Current Research in Microbial Sciences 8 (2025) 100348

Contents lists available at ScienceDirect

Current Research in Microbial Sciences

journal homepage: www.sciencedirect.com/journal/current-research-in-microbial-sciences

Chronic wounds and adaptive *Pseudomonas aeruginosa*: A phenotypic and genotypic characterization

Kasandra Buchholtz [a,1], Rie Jønsson [a,1], Rasmus L. Marvig [b], Biljana Mojsoska [a], Karen Angeliki Krogfelt [a,*]

[a] Department of Science and Environment, Roskilde University, Denmark
[b] Department of Genomic Medicine, Rigshospitalet, Copenhagen, Denmark

ARTICLE INFO

Keywords:
Pseudomonas aeruginosa
Adaptation
Phylogenetic diversity
Morphology
Chronic wound

ABSTRACT

Phenotypic and genetic diversity is found in varying prevalence in clinical populations where beneficial adaptations enable the bacteria to avoid recognition and eradication by the host immune system. This study aimed to investigate the presence of *Pseudomonas aeruginosa* in chronic venous leg ulcers wounds over an 8-week time course. This was performed using genomic and phenotypic approaches to understand the survival and persistence of *Pseudomonas* strains. The findings of this study show that the two patients were colonized with a recurring *P. aeruginosa* genotype with only minor phenotypic differences and few SNP differences, suggesting that the *Pseudomonas* isolates present in the wound can survive and proliferate in the host's hostile environment. The results provided from this study will allow us to understand *P. aeruginosa* colonization during a 8 week time period.

1. Introduction

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic human pathogen. Responsible for infecting immunocompromised patients, *P. aeruginosa* is often seen in individuals with cystic fibrosis, urinary infections, and chronic wounds (Moradali et al., 2017).

Wounds infected with *P. aeruginosa* are important in diabetic patients as they heal very slowly, and in venous leg ulcers are seen to be larger in size and associated with more severe wound outcomes compared to wounds where the pathogen is not present (Halbert et al., 1992; Madsen et al., 1996; Kirketerp-Møller et al., 2008; Gjødsbøl et al., 2006).

The large genome size and the genetic complexity of *P. aeruginosa* could explain the bacterium's pathogenicity and unique ability to persist and thrive under different environmental conditions (Stover et al., 2000). In chronic wounds, the expression of multiple virulence factors (such as pyocyanin) is regulated by quorum sensing systems such as *las, rhl,* and *pqs* (Muller et al., 2009; Serra et al., 2015). Pyocyanin is a redox-active phenazine pigment that can interact with molecular oxygen and induce oxidative stress and cell damage to the host. This release of pyocyanin has been shown to enhance the adhesion, microcolony, and biofilm formation of *P. aeruginosa*, thus promoting the ability of the bacterium to form chronic wound infections (Hall et al., 2016).

Treating *P. aeruginosa* infection is extremely difficult once established, probably due to the formation of antimicrobial-tolerant biofilms (Olivares et al., 2020). Therefore, studies are needed to investigate *P. aeruginosa* in clinically relevant environments to find novel ways of eradicating chronic infections.

This study characterized 16 clinical wound isolates of *P. aeruginosa* collected from two patients with chronic venous leg ulcers over an 8-week course for their phenotypic and genotypic diversity. We aimed to identify the clonal lineages to explore the hypothesis of whether the clinical wound isolates originated from the same hospital lineage of *P. aeruginosa.* Furthermore, we compared the strains phenotypically and genotypically to characterize the heterogeneity among the isolates during a chronic infection.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Clinical wound isolates were collected over two months from patients admitted with persistent venous leg ulcers at Copenhagen Wound

* Corresponding author.
E-mail address: karenak@ruc.dk (K.A. Krogfelt).
[1] These authors contributed equally to the work, and each reserves the right to put their name first on their respective CVs.

https://doi.org/10.1016/j.crmicr.2025.100348

Available online 3 February 2025

Healing Centre, Bispebjerg Hospital in Denmark (Gjødsbøl et al., 2006). Isolates were collected using filter paper pads, biopsies, and charcoal swabs throughout eight weeks of regular wound observations. All samples were spread on 5 % blood agar, chocolate agar, chocolate agar with cysteine and K-vitamin; and a blue agar plate and incubated at 37 °C under anaerobic and aerobic conditions. Two patients (one male and one female) with repeated *P. aeruginosa* infection were chosen (Table 1). None of the patients had received antibiotics at least 14 days before or during the study period (Gjødsbøl et al., 2006).

All samples were streaked and identified (VITEK GNI+, ID 32 Kits, BioMerieux, Marcy L'Etoile, France), and bacterial isolates were stored at −80 °C in Luria-Broth (LB) with 10 % glycerol. All strains were grown at 37 °C. The clinical isolates used are listed in Table 2. Reference strains *P. aeruginosa* PAO1 and PA14 were used for comparison (Stover et al., 2000; He et al., 2004).

2.2. Growth rate determination

Overnight cultures diluted in LB-broth (Merck, Denmark) to OD_{600} 0.03 were used for growth rate measurements in a conical flask. All strains' growth rates were measured by measuring the optical density during growth (~20 min) in 50 ml LB-broth in 250-ml flasks at 180 RPM at 37 °C. Growth rates were expressed as generation times in minutes. Three biological replicas for each strain were made.

2.3. Quantification of pyocyanin

A serial dilution of pyocyanin in LB-broth (100, 50, 25, 12, 6 μM) was made from 1 mg/ml of pyocyanin as a reference. UV–vis spectrophotometry was used to quantify pyocyanin in LB media. One colony of each clinical isolate of *P. aeruginosa* and PAO1 was inoculated in 5 ml of LB-broth and incubated overnight (O.N.) in a water bath at 37 °C, 180 RPM. After that, bacterial supernatants were filtered using a 0.45 μm filter (Frisenette Aps). The production of the quorum-sensing pigment was determined by measuring the absorption at 311 nm. The absorption at 311 nm was converted into a concentration using pyocyanin's extinction coefficient in LB media. For normalization, a colony forming units (CFU) assay was performed from the overnight culture.

2.4. Determination of the minimum inhibition concentration

Clinically relevant antimicrobials were used to determine the phenotypic antimicrobial susceptibility (tobramycin, ciprofloxacin, meropenem, and polymyxin B) ($n = 3$). A cation-adjusted Mueller-Hinton Borth (MHIIB) (Merck, Denmark) was used to test the susceptibility to tobramycin (Wiegand et al., 2008). The concentration range of the antibiotics used in this study was as follows: tobramycin 32–0.05 μg/ml; ciprofloxacin 20–0.031 μg/ml; meropenem, and polymyxin B, 40–0.062 μg/ml. The plate was sealed and incubated O.N. at 37 °C. CFU assay was performed on an exponentially growing culture with 1:500 diluted culture to estimate the final concentration. The expected concentration range was 2×10^5–8×10^5 CFU/ml. The results were obtained 24 h after incubation.

Table 2
Clinical wound isolates were used in this study.

Isolate no.	Patient id.	Collected at week no.	Sample
911 a	3	1	Biopsy
909 a	3	1	Filter
909 c	3	1	Filter
925 a	3	2	Filter
953 c	3	4	Biopsy
952 d	3	4	Filter
952 b	3	4	Filter
977 b	3	6	Filter
9000 a	3	8	Filter
907 a	5	1	Biopsy
907 c	5	1	Biopsy
906 a	5	1	Filter
921 a	5	2	Filter
945 a	5	4	Biopsy
944 a	5	4	Filter
989 a	5	8	Filter
PAO1			

2.5. Swarming activity

The swarming motility of the *P. aeruginosa* strains was assessed as previously described (Song et al., 2019). Briefly, 5 μl O.N. cultures were plated from each culture in the center of a swarming plate, which contained M9 medium supplemented with $MgSO_4$ (1mM), 0.5 % casamino acids, 0.2 % glucose and solidified with 0.5 % Bacto agar. Plates were incubated at 37 °C for 24 h and plates were evaluated visually for swarming capabilities "+" or no swarming capabilities "-". Each swarming experiment was carried out with at least three biological replicates.

2.6. Extraction of chromosomal DNA for genome sequencing

The extraction of genomic DNA was prepared according to a previously described protocol (Rasool et al., 2021) The concentration was measured on NanoDrop, and the samples were run on 0.7 % agarose gel (Sigma) together with a Generuler 1 kb ladder (Thermo Scientific) to check for impurities. The sample was sent to Novogene for whole genome sequencing using the Illumina NovaSeq 6000 platform.

2.7. Genomic assembling

BBDuk version 36.49 was used to quality trim the sequence reads, and de novo assembly and annotation were created using the Shovill version 1.0.4 (https://github.com/tseemann/shovill. *In silico* analysis was conducted using ResFinder to identify resistance genes within the genome, and Multi-Locus sequence typing was performed using MLST 2.0. (Database version 24.04.2021)(Centre for Genomic Epidemiology (CGE)). Moreover, SNPs were identified in each isolate and compared across the isolate within the same sequence type using BacDist (https://github.com/MigleSur/BacDist). The BacDisk pipeline was set to filter and retain SNPs with coverage of at least ten reads in all clones and exclude mutation in clones with more than 80 % non-reference reads. The reference genome used in this study was *Pseudomonas aeruginosa,* Stain: PAO1 (RefSeq assembly accession number: GCF_00006765.1).

Table 1
. Patient characteristics.

	Sex	Age	Duration of infection prior to inclusion (months)	Wound size (cm^2) week 1	Wound size (cm^2) week 8	% difference in wound size In the 8 weeks	Occasionally isolated organisms identified besides *P. aeruginosa*
Patient Id.3	Male	87	36	19.67	29.62	2.39	*E. faecalis, S. aureus, S. maltophilia, S. cohnii* ssp. *urealyticus*
Patient Id.5	Female	81	50	98.34	79.55	−6.37	*Yeast, S. maltophilia, E. coli, E. faecalis, Moraxella* spp.

The Phylogenetic tree was visualized using the bioinformatic tool Tree of Life Tool - iTOL (version 6). The whole-genome sequences have been deposited in Genbank under the BioProject accession number 'PRJNA1133749'.

2.8. Single-nucleotide-variant (SNV)-based phylogenetic tree

We used parnsp version 1.2 (Treangen et al., 2014) with default settings to make core genome single-nucleotide-variant (SNV)-based phylogenetic tree of assembled genomes.

2.9. Statistics

Statistical analyses were performed in GraphPad Prism 9.3 (GraphPad Software Inc., CA, USA). Doubling times were compared by a one-way ANOVA and Tukey's multiple-comparisons test. P value below 0.05 ($p < 0.05$) was considered statistically significant. Further levels of significance are indicated with asterisks *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

2.10. Ethics statement

The Danish Scientific Ethical Board approved the study, and samples were obtained after patients' written consent (Gjødsbøl et al., 2006).

3. Results

To investigate *P. aeruginosa* diversity during chronic wound colonization, isolates from two patients (patient id. 3 and patient id. 5) with repeated *P. aeruginosa* infection were chosen. It was noted that the patients were co-infected with other bacteria in the wound. Table 1 summarizes the patient characteristics as well as Supplementary Table 1.

3.1. No differences in growth rates between the isolated strains

Long-term infection of *P. aeruginosa* can sometimes be accompanied by a reduction in growth rate for a pathogen to adapt to its environment (la Rosa et al., 2021). This was tested by measuring the growth rate of clinical wound isolates and comparing it to the reference strain PAO1. The linear regression result showed the doubling time for the clinical isolate from patient id. 3 had a doubling time that varied from 39 to 45 min, whereas for patient id. 5, the doubling time varied from 37 to 55 min (Table 3). In comparison, PAO1 had a doubling time of 37 min. A one-way ANOVA was used to compare each patient's wound isolates. The result showed no significant difference in the doubling time between the isolates from patient id. 3 nor patient id. 5.

3.2. Small phenotypic differences were observed in patient id. 3 for both swarming and colony morphology

P. aeruginosa harbors polar flagella, which enables it to swarm on semi-solid agar. This complex behavior allows it to move across a surface and is suggested to play a role in the initiation of biofilm formation (Yeung et al., 2009; Overhage et al., 2008; Shrout et al., 2006). To assess the swarming abilities of the strains, overnight cultures were spotted on 0.5 % M9 agar plates supplemented with casamino acids and glucose. The results showed that six of the strains from patient id. 3 were not able to swarm, except for three strains (909a (week 1), 909c (week 1) and 953c (week 4) (Fig. 1). In patient id. 5, all strains could swarm. There was no correlation between the colony phenotype and the isolation method, i.e., filter and biopsy. The colony morphology analysis observed that the wound isolates 952d and 909a (weeks 1 and 3) from patient id. 3 were non-mucoid, whereas all the other clinical wound isolates from patient id. 3 and id. 5 and were mucoid, together with PAO1 (Table 3).

3.3. Strain 953c id. 3 (week 4) produces significantly more pyocyanin compared to the other strains

To investigate the phenotypic diversity of the clinical wound isolates *of P. aeruginosa* and PAO1, spectrophotometric analysis was performed to estimate the amount of quorum-sensing pigment pyocyanin produced by each clinical isolate and PAO1. The result of ANOVA analysis followed post hoc Turkeys multiple comparison tests showed a significant difference between isolate 953c and isolate 911a, 952d, 952b and 977b ($p < 0.05$) for patient id. 3 (Fig. 2). The test results for patient id. 5 showed no significant difference between the isolates.

3.4. The patients carried the same MLST type during the 8 weeks, yet different MLSTs

The phylogenetic diversity among the isolates was determined by measuring the single nucleotide polymorphism (SNPs) present in each genome, thereby revealing the evolutionary relationship between the wound isolates. The MLST analysis showed that the seven wound

Table 3
. Summary of the phenotypic analysis performed on *Pseudomonas aeruginosa* wound isolates from patients id 3 and 5 and reference strain PAO1 and PA14. In the colony morphology analysis, they were distinguished between mucoid and non-mucoid. The doubling time is minutes (± SD; $n = 3$). Swarming was evaluated visually, where '+' indicates swarming capabilities, and '-' is when no swarming is observed. In the quantification of quorum sensing pigment, pyocyanin was used as a reference molecule, and the amount of pigment produced is given in μM/CFU ml^{-1}.

isolate no.	Patient ID	Week no.	Sample	Swarming	Colony morphology	Doubling time (min) ± sd	Pyocyanin/colony (μM)
911 a	3	1	Biopsy	–	Mucoid	39.81 ± 1.90	2.23E-07 ± 2.35E-08
909 a	3	1	Filter	+	Non-mucoid	43.31 ± 2.31	2.76E-07 ± 1.23E-07
909 c	3	1	Filter	+	Mucoid	37.97 ± 2.35	2.26E-07 ± 9.36E-08
925 a	3	2	Filter	–	Mucoid	43.96 ± 3.45	2.05E-07 ± 1.12E-08
953 c	3	4	Biopsy	+	Mucoid	42.82 ± 1.57	4.55E-07 ± 1.74E-07
952 d	3	4	Filter	–	Non-mucoid	45.47 ± 3.49	2.26E-07 ± 4.48E-08
952 b	3	4	Filter	–	Mucoid	42.98 ± 1.06	2.13E-07 ± 4.47E-08
977 b	3	6	Filter	–	Mucoid	40.58 ± 2.84	1.79E-07 ± 4.90E-08
9000 a	3	8	Filter	–	Mucoid	45.78 ± 7.11	1.08E-07 ± 1.47E-08
907 a	5	1	Biopsy	+	Mucoid	43.82 ± 2.94	1.12E-07 ± 2.49E-08
907 c	5	1	Biopsy	+	Mucoid	55.59 ± 12.73	7.69E-08 ± 1.69E-08
906 a	5	1	Filter	+	Mucoid	48.64 ± 0.24	7.31E-08 ± 3.06E-10
921 a	5	2	Filter	+	Mucoid	51.37 ± 1.52	1.24E-07 ± 4.91E-08
945 a	5	4	Biopsy	+	Mucoid	50.05 ± 6.38	1.38E-07 ± 2.40E-08
944 a	5	4	Filter	+	Mucoid	48.07 ± 1.91	1.77E-07 ± 3.52E-08
989 a	5	8	Filter	+	Mucoid	43.43 ± 1.14	9.14E-08 ± 4.46E-08
PAO1				+	Mucoid	37.47 ± 3.93	1.84E-07 ± 5.51E-08
PA14				+			

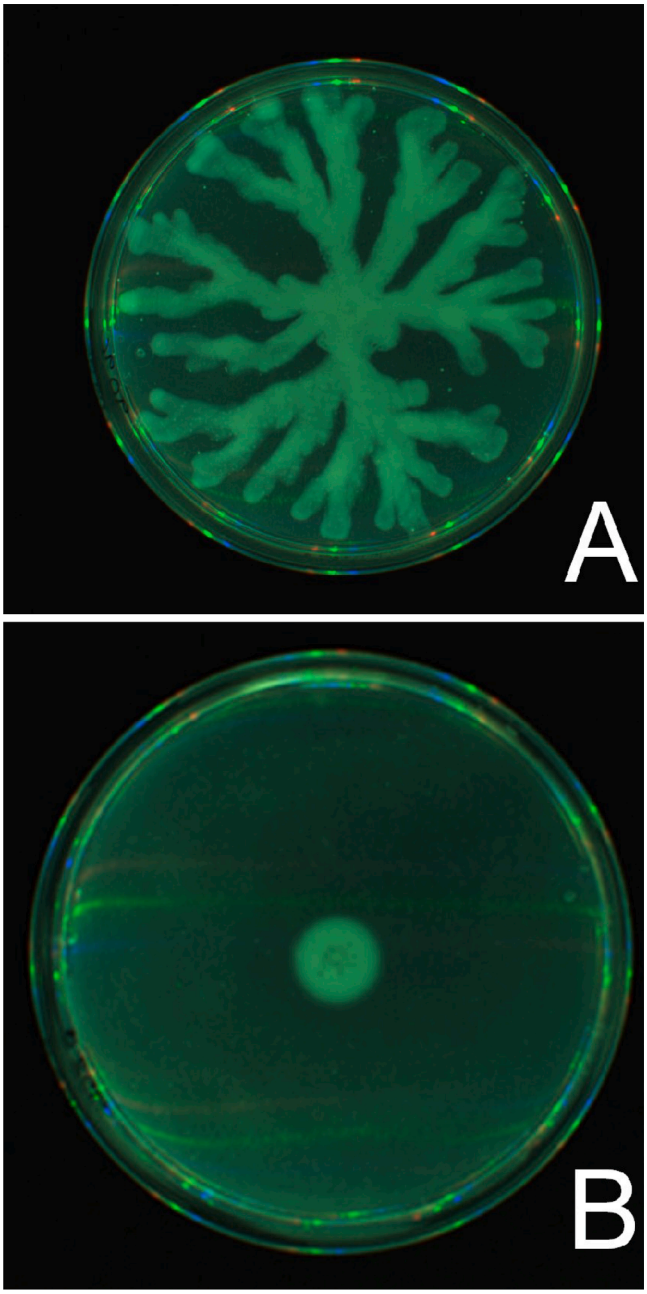

Fig. 1. Some isolates were capable of swarming in patient id. 3 ((A) isolate 909c, week 1), whereas other isolates from the same patient (e.g. (B) 952d, week 4) showed no swarming capabilities.

isolates were collected from patient id. 3 had the same sequence type profile ST132. The same observations were seen for the nine wound isolates collected from patient id. 5, with the sequence type profile ST3244, and for PAO1, the sequence type profile was ST549 (Fig. 3).

The total number of SNPs found in ST132 ($n = 9$) was 18; in ST3244 ($n = 7$), the total number of SNPs was 20. Moreover, the phylogeny results showed a close genetic relationship between all ST132 wound isolates, with 0–8 SNPs differences (median 4 SNPs differences) with 12 non-synonymous- and 6 synonymous mutations. The same minor SNPs difference was observed between all ST3244 wound isolates, with 0–15 SNPs differences (median 7 SNPs differences) where 13 of the mutations were non-synonymous and 8 were synonymous mutations. The phylogenetic diversity is illustrated in Fig. 3. When we aligned the genomes of our 17 isolates to a collection of 448 *P. aeruginosa* genomes (446 genomes from Gabrielaite et al., 2020) (and genomes of reference strains PAO1 (NC_002516.2) and PA14 (NC_008463.1)), we found that genomes of ST132 (patient id. 3) were within PAO1 main group whereas genomes of ST3244 (patient id. 5) were not part of either PAO1 or PA14 main groups (Supplementary Fig. 1).

3.5. Intra-lineage genetic diversity in virulence genes

To investigate whether intra-lineage genetic diversity was the cause of the phenotypic differences, we invstigated the SNPs which are essential for colonization, virulence, and biofilm formation. The intra-lineage mutations were in genes such as *pilA, amrZ, hcp3, hcpC, mvfR, fleQ, chpA,* and *impA* all shown in Table 4.

3.6. No phenotypic antimicrobial resistance against the antibiotics tested

The results from the bioinformatic analysis revealed that all wound isolates harbored resistance genes. This included fosfomycin (*fosA)*, ciprofloxacin (*crpP)*, chloramphenicol (*cat7B*), a carbapenem ($bla_{OXA-396}$/$bla_{OXA-486}$), aminoglycoside (*aph(3′)-IIb*), and β-lactam (bla_{POA}). However, when performing minimum inhibitory concentration testing on the strains, the strains showed all to be susceptible to common antibiotics used for *P. aeruginosa* infections, such as tobramycin (aminoglycoside), ciprofloxacin (fluoroquinolone), meropenem (carbapenem (β-lactam)), and polymyxin B (Table 5).

4. Discussion

Residing in a laboratory culture flask is a stark contrast to inhabiting a chronic wound, where bacteria endure ongoing environmental stressors, including challenges from the host's immune system, pH levels, temperature fluctuations, bacterial diversity, and limited access to nutrients and oxygen. These conditions are believed to prompt the development of adaptive strategies in bacteria to thrive in chronic infection. This study investigated the complexity and stability of *P. aeruginosa* populations in 2 chronic wounds during an 8-week course. Sequencing of the isolates revealed that each patient was colonized with one genotype each and different for each other. The isolates from each patient remained relatively stable both phenotypically and genotypically during the 8 weeks.

Bacteria isolated from chronic infections have been previously shown to lose function of genes involved in virulence or alter their virulence (Darch et al., 2015; Mowat et al., 2011; Winstanley et al., 2016; Smith et al., 2006a).

Patient id. 3 harbored the ST-type ST132, that is found to be highly prevalent in blood cultures from eastern European hospitals and associated with multidrug-resistance. Furthermore, the sequence type is also frequently added to the *P. aeruginosa* MLST database (http://pubmlst.org/paeruginosa). The molecular epidemiology of *P. aeruginosa* differs between isolation sites but sequence types (STs) such as ST235, ST175, and ST111 are frequently isolated from wounds (Vaez et al., 2017; Tahmasebi et al., 2022). These are considered high-risk clones due to their capacity to acquire antibiotic resistance mechanisms.

The other patient (id. 5) harbored ST3244, which has only been reported once, from a horse's wound (Pottier et al., 2022). Notably, the two patients were treated in the same hospital setting and during the same period of time. This fact suggests that the patients were colonized by an isolate that was adapted to the patient itself, and it was not a hospital-acquired infection.

It is of interest to note that the presence of *P. aeruginosa* was consistent in all samples, whereas other species were not present in the full time period. The wounds were colonized with species which are typically isolated from wounds such as *S. maltophilia, S. aureus* and *E. coli*.

The total number of SNPs found in ST132 was 18, and in ST3244, the number was 20 with a high number of non-synonymous mutations, suggesting that the strains have been under positive selection. In

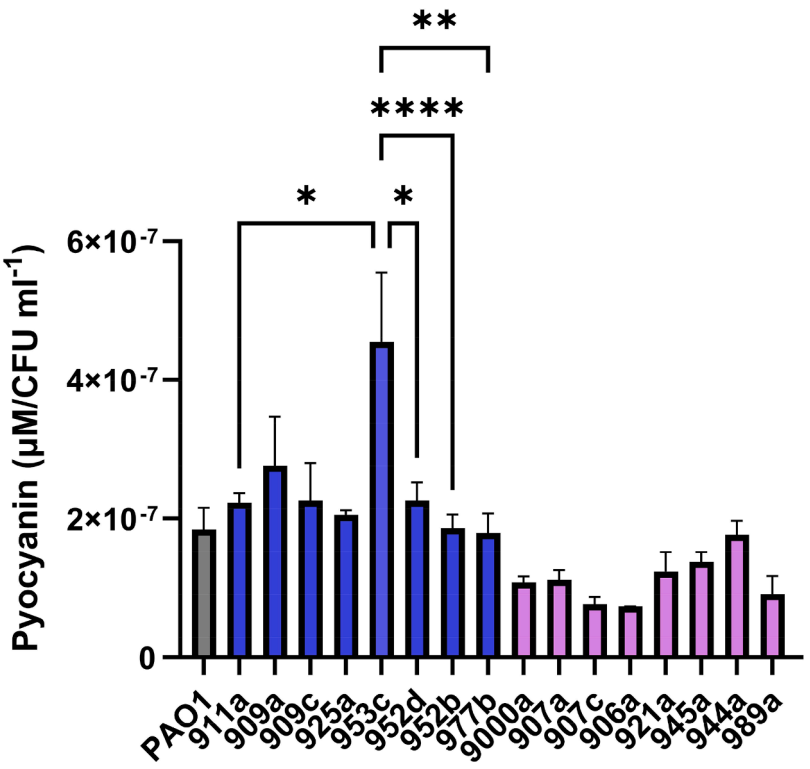

Fig. 2. Graphic representation of the quorum sensing pigment produced by each clinical isolate of *Pseudomonas aeruginosa* from patient id. 3 (blue) and id. 5 (purple) together with PAO1 (Gray)., The data was obtained using UV–Vis spectroscopy using Pyocyanin as a reference molecule and 311 nm absorption wavelength. The result was normalized to represent the concentration µM per CFU/ml. (± SEM; $n = 3$).

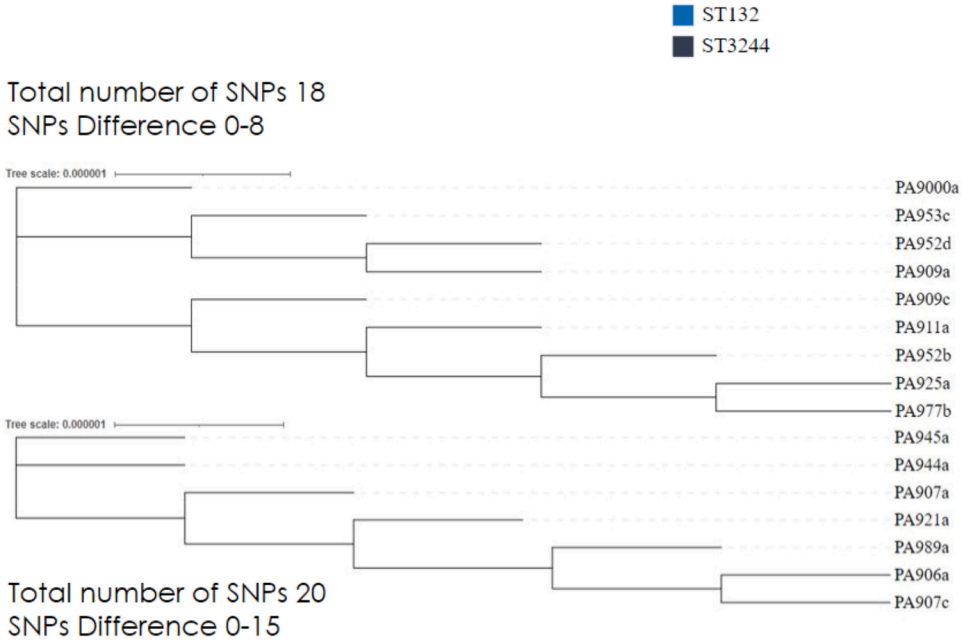

Fig. 3. Phylogenetic tree of the strains. The phylogenetic tree was generated by analyzing the single nucleotide polymorphisms (SNPs). The tree scale indicates the substitution per site.

comparison, a study performed on *P. aeruginosa* in cystic fibrosis patients showed 41 SNPs over a 7.5 year period (Smith et al., 2006b), whereas another study showed 15 SNPs during 15 years (Cramer et al., 2011).

Previous studies have shown that diversity in a bacterial population *in vivo* is often related to biofilm formation, growth rate, and motility. Among growth rates, no difference was observed between the strains of patient id. 3 and id. 5. Interestingly, other phenotypic differences were observed between the strains such as mucoidity, swarming and pyocyanin production. Some of the isolates from each patient could not swarm on semi-solid agar, suggesting diversity among them. The ability to swarm is probably essential in the initial colonization, whereas when the pathogen has established itself in the chronic wound, swarming

Table 4
Summary of the mutations related to virulence genes from WGS.

	hcpC	*pilA*	*impA*	*metH*	*hcp3*	*amrZ*	*chpA*	*mvfR*	*fleQ*
					Patient id. 3				
911 a	–	NS	–	–	–	–	–	–	–
909 a	S	NS	NS	–	–	NS	–	–	–
909 c	–	–	–	NS	–	–	–	–	–
925 a	–	NS	–	–	–	–	–	–	–
953 c	S	NS	–	–	–	NS	–	–	–
952 d	S	NS	–	–	–	NS	–	–	–
952 b	–	NS	–	–	–	–	–	–	–
977 b	–	NS	–	–	S	–	–	–	–
9000 a	S	NS	–	–	–	NS	–	–	–
Patient id. 5									
907 a	–	–	–	–	–	–	NS	–	–
907 c	–	–	–	–	NS	–	–	NS	–
906 a	–	–	–	–	NS	–	–	NS	NS
921 a	–	–	–	–	NS	–	NS	–	–
945 a	–	–	–	–	NS	–	NS	–	–
944 a	–	–	–	–	NS	–	NS	–	–
989 a	–	–	–	–	NS	–	–	NS	–

NS: Non-synonymous, S: Synonymous or intergenic variants, '%' indicates an absent gene.

Table 5
The minimum inhibition concentration was determined with the use of tobramycin (3.2–0.005 μg/ml), Meropenem (4–0.0062 μg/ml), ciprofloxacin (2–0.0031 μg/ml), and Polymyxin B (4–0.0062 μg/ml). The MIC breakpoint for *P. aeruginosa*, according to European Committee on Antimicrobial Susceptibility Testing (EUCAST), is for tobramycin ($S \leq 2$; $R > 2$), meropenem ($S \leq 2$; $R > 8$), ciprofloxacin ($S \leq 0.001$; $R > 0.5$) and for Polymyxin B ($S \leq 2$; $R > 2$). The capital letter S and R stand for susceptibility and resistance, respectively.

Isolate no.	Tobramycin MIC (μg/mL)	Meropenem	Ciprofloxacin	Polymyxin B
Patient id 3				
911 a	0.4	20	0.25	1/0.5
909 a	0.4	0.025	0.25	0.1
909 c	0.4	0.05	0.25	0.1
925 a	0.4	0.5	0.25	0.5
953 c	0.4	0.05	0.25	0.2
952 d	0.4	0.05	0.25	0.2
952 b	0.4	0.05	0.25	0.2/0.1
977 b	0.4	0.05	0.25	0.1
9000 a	0.4	2	0.25	4-1
Patient id. 5				
907 a	0.8	0.05	0.5	0.1
907 c	0.4	0.025	0.25	0.1
906 a	0.4	2	0.25	2
921 a	0.4	0.05	0.5	0.1
945 a	0.4	2	0.5	0.5
944 a	0.4	0.1	0.25/1	0.1
989 a	0.4	2	0.25	1
Reference strain				
PAO1	0.4	20	2	0.25

might not be as important. Differences within an isogenic population could result from variations in the cells' ability to adapt to environmental changes (Vermeersch et al., 2019). This means that even though differences in phenotypes are observed between the wound isolates, they still originate from the same clonal lineage. This could be due to phase variation, differences in lag phase, or presence of a SNP which is related to protein expression or protein stability (Goldberg et al., 2014). Mucoidity could not be explained from the sequences, as no differences were observed (e.g. on MucA) and several of the strains still had the capabilities even though some studies report the loss of this function in chronic infections (Vaez et al., 2017; Tahmasebi et al., 2022). Some mutations in swarming-related genes were observed (Table 4), but other isolates who were swarming also harbored the same mutation. Previous studies (Overhage et al., 2008; Tremblay and Déziel, 2010) showned that over 378 genes are involved in swarming motility, which suggests that deficiency in swarming motility can be challenging to identify genotypically.

The virulence factor pyocyanin was produced by all isolates, though in higher amounts in the ST132 representing the clinical isolates. Yet, it has previously been suggested that a lysophospholipid (MPPA), which is generated by the secretory phospholipase A2 and accumulates in inflammatory exudates, inhibits the production of virulence factors by inhibiting the Quorum sensing system (Laux et al., 2002)

The study shows that the two patients were colonized with the same *P. aeruginosa* isolates over time with minor phenotype differences. Compared to the studies shown in cystic fibrosis lungs, adaptations in a chronic wound may be less extensive, as demonstrated by a study on a murine wound model (Vanderwoude et al., 2020). However, given the knowledge that the infection was between 36 and 50 months at the inclusion of this study, caution must be exercised since the precise time for the first colonization by *P. aeruginosa* is not known. Therefore, further research is needed to gain a clearer view of the strains' evolution by sampling much earlier and collecting isolates over a longer duration than 8 weeks. In conclusion, our findings suggest that *P. aeruginosa* can colonize and persist in chronic wounds for extended periods. During these 8 weeks, only minor differences were observed between the isolates in the respective wounds. More studies are needed to better understand the diversity and evolutionary adaptations of *P. aeruginosa* during chronic wound infections.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT-3.5 in order to improve readability. After using this tool/service, the author (s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

CRediT authorship contribution statement

Kasandra Buchholtz: Conceptualization, Methodology, Software, Validation, Formal analysis, Data curation, Writing – original draft, Visualization, Investigation, Writing – review & editing. **Rie Jønsson:** Conceptualization, Methodology, Software, Validation, Formal analysis, Data curation, Writing – original draft, Visualization, Investigation, Writing – review & editing. **Rasmus L. Marvig:** Conceptualization, Methodology, Software, Validation, Formal analysis, Data curation, Writing – original draft, Visualization, Investigation, Writing – review & editing. **Biljana Mojsoska:** Conceptualization, Methodology, Software, Validation, Formal analysis, Writing – review & editing. **Karen Angeliki**

Krogfelt: Conceptualization, Methodology, Software, Validation, Formal analysis, Writing – review & editing.

Declaration of competing interest

All Authors have no competing interests to declare

Acknowledgment

Thanks to Kristine Gjødsbøl for providing culture data from the patient's samples. Also, thanks to project nurses Lone Haase and Hanne Vogensen, Copenhagen Wound Healing Center, and Bispebjerg University Hospital for collecting samples and recruiting patients.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2025.100348.

Data availability

Data will be made available on request.

References

Cramer, N., Klockgether, J., Wrasman, K., Schmidt, M., Davenport, C.F., Tümmler, B., 2011. Microevolution of the major common *Pseudomonas aeruginosa* clones C and PA14 in cystic fibrosis lungs. Environ. Microbiol. 13, 1690–1704.

Darch, S.E., McNally, A., Harrison, F., Corander, J., Barr, H.L., Paszkiewicz, K., et al., 2015. Recombination is a key driver of genomic and phenotypic diversity in a *Pseudomonas aeruginosa* population during cystic fibrosis infection. Sci. Rep. 5, 7649.

Gabrielaite, M., Johansen, H.K., Molin, S., Nielsen, F.C., Marvig, R.L., 2020. Gene loss and acquisition in lineages of *Pseudomonas aeruginosa* evolving in cystic fibrosis patient airways. mBio 11.

Gjødsbøl, K., Christensen, J.J., Karlsmark, T., Jørgensen, B., Klein, B.M., Krogfelt, K.A., 2006. Multiple bacterial species reside in chronic wounds: a longitudinal study. Int. Wound J. 3, 225–231.

Goldberg, A., Fridman, O., Ronin, I., Balaban, N.Q., 2014. Systematic identification and quantification of phase variation in commensal and pathogenic Escherichia coli. Genome Med. 6, 112.

Halbert, A.R., Stacey, M.C., Rohr, J.B., Jopp-Mckay, A., 1992. The effect of bacterial colonization on venous ulcer healing. Australas. J. Dermatol. 33, 75–80.

Hall, S., McDermott, C., Anoopkumar-Dukie, S., McFarland, A., Forbes, A., Perkins, A., et al., 2016. Cellular effects of pyocyanin, a secreted virulence factor of *Pseudomonas aeruginosa*. Toxins 8, 236 (Basel).

He, J., Baldini, R.L., Déziel, E., Saucier, M., Zhang, Q., Liberati, N.T., et al., 2004. The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. Proc. Natl. Acad. Sci. 101, 2530–2535.

Kirketerp-Møller, K., Jensen, P.Ø., Fazli, M., Madsen, K.G., Pedersen, J., Moser, C., et al., 2008. Distribution, organization, and ecology of bacteria in chronic wounds. J. Clin. Microbiol. 46, 2717–2722.

la Rosa, R., Rossi, E., Feist, A.M., Johansen, H.K., Molin, S., 2021. Compensatory evolution of *Pseudomonas aeruginosa*'s slow growth phenotype suggests mechanisms of adaptation in cystic fibrosis. Nat. Commun. 12, 3186.

Laux, D.C., Corson, J.M., Givskov, M., Hentzer, M., Møller, A., Wosencroft, K.A., et al., 2002. Lysophosphatidic acid inhibition of the accumulation of *Pseudomonas aeruginosa* PAO1 alginate, pyoverdin, elastase and LasA. Microbiology 148, 1709–1723 (N. Y).

Madsen, S.M., Westh, H., Danielsen, L., Rosdahl, V.T., 1996. Bacterial colonization and healing of venous leg ulcers. APMIS 104, 895–899.

Moradali, M.F., Ghods, S., Rehm, B.H.A., 2017. *Pseudomonas aeruginosa* lifestyle: a paradigm for adaptation, survival, and persistence. Front. Cell Infect. Microbiol. 7.

Mowat, E., Paterson, S., Fothergill, J.L., Wright, E.A., Ledson, M.J., Walshaw, M.J., et al., 2011. *Pseudomonas aeruginosa* population diversity and turnover in cystic fibrosis chronic infections. Am. J. Respir. Crit. Care Med. 183, 1674–1679.

Muller, M., Li, Z., Maitz, P.K.M., 2009. Pseudomonas pyocyanin inhibits wound repair by inducing premature cellular senescence: role for p38 mitogen-activated protein kinase. Burns 35, 500–508.

Olivares, E., Badel-Berchoux, S., Provot, C., Prévost, G., Bernardi, T., Jehl, F., 2020. Clinical impact of antibiotics for the treatment of *Pseudomonas aeruginosa* biofilm infections. Front. Microbiol. 10.

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.

Pottier, M., Castagnet, S., Gravey, F., Leduc, G., Sévin, C., Petry, S., et al., 2022. Antimicrobial resistance and genetic diversity of *Pseudomonas aeruginosa* strains isolated from equine and other veterinary samples. Pathogens. 12, 64.

Rasool, F.N., Saavedra, M.A., Pamba, S., Perold, V., Mmochi, A.J., Maalim, M., et al., 2021. Isolation and characterization of human pathogenic multidrug resistant bacteria associated with plastic litter collected in Zanzibar. J. Hazard. Mater. 405, 124591.

Serra, R., Grande, R., Butrico, L., Rossi, A., Settimio, U.F., Caroleo, B., et al., 2015. Chronic wound infections: the role of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Expert Rev. Anti Infect. Ther. 13, 605–613.

Shrout, J.D., Chopp, D.L., Just, C.L., Hentzer, M., Givskov, M., Parsek, M.R., 2006. The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* biofilm formation is nutritionally conditional. Mol. Microbiol. 62, 1264–1277.

Smith, E.E., Buckley, D.G., Wu, Z., Saenphimmachak, C., Hoffman, L.R., D'Argenio, D.A., et al., 2006a. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc. Natl. Acad. Sci. 103, 8487–8492.

Smith, E.E., Buckley, D.G., Wu, Z., Saenphimmachak, C., Hoffman, L.R., D'Argenio, D.A., et al., 2006b. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc. Natl. Acad. Sci. 103, 8487–8492.

Song, Y., Yang, C., Chen, G., Zhang, Y., Seng, Z., Cai, Z., et al., 2019. Molecular insights into the master regulator CysB-mediated bacterial virulence in *Pseudomonas aeruginosa*. Mol. Microbiol. 111, 1195–1210.

Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., et al., 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 406, 959–964.

Tahmasebi, H., Dehbashi, S., Nasaj, M., Arabestani, M.R., 2022. Molecular epidemiology and collaboration of siderophore-based iron acquisition with surface adhesion in hypervirulent *Pseudomonas aeruginosa* isolates from wound infections. Sci. Rep. 12, 7791.

Treangen, T.J., Ondov, B.D., Koren, S., Phillippy, A.M., 2014. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol. 15, 524.

Tremblay, J., Déziel, E., 2010. Gene expression in *Pseudomonas aeruginosa* swarming motility. BMC Genom. 11, 587.

Vaez, H., Safaei, H.G., Faghri, J., 2017. The emergence of multidrug-resistant clone ST664 *Pseudomonas aeruginosa* in a referral burn hospital, Isfahan, Iran. Burns Trauma 5.

Vanderwoude, J., Fleming, D., Azimi, S., Trivedi, U., Rumbaugh, K.P., Diggle, S.P., 2020. The evolution of virulence in *Pseudomonas aeruginosa* during chronic wound infection. Proc. R. Soc. B Biol. Sci. 287, 20202272.

Vermeersch, L., Perez-Samper, G., Cerulus, B., Jariani, A., Gallone, B., Voordeckers, K., Steensels, J., Verstrepen, K.J., 2019. On the duration of the microbial lag phase. Curr Genet. 65, 721–727.

Wiegand, I., Hilpert, K., Hancock, R., 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat. Protoc. 3, 163–175.

Winstanley, C., O'Brien, S., Brockhurst, M.A, 2016. *Pseudomonas aeruginosa* evolutionary adaptation and diversification in cystic fibrosis chronic lung infections. Trends Microbiol. 24, 327–337.

Yeung, A.T.Y., Torfs, E.C.W., Jamshidi, F., Bains, M., Wiegand, I., Hancock, R.E.W., et al., 2009. Swarming of *Pseudomonas aeruginosa* is controlled by a broad spectrum of transcriptional regulators, including MetR. J. Bacteriol. 191, 5592–5602.