93-98. doi: https://doi.org/10.1053/jhin.2002.1279

[11] English, E. L., Schutz, K. C., Willsey, G. G., & Wargo, M. J. (2018). Transcriptional Responses of *Pseudomonas aeruginosa* to Potable Water and Freshwater. Applied and Environmental Microbiology, 84(6). doi: https://doi.org/10.1128/ AEM.02350-17

[12] Hahn, M. W., & Höfle, M. G. (2001). Grazing of protozoa and its effect on populations of aquatic bacteria. FEMS Microbiology Ecology, 35(2), 113-121. doi: https://doi.org/10.1111/j.1574-6941.2001.tb00794.x [

13] Abalos, A., Viñas, M., Sabaté, J., Manresa, M. A., & Solanas, A. M. (2004). Enhanced Biodegradation of Casablanca Crude Oil by a Microbial Consortium in Presence of a Rhamnolipid Produced by *Pseudomonas aeruginosa* AT10. Biodegradation, 15(4), 249-260. doi: https://doi.org/10.1023/B:BIOD.0000042915.28757.fb

[14] Benie, C. K. D., Dadié, A., Guessennd, N., N'gbesso-Kouadio, N. A., Kouame, N. D., N'golo, D. C., Aka, S., Dako, E., Dje, K. M., & Dosso, M. (2017). Characterization of virulence potential of *Pseudomonas aeruginosa* isolated from bovine meat, fresh fish, and smoked fish. European Journal of Microbiology and Immunology, 7(1), 55-64. doi: https://doi. org/10.1556/1886.2016.00039

[15] Das, K., & Mukherjee, A. K. (2007). Crude petroleum-oil biodegradation efficiency of Bacillus subtilis and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East India. Bioresource Technology, 98(7), 1339-1345. doi: https://doi.org/10.1016/j.biortech.2006.05.032

[16] Gupta, B., Kunal, Rajor, A., & Kaur, J. (2018). Isolation, Characterisation of Novel *Pseudomonas* and Enterobacter sp. From Contaminated Soil of Chandigarh for Naphthalene Degradation. In S. K. Ghosh (Ed.), Utilization and Management of Bioresources (pp. 175-186). Springer Singapore. doi: https://doi.org/10.1007/978-981-10-5349-8_17 8.

[17] Kotresha, D., & Vidyasagar, G. M. (2008). Isolation and characterisation of phenol-degrading *Pseudomonas aeruginosa* MTCC 4996. World Journal of Microbiology and Biotechnology, 24(4), 541-547. doi: https://doi.org/10.1007/s11274-007-9508-2

[18] Tortell, P. D., Maldonado, M. T., & Price, N. M. (1996). The role of heterotrophic bacteria in iron-limited ocean ecosystems. Nature, 383(6598), 330-332. doi: https://doi.org/10.1038/383330a0

[19] Wei, Y.-H., Chou, C.-L., & Chang, J.-S. (2005). Rhamnolipid production by indigenous *Pseudomonas aeruginosa* J4 originating from petrochemical wastewater. Biochemical Engineering Journal, 27(2), 146-154. doi: https://doi.org/10.1016/j.bej.2005.08.028

[20] Bert, F., Maubec, E., Bruneau, B., Berry, P., & Lambert-Zechovsky, N. (1998). Multi-resistant *Pseudomonas aeruginosa* outbreak associated with contaminated tap water in a neurosurgery intensive care unit. Journal of Hospital Infection, 39(1), 53-62. doi: https://doi.org/10.1016/S0195-6701(98)90243-2

[21] Meyer, B. (2003). Approaches to prevention, removal and killing of biofilms. International Biodeterioration & Biodegradation, 51(4), 249-253. doi: https://doi.org/10.1016/S0964-8305(03)00047-7

[22] Chatterjee, M., Anju, C., Biswas, L., Anil Kumar, V., Gopi Mohan, C., & Biswas, R. (2016). Antibiotic resistance in *Pseudomonas aeruginosa* and alternative therapeutic options. International Journal of Medical Microbiology: IJMM, 306(1), 48-58. doi: https://doi.org/10.1016/j.ijmm.2015.11.004

[23] Ruiz-Garbajosa, P., & Cantón, R. (2017). Epidemiology of antibiotic resistance in *Pseudomonas aeruginosa*. Implications for empiric and definitive therapy. Revista Espanola De Quimioterapia, 30 Suppl 1, 8-12. doi: https.//doi. org/10.13039/100004325

[24] Subedi, D., Vijay, A. K., & Willcox, M. (2018). Overview of mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*: An ocular perspective: Mechanism of antimicrobial resistance in *P. aeruginosa*. Clinical and Experimental Optometry, 101(2), 162-171. doi: https://doi.org/10.1111/cxo.12621

[25] Sokurenko, E. V., Gomulkiewicz, R., & Dykhuizen, D. E. (2006). Source–sink dynamics of virulence evolution. Nature Reviews Microbiology, 4(7), 548-555. doi: https://doi.org/10.1038/nrmicro1446

[26] Dettman, J. R., Rodrigue, N., Aaron, S. D., & Kassen, R. (2013). Evolutionary genomics of epidemic and nonepidemic strains of *Pseudomonas aeruginosa*. Proceedings of the National Academy of Sciences, 110(52), 21065-21070. doi: https://doi.org/10.1073/pnas.1307862110

[27] Dettman, Jeremy R., Rodrigue, N., & Kassen, R. (2015). Genome-Wide Patterns of Recombination in the OpportunisticHuman Pathogen *Pseudomonas aeruginosa*. Genome Biology and Evolution, 7(1), 18-34. doi: https://doi.org/10.1093/gbe/evu260

[28] Pukatzki, S., Kessin, R. H., & Mekalanos, J. J. (2002). The human pathogen *Pseudomonas aeruginosa* utilizes conserved virulence pathways to infect the social amoeba Dictyostelium discoideum. Proceedings of the National Academy of Sciences of the United States of America, 99(5), 3159-3164. doi: https://doi.org/10.1073/pnas.052704399

[29] Wolfgang, M. C., Kulasekara, B. R., Liang, X., Boyd, D., Wu, K., Yang, Q., Miyada, C. G., & Lory, S. (2003). Conservation of genome content and virulence determinants among clinical and environmental isolates of *Pseudomonas aeruginosa*. Proceedings of the National Academy of Sciences, 100(14), 8484-8489. doi: https://doi.org/10.1073/pnas.0832438100

[30] Abd, H., Wretlind, B., Saeed, A., Idsund, E., Hultenby, K., & Sandstrã–M, G. (2008). *Pseudomonas aeruginosa* Utilises Its Type III Secretion System to Kill the Free-Living Amoeba Acanthamoeba castellanii. Journal of Eukaryotic Microbiology, 55(3), 235-243. doi: https://doi.org/10.1111/j.1550-7408.2008.00311.x

[31] Matz, C., Moreno, A. M., Alhede, M., Manefield, M., Hauser, A. R., Givskov, M., & Kjelleberg, S. (2008). *Pseudomonas aeruginosa* uses type III secretion system to kill biofilm-associated amoebae. The ISME Journal, 2(8), 843-852. doi: https://doi.org/10.1038/ismej.2008.47

[32] Hauser, A. R. (2009). The typ e III secretion system of *Pseudomonas aeruginosa*: Infection by injection. Nature Reviews Microbiology, 7(9), 654-665. doi: https://doi.org/10.1038/nrmicro2199

[33] Epelman, S., Stack, D., Bell, C., Wong, E., Neely, G. G., Krutzik, S., Miyake, K., Kubes, P., Zbytnuik, L. D., Ma, L. L., Xie, X., Woods, D. E., & Mody, C. H. (2004). Different Domains of *Pseudomonas aeruginosa* Exoenzyme S Activate Distinct TLRs. The Journal of Immunology, 173(3), 2031-2040. ttps://doi.org/10.4049/jimmunol.173.3.2031

[34] Teitzel, G. M., Geddie, A., De Long, S. K., Kirisits, M. J., Whiteley, M., & Parsek, M. R. (2006). Survival and Growth in the Presence of Elevated Copper: Transcriptional Profiling of Copper-Stressed *Pseudomonas aeruginosa*. Journal of Bacteriology, 188(20), 7242-7256. doi: https://doi.org/10.1128/JB.00837-06

[35] Schalk, I. J., & Guillon, L. (2013). Pyoverdine biosynthesis and secretion in *Pseudomonas aeruginosa*: Implications for metal homeostasis: Pyoverdine biosynthesis. Environmental Microbiology, 15(6), 1661-1673. doi: https://doi.org/10.1111/1462-2920.12013

[36] Meyer, J. M., Neely, A., Stintzi, A., Georges, C., & Holder, I. A. (1996). Pyoverdin is essential for virulence of *Pseudomonas aeruginosa*. Infection and Immunity, 64(2), 518-523. doi: https://doi.org/10.1128/iai.64.2.518-523.1996

[37] Touati, D. (2000). Iron and Oxidative Stress in Bacteria. Archives of Biochemistry and Biophysics, 373(1), 1-6. doi: https://doi.org/10.1006/abbi.1999.1518

[38] Silby, M. W., Winstanley, C., Godfrey, S. A. C., Levy, S. B., & Jackson, R. W. (2011). *Pseudomonas* genomes: Diverse and adaptable. FEMS Microbiology Reviews, 35(4), 652-680. doi: https://doi.org/10.1111/j.1574-6976.2011.00269.x

[39] Pirnay, J.-P., Bilocq, F., Pot, B., Cornelis, P., Zizi, M., Van Eldere, J., Deschaght, P., Vaneechoutte, M., Jennes, S., Pitt, T., & De Vos, D. (2009). *Pseudomonas aeruginosa* Population Structure Revisited. PLoS ONE, 4(11), e7740. doi: https://doi.org/10.1371/journal.pone.0007740

[40] Parcell, B. J., Oravcova, K., Pinheiro, M., Holden, M. T. G., Phillips, G., Turton, J. F., & Gillespie, S. H. (2018). *Pseudomonas aeruginosa* intensive care unit outbreak: Winnowing of transmissions with molecular and genomic typing. Journal of Hospital Infection, 98(3), 282-288. doi: https://doi.org/10.1016/j.jhin.2017.12.005

[41] Quick, J., Cumley, N., Wearn, C. M., Niebel, M., Constantinidou, C., Thomas, C. M., Pallen, M. J., Moiemen, N. S., Bamford, A., Oppenheim, B., & Loman, N. J. (2014). Seeking the source of *Pseudomonas aeruginosa* infections in a

recently opened hospital: An observational study using whole-genome sequencing. BMJ Open, 4(11), e006278. doi: https://doi.org/10.1136/bmjopen-2014-006278

[42] Sánchez, D., Gomila, M., Bennasar, A., Lalucat, J., & García-Valdés, E. (2014). Genome Analysis of Environmental and Clinical *Pseudomonas aeruginosa* Isolates from Sequence Type-1146. PLoS ONE, 9(10), e107754. doi: https://doi.org/10.1371/journal.pone.0107754

[43] Lucchetti-Miganeh, C., Redelberger, D., Chambonnier, G., Rechenmann, F., Elsen, S., Bordi, C., Jeannot, K., Attrée, I., Plésiat, P., & de Bentzmann, S. (2014). *Pseudomonas aeruginosa* Genome Evolution in Patients and under the Hospital Environment. Pathogens, 3(2), 309-340. doi: https://doi.org/10.3390/pathogens3020309

[44] Mahenthiralingam, E., Campbell, M. E., & Speert, D. P. (1994). Nonmotility and phagocytic resistance of *Pseudomonas aeruginosa* isolates from chronically colonized patients with cystic fibrosis. Infection and Immunity, 62(2), 596-605.

[45] Diaz Caballero, J., Clark, S. T., Coburn, B., Zhang, Y., Wang, P. W., Donaldson, S. L., Tullis, D. E., Yau, Y. C. W., Waters, V. J., Hwang, D. M., & Guttman, D. S. (2015). Selective Sweeps and Parallel Pathoadaptation Drive *Pseudomonas aeruginosa* Evolution in the Cystic Fibrosis Lung. MBio, 6(5). e00981-15 doi: https://doi.org/10.1128/mBio.00981-15

[46] Tamburini, F. B., Andermann, T. M., Tkachenko, E., Senchyna, F., Banaei, N., & Bhatt, A. S. (2018). Precision identification of diverse bloodstream pathogens in the gut microbiome. Nature Medicine, 24(12), 1809-1814. doi: https://doi.org/10.1038/s41591-018-0202-8

[47] Cholley, P., Thouverez, M., Hocquet, D., van der Mee-Marquet, N., Talon, D., & Bertrand, X. (2011). Most Multidrug-Resistant *Pseudomonas aeruginosa* Isolates from Hospitals in Eastern France Belong to a Few Clonal Types. Journal of Clinical Microbiology, 49(7), 2578-2583. doi: https://doi.org/10.1128/JCM.00102-11

[48] Bhagirath, A. Y., Li, Y., Somayajula, D., Dadashi, M., Badr, S., & Duan, K. (2016). Cystic fibrosis lung environment and *Pseudomonas aeruginosa* infection. BMC Pulmonary Medicine, 16(1). doi: https://doi.org/10.1186/s12890-016-0339-5

[49] Williams, B. J., Dehnbostel, J., & Blackwell, T. S. (2010). *Pseudomonas aeruginosa*: Host defense in lung diseases. Respirology, 15(7), 1037-1056. doi: https://doi.org/10.1111/j.1440-1843.2010.01819.x

[50] Perron, G. G., Gonzalez, A., & Buckling, A. (2007). Source-sink dynamics shape the evolution of antibiotic resistance and its pleiotropic fitness cost. Proceedings of the Royal Society B: Biological Sciences, 274(1623), 2351-2356. doi: https://doi.org/10.1098/rspb.2007.0640

[51] Holt, R. D., Barfield, M., & Gomulkiewicz, R. (2004). Temporal Variation Can Facilitate Niche Evolution in Harsh Sink Environments. The American Naturalist, 164(2), 187-200. doi: https://doi.org/10.1086/422343 10 *Pseudomonas aeruginosa*transition from environmental generalist to human pathogenVol. 13, nro. 1 ID: 2225

[52] Kassen, R. (2002). The experimental evolution of specialists, generalists, and the maintenance of diversity: Experimental evolution in variable environments. Journal of Evolutionary Biology, 15(2), 173-190. doi: https://doi.org/10.1046/j.1420-9101.2002.00377.x

[53] Juárez-Vázquez, A. L., Edirisinghe, J. N., Verduzco-Castro, E. A., Michalska, K., Wu, C., Noda-García, L., Babnigg, G., Endres, M., Medina-Ruíz, S., Santoyo-Flores, J., Carrillo-Tripp, M., Ton-That, H., Joachimiak, A., Henry, C. S., & Barona-Gómez, F. (2017). Evolution of substrate specificity in a retained enzyme driven by gene loss. eLife, 6. doi: https://doi.org/10.7554/eLife.22679

[54] Zhang, X., Liu, X., Liang, Y., Guo, X., Xiao, Y., Ma, L., Miao, B., Liu, H., Peng, D., Huang, W., Zhang, Y., & Yin, H. (2017). Adaptive Evolution of Extreme Acidophile Sulfobacillus thermosulfidooxidans Potentially Driven by Horizontal Gene Transfer and Gene Loss. Applied and Environmental Microbiology, 83(7). e03098-16. doi: https://doi.org/10.1128/AEM.03098-16

[55] Moran, N. A. (2002). Microbial minimalism: Genome reduction in bacterial pathogens. Cell, 108(5), 583-586. doi: https://doi.org/10.1016/S0092-8674(02)00665-7

[56] Andersen, S. B., Ghoul, M., Griffin, A. S., Petersen, B., Johansen, H. K., & Molin, S. (2017). Diversity, Prevalence, and Longitudinal Occurrence of Type II Toxin-Antitoxin Systems of *Pseudomonas aeruginosa* Infecting Cystic Fibrosis Lungs. Frontiers in Microbiology, 8, 1108 doi: https://doi.org/10.3389/fmicb.2017.01180

[57] Barth, A. L., & Pitt, T. L. (1996). The high amino-acid content of sputum from cystic fibrosis patients promotes growth of auxotrophic *Pseudomonas aeruginosa*. Journal of Medical Microbiology, 45(2), 110-119. doi: https://doi.org/10.1099/00222615-45-2-110

[58] Thomas, S. R. (2000). Increased sputum amino acid concentrations and auxotrophy of *Pseudomonas aeruginosa* in severe cystic fibrosis lung disease. Thorax, 55(9), 795-797. doi: https://doi.org/10.1136/thorax.55.9.795

[59] Huse, H. K., Kwon, T., Zlosnik, J. E. A., Speert, D. P., Marcotte, E. M., & Whiteley, M. (2010). Parallel Evolution in *Pseudomonas aeruginosa* over 39,000 Generations In Vivo. MBio, 1(4). doi: https://doi.org/10.1128/mBio.00199-10

[60] Nguyen, A. T., O'Neill, M. J., Watts, A. M., Robson, C. L., Lamont, I. L., Wilks, A., & Oglesby-Sherrouse, A. G. (2014). Adaptation of Iron Homeostasis Pathways by a *Pseudomonas aeruginosa* Pyoverdine Mutant in the Cystic Fibrosis Lung. Journal of Bacteriology, 196(12), 2265-2276. doi: https://doi.org/10.1128/JB.01491-14

[61] Ghio, A. J., Roggli, V. L., Soukup, J. M., Richards, J. H., Randell, S. H., & Muhlebach, M. S. (2013). Iron accumulates in the lavage and explanted lungs of cystic fibrosis patients. Journal of Cystic Fibrosis, 12(4), 390-398. doi: https://doi.org/10.1016/j.jcf.2012.10.010

[62] Wegiel, B., Nemeth, Z., Correa-Costa, M., Bulmer, A. C., & Otterbein, L. E. (2014). Heme Oxygenase-1: A Metabolic Nike. Antioxidants & Redox Signaling, 20(11), 1709-1722. doi: https://doi.org/10.1089/ars.2013.5667

[63] Huse, H. K., Kwon, T., Zlosnik, J. E. A., Speert, D. P., Marcotte, E. M., & Whiteley, M. (2013). *Pseudomonas aeruginosa* Enhances Production of a Non-Alginate Exopolysaccharide during Long-Term Colonization of the Cystic Fibrosis Lung. PLoS ONE, 8(12), e82621. doi: https://doi.org/10.1371/journal.pone.0082621

[64] Smith, E. E., Buckley, D. G., Wu, Z., Saenphimmachak, C., Hoffman, L. R., D'Argenio, D. A., Miller, S. I., Ramsey, B. W., Speert, D. P., Moskowitz, S. M., Burns, J. L., Kaul, R., & Olson, M. V. (2006). Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proceedings of the National Academy of Sciences, 103(22), 8487-8492. doi: https://doi.org/10.1073/pnas.0602138103

[65] Oliver, A., Cantón, R., Campo, P., Baquero, F., & Blázquez, J. (2000). High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. Science, 288(5469), 1251-1254. doi: https://doi.org/10.1126/science.288.5469.1251

[66] Mena, A., Smith, E. E., Burns, J. L., Speert, D. P., Moskowitz, S. M., Perez, J. L., & Oliver, A. (2008). Genetic Adaptation of *Pseudomonas aeruginosa* to the Airways of Cystic Fibrosis Patients Is Catalyzed by Hypermutation. Journal of Bacteriology, 190(24), 7910-7917. doi: https://doi.org/10.1128/JB.01147-08

[67] Lam, J., Chan, R., Lam, K., & Costerton, J. W. (1980). Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. Infection and Immunity, 28(2), 546-556. doi: https://doi.org/10.1128/iai.28.2.546-556.1980

[68] Sriramulu, D. D. (2005). Microcolony formation: A novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung. Journal of Medical Microbiology, 54(7), 667-676. doi: https://doi.org/10.1099/jmm.0.45969-0

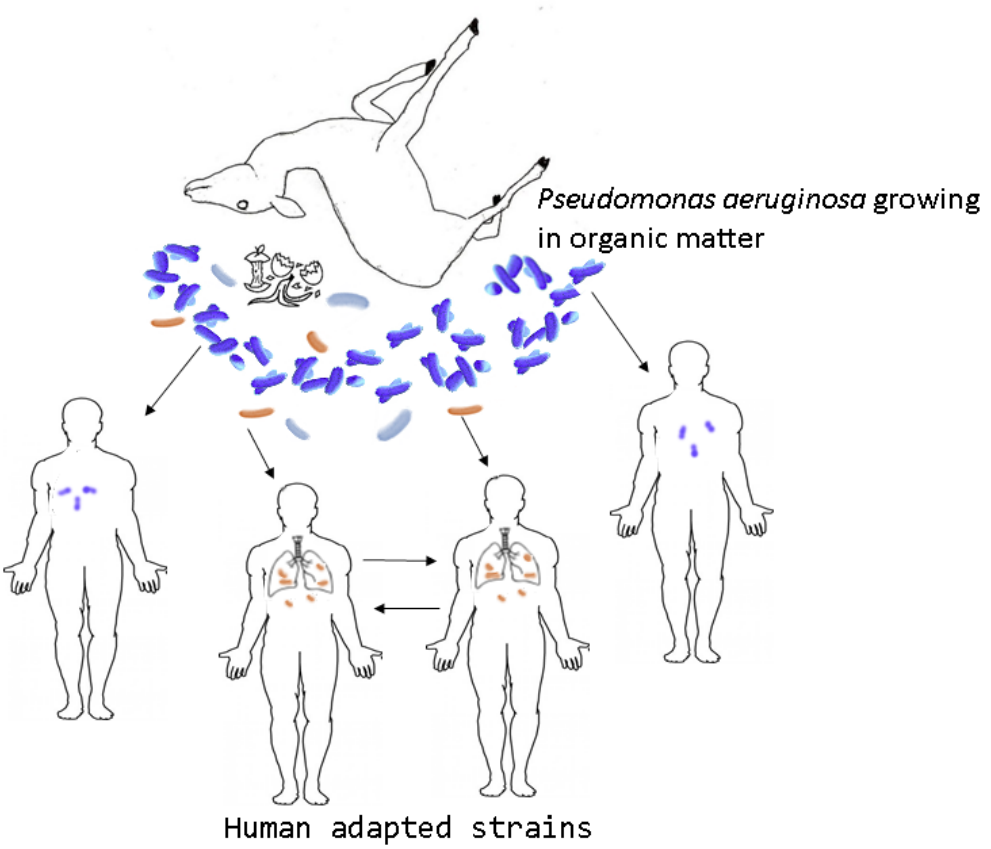
[69] Treepong, P., Kos, V. N., Guyeux, C., Blanc, D. S., Bertrand, X., Valot, B., & Hocquet, D. (2018). Global emergence of the widespread *Pseudomonas aeruginosa* ST235 clone. Clinical Microbiology and Infection, 24(3), 258-266. doi: https://doi.org/10.1016/j.cmi.2017.06.018

[70] Yang, L., Jelsbak, L., Marvig, R. L., Damkiaer, S., Workman, C. T., Rau, M. H., Hansen, S. K., Folkesson, A., Johansen, H. K., Ciofu, O., Hoiby, N., Sommer, M. O. A., & Molin, S. (2011). Evolutionary dynamics of bacteria in a human host environment. Proceedings of the National Academy of Sciences, 108(18), 7481-7486. doi: https://doi.org/10.1073/pnas.1018249108

[71] Kawecki, T. J., & Ebert, D. (2004). Conceptual issues in local adaptation. Ecology Letters, 7(12), 1225-1241. doi: https://doi.org/10.1111/j.1461-0248.2004.00684.x[72] Chatterjee, P., Davis, E., Yu, F., James, S., Wildschutte, J. H., Wiegmann, D. D., Sherman, D. H., McKay, R. M., LiPuma,J. J., & Wildschutte, H. (2017). Environmental Pseudomonads Inhibit Cystic Fibrosis Patient-Derived *Pseudomonas aeruginosa*.

Applied and Environmental Microbiology, 83(2). doi: https://doi.org/10.1128/AEM.02701-16

Figure 1. Diverse *Pseudomonas aeruginosa* strains (blue bacteria) thrive in the environment degrading organic matter. Some strains have improved aptitude to grow in human tissue (brown bacteria); a subset of them have evolved additional adaptations and can be transmitted from human to human.



CHAPTER III

Endogenous origin of *Pseudomonas aeruginosa* infecting hospitalized patients in Ecuador

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Whole genome sequencing

Summary

Recent evidence suggests that *Pseudomonas aeruginosa*, a bacterium that causes deadly infections in hospitalized patients, could originate in the patient's intestine. We employed the Oxford Nanopore platform to obtain whole genome sequences (WGS) from clinical and rectal strains belonging to 15 patients from two hospitals. Our study found evidence that clinical and rectal isolates were clonal, with some evidence suggesting that the infecting strain was present in the intestine at the time of admission, ruling out hospital acquisition. The use of WGS analysis is crucial to detect alternative sources of *P. aeruginosa* to develop new preventive measures against these serious infections.

1. Introduction

Pseudomonas aeruginosa is a priority pathogen by the World Health Organization [1] as part of the ESKAPE pathogens by the Infectious Diseases Society of America, due to their role in nosocomial infections and resistance to last-resort antimicrobials [2]. It is generally accepted that *P. aeruginosa* infections are primarily contracted within hospitals through contact with inanimate objects, such as fixtures and wet surfaces [3]. However, many studies have revealed that in some cases, there is no clonal relationship between the infecting strains in the same hospital, even in infections from temporally related patients [4]. Other studies show that strains causing infections are constantly changing over time within the same hospital [5]. Moreover, recent research suggests that these infections may originate from *P. aeruginosa* colonizing the patient’s intestine before the disease [6].

Enhanced genotyping methods are necessary to reliably identify the sources of *P. aeruginosa*. In this study, we employed phylogenetic analysis of whole genome sequences (WGS), core genome Single Nucleotide Polymorphisms (SNPs), and Average Nucleotide Identity (ANI) to investigate the relationship between intestinal and clinical *P. aeruginosa* isolates from 15 hospitalized patients in Quito.

2. Materials and methods

For one year, we recruited adult patients suffering from *P. aeruginosa* infection at two general hospitals (Hospital General Docente de Calderón-Hospital_1C and Hospital Padre Carollo Un Canto Para la Vida-Hospital_2P) in Quito City, Ecuador. All participants provided informed consent approved by the Comité de Ética de Investigación en Seres Humanos de la Universidad San Francisco de Quito USFQ (CEISH-USFQ). We recruited the patients after the *P. aeruginosa* infection was confirmed by the hospital's clinical laboratory, except for 2 patients for whom faecal samples were obtained (and submitted to culture) at the time of admission as part of the clinical procedures. Clinical samples were obtained from the hospital's clinical laboratory, and rectal swab samples were collected from each patient using Stuart transport media (BD). Samples were streaked on Cetrimide agar plates (Difco, BD) to obtain up to 5 green-blue colonies, and after biochemical testing, ultimately confirming *P. aeruginosa* isolates.

We extracted DNA from samples using the Wizard® Genomic DNA Purification Kit. For some isolates, DNA quality did not meet the sequencing requirements, and we used the Qiagen DNeasy Blood & Tissue Kit columns (both DNA extraction kits included RNAse treatment). To reduce sequencing costs, we used nucleotide sequences of two genes to

detect possible clonal strains; we PCR amplified, and Sanger sequenced (Macrogene, South Korea) the *acsA* and *aroE* genes according to PubMLST.org *P. aeruginosa* protocols of all the isolates. Isolates with identical *acsA* and *aroE* sequences from the same patient were selected for whole-genome sequencing.

To obtain draft bacterial genomes, we used Oxford Nanopore long-read sequencing. The library preparation was performed using the Rapid Barcoding Kit 96 SQK-RBK110.96, following the manufacturer's protocol. Whole Genome Sequencing was conducted using R9.4.1 flow cells (FLO-MIN106) on a GridION Mk1 platform from Oxford Nanopore Technologies.

We obtained the WGS assemblies of all the isolates and used them for the phylogenetic analysis. We built a WGS tree, a core SNPs tree, an SNPs matrix, and a whole genome Average Nucleotide Identity (ANI) matrix. We also obtained the sequence types (STs) from the *P. aeruginosa* website on PubMLST.org. Bioinformatic analysis was conducted using the programs and workflows summarized in Table S1. We used the AE004091.2 *P. aeruginosa* PAO1 complete genome reference GenBank accession GCA_000006765.1 (size 6,26 Mbps) as the reference genome. For quality purposes, one isolate (Rectal_P2) underwent duplicate sequencing.

We obtained the phenotypic resistances of the strains using the Kirby-Bauer method, according to Clinical and Laboratory Standards Institute M-100 2022 standards. We tested the β-lactams cefepime, ceftazidime, imipenem, meropenem, and piperacillin-tazobactam, the fluoroquinolones ciprofloxacin and levofloxacin, and the

aminoglycosides amikacin and gentamicin. We used the genomic assemblies of the isolates to perform an analysis of the presence of antibiotic-resistance genes or markers according to three genotype resistance databases (Table S1).

Statistical analysis was performed in JASP software version 0.16.4.0.

3. Results

We enrolled 40 patients during the study between November 2021 and October 2022. We obtained rectal samples from 22 patients and found the presence of *P. aeruginosa* in 16 of them. We obtained genomic information from isolates (n = 52) from 15 patients, of which 21 were clinical and 31 were rectal isolates (Table 1, S2 metadata).

The alignment information for the phylogenetic tree construction covered 5.29 million base pairs (Mbps), which represents 77.9% of the *P. aeruginosa* reference strain genome size (range from 5.5 to 7 Mbps). The phylogenetic analysis of draft genomes showed clustering of isolates (clinical and rectal) from each patient (Figure 1). However, 12 isolates from 5 patients (P3, P9, and P12 from Hospital_1C and P2, and P11 from Hospital_2P) showed genetic similarity (WGS phylogeny, core SNPs, and ANI, Figures 1, S1, and S2) and belonged to the clone ST-253. These patients were not hospitalized during the same period, however, their persistence and abundance among the strains may be compatible with transmission within the hospital (Figure S3).

The isolates within the same clade (clinical and faecal isolates from the same patient) had an SNPs average difference of 0.053% (ranging from 0.0077% to 0.1%). The average

difference across all genomes was 0.71%, thirteen times higher than the clade SNPs differences. Additionally, the 6.02 Mbps alignment of the two sequences from the same isolate (quality control) showed 0.0058% SNPs of difference, comparable to those observed in isolates (faecal and clinical) from the same patient (clade) (Figure S4).

Due to bioethics considerations, we obtained the rectal samples, for most cases, after the *P. aeruginosa* infection. However, in patients P7 and P15, we obtained the rectal samples on the first day (due to high-risk assessment hospital policies at admission). In P7, the clinical isolate was obtained six days after hospital admission (Figure 1, double asterisks**). There were *P. aeruginosa* infection recurrences in two cases: patients P1 and P12; in both cases, the isolates from the recurrent infection were genetically related to the other isolated from the same patient (Figure 1, asterisks*).

The clinical and rectal isolates from the same patient had comparable phenotypic resistances within them in most cases (Figure 1, circles). We utilized three genotypic resistance analysis databases; each detected or lost some genotypic resistances (Table S3). Our findings showed that $bla_{\text{PDC-34}}$ and $bla_{\text{OXA-488}}$ were associated with resistance to cefepime, imipenem, meropenem, piperacillin-tazobactam, and ST-253 strains (Fisher's exact tests $p < 0.05$). Additionally, $bla_{\text{VIM-2}}$ was associated with ceftazidime, imipenem, meropenem, piperacillin-tazobactam resistances, and ST-389 strains (Fisher's exact tests $p < 0.05$). We found a significant association of *gyrA*_T83I mutation with fluoroquinolone's ciprofloxacin and levofloxacin resistances. Lastly, for aminoglycosides amikacin and gentamicin resistances, we found an association with genes *aac*(6')-II, *ant*(2")-Ia, and *aph*(3')-VIa, and for gentamicin only, the *aadA6* gene. Other genes were

statistically associated with phenotypic resistance to different antibiotic classes, including the *sul1*, *qacEΔ1*, ArmM-MexR, and *OprM* efflux pump encoding genes (Table S4).

4. Discussion

We present genetic evidence through a comprehensive approach involving WGS (>5 Mbps), core SNPs, and ANI that intestinal and clinical isolates from the same patient have a clonal relationship. Moreover, in two patients, *P. aeruginosa*-positive rectal samples were obtained on the same day of hospital admission, which rules out the possibility of nosocomial transmission. These results suggest that the patient's gastrointestinal tract was the source of many *P. aeruginosa* infections. Previous studies have found that intestinal carriage of *P. aeruginosa* increases the risk of intensive care infection by this bacterium [6, 7], and in most cases, the intestinal and the clinical strains share the same PFGE genotype [6].

However, we also found evidence that ST-253 strains may be transmitted within the hospital, even when these cases were not temporally related. We hypothesize that a hospital facility or intestinal colonization (in patients or personnel) may be the source of these strains.

In most cases, the phenotypic resistance was the same between faecal and clinical isolates (Figure 1). Also, genomic data revealed that antimicrobial resistance genes were not always associated with phenotypic resistance to the respective antimicrobial and

vice versa. These discrepancies may be related to mutations that affect the resistance genes or other epistatic events that cause resistance [8].

Some studies of environmental, faecal, and clinical *P. aeruginosa* isolates in hospitals have used single-gene sequencing, Multilocus Sequence Typing (MLST) with seven genes, and Pulsed Field Gel Electrophoresis (PFGE), which analysed around 30 restriction sites. These techniques produce reliable results only when used to find clonal relationships in *P. aeruginosa* isolates from outbreaks within the same hospital but show spurious associations when used to study clones from different timelines or between hospitals [9].

Even though we present an analysis of draft genomes (as opposed to closed genomes), the accuracy of our sequences was assessed by sequencing twice the same strain (Rectal_P2), and the results were within the parameters described previously [10]. Another limitation of our study was the low number of isolates. Nevertheless, our results provide strong evidence of the endogenous nature of these *P. aeruginosa* infections. Our study underlines the need for investigations with a larger number of patients and using WGS to determine the prevalence of endogenous *P. aeruginosa* transmission. High frequency of endogenous infections may warrant profound changes in *P. aeruginosa* prevention protocols, such as faecal-carriage screening for patients upon hospital admission and strain-specific decolonization, which could include bacteriophage therapy for individuals identified as high-risk cases. The origin of *P. aeruginosa* acquired outside the hospitals remains unknown. Human-to-human transmission, particularly of high-risk clones, and environmental sources need to be

addressed in future studies. Finally, this study shows the utility of Oxford Nanopore (a low-cost sequencing technology) to study phylogenetic relations between bacterial isolates in countries with limited resources.

Conflict of interest statement

All authors have no conflicts of interest to declare.

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Credit Author Statement

Gabriela Vasco: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Writing-Reviewing, and Editing. Mishell Achig, Belén Prado-Vivar, Maritza Páez, Franklin Espinosa and, Evelyn Espinoza: Investigation, Resources. Danny Quinancela: Investigation, Data Curation. Paul Cardenas: Validation, Supervision. Gabriel Trueba: Conceptualization, Supervision, Visualization, Writing-Reviewing, and Editing.

Ethics approval and consent to participate

The study was approved by the Comité de Ética de Investigación en Seres Humanos de la Universidad San Francisco de Quito USFQ (CEISH-USFQ) reference 2021-090M.

Data availability

Whole genome sequences from this study are available in the NCBI repository under BioProject PRJNA946810.

References

[1] World Health Organization. Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug-resistant bacterial infections, including tuberculosis. Geneva: 2017.

[2] Rice LB. Federal Funding for the Study of Antimicrobial Resistance in Nosocomial Pathogens: No ESKAPE. J Infect Dis 2008;197:1079–81. https://doi.org/10.1086/533452.

[3] Juan C, Peña C, Oliver A. Host and Pathogen Biomarkers for Severe *Pseudomonas aeruginosa* Infections. J Infect Dis 2017;215:S44–51. https://doi.org/10.1093/infdis/jiw299.

[4] Berthelot P, Grattard F, Mahul P, Pain P, Jospé R, Venet C, et al. Prospective study of nosocomial colonization and infection due to *Pseudomonas aeruginosa* in mechanically ventilated patients. Intensive Care Med 2001;27:503–12. https://doi.org/10.1007/s001340100870.

[5] Bonten MJM, Bergmans DCJJ, Speijer H, Stobberingh EE. Characteristics of Polyclonal Endemicity of *Pseudomonas aeruginosa* Colonization in Intensive Care Units. Am J Respir Crit Care Med 1999;160:1212–9. https://doi.org/10.1164/ajrccm.160.4.9809031.

[6] Gómez-Zorrilla S, Camoez M, Tubau F, Cañizares R, Periche E, Angeles Dominguez M, et al. Prospective Observational Study of Prior Rectal Colonization Status as a Predictor for Subsequent Development of *Pseudomonas aeruginosa* Clinical Infections 2015. https://doi.org/10.1128/AAC.04636-14.

[7] Venier A-G, Leroyer C, Slekovec C, Talon D, Bertrand X, Parer S, et al. Risk factors for *Pseudomonas aeruginosa* acquisition in intensive care units: a prospective multicentre study. Journal of Hospital Infection 2014;88:103–8. https://doi.org/10.1016/j.jhin.2014.06.018.

[8] López-Causapé C, Oliver A. Insights into the evolution of the mutational resistome of *Pseudomonas aeruginosa* in cystic fibrosis. Future Microbiol 2017;12:1445–8. https://doi.org/10.2217/fmb-2017-0197.

[9] Quainoo S, Coolen JPM, van Hijum SAFT, Huynen MA, Melchers WJG, van Schaik W, et al. Whole-Genome Sequencing of Bacterial Pathogens: the Future of Nosocomial Outbreak Analysis. Clin Microbiol Rev 2017;30:1015–63. https://doi.org/10.1128/CMR.00016-17.

[10] Klockgether J, Cramer N, Wiehlmann L, Davenport CF, Tümmler B. *Pseudomonas aeruginosa* Genomic Structure and Diversity. Front Microbiol 2011;2. https://doi.org/10.3389/fmicb.2011.00150.

Table 1. Information on patients, origin and type of infection, and discrepancies between clinical and rectal samples.

Patient	Hospital	Isolate ST	Sample origin	Infection Environment	Days staying in a previous hospital	Days between admission to sample collection	Days between clinical and rectal samples
P1	2P	274	Abdominal wound	Hospital	1	10	4
		274	Rectal			14	
		274	Abdominal discharge	Hospital		21*	2
		274	Rectal			23*	
P2	2P	253	Tracheal discharge	Hospital	29	1	11
		253	Rectal			11	
P3	1C	253	Sputum	Hospital	-	55	3
		253	Rectal			58	
P4	1C	n1	Sputum	Hospital (?)	-	25	0
		n1	Rectal			25	
P5	1C	n2	Tracheostome secretion	Hospital (?)	-	6	3
		n2	Rectal			9	
P6	1C	17	Blood	Hospital (?)	-	12	3
		17	Rectal			15	
P7	1C	389	Wound	Community	-	6	-6
		389	Rectal			0**	
P8	2P	n3	Tracheal discharge	Community	-	1	3
		n3	Rectal			4	
P9	1C	253	Tracheal discharge	Hospital	-	17	4
		253	Rectal			21	
P10	1C	n4	Urine	Hospital (?)	-	18	3
		n4	Rectal			21	
P11	2P	253	Blood	Hospital	20	13	1
		253	Rectal			14	
P12	1C	309	Wound	Hospital (?)	-	20	
		253	Wound	Hospital	-	53	17
		253	Urine	Hospital	-	70*	0
		253	Rectal			70	
P13	1C	n5	Acetabulum	Hospital (?)	-	64	0
		n5	Femur	Hospital (?)	-	64	
		n5	Wound	Hospital (?)	-	64	
		n5	Rectal			64	
P14	1C	3142	Urine	Community	-	0	1
		3142	Wound	Community	-	1	
		3142	Rectal			1	
P15	1C	2433	Wound	Community	-	0	0
		2433	Rectal			0**	

"n" represents a new ST, not listed in PubMLST. Single asterisks represent recurrence isolates; double asterisks represent rectal samples obtained on admission to the hospital. Dash indicates no previous hospitalization. Question marks indicate the most likely environment.

Table S1. Bioinformatic tools employed.

Analysis	Tools	Options	References
Basecalling	MinKNOW software version 22.08.9	High-accuracy model	Wang Y, Zhao Y, Bollas A, Wang Y, Au KF. Nanopore sequencing technology, bioinformatics and applications. Nat Biotechnol 2021;39:1348–65. https://doi.org/10.1038/s41587-021-01108-x.
Assembly, polishing, and annotation	EPI2MElabs software version 4.1.1. The workflow includes the software Pysam 0.20.0, Fastcat 0.7.0, Medaka 1.7.2, Mosdepth 0.3.3, Flye 2.9.1-b1780, Pomoxis 0.3.11, and dna_features_viewer 3.1.2	wf-bacterial genomes, Nextflow workflow version 0.2.11, denovo assembly	Ewels PA, Peltzer A, Fillinger S, Patel H, Alneberg J, Wilm A, et al. The nf-core framework for community-curated bioinformatics pipelines. Nat Biotechnol 2020;38:276–8. https://doi.org/10.1038/s41587-020-0439-x. Kolmogorov M, Bickhart DM, Behsaz B, Gurevich A, Rayko M, Shin SB, et al. metaFlye: scalable long-read metagenome assembly using repeat graphs. Nat Methods 2020;17:1103–10. https://doi.org/10.1038/s41592-020-00971-x. Oxford Nanopore Technologies. Medaka. Medaka 2023. https://github.com/nanoporetech/medaka (accessed February 25, 2023). Oxford Nanopore Technologies. Pomoxis- bioinformatics tools for nanopore research 2023. Pysam-developers. Pysam 2023. Zulkower V, Rosser S. DNA Features Viewer: a sequence annotation formatting and plotting library for Python. Bioinformatics 2020;36:4350–2. https://doi.org/10.1093/bioinformatics/btaa213.
Alignment	Parsnp Rapid core genome multi-alignment version 1.7.2 by Harvest Tools	Default	Treangen TJ, Ondov BD, Koren S, Phillippy AM. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol 2014;15:524. https://doi.org/10.1186/s13059-014-0524-x.
Phylogenetic tree	IQTREE software version 2.1.4-beta COVID-edition for Linux 64-bit	Maximum likelihood, ModelFinder option, transition model GTR+F+R2 and, bootstrap 1000	Chernomor O, von Haeseler A, Minh BQ. Terrace Aware Data Structure for Phylogenomic Inference from Supermatrices. Syst Biol 2016;65:997–1008. https://doi.org/10.1093/sysbio/syw037. Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat Methods 2017;14:587–9. https://doi.org/10.1038/nmeth.4285. Soubrier J, Steel M, Lee MSY, Der Sarkissian C, Guindon S, Ho SYW, et al. The Influence of Rate Heterogeneity among Sites on the Time Dependence of Molecular Rates. Mol Biol Evol 2012;29:3345–58. https://doi.org/10.1093/molbev/mss140. Yang Z. A space-time process model for the evolution of DNA sequences. Genetics 1995;139:993–1005. https://doi.org/10.1093/genetics/139.2.993.
Tree annotations	Tree of Life iTOL annotation editor v1.6		Letunic I, Bork P. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res 2021;49:W293–6. https://doi.org/10.1093/nar/gkab301.
Multi-locus sequence typing	PubMLST.org *Pseudomonas aeruginosa*	WGS fasta files	Jolley KA, Bray JE, Maiden MCJ. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Wellcome Open Res 2018;3:124. https://doi.org/10.12688/wellcomeopenres.14826.1.
Core SNPs tree	kSNP version 4.1 software	-ML, Jukes-Cantor, Generalized Time-Reversible, k 25, and core SNPs	Gardner SN, Slezak T, Hall BG. kSNP3.0: SNP detection and phylogenetic analysis of genomes without genome alignment or reference genome. Bioinformatics 2015;31:2877–8. https://doi.org/10.1093/bioinformatics/btv271.
SNPs matrix	snpdists GLP version 3 software	Default	Seemann T. Source code for snp-dists software (0.6.2). 2018. https://doi.org/https://doi.org/10.5281/zenodo.1411986.
Whole genome Average Nucleotide Identity matrix	ANIclustermap version 1.2.0	Skani	Shimoyama Y. 2022. ANIclustermap: A tool for drawing ANI clustermap between all-vs-all microbial genomes [Computer software]. https://github.com/moshi4/ANIclustermap
Genotypic resistance	National Center for Biotechnology Information NCBI AMRFinder		Feldgarden M, Brover V, Haft DH, Prasad AB, Slotta DJ, Tolstoy I, et al. Validating the AMRFinder Tool and Resistance Gene Database by Using Antimicrobial Resistance Genotype-Phenotype Correlations in a Collection of Isolates. Antimicrob Agents Chemother 2019;63. https://doi.org/10.1128/AAC.00483-19.
	Comprehensive Antibiotic Resistance Database (CARD) Resistance Gene Identifier (RGI)		Jia B, Raphenya AR, Alcock B, Waglechner N, Guo P, Tsang KK, et al. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. Nucleic Acids Res 2017;45:D566–73. https://doi.org/10.1093/nar/gkw1004.
	Center for Genomic Epidemiology ResFinder version 4.3.3		Florensa AF, Kaas RS, Clausen PTLC, Aytan-Aktug D, Aarestrup FM. ResFinder – an open online resource for identification of antimicrobial resistance genes in next-generation sequencing data and prediction of phenotypes from genotypes. Microb Genom 2022;8. https://doi.org/10.1099/mgen.0.000748.

Table S2. Metadata of the clinical history of the included patients.

Patient	Hospital	Patient Age (years)	Sex	Admission date at other hospital referral from	ICD-10 DIAGNOSIS AT ADMISSION	ADMISSION DATE	DISCHARGE DATE	TOTAL HOSPITAL STAY DAYS	HOSPITAL DISCHARGE CONDITION	ICD-10 DIAGNOSIS AT SAMPLING DATE	CLINICAL SAMPLE	Clinical sample date	*Pseudomonas* sequence type ST	Rectal sample date
P1	Hospital_2P	61	Female	31/10/2021	K92.2 Gastrointestinal haemorrhage, unspecified	01/11/2021	01/12/2021	30	Dead	K65.0 Acute peritonitis K57.3 Diverticular disease of large intestine without perforation or abscess R57.2 Septic shock	Abdominal wound	11/11/2021	274	15/11/2021
P2	Hospital_2P	61	Female	04/11/2021	R57.2 Septic shock R65.1 Systemic Inflammatory Response Syndrome of infectious origin with organ failure T81.3 Disruption of operation wound, not elsewhere classified K63.2 Fistula of intestine	03/12/2021	18/12/2021	15	Dead	R57.2 Septic shock R65.1 Systemic Inflammatory Response Syndrome of infectious origin with organ failure I47.2 Ventricular tachycardia K43.2 Incisional hernia without obstruction or gangrene N17.9 Acute renal failure, unspecified	Abdominal discharge Tracheal discharge	22/11/2021 04/12/2021	274 253	24/11/2021 14/12/2021
P3	Hospital_1C	49	Male	NA	R57.1 Hypovolaemic shock S06.9 Intracranial injury, unspecified S02.1 Fracture of base of skull S01.9 Open wound of head, part unspecified	04/12/2021	25/02/2022	83	Recovered		Sputum	28/01/2022	253	31/01/2022
P4	Hospital_1C	85	Female	NA	I60.6 Subarachnoid haemorrhage from other intracranial arteries I11.9 Hypertensive heart disease without (congestive) heart failure	24/12/2021	10/02/2022	48	Dead	G91.9 Hydrocephalus, unspecified N39.0 Urinary tract infection, site not specified, catheter	Sputum	18/01/2022	n1	18/01/2022
P5	Hospital_1C	18	Female	NA	U07.2 COVID-19	05/02/2022	23/02/2022	18	Recovered	J15.1 Pneumonia due to *Pseudomonas* E87.1 Hypo-osmolality and hyponatraemia	Tracheostomy secretion	11/02/2022	n2	14/02/2022
P6	Hospital_1C	74	Female	NA	I46.0 Cardiac arrest with successful resuscitation	23/02/2022	12/03/2022	17	Dead	J18.0 Bronchopneumonia, unspecified	Blood	07/03/2022	17	10/03/2022
P7	Hospital_1C	90	Male	NA	L89.X Decubitus ulcer and pressure area	03/03/2022	19/03/2022	16	Dead	I21.9 Acute myocardial infarction, unspecified	Wound	09/03/2022	389	03/03/2022
P8	Hospital_2P	75	Male	NA	J18.9 Pneumonia, unspecified N17.9 Acute renal failure, unspecified	20/03/2022	31/03/2022	11	Recovered	R57.2 Septic shock I49.0 Ventricular fibrillation and flutter	Tracheal discharge	21/03/2022	n3	24/03/2022
P9	Hospital_1C	60	Male	NA	A41.9 Sepsis, unspecified	03/05/2022	31/05/2022	28	Recovered	J15.1 Pneumonia due to *Pseudomonas* E87.6 Hypokalaemia	Tracheal discharge	20/05/2022	253	24/05/2022
P10	Hospital_1C	70	Male	NA	I67.9 Cerebrovascular disease, unspecified N17 Acute renal failure E87.0 Hyperosmolality and hypernatraemia D45 Polycythaemia vera L97 Ulcer of lower limb, not elsewhere classified	25/06/2022	11/08/2022	47	Referral to other hospital	D33.2 Brain, unspecified neoplasm	Urine	13/07/2022	n4	16/07/2022
P11	Hospital_2P	55	Female	13/04/2022	M05.3 Rheumatoid arthritis with involvement of other organs and systems. Methotrexate pneumonitis J18.9 Pneumonia, unspecified E87.6 Hypokalaemia N17 Acute renal failure	03/05/2022	20/05/2022	17	Dead	M05.2 Rheumatoid vasculitis	Blood	16/05/2022	253	17/05/2022
P12	Hospital_1C	56	Male	NA	L02.3 Cutaneous abscess, furuncle and carbuncle of buttock E11 Type 2 diabetes mellitus	17/06/2022	28/08/2022	72	Dead	L02.3 Cutaneous abscess, furuncle and carbuncle of buttock E11 Type 2 diabetes mellitus	Wound	07/07/2022	309	
										N49.8 Inflammatory disorders of other specified male genital organs N17 Acute renal failure R57.2 Septic shock K63.1 Perforation of intestine (nontraumatic)	Wound	09/08/2022	253	26/08/2022
P13	Hospital_1C	34	Male	NA	L89.X Decubitus ulcer and pressure area	11/07/2022	04/10/2022	85	Recovered	M86 Osteomyelitis femur	Urine Acetabulum Femur Wound	26/08/2022 13/09/2022 13/09/2022 13/09/2022	253 n5 n5 n5	26/08/2022 13/09/2022 13/09/2022 13/09/2022
P14	Hospital_1C	84	Male	NA	L89.X Decubitus ulcer and pressure area R33 Retention of urine K59.0 Constipation	18/09/2022	28/09/2022	10	Recovered		Urine Wound	18/09/2022 19/09/2022	3142 3142	19/09/2022 19/09/2022
P15	Hospital_1C	30	Male	NA	N10 Acute tubulo-interstitial nephritis L93 Lupus erythematosus L89.X Decubitus ulcer and pressure area M32.1 Systemic lupus erythematosus with organ or system involvement	26/10/2022	11/11/2022	16	Referral to other hospital	M32.1 Systemic lupus erythematosus with organ or system involvement N31.1 Reflex neuropathic bladder, not elsewhere classified N06.7 Isolated proteinuria with specified morphological lesion: diffuse crescentic glomerulonephritis G03.1 Chronic meningitis G37.3 Acute transverse myelitis in demyelinating disease of central nervous system L89.2 Stage III decubitus ulcer	Wound	26/10/2022	2433	26/10/2022

Table S3. Number of isolates carrying resistant genes detected by at least two out of three databases Comprehensive Antibiotic Resistance Database (CARD) Resistance Gene Identifier (RGI), National Center for Biotechnology Information NCBI AMRFinder, and the Center for Genomic Epidemiology Resfinder.

Gene	RGI n=52 (%)	AMRFinder n=52 (%)	ResFinder n=52 (%)	Drug class
aac(6')-29	0 (0)	2 (3.85)	2 (3.85)	Aminoglycosides
aac(6')-Il	0 (0)	3 (5.77)	3 (5.77)	Aminoglycosides
aadA6	7 (13.46)	12 (23.08)	12 (23.08)	Aminoglycosides
ant(2'')-Ia	1 (1.92)	2 (3.85)	3 (5.77)	Aminoglycosides
aph(3')-IIb	32 (61.54)	50 (96.15)	52 (100)	Aminoglycosides
aph(3')-VIa	0 (0)	3 (5.77)	3 (5.77)	Aminoglycosides
arr-2	1 (1.92)	3 (5.77)	3 (5.77)	Rifamycin
bla$_{\text{OXA-486}}$	4 (7.69)	4 (7.69)	4 (7.69)	β-lactams
bla$_{\text{OXA-488}}$	9 (17.31)	12 (23.08)	14 (26.92)	β-lactams
bla$_{\text{OXA-50}}$	23 (44.23)	22 (42.31)	26 (50)	β-lactams
bla$_{\text{OXA-851}}$	2 (3.85)	2 (3.85)	0 (0)	β-lactams
bla$_{\text{PDC-188}}$	3 (5.77)	3 (5.77)	0 (0)	β-lactams
bla$_{\text{PDC-24}}$	3 (5.77)	4 (7.69)	0 (0)	β-lactams
bla$_{\text{PDC-3}}$	8 (15.38)	12 (23.08)	0 (0)	β-lactams
bla$_{\text{PDC-34}}$	10 (19.23)	12 (23.08)	0 (0)	β-lactams
bla$_{\text{VIM-2}}$	5 (9.62)	5 (9.62)	5 (9.62)	β-lactams
catB3	1 (1.92)	3 (5.77)	3 (5.77)	Phenicol
catB7	34 (65.38)	50 (96.15)	52 (100)	Phenicol
crpP	0 (0)	36 (69.23)	37 (71.15)	Fluoroquinolones
dfrA22	1 (1.92)	3 (5.77)	3 (5.77)	Sulfonamides
fosA	47 (90.38)	51 (98.08)	52 (100)	Fosfomycin
*gyrA*_T83I	11 (21.15)	13 (25)	0 (0)	Fluoroquinolones
qacEΔ1	1 (1.92)	14 (26.92)	0 (0)	Efflux pump
qnrVC1	2 (3.85)	3 (5.77)	3 (5.77)	Fluoroquinolones
sul1	13 (25)	15 (28.85)	15 (28.85)	Sulfonamides
tmexD2	0 (0)	3 (5.77)	3 (5.77)	Efflux pump

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Table S4. Phenotypic and genotypic antibiotic resistance analysis of relatedness by Fisher´s exact test and OR-Fisher with 95% confidence interval. Bold numbers represent a statistically significant relation between the phenotype and the genotype.

		Antibiotic→	Cefepime		Ceftazidime		Imipenem		Meropenem		Piperacillin-Tazobactam		Ciprofloxacin		Levofloxacin		Amikacin		Gentamicin	
		Resistant Phenotype n=52 →	13		11		25		22		12		22		23		7		9	
Gene↓	**Database↓**	Positive genotype n=52↓	OR-Fisher (p)	CI (95%)	OR-Fisher (p)	CI (95%)	OR-Fisher (p)	CI (95%)	OR-Fisher (p)	CI (95%)	OR-Fisher (p)	CI (95%)	OR-Fisher (p)	CI (95%)	OR-Fisher (p)	CI 95%	OR-Fisher (p)	CI 95%	OR-Fisher (p)	CI 95%
	RGI	10	**26.44 (<0.001)**	**3.92-322**	3.24 (0.19)	0.53-18.53	13.95 (0.004)	1.68-662	18.93 (<0.001)	18.93-904	4.08 (0.039)	0.86-27.88								
bla_{PDC-34}	AMRFinder	12	**51.98 (<0.001)**	**7.16-710**	3.91 (0.1)	0.73-21	**19.328 (<0.001)**	**2.38-907**	**27.03 (<0.001)**	**3.27-1279**	**5.42 (0.02)**	**1.07-29.43**								
	ResFinder	-																		
	RGI	5	2.14 (0.58)	0.16-21	**20.8 (0.005)**	**1.74-1142**	**∞ (<0.02)**	**1.082-∞**	**∞ (0.01)**	**1.39-∞**	**∞ (<0.001)**	**3.99-∞**								
bla_{VIM-2}	AMRFinder	5	2.14 (0.58)	0.16-21	**20.8 (0.005)**	**1.74-1142**	**∞ (<0.02)**	**1.082-∞**	**∞ (0.01)**	**1.39-∞**	**∞ (<0.001)**	**3.99-∞**								
	ResFinder	5	2.14 (0.58)	0.16-21	**20.8 (0.005)**	**1.74-1142**	**∞ (<0.02)**	**1.082-∞**	**∞ (0.01)**	**1.39-∞**	**∞ (<0.001)**	**3.99-∞**								
	RGI	9	**19.64 (<0.001)**	**2.89-236**	2.15 (0.37)	0.28-13	**11.71 (0.01)**	**1.36-561**	**15.72 (0.003)**	**1.81-756**	**6.123 (0.022)**	**1.03-39.89**								
$bla_{OXA-488}$	AMRFinder	15	**51.98 (<0.001)**	**7.18-710**	3.91 (0.1)	0.73-21	**19.32(<0.001)**	**2.38-907**	**27.03 (<0.001)**	**3.27-1279**	**5.42 (0.02)**	**1.07-29**								
	ResFinder	14	**26.05 (<0.001)**	**4.51-215**	2.89 (0.141)	0.56-14.69	**6.05 (0.012)**	**1.3-39**	**8.57 (0.003)**	**1.8-57.19**	3.87 (0.063)	0.8-19								
	RGI	11											**32.69 (<0.001)**	**3.82-1573**	**28.72 (<0.001)**	**3.39-1375**				
gyrA_T83I	AMRFinder	13											**49.29 (<0.001)**	**5.76-2375**	**42.28 (<0.001)**	**5.027-2021**				
	ResFinder	-																		
	RGI	-																		
aac(6')-Il	AMRFinder	3															**∞ (0.002)**	**3.24-∞**	**∞ (0.004)**	**2.28-∞**
	ResFinder	3															**∞ (0.002)**	**3.24-∞**	**∞ (0.004)**	**2.28-∞**
	RGI	1															∞ (0.13)	0.165-∞	∞ (0.22)	0.09-∞
ant(2")-Ia	AMRFinder	1															∞ (0.13)	0.165-∞	∞ (0.22)	0.09-∞
	ResFinder	2															**∞ (0.002)**	**3.24-∞**	**∞ (0.004)**	**2.28-∞**
	RGI	-																		
aph(3')-VIa	AMRFinder	3															**∞ (0.002)**	**3.24-∞**	**∞ (0.004)**	**2.28-∞**
	ResFinder	3															**∞ (0.002)**	**3.24-∞**	**∞ (0.004)**	**2.28-∞**
	RGI	7															3.1 (0.23)	0.23-26.93	**22.65 (<0.001)**	**2.73-311**
aadA6	AMRFinder	12															2.92 (0.33)	0.36-20.97	**11.45 (0.003)**	**1.87-91.48**
	ResFinder	12															2.92 (0.33)	0.36-20.97	**11.45 (0.003)**	**1.87-91.48**
	RGI	35	1.84 (0.5)	0.38-12.16	1.37 (1)	0.27-9.3	**4.72 (0.019)**	**1.15-24**	**9.57 (0.002)**	**1.8-98.98**	7.11 (0.076)	0.87-334	**6.84 (0.014)**	**1.28-70**	**7.64 (0.007)**	**1.43-78.8**	∞ (0.081)	0.75-∞	**∞ (0.023)**	**1.09-∞**
OprM	AMRFinder	-																		
	ResFinder	-																		
	RGI	23	**28.45 (<0.001)**	**3.49-1339**	**4.48 (0.044)**	**0.9-30.27**	**4.9 (0.011)**	**1.34-19.81**	**5.66 (0.005)**	**1.52-23**	**9.89 (0.003)**	**1.75-105**	**18.32 (<0.001)**	**3.83-126**	**13.36 (<0.001)**	**3.07-74.14**	3.65 (0.219)	0.52-42.308	**14.21 (0.007)**	**1.65-682**
ArmM-MexR	AMRFinder	-																		
	ResFinder	-																		
	RGI	1	0 (1)	0-116	∞ (0.212)	0.09-∞	∞ (0.48)	0.028-∞	∞ (0.42)	0.03-∞	∞ (0.23)	0.085-∞	∞ (0.36)	0.045-∞	∞ (0.385)	0.041-∞	∞ (0.13)	0.16-∞	∞ (0.17)	0.123-∞
qacEΔ1	AMRFinder	14	**55.54 (<0.001)**	**7.8-748**	**8.03 (0.005)**	**1.56-48**	**26.38 (<0.001)**	**3.29-1232**	**38.43 (<0.001)**	**4.67-1817**	**10.6 (<0.001)**	**2.09-65**	**61.08 (<0.001)**	**7.08-2969**	**51.69 (<0.001)**	**6.1-2479**	9.42 (0.01)	1.29-114	**43.72 (<0.001)**	**4.65-2209**
	ResFinder	-																		
	RGI	13	**17.95 (<0.001)**	**3.31-126**	**9.57 (0.003)**	**1.81-60**	**22.59 (<0.001)**	**2.8-1057**	**32.2 (<0.001)**	**3.92-1522**	**24.15 (<0.001)**	**4.09-202**	**49.29 (<0.001)**	**5.76-2375**	**42.28 (<0.001)**	**5.02-2021**	**29.42 (<0.001)**	**2.9-1519**	**∞ (<0.001)**	**12.2-∞**
sul1	AMRFinder	15	**41.87 (<0.001)**	**6.32-514**	**12.08 (<0.001)**	**2.23-89**	**30.8 (<0.001)**	**3.85-1438**	**46.09 (<0.001)**	**5.58-2185**	**15.67 (<0.001)**	**2.9-117**	**77.23 (<0.001)**	**8.78-3789**	**63.92 (<0.001)**	**7.45-3083**	**22.15 (0.001)**	**2.27-1124**	**∞ (<0.001)**	**8.65-∞**
	ResFinder	15	**41.87 (<0.001)**	**6.32-514**	**12.08 (<0.001)**	**2.23-89**	**30.8 (<0.001)**	**3.85-1438**	**46.09 (<0.001)**	**5.58-2185**	**15.67 (<0.001)**	**2.9-117**	**77.23 (<0.001)**	**8.78-3789**	**63.92 (<0.001)**	**7.45-3083**	**22.15 (0.001)**	**2.27-1124**	**∞ (<0.001)**	**8.65-∞**

Figure 1. Phylogenetic tree using IQTREE software (maximum likelihood) of 5.29 Mbps length alignment of 53 *P. aeruginosa* isolates (including the reference sequence). Blue branches represent isolates from Hospital_1C. Red branches represent isolates from Hospital_2P. Shaded boxes represent each patient's isolates. The yellow shade shows the ST-253 epidemic clade. The numbers in nodes indicate bootstrap values using 1000 pseudo-replicates; the bootstrap number is shown as a percentage (%). Resistance phenotypes are shown on the right. Resistant strains with at least one genetic marker statistically significantly related to its phenotype are drawn in filled circles, and the ones with the resistant phenotype but not the genetic feature are in empty circles. Susceptible isolates are not illustrated. A single asterisk denotes the recurrence isolates. Double asterisks denote rectal isolates collected on the admission date.

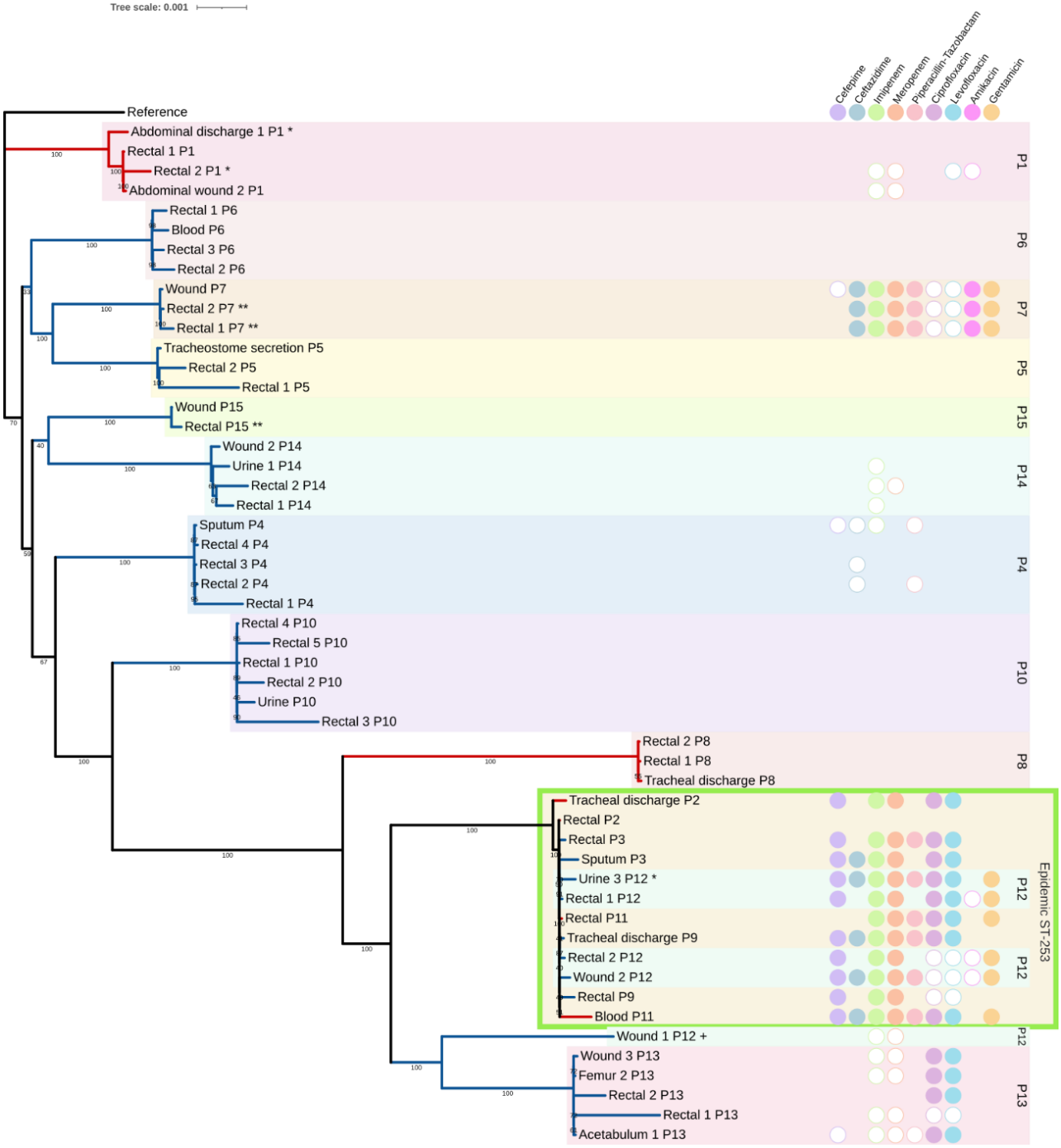


Figure S1. Whole genome core SNPs phylogenetic tree obtained by kSNPs software using maximum likelihood GTR model. Number of core SNPs from 53 sequences (including the reference sequence): 49188. The clades resemble the clustering from Figure 1. The bootstrap is a percentage.

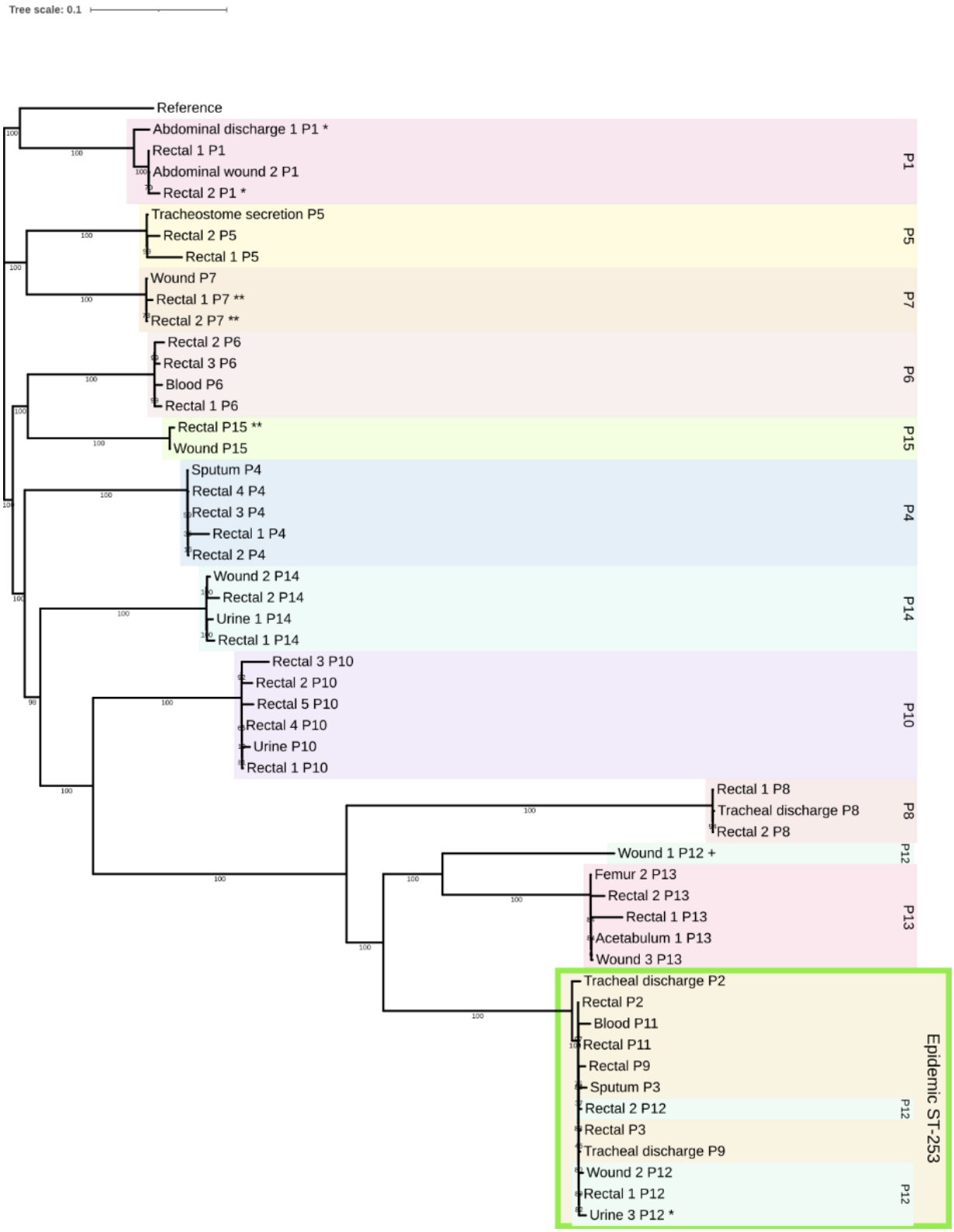


Figure S2. Average Nucleotide Identity phylogenetic tree and matrix obtained by ANIclustermap software, skani. The matrix denotes the closely related strains in darker squares, resembling the phylogenetic tree from Figure 1.

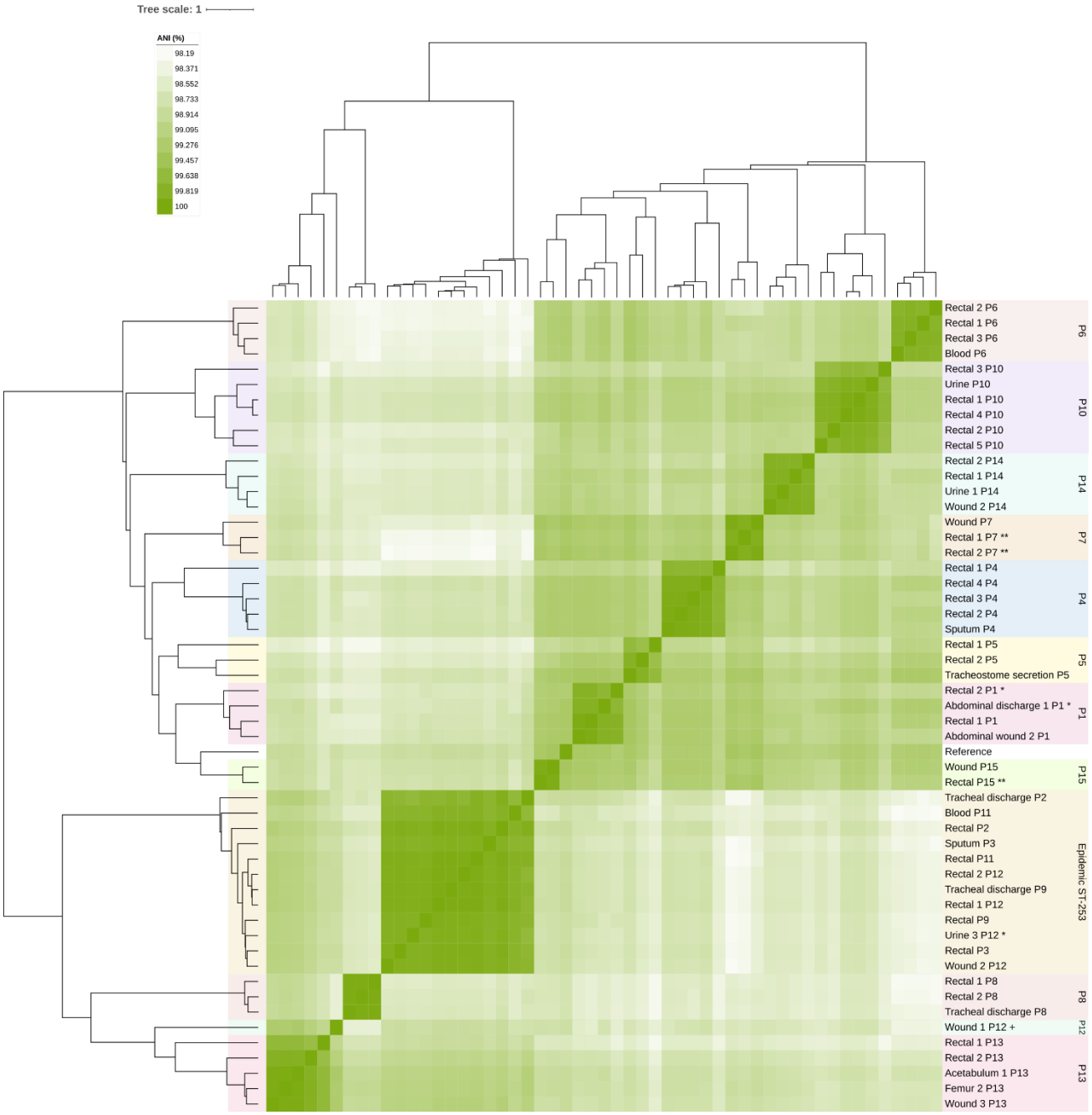


Figure S3. Timeline of the ST strains isolated from Hospital_1C (blue) and Hospital_2P (red). Horizontal bars correspond to the stay length at the hospital from each patient. The circle's position shows the date when the clinical sample was taken.

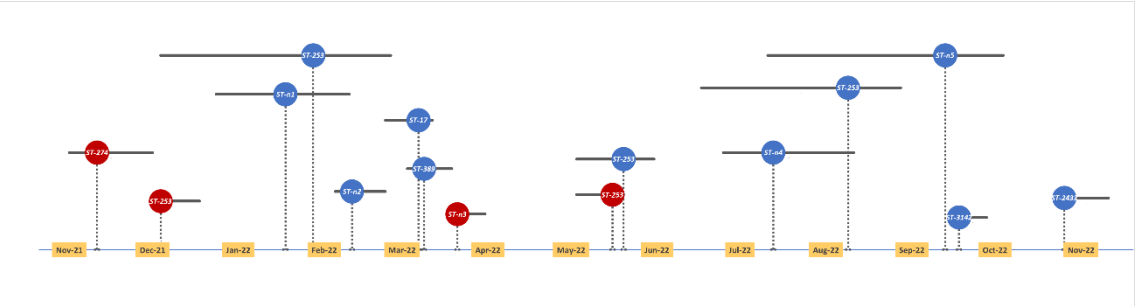
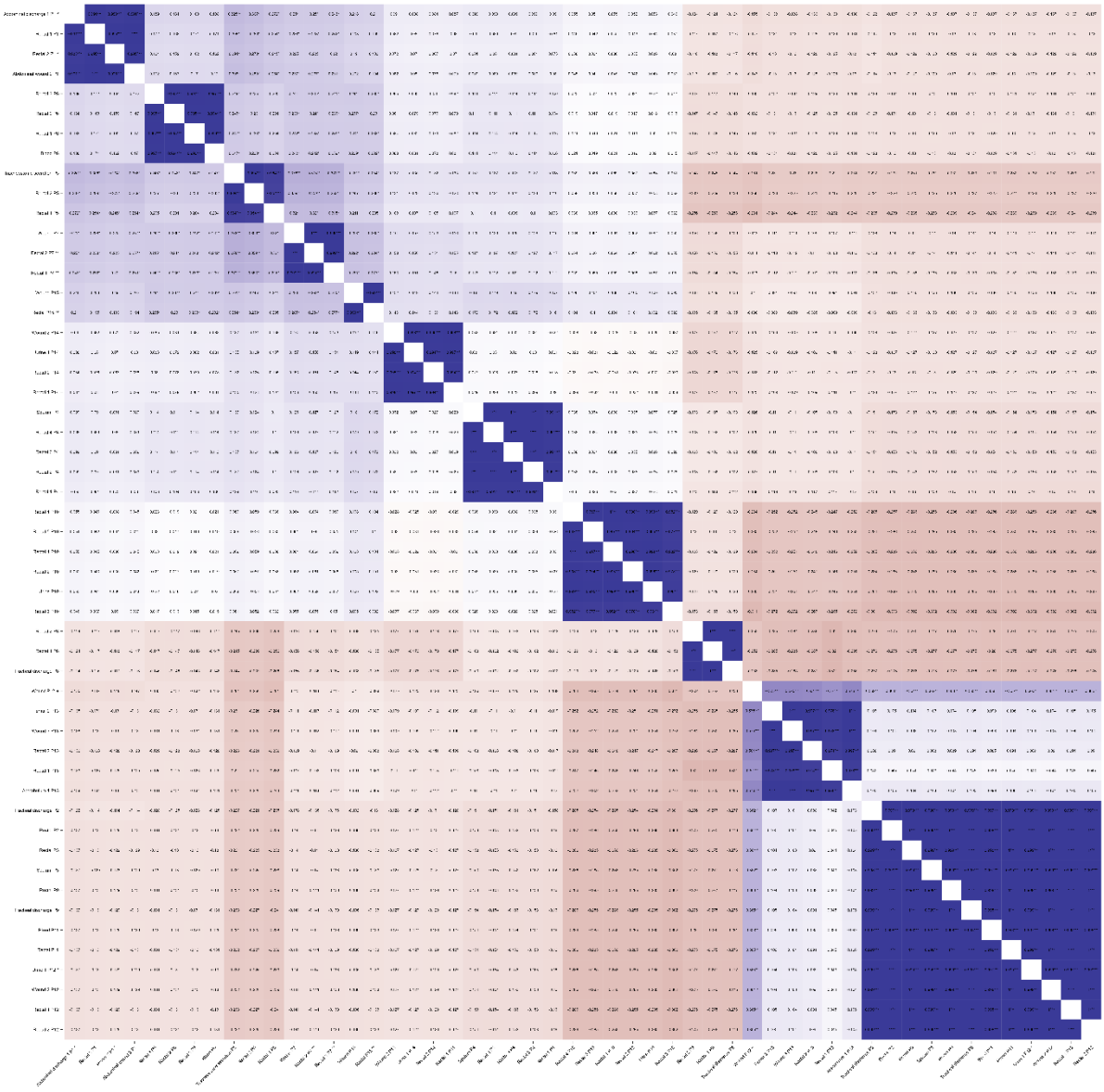


Figure S4. Partial Pearson's r heatmap, conditioned on the variable of the number of SNPs per million base pairs between the genomes of the isolates and the reference sequence *P. aeruginosa* AE004091.2. *p < 0.05 **p < 0.01 ***p <0.001.



Not published Annex Table 1. Resistance genotypes and phenotypes discrepancies between bacteria from the same clade (Highlighted cells). ResFinder database profiles were confirmed by AMRFinder database profiles. 1=gene presence, 0=gene absence, R=resistant phenotype, S=sensitive phenotype.

			RESISTANCE GENOTYPE																													RESISTANCE PHENOTYPE								
New Name	ST	Isolation date	*aac(6')-29a*	*aac(6')-29b*	*aac(6')-Il*	*aadA6*	*ant(2")-Ia*	*aph(3')-IIb*	*aph(3')-VIa*	*ARR-2*	*blaOXA-395*	*blaOXA-486*	*blaOXA-488*	*blaOXA-494*	*blaOXA-396*	*blaOXA-50*	*blaPDC-34*	*blaCTX-M-2*	*blaTEM-1b*	*blaOXA-2*	*blaIMP-15*	*blaVIM-2*	*catB3*	*catB7*	*crpP*	*dfrA22*	*fosA*	*gyrA_T83I*	*qnrVC1*	*sul1*	*tmexC3*	*tmexO2*	*TOprI3*	Amikacin	Cefepim	Ceftazidim	Ciprofloxacin	Gentamicin	Imipenem	Levofloxacin	Meropenem	Piperacillin-Tazobactam
Tracheal discharge P2	253	4/12/2021	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	1	1	0	1	0	0	0	S	R	S	R	S	R	R	R	S
Rectal P2	253	14/12/2021	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	1	1	0	1	0	0	0	S	S	S	S	S	S	S	S	S
Sputum P3	253	28/1/2022	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	0	1	0	0	0	S	R	R	R	S	R	R	R	S
Rectal P3	253	31/1/2022	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	0	1	0	0	0	S	R	S	R	S	R	R	R	I
Blood P11	253	16/5/2022	1	1	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	1	0	1	1	0	1	0	0	0	S	I	R	R	R	R	R	R	I
Rectal P11	253	17/5/2022	1	1	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	1	0	1	1	0	1	0	0	0	S	S	S	I	R	R	R	R	I
Tracheal discharge P9	253	20/5/2022	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	0	1	0	0	0	S	R	R	R	S	R	R	R	I
Rectal P9	253	24/5/2022	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	0	0	1	0	0	0	S	R	S	R	S	R	R	R	S
Wound 2 P12	253	9/8/2022	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	0	0	1	0	0	0	R	R	I	R	R	R	R	R	I
Urine 1 P12	253	26/8/2022	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	0	1	0	0	0	S	R	R	R	I	R	R	R	I
Rectal 1 P12	253	26/8/2022	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	0	1	0	0	0	R	R	S	R	R	R	R	R	S
Rectal 2 P12	253	26/8/2022	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	0	1	0	0	0	R	R	S	R	R	R	R	R	S
Abdominal wound 2 P1	274	11/11/2021	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	S	S	S	S	S	R	S	R	S
Rectal 1 P1	274	15/11/2021	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Abdominal discharge 1 P1	274	22/11/2021	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Rectal 2 P1	274	24/11/2021	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	I	S	S	S	S	R	R	R	S
Rectal 1 P7	389	3/3/2022	0	0	1	0	1	1	1	1	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	1	1	0	1	1	1	1	1	R	S	R	R	R	R	R	I	I
Rectal 2 P7	389	3/3/2022	0	0	1	0	1	1	1	1	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	1	1	0	1	1	1	1	1	R	S	R	I	R	R	R	I	I
Wound P7	389	9/3/2022	0	0	1	0	1	1	1	1	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	1	1	0	1	1	1	1	1	R	R	R	R	R	R	R	I	I
Wound P15	2433	26/10/2022	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Rectal P15	2433	26/10/2022	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Urine 1 P14	3142	18/9/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Wound 2 P14	3142	19/9/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Rectal 1 P14	3142	19/9/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	R	S	S	S
Rectal 2 P14	3142	19/9/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	R	S	I	S
Rectal 1 P4	n1	18/1/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Rectal 2 P4	n1	18/1/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	R	S	S	S	S	S	I
Rectal 3 P4	n1	18/1/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	I	S	S	S	S	S	S
Rectal 4 P4	n1	18/1/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Sputum P4	n1	18/1/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	R	R	S	S	I	S	S	R
Tracheostome secretion P5	n2	11/2/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Rectal 1 P5	n2	14/2/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Rectal 2 P5	n2	14/2/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Blood P6	n3	7/3/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Rectal 1 P6	n3	10/3/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Rectal 2 P6	n3	10/3/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Rectal 3 P6	n3	10/3/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Tracheal discharge P8	n4	21/3/2022	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Rectal 1 P8	n4	24/3/2022	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Rectal 2 P8	n4	24/3/2022	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Urine P10	n5	13/7/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	I	S	S	S
Rectal 1 P10	n5	16/7/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Rectal 2 P10	n5	16/7/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Rectal 3 P10	n5	16/7/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Rectal 4 P10	n5	16/7/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Rectal 5 P10	n5	16/7/2022	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	S	S	S	S	S	S	S	S	S
Acetabulum 1 P13	n6	13/9/2022	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	S	R	S	R	S	R	R	R	I
Femur 2 P13	n6	13/9/2022	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	S	S	S	R	S	R	R	R	S
Wound 3 P13	n6	13/9/2022	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	S	S	S	R	S	R	R	R	S
Rectal 1 P13	n6	13/9/2022	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	S	S	S	R	S	R	R	R	S
Rectal 2 P13	n6	13/9/2022	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	S	S	S	R	S	S	R	S	S

Not published Annex Table 2. Patients not included in the phylogenetic analysis. Resistance phenotypes and origin of samples. R=resistant phenotype, S=sensitive phenotype.

Patient	Origin of the sample	Sex	Age	RESISTANCE PHENOTYPE								
				Amikacin	Cefepim	Ceftazidim	Ciprofloxacin	Gentamicin	Imipenem	Levofloxacin	Meropenem	Piperacillin-Tazobactam
16	Catheter tip	F	62	S	S	I	R	R	R	R	R	I
17	Sputum	M	60	S	R	R	R	S	R	R	R	I
18	Urine	M	25	I	R	R	S	R	R	S	S	S
19	Peritoneal liquid	M	35	S	S	S	S	S	S	S	S	S
20	Wound	M	56	S	S	S	S	S	R	S	R	S
21	Traqueal secretion	M	31	S	R	R	S	R	R	I	S	S
22	Urine	M	70	S	S	S	S	S	I	S	S	S
23	Wound	F	58	R	R	S	R	R	R	R	R	R
24	Urine	M	90	S	R	R	R	S	R	R	R	I
25	Head Burn	F	67	S	S	S	S	S	S	S	S	S
26	Sputum	M	46	S	S	S	S	S	-	-	-	S
27	Sputum	M	23	S	S	S	S	S	R	R	R	S
28	Urine	F	78	S	S	S	S	S	-	-	-	-
29	Urine	F	61	S	R	-	R	S	I	R	R	S
30	Sputum	M	81	S	-	S	S	S	-	-	-	-
31	Sputum	F	61	S	-	-	S	S	R	S	R	I
32	Bone Tissue	F	50	S	I	S	R	R	R	-	R	I
33	Sputum	M	32	S	-	S	S	S	-	-	S	S
34	Blood	M	40	S	-	S	-	-	S	-	-	S
35	Catheter tip	M	46	S	-	S	-	-	S	-	-	S
36	Tracheal secretion	F	27	S	S	S	I	S	I	-	I	I
37	Abscess	M	35	S	S	S	S	S	S	-	S	S
38	Urine	F	80	S	S	S	S	-	-	-	I	S
39	Urine	M	84	S	S	S	S	S	-	-	-	-
40	Blood	F	67	S	S	S	S	-	-	-	S	S

CHAPTER IV

Insights into Physiological Adaptations of *Pseudomonas aeruginosa*: Contrasting Clinical and Environmental Isolates in a Hospital Setting

In preparation for publishing

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Abstract

Pseudomonas aeruginosa is a versatile bacterium associated with often lethal nosocomial infections. We investigated some physiological characteristics of clinical and environmental *P. aeruginosa* strains (from the same hospital). We observed that clinical isolates outperformed environmental ones in growth under nutrient deprivation, ciliate predation resistance, bacterial antagonism, swarming, and swimming. Unexpectedly, clinical strains showed lower resistance to cefepime and piperacillin/tazobactam than environmental ones. In conclusion, our study shows that clinical isolates of *P. aeruginosa* have special physiological adaptations than most of their environmental counterparts.

Keywords

Pseudomonas aeruginosa, physiology, clinical, environmental, hospital

1. Introduction

Pseudomonas aeruginosa, a ubiquitous Gram-negative bacterium, is one of the six highly virulent antimicrobial-resistant bacteria known as ESKAPE (Rice, 2008), constituting an important threat in hospitals (Pendleton et al., 2013). Studies have shown that *P. aeruginosa* infections are particularly prevalent among immunocompromised patients, producing a variety of opportunistic diseases such as wound infections, urinary tract infections, pneumonia, bacteremia, and sepsis (Hernández-Jiménez et al., 2022; Juan et al., 2017). The widespread distribution of *P. aeruginosa* in diverse environments, such as bodies of water, soil, and animals including humans (Gómez-Zorrilla et al., 2015), enables contamination of healthcare settings.

Pseudomonas also has the ability to form biofilms on surfaces, including medical equipment and catheters, and tolerates various stressors such as starvation, oxidative reactive molecules, etc., enhancing its persistence and resistance to disinfection procedures (Juan et al., 2017; Vasco and Trueba, 2021).

This predominantly environmental bacterium plays a role in natural ecosystems in decomposing organic matter, including pollutants (Mortimer et al., 2016; Werlin et al., 2011). In the environment, this bacterium competes with other bacterial species and faces predation by protozoa (Hilbi et al., 2007). Adapting to these challenges can occur through exchanging mobile genetic elements (MGEs) or selecting mutants (Sommer et

al., 2020). Some of these adaptations such as the formation of small colony variants, alterations in the biofilm structure, or expression of secretion systems, are defense mechanisms (Liu et al., 2018; Scherwass et al., 2016). These adaptations may be a result of environmental evolution and some virulence factors may be a spin-off of factors enabling survival in the environment.

Understanding the physiological and genetic diversity of *P. aeruginosa* and the relationship between clinical and environmental isolates is crucial to developing a system to monitor and control these infections. We investigated the physiological differences between *P. aeruginosa* strains isolated from the environment and infections within the same hospital.

2. Materials and Methods

2.1. Hospital surveillance and strain isolation

We conducted surveillance at Hospital Pablo Arturo Suárez, a public hospital in Quito City, Ecuador, with 241 beds. The sampling was performed for 12 continuous months, from August 2017 to July 2018. Clinical strains were obtained from the hospital's clinical laboratory, while environmental isolates were collected by swabbing the sinks (including the aerator, spout, or pop-up stopper) at various hospital services.

The environmental samples were transported in Stuart media and inoculated on Cetrimide agar (BD Difco) on the same collection day. Incubation was carried out at 37°C for 48 hours, with monitoring of bacterial growth every 24 hours for the presence

of blue-green colonies. Further tests included assessing positive oxidase reactions, UV light fluorescence, and growth at 42°C. Positive isolates were subjected to DNA extraction using the Wizard® Genomic DNA Purification Kit (Promega) and tested for *aroE* (Shikimate dehydrogenase) through PCR and Sanger sequencing to confirm *P. aeruginosa* strains (Curran et al., 2004). The isolates were stored at -80°C in brain heart infusion (BHI) supplemented with 15% glycerol until further use. We used the *aroE* allele sequences to build a phylogenetic tree using the UPGMA method (Mega11 version 11.0.13, options bootstrap 100, maximum composite likelihood) (Tamura et al., 2021).

2.2. Water Survival

We reactivated the strains on a 10 ml BHI tube and incubated them at 37°C for 18 hours. We subcultured and streaked in a Cetrimide agar and incubated at 37°C for 24 hours. One colony from each plate was selected and inoculated in a 10 ml tube of BHI, which was incubated at 37°C for 18 hours. After incubation, each tube was vortexed for one minute, followed by two washes with sterile distilled water through centrifugation at 1000 x g force for 10 minutes. The resulting pellet was diluted in a 50 ml tube with 40 ml sterile distilled water to achieve a concentration of around 1.3 per 108 cells. We made aliquots of 1 ml in 1.8 ml tubes, which were tightly closed and stored at room temperature, covered from the light.

We took one aliquot and cultured the water suspensions at different times (days 0, 14, 28, 42, 56, 69, 83, 98, 113, 130, 148, and 592 days). The bacterial suspensions were vortexed for 1 minute, performed 10-fold serial dilutions, and 100 µl of each dilution

was plated onto a Cetrimide agar in triplicate and in a Trypticase Soy agar (BD Difco) to rule out contamination. The agar plates were then incubated at 37°C for 24 hours, and the plates with 15-150 colonies were counted.

For the statistical analysis, we used two tails Student's t-test (of the natural logarithm of the counts), utilizing the grouping variable "type" with two categories: "environmental" and "clinical."

2.3. Ciliate predation

To investigate the susceptibility of *P. aeruginosa* to the ciliate bacteria grazer *Tetrahymena pyriformis* (Ward's® Science), we co-cultured both organisms. We monitored their densities at six specific time points: days 1, 2, 6, 10, 16, and 61.

T. pyriformis was cultured in 1% bacto peptone sterile non-filtered solution (BD Difco) media and incubated at room temperature for approximately seven days or until the culture reached 5000 ciliates per ml density.

Bacteria for this assay were inoculated into 10 ml of BHI and incubated at 37°C for 18 hours, vortexed for one minute, and washed twice by centrifugation at 1000 x g for 10 minutes using Page's Amoeba Saline 1X (PAS) as the washing solution. The resulting pellet was resuspended in 5 ml of PAS, resulting in a bacterial density of approximately 2x107 CFU/ml.

We prepared 1.8 ml tubes containing 700 µl of sterile PAS for the predation assay. Each tube was inoculated with 100 µl of the washed bacteria and 200 µl of the *T. pyriformis* culture, resulting in a final volume of 1000 µl and a bacteria-to-ciliate ratio (MOI) of 2000:1. The tubes were gently homogenized by inversion, tightly closed, and incubated at room temperature. For the bacterial controls, 100 µl of each washed bacterial cells were placed in a 1.8 ml tube with 900 µl of PAS. Seven bacterial control tubes were prepared for each bacterial strain, including one tube for each time point and one for the initial inoculation count. The control for the ciliates consisted of 200 µl of *T. pyriformis* placed into a 1.8 ml tube with 800 µl of PAS. A total of 12 tubes were prepared for the ciliate's controls, with two tubes per time point.

We gently homogenized the tubes by inverting them ten times to quantify the ciliates. From each survival-test tube and two non-survival-test ciliate control tubes per time point, we took a 10 µl volume and placed it in a Neubauer-improved chamber with a depth of 0.1 mm. Each sample was analyzed in duplicate. Using 400X magnification, we counted the number of motile ciliates in the nine large squares (each with a surface area of 1 mm^2). The number of ciliates was divided by nine and multiplied by 104 to obtain the ciliates/ml count.

To enumerate the bacteria, we vortexed the tubes and performed 10-fold dilutions. Subsequently, we spread 100 µl of each dilution onto Cetrimide agar plates in duplicate. The agar plates were then incubated at 37°C for 24 hours. We counted the plates containing 15 to 150 colonies. Additionally, we cultured the bacterial suspensions on Trypticase Soy agar plates to check for any contaminant bacteria.

For the statistical analysis, we calculated the natural logarithm (LN) of the CFU/ml for *P. aeruginosa*, the LN of the ciliates/ml, and the LN of the MOI. We then performed an Independent Samples Student's t-test (two tails) using the grouping variable "type" with two categories: "environmental" and "clinical."

2.4. Antibiotic resistance

We conducted antibiotic resistance testing for two primary purposes. Firstly, we aimed to investigate potential differences in antibiotic resistance patterns between the clinical and environmental isolates of *P. aeruginosa*. Secondly, we used the resistance profiles of the strains to identify and control for any contaminating bacteria during the water survival and ciliate survival-test experiments.

We employed the Kirby Bauer method outlined in the Clinical and Laboratory Standards Institute (CLSI) M100 manual to determine antibiotic resistance. A total of nine antibiotics were tested, to know: amikacin (30 µg), aztreonam (30 µg), cefepime (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), imipenem (10 µg), levofloxacin (5 µg), meropenem (10 µg) and piperacillin-tazobactam (100-10 µg) (Clinical and Laboratory Standards Institute, 2022). In each assay, we included the *P. aeruginosa* ATCC® 27853 strain as a quality control reference. For analysis, we reported the zone diameters of inhibition for each antibiotic and categorized the results as sensitive, intermediate, or resistant based on established interpretive criteria.

We calculated the Student's t-test (two tails) using the zone diameters to assess the differences in antibiotic resistance between the clinical and environmental isolates. Additionally, we employed the chi-square test (X^2) using the interpretive categories to confirm any observed differences.

Antibiotic resistance testing, including the water survival and ciliate survival assays, was performed before the experiments. Furthermore, we conducted resistance testing after the completion of the experiments using the final bacterial isolates. If the interpretive categories of a particular bacterium did not match between the pre and post-experiment testing, we excluded the results of that bacterium from the corresponding analysis.

2.5. Antagonism of *P. aeruginosa* strains

We conducted *in-vitro* competitions to investigate the clinical and environmental strains' aptitude to compete with other *P. aeruginosa* strains based on the Chatterjee P. et al. competition plate assay (Chatterjee et al., 2017). We made confluent cultures of different *P. aeruginosa* strains, obtained by swabbing a 0.5 McFarland standard bacterial density solution onto two different agar media: Mueller Hinton agar (BD Difco) for optimal nutrient growth conditions (36 strains) and M9 supplemented with 0.4% glucose and 1.5% agar for minimal growth conditions (17 strains). The confluent culture was spotted with environmental or clinical strains (single colony diluted in 0.85% saline - 0.5 McFarland standard density, volume of 1 ul), and the plates were incubated at 37°C for 24 hours. These assays were performed in triplicate. If there was no growth in the spot, we categorized the reaction as inhibitory (lysis); if a clear zone appeared around

the edges of the spotted bacteria, we named it as an antagonist activity "halo," and if no visible clear zones were observed around the spot, we called it as "none" (Fig. 4 A). We recorded the mean number of inhibitory or antagonist spots per bacterium.

2.7. Swarming

To examine the swarming motility of the bacteria, we prepared an M9 minimal medium supplemented with 0.4% glucose and 0.45% agar. We poured the media into 90 mm Petri dishes to create a viscous surface for bacterial movement, as described by O'Toole G. and Kolter (O'Toole and Kolter, 1998). Using a toothpick, we inoculated the bacteria by gently touching the surface of the M9 agar with the tip. The dishes were then incubated at 35°C for 20 hours for bacterial growth and motility. After incubation, we measured the swarming zone's diameter, representing the extent of bacterial migration across the agar surface. The diameter was measured in millimeters, providing a quantitative assessment of the swarming motility capability of the bacteria.

2.8. Twitching motility

To assess twitching motility, we used the protocol described by O'Toole G. and Kolter (O'Toole and Kolter, 1998) and prepared Petri dishes containing Lysogenic Broth (LB, BD Difco) supplemented with 1.5% agar. We inoculated the bacteria using a toothpick by gently touching the agar surface. The plates were then incubated at 35°C for 16 hours, followed by an additional incubation at 25°C for 24 hours. After incubation, we measured the motility area's diameter, representing the extent of bacterial movement through surface-associated twitching. The diameter was measured in millimeters.

2.9. Swimming

We based on the protocol from Ha D et al. to observe swimming motility (Ha et al., 2014). We prepared 90 mm Petri dishes with LB supplemented with 0.3% agar. We inoculated the bacteria using a toothpick by gently pointing it on the agar surface. The plates were incubated at 30°C for 20 hours to allow the bacteria to "swim" in the agar. After incubation, we measured the diameter of the motility zone in millimeters, which indicates the extent of bacterial movement in the medium.

3. Results

3.1. Hospital surveillance

Over twelve months, 643 isolates were obtained, consisting of 27 clinical *P. aeruginosa* isolates from 19 patients and 337 confirmed environmental strains (Table 1). *P. aeruginosa* was present ubiquitously throughout the hospital, with a high colonization rate of 80% in the sampled sinks. The phylogenetic analysis of the *aroE* gene demonstrated a polyphyletic origin of the bacterial strains (Fig. S1).

3.2. Water Survival

A representative subsample of the isolates was obtained for this assay by matching the environmental and clinical samples according to the isolation dates of the clinical strains. For this analysis, we tested 20 clinical and 19 environmental bacterial strains. The survival of bacteria was assessed by obtaining the mean of three replicates CFU/ml at various time points: day 0, 14, 28, 42, 56, 69, 83, 98, 113, 130, 148, and 592 days.

Among the environmental strains, the mean survival dropped from day 0 to day 28, followed by no significant variations until day 148. However, a consistent decline in survival was observed from day 130 onwards, continuing until day 592 (Pearson's r = 0.396, p = 0.116).

In contrast, the clinical bacteria exhibited an increase in density from day 0 to day 14 (Pearson's r = 0.589, p = 0.095), showing a significant difference in the bacterial counts (t = -2.588, p = 0.014). A statistically significant difference was observed on day 28, day 42, day 69, and day 83 (t = -2.9, p = 0.006; t = -2.54, p = 0.015; t = -2.67, p = 0.01; t = -2.08, p = 0.045) (Fig. 1). Subsequently, no other significant variations in clinical bacterial counts were observed, except for a substantial decrease from day 148 to day 592 (Pearson's r = 0.076, p = 0.765).

3.3. Ciliate predation survival-test

We tested 23 environmental bacteria and 19 clinical strains matched by their isolation dates. We conducted a survival-test of *P. aeruginosa* and the ciliates (*T. pyriformis*). The CFU/ml was measured at various time points: days 0, 1, 2, 6, 10, 16, and 61.

In both clinical and environmental bacteria, we observed an early drop in *P. aeruginosa* density from day 1 to 6, followed by a subsequent increase on day 10, compared to the respective bacterial controls (Fig. 2 D). The bacterial density was lower at the early drop on day 1 for the environmental bacteria than the clinical bacteria (t = -2.253, p = 0.031, Fig. 2 B). It should be noted that by days 16 and 61, there were no differences in the

density of *P. aeruginosa* between the tubes with ciliates and bacterial controls, which we attribute to the significant loss of ciliates.

The early drop in *P. aeruginosa* density coincided with an increase in *T. pyriformis* density (Fig. 2 C), and the subsequent rise in *P. aeruginosa* on day 10 coincided with a decrease in ciliate density compared to the non-survival-test ciliate controls. The trend of the survival-test curve was similar between the two types of bacteria, except for day 61, where the ciliate density was lower in the survival-test with the clinical strains ($t = 2.065$, $p = 0.048$).

In this analysis, we observed a parabolic tendency in the MOI (bacteria:ciliate, Fig. 2 E), with an initial decrease dependent on the drop in bacterial densities and an increase in ciliate densities, followed by an increase in the MOI due to the decline in the ciliate density. Initially, the ciliates may utilize the bacteria as a nutrient source; however, at a certain point during the survival-test, the growth conditions may be detrimental for the ciliates and favorable for the bacteria.

3.4. Antibiotic resistance

In this analysis, we obtained antibiotic resistance patterns from a subsample matched by isolation time: 25 environmental and 19 clinical isolates. We observed statistically significant differences in the halo diameter between the clinical and environmental strains for cefepime ($t = -2.858$, $p = 0.007$) and piperacillin-tazobactam ($t = -2.136$, $p = 0.039$). In both cases, unexpectantly, the clinical strains exhibited greater sensitivity than the environmental bacteria (Fig. 3). The analysis of interpretative categories

confirmed these findings for cefepime (chi-square X2 = 7.256, p = 0.027) and piperacillin-tazobactam (chi-square X2 = 8.504, p = 0.014), supporting the prevalence of sensitive phenotypes among the clinical strains.

No significant differences were observed for aztreonam, ceftazidime, ciprofloxacin, imipenem, levofloxacin, meropenem, and amikacin. However, the Brown-Forsythe test indicated significance ($p < 0.05$) for cefepime, ceftazidime, ciprofloxacin, meropenem, and piperacillin/tazobactam, suggesting a violation of the equal variance assumption (Supplementary Table 1). The clinical strains exhibited larger halo diameters in all these cases than the environmental bacteria.

3.5. Antagonism of *P. aeruginosa* strains

We tested a subsample of 19 clinical and 40 environmental strains for this analysis. We observed higher mean times of antagonist activity (halos) formed by the clinical isolates compared with the environmental ones under nutrient growth conditions (mean times per bacteria: 2.158 versus 0.475, $t = -3.932$, $p < 0.001$) and even under minimal growth conditions (mean times per bacteria: 2.053 versus 0.3, $t = -6.271$, $p < 0.001$, Fig. 4B).

Also, the mean number of inhibitory activities (lysis) was higher under nutrient conditions than minimal conditions for both the environmental and the clinical strains. In the case of the environmental bacteria, the inhibitory activity (lysis) recorded was 2.3 mean times higher under nutrient growth conditions than under minimal growth (mean times per bacteria: in nutrient conditions 2.025 versus in minimal conditions 0.85). For the clinical bacteria, lysis was 4.5 times higher under the nutrient growth conditions

than under minimal conditions (mean times per bacteria: 2.158 versus 0.474). However, the mean number of inhibitory effects (lysis) did not differ significantly between the clinical and environmental isolates in either nutrient or minimal conditions (nutrient condition: $t = -0.245$, $p = 0.807$; minimal condition: $t = 1.101$, $p = 0.276$).

The antagonist halo formation may indicate the dominance of one strain (top) over the other (confluent cultured), inhibiting the bacterium at the bottom (Chatterjee et al., 2017). On the other hand, the inhibitory lysis zones appeared due to the competition between the top and the bottom strains, which depends on nutrient availability. We do not rule out bacteriophage infection expression or *P. aeruginosa* responses to antagonism due to the bacterial competition stresses (LeRoux et al., 2015).

3.6. Swarming

Swarming motility was measured for clinical (n=25) and environmental (n=80) bacteria. The mean diameter of the motility zone was significantly wider for the clinical strains (mean 37.32 mm, SD 19.86) compared to the environmental strains (mean 11.81 mm, SD 8.006) ($t = -9.37$, $p < 0.001$, Fig. 5).

3.7. Twitching

Twitching motility was measured for 166 environmental and 23 clinical strains. The mean diameter of twitching zones was not significantly different between the two types (environmental: mean 9.88 mm, SD 5.08; clinical: mean 11.13 mm, SD 5.13 mm; $t = -1.1$, $p = 0.273$, Fig. 5).

3.8. Swimming

For swimming motility, we tested 144 environmental and 18 clinical strains. The mean diameter of the motility zone was wider for the clinical strains (mean 31.611, SD 10.387) compared to the environmental strains (mean 21.931, SD 12.931), showing a statistically significant difference ($t = -3.052$, $p = 0.003$, Fig. 5).

4. Discussion

Under nutrient-deprived conditions, clinical isolates initially exhibited a density increase followed by a plateau phase, whereas environmental bacteria showed a slower decline in density over the long term. This finding was unexpected because environmental *P. aeruginosa* is more likely to face starvation than clinical isolates. *P. aeruginosa* maintains high densities even under starvation conditions (Favero et al., 1971). In agreement with these findings, other studies have found that starvation strategies vary even at the strain level (Bergkessel and Delavaine, 2021; Lewenza et al., 2018). Long-term water survival also raises concerns about potential long-term sources of contamination (Legnani et al., 1999).

We found that clinical isolates showed higher resistance to ciliate predation (Fig. 2). This observation may indicate that clinical isolates have selected mechanisms that protect against predatory protists such as type III secretion systems that neutralize phagocytic killing mechanisms, which may also protect against phagocytic cells in the human tissues (Vasco and Trueba, 2021). Early biofilm production among clinical strains has been documented for acute and chronic infections (Weitere et al., 2005). The biofilm

production may depend on quorum sensing signaling, siderophores production, diguanylate cyclase activity, fatty acid-mediated signaling, etc. (Al-Wrafy et al., 2017). The host immune system may be unable to penetrate *P. aeruginosa* biofilm, and even exopolysaccharide alginate biofilms may protect *P. aeruginosa* from host phagocytosis and antibodies (Leid et al., 2005).

Our study also revealed differences in antibiotic susceptibility between clinical and environmental strains. Clinical strains were less resistant to cefepime and piperacillin-tazobactam. Pathoadaptive pressures can lead to changes in bacterial physiology, including accumulating chromosomal mutations or acquiring (or losing) mobile genetic elements (Sommer et al., 2020). The clinical condition may impose constraints on resistance acquisition phenomena. In contrast, environmental bacteria may experience harsher conditions that select for resistant phenotypes, prevent gene loss, or possess more efficient compensation mechanisms for gene acquisition (Sommer et al., 2020). This hypothesis needs to find genetic approaches to elucidate the corresponding mechanisms. In agreement with this finding, Gad et al. also found that environmental strains were more able to produce β-lactamases and be more resistant to β-lactams (including cefepime) than their clinical counterparts (Gad et al., 2007).

Interestingly, the clinical strains exhibited higher dominance (antagonist activity, Fig. 4) over other bacteria than the environmental strains and were more influenced by nutritional conditions in competition assays (lysis activity, Fig. 4). This feature may contribute to a predominance of the strain into its environmental source, or even to improve its capability to survive under stressful circumstances, such as intestinal

colonization or host infection (Chatterjee et al., 2017). Antagonistic properties may be necessary to survive in the intestinal environment which is populated by highly competitive (and antagonistic) bacteria (Peterson et al., 2020).

We also observed significant differences in the clinical strain's swarming and swimming motility compared to environmental strains. Clinical strains exhibited wider zones of motility, indicating potential physiological disparities between the two groups (Fig. 5). Greater swarming may indicate greater potential to move in viscous environments such as biofilms. Also, gene expression studies have shown that bacteria growing on viscous surfaces upregulate chaperones, heat shock proteins, secreted factors, protein secretion/export apparatus, and other genes known to be virulence factors (Overhage et al., 2008). Furthermore, swimming motility, which relies on flagella, is also considered a virulence factor in *Pseudomonas* (Murray and Kazmierczak, 2006; Tian et al., 2022). However, we did not observe a significant difference in twitching motility mediated by Type IV pili. Twitching motility is also a bacterial mechanism for bacterial movement in viscous environments, which may be necessary for environmental lifestyle. However, the mechanisms that involve this motility feature do not share with swarming or swimming ones (Burrows, 2012).

We found a permanent and diverse population of *P. aeruginosa* strains in this hospital setting (Fig. S1) which may indicate multiple introductions of this bacterium. Human activities may facilitate their introduction into the hospital setting, including pathoadapted strains (Hutchins et al., 2017). Several studies have demonstrated that while *P. aeruginosa* can be found in various natural environments, its density tends to

be higher in settings with anthropogenic activities (Crone et al., 2020). Factors such as the abundance of organic matter generated during human activities, which can serve as a nutrient source for the bacteria, may contribute to this observation (Crone et al., 2020). The higher density of *P. aeruginosa* in such environments may favor the evolution of pathoadapted strains (Sommer et al., 2020). For example, *P. aeruginosa* human colonization may be present before hospitalization and even before the development of an infection caused by the same genotype (Berthelot et al., 2001; Gómez-Zorrilla et al., 2015; Valenza et al., 2015). These findings support the idea that humans may act as reservoirs of pathoadapted strains in the hospital setting. This cycle of transmission and adaptation may contribute to the emergence of clinical strains within hospitals and the wider community. Under these assumptions, controlling its spread presents as highly complicated to achieve.

In summary, our study provides evidence for physiological differences in strains adapted to humans which may indicate that strains able to colonize humans may have been subjected to different selective forces. Higher antagonism, enhanced motility, and enhanced resistance to predators may indicate the adaptation to prosper in intestinal communities.

While this study provides valuable insights into the physiological differences and potential adaptation of *P. aeruginosa* strains in a hospital setting, several limitations should be considered. The study was conducted in a single hospital facility, which may limit the generalizability of the findings to other healthcare settings or geographic locations. Different hospitals may have varying environmental conditions and patient

populations that could influence the behavior of *P. aeruginosa* strains. The number of infected patients in the study was relatively low, which may affect the statistical power and ability to draw definitive conclusions. Furthermore, the genotyping analysis focused on the *aroE* gene, which may not fully capture the genetic diversity and relatedness of the strains. Examining a broader range of genetic markers or employing whole-genome sequencing would provide a more comprehensive understanding of strain diversity and evolutionary relationships. Finally, the study primarily focused on physiological differences and did not assess the impact of these differences on clinical outcomes, such as infection rates or patient outcomes. Further studies integrating clinical data would enhance understanding of the clinical significance of the observed strain characteristics.

Our study revealed several key findings regarding the physiological characteristics of *P. aeruginosa* strains in the hospital setting. Throughout the surveillance period, *P. aeruginosa* was ubiquitous at the hospital facilities, highlighting its long-term survival capabilities in this environment (Chatterjee et al., 2017; Hurst and Sutter, 1966; Hutchins et al., 2017)).

5. Conclusion

In conclusion, our study highlights show evidence of differences between *P. aeruginosa* strains from the hospital environment and patients While some adaptations may be detrimental to their survival outside the host, others appear to enhance their clinical potential, such as improved motility associated with the expression of virulence factors or subtle variations in survival rates and competitive abilities. Further research is needed to elucidate the underlying molecular mechanisms driving these findings.

Understanding the factors contributing to the emergence and persistence of clinical strains is crucial for developing effective strategies to control and prevent *P. aeruginosa* infections.

Plain text

Extensive diversification within *Pseudomonas aeruginosa* strains has been observed as a result of distinct lifestyles and selective pressures. Through our research, we have identified notable physiological differences between clinical (patient-derived) and environmental strains. Specifically, we have observed an increased level of antagonistic activity against other *P. aeruginosa* strains, swarming and swimming motility, and resistance to ciliate predation among clinical strains, which may contribute to their pathogenic potential. These findings could uncover molecular mechanisms that may become targets for improved preventive strategies against clinically relevant strains.

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Authors contributions

VG designed the study, took the environmental samples, conducted the assays, and wrote the manuscript. MR and ND identified the clinical strains. VK made statistical analysis figures and wrote the manuscript. TG supervised the study and wrote the manuscript.

Ethics approval and consent to participate

The study was approved as exempt from the Universidad San Francisco de Quito ethics committee CEISH-USFQ (reference 2017-063IN).

Consent for publication

Not applicable.

Data availability statement

This article and its supplementary material files include all data generated or analyzed during this study. Further inquiries can be directed to the corresponding author.

Declaration of Competing Interest

All authors have no conflicts of interest to declare.

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References

Al-Wrafy, F., Brzozowska, E., Górska, S., Gamian, A., 2017. Pathogenic factors of *Pseudomonas aeruginosa* – the role of biofilm in pathogenicity and as a target for phage therapy. Postepy Hig Med Dosw 71, 78–91. https://doi.org/10.5604/01.3001.0010.3792

Bergkessel, M., Delavaine, L., 2021. Diversity in Starvation Survival Strategies and Outcomes among Heterotrophic Proteobacteria. Microb Physiol 31, 146–162. https://doi.org/10.1159/000516215

Berthelot, P., Grattard, F., Mahul, P., Pain, P., Jospé, R., Venet, C., Carricajo, A., Aubert, G., Ros, A., Dumont, A., Lucht, F., Zéni, F., Auboyer, C., Bertrand, J.-C., Pozzetto, B., 2001. Prospective study of nosocomial colonization and infection due to *Pseudomonas aeruginosa* in mechanically ventilated patients. Intensive Care Med 27, 503–512. https://doi.org/10.1007/s001340100870

Burrows, L.L., 2012. *Pseudomonas aeruginosa* Twitching Motility: Type IV Pili in Action. Annu Rev Microbiol 66, 493–520. https://doi.org/10.1146/annurev-micro-092611-150055

Chatterjee, P., Davis, E., Yu, F., James, S., Wildschutte, J.H., Wiegmann, D.D., Sherman, D.H., McKay, R.M., LiPuma, J.J., Wildschutte, H., 2017. Environmental Pseudomonads Inhibit Cystic Fibrosis Patient-Derived *Pseudomonas aeruginosa*. Appl Environ Microbiol 83. https://doi.org/10.1128/AEM.02701-16

Clinical and Laboratory Standards Institute, 2022. Performance Standards for Antimicrobial Susceptibility Testing, 32nd ed. Clinical and Laboratory Standards Institute (CLSI).

Crone, S., Vives-Flórez, M., Kvich, L., Saunders, A.M., Malone, M., Nicolaisen, M.H., Martínez-García, E., Rojas-Acosta, C., Catalina Gomez-Puerto, M., Calum, H., Whiteley, M., Kolter, R., Bjarnsholt, T., 2020. The environmental occurrence of *Pseudomonas aeruginosa*. APMIS 128, 220–231. https://doi.org/10.1111/apm.13010

Curran, B., Jonas, D., Grundmann, H., Pitt, T., Dowson, C.G., 2004. Development of a Multilocus Sequence Typing Scheme for the Opportunistic Pathogen *Pseudomonas aeruginosa*. J Clin Microbiol 42, 5644–5649. https://doi.org/10.1128/JCM.42.12.5644-5649.2004

Favero, M.S., Carson, L.A., Bond, W.W., Petersen, N.J., 1971. *Pseudomonas aeruginosa* : Growth in Distilled Water from Hospitals. Science (1979) 173, 836–838. https://doi.org/10.1126/science.173.3999.836

Gad, G.F., El-Domany, R.A., Zaki, S., Ashour, H.M., 2007. Characterization of *Pseudomonas aeruginosa* isolated from clinical and environmental samples in Minia, Egypt: prevalence, antibiogram and resistance mechanisms. Journal of Antimicrobial Chemotherapy 60, 1010–1017. https://doi.org/10.1093/jac/dkm348

Gómez-Zorrilla, S., Camoez, M., Tubau, F., Cañizares, R., Periche, E., Angeles Dominguez, M., Ariza, J., Peña, C., 2015. Prospective Observational Study of Prior Rectal Colonization Status as a Predictor for Subsequent Development of *Pseudomonas aeruginosa* Clinical Infections. https://doi.org/10.1128/AAC.04636-14

Ha, D.-G., Kuchma, S.L., O'Toole, G.A., 2014. Plate-Based Assay for Swimming Motility in *Pseudomonas aeruginosa*. pp. 59–65. https://doi.org/10.1007/978-1-4939-0473-0_7

Hernández-Jiménez, P., López-Medrano, F., Fernández-Ruiz, M., Silva, J.T., Corbella, L., San-Juan, R., Lizasoain, M., Díaz-Regañón, J., Viedma, E., Aguado, J.M., 2022. Risk Factors and Outcomes for Multidrug Resistant *Pseudomonas aeruginosa* Infection in

Immunocompromised Patients. Antibiotics 11, 1459. https://doi.org/10.3390/antibiotics11111459

Hilbi, H., Weber, S.S., Ragaz, C., Nyfeler, Y., Urwyler, S., 2007. Environmental predators as models for bacterial pathogenesis. Environ Microbiol 9, 563–575. https://doi.org/10.1111/j.1462-2920.2007.01238.x

Hurst, V., Sutter, V.L., 1966. Survival of *Pseudomonas aeruginosa* in the Hospital Environment. J Infect Dis 116, 151–154.

Hutchins, C.F., Moore, G., Thompson, K.-A., Webb, J., Walker, J.T., 2017. Contamination of hospital tap water: the survival and persistence of *Pseudomonas aeruginosa* on conventional and 'antimicrobial' outlet fittings. Journal of Hospital Infection 97, 156–161. https://doi.org/10.1016/j.jhin.2017.06.005

Juan, C., Peña, C., Oliver, A., 2017. Host and Pathogen Biomarkers for Severe *Pseudomonas aeruginosa* Infections. J Infect Dis 215, S44–S51. https://doi.org/10.1093/infdis/jiw299

Larsson, D.G.J., Flach, C.-F., 2022. Antibiotic resistance in the environment. Nat Rev Microbiol 20, 257–269. https://doi.org/10.1038/s41579-021-00649-x

Legnani, P., Leoni, E., Rapuano, S., Turin, D., Valenti, C., 1999. Survival and growth of *Pseudomonas aeruginosa* in natural mineral water: a 5-year study. Int J Food Microbiol 53, 153–158. https://doi.org/10.1016/S0168-1605(99)00151-8

Leid, J.G., Willson, C.J., Shirtliff, M.E., Hassett, D.J., Parsek, M.R., Jeffers, A.K., 2005. The Exopolysaccharide Alginate Protects *Pseudomonas aeruginosa* Biofilm Bacteria from IFN-γ-Mediated Macrophage Killing. The Journal of Immunology 175, 7512–7518. https://doi.org/10.4049/jimmunol.175.11.7512

LeRoux, M., Kirkpatrick, R.L., Montauti, E.I., Tran, B.Q., Peterson, S.B., Harding, B.N., Whitney, J.C., Russell, A.B., Traxler, B., Goo, Y.A., Goodlett, D.R., Wiggins, P.A., Mougous, J.D., 2015. Kin cell lysis is a danger signal that activates antibacterial pathways of *Pseudomonas aeruginosa*. Elife 4. https://doi.org/10.7554/eLife.05701

Lewenza, S., Abboud, J., Poon, K., Kobryn, M., Humplik, I., Bell, J.R., Mardan, L., Reckseidler-Zenteno, S., 2018. *Pseudomonas aeruginosa* displays a dormancy phenotype during long-term survival in water. PLoS One 13, e0198384. https://doi.org/10.1371/journal.pone.0198384

Liu, J., Dong, Y., Wang, N., Li, S., Yang, Y., Wang, Y., Awan, F., Lu, C., Liu, Y., 2018. *Tetrahymena* thermophila Predation Enhances Environmental Adaptation of the Carp Pathogenic Strain Aeromonas hydrophila NJ-35. Front Cell Infect Microbiol 8. https://doi.org/10.3389/fcimb.2018.00076

Mortimer, M., Petersen, E.J., Buchholz, B.A., Orias, E., Holden, P.A., 2016. Bioaccumulation of Multiwall Carbon Nanotubes in *Tetrahymena* thermophila by Direct

Feeding or Trophic Transfer. Environ Sci Technol 50, 8876–8885. https://doi.org/10.1021/acs.est.6b01916

Murray, T.S., Kazmierczak, B.I., 2006. FlhF Is Required for Swimming and Swarming in *Pseudomonas aeruginosa*. J Bacteriol 188, 6995–7004. https://doi.org/10.1128/JB.00790-06

O'Toole, G.A., Kolter, R., 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol Microbiol 30, 295–304. https://doi.org/10.1046/j.1365-2958.1998.01062.x

Overhage, J., Bains, M., Brazas, M.D., Hancock, R.E.W., 2008. Swarming of *Pseudomonas aeruginosa* Is a Complex Adaptation Leading to Increased Production of Virulence Factors and Antibiotic Resistance. J Bacteriol 190, 2671–2679. https://doi.org/10.1128/JB.01659-07

Pendleton, J.N., Gorman, S.P., Gilmore, B.F., 2013. Clinical relevance of the ESKAPE pathogens. Expert Rev Anti Infect Ther 11, 297–308. https://doi.org/10.1586/eri.13.12

Peterson, S.B., Bertolli, S.K., Mougous, J.D., 2020. The Central Role of Interbacterial Antagonism in Bacterial Life. Current Biology 30, R1203–R1214. https://doi.org/10.1016/j.cub.2020.06.103

Rice, L.B., 2008. Federal Funding for the Study of Antimicrobial Resistance in Nosocomial Pathogens: No ESKAPE. J Infect Dis 197, 1079–1081. https://doi.org/10.1086/533452

Scherwass, A., Erken, M., Arndt, H., 2016. Grazing Effects of Ciliates on Microcolony Formation in Bacterial Biofilms, in: Microbial Biofilms - Importance and Applications. InTech. https://doi.org/10.5772/63516

Sommer, L.M., Johansen, H.K., Molin, S., 2020. Antibiotic resistance in *Pseudomonas aeruginosa* and adaptation to complex dynamic environments. Microb Genom. https://doi.org/10.1099/mgen.0.000370

Tamura, K., Stecher, G., Kumar, S., 2021. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol Biol Evol 38, 3022–3027. https://doi.org/10.1093/molbev/msab120

Tian, M., Wu, Z., Zhang, R., Yuan, J., 2022. A new mode of swimming in singly flagellated *Pseudomonas aeruginosa*. Proceedings of the National Academy of Sciences 119. https://doi.org/10.1073/pnas.2120508119

Tremblay, J., Déziel, E., 2010. Gene expression in *Pseudomonas aeruginosa* swarming motility. BMC Genomics 11, 587. https://doi.org/10.1186/1471-2164-11-587

Valenza, G., Tuschak, C., Nickel, S., Krupa, E., Lehner-Reindl, V., Höller, C., 2015. Prevalence, antimicrobial susceptibility, and genetic diversity of *Pseudomonas*

aeruginosa as intestinal colonizer in the community. Infect Dis 47, 654–657. https://doi.org/10.3109/23744235.2015.1031171

Vasco, G., Trueba, G., 2021. *Pseudomonas aeruginosa* transition from environmental generalist to human pathogen. ACI Avances en Ciencias e Ingenierías 13, 11. https://doi.org/10.18272/aci.v13i1.2225

Weitere, M., Bergfeld, T., Rice, S.A., Matz, C., Kjelleberg, S., 2005. Grazing resistance of *Pseudomonas aeruginosa* biofilms depends on type of protective mechanism, developmental stage and protozoan feeding mode. Environ Microbiol 7, 1593–1601. https://doi.org/10.1111/j.1462-2920.2005.00851.x

Werlin, R., Priester, J.H., Mielke, R.E., Krämer, S., Jackson, S., Stoimenov, P.K., Stucky, G.D., Cherr, G.N., Orias, E., Holden, P.A., 2011. Biomagnification of cadmium selenide quantum dots in a simple experimental microbial food chain. Nat Nanotechnol 6, 65–71. https://doi.org/10.1038/nnano.2010.251

Table 1. Description of the environmental samples taken during the surveillance and the number of *Pseudomonas aeruginosa* isolates.

Service	Number of sinks	Number of sink samples	Number of positive samples	Samples per month	Number of isolates obtained
Internal Medicine	19	36	36	3	144
Surgery	19	30	18	2,5	48
Infectiology	16	14	2	1,17	5
Intensive Care	13	47	43	3,9	142
Traumatology	14	12	12	1	34
Total	81	139	111	11,57	337

Supplementary Table 1. Two tails T-test *t* values and correspondent *p* values from the difference between the zone diameter area of inhibition due to antibiotic resistance Kirby-Bauer technique for nine antibiotics of environmental versus clinical isolates.

Independent Samples T-Test

	t	df	*p*
Amikacin	-1.184	40	0.243
Aztreonam	0.289	41	0.774
Cefepime	-2.837	42	0.007 [a]
Ceftazidime	-1.897	42	0.065 [a]
Ciprofloxacin	-1.986	42	0.054 [a]
Imipenem	-1.793	42	0.080
Levofloxacin	-1.991	39	0.054 [a]
Meropenem	-0.865	42	0.392 [a]
Piperacillin/Tazobactam	-2.145	42	0.038 [a]

Note. Student's t-test.

The Brown-Forsythe test is significant ($p < .05$), suggesting a violation of the equal variance assumption.

Figure S1. UPGMA phylogenetic tree of the *aroE* allele's sequences from pathogenic and environmental isolates. Seventy-seven sequences: 9 from PubMLST allele reference sequences and 68 from this study were included in the tree, and 499 nucleotide sites were used. The clinical isolates are drawn as orange-filled squares (Type). The bootstraps are annotated as percentages; branch lengths are annotated upside the bootstrap value.

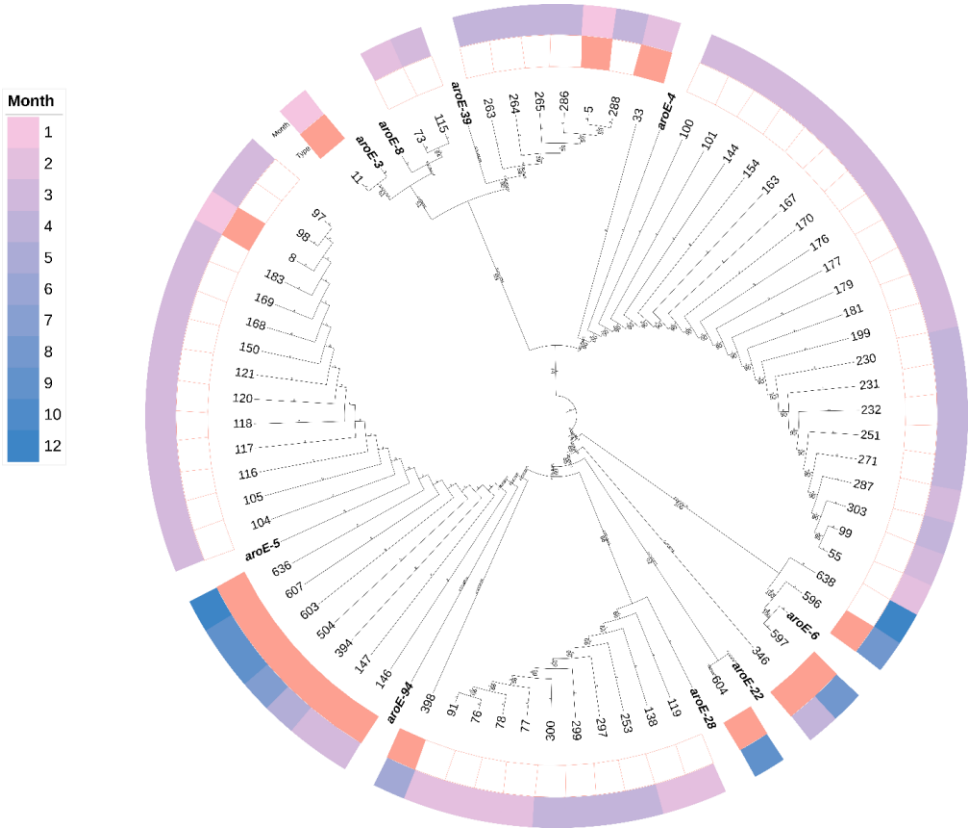


Figure 1. Water survival bacterial counts, CFU/ml transformed to LN of clinical and environmental strains at 12 time points. Error bars represented 95% confidence intervals.

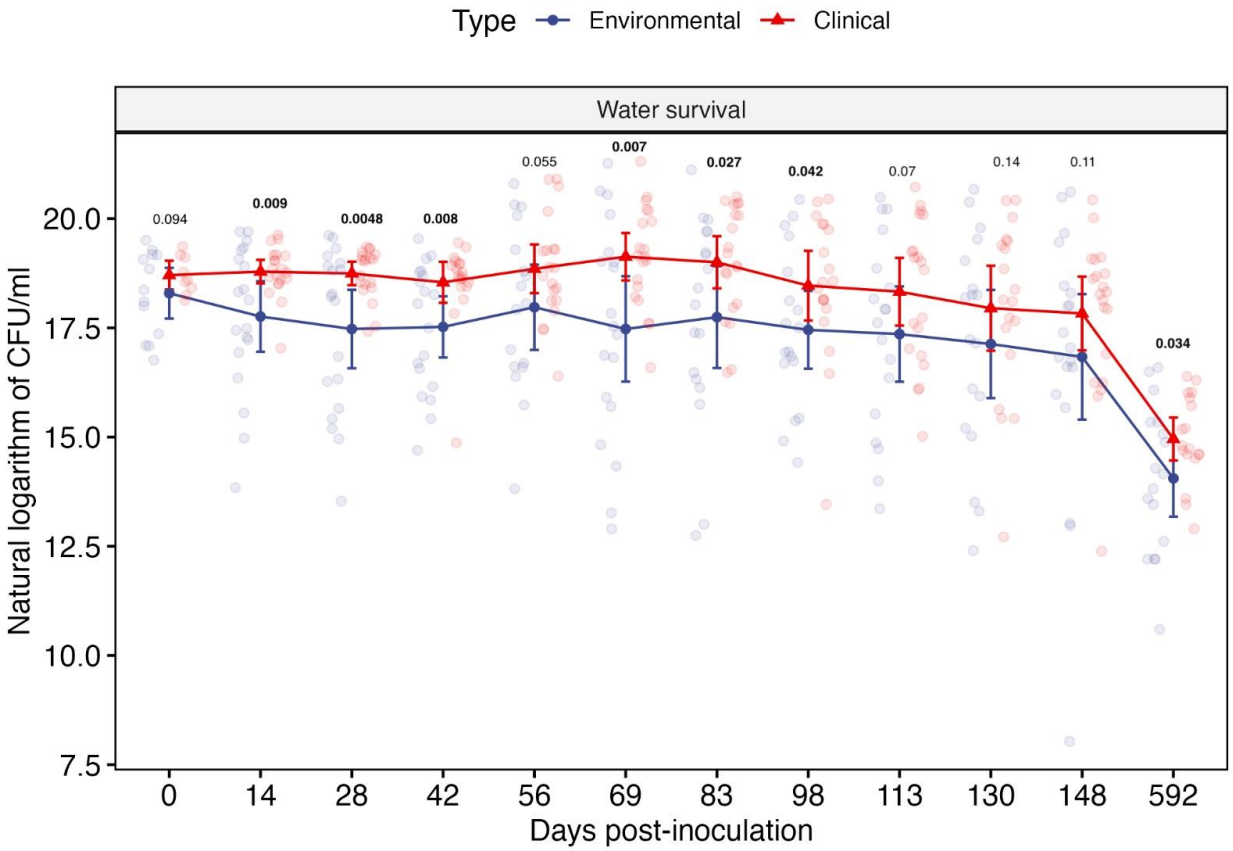


Figure 2. Survival differences among clinical and environmental *P. aeruginosa* strains with the bacteria grazer *T. pyriformis* host at different time points. A. Box plots showing the count on bacterial density without ciliates CFU/ml (control). B. Box plots showing the count on bacterial density with the ciliate *T. pyriformis* CFU/ml (test). C. Box plots showing the ciliate density of the tests in cells/ml. D. Box plots showing the comparison of the counts of bacteria density in CFU/ml between the controls and the tests. E. Box plots showing the MOI changes in time intervals (bacteria:ciliates). Error bars 95% confidence intervals. Counts display as the natural logarithm LN.

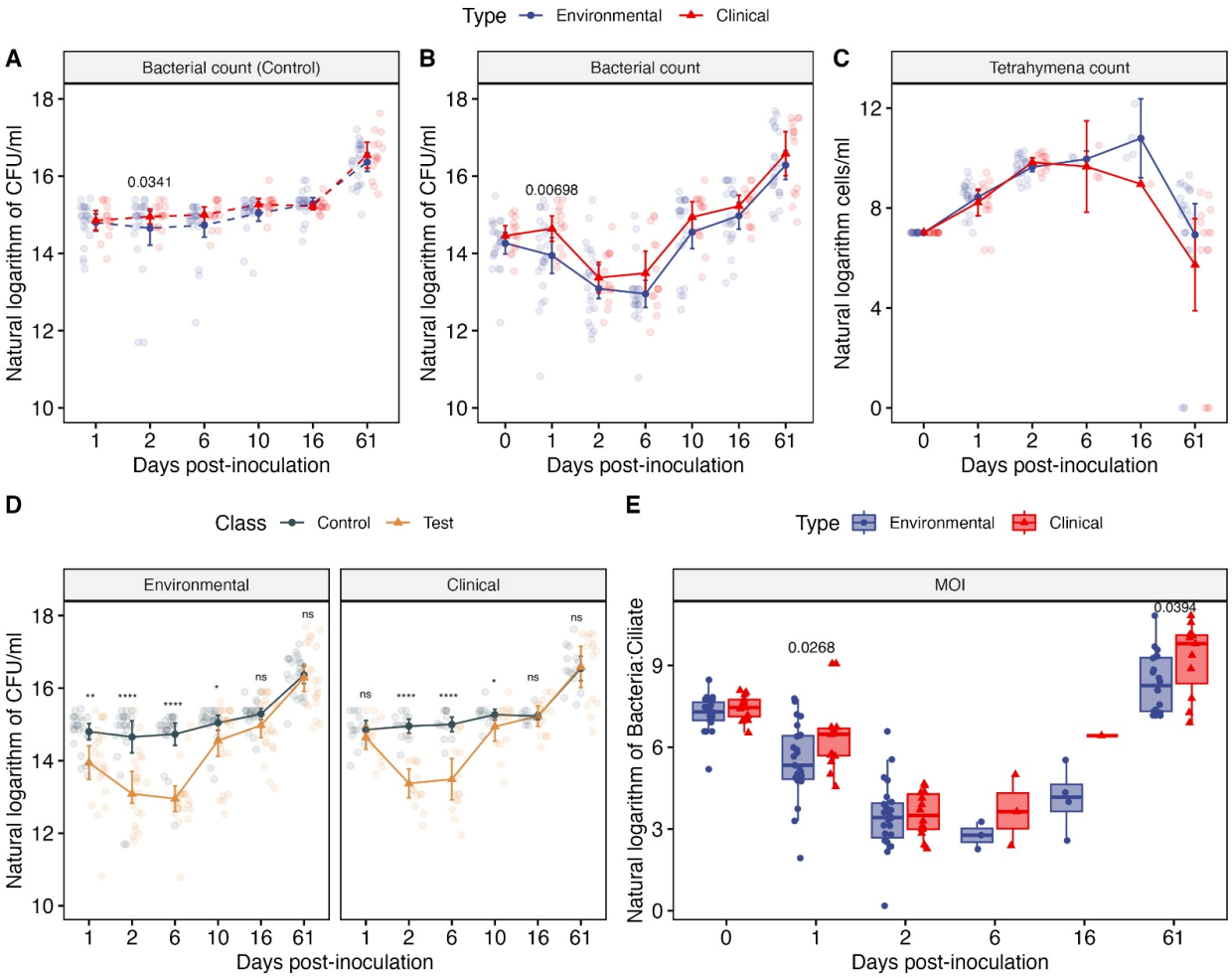


Figure 3. Antibiotic resistance boxplots of the zone diameters differences among clinical and environmental strains under antibiotic resistance Kirby-Bauer test for nine antibiotics. Error bars 95% confidence interval.

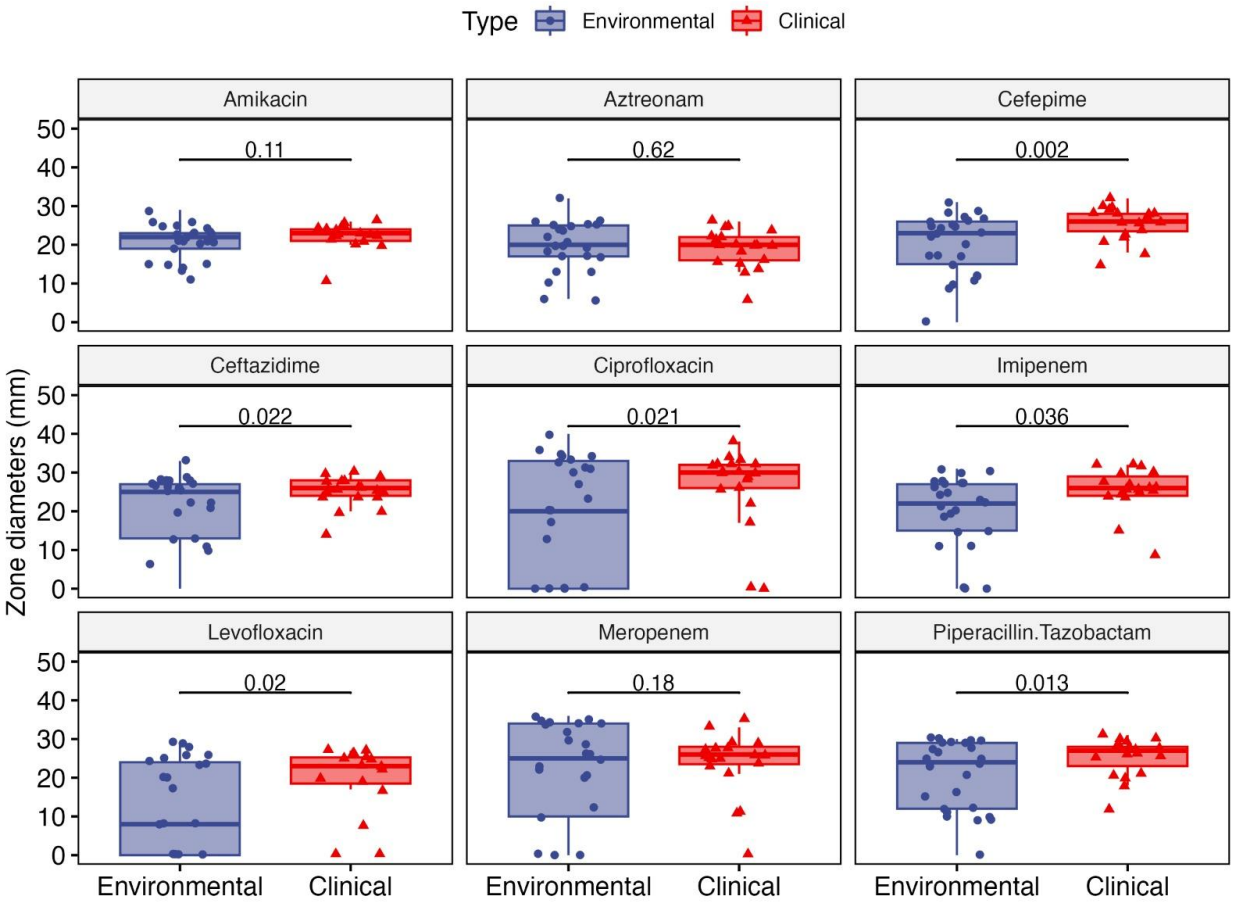
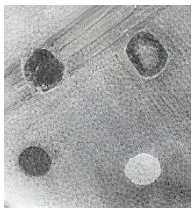


Figure 4. *In-vitro* competitions. A. Photo improved by artificial intelligence of the left=lysis, right-up corner=halo, right-down=no inhibition. B. Bar Plots of the mean halo or lysis activity times per bacteria under nutrient and minimal growth conditions.

A.



B.

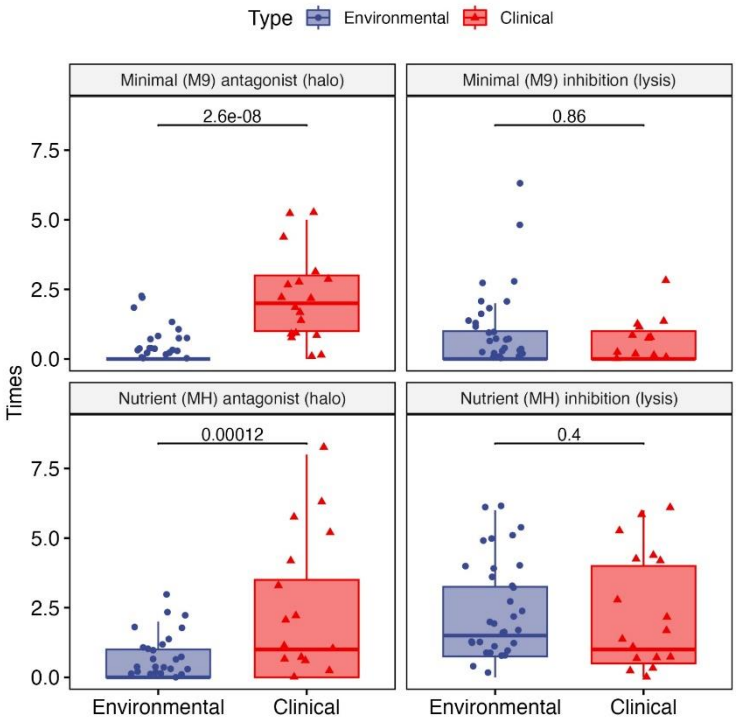
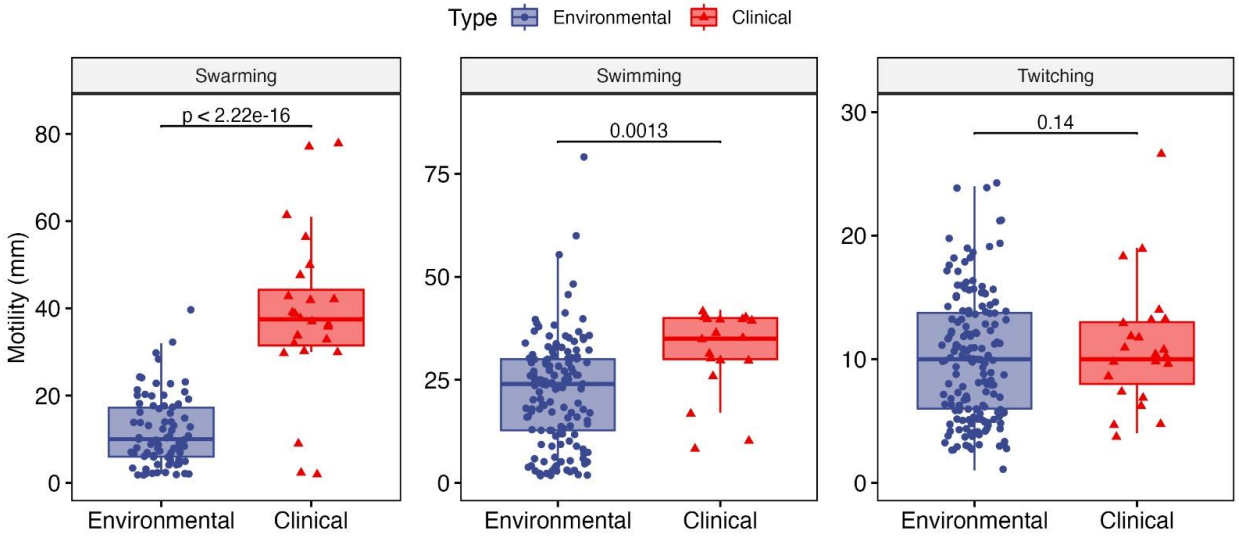
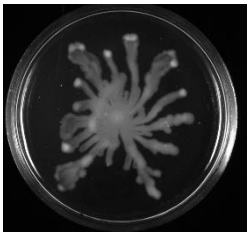


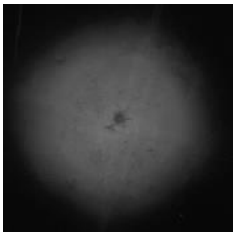
Figure 5. Swarming, swimming, and twitching motility diameter in clinical versus environmental strains (mm). Error bars 95% confidence interval. Photos under the boxplots represent the motility differences on agar tests.



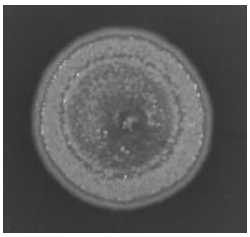
Swarming



Swimming



Twitching



CHAPTER V

GENERAL CONCLUSION

The emergence of new human pathogens from the environment is a priority for public health [1]. However, it is crucial to understand the conditions that underlie their niches and, for instance, the species living in them [2] to study possible sources of strains that can adapt to human hosts and to identify evolutionary features that may enable future approaches against this threat.

This project used genomic and physiological approaches to investigate evolutionary adaptations in the opportunistic bacteria *Pseudomonas aeruginosa*. First, we demonstrated the presence of the same *P. aeruginosa* strain in the intestines before it was detected in clinical infection in immunocompromised individuals. This feature may be crucial because the microbiota is a complex and antagonistic environment, and this bacteria's survival strategies may have evolved outside the human host. The environment outside the host presents strong antagonism activity, given by the diversity of niches, nutrient availability, and the presence of grazing protozoa [3,4]. *Pseudomonas* itself is considered an antagonistic bacterium from soil [4]. Thus, *P. aeruginosa* survival adaptations in the environment may be repurposed to enable their colonization of the intestines. For example, adapting T3SS and effector proteins in bacteria allows them to survive in various environments, including the ability to evade the human immune system [3]. The T3SS apparatus is activated when the bacterial cell is in proximity to a eukaryotic protozoa host. This allows the bacteria to attach to the host's cell and transfer effector proteins (such as exotoxins) into its interior. These

proteins, such as ExoU, ExoS, and ExoT, may act as phospholipases or trigger cytotoxic effects in the eukaryotic cell [5]. During human infection, the same apparatus and effector proteins disrupt cell functions and ultimately kill macrophages [3].

Identifying the source of bacteria during colonization can pose unique challenges. Bacteria genotypes acquired before hospitalization do not usually share clonal relationships, nor are they related to strains acquired in the hospital [6]. This diversity in genotypes may reflect the diversity of the host's environment outside the hospital. However, when a patient acquires colonization/infection during hospitalization, the genotypes are more likely to be shared by many patients, such as in the case of *P. aeruginosa* ST-253 strains seen in this study. In such instances, the most probable source could be cross-contamination with bacteria consistently present in other patients, hospital staff, or even environmental sources. Future approaches must address whether another host developing colonization but not infection may act as a masked source.

One of the more critical barriers to pathogenicity is the ability to colonize the intestines. Not many bacteria can be successful colonizers, mainly because the intestinal microbiota represents a harsh and antagonistic force and intense competition for nutrients [7]. For this reason, bacteria that can colonize the human microbiota must have evolved in another, equally (or more) antagonistic environment. One factor that may impact colonization is the disruption of the microbiota or host immunological barriers caused by disease. Additionally, in accordance with the bacteria's ability to

colonize, it must have host immunological evasion strategies and bacterial virulence factors that enable its ability to cause disease.

The physiological approach confirmed a higher antagonistic bacterial activity among clinical strains compared to environmental counterparts. This feature may reflect the population diversity in *P. aeruginosa* species, where the clinical strains are selected for their ability to survive under hostile biological conditions (presence of other bacteria, bacteriophages, and grazing protozoa). Given the importance of immune evasion, it is feasible to hypothesize that evolution in the environment with grazing protozoa may select strains able to evade phagocytosis, which shares mechanisms with the macrophages [3]. Such a condition is present in the intestines, where the bacteria must survive the strong antagonistic microbiota activity and the innate immunological barriers. Clinical strains showed higher resistance to ciliate predation, confirming that the bacteria may have repurposed its abilities to compete with other hosts for the ability to support human host immune system responses, as we explained before. The clinical strains of *P. aeruginosa* also demonstrated the ability to stay long under starvation. The complexity of the intestine's niche regarding cohesive speciation or nutrient availability of critical elements such as iron may underline this feature. Clinical strains were less resistant to cefepime and piperacillin-tazobactam than their environmental counterparts, which may represent clinical bacteria constraints in antibiotic-resistant acquisitions. The host adaptation may induce the loss of compensatory mechanisms that may enable resistance acquisition. However, this last feature may also reflect the outcompete problems that human-adapted strains may face once they return to the environment. Finally, the conservation of swarming and

swimming motility was a feature of the clinical strains, thus demonstrating that motility in viscous environments may be a vital adaptation of pathoadapted strains.

FUTURE WORK

I will present recommendations for future experiments that corroborate the findings of this thesis.

Strategies for the P. aeruginosa colonization eradication as a tool for the prevention of clinical infections in immunocompromised patients

Our research indicates that the colonization of bacteria in the intestines can act as a selective force for the selection of pathogenic strains. To further investigate this, I propose conducting clinical trials with a prospective multicentric cohort of patients admitted to hospitalization rooms, such as intensive care units. I will collect stool or rectal samples from these patients to analyze the presence of *P. aeruginosa* colonization. Patients who are colonized but do not show any apparent disease will be recruited for the cohort and divided into two groups: Group 1 will receive an intervention. At the same time, Group 2 will serve as the control group. Patients who are not colonized at admission will be part of Group 3. All group participants will be screened weekly for *P. aeruginosa* rectal colonization. Additionally, all groups will be assessed for new infections acquired after hospitalization.

The intervention for Group 1 will involve determining the strain susceptibility to at least two out of six or more bacteriophages present in a bacteriophage cocktail or in a strain-specific cocktail. The cocktail will then be administered to the patient to eradicate the

colonization. This strategy will measure (a) the safety of the treatment (adverse events, serious adverse events, and adverse events of special interest), (b) the risk of developing a *P. aeruginosa* infection during the hospital stay (absolute risk-incidence, relative risk-risk ratio), (c) the risk of developing an infection by the same genotype present in the intestine, (d) the decolonization rate, (e) the time frame that the decolonization is effective in reducing the infection rate, (f) the risk of acquiring new intestinal colonization, (g) the morbidity and mortality rates, (h) the bacteria resistance *in-vivo* to the bacteriophage treatment, and (i) the infection failure in cases where there is an infection process.

Changes in microbiota when colonization of P. aeruginosa occurs and when infection occurs [7]

To test this hypothesis, I propose conducting a surveillance study on a cohort of patients who will be admitted to intensive healthcare units. They should be screened for *P. aeruginosa* intestinal colonization from the time of admission to their discharge, regardless of whether they have been diagnosed with an infection or if they develop an infection related to the bacteria during their hospital stay. The screening can identify whether (a) they are newly colonized during their hospital stay, (b) the changes in the strains that can colonize their intestines, and (c) in cases where patients develop an infection by the same intestinal strain, to determine if the treatment for the infection may disrupt their colonization status. Additionally, I will conduct a functional study to understand the changes in the expression of the T6SS, a bacterial apparatus closely related to the antagonism activity between bacteria in the intestines. Through this approach, I hope to examine if (a) colonization is related to a lesser harsh antagonism

due to the patient's disease status, antibiotic, and anti-inflammatories consumption, which may enable *P. aeruginosa* colonization, (b) whether colonization induces T6SS expression, and if (c) these changes are reflected in the taxonomy and functional activities in the microbiota. Follow-up with the surviving patients will be necessary to understand if (a) microbial functionality can be restored after hospital discharge and (b) if *P. aeruginosa* colonization has been overcome.

Antibiotic resistance acquisition

The study demonstrated significant differences in antibiotic resistance between clinical and environmental strains. I recommend conducting *in-vitro* experiments to explore bacterial fitness changes in clinical and environmental strains that become resistant due to transformation, conjugation, or mutation. Additionally, creating a library of mutants may make it possible to identify viable epistatic mutants and determine if they contribute to resistance acquisition to clinical and environmental strains.

Environmental counterparts may outcompete clinical-adapted strains outside the host

Studying co-evolution can help demonstrate the hypothesis. Cultivating and co-cultivating bacteria under nutrient deprivation and richness conditions can determine if bacterial density is affected. Survival strategies employed by clinical strains under nutrient deprivation can be more effective than their environmental counterparts in a clinical context; however, they may be detrimental when competition happens outside the host. Suppose there is a difference in survival strategies. In that case, it is essential to address their mechanisms as they may have the potential to be exploited for identifying pathogenic-like strains and developing antibacterial treatments. Additionally,

suppose outcompeting is demonstrated in clinical bacteria, which may reveal the possibility that potential pathogenic *P. aeruginosa* may be rising in nature. In that case, it is possible to determine if intestinal carriage and human-to-human transmission could be a potential source of such strains, thus enhancing the need for strategies to treat colonization in patients.

Finding bacteriophages to treat P. aeruginosa colonization

Antibiotics have been known to impact the microbiota's functionality and taxonomy significantly. However, linking these changes to patient morbidity and mortality is still challenging. Nevertheless, they have been found to contribute strongly to the selection of antibiotic-resistant bacteria, which can become new colonizers and be spread to other humans or the environment, causing infections that are difficult to eliminate and posing a significant threat. This has led to exploring alternative antibacterial strategies, such as bacteriophage treatment. Bacteriophages are strain-specific and have a lower spectrum than antibiotics, meaning they are less likely to disrupt the microbiota's taxonomy and functionality. By isolating bacteriophages from the environment and developing practices to formulate bacteriophage cocktails to treat colonization, it may be possible to prevent infections and reduce the impact on microbiota functionality at a lower cost. The bacteriophage approach can also address de-colonization regardless of multidrug-resistant strains.

Selection and diversification of antagonistic strains in a gradient of antagonism activity

I believe the evolutionary approach is promising for understanding niche origins, as supported by Baquero et al. [2]. A niche can have various gradients of nutrients, water,

and predators, which allow for diversifying evolution in the strains that live within it according to the selective forces acting upon each gradient. This diversification may also assist in creating new niches for more diversified strains. It will be interesting to see how antagonism works as a selective force among niches and contributes to the emergence of new ones.

Summarizing, environmental bacteria growing in complex niches (antagonistic, nutrient deprivation, protozoan host presence, viscosity) are significant threats to selecting potential pathoadapted strains. Genomic and physiological approaches are necessary to understand the underlying mechanisms of the origin of potentially pathogenic strains. Alternative prevention methods can also be developed to prevent colonization of the intestines by *P. aeruginosa* in patients at risk of infection as a prophylactic approach.

References

[1] World Health Organization. Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug-resistant bacterial infections, including tuberculosis. Geneva: 2017.

[2] Baquero F, Coque TM, Galán JC, Martinez JL. The Origin of Niches and Species in the Bacterial World. Front Microbiol 2021;12. https://doi.org/10.3389/fmicb.2021.657986.

[3] Vasco G, Trueba G. *Pseudomonas aeruginosa* transition from environmental generalist to human pathogen. ACI Avances En Ciencias e Ingenierías 2021;13:11. https://doi.org/10.18272/aci.v13i1.2225.

[4] Peterson SB, Bertolli SK, Mougous JD. The Central Role of Interbacterial Antagonism in Bacterial Life. Current Biology 2020;30:R1203–14. https://doi.org/10.1016/j.cub.2020.06.103.

[5] Abd H, Wretlind B, Saeed A, Idsund E, Hultenby K, Sandström Gunnar. *Pseudomonas aeruginosa* Utilises Its Type III Secretion System to Kill the Free-Living Amoeba Acanthamoeba castellanii. Journal of Eukaryotic Microbiology 2008;55:235–43. https://doi.org/10.1111/j.1550-7408.2008.00311.x.

[6] Berthelot P, Grattard F, Mahul P, Pain P, Jospé R, Venet C, et al. Prospective study of nosocomial colonization and infection due to *Pseudomonas aeruginosa* in mechanically ventilated patients. Intensive Care Med 2001;27:503–12. https://doi.org/10.1007/s001340100870.

[7] Kim S, Covington A, Pamer EG. The intestinal microbiota: Antibiotics, colonization resistance, and enteric pathogens. Immunol Rev 2017;279:90–105. https://doi.org/10.1111/imr.12563.

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