The Journal of Microbiology, October 2005, p.443-450 Vol. 43, No. 5

Conserved Virulence Factors of *Pseudomonas aeruginosa* are Required for Killing *Bacillus subtilis*

Shin-Young Park[1], Yun-Jeong Heo[1], Young-Seok Choi[1], Eric Déziel[2] and You-Hee Cho[1,*]

[1]*Department of Life Science, Sogang University, Seoul 121-742, Republic of Korea*
[2]*INRS-Institut Armand-Frappier, Laval, Québec H7V 1B7, Canada*

(Received August 12, 2005 / Accepted September 26, 2005)

The multi-host pathogen, *Pseudomonas aeruginosa*, possesses an extraordinary versatility which makes it capable of surviving the adverse conditions provided by environmental, host, and, presumably, competing microbial factors in its natural habitats. Here, we investigated the *P. aeruginosa-Bacillus subtilis* interaction in laboratory conditions and found that some *P. aeruginosa* strains can outcompete *B. subtilis* in mixed planktonic cultures. This is accompanied by the loss of *B. subtilis* viability. The bactericidal activity of *P. aeruginosa* is measured on *B. subtilis* plate cultures. The bactericidal activity is attenuated in *pqsA*, *mvfR*, *lasR*, *pilB*, *gacA*, *dsbA*, *rpoS*, and *phnAB* mutants. These results suggest that *P. aeruginosa* utilizes a subset of conserved virulence pathways in order to survive the conditions provided by its bacterial neighbors.

Key words: *Bacillus, Pseudomonas aeruginosa,* survival, virulence

Many bacteria spend most of their lives in a starving or non-growing state because of the various life-threatening conditions imposed by their natural habitats. In order to survive harsh, oligotrophic environments and successfully compete with neighboring microorganisms, bacteria have developed highly sophisticated regulatory networks to govern stress responses and physiological and/or morphological differentiations, some of which are accompanied by genome evolution. In general, all bacteria are equipped with a set of survival mechanisms and their corresponding regulatory cascades. However, the potentials, as well as the proclivities to enhance them, may be variable, primarily due to the variety of the natural habitats and ecological niches occupied by the bacterial species.

Bacteria of the genus *Pseudomonas* are γ-proteobacteria which are commonly found in aquatic and soil habitats; these bacteria display extraordinary versatility for surviving their natural habitats, which involves unusual primary metabolisms, such as aromatic hydrocarbon degradation (Ramos *et al*., 1998), biochemical pathway evolution (Ornston and Parke, 1977), production of antibiotic secondary metabolites (Lépine *et al*., 2003; Lau *et al*., 2004), social behaviors of forming biofilm (Costerton *et al*., 1995), phenotypic variations (Boles *et al*., 2004; Déziel *et al*., 2001), synthesis of mucoid capsules (Deretic *et al*., 1995), and horizontal gene transfer and subsequent genome diversity (Hacker and Kaper, 2000). All of these salient features enable these bacteria to be successfully adapted to unfavorable conditions with extreme resistance to harsh environmental changes and the presence of competing organisms in their natural habitats. Among them, *P. aeruginosa* has been studied as the primary cause of mortality in individuals with cystic fibrosis and as the most common bacterial cause of sepsis in burn patients (Gang *et al*., 1999). It is not only an opportunistic human pathogen, but also pathogenic toward diverse eukaryotic non-mammalian model hosts, such as plants (Rahme *et al*., 1995), insects (Jander *et al*., 2000; D'Argenio *et al*., 2002), nematodes (Tan *et al*., 1999), and amoeba (Cosson *et al*., 2002; Pukatzki *et al*., 2002). One important feature to note is that the virulence pathways that *P. aeruginosa* exploits are relatively conserved. These observations have delineated the concepts of multi-host pathogenesis and the conservation of host defense responses (Rahme *et al*., 2000). Recently, an imperfect fungus, *Candida albicans*, was shown to be susceptible to *P. aeruginosa*, in which several virulence factors, including *N*-acyl-L-homoserine lactone-dependent quorum sensing systems, play important roles (Hogan and Kolter, 2002; Hogan *et al*., 2004). These results suggest that *P. aeruginosa* utilizes a limited number of virulence/survival mechanisms to overcome adverse environments, resulting in its persistence in its natural habitats or within host tissues.

As an initial step to broaden our knowledge of bacterial

* To whom correspondence should be addressed.
(Tel) 82-2-705-8793; (Fax) 82-2-704-3601
(E-mail) youhee@sogang.ac.kr

pathogenesis and survival, the interaction between *P. aeruginosa* and a well-characterized Gram-positive soil bacterium, *Bacillus subtilis*, was investigated using liquid and plate co-culture methods. Wild type *P. aeruginosa* cells spotted onto a plate covered with a lawn of *B. subtilis* cells resulted in the formation of a clear zone, indicating the inhibition of *B. subtilis* growth. Assays with virulence-attenuated mutants suggested that *P. aeruginosa* utilizes conserved virulence pathways in order to survive the interaction with *B. subtilis*.

Materials and Methods

Bacterial strains and culture conditions

The *P. aeruginosa* strains used in this study are listed in Table 1. *Bacillus* strains used are *B. thuringiensis* KCTC-1507 and *B. subtilis* PS382 (Shin *et al.*, 2005). All strains were grown overnight (for 14 - 18 h) at 37°C in Luria-Bertani (LB) broth as described previously (Lee *et al.*, 2003; Heo *et al.*, 2005). Overnight cultures were used to inoculate the LB broth (the inoculum size of 1.6×10^7 CFU/ml), and were grown at 37°C for 3-5 h with agitation to the early stationary phase. Likewise, the mixed cultures were made using mixtures of cells that had been grown separately, with an initial inoculum size of 1.6×10^7 CFU/ml (unless otherwise indicated) for each strain. Rifampin (Rif, 100 μg/ml), tetracycline (Tc, 100 μg/ml or 20 μg/ml), kanamycin (Km, 100 μg/ml or 50 μg/ml), ampicillin (Ap, 50 μg/ml), and carbenicillin (Cb, 200 μg/ml) were supplemented as required.

Plate killing assay and the B. subtilis killing index

Competing bacteria (*B. subtilis*, unless otherwise indicated) were grown in LB broth prior to the initiation of sporulation (OD_{600}= 2.0). LB (unless otherwise indicated), BDT (Bushnell-Haas salt media containing 0.2% dextrose and 0.5% tryptone), or M9 (M9 minimal media containing M9 salt and 0.4% glucose) plates were overlaid with 0.8% top agar containing 30-100 μl of *B. subtilis* cultures and were then dried for 1 h under sterile air blowing. The bacterial lawn was then spotted with 3 μl droplets of a *P. aeruginosa* bacterial suspension that contained 10^6 CFU of early stationary growth phase (OD_{600}= 3.0). Plates were incubated at 30°C for 18 h (unless otherwise indicated). The size of the halo was measured on the digitally-magnified photographs. The *B. subtilis* killing index indicates the percentage of the halo size relative to the PA14 halo size (0.35 ± 0.01 mm).

DNA manipulations

Restriction and modifying enzymes were used according to the manufacture's recommendations. Standard recombinant DNA methods were used (Sambrook and Russel, 2001). Competent cells of *Escherichia coli* DH5α and *E.*

Table 1. *Pseudomonas aeruginosa* strains used in this study

Strain	Relevant characteristics[a]	Source or reference
PAO1	Prototrophic, laboratory strain	Holloway, 1969
PAK	Prototrophic, laboratory strain	Frost and Paranchych, 1977
PA14	Prototrophic, clinical isolate; Rif^R	Rahme *et al.*, 1995
57RP	Prototrophic, environmental isolate	Déziel *et al.*, 2001
PA2	Human clinical isolate; Rif^R	Lau *et al.*, 2003
gacA	PA14 *gacA*::Tn*phoA*; Rif^R, Km^R	Rahme *et al.*, 1995
mvfR	PA14 *mvfR*::Tn*phoA*; Rif^R, Km^R	Cao *et al.*, 2001
pqsA	PA14 *pqsA*::Tn*phoA*; Rif^R, Km^R	Kim, 2004
pilB	PA14 *pilB*:: Tn*phoA*; Rif^R, Km^R	Kim, 2004
lasB	PA14 *lasB*::Tn*phoA*; Rif^R, Km^R	MGH-PGA[b]
dsbA	PA14 *dsbA*::Tn*phoA*; Rif^R, Km^R	MGH-PGA
phnAB	PA14 Δ*phnAB*; Rif^R	Mahajan-Miklos *et al.*, 1999
toxA	PA14 Δ*toxA*; Rif^R	Rahme *et al.*, 1995
plcS	PA14 Δ*plcS*; Rif^R	Rahme *etal.*, 1995
flgK	PA14 Δ*flgK*; Rif^R	Heo and Cho, unpublished
lasR	PA14 Δ*lasR*; Rif^R	This study
rhlR	PA14 Δ*rhlR*; Rif^R	This study
lasRrhlR	PA14 Δ*lasR*Δ*rhlR*; Rif^R	This study
rpoS	PA14 Δ*rpoS*; Rif^R	This study
phzC1	PA14 *phzC1*::pEX18T; Rif^R, Cb^R	This study

a: Rif^R, ripampicin-resistant; Km^R, kanamycin-resistant; Cb^R, carbenicillin-resistant

b: PA14 transposon insertion mutant library supported by Massachusetts General Hospital-ParaBioSys/National Heart, Lung, and Blood Institute Program for Genomic applications (http://pga.mgh.harvard.edu/cgi-bin/pa14/mutants/retrieve.cgi)

coli S17-1 (Simon *et al*., 1983) were used for general purpose cloning and conjugal transfer, respectively.

Detection of an aiiA gene

PCR primer pairs (N0, 5′-TAC CAT ATG ACA GTA AAG AAG C-3′ and C1, 5′-GCT CAA CAG GAT CCT CCT AAT G-3′) were used to amplify an 898 bp DNA fragment encompassing the full-length *aiiA*-coding region of the *B. thuringiensis* strains (Jin *et al*., 2004). The amplified DNA fragment was verified by nucleotide sequencing, exhibiting 99% identity to the *aiiA* gene from the *B. thuringiensis* B2 strain (Dong *et al*., 2002). The sequence of the *aiiA*-coding region from the KCTC1507 strain has been deposited under the accession number AY300026.

Creation of deletion and insertion mutants

The in-frame deletion mutants (*lasR*, *rhlR*, *lasRrhlR,* and *rpoS*) were created as described previously (Lee *et al*., 2005). In brief, the oligonucleotide primers that had been designed based on the sequences from the PAO1 strain (Stover *et al*., 2000) and the 5′ and 3′ segments from the *lasR*, *rhlR*, and *rpoS* genes were amplified using appropriate primer pairs and the PA14 chromosome as a template. More than 60% of the coding regions were deleted in the mutant constructs by the joining of both amplicons using engineered restriction sites. The double crossover deletion mutants were obtained by sucrose resistance selection from the verified single crossover cointegrates (Hoang *et al*., 1998). Using the internal DNA fragments (474 bp) from the *phzC1* coding region (1215 bp), the insertion mutant (*phzC1*) was generated by single crossover. All of the mutants were verified by PCR and Southern blot analyses, as well as by the associated phenotypes, such as motilities, pyocyanin, or elastase productions, as described by Déziel *et al*.(2001). The double null *lasRrhlR* mutant was created from the *rhlR* mutant by deleting the *lasR* gene.

Results

Pseudomonas aeruginosa strains kill Bacillus subtilis.

Bacillus species have been selected as the competing counterparts for *P. aeruginosa* because they grow as well as *P. aeruginosa* in laboratory conditions and are ubiquitous soil bacteria, presumably with a similar ecological niche. Lactonase-mediated quorum-quenching activities of *Bacillus* species, such as *B. thuringiensis* (Dong *et al*., 2002), may represent the interactions of the species with *N*-acyl-L-homoserine lactone (AHL)-utilizing proteobacteria. We observed that *P. aeruginosa* strain PA14 (PA14) outcompeted *B. subtilis* PS382 when they were co-cultured in LB broth, as described previously (Heo *et al*., 2005). Almost no viable *B. subtilis* cells remained after a 24 h co-culture with a similar or lower (1/10 fold) starting PA14 cell concentration (data not shown). This suggested that PA14 might inhibit the growth (or outcompete through more efficient nutrient uptake) and survival of the initially inoculated *B. subtilis* cells in the co-culture.

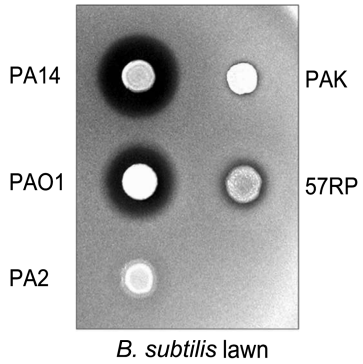

Fig. 1. Visualization of the *P. aeruginosa*-*B. subtilis* interaction. The *Bacillus* plate killing assay was performed on an LB plate, using *P. aeruginosa* strains (PA14, PAO1, PAK, 57RP, and PA2) as described in Materials and Methods.

We postulated that PA14 could be killing *B. subtilis* and devised a plate assay in order to visualize and quantify the inhibitory effect of *P. aeruginosa* strains upon *B. subtilis*. LB plates were overlaid with 0.8% top agar containing ~10^7 CFU of *B. subtilis* grown in LB broth before the initiation of sporulation, and this bacterial lawn was spotted with droplets of cell suspensions containing ~10^6 CFU of *P. aeruginosa* cells grown to the early stationary growth phase. As shown in Fig. 1, PA14 and, to a lesser extent, PAO1 antagonize the growth of *B. subtilis* presumably by secreting diffusible inhibitory factors, resulting in a visible halo around the growing cell spots. In contrast to strains PA14 and PAO1, *P. aeruginosa* strains PAK, 57RP, and PA2 do not exhibit such inhibitory effects. Furthermore, *Escherichia coli* and *Vibrio* species (*V. cholerae*, *V. vulnificus*, and *V. parahemolyticus*) are totally non-inhibitory (data not shown). These results suggest that the *P. aeruginosa*-mediated growth inhibition of *B. subtilis* is a specialized survival mechanism in some strains of *P. aeruginosa* that may be occasionally utilized when confronting *B. subtilis* in natural habitats, since the species possess similar ecological niches.

Although the spent medium from the stationary phase culture of PA14 was capable of inhibiting the growth of *B. subtilis* to some extent, the spotting with PA14 cultures or the same amount of cell pellets resuspended in fresh LB gave the best results. This implied an increasing accumulation and secretion of the inhibitory factor(s) during the growth of *P. aeruginosa* upon *B. subtilis* cells (data not shown).

B. thuringiensis is less sensitive than B. subtilis to killing by P. aeruginosa.

In addition to *B. subtilis*, we also tested *B. thuringiensis* with the plate killing assay, since it usually has quorum-quenching activity involving a lactonase encoded by *aiiA*

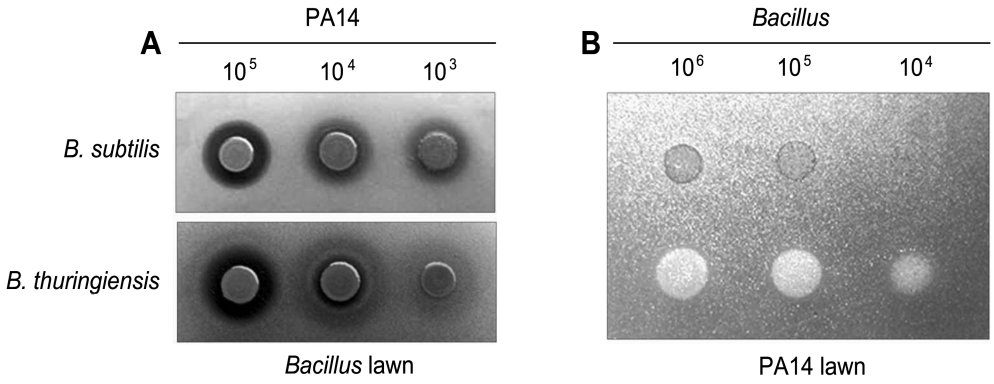

Fig. 2. Interaction between *P. aeruginosa* and *Bacillus spp.* (A) The *Bacillus* plate killing assay was done with serially-diluted PA14 culture suspensions spotted on either *B. subtilis* (upper) or *B. thuringiensis* (lower) lawns. The numbers (10^5, 10^4, and 10^3) indicate the CFU in the PA14 cell spots. (B) A reversed plate killing assay was done with serially-diluted *B. subtilis* (upper) and *B. thuringiensis* (lower) culture suspensions spotted on the PA14 lawn. The numbers (10^6, 10^5, and 10^4) indicate the CFU in the *Bacillus* cell spots.

(Dong *et al.*, 2001). *B. thuringiensis* strain KCTC1507, which contains the *aiiA* gene on its genome, was subjected to the plate killing assay with serial dilutions of PA14 cultures (Fig. 2A). The growth of *B. thuringiensis* was not inhibited by spotting ~10^3 CFU of PA14, which could sufficiently hamper the growth of *B. subtilis*. Furthermore, the plate killing assay was reversed by making PA14 lawns and spotting with serial dilutions of the *Bacillus* cells for a side-by-side comparison (Fig. 2B). *B. thuringiensis* cells seemed to be more resistant on the PA14 lawns than *B. subtilis* cells of the same CFU. These results suggest that *Bacillus* species have differential susceptibilities to *P. aeruginosa* cells, based on species-specific factors.

***P. aeruginosa* killing of *B. subtilis* requires the QS regulator, LasR.**

Since the susceptibilities of *Bacillus* species to killing by *P. aeruginosa* are attributed to some *Bacillus* species-specific factors, we first determined whether AHL-mediated quorum sensing (QS) is involved in the *P. aeruginosa*-induced killing of *Bacillus* strains, rather than using the *B. thuringiensis* strain deficient for quorum-quenching activity. The isogenic deletion mutants for one or both of the QS regulator genes, *lasR* and *rhlR,* were created (*lasR*, *rhlR,* and *lasRrhlR*) and tested for their *B. subtilis* killing activity. As shown in Fig. 3, the *lasR* mutant was severely attenuated in *B. subtilis* killing, whereas the *rhlR* mutant was not. The killing activity by the *lasRrhlR* double mutant was identical to that by the *lasR* mutant (data not shown). This result suggests that the Las system involving *N*-3-oxo-dodecanoyl-L-homoserine lactone, but not the Rhl system involving *N*-butyryl-L-homoserine lactone, is crucial for the killing of *B. subtilis* by *P. aeruginosa.*

To determine whether or not the *B. subtilis* killing by the QS mutants might be affected by growth conditions, two other media, BDT and M9, were tested for a growth inhibition assay. The QS mutants grew well in both media

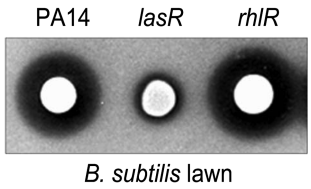

Fig. 3. Involvement of *N*-acyl-L-homoserine lactone (AHL)-dependent quorum sensing (QS) systems in *B. subtilis* killing. The *Bacillus* plate killing assay was performed on an LB plate using the PA14 mutants defective in AHL-dependent QS systems (*lasR* and *rhlR*), as in Fig. 1.

and BDT gave results similar to LB (data not shown). In contrast, the mutants were as inhibitory as PA14 on M9 (data not shown), although the halos were not as clear as those on the LB plates, and a white concentric zone appeared around the halo. These results might be related to the differences of both growth conditions, primarily in terms of growth characteristics and/or nutritional requirements for *B. subtilis* and *P. aeruginosa* which may affect the synthesis of the killing activities, although it remains to be deciphered which condition is more analogous to those provided by host tissues and natural habitats.

***P. aeruginosa* killing of *B. subtilis* requires a subset of virulence factors.**

We further investigated whether other factors involved in virulence or survival pathways play critical roles in the killing of *B. subtilis* by *P. aeruginosa*. We used several PA14 mutants defective in 1) regulator classes other than LasR and RhlR (*rpoS*, *dsbA*, *gacA*, *mvfR*), 2) QS-regulated secreted factors (*pqsA*, *phnAB*, *plcS*, *toxA*, *lasB*, and *phzC1*), 3) surface structure (*pilB* and *flgK*), which were subjected to the *B. subtilis* killing assay on LB plates. Fig. 4A shows the *B. subtilis* killing indices of the mutant strains used in this study, which are classified into 4 groups; group 1 (highly killing), group 2 (moderately killing), group 3 (weakly killing), and group 4 (unkilling). Eight out of 14 mutants (~57%) displayed significantly

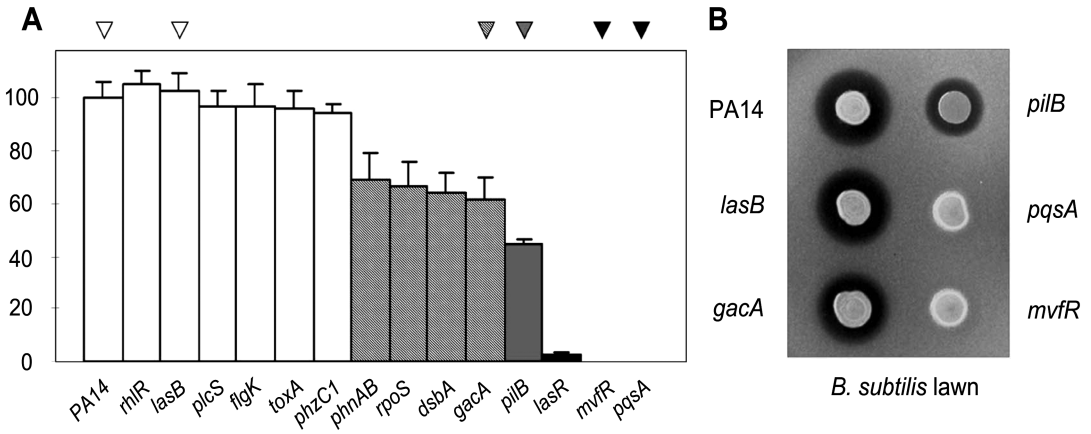

Fig. 4. Involvement of virulence factors in *B. subtilis* killing. (A) The *B. subtilis* killing indices of the PA14 mutants are represented as the relative value to the size of the PA14-derived halo (see Materials and Methods). The size of the halos was measured in five independent experiments. Based on these indices, *P. aeruginosa* strains and PA14 mutants were classified into four groups: group 1 (empty bar), highly killing (PA14, *rhlR*, *lasB*, *plcS*, *flgK*, *toxA*, and *phzC1*); group 2 (stripe bar), moderately killing (*phnAB*, *rpoS*, *dsbA*, and *gacA*); group 3 (gray bar), slightly killing (*pilB*); group 4 (black bar), unkilling (*lasR*, *mvfR*, and *pqsA*). The values indicate the averages of five independent experiments with the error bars representing standard deviations. (B) Several PA14 mutants (arrowheads in A) representing each group were subjected to the *Bacillus* plate killing assays on LB media. *lasB* (group 1), *gacA* (group 2), *pilB* (group 3), *mvfR*, and *pqsA* (group 4) mutants were included, with PA14 as the control.

reduced *B. subtilis* killing indices. As shown in Fig. 4B, the *B. subtilis* killing by the group 2 mutants (*rpoS*, *phnAB*, *gacA*, and *dsbA*) was slightly, but consistently distinguishable from that by PA14. Nevertheless, group 3 (*pilB*) and group 4 mutants (*lasR*, *mvfR*, and *pqsA*) were clearly defective in terms of their *B. subtilis* killing capabilities. The apparent interrelation between the virulence and the *B. subtilis* killing imply that *P. aeruginosa* may deploy relatively conserved virulence pathways in order to resist the possible interaction with the competing bacterial species in the natural habitats.

Discussion

We demonstrate here that the relatively conserved virulence mechanisms of *P. aeruginosa* are crucial for competition with the Gram-positive bacterium, *B. subtilis*. It will be worthwhile to determine whether other virulence factors can also be systematically analyzed in this *B. subtilis* competition system in order to obtain quantitative information regarding the conservation of virulence/survival mechanisms.

Based on the *B. subtilis* killing results in this study and in previous works (Machan *et al*., 1992; Lépine *et al*., 2003; Déziel *et al*., 2004), we cautiously propose that the *P. aeruginosa* killing of *B. subtilis* may preponderantly require functional synthesis of 4-hydroxyl-2-alkyl-quinoline (HAQ) compounds, including PQS (*Pseudomonas* quinolone signal), 2-heptyl-4-hydroxyquinoline (HHQ), and 2-heptyl-4-hydroxyquinoline N-oxide (HQNO). This is due to the fact that, among the six QS-regulated secreted factor mutants(*pqsA*, *phnAB*, *plcS*, *toxA*, *lasB*, and *phzC1*), *pqsA* is the only one which is completely defective in the killing of *B. subtilis*. The *pqsABCDE* operon encodes enzymes required for the synthesis of HAQs (Déziel *et al*., 2004). The fact that LasR controls *mvfR* (Hentzer *et al*. 2003; Schuster *et al*., 2003; Wade *et al*., 2005), which is responsible for the transcription of *pqsABCDE* and *phnAB* (Cao *et al*., 2003; Déziel *et al*., 2004), explains why these two mutants are also defective in the killing of *Bacillus*. Phospholipase C (*plcS*) and exotoxin A (*toxA*) are not involved in *B. subtilis* killing, which is intuitively understandable, since both are effective in eukaryotic cells. Elastase (*lasB*) and phenazines (*phzC1*), such as pyocyanin, also are not implicated. The moderate killing by the *phnAB* mutant for anthranilate synthase can likely be attributed to the functional presence of another anthranilate synthase encoded by *trpEG* (Essar *et al*., 1990), which allows residual production of HAQs (Déziel *et al*., 2004). The importance of HAQ biosynthesis and secretion involving *pqs* and *phn* operons under the control of MvfR (Déziel *et al*., 2005) in *B. subtilis* killing on LB plates is partially supported by the finding that HQNO displays killing activity against *B. subtilis* (Déziel *et al*., 2004) and *Staphylococcus aureus* (Machan *et al*., 1992). Furthermore, the inability of PAK to kill *B. subtilis* might be also attributed to the undetectability of these compounds in this strain (Lépine *et al*., 2003).

We found that *gacA*, *rpoS*, and *dsbA* might play some roles in the killing of *B. subtilis*, whether or not they directly affect the production and secretion of HQNO as global regulators that may have pleiotropic effects on survival/virulence pathways. Most importantly, since it is unpredictable, *pilB*-dependent cell surface structure appears to also be involved in the killing of *B. subtilis*. This implies the possible involvement of pili-mediated

cell-cell recognition in local communities of *P. aeruginosa* cells to facilitate the social behavior that may occur during the multicellular interactions, although a single *B. subtilis* cell might not serve as the proper surface which would trigger the initial attachment by *P. aeruginosa* cells. The mechanisms by which the *pilB*-dependent surface structure influences the killing of *B. subtilis* have not yet been elucidated, but these mechanisms might involve pilus movement which includes retraction and extension to affect the local concentration of PA14 cells or the use of pili as sensors that signal the presence of competing microorganisms, as suggested by Hogan and Kolter (2002).

About 50% correlation between the killing of *B. subtilis* and the mortality of *Drosophila* (Park and Cho, unpublished) suggests that *B. subtilis* might be used as an even simpler alternative interaction model for studying the virulence or survival factors of *P. aeruginosa*. Other important features are of concern: first, the factors involved in *B. subtilis* killing are most likely related with upstream signals that may be more conserved than the downstream effectors, which are prone to be diversified depending on the host types; second, the extracellular complementation between unkilling mutants is simply assessed and helps to uncover the secreted signaling molecules and intermediates, as well as the related regulatory networks, which are primarily involved in the QS circuitry (Park and Cho, unpublished). Therefore, *B. subtilis* represents a useful genetic system which could be used to identify the conserved core survival/virulence factors and to analyze the associated extracellular signaling networks of *P. aeruginosa*.

Our results suggest a link between the competition with the Gram-positive bacteria and the mechanisms of virulence of *P. aeruginosa*, both of which produce similar panoply of common virulence/survival factors. We can understand the bacterial pathogenesis as a result of the bacterial survival within the host environments. In accordance with the suggestion by Hogan and Kolter (2002) which referred to biofilm formation and interaction with fungi, we can postulate that the interspecies interaction of competitive bacteria has contributed to the evolution and maintenance of many survival factors, which are also required for the virulence mechanisms in the host environments. Therefore, we propose that the pathogenesis of *P. aeruginosa* is a specialized type of survival strategy, sharing some critical determinants in common, as exemplified by common virulence factors in multi-host pathogenesis studies and in the present study. They are prone to position at the upstream of the regulatory cascades in the survival pathways, affecting the effector molecules that are more likely to be congruously diversified with their interaction partners. Further investigation of the interaction between *P. aeruginosa* and *B. subtilis* and its utilization in screening for new virulence factors and extracellular signaling molecules of *P. aeruginosa* may help to elucidate the molecular basis which underlies the versatility of this bacterium and its ability to survive interactions with biotic and abiotic environments.

Acknowledgments

We are grateful to the Massachusetts General Hospital-ParaBioSys/National Heart, Lung, and Blood Institute Program for Genomic applications, Massachusetts General Hospital, and to Harvard Medical School, Boston, MA (http://pga.mgh.harvard.edu/cgi-bin/pa14/mutants/retrieve.cgi), for the Tn*phoA* mutants. This work was supported by a grant from the 21C Frontier Microbial Genomics and Applications Center Program of the Ministry of Science and Technology to Y.-H. Cho (MG05-0104-05-0).

References

Boles, B.R., M. Thoendel, and P.K. Singh. 2004. Self-generated diversity produces "insurance effects" in biofilm communities. *Proc. Natl. Acad. Sci. USA*. 101, 16630-16635.

Cao, H., G. Krishnan, B. Goumnerov, J. Tsongalis, R. Tompkins, and L.G. Rahme. 2001. A quorum sensing-associated virulence gene of *Pseudomonas aeruginosa* encodes a LysR-like transcription regulator with a unique self-regulatory mechanism. *Proc. Natl. Acad. Sci. USA*. 98, 14613-14618.

Cosson, P., L. Zulianello, O. Join-Lambert, F. Faurisson, L. Gebbie, M. Benghezal, C. van Delden, L.K. Curty, and T. Kohler. 2002. *Pseudomonas aeruginosa* virulence analyzed in a *Dictyostelium discoideum* host system. *J. Bacteriol*. 184, 3027-3033.

Costerton, J.W., Z. Lewandowski, D.E. Caldwell, D.R. Korber, and H.M. Lappin-Scott. 1995. Microbial biofilms. *Ann. Rev. Microbiol*. 49, 711-745.

D'Argenio, D.A., L.A. Gallagher, C.A. Berg, and C. Manoil. 2001. *Drosophila* as a model host for *Pseudomonas aeruginosa* infection. *J. Bacteriol*. 183, 1466-1471.

D'Argenio, D.A., M.W. Calfee, P.B. Rainey, and E.C. Pesci. 2002. Autolysis and autoaggregation in *Pseudomonas aeruginosa* colony morphology mutants. *J. Bacteriol*. 184, 6481-6489.

Deretic, V., M.J. Schurr, and H. Yu. 1995. *Pseudomonas aeruginosa*, mucoidy and the chronic infection phenotype in cystic fibrosis. *Trends Microbiol*. 3, 351-356.

Déziel, E., F. Lépine, S. Milot, J. He, M.N. Mindrinos, R.G. Tompkins, and L.G. Rahme. 2004. Analysis of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. *Proc. Natl. Acad. Sci. USA*. 101, 1339-1344.

Déziel, E., S. Gopalan, A.P. Tampakaki, F. Lépine, K.E. Padfield, M. Saucier, G. Xiao, and L.G. Rahme. 2005. The contribution of MvfR to *Pseudomonas aeruginosa* pathogenesis and quorum sensing circuitry regulation: multiple quorum sensing-regulated genes are modulated without affecting *lasRI*, *rhlRI* or the production of *N*-acyl-L-homoserine lactones. *Mol. Microbiol*. 55, 998-1014.

Déziel, E., Y. Comeau, and R. Villemur. 2001. Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpiliated and highly adherent phenotypic

variants deficient in swimming, swarming, and twitching motilities. *J. Bacteriol.* 183, 1195-1204.

Dong, Y.H., A.R. Gusti, Q. Zhang, J.L. Xu, and L.H. Zhang. 2002. Identification of quorum-quenching *N*-acyl homoserine lactonases from *Bacillus* species. *Appl. Environ. Microbiol.* 68, 1754-1749.

Dong, Y.H., L.H. Wang, J.L. Xu, H.B. Zhang, X.F. Zhang, and L.H. Zhang. 2001. Quenching quorum-sensing-dependent bacterial infection by an *N*-acyl homoserine lactonase. *Nature* 411, 813-817.

Essar, D.W., L. Eberly, A. Hadero, and I.P. Crawford. 1990. Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. *J. Bacteriol.* 172, 884-900.

Frost, L.S. and W. Paranchych. 1977. Composition and molecular weight of pili purified from *Pseudomonas aeruginosa* K. *J. Bacteriol.* 131, 259-269.

Gallagher, L.A., S.L. McKnight, M.S. Kuznetsova, E.C. Pesci, and C. Manoil. 2002. Functions required for extracellular quinolone signaling by *Pseudomonas aeruginosa*. *J. Bacteriol.* 184, 6472-6480.

Gang, R.K., R.L. Bang, S.C. Sanyal, E. Mokaddas, and A.R. Lari. 1999. *Pseudomonas aeruginosa* septicaemia in burns. *Burns* 25, 611-616.

Hacker, J. and J.B. Kaper. 2000. Pathogenicity islands and the evolution of microbes. *Ann. Rev. Microbiol.* 54, 641-679.

Haüßler, S. 2004. Biofilm formation by the small colony variant phenotype of *Pseudomonas aeruginosa*. *Environ. Microbiol.* 6, 546-551.

Hentzer, M., H. Wu, J.B. Andersen, K. Riedel, T.B. Rasmussen, N. Bagge, N. Kumar, M.A. Schembri, Z. Song, P. Kristoffersen, M. Manefield, J.W. Costerton, S. Molin, L. Eberl, P. Steinberg, S. Kjelleberg, N. Hoiby, and M. Givskov. 2003. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *EMBO J.* 22, 3803-3815.

Heo, Y.-J., K.S. Ko, J.-H. Song, and Y.-H. Cho. 2005. Profiling pyocins and competitive growth advantages in various *Pseudomonas aeruginosa* strains. *J. Microbiol. Biotech. in press.*

Hoang, T.T., R.R. Karkhoff-Schweizer, A.J. Kutchma, and H.P. Schweizer HP. 1988. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene. 212, 77-86.

Hogan, D.A. and R. Kolter. 2002. *Pseudomonas-Candida* interactions: an ecological role for virulence factors. *Science* 296, 2229-2232.

Hogan, D.A., A. Vik, and R. Kolter. 2004. A *Pseudomonas aeruginosa* quorum-sensing molecule influences *Candida albicans* morphology. *Mol. Microbiol.* 54, 1212-1223.

Holloway, B.W. 1969. Genetics of *Pseudomonas*. *Bacteriol. Rev.* 33, 419-443.

Jander, G., L.G. Rahme, and F.M. Ausubel. 2000. Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. *J. Bacteriol.* 182, 3843-3845.

Jin, U.-H., S.-H. Cho, M.-G. Kim, S.-D. Ha, K.-S. Kim, K-H. Lee, K.-Y. Kim, D.H. Chung, Y.-C. Lee, and C.-H. Kim. 2004. PCR method based on the *ogdH* gene for the detection of *Salmonella* spp. from chicken meat samples. *J. Microbiol.* 42, 216-222.

Kim, S.-H. 2004. Roles of virulence factors in the interaction between *Pseudomonas aeruginosa* and *Bacillus subtilis*. M.S. thesis. Sogang University, Seoul, Korea.

Lau, G.W., B.C. Goumnerov, C.L. Walendziewicz, J. Hewitson, W. Xiao, S. Mahajan-Miklos, R.G. Tompkins, L.A. Perkins, and L.G. Rahme. 2003. The *Drosophila melanogaster* Toll pathway participates in resistance to infection by the gram-negative human pathogen *Pseudomonas aeruginosa*. *Infect. Immun.* 71, 4059-4066.

Lau, G.W., D.J. Hassett, H. Ran, and F. Kong. 2004. The role of pyocyanin in *Pseudomonas aeruginosa* infection. *Trends Mol. Med.* 10, 599-606.

Lee, J.-S., S.-H. Kim, and Y.-H. Cho. 2004. Dithiothreitol attenuates the pathogenic interaction between *Pseudomonas aeruginosa* and *Drosophila melanogaster*. *J. Microbiol. Biotech.* 14, 367-372.

Lee, J.-S., Y.-J. Heo, J.K. Lee, and Y.-H. Cho. 2005. KatA, the major catalase, is critical for osmoprotection and virulence in *Pseudomonas aeruginosa* PA14. *Infect. Immun.* 73, 4399-4403.

Lee, Y.K., K.-K. Kwon, K.H. Cho, H.W. Kim, J.H. Park, and H.K. Lee. 2003. Culture and identification of bacteria from marine biofilms. *J. Microbiol.* 41, 183-188.

Lépine, F., E. Déziel, S. Milot, and L.G. Rahme. 2003. A stable isotope dilution assay for the quantification of the *Pseudomonas* quinolone signal in *Pseudomonas aeruginosa* cultures. *Biochim. Biophys. Acta.* 1622, 36-41.

Machan, Z.A., G.W. Taylorm T.L. Pitt, P.J. Cole, and R. Wilson. 1992. 2-Heptyl-4-hydroxyquinoline N-oxide, an antistaphylococcal agent produced by *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 30, 615-623.

Mahajan-Miklos, S., M.W. Tan, L.G. Rahme, and F.M. Ausubel. 1999. Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogenesis model. *Cell* 96, 47-56.

Mashburn, L.M., A.M. Jett, D.R. Akins, and M. Whiteley. 2005. *Staphylococcus aureus* serves as an iron source for *Pseudomonas aeruginosa* during in vivo coculture. *J. Bacteriol.* 187, 554-566.

Ornston, L.N. and D. Parke. 1977. The evolution of induction mechanisms in bacteria: insights derived from the study of the β-ketoadipate pathway. *Curr. Top. Cell Regul.* 12, 209-62.

Pukatzki, S., R.H. Kessin, and J.J. Mekalanos. 2002. The human pathogen *Pseudomonas aeruginosa* utilizes conserved virulence pathways to infect the social amoeba *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. USA.* 99, 3159-3164.

Rahme, L.G., E.J. Stevens, S.F. Wolfort, J. Shao, R.G. Tompkins, and F.M. Ausubel. 1995. Common virulence factors for bacterial pathogenicity in plants and animals. *Science* 268, 1899-1902.

Rahme, L.G., F.M. Ausubel, H. Cao, E. Drenkard, B.C. Goumnerov, G.W. Lau, S. Mahajan-Miklos, J. Plotnikova, M.W. Tan, J. Tsongalis, C.L. Walendziewicz, and Tompkins, R.G. 2000. Plants and animals share functionally common bacterial virulence factors. *Proc. Natl. Acad. Sci. USA.* 97, 8815-8821.

Ramos, J.L., E. Duque, P. Godoy, and A. Segura. 1998. Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E. *J. Bacteriol.* 180, 3323-3329.

Sambrook, J. and D.W. Russel. 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, New York.

Schuster, M., C.P. Lostroh, T. Ogi, and E.P. Greenberg. 2003. Iden-

tification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J. Bacteriol.* 185, 2066-2079.

Shin, I., H.-B. Ryu, H.-S. Yim, and S.-O. Kang. 2005. Cytochrome c_{550} is related to inhibition of sporulation in *Bacillus subtilis*. *J. Microbiol.* 43, 244-250.

Simon, R., U. Priefer, and A. Pühler. 1983. A broad-host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. *Biotechnology* 1, 784-791.

Tan, M.-W, L.G. Rahme, J.A. Sternberg, R.G. Tompkins, and F.M. Ausubel. 1999. *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. *Proc. Natl. Acad. Sci. USA*. 96, 2408-2413.

Van Delden, C. and B.H. Iglewski. 1998. Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. *Emerg. Infect. Dis.* 4, 551-560.

Wade, D.S., M.W. Calfee, E.R. Rocha, E.A. Ling, E. Engstrom, J.P. Coleman, and E.C. Pesci. 2005. Regulation of *Pseudomonas* quinolone signal synthesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* 187, 4372-4380.