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Co-culture biofilm patterns among different Pseudomonas aeruginosa clones from cystic fibrosis patients

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ABSTRACT

Background: Pseudomonas aeruginosa chronic lung infection is the leading cause of death in the cystic fibrosis (CF) population. The high genome versatility of this microorganism allows it to adapt to the hostile CF lung where the same clone can persist for decades. Paranasal sinuses serve as a reservoir for bacterial adaptation before lung infection. Our study investigates biofilm compatibility among identical and different *P. aeruginosa* genotypes from sinus and lungs of CF patients. Strains were further characterized by whole genome sequencing and motility assays were performed.

Methodology: Motility, gentamicin susceptibility and growth rates were assessed in four strains coming from three CF patients. The strains were subjected to whole genome sequencing with the Illumina MiSeq platform. Conjugation assays using the mini Tn7 transposon were performed in order to tag bacteria with the fluorescent proteins YFP (yellow) and CFP (cyan). Biofilm experiments were carried out in a flow cell system and images were acquired using a confocal laser microscope (CLSM) on days 3 and 5. Four experiments were performed: Experiment 1 with two clonal isolates from sinus and lungs from patient P01 (CF430-142, CF430-11621); experiments 2 (CF430-11621 + 75885-B) and 3 (CF430-11621 + 80271-B) with two lung isolates belonging to two different clones from different patients (P02, P03) and experiment 4 with one lung strain (CF430-11621) and *P. aeruginosa* PAO1 reference strain.

Results: P. aeruginosa clonal isolates coming from paranasal sinuses and lungs from the same patient were able to form mixed biofilm. When different clones were employed no mixed biofilms were observed. Similar results were observed when combining the lung strain and the reference strain PAO1. Biofilms of both strains were observed in the flow-cell channels but no mixed biofilms of them were observed, with the exception of strain 75887-B which did not appear to form any biofilm when mixed with strain CF430-11621. All strains performed swarming while strains CF430-142 and 75887B lacked twitching motility. An aminoacidic change in SadB was observed in the strain 75887B.

Conclusion: Mixed biofilms were only observed when identical clones from the same patient were cultured together. Our experiments indicate that twitching motility does not significantly affect biofilm formation or architecture in our isolates.

1. Background

Pseudomonas aeruginosa is an opportunistic pathogen that is found both in the environment as well as causing both chronic and acute infections [1]. The genome of *P. aeruginosa* has close to 6000 genes and more than 500 regulatory genes. This extended metabolic repertoire and its high versatility allows it to infect and adapt to many different ecological niches [2]. Furthermore, *P. aeruginosa* genome has also a big

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repertoire of virulence genes, important elements for establishing initial infections in animals, humans and plants [2,3].

P. aeruginosa is well known for its capacity to chronically infect the lungs and paranasal sinuses of cystic fibrosis (CF) patients, being the predominant pathogen causing chronic infection [4]. CF patients are intermittently colonized by *P. aeruginosa* in the early stages of the disease [5]. This intermittent colonization is an opportunity to eradicate *P. aeruginosa* with aggressive antibiotic therapy, combining oral ciprofloxacin and nebulized colistin [6]. However, after eradication, patients are often recolonized by *P. aeruginosa*. Interestingly, it has been reported that in 25 % of the cases the same *P. aeruginosa* genotype causes the recolonization in the lungs [4]. The same clone that recolonizes the lower respiratory tract could be present either in a persistent source in the environment or in a patient's reservoir in the paranasal sinuses [5].

Chronic infection in CF patients is a major cause of morbidity and mortality [7,8] and cannot be eradicated once biofilm mode of life is established by a mucoid phenotype [6]. The only strategy consists of giving suppressive antibiotic therapy for decades in order to maintain lung function [9]. Genetic adaptation and microevolution in the CF airways play an important role in the development of chronic infection [10]. The high capacity of *P. aeruginosa* to accumulate mutations leads to a phenotypic change that allows the bacteria to adapt to the paranasal sinuses (microaerophilic conditions) and the CF-lungs environment both the conductive part (bronchi, anaerobic conditions) and the respiratory part (respiratory bronchioles and alveolus, aerobic conditions) [4]. The changes that are related to the chronic infection includes loss of motility, loss of proteins of the type III secretion system, loss of quorum sensing, conversion to the mucoid phenotype, reduced virulence, increased antibiotic resistance and biofilm formation [11]. Several studies have addressed the different genotypic and phenotypic changes that confer adaptative advantages in the CF environment in *P. aeruginosa* strains isolated from both the paranasal sinuses and the lungs. Changes that confer adaptative advantages like mutations in *mucA* or *lasR* genes have been reported in *P. aeruginosa* strains from the paranasal sinuses of CF children before chronic lung infection is established [4]. Furthermore, identical phenotypes have been reported in *P. aeruginosa* strains isolated from paranasal sinuses and from lungs before and after lung transplantation [11]. These results demonstrate that the sinuses serve as an important environment that allows bacterial adaptation before the chronic lung infection is established.

Given the fact that the same clone from the paranasal sinuses by aspiration establish chronic infection in the lungs with a biofilm mode of growth, this suggest that two distinct clones of *P. aeruginosa* are in competition regarding biofilm formation and the original clone would persist due to its superior fitness. In the present study we examined co-culture biofilm patterns between same and different *P. aeruginosa* genotypes isolated from sinus and lungs of three different CF patients that were genotypically characterized through whole genome sequencing (WGS).

2. Methodology

Strain selection and growth conditions: Four clinical *P. aeruginosa* strains from Danish CF patients followed at the CF center at Rigshospitalet, Copenhagen, were chosen for the study. Two of these strains, CF430-142 and CF430-11621 [12], were isolated from the sinus and lungs of the intermittently colonized patient P01 and belonged to the sequence type (ST) 179. The remaining two strains were isolated from the lungs of two different *P. aeruginosa* chronically infected CF patients. The strain 75887-B (ST792) was isolated from patient P02 and the strain 80271-B (ST155) was isolated from patient P03. Strains from patients P02 and P03 belonged to the DK1 lineage which is highly prevalent in Danish CF patients due to previous occurrence of cross-infections [13]. Additionally, the laboratory reference strain PAO1 was included in the analysis.

Strain selection for the biofilm experiments was based on bacterial growth. For testing growth rates, the absorbance of the bacteria was measured at 600 nm for 24 h using a TECAN plate reader. Additionally, all the strains were tested for gentamicin susceptibility before conjugation experiments.

All *P. aeruginosa* strains were routinely cultured on Lúria Bertani (LB) agar (2 %) plates and incubated overnight at 37 °C. LB media was also used for measuring growth rates. AB-trace minimal media was used for conjugation and biofilm experiments (1 mM $MgCl_2$, 0.1 mM $CaCl_2$, nonchelated trace elements, 2 g of $(NH_4)_2SO_4$ per liter, 6 g of $Na_2HPO_4 \cdot 2H_2O$ per liter, 3 g of KH_2PO_4 per liter, 3 g of NaCl per liter) [14].

Motility assays: Swarming and twitching motility were assessed. For swarming cells were transferred to a semi-solid LB medium with 0.5 % bacteriological agar. Plates for swarming motility assays were inoculated with a 5 µl aliquot from an overnight culture in LB broth, onto the top of the agar and incubated at 37 °C for 48 h. For twitching LB broth solidified with 1.2 % bacteriological agar was used. The plates were stab inoculated with a sharp toothpick to the bottom of a Petri dish using an overnight culture grown on LB Agar at 37 °C. Plates were incubated at 37 °C for 48 h.

Tagging of *P. aeruginosa* with YFP and CFP proteins: The *P. aeruginosa* strains were chromosomally tagged with cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) using the mini-Tn7 transposon delivery vectors miniTn7 (Gm)PA1/04/03-ecfp-a or miniTn7(Gm)PA1/04/03-eyfp-a, respectively, essentially as previously described [15]. Prior to transformation, gentamicin susceptibility was verified as the mini-Tn7 transposons carry a gentamicin resistance gene for selection. Strains were plated on LB agar containing 60 µg/ml gentamicin and monitored for growth. After transformation, one single colony from each tagging was selected and fluorescence was confirmed by confocal microscopy using Zeiss LSM 880 (Carl Zeiss, Jena, Germany).

Biofilm formation: In total, four mixed biofilms were grown with two different strains in each one. Mixed biofilms were carried out first using the two sinus and one lung strains from patient P01 (CF430-142, CF430-11621; ST179). Afterwards, two more biofilm experiments were carried out using the lung strain CF430-11621 mixed with two different *P. aeruginosa* lung strains coming from patient P02 (75887-B; ST792) and patient P03 (8271-B; ST155). Finally, a mixed biofilm with the lung strain CF430-11621 and the reference laboratory strain PAO1 was also performed (Fig. 1).

Biofilms were grown in continuous-culture flow cells with three parallel channels. Flow cells were attached to flexible silicone tubing that connected to the medium supply and a Watson Marlow 250S peristaltic pump was employed to drag medium through the system [16]. The system was sterilized by flushing the tubing and flow-cells with a 0.5 % sodium hypochlorite solution and then washed with sterile water. The flow chambers were directly inoculated by injecting 350 µl of overnight culture diluted to an OD600 of 0.01 in saline into each flow channel with a small syringe. The first two channels were inoculated with individual strains and the third channel was inoculated with a mix

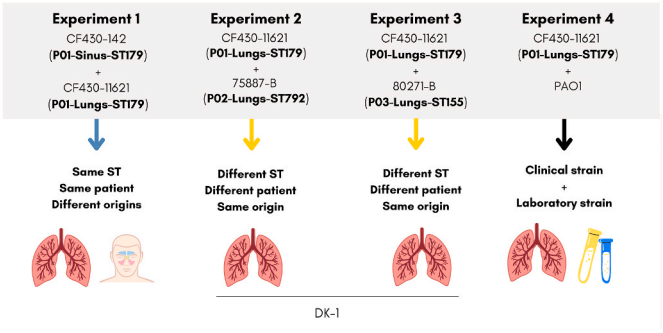

Fig. 1. Schematic representation of the four biofilm experiments performed in the study and the different *P. aeruginosa* strains used.

of both strains in the same proportion (1:1). After inoculation flow channels were inverted and left without flow for 1h to allow attachment of the bacteria to the substratum. Subsequently, the flow-cells were irrigated with AB-trace minimal medium supplemented with 0.02 % Casamino Acids, and 0.2 % of glucose at a mean flow velocity of (0.2 mm s-1). The biofilms were incubated at room temperature for five days.

Microscopy and image acquisition: All microscopic observations and image acquisitions were done with a Zeiss LSM 880 CLSM (Carl Zeiss, Jena, Germany) equipped with detectors and filter sets for monitoring of Cfp (excitation at 434 nm and emission peak at 474 nm) and Yfp (excitation at 514 nm and emission peak at 527) fluorescence. Images of the biofilms were taken at time 72h (day 3) and at time 120h (day 5). Z-stacks were generated with 1-μm intervals; the number of slices was chosen individually for each imaged area to cover the whole biofilm. Three z-stacks were taken for each flow cell channel and time point for the mixed biofilms and two images were taken for the single biofilms. Image processing was done using the IMARIS software package (Bitplane AG).

Statistical analysis: The biomass, biovolume and surface coverage of all the mixed biofilms were visualized and analyzed using GraphPad Prism 9.4.1. (GraphPad Software, San Diego, CA, USA). Statistical analysis was performed using Student's t-test, considering a p-value <0.05 as statistically significant.

DNA extraction and whole genome sequencing: DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) and quantified with a Qubit 4 Fluorometer (Thermo Fisher Scientific). The libraries were prepared using the DNA prep Library Preparation Kit (Illumina) followed by paired-end sequencing (2x250) on a MiSeq platform (Illumina). FastQ sequences were assembled with INNUca v4.2.0 pipeline (github.com/B-UMMI/INNUca) using default parameters.

Bioinformatic analysis: MLST was determined in *P. aeruginosa* genomes using the MLST v2.4 software (github.com/tseemann/mlst). Screening of mutations was assessed using Geneious R9 (version 9.1.7), using the sequence of *P. aeruginosa* PAO1 (NZ_CP129519.1) as reference. For phylogenetic analysis, the genome SNP alignment was obtained with Snippy's core module (github.com/tseeman/Snippy) and subjected to the prediction and removal of recombinant regions using the Gubbins v2.3.1 software [17]. Phylogenetic tree was constructed using strain *P. aeruginosa* PAO1 (NZ_CP129519.1) as reference. Phylogenetic tree visualization was performed using Microreact.

3. Results

3.1. Characterization of P. aeruginosa strains

P. aeruginosa strains used in the study were isolated from patients with CF. Strains coming from patient P01 were colonizing intermittently while the strains isolated from patients P02 and P03 showed a chronic infection.

Phenotypic characterization was assessed including motility, gentamicin resistance, growth rates and mucoid phenotype. All the strains showed a non-mucoid phenotype and were susceptible to gentamicin.

Table 1
Phenotypic and genotypic characteristics of the *P. aeruginosa* strains included in the experiments. *ST: Sequence type

Isolate	CF430-11621	CF430-142	75887B	80271B
Patient-ID	P01	P01	P02	P03
Origin	Lung	Sinus	Lung	Lung
Colonization	Intermittent	Intermittent	Chronic	Chronic
ST*	179	179	792	155
Mucoid	No	No	No	No
Doubling time	3.46	2.90	6.15	4.02
Growth rate	0.20	0.24	0.11	0.17
Twitching	Yes	No	No	Yes
Swarming	Yes	Yes	Yes	Yes

Furthermore, all the strains performed swarming motility while on the contrary two of them had lost twitching motility (Table 1).

Strains were further characterized genotypically by whole genome sequencing. A phylogenetic tree was performed to observe the genetic distance between the strains from the three different patients (Fig. 2).

Mutations and aminoacidic changes were screened in several genes related to mucoid phenotype, quorum sensing (QS) regulation, hypermutator phenotype and biofilm formation. No mutations in *mucABC* genes were observed in any of the strains, suggesting that none of them is a mucoid revertant. Moreover, no changes in LasR or RpoN were observed in any of the strains, as well as in the mismatch repair system genes *mutS/mutL* (Supplementary Table 1).

For conducting competitive assays, the main idea was to select strains with similar growth rates. For this purpose, growth curves were analyzed. Accordingly, two strains isolated from the sinus and lung of the patient P01, exhibiting similar growth kinetics, were chosen. Strain from the sinus (CF430-142) had a growth rate of 0.24 while strain from the lungs (CF430-11621) had a growth rate of 0.20. Additionally, growth rates of two DK-1 strains isolated from patients P02 (75887-B) and P03 (80271-B) were assessed. Strain 80271-B showed a similar growth rate when compared to the previously selected strains (0.17), while the strain 75887-B exhibited a lower growth rate (0.11) (Fig. 3). In order to explore how differing growth rates might influence the formation of mixed biofilms, both strains from the DK-1 lineage were deliberately chosen, with one exhibiting a higher growth rate and the other a lower growth rate, to assess their potential impact on biofilm dynamics.

3.2. Characterization of P. aeruginosa biofilm structures

3.2.1. Biofilm development with the same P. aeruginosa clones

Top views of the CLSM images of the flow chamber cultivations are shown in Fig. 4. Images were acquired on days 3 (72 h) and 5 (120 h) at random positions in the flow channels. Visual inspection revealed that both strain CF430-142 and CF430-11621 were able to form biofilms separately on day 3. At day 3, microcolonies were observed in both channels with no significant differences between both strains. The lung strain CF430-11621 formed big biofilms that were observed at day 3. When comparing day 3 with day 5, it was observed that additionally to the previous microcolonies present on day 3, new cell aggregates appeared forming a continuous layer that almost covered the entire surface of the channel (Fig. 4A). Regarding sinus strain, abundant microcolonies were observed in the day 3 in all the channel. At day 5, biofilms became thicker and bigger but the same structures were found. No flat biofilms were found so the surface was not covered as in the case

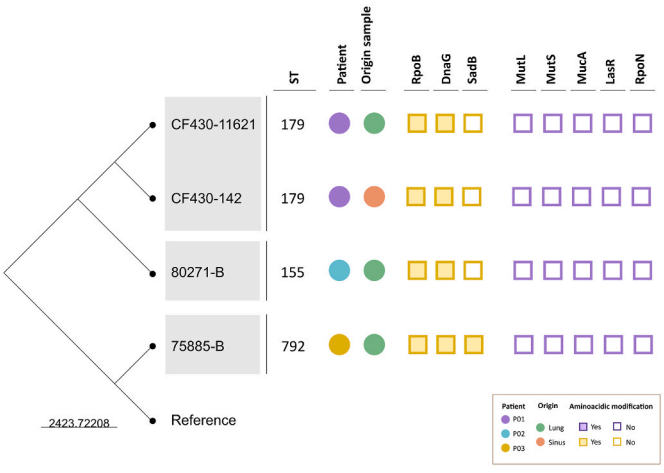

Fig. 2. Phylogenetic tree of the four *P. aeruginosa* strains present in the study and genotypic characteristics. *P. aeruginosa* PAO1 was used as reference.

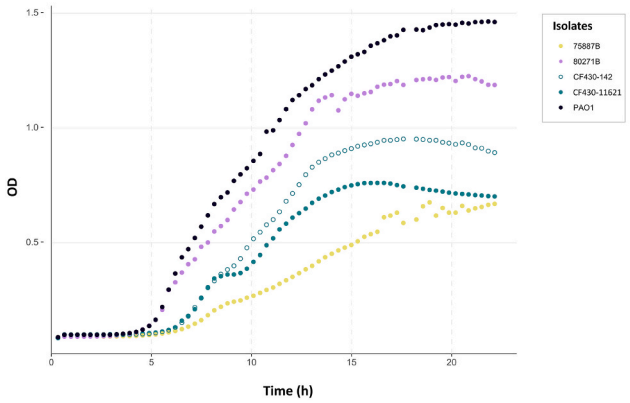

Fig. 3. Growth curves of *P. aeruginosa* strains from sinus (CF430-142) and lungs (CF430-11621, 75887B, 80271B). PAO1 was used as a reference.

of biofilms formed by strain CF430-11621 (Fig. 4B).

When looking at the channel inoculated with both strains (1:1) the results revealed that the two clonal *P. aeruginosa* strains isolated from the sinus and lungs of the same patient were able to form mixed biofilms (Fig. 4C). These mixed biofilms showed heterogeneity. Both strains were found forming the layer of the microcolonies structures as well as the caps. To further investigate the dynamics of mixed biofilm formation, we visualized the total biofilm volume for each strain. Statistical comparisons revealed no significant differences in the contribution of each strain to the total biofilm volume within mixed biofilms formed by clones isolated from the same patient ($p > 0.05$) (Fig. 4D). When looking at biomass and surface coverage the same results were observed (Supplementary Figs. 1 and 2), indicating that both strains grew equally within these mixed biofilms.

3.2.2. *Biofilm development with different P. aeruginosa clones*

3.2.2.1. Mixed biofilm CF430-11621 + 75887-B. Top views of the CLSM images of the flow chamber cultivations are shown in Fig. 5. Visual inspection of the channels inoculated with the strains separately revealed that the *P. aeruginosa* isolates formed biofilms that were significantly different from each other. The strain CF430-11621 was able to form biofilms as observed previously in experiment 1. The same microcolonies structures were observed both in days 3 and 5 (Fig. 5A). On the contrary, the strain 75887-B formed little and a smaller number of biofilms only in the edges of the flow cell channels. The biofilms consisted of irregular cell clusters that did not fully coat the entire surface throughout the duration of the experiment (Fig. 5B).

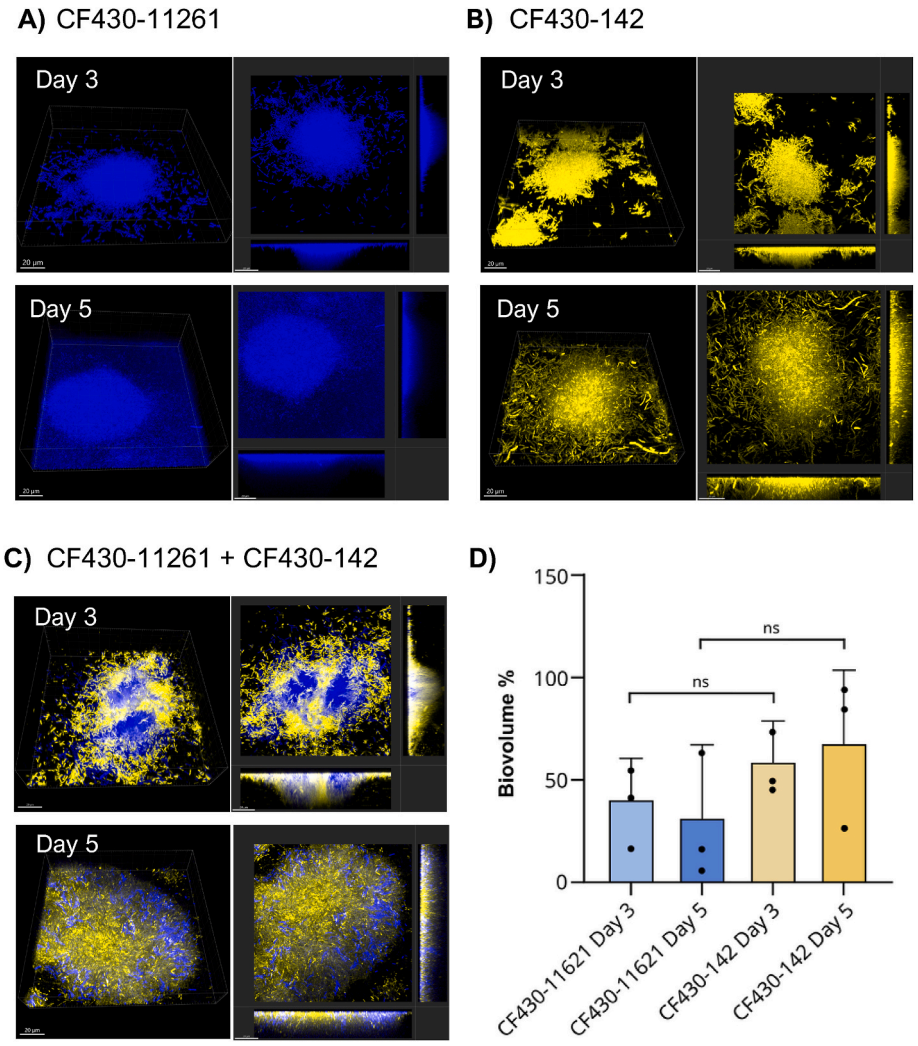

Fig. 4. CLSM images of the flowcells inoculated with strains (A) CF430-11621, (B) CF430-142 and (C) both strains (1:1). Images were acquired at days 3 and 5 after inoculation one replicate per experiment was performed. (D) Biovolumes (%) of each strain in the mixed biofilm. Significance was assumed for p-values below or equal to 0.05, indicated as follows: * <0.05, ** <0.01, *** <0.001, **** <0.0001. **ns: no statistically significant.*

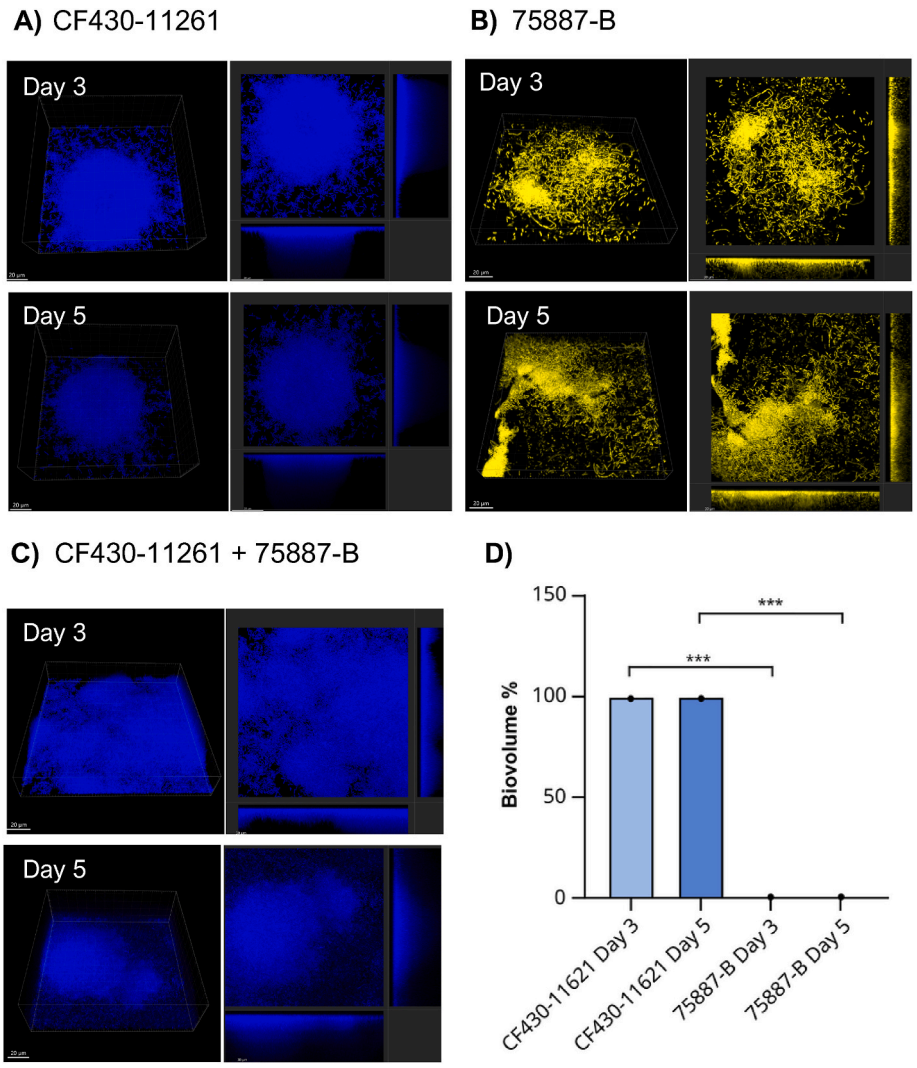

Fig. 5. CLSM images of the flowcells inoculated with strains (A) CF430-11621, (B) 75887-B and (C) both strains (1:1). Images were acquired at days 3 and 5 after inoculation and one replicate per experiment was performed. (D) Biovolumes (%) of each strain in the mixed biofilm. Significance was assumed for p-values below or equal to 0.05, indicated as follows: * <0.05, ** <0.01, *** <0.001, **** <0.0001. **ns: no statistically significant.*

Regarding the channel inoculated with both strains (1:1), only one strain CF430-11621 was able to establish a biofilm (Fig. 5C), resulting in a single-species biofilm where the dominant strain occupied 100 % of the volume, while the other strain contributed 0 %. The difference in volume between the two strains was statistically significant ($p < 0.05$) (Fig. 5D).

3.2.2.2. Mixed biofilm CF430-11621 + 80271-B. Top views of the CLSM images of the flow chamber cultivations are shown in Fig. 6. When looking at the channel inoculated with the strain CF430-11621, the same results were observed as those seen in the previous experiments (Fig. 6A). The strain 80271-B was also able to form a large number of biofilms in all the channel. Big bacterial aggregates that formed flat biofilms were observed on day 3. At day 5 these flat biofilms formed a continuous layer that covered the substratum (Fig. 6B).

Fig. 6C shows the structures of the biofilms formed by both strains. At day 3, no mixed biofilms were observed. The surface of the channel was covered by flat biofilms formed by the strain 80271-B, while no biofilms formed by the strain CF430-11261 were observed. Interestingly, at day 5, biofilms formed by the strain CF430-11621 appeared in the channel and biofilms of both strains were found in the same locations but no mixture of the clones was observed in the microcolonies. Statistical analysis of biofilm volumes revealed that at day 3, strain 80271-B accounted for 100 % of the biofilm volume ($p < 0.05$) as observed in the CLSM images, while at day 5, both strains contributed to the biofilm, with no significant differences in their respective volumes ($p > 0.05$). Similar results were obtained when analyzing surface coverage and biomass, as detailed in the Supplementary Figs. 1 and 2. However, despite both strains forming biofilms in similar amounts, no mixed biofilms were observed, as the strains remained spatially separated within the channel.

3.2.3. Biofilm development with P. aeruginosa clinical strain vs P. aeruginosa PAO1

Finally, mixed biofilms with the clinical strain CF430-11621 and the reference strain PAO1 were performed. As expected, biofilm structures were observed in both strains separately (Fig. 7A and B). When looking at the channel inoculated with both strains, the images revealed biofilms of both strains in different locations of the flow cells at day 3. At day 5 biofilm structures of both strains were observed in the same locations but no mixed biofilms were observed (Fig. 7C). At day 5, microcolonies of PAO1 strain were observed occupying a large part of the flow cell surface, at the same time the strain CF430-11621 formed smaller biofilm structures that were found occupying the free spaces. Statistical analysis of biofilm volumes revealed no significant differences in the biofilm volume of either strain at day 3 or day 5 ($p > 0.05$) (Fig. 7D). Similarly,

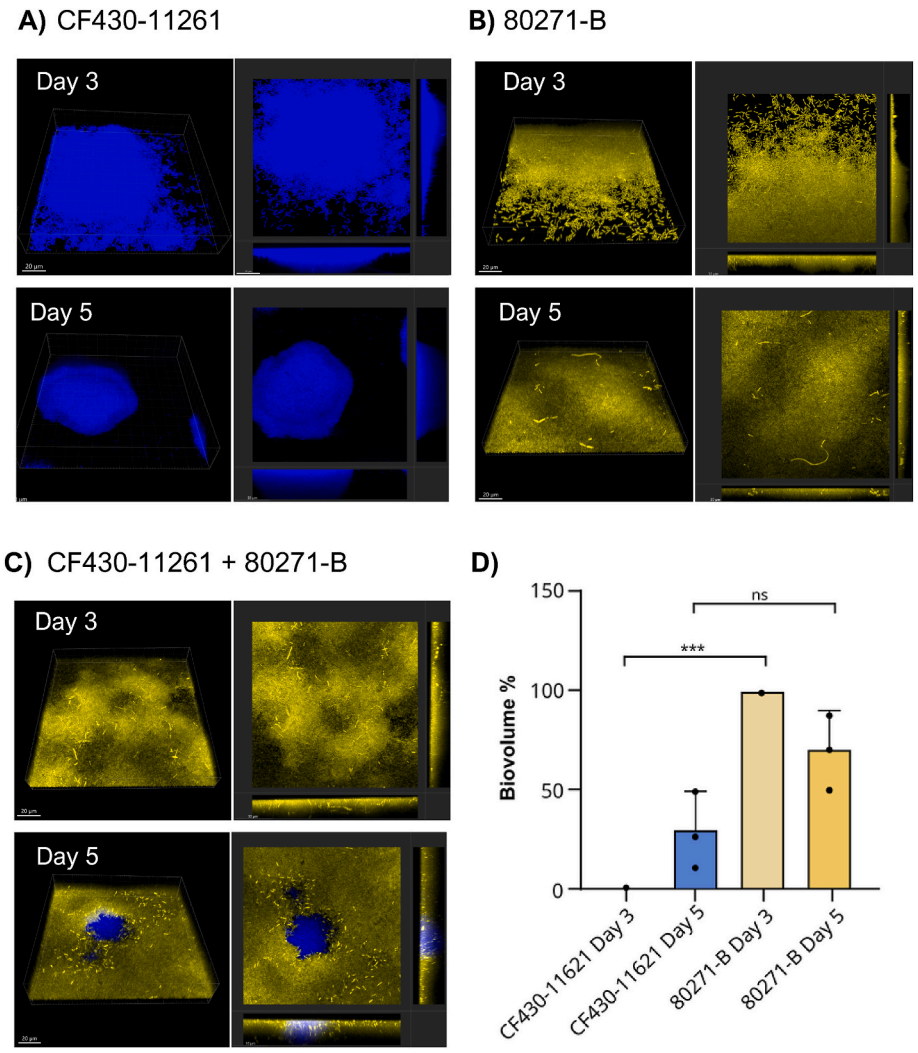

Fig. 6. CLSM images of the flowcells inoculated with strains (A) CF430-11621, (B) 80271-B and (C) both strains (1:1). Images were acquired at days 3 and 5 after inoculation one replicate per experiment was performed. (D) Biovolumes (%) of each strain in the mixed biofilm. Significance was assumed for p-values below or equal to 0.05, indicated as follows: * <0.05, ** <0.01, *** <0.001, **** <0.0001. **ns: no statistically significant.*

surface coverage and biomass measurements showed no significant differences between the strains ($p > 0.05$) (Supplementary Figs. 1 and 2). As in the previous experiment, although both strains were present in similar proportions, no mixed biofilms were formed, and the strains remained spatially separated within the flow cell.

3.3. Comparison of biofilm architectures formed by P. aeruginosa strains with motility properties

In order to determine if motility had a correlation with biofilm structures, swarming and twitching assays were performed. As described before, all the strains were able to perform swarming motility. On the contrary, the strains CF430-142 and 75887-B lacked twitching motility (Supplementary Table 2). The strain 75887-B formed a weak biofilm that was not able to fully cover the surface and was not able to form any biofilms when it was inoculated with a second strain. On the contrary, the strain CF430-142 formed strong and big biofilms on day 3. Furthermore, when mixed biofilms were observed, CF430-142 was present both forming the layer and the cap of the biofilms. Our results did not show any correlation between the lack of twitching motility and the biofilm architecture.

Therefore, we aimed to look for aminoacidic changes that could explain the weak biofilm formation of the strain 75887-B. With that purpose, several genes related to metabolism and biofilm development were screened for mutations. Mutations in genes like *dnaB* and *rpoB*, linked to slow growth in *P. aeruginosa*, were screened in order to determine if they could explain the observed differences in biofilm formation. However, amino acid changes were identified in all strains, suggesting they are not responsible for the variations in biofilm formation. Notably, amino acid alterations in SadB, a protein that plays a key role in transitioning from reversible to irreversible attachment during biofilm formation, were identified only in strain 75887-B at positions K83E and T424P.

4. Discussion

The present study shows that the same *P. aeruginosa* clones isolated from both the paranasal sinuses and the lungs of the same patient are able to form a mixed biofilm. It has been reported that the same *P. aeruginosa* clones present in the upper airways colonizes the lungs. When sinus colonization occurs the microbiota and microbiome of the lungs and the paranasal sinuses are similar, and furthermore, the same genotypes of *P. aeruginosa* are isolated from both sites [18–20]. Therefore, it is reasonable that the identical *P. aeruginosa* clones that had gone through similar evolutionary adaptation processes can collaborate forming mixed biofilms. On the contrary, when two distinct clones

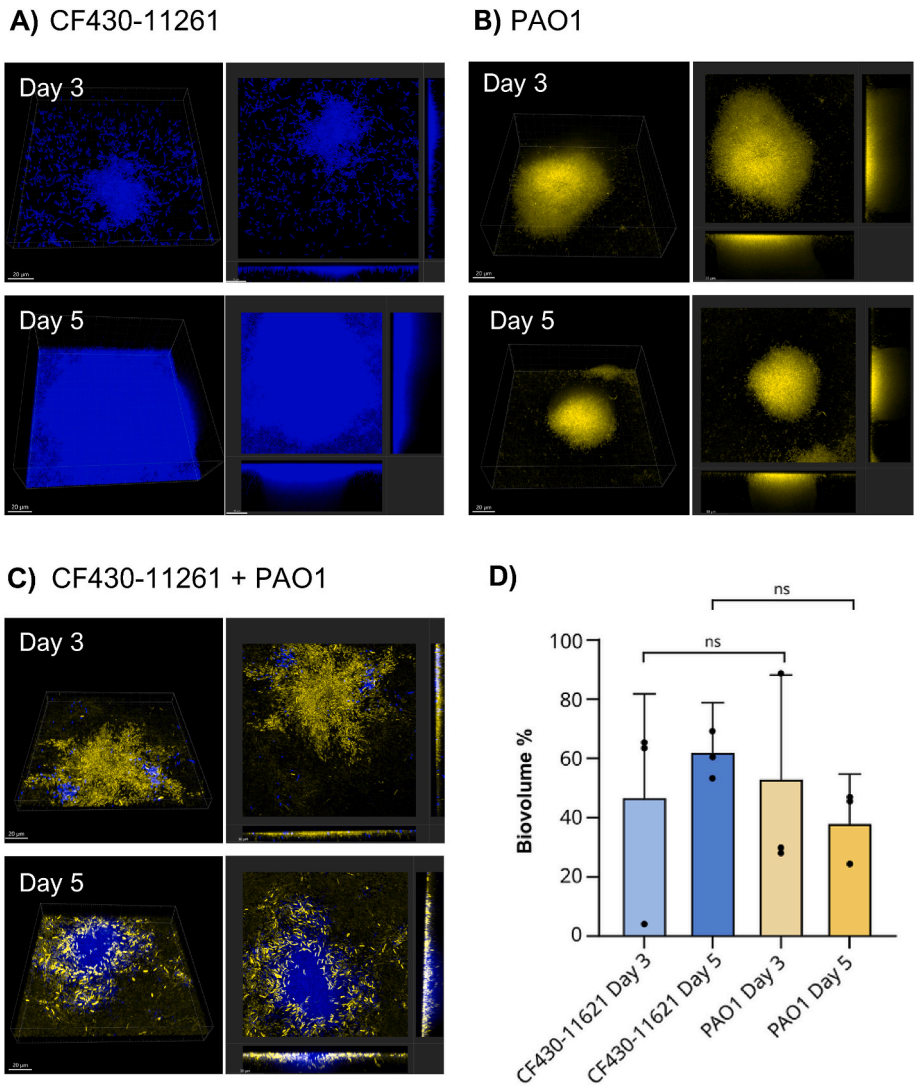

Fig. 7. CLSM images of the flowcells inoculated with strains (A) CF430-11621, (B) PAO1 and (C) both strains (1:1). Images were acquired at days 3 and 5 after inoculation and one replicate per experiment was performed. (D) Biovolumes (%) of each strain in the mixed biofilm. Significance was assumed for p-values below or equal to 0.05, indicated as follows: * <0.05, ** <0.01, *** <0.001, **** <0.0001. **ns: no statistically significant.*

isolated from different CF patients were cultivated no mixed biofilms were observed in the experiments. It is well-known that a single *P. aeruginosa* clone establishes chronic infection in the lungs of CF patients after a period of intermittent colonization [6,21]. We observed that different genotypes do not collaborate in forming mixed biofilms, interestingly, the fact that both strains were present in equal proportions within the co-cultured biofilms suggests that the ability to form a mixed biofilm in this case is not related to differences in growth rate or biofilm formation capacity. This characteristic is likely influenced by other genetic factors. Additional research is required to investigate the underlying mechanisms, which could include bacteriophages and/or pyocins produced by the distinct genotypes.

Regarding biofilm architectures, when mixed cultures with the same *P. aeruginosa* clones were grown, heterogeneous biofilms structures were observed at day 3. Both strains were observed forming both the stalks and the caps of the microcolonies. Different biofilms were observed in CLSM image acquisition, therefore, the formation of mixed biofilms exhibited heterogeneity and variability in biomass. The same pattern was observed when mixed cultures with different clones were performed, with the exception of strain 75887-B. This correlates with a previous study that showed high heterogeneity in biofilm formation among non-mucoid *P. aeruginosa* strains from CF patients [22].

Looking at motility properties, all the strains performed swarming motility. On the other hand, the strains CF430-142 and 75887-B lacked twitching motility. Strain CF430-142 was able to form biofilms structures in all sides of the flow-cell, while strain 75887-B formed very weak biofilms when cultivated alone and was not able to form any biofilm structure when cultivated with another isolate. It has been reported that flagella are involved in the attachment process of biofilm formation and that type IV pili-driven motility is required in microcolony formation and formation of larger macrocolony structures [23]. Furthermore, previous studies showed that motility driven by type IV pili is required in the formation of the caps on the mushroom-shaped biofilms [24]. Our results are not in concordance with these studies as strain CF430-142 was able to form microcolony structures since day 3 alone and together with the strain CF430-11621 despite lacking twitching motility. On the other hand, it is important to highlight that recent studies indicate that type IV pili are not always essential for surface attachment and microcolony formation. Other factors like the carbon source [15] and extracellular DNA [25] also play significant roles in biofilm formation. Therefore, we can suggest that in this scenario, other factors are likely to be more important in biofilm architectures than motility driven by type IV pili.

Biofilm formation is controlled not only by the carbon source or

extracellular DNA, but also by a complex regulatory system [26]. Since strain 75887-B had a lower growth rate and formed weak biofilm compared to the rest of the strains, we aimed to investigate mutations and aminoacidic changes that could explain this phenotype. Several regulatory genes have been reported to play a role in the slow growth rate that is characteristic in later stages of the disease in CF-patients (e.g. *rpoB, dnaG, topA*) [27] and consequently could probably affect biofilm formation. No aminoacidic modifications were found in these genes and in some other genes that were reported to have an impact on biofilm formation like *amrZ* [28] or *pmtA* [29]. However, aminoacidic modifications were observed in SadB in the strain 75887-B. SadB has been demonstrated to play an essential role in biofilm attachment in *P. aeruginosa*, furthermore, the amino acid modification K83E is located in the α/β domain. This domain was demonstrated to be necessary for the protein function and thus to biofilm irreversible attachment [30], then suggesting that this protein could be affecting the biofilm formation properties of the strain 75887-B. However, metabolic pathways in *P. aeruginosa* are complex and more genes are proteins could be involved in the phenotype of strain 75887-B.

Additional genes related to adaptation to cystic fibrosis environment were screened. While the hypermutable phenotype is frequent in chronic strains of *P. aeruginosa* among cystic fibrosis patients [31–33], no alterations were detected in the mismatch repair system (MutL, MutS, UvrD) [34] of any of the strains. Regarding LasR mutants [35,36], no modifications were found either. Finally, the four strains were non-mucoid, when looking at *muc* genes no modifications were found, suggesting that none of them is a mucoid-revertant [21].

While this study provides valuable insights, there are several limitations that should be addressed in future research. First, as one replicate per experiment was performed, additional replicates are required to confirm these findings. Moreover, the study focused on a limited number of *P. aeruginosa* strains from specific CF patients, so experiments with clinical strains from other patients are necessary to determine whether the observed interactions are generalizable. Furthermore, the underlying mechanisms of strain interactions during biofilm formation remain unclear and require further investigation to fully understand how these processes may vary between different genotypes.

5. Conclusions

Our findings suggest that different *P. aeruginosa* clones isolated from different CF patients do not collaborate to form mixed biofilms. In contrast, same clonal types isolated from the same patient appear to form mixed biofilms when cultivated together. Twitching motility did not seem to have a substantial impact on biofilm formation in our experiments.

CRediT authorship contribution statement

Irene Cadenas-Jiménez: Writing – original draft, Visualization, Methodology, Investigation. **Morten Levin Rybtke:** Writing – review & editing, Resources, Methodology. **Doaa Higazy:** Writing – review & editing, Resources, Methodology. **Sara Martí-Martí:** Writing – review & editing, Project administration, Conceptualization. **Tim Tolker-Nielsen:** Writing – review & editing, Project administration, Conceptualization. **Oana Ciofu:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Niels Høiby:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization.

Transparency declarations

The authors declare that there are no conflicts of interest.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2025.100257.

Data availability

Data will be made available on request.

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