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Characterization of clinically relevant model bacterial strains of *Pseudomonas aeruginosa* for anti-biofilm testing of materials

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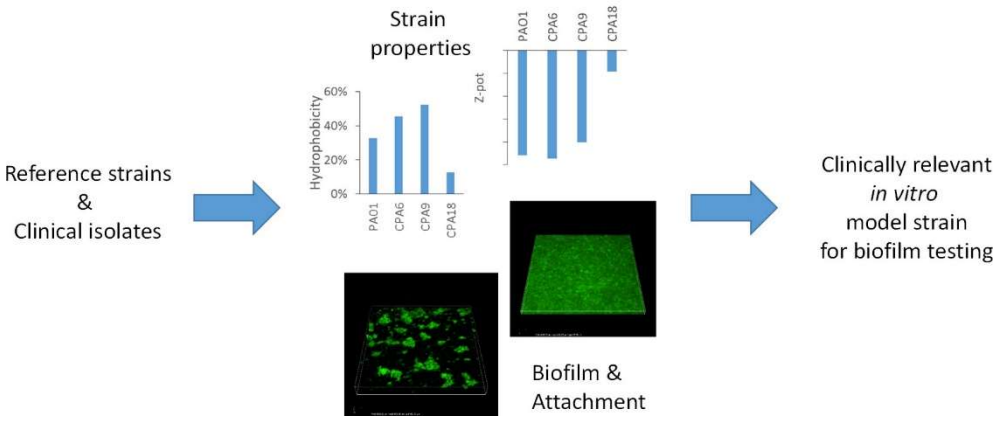
Abstract

There is a great interest in developing novel anti-biofilm materials in order to decrease medical device-associated bacterial infections causing morbidity and high healthcare costs. However, the testing of novel materials is often done using bacterial lab strains that may not exhibit the same phenotype as clinically relevant strains infecting medical devices. Furthermore, no consensus of strain selection exists in the field, making results very difficult to compare between studies. In this work, 19 clinical isolates of *Pseudomonas aeruginosa* originating from intubated patients in an intensive care unit have been characterized and compared to the lab reference strain PAO1 and a *rmlC* lipopolysaccharide mutant of PAO1. The adhesion and biofilm formation was monitored, as well as cell properties such as hydrophobicity, zeta potential and motility. Two groups of isolates were observed: one with high adhesion to polymer surfaces and one with low adhesion (the latter including PAO1). Furthermore, detailed biofilm assays in a flow system were performed using five characteristic isolates from the two groups. Confocal microscopy showed that the adhesion and biofilm formation of four of these five strains could be reduced dramatically on zwitterionic surface coatings. However, one isolate with pronounced swarming colonized and formed biofilm also on the antifouling surface. We demonstrate that the biofilm properties of clinical isolates can differ greatly from that of a standard lab strain and propose two clinical model strains for testing of materials designed for prevention of biofilm formation in the respiratory tract. The methodology used could beneficially be applied for screening of other collections of pathogens to identify suitable model strains for *in vitro* biofilm testing.

Keywords: clinical isolates, *Pseudomonas aeruginosa*, antifouling, model strain, surface chemistry

Graphical abstract



1. Introduction

Microbial biofilms are well-organized communities of microorganisms generally attached to a surface and embedded in a matrix of extracellular substances such as polysaccharides, proteins, DNA. These biofilms cause very large problems in health care such as increased suffering, raised usage of antibiotics, prolonged hospital stays, recurring transplantations, chronic infections and mortality. Many of these infections are related to biofilm on medical devices such as implants, catheters and stents. The World Health Organization (WHO) estimated in 2011 that the direct costs (only) for hospital-acquired infections (HAI) were € 7 billion annually in Europe. A large majority (e.g. 97 % of urinary tract infections and 83 % of hospital-acquired pneumonia) was associated with invasive devices [1]. Bacteria within biofilms have shown to tolerate up to 1 000 times more concentrated antibiotics compared to planktonic bacteria [2] leading to problems in eradicating biofilms on medical devices using antibiotics, as well as promoting development of antibiotic resistance [3]. This illustrates the great importance of finding ways to better understand, prevent and treat biofilm infections, and most likely several approaches are needed in order to successfully reduce incidences of device-related infections. One attractive option often reported in the literature is to cover surfaces of devices with antimicrobial or antibiofilm coatings [4, 5]. To successfully evaluate their microbial performance, however, it is of great importance that good *in vitro* protocols are followed using bacterial strains that have the ability to predict the outcome of a new anti-biofilm material in animal models as well as in patients. In the quest of identifying such model strains, this work is investigating clinical isolates from intubated patients in an intensive care unit (ICU). We show that many of the clinical isolates have very different phenotype compared to lab strains, indicating that testing using lab strains may produce false positives e.g. when testing antibiofilm materials.

1.1. Ventilator-associated infections

Patients in intensive care in need of mechanical lung ventilation have an increased risk of acquiring infections of the airways. These infections are often considered as two categories where ventilator-associated tracheobronchitis (VAT), with an incidence rate between 1 – 19 %, is considered as a condition representing an intermediate phase in the continuum between bacterial colonization and ventilator-associated pneumonia (VAP) [6]. However, the boundaries between these infections are not clear [6]. Patients with VAT have been described to have a two-fold higher incidence rate of VAP than those without VAT [6]. Both types of infection give rise to longer ICU stay, longer time period with mechanical ventilation and higher hospital costs [6]. Pneumonia has been reported to be the second most common infection acquired at hospitals after urinary tract infections [7]. Approximately 15 % of HAI have been described as pneumonia, and

pneumonia represents a quarter of all infections in the ICU [7]. Patients with mechanical lung ventilation run higher risk of developing pneumonia compared to non-ventilated patients [8, 9] resulting in approximately 10 % of patients with mechanical ventilation diagnosed with VAP [10]. This condition is associated with higher fatality rates, reaching 20 – 50 %, compared to other HAI [7, 9, 10]. Several bacteria have been identified causing VAP and VAT in ICU, *e.g.*, *Pseudomonas aeruginosa, Acinetobacter* spp and *Staphylococcus aureus* [6, 7, 10]. *P. aeruginosa* is one of the most frequently isolated pathogens, found in 22 % - 24 % of patients with VAP [9, 11] although lower rates are reported from the US, 10-20 % [10]. High mortality rates, reaching 73 %, have been reported when infections are caused by this organism or by *Acinetobacter* spp. [7, 12]. These bacteria have been shown to be capable of evading antibiotic treatment due to biofilm formation or antibiotic resistance mechanisms, giving rise to relapsing episodes of pneumonia [9, 11]. In the ICU, bacteria are believed to enter into the lower regions of the lung from the pharynx. They may migrate to the lower respiratory tract via microaspirations, inhalation of contaminated aerosols, or may originate from gastric reflux. However, microaspirations have been stated as the most important route [7]. The presence of devices for mechanically assisted ventilation, such as an endotracheal tube, hinders the patients normal cough reflex as well as the normal mucociliary clearance of secretions, and thereby produces a facilitated entry route for microorganisms into the lungs [9, 13]. The transport of bacteria may be through movement on the tube surface (at insertion or after) or build-up of biofilm that later is dislodged and transported into the lung during ventilation or tube handling [7, 9]. Another route of infection consists of contaminated secretions that may build up around the cuff of the endotracheal tube and thereafter leak down into the airways [9, 13]. To diagnose VAP in the airways, tracheal aspirates are routinely sampled for microorganisms. However, the distinction between colonization and true infection of the airways is still an unresolved issue. Thus in collections of clinical isolates from aspirates we do not know which ones are causing problems to the patients, illustrating that not all clinical isolates will present good model strains for medical-device associated infections.

In general, many medical devices such as endotracheal tubes are made from hydrophobic materials such as polyvinyl chloride (PVC), silicone or polyurethane [14]. These materials may easily become preconditioned by proteins and colonized by bacteria [15, 16]. Several attempts have been described in the literature to reduce bacterial colonization of medical devices and render their surface non-fouling. This can be achieved by e.g.surface modifications changing the physicochemical properties of the top layer of the material, or application of coatings [17]. Introduction of surface morphology and topography has also been suggested as a promising route

to tailor bacterial adhesion to surfaces, where bacterial adhesion has been shown to both be enhanced and repelled by different types of physical structuring [18, 19]. One promising approach presented in the literature has been to coat surfaces with thin polymer coatings called polymer brushes [20]. Polymer brushes are polymer films where each polymer chain is covalently tethered to the surface, giving a brush like structure. The chemical composition of these coatings can be altered in a modular way during synthesis. Therefore they have been investigated to a large extent as model systems both *in vitro* and *in vivo* for creating surface coatings that do not allow for bacterial adhesion or biofilm formation [20]. Thus, it has been shown that motility, adhesion and biofilm formation of the lab strain *P. aeruginosa* PAO1 can be strongly reduced on negatively charged poly (3-sulphopropylmethacrylate) (pSPM) and zwitterionic poly (2-(methacryloyloxy)ethyl)dimethyl-3-sulphoproyl) ammonium hydroxide) (pMEDSAH) coatings [21]. Antifouling polymer brushes from poly[oligo(ethylene glycol)methyl ether methacrylate] (pOEGMA) and poly[*N*-(2-hydroxypropyl)methacrylamide] (pHPMA) also dramatically decreased attachment and biofilm formation of the two *P. aeruginosa* strains PAO1 and 30, but only slightly hindered colonization of the multi-resistant PA49 strain [22].

1.2.Diversity of bacterial strains

Studies of clinical isolates, as well as laboratory strains, of *P. aeruginosa* have shown that they differ significantly in their capacity to adhere to material surfaces and form biofilm [23, 24]. Sanchez et al. [23] found that 83 % of *P. aeruginosa* clinical isolates exhibited high or average levels of biofilm formation. They further showed initial clinical data supporting the hypothesis that the presence of biofilm is contributing to cases of relapsing infections or persistent colonization and microbial adaptation [23]. In the light of variation in bacterial phenotypes, it becomes important to understand how this diversity is connected to biofilm formation as well as clinical outcome. The range of strain properties also demands that preventive measures should be efficient on several different phenotypes. Previous studies using lab strains derived from PAO1 have shown that several physicochemical parameters of the bacterial cell are highly correlated to enhanced biofilm formation *in vitro* [21, 25]. It was shown that strains with more hydrophobic surface and near neutral zeta potential (reducing electrostatic repulsion to negatively charged surfaces) displayed higher levels of biofilm formation onto several types of surfaces, both hydrophobic and hydrophilic [21, 25]. It was also suggested that motility of the bacterium exhibited almost similar level of importance as hydrophobicity and near neutral zeta potential [25]. Previous studies of clinical collections of *P aeruginosa* isolates have shown variability between genotype as well as phenotype, the latter especially with respect to adhesion onto

polystyrene, biofilm forming ability, motility and hydrophobicity [26-28]. It has been shown that twitching motility is positively correlated to higher amount of biofilm in clinical isolates of *P. aeruginosa* from a range of hospital environments including urine, bronchial washings, stool, blood and wounds. It has also been shown that association between phenotype differences and differences in genotype may be lacking in clinical strains [29], illustrating the need to characterize bacterial phenotype when identifying good model strains for testing protocols.

In this work, we present a detailed characterization of physicochemical properties of 19 clinical isolates of *P. aeruginosa* isolated from intubated patients in an intensive care unit. We compare these strains to the PAO1 reference strains as well as laboratory strains that we have previously investigated [21, 25]. We thereafter investigate the correlation between the physicochemical parameters and their adhesion after 30 min, as well as biofilm formation both in multi-well plates and in flow-chamber experiments.

2. Materials and Methods

2.1.Bacterial strains and culture conditions

P. aeruginosa strains CPA1 – CPA19 (Table 1) were isolated from respiratory samples collected as part of clinical and microbiological routine diagnosis procedures from ICU patients at the Hospital Universitari Germans Trias i Pujol (Badalona, Spain). Ethical approval was provided by the Institutional Review Board: Comitè d' Ètica de la Investigació de l'Hospital Germans Trias i Pujol. At the time of sample collection and strain isolation ICU patients were under mechanical ventilation through an orotracheal tube meaning that they were in contact with the medical device and *P. aeruginosa* infection can be considered as a medical device-associated infection. Strains were isolated according to the standard clinical procedure and the patients were classified according to clinical/radiological and microbiological criteria (Table 1). These clinical strains (CPA1 – CPA19) have been deposited in a culture collection and are available at the Culture Collection Göteborg University (CCUG, http://www.ccug.se/). *P. aeruginosa* PAO1 wild type and PAO1 *ΔrmlC* [30] kindly provided by Prof. Joseph Lam, University of Guelph, Canada were used as controls. The *ΔrmlC* mutant displays a truncated lipopolysaccharide structure and has, in previous research, been observed to display different surface properties and biofilm formation characteristics compared to the PAO1 parent strain [21, 25]. Thus, it was included to serve as a second control strain enabling comparisons to previous studies. Additionally a third control, the PAO1 double mutant of *ΔfliC ΔpilA,* was introduced that has impaired motility. It was used as a negative control for twitching and swarming, and was also included as a third reference in the

attachment experiment [25, 31]. All strains were routinely cultivated on blood agar. In case any phage-damaged colonies were found (visual inspection) it was reported in Table 2.

Table 1. Description of clinical isolates

CPA strain No	CCUG Strain No	Study group of patients	CPA strain No	CCUG Strain No	Study group of patients
1	71595	colonization	11b	71605	tracheobronchitis
2	71596	bronchial aspiration	12	71606	pneumonia
3a	71597	tracheobronchitis	13	71607	tracheobronchitis
4	71598	colonization	14b	71608	tracheobronchitis
5	71599	pneumonia	15	71609	tracheobronchitis / pneumonia
6	71600	tracheobronchitis	16	71610	pneumonia
7a	71601	tracheobronchitis	17	71611	tracheobronchitis
8	71602	tracheobronchitis	18	71612	colonization
9	71603	tracheobronchitis	19	71613	pneumonia
10	71604	bronchial aspiration			

2.2. Cell surface physicochemical parameters

To determine zeta potential, bacteria grown overnight on blood agar were transferred into tubes with 2 mL of PBS buffer, placed on icebath and diluted to optical density at 600 nm (OD600) = 1.0. Cell size (size of aggregates/cells) and z-potential were measured with Zetasizer Nano ZS, Malvern Instruments. For all measurements three measurements replica were performed for each biological replica and at least two biological replica were performed. The data presented represent an average.

Cell surface hydrophobicity was analyzed using the MATH-assay [21, 32]. Cells were re-suspended in 3 mL of PBS. 1 mL of hexadecane was added to these 3 mL of cell suspension (OD600 ~ 0.5) in test tubes, and the mixtures were agitated vigorously by Vortex for 1 min at room temperature. Samples were left until two layers completely separated, and OD600 of the aqueous phase was measured with a spectrophotometer. Results were calculated as a percentage of transfer to hexadecane layer, by the formula [(A0 - A)/A0] x 100, where A0 is the optical density of the initial cell suspension and A is that of the aqueous phase after agitation with hexadecane. The reported data represent an average of three replica.

2.3.Motility assays

Motility was assessed using a solid plate assay containing 20 % Iso-sensitest medium. For swimming 0.3 % agar was added to the plates. For swarming and twitching 0.5 % and 1 % of agar was used respectively. A volume of 5 µL bacterial suspension with OD =1.0 (prepared from cultures on blood agar plates suspended in PBS) was added to the plates. For swimming and twitching the diameter of bacterial spreading was measured after 24 h of incubation at 37 ºC. For swarming, the assessment "no"; "yes" and "strong" was established (Suppl. Figure S1). The experiments were repeated in triplicate during three different days (i.e. using three different cultures of each strain). Thus each experimental day provided one replica for the entire collection tested. In case of swarming, if this type of motility was not evident in at least one of three separate experiments, the swarming of this strain was considered "weak".

2.4. Attachment and biofilm formation

For the attachment experiments, bacterial cells were suspended in 0.9 % NaCl (OD600 = 1.0) and 100 µL of the resulting suspensions were transferred to two types of 96-well plates (Corning®, product N 3799 and 3795, Sigma-Aldrich): hydrophilic plates made of plasma-treated polystyrene and hydrophobic plates made of untreated polystyrene (description according to the manufacturer, no further modification was performed). In general, the water-contact angle of plasma-treated polystyrene has been reported to range from 30° to 40° and be ~90° for untreated polystyrene [33]. Plates were incubated for 30 min at 37 ºC. For each strain 12 replica were done: representing three different cultures and four wells incubated for each culture (n = 12). For the biofilm formation studies, an aliquot of 20 µL of bacterial cells suspension (~ 1 x 10^4 CFU/mL) was added to 180 µL of Iso-sensitest broth in 96-well plates. Plates were incubated overnight at 37 ºC at static conditions. For each strain eight wells were inoculated giving n = 8. After incubation, in both cases, plates were analyzed using crystal violet assay [34]: attached cells and biofilm in each well were stained with 0.1 % of crystal violet and incubated for 10 min at room temperature. After incubation plates were washed with PBS and air dried. Crystal violet was eluted with 33 % acetic acid and optical absorbance was measured at 595 nm. Statistical analysis of the data was performed using Microsoft Excel, Students T-test (two-tailed distribution, two samples with equal variance) and $p < 0.01$.

2.5.Biofilm formation in a continuous culture system

Continuous culture systems allow monitoring development of biofilms in stable conditions during long time period comparing to batch cultures where nutrients are limited. To create

continuous biofilm culture, a flow chamber set-up was used as described previously [21]. Ten times diluted Iso-sensitest medium was used as culture medium. Clinical strains were collected during exponential growth phase, rinsed in PBS and injected into the flow chamber using a syringe to give an inoculum of 2×10^9 CFU /mL . The flow system consisted of a flow chamber (BST FC270), a glass flow break (FB50), and a bubble trap (FC34) from Biosurface Technology Corporation, USA connected with silicon tubing, 2 mm bore diameter. Connections to the pump head were through Marprene tubing, 0.5 mm bore diameter (Alitea, Sweden). After the flow chamber was autoclaved, functionalized glass slides sterilized in 70 % ethanol were inserted sterile. The entire flow setup was kept at 37 °C throughout the experiment and pH was controlled by the circulating medium (Iso-sensitest) that includes phosphate. Bacterial cells were allowed to attach to the glass or polymer coated glass surfaces for 30 min before the flow was applied and the chamber was operated for 48 h. The flow through the flow system was kept at 1.2 mL/min using a 405U/L2 double-channel pump (Watson Marlow, Alitea, Sweden). Antifouling and zwitterionic poly (2-(methacryloyloxy)ethyl)dimethyl-3-sulphoproyl) ammonium hydroxide) (pMEDSAH) was used representing a hydrophilic surface coating [21]. This polymer brush display contact angles of 39 °, for a water drop at neutral pH [21]. After 48 h, biofilms on each glass were stained with Syto-9 fluorescent dye (Invitrogen, Molecular Probes, USA) and analyzed using confocal-laser scanning microscopy as described previously [21]. In brief, 1 mL of media containing 5 nM Syto-9 was injected into each channel and the flow was stopped for 10 min to allow staining. Confocal (3-D) images were recorded using a Nikon Eclipse90i fluorescent microscope equipped with Nikon D-eclipse C1+ laser system (Nikon Corporation, Japan). Flow chamber experiments were performed at least in duplicates and images were captured at several positions on each slide. The time period of two days for the flow chamber experiments were chosen in order to grow more biomass at the surface. We have previously shown that the structure of the biofilm after 18 h, 72 h and 96 h is similar but the biomass increases making it easier to image bacterial strains that form small quantities of biofilm [21].

3. Results

3.1. Characterization of clinical isolates

The clinical isolates were characterized with respect to their zeta potential, hydrophobicity, motility and colony morphology. The strains had different phenotypes and showed variation in all parameters analyzed (Table 2). For the majority of strains, high hydrophobicity was combined with a marked negative zeta potential and vice versa. Motility did not appear to be linked to

hydrophobicity or zeta potential, which is not surprising, since motility depends largely on cell wall structures such as flagella and pili. Among our isolates, five isolates exhibited higher twitching or swimming than the reference strain PAO1. Six of the strains did not exhibit any swarming and four only had weak swarming (Table 2 and Supplement, Figure 1).

Table 2. Characterization of clinical isolates comparing to reference strains.

CPA strain No	Presence of phages	Haemolysis	Pigmentation of colonies	Hydrophobicity		Zeta potential		Twitching		Swarming	Swimming	
				ave (%)	st dev	ave (mV)	st dev	ave (mm)	st dev		ave (mm)	st dev
1	yes	No	white	9	1	-6	1.0	4.3	2.9	no	16	3.0
2c	yes	No	white	14	3	-6	1.1	5.7	2.5	no	18	2.0
3a	no	Yes	green	51	9	-20	1.2	10	1.0	yes	23	3.2
4	**yes**	**Yes**	**green**	**51**	**17**	**-23**	**0.9**	**7.3**	**1.5**	**yes**	**30**	**4.0**
5	no	No	green*	15	3	-5	0.6	8.7	1.5	yes	28	3.0
6	**no**	**No**	**green**	**45**	**8**	**-24**	**2.1**	**9.3**	**1.2**	**yes**	**22**	**4.2**
7a	no	Yes	green	39	18	-20	1.0	8.7	1.5	yes	21	3.5
8	yes	No	white	5	2	-7	0.8	4	3.0	no	18	2.9
9	**no**	**Yes**	**green**	**52**	**17**	**-20**	**1.7**	**4.7**	**3.2**	**strong**	**30**	**6.1**
10	yes	No	green*	14	10	-24	1.5	7.3	0.6	yes	31	5.5
11b	no	No	green	34	13	-28	1.5	4.3	2.9	yes	16	2.0
12c	yes	No	white	18	3	-5	0.9	5	1.7	no	19	2.1
13	no	Yes	green*	32	17	-7	1.9	6.7	5.1	weak	16	1.0
14b	no	No	green	62	10	-29	1.8	5.7	1.5	weak	16	2.1
15	yes	No	white	13	9	-9	1.1	4.7	1.5	weak	12	0.6
16	yes	No	green	46	15	-33	1.4	4.3	0.6	weak	11	1.5
17	no	No	green*	42	16	-5	0.6	9	1.0	strong	25	3.2
18	**yes**	**No**	**white**	**13**	**9**	**-5**	**0.9**	**4.3**	**1.5**	**no**	**20**	**1.5**
19	**yes**	**No**	**white**	**6**	**3**	**-7**	**1.9**	**4.7**	**1.5**	**no**	**16**	**3.2**
Ref strains												
PAO1	no	No	green	33	9	-23	1.4	8	2.0	yes	23	3.5
rmlC	no	No	green*	3	1	-43	3.7	5.3	0.6	no	16	3.1
fliC pilA								4.7	0.6	yes	-	-

Abbreviations used: ave = average, st dev = standard deviation; pale red = high absolute value, grey = low absolute value; swimming and twitching colours relate to the motility of the reference PAO1, low twitching refer to twitching levels of the PAO1 *fliC pilA* mutant (4.7 mm); Swarming levels shown in supplement Figure 1; CPA isolates written in bold were used in flow chamber experiments; CPA7 and CPA3 were isolated from the same patient 4 days apart (we hypothesize it is the same lineage, future genetic analysis will resolve this question). CPA14 and CPA11 also originated from the same patient. CPA2 and CPA12 displayed the same pattern in pulsed field gel electrophoresis indicating relatedness. green* = strains with unexpected hydrophobicity or zeta potential following colony pigmentation.

The strains can be grouped into two phenotypes based on the observations in Table 2. Higher hydrophobicity, ranging from 32 % to 62 %, was characteristic of the strains with green pigmentation, while non-pigmented strains had lower hydrophobicity ranging from 6 %– 15 % (Figure 1). Most of the non-pigmented strains had weak motility (no swarming, weak swimming and twitching) (Table 2). However, the green pigmented group varied in their motility phenotype and contained both strains with strong and weak swarming as well as twitching and swimming. The majority of the patients with tracheobronchitis (6/8) had isolates that were hydrophobic and belonged to this group with a green pigmentation. The zeta potential ranged from -5 to -9 mV for

the non-pigmented strains and was more negative for the green pigmented strains (-5 to-33 mV). None of the clinical strains had as negative zeta potential as the reference strain PAO1 Δ*rmlC* that previously have shown to have a pronounced negative zeta potential [21]. Whereas several strains showed similar zeta potential to PAO1.

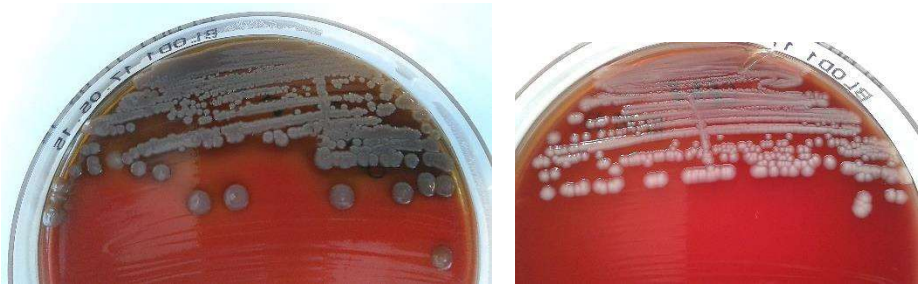


Figure 1. Colony morphology of the strain CPA7 (left) with green pigmentation and the strain CPA8 (right) with no pigmentation (white colonies).

3.2. Adhesion and biofilm formation

To investigate if the different phenotypes of the clinical isolates were reflected in their attachment and biofilm formation, three sets of assays were performed: attachment in multi-well plates after 30 min (Figure 2a), 24 h biofilm formation in 96-well plates (static) (Figure 2b), as well as biofilm formation under flow on two types of surfaces in a flow chamber. The surfaces used were a hydrophilic and antifouling pMEDSAH brush and a reference glass (Figure 3). The glass has previously shown identical biofilm formation compared to hydrophobic polymethylmethacrylate (pMMA) brush surfaces for PAO1 [21].

Based on the attachment ability, clinical strains in our study can be divided in two groups. Weak attachment group included: CPA1, CPA2, CPA5, CPA8, CPA10, CPA12, CPA15, CPA16, CPA17, CPA18, and CPA19 (Figure 2a). Strong attachment group included: CPA3, CPA4, CPA6, CPA7, CPA9, CPA11, CPA13, and CPA14 (Figure 2a). The high-attachment group all displayed comparably high hydrophobicity (more than 30 %, Table 2) and all, except one (CPA13), had negative zeta potentials at -20 mV or lower. They also exhibited green pigmentation and the five strains that demonstrated haemolysis were all in this group. Thus, this high-attachment group seemed to share many characteristic physicochemical properties even if their motility phenotypes differed.

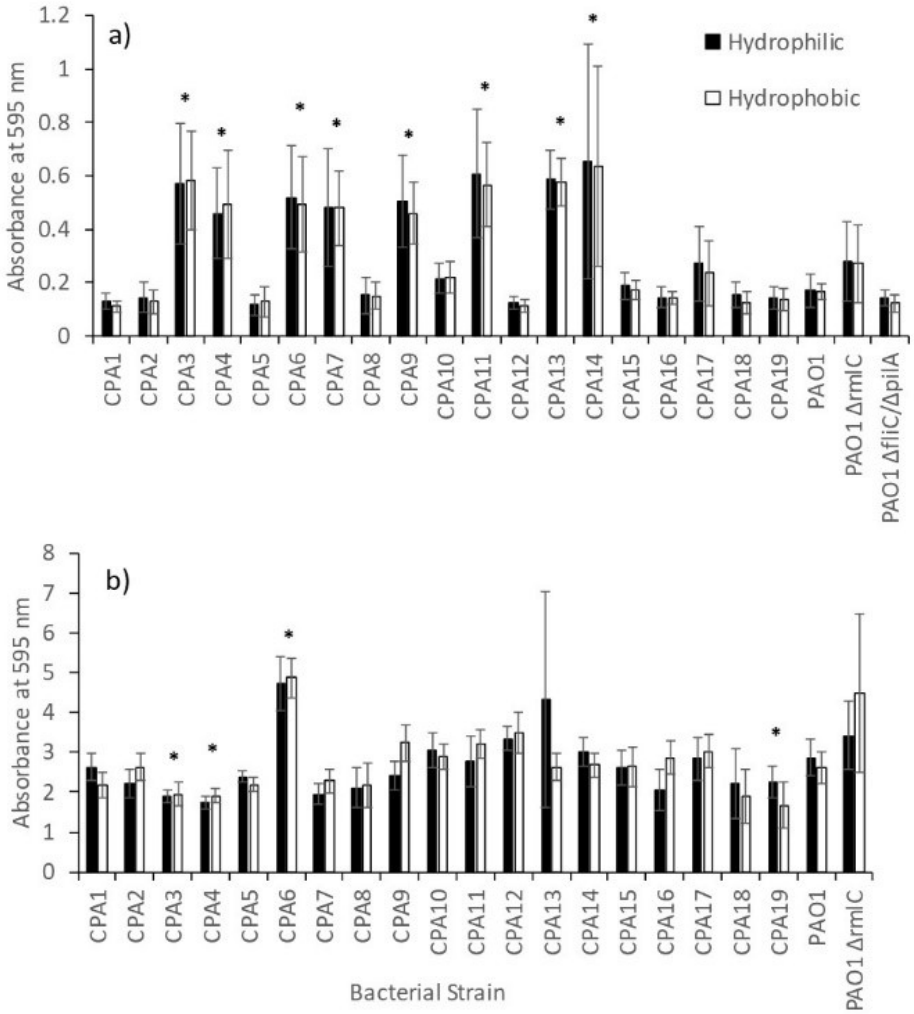


Figure 2. a) Attachment of clinical isolates of P. aeruginosa *and reference strains after 30 min in two types of commercial 96-well plates; black bars - hydrophilic plates; white bars - hydrophobic plates; the data represent an average of 3 independent experiments with at least 4 replicates in each experiment; *= attachment significantly different from PAO1 on both types of plates ((t-test, P ≤0.01, n=12); b) Biofilm formation of clinical isolates and reference strains on two types of commercial 96-well plates after 24h with an initial bacterial loading of 10^4 CFU/mL; black bars - hydrophilic plates; white bars - hydrophobic plates. * = Biofilm formation was significantly different from PAO1 on both types of plates (t-test, p<0.01, n=8).*

The low-attachment group had a more heterogeneous composition consisting of both pigmented and non-pigmented strains, but the large majority of strains demonstrated lower hydrophobicity and a more neutral zeta potential. CPA16 was an exception that showed both high hydrophobicity and strongly negative zeta potential, but displayed low motility. The initial attachment for the high-attachment group was much higher than for the reference strains used in this study.

The crystal-violet assay was used to screen biofilm formation of the different clinical strains using 96-well plates of two different types that are described by the manufacturer as hydrophilic or hydrophobic. For this set of strains, the multi-well assay of biofilm did not show any distinct correlation between the strain phenotype and amount of biofilm formed (Figure 2b) and no difference was observed between the two commercial multi-well plates. However, strain CPA6 produced significantly more biofilm (t-test, $p<0.01$, $n=8$ where n represent number of wells) compared to the other isolates (with the exception of the CPA13 strain on the hydrophilic plate and the PAO1 *rmlC* reference strain).

Based on the data from the multi-well plates, we selected CPA4, CPA6, CPA9 CPA18 and CPA19 as representative strains for more detailed studies using a flow-chamber set up. Strains CPA4 and CPA9 exhibited high attachment, while strains CPA18 and CPA19 showed low attachment. Strain CPA6 was included since it produced the highest amount of biofilm among all strains studied in the overnight multi-well assay. To produce a worst-case scenario biofilm, high loadings of bacterial cells were used in all flow-chamber experiments, *i.e.*, 10^9 CFU/mL. After 48 h, all five strains produced biofilm covering the surface of the reference glass (Figure 3). Certain difference in the structure of the biofilm on glass could be seen between the isolates indicating an influence of the different phenotypes (Figure 3). The low-attachment strains CPA18 and CPA19 produced patchy biofilm.

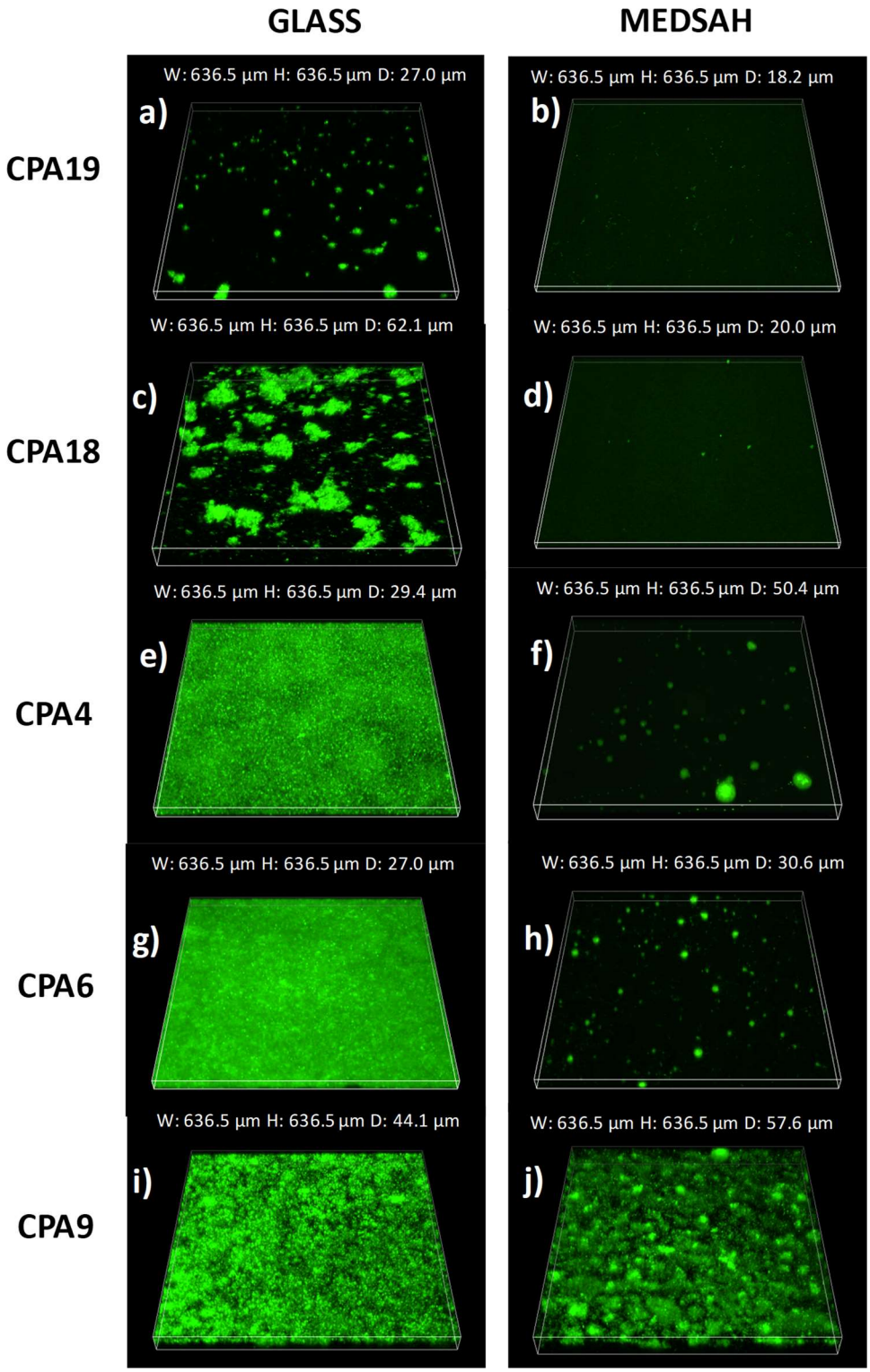


Figure 3. Biofilm formation on reference glass surfaces and pMEDSAH surfaces after 48 h in a flow chamber by strains from the low adhesion group (CPA18 and CPA19) as well as high adhesion group. a) CPA19 glass, b) CPA19 pMEDSAH, c) CPA18 glass, d) CPA18 pMEDSAH, e) CPA4 glass, f) CPA4 pMEDSAH, g) CPA6 glass, h) CPA6 pMEDSAH, i) CPA9 glass, j) CPA9 pMEDSAH.

In the next step, the biofilm formation was investigated on antifouling pMEDSAH coatings known to reduce biofilm formation by bacteria [20]. Interestingly, on these highly hydrophilic and zwitterionic brush surface, the amount of biofilm was greatly reduced for four of the five strains tested, and only small mushroom-shaped biofilm clusters were formed, similar to what has been described for the reference strain PAO1 [21].

4. Discussion

The characterization of the 19 isolates confirmed previous observations that motility in clinical isolates correlate with their ability to form biofilm [27, 29, 35]. An important observation to note in the context of testing bacterial biofilm formation onto materials is that the reference strains did not exhibit as pronounced adhesion as many of the clinical isolates did (Figure 2a). This indicates that *in vitro* studies using reference strains may underestimate the ability of bacteria to attach in clinical settings.

The multi-well plate assay for biofilm formation reflects a colonization process where bacteria can adhere any time during 24 h. Thus, the adhesion differences observed between the strains initially were not resolved after 24h using this assay (Figure 2b). However, strain CPA6 had a pronounced biofilm formation in this assay that was significantly different from the others and the reference. In the strain characterization, this strain exhibited high levels of attachment and had high motility. However, it did not appear to differ from the other strains in the high attachment group (Table 2). Thus, the reason for the increased biofilm formation of this strain in this assay remains an open question. The reported inverse correlation between swarming and biofilm formation from the study by Murray et al [35] is not confirmed in this study as the strains with high attachment all exhibited swarming including strain CPA6.

Monitoring bacterial biofilms using crystal violet is a standard and well-established assay. However, as the bacteria are grown in batch conditions, the nutrients become limited and the products of bacterial metabolism are accumulated with time. Around a medical device, the scenario may be very different as there is often no limitation of nutrients (they are constantly provided by the host). Furthermore, there is often a flux of solution that will transport the products of bacterial metabolism and even bacterial cells away from the surface unless they adhere to it. The use of a flow chamber allows for these types of processes to be mimicked as well as enabling long-term biofilm studies. Using this experimental set-up, the difference in biofilm formation, that were not possible to separate in the multi-well plate experiment, were easily distinguished (Figure 3). The data clearly show that the five strains analyzed fall into three

categories based on their biofilm forming ability on glass and on pMEDSAH; Strains CPA18 and CPA19 formed weak biofilms on the reference and could easily be prevented to form biofilm on the antifouling surface, strains CPA4 and CPA6 formed intact well-developed biofilms on glass and were prevented to large extent from forming biofilms on pMEDSAH, and strain CPA9 formed biofilm on both glass and the antifouling polymer coating. The patchy biofilms observed for CPA18 and CPA19 have been observed previously for strains such as PAO1 *ΔrmlC* that have reduced swimming and no swarming motility [21] (Table 2). Hence, this type of biofilm structure may be explained by reduced motility. Strains CPA18 and CPA19, similarly to PAO1 *ΔrmlC,* had reduced swimming, no swarming and low levels of twitching (Table 2) whereas the other three strains all exhibited swarming and two of them had higher levels of twitching compared to PAO1. The strain CPA9 had lower levels of twitching compared to PAO1, but instead exhibited strong swarming and swimming, which may have compensated for the low levels of twitching. It has been described that swarming, twitching and initial adhesion are tightly co-regulated in *P. aeruginosa* through c-di-GMP-regulated processes building up bacterial biofilms [36], thus illustrating that these processes may be highly interlinked.

From the flow cell data on pMEDSAH, it can be concluded that our previous observations of the *P aeruginosa* lab strain PAO1 [21] holds true for clinical strains with a more adhesive phenotype. However, this phenomenon would probably not have been observed if strains with very low biofilm formation ability had been used. The highly motile strain CPA9 from the high adhesion group appeared to be able to colonize also the antifouling coatings similarly to glass. Further studies will reveal if it is only the pronounced motility in the form of swarming that enables this enhanced colonization ability or if this is linked to other factors promoting biofilm formation.

5. Conclusions

The characterization of the clinical isolates presented in this study suggests that suitable model strains for assessing biofilm formation on biomaterials in the airways would be the strains CPA6 and CPA9. Both are good biofilm producers and share several phenotype features with the high-attachment group of isolates and formed a good biofilm in all experiments. These strains do not appear to be infected by phage indicating that they should be stable model strains for routine testing. Strain CPA9 is interesting as it showed an enhanced capability to form biofilm on highly hydrophilic polymer surfaces that strain CPA6 were less able to colonize. Thus, we suggest that these two strains could function as model strains for testing biofilm formation on medical devices and would have a larger clinical relevance for studies of airway devices than for example

the *P. aeruginosa* lab strain PAO1. Furthermore, we suggest that the methodology used here to identify clinically relevant strains for testing medical devices could be used on other collections of clinical isolates. This could provide the scientific community with the "next generation" model strains tailored for different device areas of the human body, enabling an increased clinical relevance of biomaterials testing with respect to biofilm formation.

The data on biofilm formation on the zwitterionic model surface is very interesting as it suggests that coating of an endotracheal tube with a highly hydrophilic coating may reduce the amount of biofilm formed by the majority of the bacterial strains tested even at the very high inoculum used in this study. However, despite the promising results for biofilm reduction in four out of five strains, one strain was able to colonize the surface and form a biofilm. Further studies will reveal the mechanism behind this enhanced colonization on this antifouling pMEDSAH surface and how it depends on bacterial load. Future studies may also shed light on how a wider variety of polymer surfaces may be colonized or are able to prevent colonization from these new model strains.

An intriguing clinical aspect of this library of isolates, deserving future investigation, is the link between phenotype observed and likelihood to cause infection versus colonization. If such prediction power could be obtained this could prove to be of great use for clinics working to treat infected patients. A future "next generation" collection of clinical model strains from several device areas would give a good basis for building such enhanced understanding. This could facilitate the identification of "problem strains" in clinic as well as open up opportunities to tailor device surfaces to prevent colonization of these specific problem strains while neglecting harmless strains.

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