RESEARCH LETTER

Swarming of *Pseudomonas aeruginosa* PAO1 without differentiation into elongated hyperflagellates on hard agar minimal medium

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Abstract

Polar flagellated *Pseudomonas aeruginosa* PAO1 demonstrated extensive spreading growth in 2 days on 1.5% agar medium. Such spreading growth of *P. aeruginosa* PAO1 strains was absent on Luria–Bertani 1.5% agar medium, but remarkable on Davis minimal synthetic agar medium (especially that containing 0.8% sodium citrate and 1.5% Eiken agar) under aerobic 37 °C conditions. Analyses using isogenic mutants and complementation transformants showed that bacterial flagella and rhamnolipid contributed to the surface-spreading behavior. On the other hand, a type IV pilus-deficient *pilA* mutant did not lose the spreading growth activity. Flagella staining of PAO1 T cells from the frontal edge of a spreading colony showed unipolar and normal-sized rods with one or two flagella. Thus, the polar flagellate *P. aeruginosa* PAO1 T appears to swarm on high-agar medium by producing biosurfactant rhamnolipid and without differentiation into an elongated peritrichous hyperflagellate.

Introduction

Bacterial swarming has been recognized as flagellum-dependent spreading growth on solid medium surfaces (0.5–2.5% agar) (Harshey, 2003) and is considered to be a virulence factor that enables broader infection of plant and animal body surfaces (Rashid & Kornberg, 2000; Harshey, 2003). Swarming bacteria have been classified into two types (Rashid & Kornberg, 2000): strong swarmers (e.g. *Proteus mirabilis*) and weak swarmers (e.g. *Salmonella typhimurium*). Strong swarmers are able to translocate on high-agar (1.5–2.5%) medium by differentiation into remarkably elongated hyperflagellates (Allison & Hughes, 1991) from short peritrichous rods (swimmers) in liquid medium. Although weak swarmers are also able to differentiate moderately into elongated hyperflagellates (Harshey & Matsuyama, 1994), their swarming has only been observed on low-agar surfaces (< 0.8%) (Harshey, 2003).

On 2.0% agar medium, *Vibrio parahaemolyticus* has been reported to translocate as a strong swarmer by differentiating into an elongated peritrichous hyperflagellate from the monotrichous polar swimmer observed in liquid medium (McCarter, 1999). *Pseudomonas aeruginosa* has been reported to perform rhamnolipid-dependent swarming as a slightly elongated rod with a few polar flagella (Köhler *et al.*, 2000; Déziel *et al.*, 2003). In contrast to the report by Rashid & Kornberg (2000), Köhler *et al.* (2000) have reported that type IV pili were also required for the swarming of *P. aeruginosa*. However, our *pilA* mutant of *P. aeruginosa* exerted distinct swarming on 0.5% agar Luria–Bertani (LB) medium as reported already with various type IV pili mutants of *P. aeruginosa* (Toutain *et al.*, 2005).

Because the previously reported agar concentrations permitting swarming of *P. aeruginosa* have been 0.6% or less, it seems to be a weak swarmer. In the present study, however, an ability of *P. aeruginosa* PAO1 to develop thin expanding colonies on 1.5% agar medium in 2 days was demonstrated. For surface-spreading growth on hard agar medium, three independent bacterial factors have been elucidated so far: flagella for swarming (Allison & Hughes, 1991), pili for twitching motility (Semmler *et al.*, 1999), and

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Sources/references
P. aeruginosa		
PAO1 T	Wild type	Hasegawa *et al*. (2003)
PAO1 TR	*rhlA*::Gm^r	Nozawa *et al*. (2007)
PAO1 TF2	*fliC*::Cm^r	Nozawa *et al*. (2007)
PAO1 TP2	*pilA*::Cm^r	Hasegawa *et al*. (2007)
PAO1 TF2P3	*fliC*::Cm^r, *pilA*::Gm^r	This study
E. coli		
JM109	Used for propagation of pUC19 and pME6032	Yanisch-Perron *et al*. (1985)
S17-1λ*pir*	Used for propagation of pFS200GM	Suzuki *et al*. (2001)
Plasmids		
pBR322	Ap^r, Tc^r, cloning vector	Sutcliffe (1979)
pBSL182	*ori* R6K *mob*RP4 Ap^r mini-Tn*10* Gm^r	Alexeyev & Shokolenko (1995)
pFS200GM	pFS200, Gm^r, delivery vector	Hasegawa *et al*. (2007)
pFPIL	pFS200GM carrying SphI–XbaI fragment containing *pilA*	Hasegawa *et al*. (2007)
pFPILGM	pFS200GM carrying Gm^r gene-inserted *pilA*	This study
pFPILGMt	pFPILGM carrying Tc^r gene	This study
pME6032	Tc^r, cloning vector	Heeb *et al*. (2002)
pMF	pME6032 carrying *fliC*	This study
pMP2	pME6032 carrying *pilA*	Hasegawa *et al*. (2007)
pMR2	pME6032 carrying *rhlAB*	Nozawa *et al*. (2007)

biosurfactants for slow-spreading growth of *Serratia marcescens* (Matsuyama *et al*., 1989) and *P. aeruginosa* (Nozawa *et al*., 2007). Thus, the involvement of these factors in the spreading growth of *P. aeruginosa* PAO1 and related cellular and environmental conditions were analyzed.

Materials and methods

Bacteria, plasmids and media

The bacterial strains and plasmids used in this study are listed in Table 1. The phenotypes of the listed and newly constructed strains were confirmed as described previously (Nozawa *et al*., 2007). Flagella staining was carried out by Leifson's method (Leifson, 1951). LB broth or agar medium was used for ordinary propagation of bacteria (Matsuyama, *et al*., 1995). Modified Davis basic salt solution (Davis & Mingioli, 1950) [7.0 g of K_2HPO_4, 3.0 g of KH_2PO_4, 0.1 g of $MgSO_4$, 1.0 g of $(NH_4)_2SO_4$ per liter] supplemented with 0.8% sodium citrate and 1.5% Eiken agar (Eiken, Tokyo) was routinely used as Davis citrate medium (pH, 7.0) for efficient bacterial spreading growth. In some experiments, varied concentrations of sodium citrate, glucose, glycerol, sodium pyruvate, or sodium succinate were used instead of 0.8% sodium citrate. After solidification, plates (internal dish diameter, 85 mm) were briefly dried at 30 °C for 40 min.

Examination of spreading growth

For examination of bacterial spreading growth on high-agar medium, 5 μL of bacterial suspension (*c*. 10^9 CFU mL^{-1} of LB broth) was point-inoculated at the center of the medium. Cultivation was carried out aerobically for 2 days at 37 °C in a sealed plastic box with wet tissue to maintain a high level of humidity (≥90%). In some special anaerobic cultivations, an ASKA anaerobic system (ASKA Diagnostics Inc., Tokyo) in a transparent plastic bag was used as described previously (Nozawa *et al*., 2007). Spreading growth was examined by measuring colony diameters. For statistical analysis, two-tailed Student's *t*-tests were used for the comparison of two groups, e.g. mutants vs. the wild type or complements. Spreading growth was also observed using a phase-contrast microscope (Nikon DIAPHOT-TMD, Tokyo) with a time-lapse camera (Canon EOS 10D, Tokyo).

DNA manipulation

Basic DNA manipulation procedures were carried out as described previously (Sambrook *et al*., 1989; Ausubel *et al*., 1990). Southern hybridization and preparation of the labeled probe were performed using AlkPhos Direct (Amersham Biosciences) as described by the manufacturer. The PCR primers used are listed in Table 2.

For genetic complementation of the *fliC* mutant TF2, a plasmid pMF was generated by insertion of SphI–XhoI-digested *fliC* DNA (PCR product generated from the primer pair FLICF/FLICR and PAO1 T chromosomal DNA template) into SphI–XhoI-digested pME6032.

The transformant TF2/pMF restored flagellation and swimming activity (a little reduced) in soft agar medium. To obtain a double-mutant TF2P3, the gentamycin resistant (Gm^r) gene (PCR product generated from the primer pair

GMFK/GMRK and pBSL182 DNA template) was inserted into the KpnI cleavage site of the *pilA* gene in pFPIL after digestion with KpnI to give pFPILGM. Then, the tetracycline-resistant (Tc^r) gene (PCR product generated from the primer pair TCFX/TCRX and pBR322 DNA template) was inserted into the XbaI cleavage site of pFPILGM after digestion with XbaI to generate pFPILGMt. Thereafter, the constructed plasmid pFPILGMt was transferred into PAO1 TF2 cells via *Escherichia coli* S17-1 λ*pir*. The transconjugant generated by homologous recombination was selected as the desired double-mutant TF2P3. Molecular genetic compositions of the double mutant were confirmed by PCR analysis and Southern hybridization with probes specific to the Cm^r and Gm^r genes.

Results

Spreading growth of *P. aeruginosa* PAO1 on high-agar medium

Point inoculation of *P. aeruginosa* PAO1 T (and several PAO1 strains from different sources, data not shown) on high-agar (1.0–2.0%) Davis synthetic medium resulted in extensive spreading growth in 2 days (Fig. 1a). Spreading giant colonies were observed with thin bacterial growth on Davis synthetic medium plates, and not observed with thick bacterial growth on LB medium plates (Fig. 1b). The Davis synthetic medium was then varied in its carbon/energy source, and different concentrations were examined for their ability to promote spreading growth (Table 3). Although poor nutrient conditions with glucose or glycerol promoted spreading growth to some extent, the most prominent spreading growth was observed with Davis synthetic medium containing a higher concentration (0.8%) of sodium citrate (Davis citrate medium). Citrate seemed to have a special enhancing effect on spreading growth. On the other hand, Davis medium containing sodium pyruvate (citrate precursor) or sodium succinate (citrate derivative in a citric acid cycle) did not induce the spreading growth of the organism (data not shown).

The cultivation temperature and gas conditions also profoundly affected the spreading growth (Table 3). This suggested a distinction from other types of spreading growth exerted by *P. aeruginosa* without flagella and type IV pili (Nozawa *et al*., 2007).

Table 2. Oligonucleotides used in this study

Primers	Sequence (5′–3′)*
FLICF	ACATGCATGCTTCAGGACCGATATTGGCGA (SphI)
FLICR	CCGCTCGAGAGTCACCGTCGATCACCTTGA (XhoI)
GMFK	GGGGTACCGACATAAGCCTGTTCGGTTC (KpnI)
GMRK	GGGGTACCCCGATCTCGGCTTGAACG (KpnI)
TCFX	GCTCTAGACATGTTTGACAGCTTATCATCG (XbaI)
TCRX	GCTCTAGATCTCCGCAAGAATTGATTGG (XbaI)

*The restriction site is underlined, and the restriction enzyme is shown in parentheses.

Morphological characterization of spreading growth

Flagella staining of cells from the frontal edge of a colony growing on high-agar medium was carried out. It was noteworthy that there were no remarkable differences in flagellation between *P. aeruginosa* PAO1 T on Davis citrate medium (cells achieving spreading growth, Fig. 1c) and LB medium (cells not achieving spreading growth, Fig. 1d). *Pseudomonas aeruginosa* PAO1 T on these two 1.5% agar media possessed only one or two polar flagella, as seen in

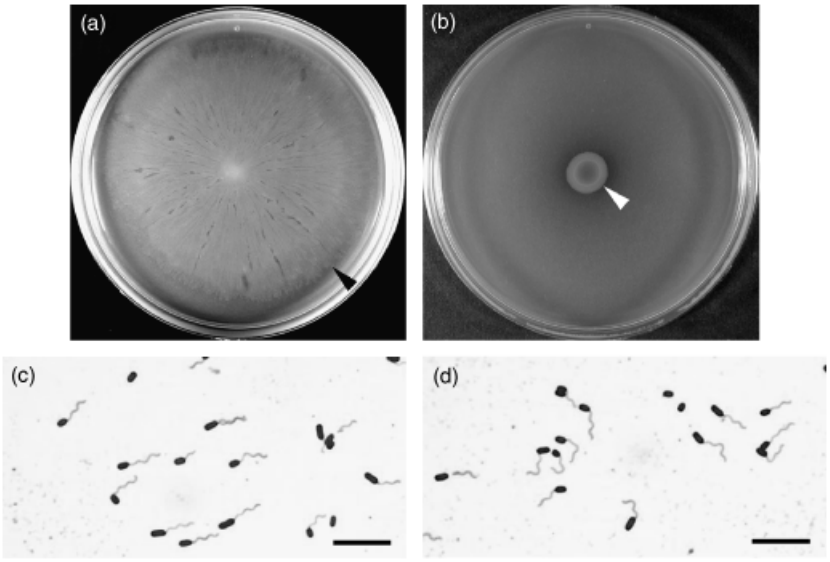

Fig. 1. Spreading and nonspreading colonies of *Pseudomonas aeruginosa* PAO1 T on 1.5% agar medium incubated at 37 °C for 2 days.
(a) A thinly spreading giant colony with a fuzzy branching frontal margin developed on Davis citrate medium. (b) A nonspreading thick colony developed on LB medium. The arrowhead indicates the frontal margin of the growing colony. (c) Flagella-stained cells taken from the spreading colony margin on Davis citrate medium. (d) Flagella-stained cells taken from the nonspreading colony margin on LB medium. Each scale bar = 5 μm.

Table 3. Environmental factors influencing the spreading growth of *Pseudomonas aeruginosa* PAO 1 T on 1.5% agar medium

Environmental factor	Colony diameter (mm)*
Medium (incubated under aerobic 37 °C conditions if not otherwise specified)	
Luria–Bertani	15.9 ± 2.7
Davis basic salt solution with	
0.1% glycerol	13.6 ± 6.0
0.2% glycerol	29.6 ± 5.8
0.8% glycerol	13.8 ± 6.7
0.1% glucose	23.8 ± 7.4
0.2% glucose	29.6 ± 0.9
0.8% glucose	14.2 ± 0.8
0.1% sodium citrate	21.9 ± 1.3
0.2% sodium citrate	21.8 ± 1.5
0.8% sodium citrate	66.7 ± 9.7
Temperature of Davis 0.8% sodium citrate medium	
30 °C	22.8 ± 3.3
37 °C	66.7 ± 9.7
Gas condition of Davis 0.8% sodium citrate medium supplemented with 0.05% $NaNO_3$†	
Aerobic	67.0 ± 6.2
Anaerobic with 8% CO_2	28.0 ± 4.3

*Mean ± SD ($n \geq 3$), after 2-day cultivation.
†Supplemented for anaerobic nitrate respiration.

organisms grown in liquid culture. In 146 cells from LB medium, 4.1% had two unipolar flagella, 87.7% had a single unipolar flagellum, and the rest (8.2%) lacked flagella; in 185 cells from Davis citrate medium, 3.8% had two unipolar flagella, 87.6% had a single unipolar flagellum, and the rest (8.6%) lacked flagella. In addition, the cell lengths of *P. aeruginosa* PAO1 T from the spreading growth front and nonspreading growth front on both 1.5% agar media were nearly identical (in the range of 1.4 ± 0.3 μm, $n = 60$).

Direct microscopic examination of the frontal area of the expanding colony showed a compactly layered cell population forming a terraced structure with randomly protruding spurs composed of dense monolayer cells (Fig. 2). Although the images seemed to be static under real-time observation, time-lapse photo recording (at 30-s intervals, supplementary material) revealed the dynamic status of the expanding cell population. The compact layered cell population steadily advanced ($6.0 \pm 1.0\,\mu m\,min^{-1}$, $n = 5$) en masse by engulfing spurs that occasionally grew in random directions (Fig. 2a and b). Such compact and slow cell dynamics were quite different from the lace-like monolayered cell population expansion by type IV pilus-dependent twitching motility that was recognizable under real-time observation [please refer to photomicrographs taken by Semmler *et al.* (1999)].

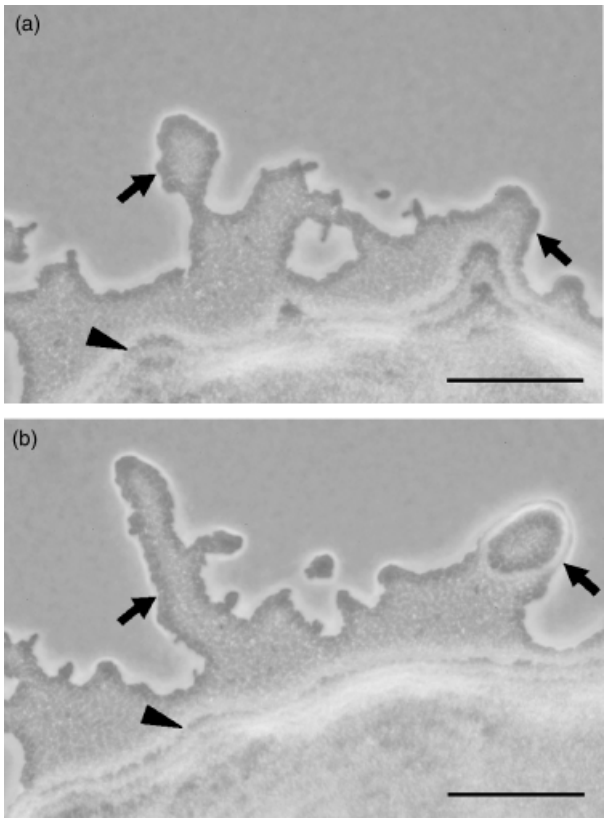

Fig. 2. Phase-contrast photomicrograph of spreading *Pseudomonas aeruginosa* PAO1 T colony front area on 1.5% agar Davis citrate medium at 37 °C. (a) A still image at time 0. (b) A still image after 5 min. The cell population is spreading upward en masse. The arrowhead indicates a compactly layered cell population with a terraced structure. The arrow indicates a spur composed of dense monolayer cells. Each scale bar = 50 μm.

Bacterial factors involved in spreading growth

Mutational analyses of the spreading growth of *P. aeruginosa* PAO1 T on high-agar medium surfaces were carried out using isogenic *fliC*, *pilA*, and *rhlA* mutants, as well as revertants made by gene complementation. The results (Fig. 3) indicated that the surface-spreading process was pilus-independent and flagellum- and rhamnolipid-dependent. Thus, spreading growth on high-agar medium was shown to be rhamnolipid-dependent swarming, which has only been reported so far for *P. aeruginosa* growing on low-agar nutrient medium. However, the swarming on hard agar medium occurred under various restricted synthetic nutrient conditions and has not been recognized in usual nutrient media such as LB (Fig. 1b).

Discussion

From morphological and mutational analyses of spreading growth, polar flagellated *P. aeruginosa* PAO1 T was shown to be strong swarmers without differentiation into peritrichous

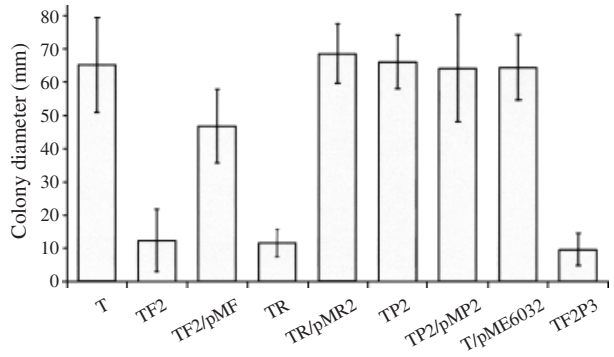

Fig. 3. Colony diameter of *Pseudomonas aeruginosa* PAO1 T, its mutants, and transformants generated with plasmids (see Table 1) measured on 1.5% agar Davis citrate medium after 2 days of cultivation at 37 °C. Vertical error bars denote SD ($n \geq 4$). Statistical analysis showed significant differences between T and TF2, TF2 and TF2/pMF, T and TR, TR and TR/pMR2 ($P < 0.001$; $n \geq 4$), and between T and TF2/pMF ($P < 0.05$; $n = 9$).

elongated hyperflagellates on high-agar medium. This is surprising when considering the distinct differentiation exerted by *V. parahaemolyticus* for surface migration on high-agar medium (McCarter, 1999). Instead of differentiation into elongated hyperflagellates, rhamnolipid was indispensable for the swarming on high-agar medium as the swarming on low-agar media (Köhler *et al.*, 2000; Déziel *et al.*, 2003). Rhamnolipid seems to have a special function in the surface spreading of *P. aeruginosa*.

Rhamnolipid activity enabling spreading growth has been reported even in *P. aeruginosa* PAO1 T mutants losing flagella or pili (Nozawa *et al.*, 2007). These effects have been attributed to rhamnolipid surfactant activity, as external application of rhamnolipid or other surfactants (e.g. Tween 80) restored the spreading growth of rhamnolipid-deficient mutant TR. In the present study, defective swarming of TR on high-agar medium (Fig. 3) was also restored by external supply of Tween 80 (data not shown). However, the rhamnolipid-dependent, but flagellum- and pilus-independent spreading growth of *P. aeruginosa* on high-agar medium, took a longer time (5 days) to develop a thin colony as large as the colony shown in Fig. 1a. In addition, CO_2-rich anaerobic and low-temperature conditions were preferential for rhammnolipid-dependent but flagellum- and pilus-independent spreading growth (Nozawa *et al.*, 2007) in contrast to rhamnolipid-dependent swarming on high-agar medium (see Table 3). Thus, rhamnolipid-dependent swarming on high-agar medium (shown in the present study) was clearly distinct from rhamnolipid-dependent but flagellum- and pilus-independent slow-spreading growth of *P. aeruginosa* PAO1 T on high-agar medium.

Interfaces between air and hydrated solid materials seem to be prevalent in nature (e.g. surfaces of living organisms) and artificial products (e.g. surfaces of food products). For micrometer-scale bacteria, however, intermolecular forces from the substrate and the surface tension of the surrounding water restrict spreading growth. Microorganisms appear to have developed special abilities to overcome such restrictions by moving as coordinated populations and production of various surfactants (Matsuyama & Matsushita, 1993).

Simple synthetic Davis medium clearly promoted the swarming of *P. aeruginosa* PAO1 on hard agar medium in contrast to nutrient-rich LB medium. Although some unknown ingredients in LB medium might inhibit cell migration on hard agar surfaces, poor nutritional conditions might induce the migration of cells that are presumably eager to escape from the uncomfortable site. As the small colony size of *P. aeruginosa* PAO1 T on high-agar LB medium was nearly identical to that of flagellum-less mutant TF2 (data not shown), bacteria on nutrient-rich media appear to have no such need for migration out. The authors have no definitive data to support such bacterial inclination, but the behavior of *P. aeruginosa* PAO1 T on Davis glycerol or glucose medium (compare colony diameters of 0.2% and 0.8% of these carbon sources in Table 3) seems to suggest this possibility. With regard to the effect of 0.8% sodium citrate on the swarming of *P. aeruginosa* PAO1 T, the related derivative (succinate) in citrate metabolism demonstrated no effect. Therefore, citrate itself seems to promote the swarming of *P. aeruginosa* PAO1 T directly on hard agar medium. Its mechanism remains to be elucidated.

For *in vitro* examination of bacterial behavior on surfaces, gelling agents such as agar for medium solidification have been used. Interfacial conditions of these solid media may be important for bacterial surface behavior. As reported previously (Harshey & Matsuyama, 1994), the colony size of swarming bacteria on Bacto-agar medium was smaller than that on Eiken agar medium. In authors' studies of rhamnolipid-dependent spreading growth, the spreading growth of *P. aeruginosa* was also retarded on Bacto-agar medium (data not shown). In experiments overlaying Eiken agar medium onto solidified Bacto-agar medium and *vice versa*, the dominance of spreading growth-inhibitory factors from Bactor-agar was confirmed (Nozawa, Master thesis, 2007). Surface conditions of solid media depend on many unknown factors, making it difficult to prepare the appropriate media consistently. In the present experiments, the droplet size of an inoculum (5 μL) has been checked on the medium surface. When the droplet horizontal diameter was smaller (lower wettability of the solid medium surface, e.g. surface of Davis citrate medium solidified with Eiken agar or agarose), the inoculated bacteria generally formed larger spreading colonies. In contrast, when the droplet diameter was larger, e.g. on the surface of LB medium or Davis citrate medium solidified with Bacto-agar or gellun gum, rhamnolipid-dependent spreading growth was negligible (Nozawa, Master thesis, 2007). Retardation of rhamnolipid-

dependent spreading growth on surfaces with higher wettability is counterintuitive but represents a real finding. This mystery related to surface translocation of bacteria seems to be suggesting novel biophysical mechanisms that remain to be elucidated.

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Authors' contribution

C.T., T.N. and T.T. contributed equally to this study.

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Supplementary material

The following supplementary material is available for this article online:

Video Clip S1. Microscopic colony expanding behavior of *P. aeruginosa* PAO1 T on 1.5% agar Davis citrate medium. The video clip is in Quicktime. Width of a screen, 220 μm. Speed, real time × 300.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2007.01057.x (This link will take you to the article abstract).

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