Diversity of biofilms produced by quorum-sensing-deficient clinical isolates of *Pseudomonas aeruginosa*

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The quorum-sensing (QS) systems control several virulence attributes of *Pseudomonas aeruginosa*. Five QS-deficient *P. aeruginosa* clinical isolates (CI) that were obtained from wound (CI-1), tracheal (CI-2, CI-3, CI-4) and urinary tract (CI-5) infections had previously been characterized. In this study, a flow-through continuous-culture system was utilized to examine in detail the biofilms formed by these isolates in comparison with the *P. aeruginosa* prototrophic strain PAO1. Analysis of the biofilms by confocal laser scanning microscopy and COMSTAT image analysis at 1 and 7 days post-inoculation showed that the isolates produced diverse biofilms. In comparison with PAO1, the CI produced biofilms that scarcely or partially covered the surface at day 1, although CI-1 produced larger microcolonies. At day 7, CI-2 and CI-4 produced mature biofilms denser than that produced by PAO1, while the biofilm formed by CI-1 changed very little from day 1. CI-1 was defective in both swarming and twitching motilities, and immunoblotting analysis confirmed that it produced a reduced level of PilA protein. The twitching-motility defect of CI-1 was not complemented by a plasmid carrying intact *pilA*. In the 48 h colony biofilm assay, the CI varied in susceptibility to imipenem, gentamicin and piperacillin/tazobactam. These results suggest that: (1) the isolates produced biofilms with different structures and densities from that of PAO1; (2) biofilm formation by the isolates was not influenced by either the isolation site or the QS deficiencies of the isolates; (3) the behaviour of CI-1 in the different biofilm systems may be due to its lack of swarming motility and type IV pilus-related twitching motility.

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INTRODUCTION

Pseudomonas aeruginosa is a versatile Gram-negative pathogen that produces severe infections in immunocompromised patients, including severely burned patients, patients with cystic fibrosis (CF) and cancer patients undergoing chemotherapy (Pollack, 2000; van Delden & Iglewski, 1998). The severity of *P. aeruginosa* infections is due to the production of different extracellular and

cell-associated virulence factors (Pollack, 2000). These factors contribute to different aspects of *P. aeruginosa* pathogenesis, including biofilm formation (Pollack, 2000; Rumbaugh *et al.*, 2000). Under certain conditions, bacteria attach to biotic and abiotic surfaces and develop into organized communities termed biofilms (Donlan & Costerton, 2002). During the formation of these biofilms, bacteria proliferate and produce exopolymers (extracellular matrix) and large complex structures, which protect the bacteria from the host immune response as well as against different antimicrobials (Costerton *et al.*, 1999; Hentzer *et al.*, 2001; Matsukawa & Greenberg, 2004; Stewart & Costerton, 2001). Biofilm formation is important in the establishment of *P. aeruginosa* infections on different host tissues, including the lung alveoli of the CF patient

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Abbreviations: 3OC₁₂-HSL, *N*-(3-oxododecanoyl) homoserine lactone; C₄-HSL, *N*-butyryl homoserine lactone; CF, cystic fibrosis; CI, clinical isolate(s); max. thickness, maximum thickness; QS, quorum sensing; rc, roughness coefficient; sbr, surface-to-biovolume ratio; ssc, substratum surface coverage.

(Donlan & Costerton, 2002; Prince, 2002). *P. aeruginosa* also forms biofilms on medical devices such as urinary catheters (Stickler, 2002). In these settings, the antibiotic resistance engendered by biofilms presents a serious challenge to the treatment of chronic *P. aeruginosa* infections (Donlan & Costerton, 2002).

Using microscopy, *in situ* reporter gene analysis and 2D electrophoretic analysis, Sauer *et al.* (2002) have suggested that biofilm development by *P. aeruginosa* occurs in five specific stages: reversible attachment, irreversible attachment, maturation-1, maturation-2 and dispersion. Flagellar motility facilitates the reversible attachment. After the initial attachment to the surface, *P. aeruginosa* cells move along the surface by their type IV pili and proliferate into small microcolonies (Klausen *et al.*, 2003; O'Toole & Kolter, 1998a). The irreversible-attachment stage is characterized by the development of these cell clusters (Sauer *et al.*, 2002). During maturation-1 and -2, the cell cluster thickness increases and reaches maximum development (Sauer *et al.*, 2002). During the dispersion stage, bacteria actively depart the cell clusters, possibly through flagellar-mediated motility (Sauer *et al.*, 2002).

Biofilm formation by *P. aeruginosa* involves the cell-to-cell communication quorum-sensing (QS) systems. QS is a cell-density-dependent mechanism through which bacteria coordinate different activities, including bioluminescence, plasmid conjugation and the production of different virulence factors (Donlan & Costerton, 2002; Rumbaugh *et al.*, 2000; Venturi, 2006). *P. aeruginosa* possesses at least two well-defined, interrelated QS systems, *las* and *rhl*, that control the production of different virulence factors (Rumbaugh *et al.*, 2000; Venturi, 2006). Each QS system consists of two components, the autoinducer synthases (LasI and RhlI, respectively) and their cognate transcriptional regulators (LasR and RhlR, respectively) (Rumbaugh *et al.*, 2000; Venturi, 2006). LasI is the synthase for the autoinducer *N*-(3-oxododecanoyl) homoserine lactone (3OC₁₂-HSL), while RhlI synthesizes the autoinducer *N*-butyryl homoserine lactone (C₄-HSL) (Rumbaugh *et al.*, 2000; Venturi, 2006). Based on the analysis of PAO1 and its QS-defective isogenic mutants, previous studies have suggested that in *P. aeruginosa*, QS is involved in both the initiation of biofilm formation and the maturation of the biofilm (Davies *et al.*, 1998; De Kievit *et al.*, 2001). The *las* QS system appears to be important during the late but not the early stages of biofilm development (De Kievit *et al.*, 2001). The mature architecture of a particular biofilm also appears to be dependent on the carbon source available. Biofilms formed in glucose minimal medium develop characteristic larger structures, towers and forms that resemble mushrooms, while those formed in citrate minimal medium are much flatter and more homogeneous (Davies *et al.*, 1998; Heydorn *et al.*, 2002; Klausen *et al.*, 2003; Stewart *et al.*, 1993). Recently, Shrout *et al.* (2006) have suggested that the nutritional requirement influences the contribution of QS to biofilm development by *P. aeruginosa*.

Many earlier *P. aeruginosa* virulence studies (both *in vitro* and *in vivo*) have utilized isogenic mutants derived from strain PAO1. Although PAO1 was originally obtained from a human infection (infected wound) (Holloway *et al.*, 1979), it has been passaged *in vitro* in different laboratories for several decades. Thus, PAO1 may no longer be similar to freshly isolated *P. aeruginosa* strains. A series of *P. aeruginosa* strains isolated from CF patients had larger genome sizes and exhibited greater genomic diversity than PAO1 (Head & Yu, 2004). Analyses of *P. aeruginosa* clinical isolates (CI) have provided further insight into the mechanisms of *P. aeruginosa* infections. Rumbaugh *et al.* (1999) have shown that compared to CI from respiratory tract infections, CI from urinary tract and wound infections produce more exotoxin A and exoenzyme S, and that prolonged infection with a strain enhances exoenzyme S production. Roy-Burman *et al.* (2001) have shown a strong correlation between the expression of type III secretion proteins by CI and patient death. Head & Yu (2004) have shown that *P. aeruginosa* isolates from CF patients differ among each other and also in comparison with non-CF isolates in many aspects of biofilm formation. In addition, Lee *et al.* (2005) have found differences in the ability of CF isolates to form biofilms. They also suggest that biofilm development may not be necessary for the longitudinal survival of non-mucoid *P. aeruginosa* during chronic infection of the CF lung, as the ability of sequential isolates to form biofilm *in vitro* decreases over time (Lee *et al.*, 2005).

We have recently characterized five QS-deficient CI of *P. aeruginosa*. The isolates produce no LasB elastase, and variable levels of exotoxin A and exoenzyme S; they also vary in their swimming and twitching motilities (Schaber *et al.*, 2004). Analysis of biofilm initiation using the crystal violet assay revealed that four of the isolates do not effectively adhere to the polystyrene surface (Schaber *et al.*, 2004). In this study, we extended our biofilm analysis of these CI to include the flow-through continuous-culture system and the colony-biofilm system. In addition, we further characterized one of the isolates with respect to pilin production.

METHODS

Bacterial strains, media and growth conditions. The prototypic *P. aeruginosa* strain PAO1 (Holloway *et al.*, 1979) was used as a positive control for biofilm formation and for different assays. Its isogenic *pilA* mutant PAOΔ*pilA* (Klausen *et al.*, 2003) was used as a negative control for the analysis of twitching motility and the PilA protein. CI-1 to CI-5 were obtained from human infections: CI-1 from a wound infection, CI-2 and CI-4 from tracheal aspirates obtained 1 month apart from a patient with lower respiratory tract infection, CI-3 from sputum of a patient with lower respiratory tract infection and CI-5 from a urinary tract infection (catheterized urine) (Schaber *et al.*, 2004). Strains were routinely grown in Luria-Bertani (LB) broth (Miller, 1972) at 37 °C with shaking (250 r.p.m.). Biofilm formation was examined using M63 minimal medium (M63GCA) (13.6 g KH₂PO₄ l⁻¹, 2.0 g (NH₄)₂SO₄ l⁻¹, 0.5 mg FeSO₄·7H₂O l⁻¹, pH 7.0) supplemented with 0.2 % (w/v) glucose,

1 mM MgSO₄·7H₂O and 0.5% (w/v) Casamino acids (Miller, 1972; O'Toole & Kolter, 1998b).

Flow-through continuous culture. Development of the *P. aeruginosa* biofilm on a glass surface (~400 mm²) was monitored using a multi-cell flow-through continuous-culture system at 37 °C (Davies *et al.*, 1998). Polycarbonate flow devices (Protofab) were sealed with glass coverslips (45 × 50 mm, 1.5 mm thickness) and secured with stainless steel brackets. The coverslips were pretreated with 0.5 M HCl for 1 h. The flow cells were attached to #16 silicone tubing and sterilized by autoclaving. Sterile M63GCA was maintained in a 10 l reservoir and pumped to the flow cell through one-eighth inch (internal diameter) silicone tubing using a six-roller-head peristaltic pump (Masterflex). The flow rate was maintained at 0.6 ml min⁻¹. The fluid residual time was ~3 min with laminar flow. M63GCA that passed through the chamber was collected in a second 10 l reservoir. Overnight cultures were subcultured (1 : 100), grown in M63GCA for ~3 h and then diluted in fresh M63GCA to OD₆₀₀ 0.02 (~10⁷ c.f.u. ml⁻¹) (DU-70 Spectrophotometer, Beckman). A 3 ml aliquot of this diluted culture was injected by syringe immediately upstream of the flow cell. After inoculation, flow was stopped for 1 h to allow the bacteria to attach to the glass surface and then followed by continuous flow until the end of the experiment.

Microscopy of the biofilms. Syto 61 red fluorescent nucleic acid stain (Invitrogen), which stains red both live and dead cells, was used according to the manufacturer's recommendations to visualize biofilms. Biofilm formation by each strain was examined in three separate experiments. Seven image stacks were obtained from random positions within the middle section of each flow cell for a total of 21 image stacks for each strain. Images were acquired at 1–3 µm intervals through the biofilms using an Olympus IX71 Fluoview 300 confocal laser scanning microscope through a UPlanApo ×40/1.00 numerical aperture oil objective and a 633 nm Red Helium Neon laser (Olympus America). Biofilm image reconstruction was performed using NIS-Elements 2.2 (Nikon Instruments).

COMSTAT analysis of biofilms. The 21 image stacks obtained per strain were analysed using the COMSTAT program (Heydorn *et al.*, 2000). We examined the following parameters (Table 1): substratum coverage (ssc), a reflection of the efficiency with which the strain colonizes the surface; mean thickness, the mean height of the biofilm; maximum (max.) thickness; roughness coefficient (rc), a measure of how much the thickness of the biofilm varies; and surface-to-biovolume

ratio (sbr), an estimate of the portion of the biofilm exposed to nutrients.

Real-time quantitative PCR. This was done as previously described (Carty *et al.*, 2003). Briefly, overnight cultures of each strain were subcultured into fresh M63GCA and grown to OD₆₀₀ 2.2–3.2. Total RNA was extracted by the hot phenol method and cDNA was synthesized using random oligonucleotides. The cDNA was then subjected to PCR using specific *lasI* and *rhlI* primers (*lasI*: forward 5'-TTCGCCATCAACTCTGGACA-3', reverse 5'-CGTACAGTCGGAA-AAGCCCA-3'; *rhlI*: forward 5'-GGCGATGAAGATATTCTGGTCC-3', reverse 5'-ACTGGCGCCCAGGTACC-3'). Detection of amplified products was accomplished using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Measurement of 16S rRNA was used as an internal standard. Each real-time quantitative PCR experiment was repeated twice using separate cultures. In each experiment, duplicate pellets were obtained for RNA extraction.

Swarming and twitching motilities. Swarming plates consisted of nutrient broth (8 g l⁻¹), glucose (5 g l⁻¹) and agar (0.5%, w/v) (Boles *et al.*, 2005). The plates were dried at room temperature for several hours, inoculated with overnight cultures of the strains and incubated at 32 °C for 48 h. Swarming motility was compared by measuring the diameters of the colonies on the plates.

Twitching motility was measured as previously described (Schaber *et al.*, 2004; Deziel *et al.*, 2001). Briefly, individual colonies of the strains were stab-inoculated through the agar to the bottom of 1% (w/v) agar plates. Following incubation at 32 °C for 24 h, the agar was removed and the bacterial growth on the plastic surface was visualized with 1% (w/v) crystal violet. Twitching motility was determined by measuring the diameter of the stained growth. Assays for both twitching and swarming motility were repeated at least three times.

Immunoblotting analysis. *P. aeruginosa* strains were grown overnight in LB broth and adjusted to equivalent OD₆₀₀ with LB broth. Whole-cell extracts were prepared from 1 ml cell pellets by resuspending the cells in 100 µl lysis-loading buffer (125 mM Tris/HCl, pH 6.8, 20%, v/v, glycerol, 4%, w/v, SDS, 0.005%, w/v, bromophenol blue and 700 mM 2-mercaptoethanol) and boiling for 5 min. Proteins were separated by 15% SDS-PAGE and transferred to PVDF membranes (Immun-Blot, Bio-Rad). Membranes were probed for PilA using rabbit polyclonal anti-PilA antibody at a dilution of 1 : 20 000. The probed membranes were treated with anti-rabbit

Table 1. Quantitative analysis of 1- and 7-day-old biofilms formed by PAO1 and the CI

The analysis was conducted using the COMSTAT program. In all experiments, images were acquired from random positions in the middle of the flow channel. Results represent the mean ± SEM of three independent experiments (seven image stacks per experiment with a fixed threshold).

Biofilm characteristic	Day	Strain					
		PAO1	CI-1	CI-2	CI-3	CI-4	CI-5
ssc (%)	1	59.02 ± 2.55	7.50 ± 0.96	6.92 ± 1.06	0.24 ± 0.03	2.78 ± 0.41	16.47 ± 2.28
	7	72.29 ± 4.73	9.62 ± 2.10	77.35 ± 5.75	19.14 ± 4.88	87.44 ± 3.74	32.52 ± 4.84
Mean thickness (µm)	1	3.09 ± 0.23	1.07 ± 0.17	0.17 ± 0.03	0.01 ± 0.00	0.07 ± 0.01	0.71 ± 0.12
	7	25.25 ± 5.33	1.12 ± 0.31	8.88 ± 1.06	2.43 ± 0.74	14.21 ± 1.02	7.02 ± 1.14
Max. thickness (µm)	1	10.62 ± 0.22	26.52 ± 0.91	7.00 ± 0.47	6.14 ± 0.24	8.00 ± 0.33	8.81 ± 0.36
	7	61.67 ± 12.44	29.76 ± 3.80	20.76 ± 1.09	23.33 ± 1.37	26.52 ± 0.98	79.81 ± 7.50
rc	1	0.66 ± 0.04	1.78 ± 0.03	1.87 ± 0.02	2.00 ± 0.00	1.94 ± 0.01	1.58 ± 0.06
	7	0.37 ± 0.07	1.78 ± 0.05	0.47 ± 0.12	1.47 ± 0.14	0.21 ± 0.05	1.41 ± 0.07
sbr (µm ⁻² µm ⁻³)	1	3.97 ± 0.12	3.38 ± 0.12	6.44 ± 0.25	6.91 ± 0.30	6.62 ± 0.07	5.30 ± 0.08
	7	3.33 ± 0.28	6.28 ± 0.45	3.44 ± 0.57	5.30 ± 0.40	2.17 ± 0.42	3.83 ± 0.38

horseradish peroxidase-conjugated IgG (Sigma-Aldrich) and developed using SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology). *P. aeruginosa* strain PAO1 was used as a positive control while its PilA-deficient isogenic mutant PAO Δ pilA (Klausen *et al.*, 2003) was used as a negative control.

Phage adsorption (efficiency of plating). Phage F116L was amplified by standard methods (Kutter & Sulakvelidze, 2005; Martin *et al.*, 1993) and serially diluted in 10-fold steps. Each tested bacterial strain was grown to OD₆₀₀ 0.5 ($\sim 10^9$ c.f.u. ml⁻¹) in LB broth. A 100 μ l aliquot of the culture was incubated with 100 μ l of each F116L phage dilution for 10 min at 37 °C. Each mixture was added to 3 ml LB soft agar (7.5 g l⁻¹) and the soft agar was poured over LB agar plates. The plates were incubated overnight at 37 °C. Efficiency of plating was calculated as mean phage titre determined for each bacterial strain divided by the mean phage titre determined for PAO1 (Kutter & Sulakvelidze, 2005). Each experiment was performed in triplicate.

Colony biofilm assay. These experiments were conducted using 25 mm diameter, 0.22 μ m pore-size black polycarbonate membrane filters (Poretics), as described elsewhere (Anderl *et al.*, 2003). Both sides of the membrane filters were exposed to UV light for 15 min and the membranes were gently pressed onto the surface of M63GCA agar (1.8 %, w/v) plates. *P. aeruginosa* strains grown overnight in LB broth at 37 °C were subcultured 1 : 100 in M63GCA and incubated at 37 °C for 3 h. The subcultures were diluted in fresh M63GCA to OD₆₀₀ 0.2 ($\sim 10^7$ c.f.u. ml⁻¹) and 10 μ l of each diluted strain was spotted in the centre of the membrane on the M63GCA agar plate and allowed to dry. Inverted plates were incubated at 37 °C for a total of 48 h, and the membranes were transferred to fresh M63GCA agar plates at 24 h. Colony biofilms were harvested by aseptically transferring the membranes to 10 ml sterile PBS (pH 7.0) and vortexing for 2 min. The cell suspensions were serially diluted in PBS and drop-plated (10 μ l aliquots) on LB agar. Plates were allowed to dry, inverted and incubated at 37 °C overnight. Numbers of micro-organisms were calculated as c.f.u. per square centimetre of the membrane.

Antibiotic susceptibility of the colony biofilms. This was determined by transferring the 48 h biofilms from the M63GCA agar plates onto LB plates supplemented with at least ten times the MIC of antibiotic previously determined for planktonic cells of CI-1 and PAO1 (data not shown; Schaber *et al.*, 2004); that is, imipenem at 40 μ g ml⁻¹, gentamicin at 40 μ g ml⁻¹ and piperacillin/tazobactam at 80 μ g ml⁻¹. After 16 h incubation at 37 °C, the membranes were lifted from the antibiotic agar plates and c.f.u. were determined as described above. Killing of the biofilm cells was calculated as log reduction according to the following formula (Anderl *et al.*, 2003):

$$-\log_{10}(\text{c.f.u. with antibiotic}/\text{c.f.u. without antibiotic}).$$

Significant differences in log reductions between PAO1 and CI-1 were calculated by unpaired *t* test using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software).

RESULTS

Biofilm formation by the QS-deficient CI

We have previously described five CI that are defective in QS, producing 2–20 % of the 3OC₁₂-HSL elaborated by PAO1 and 0–34 % of the C₄-HSL (Schaber *et al.*, 2004). Using the crystal violet biofilm assay, we showed that four of the five CI were less than 35 % as efficient as PAO1 in the initiation of biofilms, while one was 82 % as efficient (Schaber *et al.*, 2004).

The QS defects in these CI may also influence their ability to develop a mature biofilm. Therefore, to determine if one or more was able to form mature biofilms and to examine the structure of these biofilms, we monitored biofilm formation by the CI using a flow-through continuous-culture system irrigated with M63 minimal medium containing glucose as a carbon source (M63GCA) (O'Toole & Kolter, 1998b). As a control, we utilized the *P. aeruginosa* strain PAO1, which forms a well-developed characteristic mature biofilm in this medium (O'Toole & Kolter, 1998b). Using confocal laser scanning microscopy, we observed biofilm initiation at day 1 post-inoculation and maturation of the biofilm at day 7. We also analysed the biofilm quantitatively using the COMSTAT program (Heydorn *et al.*, 2000).

As shown in Fig. 1 and Table 1, and briefly described below, the day 1 and day 7 biofilms formed by the CI differed from that produced by PAO1 and from each other.

PAO1. The day 1 biofilm was characterized by the formation of a monolayer that covered much of the substratum surface (59 %) with multiple small microcolonies (rc 0.66). At day 7, PAO1 formed a more structured biofilm with regular clusters of large, differentiated microcolonies (ssc 72 %, max. thickness 61.67 μ m, rc 0.37).

CI-1. The day 1 biofilm was significantly less dense than that of PAO1 (ssc 7.5 %), and the microcolonies were fewer but larger than those of the PAO1 day 1 biofilm (max. thickness 26.52 μ m compared to 10.62 μ m). In the day 7 biofilm, the glass surface was evenly spotted with these microcolonies (ssc 9.6 %), but they had changed little in height (max. thickness 29.8 μ m). At both day 1 and day 7, this CI produced the most variable biofilm of all isolates tested (rc on both days of 1.78).

CI-2. The day 1 biofilm showed a less dense ssc than PAO1 (7 %) with small microcolonies visible (max. thickness 7 μ m). However, by day 7 the surface was more densely covered with a rougher biofilm than PAO1 (ssc 77 %, rc 0.5 compared to 0.37). In addition, large filaments were observed arching among the microcolonies (data not shown).

CI-3. At day 1, very few bacteria had attached to the surface (ssc 0.2 %), forming sparse microcolonies with a max. thickness of only 6.14 μ m, but by day 7, the strain covered 19 % of the glass surface with microcolonies reaching 23.33 μ m in height.

CI-4. This strain was a sequential isolate from the same patient and same site as CI-2. Its day 1 biofilm covered less surface area (2.8 %) than that of CI-2, but the day 7 biofilm covered more surface area (87 %). Its day 7 biofilm was thicker (max. thickness 26 μ m versus 21 μ m) but smoother (rc 0.2 compared to 0.5) than that of CI-2. The same arching filaments were observed (data not shown).

CI-5. At day 1, this strain covered the greatest surface area of any of the CI (16 %). At day 7, 32 % of the surface was

Fig. 1. Representative structures of the biofilms formed by PAO1 and the CI. The strains were inoculated in flow-through continuous-culture chambers in M63GCA. The biofilms were analysed by confocal laser scanning microscopy at days 1 and 7 post-inoculation. Magnification $\times 400$; bars, 20 μm .

covered with variably sized microcolonies, including thick towers of cells reaching 80 μm in height.

These data indicate the following features of the CI biofilms. In comparison with PAO1, all CI initiated biofilm formation significantly less efficiently (ssc 0.24–16.47%). The day 1 CI biofilms also showed higher degrees of diversity (all rc greater than that of PAO1), and except for CI-1, which was similar to PAO1, the CI produced more scattered microcolonies (sbr greater than that of PAO1). By day 7, the surface coverage of the mature biofilms of CI-2 and CI-4 exceeded that of PAO1, but similar to PAO1, CI-2 and CI-4 formed relatively homogeneous biofilms (rc 0.37, 0.47 and 0.21; sbr 3.33, 3.44 and 2.17, respectively). In contrast, CI-1 produced the least dense (ssc <10% of that

of PAO1) and most heterogeneous (rc 1.78, sbr 6.28) mature biofilm of the CI. The mature biofilm of CI-3 was also less dense and more heterogeneous than that of PAO1 (ssc 19%, rc 1.47, sbr 5.3). CI-5, which formed a mature biofilm with moderate ssc and heterogeneity, produced unique towering structures, yet its sbr was similar to that of PAO1 (3.82 versus 3.33). Next, we compared the general growth characteristics of planktonic cells of the CI with those of PAO1. As shown in Fig. 2, there were no major differences in the growth characteristics of the CI and PAO1 at the early exponential, mid-exponential or stationary phases of growth.

lasI and *rhlI* transcription in the CI

Using the flow-through continuous-culture system, Davies *et al.* (1998) suggested that the *lasI* gene is important in the development of PAO1 biofilm. In addition, De Kievit *et al.* (2001) showed that during the course of an 8 day biofilm, *lasI* expression decreases progressively, while that of *rhlI* is steady but occurs in a low percentage of cells. With the exception of CI-5, our isolates produced significantly lower levels of 3OC₁₂-HSL and C₄-HSL, yet PCR analysis of their chromosomes suggested that all the CI carried the *lasI* and *rhlI* genes that code for the 3OC₁₂-HSL and C₄-HSL synthases, respectively (Schaber *et al.*, 2004). Therefore, we used quantitative real-time PCR to determine whether *lasI* and *rhlI* were transcribed in the CI as well as the relative levels of any transcription. As shown in Fig. 3, the levels of *lasI* transcripts produced by the isolates ranged from 37–71% of that produced by PAO1. Similarly, the levels of *rhlI* transcripts ranged from 35–92% of that produced by PAO1 (Fig. 3). This suggests that the defect in the production of 3OC₁₂-HSL and C₄-HSL is not due to inefficient expression of the *lasI* or *rhlI* genes. In addition, despite the lack of functional *lasR* or *rhlR* genes (Schaber *et al.*, 2004), CI-2 and CI-4 still developed mature biofilms (Fig. 1, Table 1).

Fig. 2. Comparison of the growth rates of PAO1 and the CI. An overnight culture of each strain was inoculated into fresh LB broth to OD₅₄₀ 0.03–0.05. Cells were grown at 37 °C with shaking for 12 h. Samples were obtained at the indicated times and the OD₅₄₀ (indicative of growth rate) of each sample was determined.

Fig. 3. Relative expression of *lasI* and *rhII* in PAO1 and different CI, as determined by real-time quantitative PCR. Cells were grown in M63GCA to OD₆₀₀ 2.2–3.2. RNA extraction and PCR experiments were conducted as previously described (Carty *et al.*, 2003). Relative expression of *lasI* (black bars) and *rhII* (white bars) in the CI is reported as a percentage of that detected in PAO1 (the level of expression in PAO1 was taken as 100%). Each real-time PCR experiment was repeated twice using separate cultures. In each experiment, duplicate pellets were obtained for RNA extraction.

Examining the CI for swarming and twitching motilities

Shrout *et al.* (2006) have recently shown that the carbon source plays an important role in the ability of *P. aeruginosa* QS mutants to develop a biofilm. This effect appears to occur through the regulation of swarming motility by the QS systems (Shrout *et al.*, 2006). Therefore, we examined the CI for their swarming motility. As shown in Fig. 4, CI-2 and CI-4 swarmed better than the PAO1 control strain, while CI-5 swarmed less than PAO1. CI-3 showed a considerable reduction in its swarming motility,

Fig. 4. Swarming motility of PAO1 and the CI. Swarming motility was assayed on nutrient agar swarming plates containing 5 g glucose l⁻¹ and 0.5% (w/v) agar incubated at 32 °C for 48 h. The assay for swarming motility was repeated at least three times.

while CI-1 did not swarm (Fig. 4). We have previously reported that the CI also produce variable levels of twitching motility (Schaber *et al.*, 2004). However, in that study we did not compare the isolates with a twitching-motility-deficient mutant of PAO1 (a negative control). Thus, in this study we re-examined the CI for twitching motility in comparison with PAOΔ*pilA* using the twitching-motility plate assay (Deziel *et al.*, 2001). Compared to PAO1, which produced the typical twitching-motility phenotype, neither PAOΔ*pilA* nor CI-1 produced distinctive twitching motility (Fig. 5a). The rest of the CI were not defective in their twitching motility (data not shown).

CI-1 produced the least dense and most heterogeneous biofilm of the CI (Fig. 1, Table 1). Klausen *et al.* (2003) have suggested that after the initial phase of biofilm formation, the type IV pilus (through its contribution to twitching motility) is required for the spread of *P. aeruginosa* on the substratum. In comparison with its parent strain, the pilin-deficient mutant PAOΔ*pilA* formed a heterogeneous biofilm with irregular structure and failed to spread on the glass surface (Klausen *et al.*, 2003). Many features of the CI-1 mature biofilm resembled those of the biofilm formed by the type IV pilus-deficient mutant PAOΔ*pilA* (Fig. 1, Table 1; data not shown). To determine if the twitching-motility-negative phenotype of CI-1 is due to the loss of type IV pilin protein PilA, we examined CI-1 in immunoblotting experiments using a PilA polyclonal antibody. The 15 kDa PilA protein was detected within the whole-cell lysate of PAO1 but not PAOΔ*pilA* (Fig. 5b). The whole-cell lysate of CI-1 produced a faint band that migrated at the same distance as the pilin protein of PAO1 (Fig. 5b), indicating that CI-1 produces a reduced amount of PilA. To determine whether this reduced amount of PilA leads to surface expression of the type IV pilus, we examined the ability of phage F116L (Pemberton, 1973) to infect CI-1. Phage-adsorption experiments were conducted as described in Methods. Strain PAOΔ*pilA* was utilized as a negative control. The efficiencies of plating for PAO1, PAOΔ*pilA* and CI-1 were 1, 0 and 1.8×10^{-7} , respectively (data not shown). These results suggest that despite the presence of low levels of PilA, CI-1 lacks twitching motility because it fails to assemble type IV pili.

To determine whether the biofilm produced by CI-1 is indeed due to the deficiency in production of the type IV pilus, we attempted to complement the defect with intact *pilA*. A 776 bp fragment that carries intact *pilA* (450 bp coding sequence plus upstream and downstream regions) was amplified from the chromosome of PAO1 by PCR. The fragment was cloned into the *EcoRI*/*SacI* sites of the broad-host-range cloning vector pUCP18 (Schweizer, 1991). The resulting plasmid p8566 was introduced into PAOΔ*pilA* and CI-1 by electroporation (Smith & Iglewski, 1989). As shown in Fig. 5(c), p8566 complemented the defect in twitching motility of PAOΔ*pilA* but not CI-1. Thus, CI-1 may carry a mutation in a gene(s) that regulates the synthesis of the type IV pilus. Taken together, these results suggest that the biofilm phenotype of CI-1 may be due to

Fig. 5. Twitching motility and PilA production by PAO1, PAO Δ *pilA* and CI-1. (a) Twitching motility of the strains was determined by stab inoculation of single colonies from overnight growth on LB agar into soft LB agar plates. Plates were incubated at 32 °C for 24 h followed by an additional 48 h at room temperature. Motility was evaluated by crystal violet (1 %, w/v) staining of the growth on the plastic Petri plate under the agar. (b) Immunoblot of whole-cell lysates of PAO1, PAO Δ *pilA* and CI-1 for PilA production, as described in Methods. (c) Complementation analysis of the twitching-motility defect of PAO Δ *pilA* and CI-1 using plasmid p8566, which carries intact *pilA* from PAO1. Twitching motility was analysed as described in (a). The assays for twitching motility were repeated at least three times.

the deficiency of this isolate in both swarming and twitching motility.

Colony biofilm formation by CI-1

Depending on the type of infection and the tissue to which it is attached, *P. aeruginosa* may form different biofilms. For example, CI-1 was isolated from a wound infection

(Schaber *et al.*, 2004). It has been hypothesized that *P. aeruginosa* may form a biofilm during chronic wound infections (Costerton *et al.*, 1999), although such a biofilm would not be exposed to fluid-flow shear forces as in a urinary tract infection (Nicolle, 2005). The colony biofilm system (Borriello *et al.*, 2004; Walters *et al.*, 2003) resembles the infectious environment of a wound and can be utilized to examine biofilms *in vitro*. Thus, we examined the CI in colony biofilm experiments using polycarbonate membranes, as described elsewhere (Borriello *et al.*, 2004; Walters *et al.*, 2003). At 48 h post-inoculation, the c.f.u. cm⁻² of the CI were five- to 10-fold less than that of PAO1 (1×10^6 versus 1×10^7) (data not shown). The c.f.u. cm⁻² of CI-1 was 10-fold less than that of PAO1, which suggests that, as in the flow-through continuous-culture system, CI-1 is not as efficient as PAO1 at forming a colony biofilm.

We then examined the susceptibility of colony biofilms formed by the CI to several antibiotics. We had previously determined that the planktonic cells of the CI varied in their susceptibility to three antibiotics frequently used to treat *P. aeruginosa* infections: imipenem (carbapenem β -lactam), gentamicin (aminoglycoside) and piperacillin/tazobactam (antipseudomonal β -lactam plus β -lactamase inhibitor) (Schaber *et al.*, 2004). All CI except CI-3 were susceptible to imipenem and all CI except CI-5 were susceptible to piperacillin/tazobactam, while only CI-1 was susceptible to gentamicin (Schaber *et al.*, 2004). Planktonic cells of the PAO1 strain utilized in the present study were susceptible to all three antibiotics at ≤ 4 μ g imipenem ml⁻¹, 4 μ g gentamicin ml⁻¹ and ≤ 8 μ g piperacillin/tazobactam ml⁻¹. However, within a biofilm, the resistance to antibiotics may reach more than 10 times that of planktonic cells (Borriello *et al.*, 2004). Therefore, we compared the antibiotic resistance of the CI within a colony biofilm to that of PAO1 using imipenem, gentamicin and piperacillin/tazobactam concentrations at least 10-fold higher than those for the planktonic cells: 40, 40 (40-fold higher for CI-1) and 80 μ g ml⁻¹, respectively. Since CI-4 represents a repeated isolation of CI-2 within a 1 month period and both isolates were susceptible to all three antibiotics, we examined the antibiotic susceptibility of CI-2 colony biofilm as representative of both isolates. As shown in Fig. 6(a), the CI colony biofilms were more susceptible to imipenem than that of PAO1 (the log reductions were greater than that of PAO1), although only CI-2 and CI-3 colony biofilms were significantly more susceptible ($P < 0.01$). With respect to gentamicin, colony biofilms formed by CI-1 and CI-5 were significantly more resistant ($P < 0.001$ and 0.01, respectively) than that of PAO1, while that of CI-3 was significantly more susceptible ($P < 0.001$) (Fig. 6b). Colony biofilms formed by CI-2 and CI-3 were significantly more susceptible ($P < 0.01$) to piperacillin/tazobactam than that formed by PAO1 (Fig. 6c). It is important to note that the susceptibilities of the CI in the planktonic setting did not always correlate with their susceptibility within the biofilm.

Fig. 6. Susceptibility of PAO1 and CI biofilms to antibiotics. Strains were grown on polycarbonate membranes placed on M63GCA agar plates, as described in Methods. After 48 h growth, the membranes were transferred to LB agar containing (a) 40 μg imipenem ml^{-1} , (b) 40 μg gentamicin ml^{-1} and (c) 80 μg piperacillin/tazobactam ml^{-1} . Following 16 h exposure to the antibiotic agar, the bacteria on the membranes were resuspended in sterile PBS, diluted and plated to determine c.f.u. Values represent the mean $\pm$ SEM of three independent experiments. *, $P < 0.001$; ◆, $P < 0.01$.

DISCUSSION

The three *P. aeruginosa* factors that are considered important for biofilm formation are flagellar-mediated swimming motility, pilus-mediated twitching motility and QS. Swimming motility is required for the initial reversible attachment stage of biofilm development. Although our five CI varied in the levels of their swimming motility, none was non-motile, indicating that all are flagellated (Schaber *et al.*, 2004). However, with the exception of CI-3, there was no consistent correlation between the level of swimming motility and the initiation and early development of biofilms (1-day-old biofilm). The swimming motility of CI-3 was 23 % that of PAO1 and its ability to

initiate biofilm (by the crystal violet assay) was 29 % that of PAO1 (Schaber *et al.*, 2004). However, in the flow-through continuous-culture system, CI-3 produced an ssc of only 0.24 %, which is 0.4 % of the ssc of PAO1 on day 1 (Table 1). On the other hand, the swimming motility of CI-2 and CI-4 was 92 and 100 %, respectively, compared to PAO1, which indicates the presence of a fully functional flagellum, yet their ability to initiate biofilm formation was 20 and 33 %, respectively (Schaber *et al.*, 2004). In the flow-through system these isolates produced an ssc at day 1 of only 7.5 % (11.7 % that of PAO1) and 2.78 % (4.7 % that of PAO1), respectively (Table 1). Thus, despite the presence of a fully functional flagellum, CI-2 and CI-4 showed deficiencies in the early development of their biofilms. It is possible that CI-2 and CI-4 lack an additional factor besides swimming motility that is important for biofilm initiation. However, such a defect does not appear to interfere with the ability of either strain to form mature, differentiated biofilms (Fig. 1, Table 1).

Pilus-dependent twitching motility is required for the spread of *P. aeruginosa* after its initial attachment to the surface (irreversible attachment and microcolony formation). Our results showed a good correlation between twitching motility and the efficient spread of the CI within the 7-day-old mature biofilm, with the exception of CI-5. The least efficient in its spread was CI-1 (ssc 9.62 %, or 13.3 % that of PAO1) (Table 1). CI-1 was also 10-fold less efficient than PAO1 in forming a colony biofilm. Although we have previously reported that the twitching motility of CI-1 is 30 % that of PAO1 (Schaber *et al.*, 2004), our current analysis using a *pilA*-deficient mutant of PAO1 as a negative control revealed a considerable reduction in CI-1 twitching motility (Fig. 5a). This reduction is due to the reduced production of the PilA protein (Fig. 5b). We tried to confirm the correlation between the biofilm phenotype of CI-1 and its lack of twitching motility using complementation analysis. However, plasmid p8566, which carries the intact *pilA* gene, failed to complement the defect in twitching motility in CI-1 (Fig. 5c). Another possible cause of the inefficient spread of the mature CI-1 biofilm is its lack of swarming motility. As shown in Fig. 4, CI-1 is the only isolate that did not swarm. A recent study by Shrout *et al.* (2006) has suggested that *P. aeruginosa* swarming motility may influence the early stages of biofilm development, while Kohler *et al.* (2000) have suggested that QS, the flagellum and the type IV pili contribute to the effect of swarming motility on biofilm development.

Similar to CI-1, the reduced twitching motility of CI-3 correlated with its inefficient spread within the mature biofilm (ssc 19.14 %, or 26.5 % that of PAO1) (Table 1). In contrast, both CI-2 and CI-4 showed no reduction in either their twitching motility (100 and 92 % of PAO1, respectively) (Schaber *et al.*, 2004) or their spread within the mature biofilm (ssc 77.35 and 87.44 %, or 107 and 121 % that of PAO1) (Table 1). Despite the apparent uncompromised twitching motility of CI-5 (92 % that of PAO1) (Schaber *et al.*, 2004), its efficiency of spread within the

mature biofilm was about half that of PAO1 (ssc 32.52 %, or 44.9 % that of PAO1) (Table 1). Unlike the other CI, CI-5 produced a mature biofilm with unique features: unusually tall cell clusters (Fig. 1). Whether the twitching motility contributed to this unique feature is not known at this time. Since CI-4 had similar levels of twitching motility yet covered more substratum surface than PAO1, this suggests that there is a possible defect in the pili of CI-5, or that some factor other than twitching motility is involved in its unique architecture.

The effect of the QS systems on the different virulence attributes of *P. aeruginosa* has been documented (Davies *et al.*, 1998; De Kievit *et al.*, 2001). The findings of earlier reports vary with respect to the role of QS in biofilm formation by *P. aeruginosa*. Using a glass surface as a substratum and QS isogenic mutants of PAO1, Davies *et al.* (1998) showed that in comparison with PAO1, a *lasI* mutant produces a thin biofilm that is easily dispersed when treated with a detergent such as SDS. Using similar conditions, De Kievit *et al.* (2001) showed that during the course of an 8-day biofilm, *lasI* expression decreases progressively, while that of *rhlI* is steady but occurs in a low percentage of cells. Our analysis of the CI suggests that QS may not play a major role in biofilm initiation and the spread of *P. aeruginosa* over the substratum. We have previously demonstrated that with the exception of CI-5, the CI produce significantly lower levels of 3OC₁₂-HSL and no detectable C₄-HSL (Schaber *et al.*, 2004). In addition, the CI produce considerably lower levels of the QS-controlled factors LasB and pyocyanin (Schaber *et al.*, 2004). Despite that, the CI produced mature biofilms of variable density (Fig. 1, Table 1). Our analysis also suggests that certain components of the QS systems (*lasI*, *rhlI*, *lasR* and *rhlR*) play a role in the development of certain features of the mature biofilms. We previously showed that CI-2 and CI-4 carry deletions within the *lasR* and *rhlR* genes (Schaber *et al.*, 2004). In addition, neither CI-2 nor CI-4 produced detectable *lasR* or *rhlR* transcripts (data not shown). However, CI-2 and CI-4 formed mature biofilms that covered the glass surface more densely than that of PAO1, while those formed by CI-1, CI-3 and CI-5 were less dense (Fig. 1, Table 1). In addition, the biofilms produced by CI-1, CI-3 and CI-5 were more heterogeneous than those of PAO1, while that of CI-4 was less heterogeneous (Fig. 1, Table 1). Thus, *lasR* and *rhlR* may contribute to the heterogeneity of the biofilms.

The detection of *lasI* and *rhlI* transcripts in CI-2 and CI-4 (about 40 % that of PAO1) (Fig. 3) is puzzling. We previously indicated that CI-2 and CI-4 carry deletions in both *lasR* and *rhlR* (Schaber *et al.*, 2004). Using PCR analysis, we failed to amplify DNA fragments (either internal fragments or fragments that carry the intact genes) from the chromosomes of CI-2 and CI-4 (Schaber *et al.*, 2004). In addition, we showed that both CI-2 and CI-4 produce significantly reduced levels of 3OC₁₂-HSL and C₄-HSL in comparison (Schaber *et al.*, 2004). We had not expected to detect *lasI* and *rhlI* mRNA, as 3OC₁₂-HSL-activated LasR

and C₄-HSL-activated RhlR induce expression of *lasI* and *rhlI*, respectively (Venturi, 2006). The apparent discrepancy between the present results and those of earlier studies (Latifi *et al.*, 1996; Seed *et al.*, 1995) may be due to differences in the sensitivity of the assays and the parameters that were examined in each assay. The previous studies utilized detection of β -galactosidase activity (*lacZ* fusion system), which determines the efficiency of *lasI* and *rhlI* expression from their promoters, while we utilized more sensitive real-time PCR that measures the amount of accumulated *lasI* and *rhlI* mRNA. Alternatively, other pathways may exist whereby *P. aeruginosa* obtains *lasI* and *rhlI* transcripts in the absence of autoinducer-activated LasR and RhlR. For example, Carty *et al.* (2006) have recently suggested that the *P. aeruginosa* regulatory protein PtxR enhances transcription of *lasI* but does not affect *lasR*.

Using PAO1 and its QS-isogenic mutants, Shrout *et al.* (2006) have recently suggested that the contribution of QS to the development of a PAO1 biofilm depends on the growth medium, specifically the carbon source. In the presence of glucose in the biofilm medium, both PAO1 and its QS mutants produce thin monolayers with some cell aggregates, and PAO1 and its PAO1 QS mutants do not swarm on their glucose medium (Shrout *et al.*, 2006). In the presence of succinate, PAO1, which is able to swarm, produces a flat uniform biofilm, while the QS-mutants produce cell aggregates (Shrout *et al.*, 2006). In the present study, we utilized glucose as a carbon source in our biofilm medium also (see Methods), in which PAO1, CI-2 and CI-4 produced flat uniform mature biofilms that covered the substratum (Fig. 1, Table 1). In addition, we did not find PAO1 to be defective in its swarming motility (Fig. 4). We suggest three reasons for the observed differences between the two studies. First, different media were utilized to examine biofilm development and swarming motility. While Shrout *et al.* (2006) utilized modified FAB medium that contained either succinate or glucose as a carbon source, we utilized M63 minimal medium that was supplemented with glucose and Casamino acids (O'Toole & Kolter, 1998b). The swarming plates described by Shrout *et al.* (2006) consisted of FAB medium that was supplemented with either succinate or glucose. Our swarming plates contained glucose and nutrient broth (Boles *et al.*, 2005). Second, Shrout *et al.* (2006) examined biofilm development primarily within 48 h post-inoculation, while we examined the mature biofilm 7 days post-inoculation. Third, Shrout *et al.* (2006) utilized PAO1 isogenic mutants that were defective in either *lasI/rhlI* or *lasR/rhlR*, while our QS-deficient CI were genotypically different from PAO1.

Comparison of our results with those of Lee *et al.* (2005) provided the following observations. (1) According to Lee *et al.* (2005), twitching motility affects the architecture of the *P. aeruginosa* biofilms. Isolates that retain twitching motility produce flat, homogeneous biofilms while those lacking twitching motility produce heterogeneous biofilms with irregular microcolonies. Similar to the findings of Lee *et al.* (2005), CI-1, which is twitching-motility deficient,

produced a heterogeneous biofilm with irregular microcolonies (Fig. 1, Table 1). (2) In the Lee *et al.* (2005) study, the effect of QS on biofilm formation was not clear, since isolates that failed to produce either one or both autoinducers also lacked twitching motility. One isolate that was obtained six times from a single patient over a 23-year period showed variations in biofilm development during sequential isolation. The first three sequential isolates (positive for twitching motility and both autoinducers) formed monolayers within the first 24 h and a flat biofilm structure that covered the substratum by 7 days (Lee *et al.*, 2005). However, the last three sequential isolates (positive only for C₄-HSL) formed biofilms that consisted of irregular cell aggregates that failed to cover the entire surface (Lee *et al.*, 2005). Consequently, the failure to develop biofilms by those three isolates may be due to the loss of either 3OC₁₂-HSL (a non-functional *las* system) or twitching motility, or both. Analysis of our isolates showed that CI-2 and CI-4 are competent in their twitching motility (Schaber *et al.*, 2004). However, neither isolate produced C₄-HSL and both produced very low levels of 3OC₁₂-HSL (Schaber *et al.*, 2004), yet both isolates produced mature biofilms that covered the substratum more completely than PAO1 (Fig. 1, Table 1). Therefore, the development of biofilms by these isolates did not require fully functional *las* or *rhl* QS systems. (3) Three of the isolates described by Lee *et al.* (2005) showed biofilms with abnormal architecture. One isolate (65608a/1999) showed attachment and microcolony formation at day 3; however, at day 7, the biofilm developed into a structure with massive elevated perpendicular colonies (Lee *et al.*, 2005). The architecture of CI-5 mature biofilm was somewhat similar to that produced by 65680a/1999 (Fig. 1). The CI-5 day 7 biofilm covered only 32.5 % of the surface but contained microcolonies that reached 80 µm in height (Fig. 1, Table 1). At this time, the specific factor(s) involved in the production of these abnormal biofilm architectures is not known. Neither 65680a/1999 nor CI-5 is completely defective in autoinducer production, swimming motility or twitching motility (Lee *et al.*, 2005; Schaber *et al.*, 2004), nor is biofilm initiation likely to be a factor in the development of this architecture. While 65680a/1999 showed reduced biofilm initiation, CI-5 was not significantly defective (Lee *et al.*, 2005; Schaber *et al.*, 2004). Furthermore, the isolation site is unlikely to be a factor: 65680a/1999 was isolated from CF sputum, whereas CI-5 was isolated from urine (Lee *et al.*, 2005).

Our analysis suggests that the infection site may not influence biofilm formation by *P. aeruginosa*. For example, the respiratory isolate CI-2 and its sequential isolate CI-4 produced mature biofilms that covered more substratum than PAO1 (Table 1). However, another respiratory isolate, CI-3, produced biofilm that covered only 19 % of the substratum (Table 1). Results reported by Lee *et al.* (2005) support the above possibility. Although all of the *P. aeruginosa* strains described by Lee *et al.* (2005) were respiratory isolates, they produced biofilms with variable

structures. Whether differences in the efficiency of biofilm formation influence (directly or indirectly) the outcome of *P. aeruginosa* infection is not known. CI-2 and CI-4 were obtained from a patient on respiratory support who succumbed to *P. aeruginosa* sepsis. CI-3 on the other hand was obtained from a patient with chronic obstructive pulmonary disease who was successfully treated and discharged.

In the analysis of the mature biofilms formed by our CI, it is important to consider that while we utilized the well-differentiated biofilm formed by PAO1 as a reference point for comparison, the CI are not isogenic mutants. Thus, the different biofilms shown in Fig. 1 and described in Table 1 may not reflect any defective phenotypes. Rather, each biofilm may represent a unique adaptation of that specific CI to the environment from which it was isolated.

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