Rhamnolipid but not motility is associated with the initiation of biofilm seeding dispersal of *Pseudomonas aeruginosa* strain PA17

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Seeding dispersal is an active detachment exhibit in aging *Pseudomonas aeruginosa* biofilm. Yet, effect factors of this process in the biofilm of clinical isolated mucoid *P. aeruginosa* strain remain to be better characterized. In our previous work, one mucoid *P. earuginosa* strain PA17 was isolated from a patient with recurrent pulmonary infection. In this study, confocal scanning laser microscope combined with LIVE/DEAD viability staining revealed that PA17 biofilm exhibited earlier seeding dispersal than non-mucoid PAO1. We further compared the motility and the expression of motility-associated gene during biofilm development between PA17 and PAO1. PA17 was found to be impaired in all three kinds of motility compared to PAO1. Moreover, we investigated the expression of rhamnolipid-associated genes in PA17 and PAO1 biofilm. The expression of these genes was in accordance with the process of seeding dispersal. Our results indicated that rhamnolipid but not motility is associated with the initiation of seeding dispersal of PA17 biofilm.

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1. Introduction

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium found in a wide variety of aqueous and soil environments. It is also one of the most common opportunistic pathogens responsible for host-acquired infection, especially in immune-compromised hosts such as individuals suffering from AIDS, those severely burned or patients undergoing cancer chemotherapy (Van Delden and Iglewski 1998; Kipnis *et al.* 2006). *P. aeruginosa* causes either acute or chronic infections. During chronic infections, *P. aeruginosa* adopts a biofilm lifestyle (Høiby *et al.* 2001; Donlan 2002). Biofilm is an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material (Donlan 2002). Bacterial biofilms cause the most recalcitrant human infection (Parsek and Singh 2003).

Biofilm formation by *P. aeruginosa* progresses through multiple stages, beginning with attachment to a surface, followed by immigration and division to microcolonies, and finally maturation (Sauer *et al.* 2002; Stoodley *et al.* 2002). Bacteria within the biofilm have a number of advantages over their planktonic counterparts, including protection against the host immune system, as well as enhanced resistance to antimicrobial agents and other stresses. On the other hand, those benefits were provided by restriction of bacterial growth. The costs associated with bacteria growth make it vital that bacteria possess mechanisms to separate from the biofilm and assume planktonic life. Studies have characterized three main types of detachment processes: erosion (the continual detachment of single cells and small portions of the biofilm), sloughing (the rapid, massive loss of biofilm) (Stoodley *et al.* 2001) and seeding dispersal (Webb *et al.* 2003). The former two types of detachment are generally thought of in terms of passive, shear-dependent processes. However, seeding dispersal has been considered as an active detachment exhibit in aging biofilms. Seeding dispersal is characterized by 'hollowing' in mature microcolonies (Webb *et al.* 2003). Purevdorj-Gage *et al.* (2005) has described the characterized 'hollowing' resulted from

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motile subpopulation inside of microcolonies that found its way out of the microcolony in non-mucoid *P.aeruginosa* biofilm. So the motility is considered to be associated with seeding dispersal of non-mucoid *P. aeruginosa* biofilm. It was also reported that rhamnolipids mediated the initiation of seeding dispersal in non-mucoid *P. aeruginosa* biofilms (Boles *et al.* 2005). In mucoid *P.aeruginosa* strains, only a part of strains can detach from the biofilm by seeding dispersal (Purevdorj-Gage *et al.* 2005; Kirov *et al.* 2007). The mechanisms contribute to this process in mucoid *P.aeruginosa* biofilm remain to be better characterized.

In our previous work, one mucoid *P. earuginosa* strain PA17 was isolated from a patient with recurrent pulmonary infection. The *mucA* gene of PA17 has a new type of mutate. We observed its biofilm development by scanning electron microscope. There is some difference when compare to non-mucoid PAO1 (Ni *et al.* 2004). To further investigate the seeding dispersal of the PA17 biofilm and the effect factor, we observed the biofilm development process by confocal laser scanning microscope, and compared this process with non-mucoid PAO1. Furthermore, the motility of these two strains and motility-associated genes expression during biofilm development were compared. Moreover, we investigated the expression of rhamnolipid-associated genes in the PA17 and PAO1 biofilms. Our results suggest that rhamnolipid but not motility is associated with the initiation of seeding dispersal of the PA17 biofilm.

2. Materials and methods

2.1 *Bacterial strains and medium*

The wild-type non-mucoid *P. aeruginosa* strain PAO1 was stored in our laboratory. Mucoid strain PA17 was isolated from a patient with recurrent pulmonary infection in Tongji hospital. Luria broth (LB) was used as the growth medium for biofilms and planktonic bacteria. BM2 minimal medium plate [62 mM potassium phosphate buffer, pH 7.0, 2 mM MgSO4, 10 μM FeSO4, 0.4% (wt/vol) glucose, 0.5% (wt/vol) Casamino Acids] contained either 0.3% (wt/vol) agar for assessing swimming motility, 0.5% agar for swarming assessments, or 1% agar for twitching motility assessments (Sauer *et al.* 2004). Strains were stored at −80°C in 50% (v/v) glycerol and subcultured from storage onto LB medium.

2.2 *Static biofilm formation and microscopy*

PAO1 and PA17 were inoculated to 3.0 mL LB and grown with shaking at 37°C overnight and standardized (OD_{600}). This culture was diluted 1:100 in LB and grown in triplicate in polystyrene 24-well plate with one glass coverslip in each well at 37°C. After 1, 3, 5, 7, 9 and 11 days, the glass cover slips were stained by using the BacLight LIVE/DEAD viability kit reagents according to the manufacturer's instructions. Images were obtained by using confocal laser scanning microscope (CLSM, Olympus FV500 system).

2.3 *Motility assay*

Sterile BM2 plates with varying concentrations of agar (0.3% for swimming, 0.5% for swarming, 1.0% for twitching assay) were prepared to assay motility. Overnight cultures of PAO1and PA17 in BM2 glucose were standardized (OD_{600}) and inoculated on agar plates as 1 μL aliquots. The plates were inverted and incubated at 37°C for 24 h. The diameter of the zone of spreading from the inoculation point was then measured. For twitching motility assay, PA17 and PAO1 were stabbed using a toothpick into the bottom of a petri dish containing the above agar medium. The movement of the colony on the interface between the agar medium and the dish was observed. The zone of twitching motility between the agar and petri dish interface was visualized by staining with 1% cystal violet. All experiments were repeated five times.

2.4 *Quantitative RT-PCR*

Bacteria which were in the biofilm mode of growth at 1, 3, 5, 7, 9 and 11-day old were obtained from glass coverslips. And the planktonic bacteria were gotten from overnight cultured medium. The acid-guanidinium-phenol method (Trizol LS Reagent Invitrogen, USA) was used to extract total RNA according to the manufacture's introduction. RNA was finally dissolved in 50 μL of RNase-free water. For cDNA synthesis, each 25 μL reaction contained 2 μg of RNA, 0.5 μg of random hexamer, 5× RT buffer 5 μL, 10 mM/L dNTP 1.25 μL, 25 U of RNase inhibitor (Promega), and 200 U of MLV(Promega). cDNA synthesis was performed in a PCR Thermal Cycler (Eppendorf) according to the following procedure: after an annealing step for 5 min at 70°C, reverse transcription was carried out for 60 min at 37°C, followed by reverse transcriptase inactivation for 10 min at 95°C. The Light Cycler (Roche) was used for all quantitative RT-PCR. The primers were as following: (I) *fliC* (forward: 5′- TCA ACA GTG CCA AGG ACG A -3′, reverse: 5′-TAC GCT GCA GGA TAT TGG TG -3′); (II) *rhlA* (forward: 5′-CAG CAA CCA TCA GCA CAT-3′, reverse: 5′-TCC AGG CAA GCC AAG TAG-3′); (III) *rhlB* (forward: 5′-CAC GAC CAG TTC GAC AAT-3′, reverse: 5′-GAT ACT GTG CGG TTG TGA-3′); (IV) *algR* (forward: 5′-CCA GCA ATG GCG AAG AAG C-3′, reverse: 5′-GTC

ATG GGC CGT GCA GAA G-3′); (V) Internal reference *rpoD* (forward: 5′-TCC TCA GCG GCT ATA TCG-3′, reverse: 5′-TTC TTC CTC GTC GTC CTT-3′).

2.5 *Statistical analysis*

Data shown are the mean ± SD of triplicates and are representative of three experiments. Unpaired Student's *t*-tests were applied to determine statistical difference. A statistical difference was considered significant when $p<0.05$.

3. Results and discussion

3.1 *Biofilms formation and seeding dispersal of PA17 and PAO1*

The formation and seeding dispersal of muciod PA17 and non-mucoid PAO1 biofilm were followed 1, 3, 5, 7, 9 and 11 days by CLSM. After 24 h, PAO1 had formed small microcolonies. PA17 just exhibited individual attached bacteria. The PA17 strain was a significantly slower adherent to glass than PAO1, which was similar to that in a previous study (O'Toole and Kolter 1998). Three days post-inoculation, PAO1 had formed a layer of cells covering the entire surface. By contrast, PA17 only showed microcolonies. After this initial lag, however, biofilm mode growth occurred for both strain. Five days later, a flat biofilm covering the entire glass was formed in both strains despite the thickness of the PAO1 biofilm being more than that of PA17 (figure 1). However, by day 7 there was an apparent difference in the appearance of interior hollow in the mature cluster of PA17 biofilm. At the same time PAO1 clusters were still consistently homogeneous and showed no evidence of hollowing until day 11, which was similar to that in a previous study (Boles *et al.* 2005). The morphology of microcolony and the central hollowing in the PA17 and PAO1 biofilms was different. This may result from the morphotype variants of clinical *P. aeruginosa* strains being more diverse compared to those of PAO1 (Kirov *et al.* 2007). By using the BacLight LIVE/DEAD viability probe (Molecular Probes), cell death inside microcolonies or biofilms was observed. Dead cells (red cells) occurred in the PA17 biofilm earlier than in the PAO1 biofilm. When seeding dispersal appeared, there were dead cells in the central of microcilonies. After seeding dispersal occurred in PA17, a new biofilm was formed. Traditionally, *P. aeruginosa* detachment from biofilms has been considered a passive behaviour, largely dependant on fluid shear or starvation (Sauer *et al.* 2004). However, dispersal might also be a strategy by which bacteria proactively colonize new niches before space and nutrients become limited. Seeding dispersal was characterized by a hollowing out of some microcolonies. Some researchers suggested this is 'motility-associated dispersal' (Purevdorj-Gage *et al.* 2005). Yet other researchers demonstrated that the hollowing resulted from bacteriophage-mediated lysis as 'death-associated dispersal' (Webb *et al.* 2003). Kirov *et al.* (2007) observed both highly motility subpopulation in seeding dispersal and dispersal-associated death in their clinical isolated mucoid *P. aeruginosa* biofilm. In fact, the 'motility-associated dispersal' and the 'death-associated dispersal' cannot be separated completely since bacteriophage infect a cell through flagellum and type IV pili (Merino *et al.* 1990; Hill *et al.* 1991; Webb *et al.* 2003)

3.2 *Motility phenotypes and associated genes expression of PA17 and PAO1*

Two cell surface appendages involved in motility of *P. aeruginosa* are flagella and type IV pili. The flagella mediate near-surface swimming and surface-anchored spinning in liquid environment, while type IV pili is responsible for twitching motility across solid surface, by which the bacterium moves lengthwise with high directional persistence and high instantaneous velocity, allowing it to rapidly explore microenvironments (Klausen *et al.* 2003; Gibiansky *et al.* 2010; Conrad *et al.* 2011; Tran *et al.* 2011). *P. aeruginosa* also employs another kind of surface motility on semisolid surface, swarming, which depends on functional flagella and type IV pili (Köhler *et al.* 2000) and is currently regarded as a multicellular phenomenon (Tremblay *et al.* 2007). Firstly, we assayed the motility of PA17 and PAO1 on agar plate. Surprisingly, all the motilities of PA17 were significantly weaker than those of PAO1 (figure 2). To our knowledge, upon derepression through mutation acquired in *mucA* of PA17 (Genbank NO.AY608668) (Ni *et al.* 2004), the expression of the alternative sigma factor AlgT (AlgU) in mucoid PA17 increased significantly (data not shown). As previous study showed, AlgT repressed *P. aeruginosa* flagellum biosynthesis and impaired the swimming motility of mucoid *P. aeruginosa* (Garrett *et al.* 1999; Sauer *et al.* 2004; Tart *et al.* 2006). We detected the sequence of *fliC*, the biosynthesis gene of flagella, which was identical in PA17 and PAO1. Then we assessed the sequence of *pliA*, which is responsible for the synthesis of type IV pili. The sequence of this gene in PA17 was mutated (data not shown) compared to that in PAO1. Although we did not know exactly how these mutations affected the synthesis and function of type IV pili, we know that type IV pili driving twitching motility in PA17 was significantly less than PAO1. Swarming motility is dependent on cell-to-cell signalling and requires flagella and pili. It was reported that deficiency in type IV pili results from mutation of *pilA* leading to the impaired swarming; yet, *rhl* mutants are completely unable to swarm (Morici

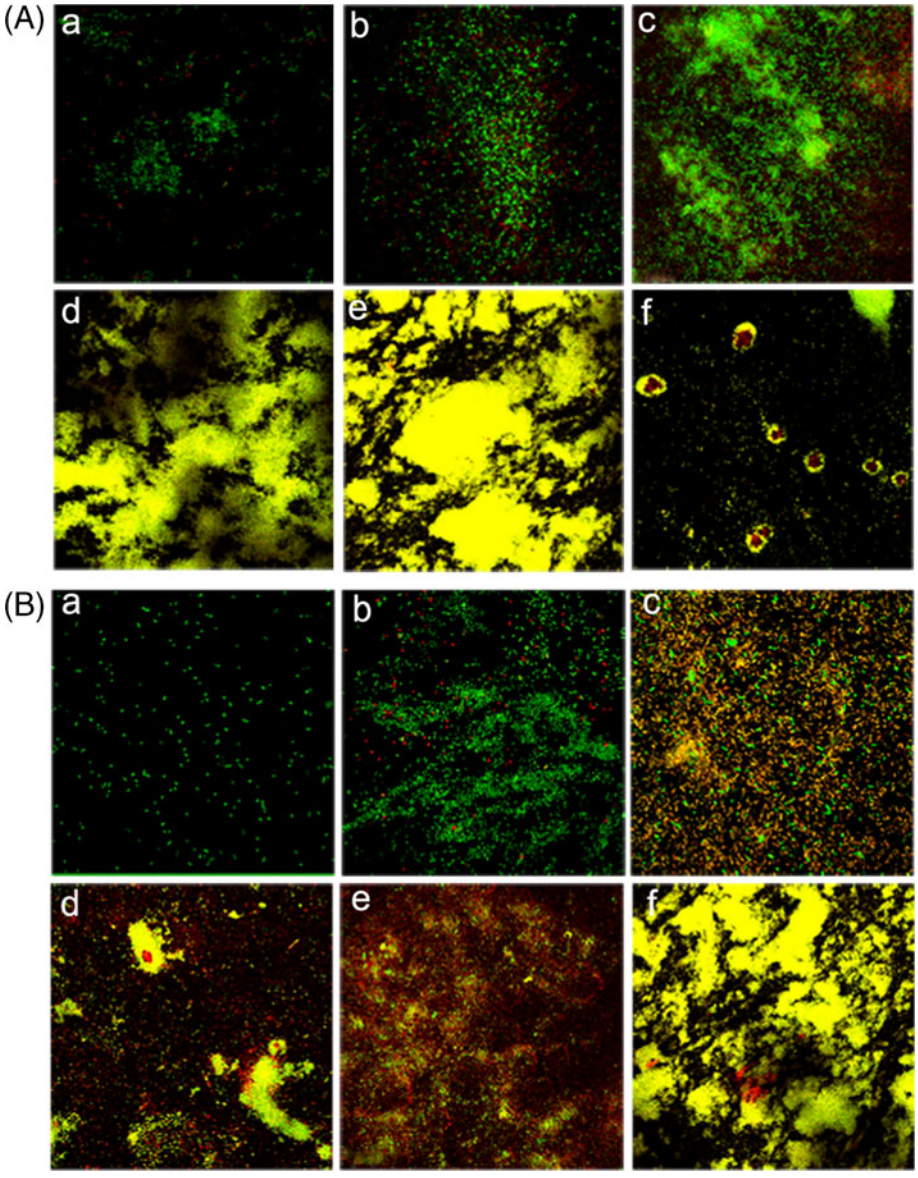

Figure 1. Biofilms formation and seeding dispersal of PAO1 and PA17. Confocal laser scanning micrographs of PAO1 (**A**) and PA17 (**B**) biofilms developed on glass coverslips, which is visualized by using the BacLight LIVE/DEAD viability stain. Green fluorescent cells are viable, whereas red fluorescent cells are dead. **a** (100×), **b** (100×), **c** (100×), **d** (40×), **e** (40×) and **f** (40×) represent 1, 3, 5, 7, 9 and 11-day-old *P. aeruginosa* biofilms respectively. Seeding dispersal occurred at day 11 in PAO1 biofilm and day 7 in PA17 biofilm via the central 'hollowing' which was associated with cell death.

et al. 2007). We also assessed the sequence of *rhlA* or *rhlB* in PA17 but did not find any mutation compared to PAO1 (data not shown). Since flagella and type IV pili both contribute to swarming motility of *P. aeruginosa*, it is easy to understand that the swarming motility of PA17 was less than that of PAO1. It has been suggested that the erosion of *P. aeruginosa* from biofilm is associated with increased expression of flagella and down-regulation of twitching motility (Sauer *et al.* 2004). Wyckoff *et al.* (2002) also demonstrated that mucoid *P. aeruginosa* can acquire flagellum-dependent motility in static growth condition. Moreover, Caiazza *et al.* (2007) observed that the swimming motility of *P. aeruginosa* changed in different viscosity medium. Thus, the results of motility assayed on agar plate may not represent the real condition in biofilms. So, we investigated the expression of *fliC* at different time point of biofilm development. The expression of *fliC* in PA17 was always lower than in PAO1 in the whole biofilm development (figure 3). It indicated that PA17 did not reacquire flagellum-dependent motility in our growth condition. So,

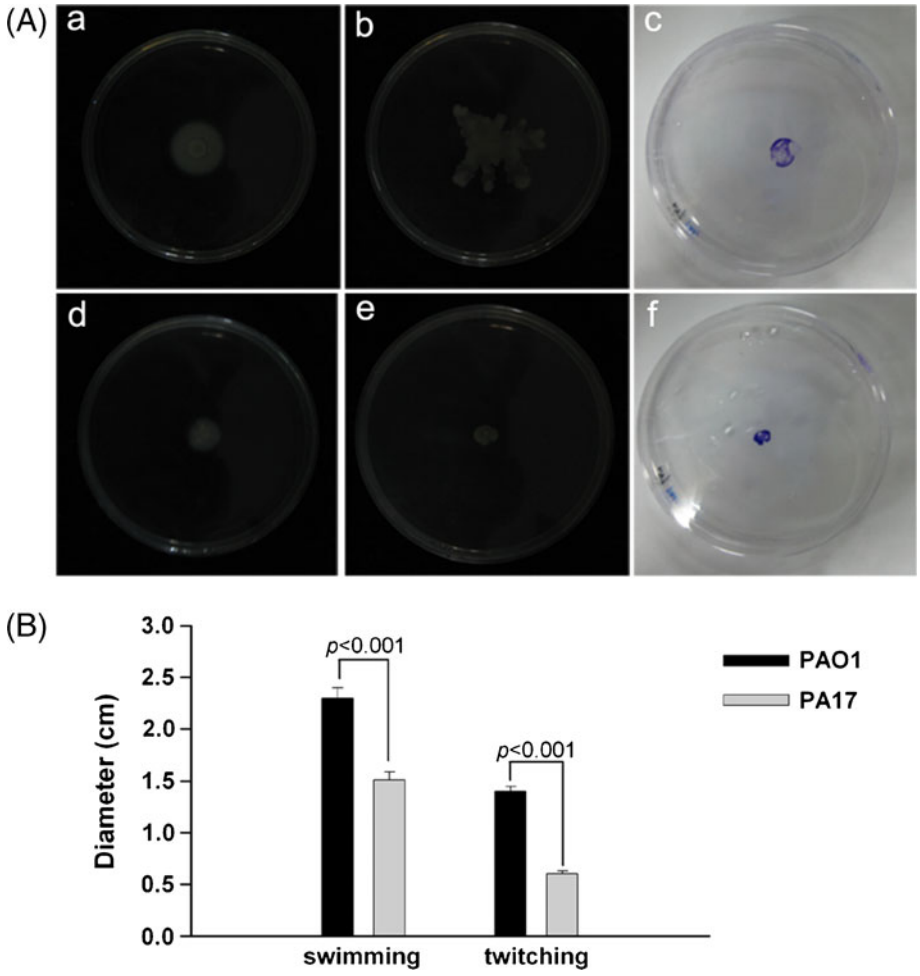

Figure 2. Mucoid PA17 displaying significant reduced swimming, swarming and twitching motility compared to nonmucoid PAO1. (**A**) **a**, **b** and **c** represent swimming, swarming and twitching motility of PAO1; **d**, **e** and **f** represent the motility of PA17. (**B**) The diameter of swimming and twitching motility of PAO1 and PA17. Data shown are mean ± SD of five experiments. A significant difference between PAO1 and PA17 was calculated by using un-paired Student's *t*-test.

the impairment of motility did not impact the time when seeding dispersal occurred in the PA17 biofilm. And it appeared in the biofilm of PA17 earlier than in PAO1, which had normal motility. These results suggest that the initiation of seeding dispersal occurred in the PA17 biofilm maybe independent of motility.

3.3 *Expression of rhamnolipid surfactant associated genes in biofilm development*

Boles *et al.* (2005) found PAO1-$^{\Delta}$*rhlAB* mutant had a much earlier seeding dispersal than PAO1 and suggested that rhamnolipids mediated the initiation of seeding dispersal in PAO1 biofilm. To investigate the role of rhamnolipids in the seeding dispersal of mucoid PA17 biofilm, we compared the expression of associated genes *rhlA* and *rhlB* in PA17 and PAO1 biofilm development (figure 4). The expression of these two genes in PA17 was less than PAO1 in planktonic condition. However, from the early stage of biofilm formation, their expression, especially *rhlB*, began to be higher than PAO1 until day 7, and then down-regulated. By contrast, their expression in PAO1 biofilm was highest at day 11, which was in agreement with the time of seeding dispersal occurred. This result suggests rhamnolipid is also associated with the initiation of seeding dispersal in mucoid PA17 biofilm. However, Purevdorj-Gage *et al.* (2005) showed that PAO1-$^{\Delta}$*rhlA* mutant had the similar seeding dispersal compared to PAO1. Our results showed the dynamic change of gene *rhlB* expression during biofilm development was much significant than that of *rhlA*. If *rhlB* play a more important role in the synthesis of rhamnolipids need further study.

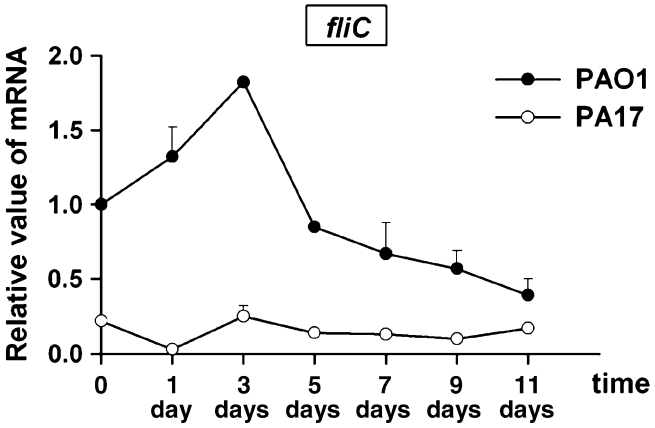

Figure 3. *fliC* expression in mucoid PA17 and non-mucoid PAO1 in planktonic condition and 1, 3, 5, 7, 9 and 11-day-old biofilms. All the results were calculated by comparison with PAO1 in planktonic condition. Data shown are mean ± SD of triplicates and representative of three independent experiments.

Bacteria biofilms formation and dispersal are both adaptation to environment changement (Shrout *et al.* 2002; Kaplan *et al.* 2003; Boles *et al.* 2005). Rhamnolipid can be induced by starvation of nutrients such as nitrogen, phosphate, magnesium, calcium, potassium, sodium, iron and trace elements (Desai and Banat 1997). *P. aeruginosa* has equipped with a large number of two-component regulatory systems to sense and quickly respond to the change of environment (Gooderham and Hancock 2009). AlgR-FimS (AlgZ) regulatory system regulates several important factors associated with biofilms development, including rhamnolipids production (Morici *et al.* 2007), twitching motility (Whitchurch *et al.* 1996), swarming motility (Overhage *et al.* 2007) and alginate biosynthesis (Deretic and Konyecsni 1989). Moreover, it was indicated that AlgR repressed the ramnolipid production in biofilm-specific manner (Morici *et al.* 2007). We also assayed *algR* expression in PA17 and PAO1 biofilm development (figure 4). Expression of *algR* in PA17 at planktonic condition was significantly higher than in PAO1. It decreased at the early stage of biofilm formation but up-regulated after day 7 in PA17 biofilm development. Whereas during PAO1 biofilm development, its expression down-regulated in the whole process. This result is in agreement with the expression of *rhlA* and *rhlB* and the time seeding dispersal occurred.

In conclusion, our results show that clinical mucoid *P. aeruginosa* strain PA17, which is impaired in motility, exhibits seeding dispersal in biofilm development earlier than non-mucoid PAO1, which suggests that motility is not associated with the initiation of seeding dispersal in the biofilm of mucoid PA17. Moreover, expression of rhamnolipid-surfactant-associated genes of PA17 is in accordance with the appearance of seeding dispersal. This result indicates that rhamnolipid is associated with the initiation of seeding dispersal in PA17 biofilm.

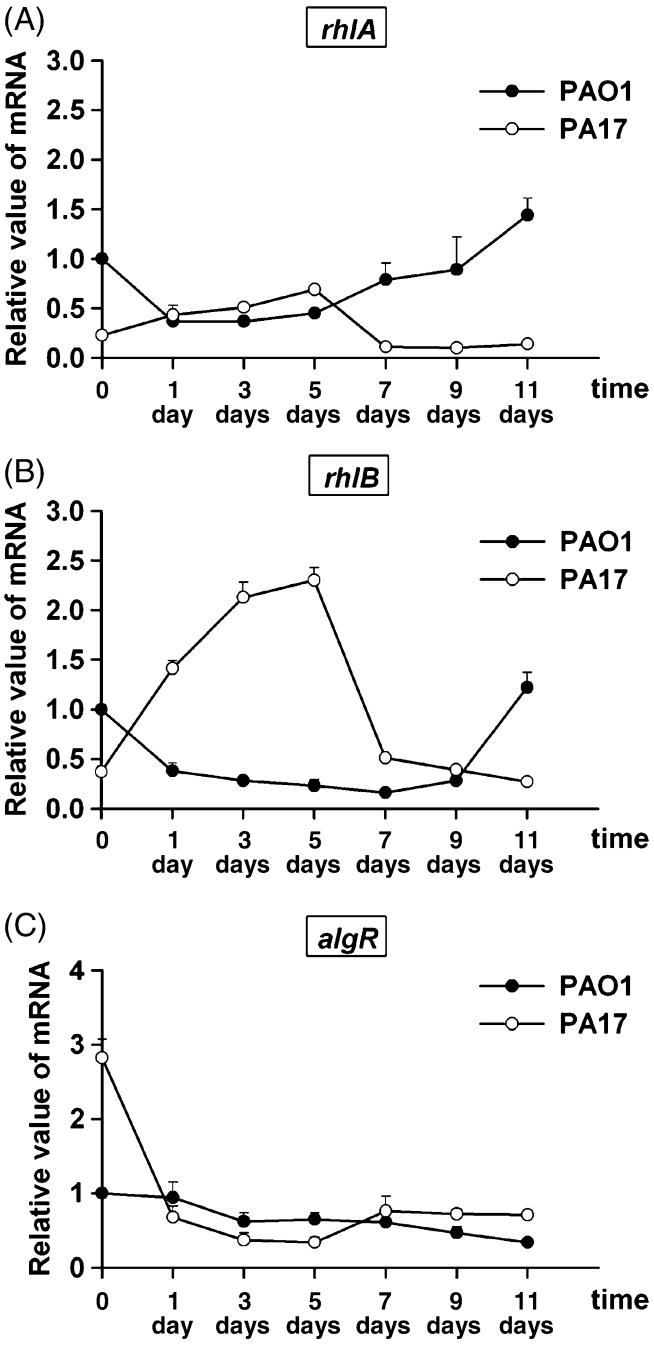

Figure 4. rhlA, rhlB and algR gene expression in mucoid PA17 and non-mucoid PAO1 in planktonic condition and 1, 3, 5, 7, 9 and 11-day-old biofilms. All the results were calculated by comparison with PAO1 in planktonic condition. Data shown are mean ± SD of triplicates and representative of three independent experiments

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