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# Mutation of the cyclic di-GMP phosphodiesterase gene in *Burkholderia lata* SK875 attenuates virulence and enhances biofilm formation

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***Burkholderia* sp. is a gram-negative bacterium that commonly exists in the environment, and can cause diseases in plants, animals, and humans. Here, a transposon mutant library of a *Burkholderia lata* isolate from a pig with swine respiratory disease in Korea was screened for strains showing attenuated virulence in *Caenorhabditis elegans*. One such mutant was obtained, and the Tn5 insertion junction was mapped to *rpfR*, a gene encoding a cyclic di-GMP phosphodiesterase that functions as a receptor. Mutation of *rpfR* caused a reduction in growth on CPG agar and swimming motility as well as a rough colony morphology on Congo red agar. TLC analysis showed reduced AHL secretion, which was in agreement with the results from plate-based and bioluminescence assays. The mutant strain produced significantly more biofilm detected by crystal violet staining than the parent strain. SEM of the mutant strain clearly showed that the overproduced biofilm contained a filamentous structure. These results suggest that the cyclic di-GMP phosphodiesterase RpfR plays an important role in quorum sensing modulation of the bacterial virulence and biofilm formation.**

***Keywords*:** quorum sensing, *Burkholderia lata*, transposon, *Caenorhabditis elegans*, c-di-GMP phosphodiesterase, BDSF

## Introduction

*Burkholderia* sp. is a Gram-negative betaproteobacteria species that inhabits remarkably diverse ecological niches, as they have been isolated from soil, plant rhizosphere, water, insects, fungi, hospital environments, and humans (Coenye and Vandamme, 2003). There are currently 118 *Burkholderia* species with validly published names (http://www.bacterio.net). *Burkholderia cepacia* complex (BCC) is a subgroup of the *Burkholderia* genus, which is currently comprised of 20 species, including a few opportunistic pathogens that can cause lung disease in immunocompromised individuals (Mahenthiralingam *et al.*, 2005). Importantly, BCC infections in cystic fibrosis patients are difficult to treat because these bacteria are resistant to most antibiotics (Coenye, 2010). Therefore, it is important to distinguish virulent BCC species and other virulent *Burkholderia* species from less-virulent *Burkholderia* soil isolates (Mahenthiralingam *et al.*, 2005).

*Burkholderia lata* is a BCC species (Vanlaere *et al.*, 2009), and according to a search of the National Center for Biotechnology Information (NCBI) database, four strains (383, FL-7-5-30-S1-D0, LK27, and LK13) of *B. lata* have been isolated from the environment. *Burkholderia lata* strain 383 was isolated from forest soil in Trinidad in 1958, and it was one of the first strains of pseudomonads described, originally named *Pseudomonas multivorans* (Stanier *et al.*, 1966).

BCC species produce various virulence factors, including cable pili, flagella, type III and type VI secretion systems, lipopolysaccharide, extracellular polysaccharide, biofilms, iron acquisition systems, etc. (Eberl, 2006). Pathogenicity and the expression of these virulence factors are known to be closely related to quorum sensing (QS) (Asad and Opal, 2008). In QS, once the concentration of the signal molecule reaches a certain threshold, it binds to its receptor and turns on the QS signaling system to activate the expression of numerous genes, including virulence factor genes. The most common QS signal molecules produced by gram-negative bacteria are *N*-acyl homoserine lactones (AHL) (Molina *et al.*, 2003; Venturi *et al.*, 2004; Steindler and Venturi, 2007). However, many other unrelated signal molecules have been identified. One type of signal molecule contains cis-2-unsaturated fatty acids, and these molecules are often referred to as diffusible signal factor (DSF) family signals (Deng *et al.*, 2011). Most strains of the genus *Burkholderia* produce both AHL- and DSF-type QS signals (Deng *et al.*, 2010; Deng *et al.*, 2011; Schmid *et al.*, 2012; Suppiger *et al.*, 2013). *Cis*-2-dodecenoic acid, also called *Burkholderia* diffusible signal factor or BDSF, is a signal molecule found in *Burkholderia*. *In vitro*, RpfR exhibits c-di-GMP phosphodiesterase activity, which is modulated by the BDSF binding to its Per/Arnt/Sim (PAS) domain (Deng *et al.*, 2012). When BDSF binds to PAS, the c-di-GMP phosphodiesterase activity of RpfR is stimulated, which lowers intracellular c-di-GMP levels.

The relationship between QS and virulence in *B. lata* is poorly understood. In this study, we investigated the pathogenicity genes related to QS in *B. lata* strain SK875, which was isolated from the respiratory tract of a pig with swine respiratory disease in Korea. A transposon (Tn) insertion mutant library was screened for strains with reduced virulence

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in *C. elegans*. In one such strain, the Tn insertion was mapped to a c-di-GMP phosphodiesterase gene that is located next to the BDSF synthase gene, *rpfF*. Then, various phenotypes related to QS, including biofilm formation, were examined in this mutant strain.

## Materials and Methods

### Bacterial strains, media, and growth conditions

A *B. lata* strain (lab stock number, SK875) isolated from the respiratory tract of a pig with swine respiratory disease was obtained from the National Veterinary Research and Quarantine Service, Anyang, Republic of Korea. *B. lata* was grown aerobically at 37°C in modified Luria-Bertani (LB) medium (Mil-Homens *et al.*, 2010) and casamino acid-peptone-glucose (CPG) medium (Karki *et al.*, 2012).

Antibiotics were used as needed at the following concentrations: kanamycin (Km) 25 μg/ml and ampicillin (Amp) 50 μg/ml. *Chromobacterium violaceum* CV026 was cultured in LB medium (McClean *et al.*, 1997). *Agrobacterium tumefaciens* NTL4 was routinely cultured in nutrient broth and inoculated into autoinducer bioassay (AB) media for experiments (Chankhamhaengdecha *et al.*, 2013). *Vibrio harveyi* BB886 was used to measure signal secretion as bioluminescence, and was cultured in LB and then inoculated to AB medium for 16 h at 30°C for assays (Bassler *et al.*, 1994). *E. coli* OP50 was used to feed *C. elegans* and was cultured in 2×YT medium (Sutphin and Kaeberlein, 2009).

### Construction of a Tn library of *B. lata* by conjugation

*E. coli* BW20767/pRL27 (Donor) and *B. lata* SK875 (Recipient) were used to construct a transposon mutagenesis library of *B. lata* (Larsen *et al.*, 2002). The donor strain was provided by William W. Metcalf, PhD of University of Illinois at Urbana-Champaign. *B. lata* SK875 is a multidrug resistant strain that shows resistance to ampicillin, carbenicillin, chloramphenicol, streptomycin, tetracycline, etc. However, it is sensitive to kanamycin. BW20767 harboring pRL27 is susceptible to ampicillin and resistant to kanamycin. Therefore, kanamycin and ampicillin were used for conjugant selection. For routine culture, *B. lata* SK875 and BW20767 harboring pRL27 were inoculated into 5 ml of LB (Amp) broth and 5 ml of LB (Km 25) broth, respectively, and the bacteria were cultured overnight at 37°C. These seed cultures were then inoculated into the same fresh broth at final concentration of 1% and cultured to an optical density at 600 nm ($OD_{600}$) of 0.5–0.7. Next, 100 μl of each culture, BW20767 harboring pRL27 and *B. lata* SK875, was plated onto an LB (Km, Amp) agar plate, which was incubated overnight at 37°C. Then, the Tn5 mutant conjugants were spread onto LB agar (Km, Amp) at dilutions aimed to obtain 300– 500 colonies, and incubated at 37°C for 16 h.

### Synchronization and virulence assay using *C. elegans*

To obtain *C. elegans* L4 stage larvae, cultures were synchronized as described previously (Tedesco *et al.*, 2016). A single colony of each Tn mutant was inoculated into a well of a 96-well plate containing 100 μl of NGM (Km, Amp). Then, 5–10 synchronized *C. elegans* worms (Khanna *et al.*, 1997) were placed into each well of the 96-well plate. The plate was incubated at 20°C, and the viability (movement or death) of the worms was checked daily under a light microscope (Correct) in a screen for Tn mutants that allow the worms to survive longer than wild-type *B. lata* SK875.

### Plasmid retrieval from the Tn5 junction region

The Tn5 junction region was retrieved as previously described (Larsen *et al.*, 2002). Briefly, chromosomal DNA was prepared from each Tn mutant culture grown in 100 ml of LB broth (Km, Amp) to retrieve the pRL27 plasmid, including the junction region. After treatment with RNase, the prepared DNA was resuspended in 200 μl of distilled water. The chromosomal DNA was digested with *Bam*HI and self-ligated. Then, the digested DNA was electroporated into *E. coli DH5α/λpir* with a Gene Pulser Xcell™ Electroporation System (Bio-Rad), which was plated on LB (Km, Amp) agar.

### DNA sequencing and homology analysis

The Tn5 insertion site in *B. lata* SK875 was sequenced using the primers P1 (AACAAGCCAGGGATGTAACG) and P2 (CAGCAACACCTTCTTCACGA). Then, a homology search was performed by using the BLAST program.

### Identification of proteins with GGDEF and/or EAL domains

A domain analysis was performed by using CCE identifier in the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) (Marchler-Bauer *et al.*, 2015).

### Motility assay

*B. lata* SK875 and the derived Tn5 mutant strain were cultured in LB for 16 h at 37°C. Cultures with the same $OD_{600}$ were used for the assay. A 3 μl aliquot of each culture was placed on an LB agar plate containing 0.3% agar to assess swimming motility. Then, the same amount of culture was placed onto a nutrient agar plate containing 0.5% agar and 0.5% glucose to assess swarming motility. Motility was observed after 32 h of incubation at 37°C.

### Congo red assay

*B. lata* SK875 and the derived Tn mutant strain were incubated in T-broth supplemented with Coomassie brilliant blue (15 μg/ml) and Congo red (40 μg/ml) for 24 h at 37°C. The Congo red plate contained T-broth without NaCl and was prepared with 1.0% agar (Friedman and Kolter, 2004). A 10 μl aliquot of each culture solution was incubated for 48 h at room temperature, and then colony morphology was observed (Kuchma *et al.*, 2007).

### Plate bioassay for AI signals in QS

The sensor strains *A. tumefaciens* NTL4 and *C. violaceum* CV026 were used in a plate bioassay for QS using *B. lata* SK875 and a derived Tn mutant. OHHL and HHL were used as controls (McClean *et al.* 1997; Shaw *et al.* 1997). For the

assay, 50 µl aliquots of *B. lata* culture supernatants were loaded into holes in an LB agar plate inoculated with *C. violaceum* CV026 (in 0.8% top agar) and an AB minimal medium agar plate inoculated with *A. tumefaciens* NTL4 (in 1.2% top agar).

### Thin-layer chromatography (TLC) assay

Wild-type *B. lata* SK875 and the Tn mutant strain were inoculated into 50 ml of LB (Km, Amp) and incubated for 16 h at 37°C. Then, 500× concentrates of the culture supernatant were prepared as follows. The two cultures were adjusted to the same $OD_{600}$ values, and then centrifuged for 10 minutes at 10,000 rpm. Then, the culture supernatant was filtered through a 0.45 µm membrane filter (PALL, Acrodisc Syringe Filter), and mixed with 10% ethyl acetate (1:1). The supernatant extract was obtained by separation using a funnel. Then, it was concentrated with a vacuum evaporator (Brinkmann Buchi RE-111) after repeating the extraction. Finally, 100 µl of methanol was added, and the final sample was concentrated 500×.

The prepared samples were separated by TLC (TLC Silica Gel60 RP-18F254S; Merck) and developed with 60% methanol for 4 h. Then, *C. violaceum* CV026 was mixed with LB medium containing 0.8% agar at 10%, and *A. tumefaciens* NTL4 was mixed with AB minimal medium containing 1.2% agar at 10%. The above mixtures were poured onto separate developed TLC plates. After air drying, the plates were incubated at 28°C for 16 h.

A 7 µl aliquot of each concentrate was loaded. The following autoinducer markers were used in the TLC assay with the *C. violaceum* CV026 reporter strain: C4, N-butyryl-homoserine lactone (880 µM), 5 µl; 3-oxo-$C_6$, *N*-3-oxo-hexanoyl-homoserine lactone (10 µM), 7 µl; $C_6$, *N*-hexanoyl-homoserine lactone (100 µM), 1 µl; $C_7$, *N*-heptanoyl-homoserine lactone (1 mM), 1 µl; and $C_8$, *N*-octanoyl-homoserine lactone (1 mM), 7 µl. The following autoinducer markers were used in the TLC bioassay with the *A. tumefaciens* NTL4 reporter strain: 3-oxo-$C_6$, *N*-3-oxo- hexanoyl-homoserine lactone (1 mM), 5 µl; $C_6$, *N*- hexanoyl-homoserine lactone (1 mM), 1 µl; $C_7$, *N*-heptanoyl-homoserine lactone (1 mM) 1 µl; $C_8$, *N*-octanoyl-homoserine lactone (1 µM), 6 µl; $C_{10}$, *N*-decanoyl-homoserine lactone (10 mM), 3 µl; and $C_{12}$, *N*-dodecanoyl-homoserine lactone (10 mM), 7 µl.

### Bioluminescence assay

Aliquots (0.4 µl) of 500× concentrated culture supernatants and a control (methanol only) were dropped into 200 µl of AB medium in a 96-well plate. Then, 15 µl of an overnight culture of *V. harveyi* BB886 was added, and the plate was incubated at 250 rpm for 2 h at 28°C. Bioluminescence intensity was measured with a luminometer (VERITAS Microplate Luminometer), and the $OD_{600}$ was measured to calculate the Relative Light Units (RLU).

### Biofilm formation and cell growth assays

*B. lata* SK875 and the Tn mutant strain were incubated in LB (Amp) medium at 37°C overnight. Then, the overnight culture was inoculated at 1% into fresh medium and incubated until an $OD_{600}$ of 0.3. An aliquot of the culture (200 µl) was transferred to a 96-well plate and incubated for 8, 20, and 32 h at 37°C with shaking at 250 rpm. Then, 25 µl of 1% crystal violet was added, and the plate was incubated at room temperature for 15 min. After incubation, the staining solution was removed, the wells were rinsed once with 200 µl of distilled water, and then 200 µl of 95% ethanol was added. The absorbance at 570 nm was measured with a microplate reader (BioTek Instruments, Inc.).

### Scanning electron microscopy (SEM) analysis

The SEM (H 3000N; Hitachi) analysis was done at the Mushroom Research Institute of Gyeonggi-Do Agricultural Research. Briefly, *B. lata* SK875 and *B. lata* SK2115 were fixed with Karnovsky's fixative at 1.0 × 1.0 cm for 24 h at 4°C as previously described (Lee *et al.*, 2013). A secondary fixation was done with 1% osmic acid, and then the samples were dehydrated in a graded ethanol series (50%, 70%, 85%, 95%, and 100%) for 1 h each after rinsing three times with 0.05 M cacodylate buffer for 10 minutes. Then, the solution was replaced with 50% and 100% isoamyl acetate solutions, and dried with a critical point dryer (HCP 2, Hitachi). For SEM, the acceleration voltage was 14.3 kV–18 kV after coating for 120 seconds with an ion sputter coater (Hitachi).

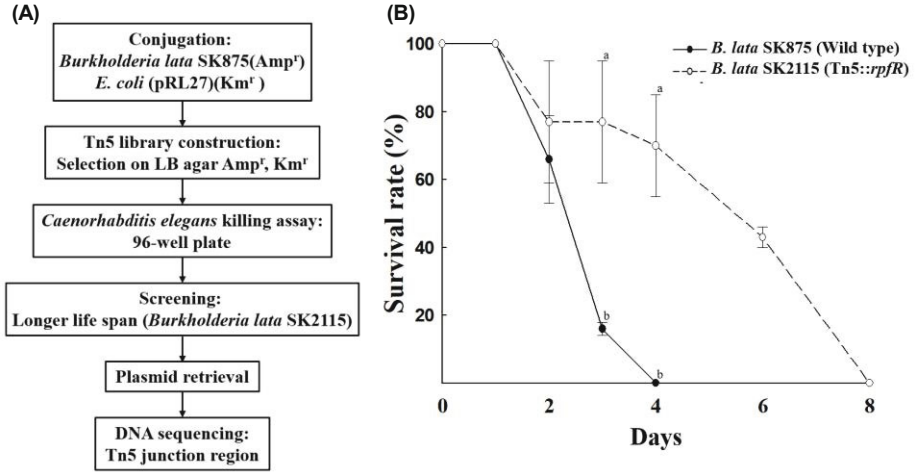


**Fig. 1. Screening for virulence factors in *Burkholderia lata* by selecting for mutants that allow a longer life span in a *C. elegans* infection model.** (A) Screening strategy for virulence factors: the selection of mutants with attenuated pathogenicity in *C. elegans*. (B) Extended life span of *C. elegans* infected with the c-di-GMP phosphodiesterase mutant strain *B. lata* SK2115. Data shown are the means of three replicates, and the error bars are the SDs. Lowercase letters (a, b) indicate significant differences ($P < 0.05$).

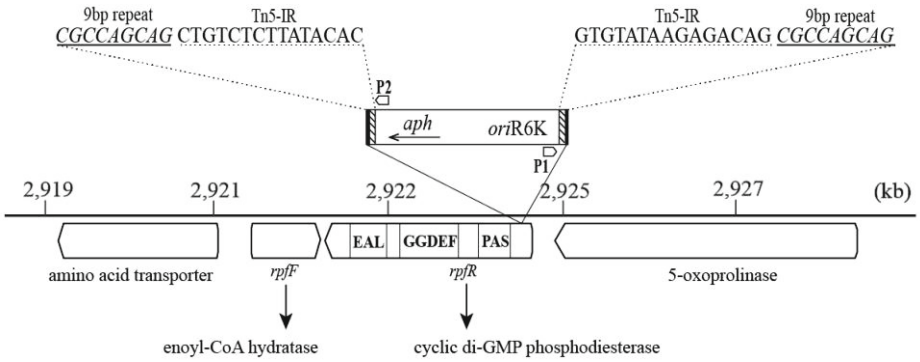


**Fig. 2. Mapping of the Tn5 insertion in the chromosomal DNA of *Burkholderia lata* 383.** The 9 bp repeated sequence and Tn5 in pRL27 were located and mapped near the 5′ end of the *rpfR* gene. The regions of the *rpfR* gene encoding the PAS, GGDEF, and EAL domains are shown in boxes.

### Statistical analysis

The data are presented as the mean ± standard deviation (SD) of triplicate or twelve replicates in 96-well plates and were analyzed with SPSS statistics for Windows (ver. 24; IBM). The significance of differences was determined by Duncan's multiple range test, and a *P* value less than 0.05 was considered statistically significant.

## Results

### Screening for *B. lata* transposon mutants with attenuated virulence in a *C. elegans* model of infection

We tested animal pathogens that produce an AI signal. *B. lata* SK875, an isolate from respiratory swine disease, produced a strong AI signal with both reporter strains, *C. violaceum* CV026 and *A. tumefaciens* NTL4 (Data not shown). Then, an *E. coli* BW20767 strain harboring pRL27 was conjugated with *B. lata* strain SK875, and the resulting Tn library was screened for attenuated virulence in *C. elegans* (Fig. 1A). About 2,000 colonies from the transposon library were replicated in 96-well plates containing NGM to screen for mutants that allow *C. elegans* to live longer than wild-type *B. lata* SK875. The *C. elegans* in eight wells of the plate appeared to survive longer than the worms in wells containing wild-type *B. lata* SK875. We reconfirmed that the worms incubated with these Tn mutants lived longer to exclude any unknown artificial effects, and only one of the mutants showed attenuated virulence (Fig. 1B). This mutant strain, *B. lata* SK2115, showed delayed death in *C. elegans*, with ~43% survival at 6 days compared to 0% for the wild-type strain *B. lata* SK875. The Tn5 junction region was retrieved from the chromosomal DNA as a plasmid and sequenced. The 700 bp sequence upstream of the Tn5 insertion showed the highest homology, with 97% nucleotide identity and 91% amino acid identity, to *B. lata* 383, although the 16S rDNA genes of the two strains were identical. The Tn5 insertion position in *B. lata* SK2115 was mapped to the c-di-GMP phosphodiesterase gene *rpfR*, which encodes a protein containing PAS, GGDEF, and EAL domains that is located on chromosome 2 of *B. lata* 383 (Fig. 2) next to the BDSF synthase gene *rpfF*.

It was previously shown that, upon interaction with BDSF, the RpfR receptor protein becomes a potent c-di-GMP phosphodiesterase. Thus, BDSF stimulates the c-di-GMP phosphodiesterase activity of the protein (Marchler-Bauer *et al.*, 2015; Suppiger *et al.*, 2016a). The conserved GGDEF and EAL domains are common features of diguanylate cyclases and phosphodiesterases, which are involved in the synthesis and degradation, respectively, of the second messenger c-di-GMP.

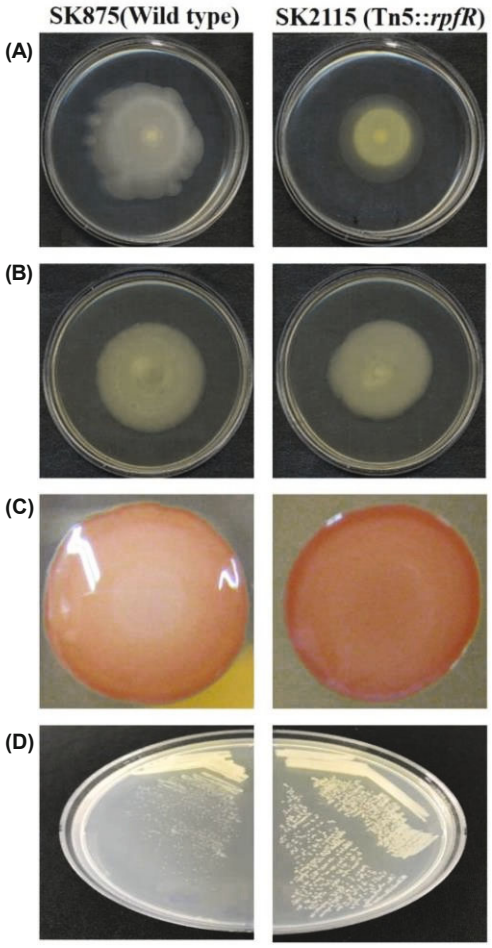


**Fig. 3. Comparison of motility, growth on Congo red, and cell growth between wild-type *B. lata* SK875 and *B. lata* SK2115 (Tn5::*rpfR*).** (A) Swimming motility. (B) Swarming motility. The plates were incubated at 37°C for 32 h. (C) Congo red assay. The plate (containing 1.0% agar) was incubated at room temperature for 48 h. (D) Growth comparison on a CPG agar plate. The plate was incubated at 37°C for 32 h.

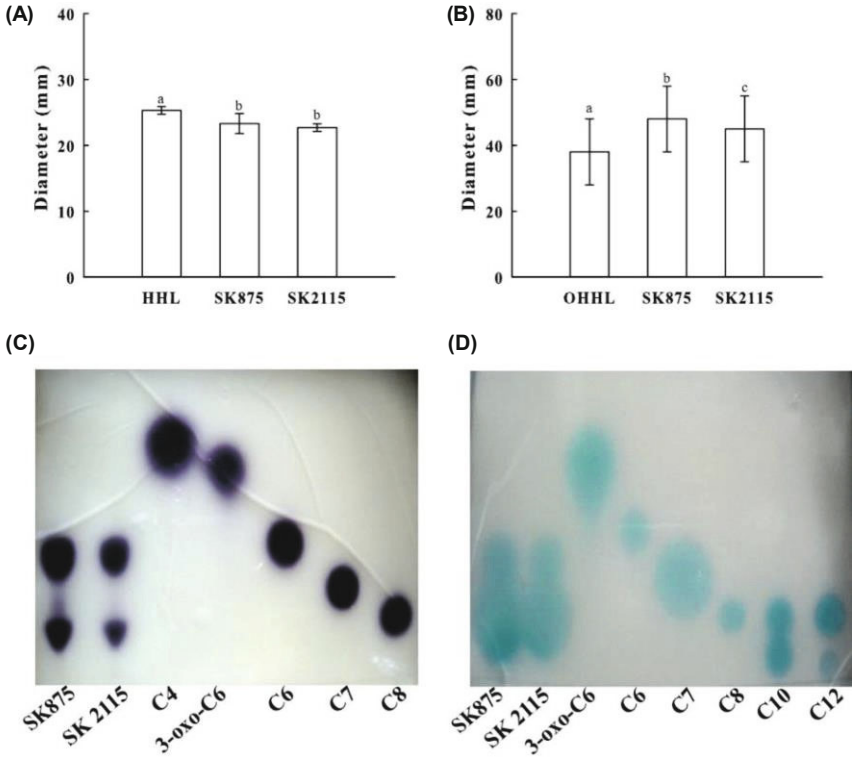


**Fig. 4. Assay for AHL signal production by wild-type *B. lata* SK875 and *B. lata* SK2115 (Tn5::*rpfR*) using *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* NTL4 as reporter strains.** (A) and (B) show the results of a bioassay on LB and AB minimal medium agar plates, respectively. (C) and (D) show TLC assays for AHL signal production. Data shown are means of three replicates, and the error bars are the SDs. Lowercase letters (a, b, c) indicate significant differences ($P < 0.05$).

### Motility, colony morphology, and growth of *B. lata* SK2115 (Tn5::*rpfR*)

The c-di-GMP phosphodiesterase mutant *B. lata* SK2115 showed reduced swimming motility when compared to that of wild-type *B. lata* SK875 (Fig. 3A). However, there was no detectable difference in swarming motility between *B. lata* SK2115 and SK875 (Fig. 3B). It was previously shown that swimming and swarming motilities are decreased by mutation of the c-di-GMP phosphodiesterase gene (Kuchma *et al.*, 2007; Lee *et al.*, 2010; Suppiger *et al.*, 2016b).

*B. lata* SK2115 displayed a different colony morphology in the Congo red assay; it was less shiny with a rougher colony morphology than colonies of the parent strain SK875 (Fig. 3C). The thick pinkish color of the c-di-GMP phosphodiesterase mutant *B. lata* SK2115 relative to that of the parent strain suggests increased production of cellulose and/or curli (Suppiger *et al.*, 2016b). Some strains of this pathogen produced dark brown pigments when they were grown on casamino-acid peptone glucose (CPG) agar medium (Karki *et al.*, 2012). However, comparison of *B. lata* SK875 and SK2115 did not reveal any altered pigmentation. Interestingly, a difference in growth (Fig. 3D) was observed between the wild-type and Tn5::*rpfR* mutant strains, as *B. lata* SK2115 clearly showed less growth on CPG agar; however, no difference was observed on LB agar.

### Reduced AI secretion in *B. lata* SK2115 (Tn5::*rpfR*)

To investigate the QS signals in *B. lata* SK2115, the effect of autoinducer (AI) secretion was examined by both agar plate and TLC assays using *C. violaceum* CV026 and *A. tumefaciens* NTL4 as reporter strains (McClean *et al.*, 1997; Shaw *et al.*, 1997). *B. lata* SK2115, the transposon c-di-GMP phosphodiesterase mutant, showed slightly decreased secretion of AI signal on LB and AB minimal medium plates compared to the parent strain *B. lata* SK875 (Fig. 4A and 4B). In the TLC analysis using *C. violaceum* CV026 as the reporter strain, the spots from *B. lata* SK875 and mutant *B. lata* SK2115 corresponded to $C_6$, *N*-hexanoyl-homoserine lactone and $C_8$, *N*-octanoyl-homoserine lactone (Fig. 4C). In the assay using *A. tumefaciens* NTL4 as the reporter strain, the spots corresponded more closely to $C_7$, *N*-heptanoyl-homoserine lactone and $C_8$, *N*-octanoyl-homoserine lactone (Fig. 4D). The spot intensity was slightly lower for *B. lata* SK2115 than for *B. lata* SK875, which was in agreement with the results from the agar plates. These results suggest that the c-di-GMP pho-

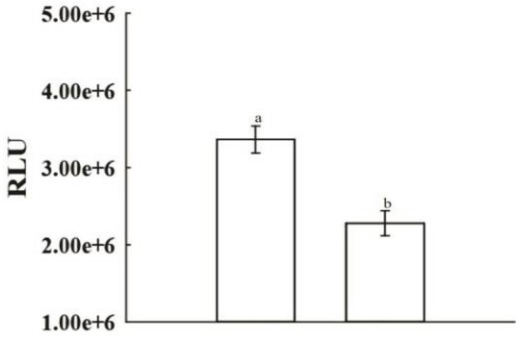


**Fig. 5. Bioluminescence assays in wild-type *B. lata* SK875 and *B. lata* SK2115 (Tn5::*rpfR*).** The intensity of Bioluminescence by the *Vibrio harveyi* BB886 reporter strain was measured. Data shown are the means of three replicates, and the error bars are the SDs. Lowercase letters (a, b) indicate significant differences ($P < 0.05$).

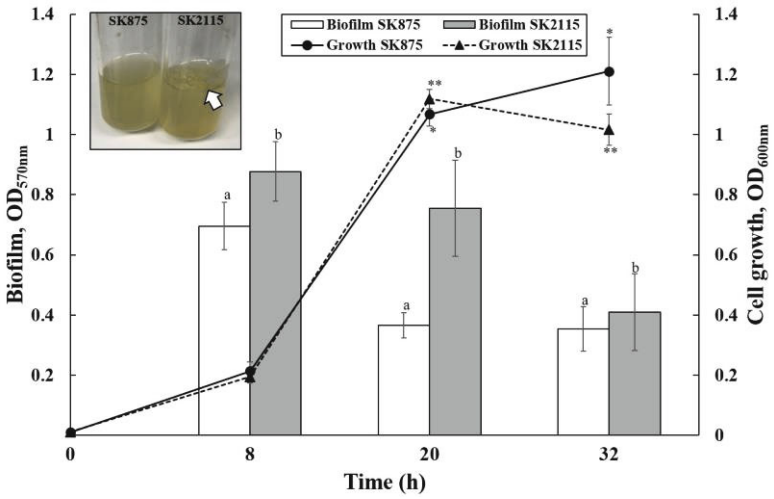


**Fig. 6. Biofilm formation assay of wild-type *B. lata* SK875 and *B. lata* SK2115 (Tn5::*rpfR*).** Biofilm formation was determined after 8, 20, and 32 h of incubation by crystal violet staining using broth cultures in a 96-well microplate, and cell growth was determined as the $OD_{600}$. Photographs of cell cultures grown on LB in glass test tubes were taken after 48 h of incubation at 37°C. Data shown are the means of twelve replicates, and the error bars are the SDs. Lower letters (a, b) and asterisks indicate significant differences ($P < 0.05$).

sphodiesterase gene indirectly controls the production or secretion of AI. The c-di-GMP phosphodiesterase mutant strain, *B. lata* SK2115 also showed relatively lower bioluminescence intensity than the wild-type strain *B. lata* SK875 (Fig. 5).

These results suggest that the QS signal BDSF controls AHL signal production by regulating AHL synthase gene transcription through modulation of intracellular levels of the second messenger c-di-GMP via its novel receptor RpfR (Deng *et al.*, 2013).

### Comparison of biofilm formation by *B. lata* SK875 (Wild type) and *B. lata* SK2115 (Tn5::*rpfR*) and SEM analysis

Biofilm formation and cell growth of the parent strain *B. lata* SK875 and the c-di-GMP phosphodiesterase mutant strain *B. lata* SK2115 were measured over time by crystal violet staining (Fig. 6). Biofilm formation peaked at 8 h, and gradually decreased thereafter for both strains. Surprisingly, the mutant strain showed more biofilm formation than the wild-type strain. In *B. lata* SK2115, a visible biofilm was detected on the surface of cell culture in glass test tube, whereas no biofilm was observed for the wild-type strain (Fig. 6). The c-di-GMP phosphodiesterase mutant, *B. lata* SK2115 showed enhanced biofilm formation during incubation. In addition, *B. lata* SK2115 grew a little faster after 20 h of incubation; however, the absorbance at 32 h was reduced significantly compared to that of the wild-type strain ($P < 0.05$).

The morphological phenotypes of wild-type *B. lata* SK875 and the c-di-GMP phosphodiesterase mutant *B. lata* SK2115

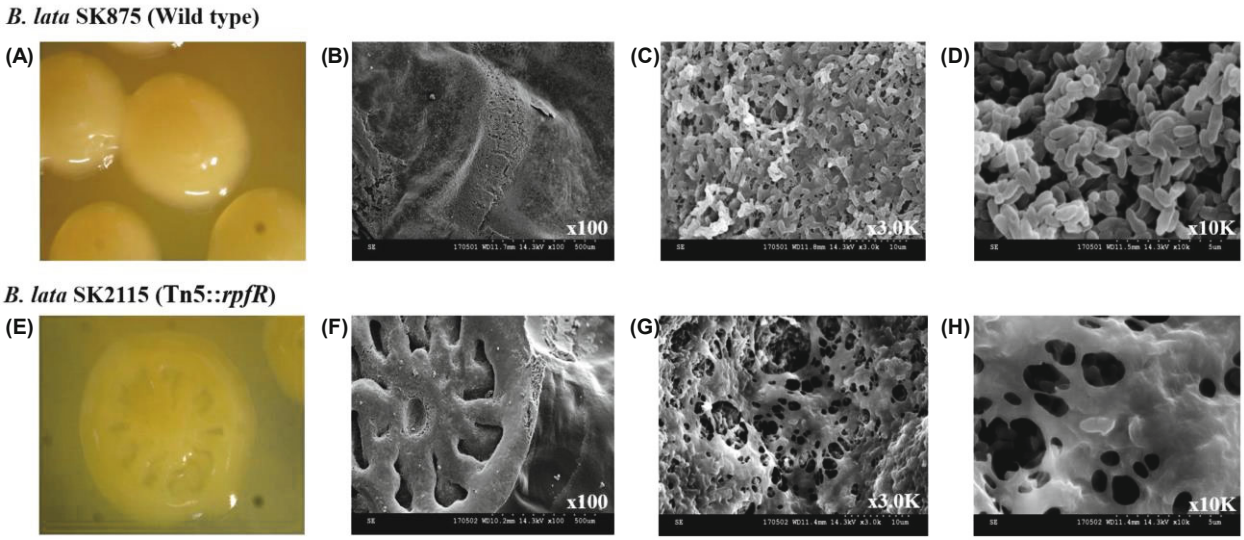


**Fig. 7. Scanning electron micrographs of agar invasion by wild-type *B. lata* SK875 and *B. lata* SK2115 (Tn5::*rpfR*).** (A) and (E) show actual photographs of colonies produced by the wild-type and c-di-GMP phosphodiesterase mutant strains, respectively. (B) and (F) show smooth and rough cell surfaces (100 ×) in a three-dimensional disposition of the wild-type and c-di-GMP phosphodiesterase mutant strains, respectively. (C) and (G) show higher magnification (3,000 ×) images of the wild-type and c-di-GMP phosphodiesterase mutant strains, respectively. (D) and (H) show even higher magnification images (10,000 ×) revealing the non-filamentous form of the wild-type strain and the biofilm-like filamentous form of the c-di-GMP phosphodiesterase mutant strains, respectively. Scale bar = (B) and (F): 500 μm; (C) and (G): 10 μm; (D) and (H): 5 μm.

were examined by SEM (Fig. 7). The colonies of the wild-type and mutant strains were clearly different (Fig. 7A and 7E); *B. lata* SK875 produced smooth colonies, whereas the c-di-GMP phosphodiesterase mutant *B. lata* SK2115 produced colonies with a rough surface and thick grids that were arranged in a three-dimensional manner (Fig. 7B and 7F). At high magnification, extracellular materials were observed on the colonies of *B. lata* SK2115, which formed a biofilm-like substance. However, this was not observed for the wild-type strain (compared Fig. 7G and H to Fig. 7C and D). In the mutant, we observed fibrils with enlarged structures connecting neighboring cells. We surmised that overproduction of these fibrils might inhibit bacterial movement, owing to their stickiness and hardness, resulting in reduced virulence in the *C. elegans* model of infection. Taken together, these data support the hypotheses that mutation of the c-di-GMP phosphodiesterase gene increased biofilm formation and decreased virulence, as described by Lee *et al.* (2010).

## Discussion

This study investigated the QS ability and virulence of a *B. lata* strain that was isolated from the respiratory tract of a pig with swine respiratory disease in Korea. Little is known about the virulence and QS mechanisms of strains in the genus *Burkholderia*, especially *B. lata*. *B. lata* SK2115 (Tn5::*rpfR*) was isolated in a screen of a transposon library, as a strain that allows *C. elegans* to live longer that the parental wild-type strain. Mutation of the cyclic di-GMP phosphodiesterase receptor protein, RpfR, decreased both AHL secretion, the ability to induce bioluminescence, and swimming ability. However, biofilm formation and the fibril structure of the mutant strain were enhanced compared to these features in the parent strain. C-di-GMP is associated with the regulation of various biological functions, including motility, biofilm formation, and virulence factor production (Deng *et al.*, 2013), and, as a second messenger, c-di-GMP controls various aspects of bacterial locomotion, including swimming and swarming motilities (Simm *et al.*, 2004).

Another important process regulated by c-di-GMP is the production of extracellular polymeric substance (EPS), which serves as an extracellular matrix for the formation and support of biofilm architecture (Tamayo *et al.*, 2007). C-di-GMP activates biofilm formation in various bacteria (Garcia *et al.*, 2004; Kirillina *et al.*, 2004; Simm *et al.*, 2004; Tischler and Camilli, 2004; Hickman *et al.*, 2005; Kim and Lee, 2016), and in *P. aeruginosa*, biofilm formation has been linked to elevated c-di-GMP levels, while dispersion is coincident with significantly reduced c-di-GMP levels. Thus, high levels of c-di-GMP are associated with the production of adhesive matrix components, cell aggregation, and increased biofilm

**Table 1. Gene position which carries GGDEF and EAL domains in *Burkholderia lata* 383**

| Chromosome | Locus tag | Description* | Amino acids | Homology (%)** | Domain | |
|---|---|---|---|---|---|---|
| 1 | BCEP18194_RS09620 | Diguanylate phosphodiesterase | 268 | 11 | | EAL |
| | BCEP18194_RS11630 | GGDEF domain-containing protein | 612 | 26 | PAS | GGDEF/EAL |
| | BCEP18194_RS11755 | EAL domain-containing protein | 427 | 13 | | EAL |
| | BCEP18194_RS12710 | GGDEF domain-containing protein | 400 | 19 | GAF | GGDEF |
| | BCEP18194_RS13740 | GGDEF domain-containing protein | 324 | 20 | | GGDEF |
| | BCEP18194_RS16075 | GGDEF domain-containing protein | 467 | 12 | | GGDEF |
| | BCEP18194_RS18645 | GGDEF domain-containing protein | 731 | 28 | | GGDEF/EAL |
| | BCEP18194_RS19525 | EAL domain-containing protein | 425 | 12 | | EAL |
| | BCEP18194_RS21605 | EAL domain-containing protein | 429 | 14 | | EAL |
| | BCEP18194_RS21755 | Phosphodiesterase | 613 | 14 | EAL | CBS /GGDEF |
| | BCEP18194_RS22730 | GGDEF domain-containing protein | 453 | 14 | | GGDEF |
| 2 | BCEP18194_RS22845 | Sensor domain-containing diguanylate cyclase | 313 | 21 | PAS | GGDEF |
| | BCEP18194_RS22915 | Sensor domain-containing diguanylate cyclase | 521 | 14 | | GGDEF |
| | BCEP18194_RS24960 | Diguanylate phosphodiesterase | 268 | 18 | | EAL |
| | BCEP18194_RS26010 | GGDEF domain-containing protein | 423 | 20 | HAMP | GGDEF |
| | BCEP18194_RS29585 | GGDEF domain-containing protein | 599 | 13 | HAMP | GGDEF |
| | BCEP18194_RS30485 | GGDEF domain-containing protein | 517 | 15 | PAS | GGDEF |
| | BCEP18194_RS30765 | GGDEF domain-containing protein | 493 | 12 | | GGDEF |
| | BCEP18194_RS31300 | GGDEF domain-containing protein | 603 | 13 | GAF/PAS/PAC | PAC/GGDEF |
| | BCEP18194_RS32840 | Diguanylate cyclase response regulator | 356 | 22 | REC | GGEDF |
| | BCEP18194_RS32845 | Bifunctional diguanylate cyclase/phosphodiesterase | 750 | 28 | GAF/PAS | PAC/GGDEF/EAL |
| | BCEP18194_RS34720 | Sensor domain-containing diguanylate cyclase | 511 | 15 | | GGDEF |
| | **BCEP18194_RS35610** | ***Cyclic di-GMP phosphodiesterase* (RpfR)*** | **668** | **100** | **PAS** | **GGDEF** |
| | BCEP18194_RS37655 | Cyclic diguanylate phosphodiesterase | 523 | 20 | | EAL |
| 3 | BCEP18194_RS01035 | GGDEF domain-containing protein | 468 | 13 | | GGDEF |
| | BCEP18194_RS02905 | GGDEF domain-containing protein | 777 | 27 | PAS | GGEDF/EAL |
| | BCEP18194_RS02910 | Bifunctional diguanylate cyclase/phosphodiesterase | 701 | 28 | PAS | GGEDF/EAL |

*Nomenclature according to GenBank file (Accession No. CP00150, CP00151, CP00152)
**Identity for amino acids comparison with cyclic di-GMP phosphodiesterase (RpfR)
***Tn insertion position of *B. lata* SK2115 was mapped on the cyclic di-GMP phosphodiesterase gene in chromosome 2 of *Burkholderia lata* 383

formation.

The *B. lata* c-di-GMP phosphodiesterase mutant SK2115 (Tn5::*rpfR*) exhibited phenotypes such as high intracellular c-di-GMP levels, with increased biofilm formation, and reduced virulence in the killing assay of *C. elegans*. These results suggest that the cyclic di-GMP phosphodiesterase RpfR plays a regulatory role in QS, virulence, and biofilm formation. Mutation of cyclic di-GMP phosphodiesterase genes was previously shown to enhance biofilm formation in various bacteria, including *V. fischeri*, *Pseudomonas aeruginosa* PA14, *Burkholderia pseudomallei*, and *Cronobacter* spp. (Kuchma *et al.*, 2007; Bassis and Visick, 2010; Lee *et al.*, 2010; Suppiger *et al.*, 2016b). However, mutation of the same gene in *Burkholderia cenocepacia* caused a reduction of biofilm formation (Deng *et al.*, 2012). As shown in our present study and in the report by Lee *et al.* (2010), enhancement of biofilm formation was associated with a reduction in virulence; however, the reason why is unclear.

Many bacterial pathogens contain either AHL- or DSF-type QS systems that coordinate bacterial physiology (Deng *et al.*, 2011; Marchler-Bauer *et al.*, 2015). However, *B. cenocepacia* contains both BDSF and AHL signaling systems, and it was shown that a set of the BDSF-regulated genes are also controlled by AHLs (Schmid *et al.*, 2012). The major signal in *Burkholderia* sp. is *cis*-2-dodecenoic acid, which is also called BDSF (Boon *et al.*, 2008; Deng *et al.*, 2009; Ryan *et al.*, 2009; Bi *et al.*, 2012). BDSF is sensed by the receptor protein BCAM0580, also called RpfR, which contains PAS-GGDEF-EAL domains (Deng *et al.*, 2012; Schmid *et al.*, 2012). It was shown that, when BDSF binds to the PAS domain, the cyclic di-GMP phosphodiesterase activity of RpfR is stimulated, which lowers intracellular levels of cyclic di-GMP (Schmid *et al.*, 2012). Intracellular levels of c-di-GMP are controlled by the opposing diguanylate cyclase (DGC) and phosphodiesterase (PDE) activities of GGDEF and EAL domain proteins, respectively (Simm *et al.*, 2004; Suppiger *et al.*, 2013). Proteins containing a PAS, GGDEF, or EAL domain or combinations of these domains are distributed in various regions of the chromosome. In this study, the genome of *B. lata* 383 was analyzed as a surrogate because the *B. lata* SK875 genome has not been sequenced. *B. lata* 383 encodes 27 proteins that contain an EAL and/or GGDEF domain (Table 1). Some proteins have a GGDEF or EAL domain alone, whereas others have one or both of these domains in conjunction with another conserved domain, like PAS. The amino acid identity of these proteins when compared with RpfR is very low (11–28%), suggesting that there was no gene duplication event, even though proteins with these domains are distributed across three chromosomes.

The Tn insertion position in *B. lata* SK2115 was mapped to the cyclic di-GMP phosphodiesterase gene *rpfR* on chromosome 2 of *B. lata* 383. Based on the number of proteins with related domains, there is a diverse array of the potential input and output signals that may regulate cyclic di-GMP phosphodiesterase activity to control c-di-GMP levels depending on the environmental or physiological conditions. It was shown that high levels of intracellular c-di-GMP antagonize QS and virulence gene expression in *B. cenocepacia* (Schmid *et al.*, 2017).

Elucidation of the growth defect of *B. lata* SK2115 (Tn5::*rpfR*) on CPG medium might help to decipher the role of cyclic di-GMP signaling in virulence and QS. A variety of pathogenic phenotypes related to BDSF signaling should be investigated using deletion constructs of individual domains or the complete *rpfR* gene and complementation tests. Furthermore, it would also be useful to determine the transcript levels of genes involved in QS and virulence in *B. lata* according to cyclic di-GMP levels.

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