Elastase LasB of *Pseudomonas aeruginosa* promotes biofilm formation partly through rhamnolipid-mediated regulation

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**Abstract:** Elastase LasB, an important extracellular virulence factor, is shown to play an important role in the pathogenicity of *Pseudomonas aeruginosa* during host infection. However, the role of LasB in the life cycle of *P. aeruginosa* is not completely understood. This report focuses on the impact of LasB on biofilm formation of *P. aeruginosa* PAO1. Here, we reported that the lasB deletion mutant (ΔlasB) displayed significantly decreased bacterial attachment, microcolony formation, and extracellular matrix linkage in biofilm associated with decreased biosynthesis of rhamnolipids compared with PAO1 and lasB complementary strain (ΔlasB'). Nevertheless, the ΔlasB developed restored biofilm formation with supplementation of exogenous rhamnolipids. Further gene expression analysis revealed that the mutant of lasB could result in the downregulation of rhamnolipid synthesis at the transcriptional level. Taken together, these results indicated that LasB could promote biofilm formation partly through the rhamnolipid-mediated regulation.

**Key words:** *Pseudomonas aeruginosa*, elastase, rhamnolipid, biofilm.

**Introduction**

*Pseudomonas aeruginosa* is an increasingly prevalent opportunistic human pathogen. It may cause acute or chronic infection in immunocompromised individuals or patients with lung cystic fibrosis (Farrell et al. 2009). It is a challenging task to treat the infections caused by the bacteria, as *P. aeruginosa* cells acquire extraordinary environmental adaptation by coordinating with each other to form a dynamic community within a highly differentiated structure known as “bacterial biofilm” (Fux et al. 2003; Walker et al. 2004; Chang et al. 2007). A biofilm is a community of microorganisms embedded within a matrix containing proteins, nucleic acids, lipids, and exopolysaccharides (EPS) (Flemming and Wingender 2001). Compared with the planktonic bacteria lifestyle, the biofilm is the predominant growth mode for bacteria in various environments and provides a way for the bacteria to escape host immunity systems, decreases their susceptibility to antibiotics, and keep cells hydrated and nutrients trapped and immobilized (Fux et al. 2003; Hu et al. 2011). Therefore, interrupting biofilm formation is beneficial in controlling the continuous infection of *P. aeruginosa*. Several secreted factors, including alginate (Boyd and Chakrabarty 1994), rhamnolipids (Boles et al. 2005) and the quorum-sensing (QS) system (De Kievit et al. 2001), have been deemed to be involved in biofilm formation. The elastase LasB, a well-defined virulence factor encoded by the lasB gene, has been well defined in its pathogenicity to the host, causing tissue damage, disruption of intercellular junctions of lung epithelia, and degradation of components of the pulmonary innate immune system (Kuang et al. 2011). However, the correlation of LasB to the survival and adaptability of bacteria under different ecological conditions has not been well studied. Hence, the present study investigated the impact of LasB on the biofilm formation by mutating the lasB gene in *P. aeruginosa*.

**Materials and methods**

**Bacterial strains and growth conditions**

The *P. aeruginosa* PAO1 strains were cultured in Mueller–Hinton broth (MHB) at 37 °C. The N-acyl homoserine lactone (AHL) biosensor strains *Escherichia coli* JM109 (pSB1075) and *E. coli* JM109 (pSB536), which were used for detection of QS signal molecules N-(3-oxo-dodecanoyl) homoserine lactones (3-oxo-C12-HSLs) and...
N-butyl-2-homoserine lactones (C4-HSLs) (Sio et al. 2006), respectively, were grown at 30 °C in Luria–Bertani broth (Yates et al. 2002). Antibiotics were used at the following concentrations: for \textit{E. coli} JM109 (pSB1075), tetracycline 10 μg/mL; for \textit{E. coli} JM109 (pSB536), ampicillin 50 μg/mL; for \textit{P. aeruginosa}, carbenicillin 300 μg/mL, gentamicin 100 μg/mL, and kanamycin 100 μg/mL.

Construction of gene deletion mutant and complementary strain

The PA01 lasB gene deletion mutant (ΔlasB) and lasB complementary strain (ΔlasB') and their green fluorescent protein (GFP) derivatives were constructed by homologous recombination as described previously (Lesic and Rahne 2008; Liang and Liu 2010).

Assays for elastolytic activity, extracellular protein level, and bacterial growth

All strains were inoculated into 200 mL of MHB medium with shaking (initial concentration of −0.05 at OD600). Every 4 h, 1 mL of culture was collected and centrifuged at 12 000g for 5 min, and the cell pellets were resuspended in the same volume of phosphate-buffered saline (PBS). The growth rate of the bacteria was monitored by measuring the OD600 (Beckman Coulter DU800, USA). Elastolytic activity in the cell culture was determined by the skim milk plate assay (an indicator for protease activity) and the elastin–Congo red assay as described previously (Sokol et al. 1979; Pearson et al. 1997). To investigate the extracellular protein level of LasB, the supernatants of the strains were subjected to SDS–PAGE, followed by Western blotting using mouse anti-LasB antibody (prepared in our laboratory).

Biofilm formation

For analysis of biofilm formation on polycarbonate membrane, the bacteria were inoculated into MHB medium with shaking overnight with shaking. Then the concentration of the culture was adjusted to 0.05 (OD600) and the bacteria in 5 mL of the diluted culture were sonicated separately, and the supernatants were collected and centrifuged at 12 000g for 5 min, and the cell pellets were resuspended in the same volume of phosphate-buffered saline (PBS). The growth rate of the bacteria was monitored by measuring the OD600 (Beckman Coulter DU800, USA). Elastolytic activity in the cell culture was determined by the skim milk plate assay (an indicator for protease activity) and the elastin–Congo red assay as described previously (Sokol et al. 1979; Pearson et al. 1997). To investigate the extracellular protein level of LasB, the supernatants of the strains were subjected to SDS–PAGE, followed by Western blotting using mouse anti-LasB antibody (prepared in our laboratory).

Biofilm quantitation

The biofilms cultured for 24–96 h on polycarbonate membranes were rinsed with deionized water and fixed with 4.0% formalin overnight, and then staining and quantified according to the method described by O’Toole and Kolter (1998).

Biofilm morphology analyses by scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM)

For SEM analysis, the biofilms on polycarbonate membrane were fixed and dehydrated as described previously (Katebian and Jiang 2012) and were observed under a Hitachi S-3400N SEM (Hitachi High Technologies, Japan). For CLSM analysis, the biofilms on coverslips were rinsed with PBS and were observed under a Leica SP5 CLSM (Microsystems, Germany).

Measurements of alginate and rhamnolipid production and their gene expression

For quantitation of alginates and rhamnolipids in biofilms, the biofilms cultured for 24–96 h were rinsed out from polycarbonate membranes with PBS. To quantify the amount of alginates and rhamnolipids produced in the medium, the bacteria were inoculated into MHB (initial concentration of −0.05 at OD600) and cultured with shaking for 24–96 h. The bacteria biofilm mixture and culture were sonicated separately, and the supernatants were then collected for following measurements. The alginates were measured by the borate–carbazole method that utilizes purified alginate (Sigma, USA) as a standard (Knutson and Jeanes 1968). The rhamnolipids in the supernatant were extracted and measured as described previously, where purified rhamnolipids (Sigma, USA) were used as a standard (Zhang and Miller 1992).

For gene expression analysis, the bacteria cultured in MHB and incubated on membranes were harvested and dissolved in Trizol (Takara Bio, Japan). RNA extraction and reverse transcription generating cDNA were performed according to the manufacturer’s instructions. The genes involved in the biosynthesis of alginates (an operon of 12 genes, e.g., \textit{algA}, \textit{algC}) and rhamnolipids (encoded by \textit{rhlA}, \textit{rhlB}, and \textit{rhlC}) were quantified by semiquantitative reverse transcription PCR. The expression levels of the genes were normalized to the housekeeping gene \textit{rpiU} (Kuchma et al. 2007), and the primer sequences are presented in Table S1.

Detection of AHLs by well-diffusion assay and thin layer chromatography (TLC)

For detection of AHLs in biofilms, the biofilms incubated for 48 h were rinsed out from polycarbonate membranes with PBS. After centrifugation, the AHLs in cell-free supernatants were extracted by ethyl acetate and dissolved in methanol. To prepare the well-diffusion plates, a mixture of the appropriate \textit{E. coli} culture (70 mL) in exponential growth phase and Luria–Bertani agar (0.8% agar, 140 mL) was immediately poured into dishes. The concentrated AHLs were pipetted into wells punched in the solidified agar, and the plates were incubated at 30 °C for 12 h. The bioluminescent zones induced by AHLs were captured by Kodak Image Station 4000R (Carestream Health, USA). For TLC analysis, the AHLs were spotted on RP-18 F254s plates (Millipore, Germany), followed by overlaying the biosensor strains on the plates as described previously (Huang et al. 2003).

Effect of rhamnolipids on biofilm formation of ΔlasB

To analyze the effect of rhamnolipids on biofilm formation of ΔlasB on polycarbonate membranes or glass coverslips, the rhamnolipids extracted from a culture supernatant of PA01 (Caiazza et al. 2005) were added to MHB plates or MHB culture of ΔlasB at a concentration of 50 μg/mL. The biofilm formation of ΔlasB was observed by SEM and CLSM as described above.

Effect of rhamnolipids on swarming of ΔlasB

Several measurements of cell motility were determined by inoculating the bacteria on agar plates containing different agar concentrations (Rashid and Kornberg 2000). Specifically, swarming (0.5% agar), swimming (0.3%), and twitching (1%) were established. To analyze the effect of rhamnolipids on swarming of ΔlasB, the rhamnolipids were added to the swarming plates at concentrations of 10, 20, and 40 μg/mL.

Statistical analysis

All data were collected from 3 independent experiments and presented as mean ± SD. Statistical analysis was performed by a Student’s \textit{t} test or ANOVA followed by a Fisher’s least significance difference test. A \textit{P} value of <0.05 was considered statistically significant.

Results

Assays for elastolytic activity, extracellular protein level, and bacterial growth

The ΔlasB and ΔlasB’ were constructed by homologous recombination. By utilizing the skim milk plate and elastin–Congo red

\footnotesize{\supplementarydata{Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjm-2013-0667.}}
assays, the elastolytic activity of the ΔlasB was determined to be completely deficient, whereas the activity of the ΔlasB⁺ strain was comparable with that of PAO1 (Figs. 1a and 1b). Furthermore, the extracellular LasB level of ΔlasB strain was completely deficient, as observed by Western blotting analysis (Fig. 1c). These results suggested that the construction of the ΔlasB and ΔlasB⁺ strains was successful. To determine whether mutation of lasB would affect the growth of the strains, the biomass of bacteria was measured every 4 h. As shown in Fig. 1d, no significant difference in growth rate of the strains was observed after 48 h of culture.

**Measurements of alginate and rhamnolipid production and their gene expression**

Alginites and rhamnolipids have been widely regarded as very important factors involved in biofilm formation. However, their production and gene expression display significant differences in biofilm and planktonic phase (Sauer et al. 2002; Waite et al. 2005). Hence, we analyzed their production and gene expression in the 2 conditions. The alginate content of the ΔlasB strain in culture supernatant was slightly decreased at 24 and 48 h (not at a significant level) compared with PAO1 and ΔlasB⁺ strains; however, that content did not show significant change over a prolonged time period (72 and 96 h) (Fig. 2a). In biofilm models, the alginate contents of the 3 strains showed no significant difference when cultured for 96 h (Fig. 2a). Moreover, no conclusions can be drawn for the difference of alginate-related gene expression both in biofilm and planktonic phase of PAO1 and ΔlasB (Table S2). The rhamnolipid quantitation assay revealed that the rhamnolipid contents (from 24 to 96 h) of ΔlasB in biofilm and planktonic phase were both significantly lower than that of PAO1 and ΔlasB⁺; even those contents showed a slightly increased trend with the extended incubation time (Fig. 2b). The gene analysis showed that the rhamnolipid-related genes (especially rhlA and rhlB) of ΔlasB were significantly decreased in both biofilm and planktonic phase at 24 h and 48 h compared with PAO1 (Table 1). This result indicated that the mutant of lasB could result in downregulation of rhamnolipid biosynthesis at the transcriptional level.

**Detection of AHLs by well-diffusion assay and TLC**

Since the genes related to rhamnolipid biosynthesis are regulated by QS at the transcriptional level (Van Delden and Iglesia 1998), we further analyzed the QS signal molecules (3-oxo-C12-HSLs and C4-HSLs) of the PAO1 strains in biofilm by well-diffusion assay and TLC. In the well-diffusion assay, the halo size of bioluminescence around the well correlates with the concentration of AHLs. As shown in Fig. 2c, there was no significant difference in the concentration of 3-oxo-C12-HSLs among these strains, while a prominent decrease in concentration of C4-HSLs was observed in ΔlasB compared with that in PAO1 and ΔlasB⁺. When analyzed by TLC, a similar trend was also obtained (Fig. 2c).

**Biofilm quantitation and morphology analysis**

In the biofilm quantitation analysis, the amount of biofilm formation was indicated by the absorbance of crystal violet staining.
Results showed that the biofilm formation of PAO1 and ΔlasB+ was increased with longer incubation time and reached its peak at 72 h. However, in the ΔlasB group, the biofilm formation was significantly lower than that of PAO1 and ΔlasB+, even though it showed a slightly increasing trend with prolonged time (Fig. 3a).

When supplemented with rhamnolipids, the ΔlasB developed significantly increased biofilm formation, which was comparable with that of PAO1 and ΔlasB+ (Fig. 3a).

In addition, the morphology analysis using SEM showed that in the PAO1, ΔlasB+, and ΔlasB supplemented with rhamnolipids groups, the biofilm thickness was significantly thicker than the ΔlasB group, and large voids could be found within the biofilm of...
Fig. 3. Biofilm quantitation and morphology analysis. The biofilm formation on polycarbonate membrane was quantified by crystal violet staining (a). The biofilm morphology was visualized by scanning electron microscopy (b). The biofilms of green fluorescent protein (GFP)-tagged strains on glass surface were visualized by confocal laser scanning microscopy (c). In the rhamnolipid-treated groups (ΔlasB+rhamnolipid), the rhamnolipids were added at a concentration of 50 μg/ml at day 1 and then the rhamnolipid-treated biofilms were cultured for the indicated day.
the former 3 groups (especially at 72 h). In the ΔlasB group, there was only a small amount of bacteria attached on the membrane and no significant matrix linkage among each bacterium especially at 24 and 48 h (Fig. 3b). Furthermore, to test whether there was a similar phenomenon of biofilm formation on glass surface, we used the GFP-tagged derivatives to analyze biofilm formation. Similarly, CLSM results showed that ΔlasB was less likely to form microcolonies (Fig. 3c) than PAO1, ΔlasB+, and ΔlasB supplemented with rhamnolipids. However, we failed to observe the GFP signal of the strains at 72 and 96 h, perhaps because of the static solid-liquid biofilm culture condition, which lacks sufficient nutrients for bacterial growth.

**Effect of rhamnolipids on cell motilities of ΔlasB**

It has been shown that the swimming, swarming, and twitching motilities are all closely related to biofilm formation (Klausen et al. 2003), thus, cell motilities of the 3 strains were also examined. As shown in Fig. 4, the ΔlasB strain displayed a more intensive dendritic-like swarming phenotype than the PAO1 and ΔlasB+ strains, while the ΔlasB strain supplemented with increased concentrations of rhamnolipids displayed a decreased or complete lack of swarming, suggesting the negative effect of rhamnolipids to swarming motility of the ΔlasB strain (Caiazza et al. 2005). However, the swimming and twitching motilities were not significantly different from PAO1 and ΔlasB+ strains (Fig. S1). 

**Discussion**

The protease LasB is one of the main extracellular virulence factors of *P. aeruginosa*, and its role on biofilm formation has received much attention. Researchers have shown that the biofilm formation of *P. aeruginosa* is attenuated when treated with LasB inhibitors such as N-mercaptoacetetyl-Phe-Tyr-amide (Cathcart et al. 2011) and tropone (Fullagar et al. 2013), indicating that LasB could promote biofilm formation. Besides, since alginate has once been regarded as one of the most important components of the EPS matrix required for biofilm formation (Flemming and Wingender 2003), LasB may promote biofilm formation by activating nucleoside diphosphate kinase, which generates GTP for the production of alginate (Kamath et al. 1998; Cathcart et al. 2011).

In this study, the mutant of lasB resulted in a significant decrease in biofilm formation of PAO1 (Fig. 3); however, the attenuated biofilm formation of ΔlasB (Fig. 3) was not associated with decreased alginate biosynthesis (Fig. 2a). These results appear to demonstrate that alginites are not involved in maintaining the biofilm formation of ΔlasB; however, further studies are required to test this effect. One evidence reported by Wozniak et al. showing that alginites are not required during the formation of non-mucoid biofilms in 2 *P. aeruginosa* strains (PAO1 and PA14) has supported this speculation (Wozniak et al. 2003). In addition, the mutant of PAO1 alginate biosynthesis gene (algD, encodes GDP mannose dehydrogenase essential for alginate biosynthesis) displayed no significant difference in the components of EPS matrix and biofilm architecture (Roychoudhury et al. 1989). Recently, researchers have also shown that apart from alginate (a polysaccharide composed of mannuronic and guluronic acid), there are at least another 2 EPS components produced by *P. aeruginosa*, Pel (a glucose-rich EPS synthesized by enzymes encoded by the pel gene cluster) and PsI (a mannose-, glucose-, and rhamnose-rich EPS produced by proteins encoded in the pel gene cluster), which are more likely to be involved in the initial stage of biofilm formation rather than alginate (Schurr 2013).

Rhamnolipids, the surface-active compounds, have been widely known to disrupt bacterial biofilms (Schooling et al. 2004; Boles et al. 2005) by changing the hydrophobicity of the physical interface and cell surface (Ron and Rosenberg 2001). Increasing studies have revealed that rhamnolipids are also needed for biofilm formation. The *P. aeruginosa rhlA* mutant deficient in synthesis of rhamnolipids is not capable of forming microcolonies and mushroom-shaped structures in the initial and later phases of biofilm formation, respectively (Pamp and Tolker-Nielsen 2007). Researchers also revealed that rhamnolipids from *P. aeruginosa* in the biofilm formation of *Listeria monocytogenes* have dual roles: (i) they help to eradicate biofilm in low concentration and in short contact time (e.g., 0.25% concentration, 2 h) and...
Fig. 5. Hypothetical model of rhamnolipid-mediated regulation of biofilm formation of ΔlasB. Grey solid arrows represent gene transcription. Black dotted arrows indicate gene translation. Black open-head arrow represents the biosynthesis of C4-HSLs by RhlR. Black solid arrow and black dashed lines represent activity promotion and repression, respectively. Grey dashed arrow indicates an uncertain regulation mechanism. The grey dotted arrows indicate the formation of a complex of RhlR and C4-HSLs.

considerably (ii) they promote biofilm growth with increased rhamnolipid concentration and contact time (e.g., 1.0% concentration, 12 h) (Zezzi do Valle Gomes and Nitschke 2012). In line with these findings, we found that the ΔlasB strain, which displayed lower levels of rhamnolipids (Fig. 2b), developed flat and weaker biofilm (Fig. 3b), whereas it displayed restored biofilm formation with the exogenous addition of rhamnolipids (Fig. 3). Our results indicate that rhamnolipids positively regulated biofilm formation of ΔlasB. However, the possible mechanism by which rhamnolipids promote biofilm formation remains elusive. Claro (2010) reported that rhamnolipids, which are to be utilized as the carbon source by P. aeruginosa, serve as the nutrient for the growth of biofilm. Besides, rhamnolipids are required for maintaining the open channels in the matured mushroom-shaped biofilm, and the voids (open channels) could serve as a tunnel for distributing nutrients and oxygen between bacterial colonies (Davey et al. 2003).

Moreover, to further understand the intercorrelation between LasB and rhamnolipids, we analyzed gene expressions of the 3 strains. Results showed that the genes (especially rhlA and rhlB) coding for rhamnolipids of ΔlasB were significantly decreased in biofilm and planktonic phase compared with PAO1 (Table 1), indicating that the mutant of lasB could result in the downregulation of rhamnolipid biosynthesis at the transcriptional level. Since the rhlAB operon (rhamnolipid biosynthesis genes) is under the control of the RhlR–C4-HSL system (Ochsner and Reiser 1995; Reis et al. 2011), we further analyzed the QS signal molecules produced by the strains. Our results showed that the ΔlasB strain displayed a decreased biosynthesis of C4-HSLs (Fig. 2c), indicating that lasB could promote biofilm formation partly through rhamnolipid up-regulation mediated by the RhlR–C4-HSL system (hypothetical model is presented in Fig. 5). However, as to why lasB could affect the biosynthesis of C4-HSLs, further investigation is still needed.

In addition, previous studies have shown that bacterial migration could prevent larger microcolony formation in biofilm (Klausen et al. 2003), indicating an inverse correlation between motility and biofilm formation (Verstraeten et al. 2008). Consistent with these findings, the ΔlasB strain, which displayed significantly reduced microcolony formation ability in biofilm (Fig. 3c), showed a more intensive swarming phenotype than the PAO1 and ΔlasB+ strains (Fig. 4). Meanwhile, we also found that rhamnolipids negatively regulated swarming motility of ΔlasB strain (Fig. 4), indicating that lasB could promote biofilm formation partly through rhamnolipid-mediated inhibition of swarming (hypothenetical model is presented in Fig. 5). As bacterial motilities are driven by flagella or (and) type IV pili (Köhler et al. 2000; Semmler et al. 1999), changes in the structure of bacterial appendages may directly affect bacterial motilities. However, there were no significant differences discovered in gene expression (data not shown) and the structure of flagella and type IV pili between ΔlasB and PAO1 strains (Fig. S2), indicating that the bacterial flagella and type IV pili were not responsible for the increased swarming of ΔlasB.

Conclusion

That the deletion mutant of the lasB gene (ΔlasB) downregulated rhamnolipid synthesis by PAO1 and that the decreased biofilm formation of the ΔlasB strain was restored with increased rhamnolipids indicates that LasB could promote biofilm formation partly through rhamnolipid-mediated regulation.

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Authors’ contributions: Kebin Zhang is the designer and guarantor of the integrity of the entire study. Hua Yu participated in the experiment design, data acquisition, analysis, and manuscript...


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