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# Effect of PEL Exopolysaccharide on the *wspF* Mutant Phenotypes in *Pseudomonas aeruginosa* PA14

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***Pseudomonas aeruginosa* is an opportunistic human pathogen that produces and secretes exopolysaccharides (EPS), in which cells are embedded to form a highly organized community structure called biofilm. Here, we characterized the role of cyclic diguanylate (c-di-GMP) and EPS (PEL) overproduction in the *wspF* mutant phenotypes of *P. aeruginosa* PA14 (wrinkly appearance, hyperadherence, impaired motilities, and reduced virulence in acute infections). We confirmed that the elevated c-di-GMP level plays a key role in all the *wspF* mutant phenotypes listed above, as assessed by ectopic expression of a c-di-GMP-degrading phophodiesterase (PvrR) in the *wspF* mutant. In contrast, PEL EPS, which is overproduced in the *wspF* mutant, was necessary for wrinkly appearance and hyperadherence, but not for the impaired flagellar motilities and the attenuated virulence of the *wspF* mutant. These results suggest that c-di-GMP affects flagellar motility and virulence, independently of EPS production and surface adherence of this bacterium.**

**Keywords:** *Pseudomonas aeruginosa*, PEL exopolysaccharide, c-di-GMP, virulence, motility

*Pseudomonas aeruginosa* is an opportunistic human pathogen that causes fatal infections in immunocompromised individuals such as hospitalized patients and those suffering from severe burns or other traumatic skin damage or from cystic fibrosis. This ubiquitous Gram-negative bacterium deploys an arsenal of diverse virulence factors to intoxicate and proliferate within hosts as varied as plants, nematodes, insects, and mammals, with a broad spectrum of pathological consequences that depends on the host and infection conditions. It is not only a successful colonizer resulting in acute infection, but also a persistent survivor as a result of chronic infection [31]. Most virulence factors associated with motility, toxin production, invasion, and secretion are usually required for acute infections, whereas the factors (so-called persistence factors; [32]) required for chronic infections have been poorly understood, except for the suggestions that biofilm growth and anaerobic metabolism are most likely implicated [13, 36].

*P. aeruginosa* cells may sense the wetness of the surface that is affecting their swarming motility and presumably the fates of their community structure [39]: one is the swarmer cells or flat biofilm and the other is the structured biofilm. The structured biofilm formation is triggered, once the swarming is restricted and known to generally involve separate factors: flagella, fimbriae, and exopolysaccharides (EPS) in initial attachment and clonal growth; type IV pili (TFP)-driven motility in microcolony formation; further growth/maturation, and cell-to-cell communications in the subsequent formation (maturation) of highly organized, mushroom-shaped structures [6, 10, 21, 34, 42, 44]. A subpopulation of nonmotile bacteria is thought to coincide with the initial attachment to form the nonmotile microcolonies by clonal growth, while another subpopulation of motile bacteria spread out on the substratum in a TFP-dependent manner [21]. The diversification of the nonmotile *vs.* motile subpopulations is currently suggested to involve the activity of the ubiquitous intracellular messenger, cyclic diguanylate (c-di-GMP) that regulates the EPS production in various microorganisms, although there is no direct evidence that c-di-GMP levels are substantially different in each subpopulation [37].

D'Argenio *et al.* [7] identified the *wspF* gene of *P. aeruginosa* strain PAO1 from an autoaggregative mutant, as a member of the *wsp* operon consisting of 8 genes (*wspABCDEFGR*). The *wsp* genes were originally identified from the wrinkly spreader (WS) morphotypes of *P. fluorescens* [41] and highly similar to the *Myxococcus xanthus frz* operon, which is a chemosensory transduction system involved in A- and S-motilities [24, 28]. The *wspF* gene encodes a methylesterase, which might act on the WspR protein that contains both CheY-like receiver (REC) and diguanylate cyclase (GGDEF) domains. The mutation of the *wspF* gene is proposed to result in the constitutive activation of WspR and the concurrent elevation of c-di-GMP

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**Table 1.** *P. aeruginosa* PA14 mutants and plasmid constructs used in this study.

| Mutant or construct | Relevant characteristics[a] | Reference |
|---|---|---|
| In-frame deletion and related mutants | | |
| *wspF* | In-frame deletion of *wspF* | This study |
| *pilA* | In-frame deletion of *pilA* | [15] |
| *flgK* | In-frame deletion of *flgK* | [15] |
| *pelA* | In-frame deletion of *pelA* | This study |
| *pslE* | *MAR2xT7* insertion in *pslE*; $Gm^R$ | [27] |
| *wspFpelA* | In-frame deletions of *wspF* and *pelA* | This study |
| *wspFpslE* | In-frame deletion of *wspF*; *MAR2xT7* insertion in *pslE*; $Gm^R$ | This study |
| Plasmid constructs | | |
| pUCP-WspF | pUCP18 with the 1.3 kb of the *wspF* gene; $Cb^R$ | This study |
| pUCP-PvrR | pUCP18 with the 1.9 kb of the *pvrR* gene; $Cb^R$ | This study |

[a]$Gm^R$, gentamicin-resistant; $Cb^R$, carbenicillin-resistant.

level [17]. The transcriptomic analysis suggests that the *psl* and *pel* genes, which determine PSL and PEL EPS production, respectively, are upregulated in the *wspF* mutant of *P. aeruginosa* strain PAO1 [17]. Several lines of evidence show that both *psl* and *pel* genes are independently involved in surface adherence and rugose colony morphology in *P. aeruginosa* strains and rugose variants [10, 11, 20]. However, it is still unclear whether the c-di-GMP-dependent overproduction of EPS is sufficient and/or necessary for the various phenotypes of the *wspF* mutant that includes wrinkly colony morphology, enhanced surface adherence, reduced virulence, and impaired motilities. In the present study, we characterized the effects of expression of a phosphodiesterase, PvrR [9, 23], and deletion of an EPS gene (*pel*) on the *wspF* mutant phenotypes.

## Materials and Methods

### Bacterial Strains and Culture Conditions

The *Escherichia coli* strains DH5α and S17-1, for general purpose cloning and conjugal DNA transfer, respectively, and the wild-type *Pseudomonas aeruginosa* strain PA14 and its derivates listed in Table 1 were used in this study. All strains were grown for 14–18 h at 37°C using Luria-Bertani (LB) broth and M63-citrate minimal medium (1.2% $NH_2PO_4$, 2.8% $K_2HPO_4$, 0.8% $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 4% citrate) or on 2% Bacto-agar (Difco) LB or cetrimide-agar (Difco) plates as described previously [16]. Overnight cultures were inoculated into the fresh LB broth with an inoculum size of $1.6\times10^7$ CFU/ml and grown at 37°C for 3–5 h with agitation to the early stationary phase and used for experiments. For colony morphology, the cell cultures were diluted with LB medium and between $10^2$ to $10^3$ colonies were plated onto the LB plate. Colony morphology was examined after incubation for 24 h at room temperature.

**Table 2.** Primers used in this study.

| Primer | Sequence (engineered enzyme site) | Purpose |
|---|---|---|
| Gene SOEing | | |
| pelA-N1 | 5'-GAGTGCGGGGCTCTTTCATTC-3' | *pelA* upstream 5' |
| pelA-UC2 | 5'-GTAGTCCATGGCGACGATGG-3' (NcoI) | *pelA* upstream 3' |
| pelA-DN2 | 5'-GCCCATGGTGCTGGAGGAGG-3' (NcoI) | *pelA* downstream 5' |
| Gene expression | | |
| wspF-N1[a] | 5'-CTACAAGCTTCGTGAGGAGGACC-3' (BglII) | WspF in pUCP18 5' |
| wspF-C1[a] | 5'-ATGGCCGGATCCTCGACAAGCAG-3' (BamHI) | WspF in pUCP18 3' |
| pvrR-N1 | 5'-ACCGAATTCAACATGCCGAACAT-3' (EcoRI) | PvrR in pUCP18 5' |
| pvrR-C1 | 5'-CAGAAGCTTCCGTAGAAATTGGC-3' (HindIII) | PvrR in pUCP18 3' |
| Gene detection and others | | |
| pUCori-F | 5'-CAAAGGCGGTAATACGGTTATCCACAG-3' | pRT733 *ori* detection 5' |
| pUCori-R | 5'-CTCATGACCAAAATCCCTTAACGTGAG-3' | pRT733 *ori* detection 3' |
| Gm-N1 | 5'-TCTCGGCTTGAACGAATTGTTAG-3 | *MAR2xT7* Gm detection 5' |
| Gm-C1 | 5'-TCGTAAACTGTAATGCAAGTAGC-3 | *MAR2xT7* Gm detection 3' |
| pslA-N1 | 5'-AACCTGCGCCATGGAACCGCAG-3' (NcoI) | *pslA* detection 5' |
| pslA-C1 | 5'-GACAAGCTTTCTACCGACTTCG-3' (HindIII) | *pslA* detection 3' |

[a]wspF-N1 and wspF-C1 were used also for the in-frame deletion of *wspF*.

**DNA Oligonucleotide Primers**
The DNA oligonucleotide primers used for gene deletion, gene expression, and gene detection in this study are listed in Table 2.

**Virulence Measurements**
Fly and mouse mortalities were determined as described elsewhere [25].

**Episomal Gene Expression**
The WspF and PvrR proteins were ectopically expressed using a multicopy plasmid, pUCP18. For WspF and PvrR, the 1.3 kb DNA fragment using wspF-N1and wspF-C1 and the 1.9 kb DNA fragment using pvrR-N1 and pvrR-C1 were amplified, respectively. These PCR products were cloned into pUCP18 as pUCP-WspF and pUCP-PvrR. All the pUCP18-based constructs were introduced into PA14 and its isogenic mutant bacteria by electroporation [4].

**Generation of In-Frame Deletion and Double Mutants**
All the in-frame deletion mutants in this study were created using pEX18T [18]. We created *pelA* and *wspFpelA* mutants by gene SOEing (splicing by overlap extension) [19] using 4 oligonucleotide primers for *pelA* deletion (Table 2). For *wspF* deletion, the 1.3 kb PCR fragment using primers wspF-N1 and wspF-C1 was cloned into pEX18T, with the 315 bp internal fragment excised by EcoRV and HincII digestion. In general, more than 30% of the coding regions were deleted for all the deletions, which were verified by PCR and by easily discernable mutant phenotypes if applicable (*e.g.*, wrinkly appearance and hyperadherence for *wspF* and no pellicle formation for *pelA* mutants). To generate *wspFpslE* double mutant, allelic exchange to transfer the *MAR2xT7* insertion region from the *pslE* transposon mutant from the PA14 NR set [27] to a new *wspF* background was performed as described elsewhere [5]. After electroporation, cells were spread on LB plates containing gentamicin (Gm; 100 μg/ml) and incubated at 37°C, until Gm-resistant colonies appeared. PCR amplifications of the Gm marker using Gm-N1 and Gm-C1 verified the introduction of the transposon.

**Biofilm Assay**
A modified abiotic solid surface assay was performed as described previously [33]. Briefly, overnight cultures were diluted to 1% in LB medium and triplicate aliquots (150 μl) were dispensed into 96-well microtiter plates, which were incubated at 30°C. After 24 h and 48 h, the culture suspension and the pellicle at the liquid/air interface were carefully removed and then the plates were washed 3 times with tap water and dried. The solid surface adherence was visualized by staining with 180 μl of crystal violet (0.1 %) for 10 min, followed by washing 3 times with distilled water to remove unbound crystal violet and quantification by $OD_{600}$ measurement.

**Motility (Swimming, Twitching, and Swarming) Assay**
For swimming motility, LB plates containing 0.3% agar were inoculated with a sterile toothpick from an overnight grown single colony on LB agar plates and incubated for 16 h at 30°C. Motility was assessed qualitatively by examining the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation. For twitching motility, an overnight grown single colony on LB agar plates was picked with a toothpick and stab inoculated through a thin (about 3 mm) LB agar layer (1.5% agar) to the bottom of the petridish. After incubation for 48 h at 30°C, a hazy zone of growth at the interface between the agar and the polystyrene surface was observed after visualization using crystal violet [8]. For swarming motility, modified M9 medium (20 mM $NH_4Cl$, 12 mM $Na_2HPO_4$, 8.6 mM NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, and 11 mM dextrose) supplemented with 0.5% casamino acids (Difco) and 0.5% Bacto-agar (Difco) was used [43]. Swarming plates were dried under laminar flow for 60 min, after which 5 μl of the stationary culture suspension ($OD_{600}$=3.0) was spotted and incubated at 30°C for 16 h. Swarming behaviors including the swarming distance and the shape of dendrites were slightly changed by pUCP18 plasmid introduction.

## RESULTS

### Altered Level of c-di-GMP in the *wspF* Mutant is Critical for its Phenotypes Such as Virulence Attenuation, Reduced Motilities, Wrinkly Appearance, and Hyperadherence

The c-di-GMP dinucleotide is a novel intracellular secondary messenger that plays a regulatory role, influencing many cellular functions and aspects of bacterial behaviors including motility and adherence [22]. The level of c-di-GMP is apparently associated with the virulence potential of *P. aeruginosa* PA14 as well, although not all mutations in the genes potentially affecting c-di-GMP level result in altered virulence, suggesting that the localized activity of c-di-GMP is more critical than the total intracellular level [23]. To understand the role of the elevated c-di-GMP in the *wspF* mutant in regards to its various phenotypes such as virulence attenuation, reduced motilities, EPS overproduction (or wrinkly colony appearance), and hyperadherence at the early stage of biofilm growth, we expressed a phosphodiesterase protein (PvrR) in the *wspF* mutant, which efficiently reduces the intracellular level of c-di-GMP [23].

We cloned the full-length coding region of the *pvrR* gene in pUCP18 and examined the phenotypes of the *wspF* in-frame deletion cells containing this construct. The wrinkly colony appearance as well as the EPS hyperproduction of the *wspF* mutant did completely disappear (data not shown). Furthermore, the attenuated virulence of the *wspF* mutant was fully restored by PvrR, as assessed by *D. melanogaster* and mouse mortality measurements (Figs. 1A and 1B), which substantiates that the elevated c-di-GMP level is critical for the virulence attenuation of the *wspF* mutant. Next, we investigated the effect of the elevated level of c-di-GMP in the reduced motilities of the *wspF* mutant. The swimming motility of the *wspF* mutant was not completely defective, compared with that of the flagellar (*flgK*) mutant (Fig. 1C). However, the swarming and twitching motilities of the *wspF* mutant appeared to be completely impaired as in the *flgK* or type IV pilus (*pilA*) mutants (Figs. 1D and 1E). The introduction of PvrR into the *wspF* mutant fully restored its swimming, swarming,

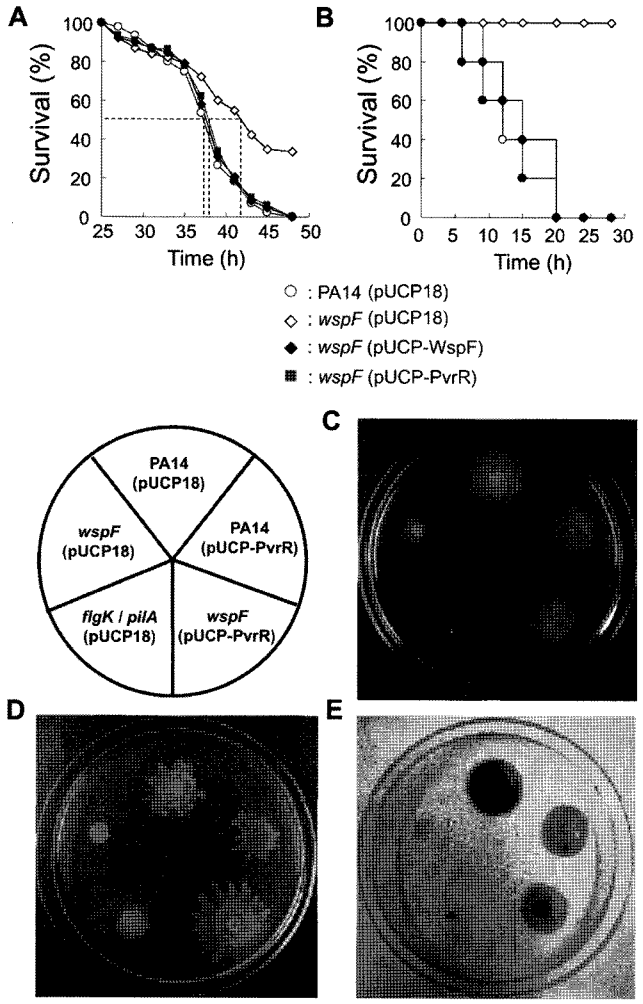


**Fig. 1.** Effect of c-di-GMP on the reduced virulence and motilities of the *wspF* mutant.

**A** and **B**. The virulence of the *wspF* in-frame deletion mutant expressing a phosphodiesterase (PvrR). Mortality curves were determined, based on the survivals in *D. melanogaster* (**A**) and mouse infections (**B**). For mouse infection, $2\times10^6$ bacterial CFU was used. Symbols: ○ and ◇, the wild type (PA14) and the *wspF* deletion mutant (*wspF*), respectively, both of which contain the pUCP18 plasmid; ◆ and ▦, the *wspF* mutant bacteria, which contain one of the pUCP18-based expression constructs for WspF (pUCP-WspF) and PvrR (pUCP-PvrR), respectively. The values are the averages of five replicate experiments for *D. melanogaster* (**A**) and three independent experiments for mouse (**B**) infections. The dotted lines in **A** represent the time required to reach 50% mortality. **C**, **D**, and **E**. The motilities of the *wspF* mutant expressing PvrR. The same bacteria as in **A** were inoculated at the 12, 2, 5, 7, and 10 o'clock directions of each motility plate as designated. One of the two mutant bacteria (*flgK*/*pilA*) containing pUCP18 was included as the negative control: a flagella-deficient mutant (*flgK*) for swimming (**C**) and swarming (**D**) motilities; a type IV pilus-deficient mutant (*pilA*) for twitching motility (**E**).

and twitching defects to the wild-type levels (or more), indicating that the elevated level of c-di-GMP is responsible for the defects in those motilities of the *wspF* mutant. Interestingly, the swimming and swarming motilities, but not twitching motility, of the wild-type bacteria were slightly decreased by the expression of PvrR (Figs. 1C, 1D, and 1E). These results simply indicate that the swarming and swimming motilities require a certain level of c-di-GMP, unlike the twitching motility. Since both swimming and swarming motilities of *P. aeruginosa* requires the appropriate flagellar function [30], which is dispensable for twitching [38], we propose that some flagellar dysfunction could be tightly associated with the altered (*i.e.*, increased and decreased) level of c-di-GMP.

### The *wspF* Mutant Requires *pel* Gene, but Not *psl* Gene for Wrinkly Appearance and Hyperadherence

One of the most prominent phenotypic characteristics of the *wspF* mutant is a wrinkly colony appearance [7], which is most likely resulted from the hyperproduction of EPS materials. Although *P. aeruginosa* strain PAO1 has three EPS gene clusters on its genome, there are several evidences that two EPS gene (*psl* and *pel*) clusters may independently contribute to the wrinkly morphology and biofilm formation of *P. aeruginosa* strains including colony variants and mutants, whereas the *alg* gene cluster is responsible for mucoidy: Both genes are required for the wrinkly colony morphology in *P. aeruginosa* strain ZK2870 [10]; pellicle formation was completely abolished in the PA14 *pel* mutants [11]; the disruption of a *psl* gene abolished the rugosity of rugose colony variants isolated from PAO1 biofilms, where the *psl* gene expression level is elevated in the rugose variant [20]; and the transcriptomic analyses of the *wspF* mutant of PAO1 revealed the hyperexpression of both *psl* and *pel* gene clusters [17]. However, we noticed that the PA14 lacks the *pslA-D* (PA2231 to PA2234) genes, with a genomic island inserted between PA2217 and PA2235 (*pslE*). Based on these and the observation that the major *psl* promoter is the *pslA* promoter [35], we suppose that the *psl* gene cluster may not be functional in PA14 and that only the *pel* cluster is involved in the adherence-associated EPS production in the *wspF* mutant.

To investigate whether the wrinkly colony appearance (*i.e.*, EPS overproduction) and the hyperadherence of the *wspF* mutant requires the presence of functional *pel* genes, we created *wspFpelA* and *wspFpelB* double mutants. Both *wspFpelA* and *wspFpelB* mutants exhibited a smooth colony appearance (Fig. 2A; data not shown) and the pellicle formation as well as hyperadherence were completely gone (Fig. 2B). To exclude the possibility of the requirement of *psl* in the hyperadherence of the PA14 *wspF* mutant, we included the *wspFpslE* double mutant as well. We were unable to find any difference between the *wspF* and *wspFpslE* mutants of PA14 strain in terms of colony morphology and surface adherence (Fig. 2). These results suggest that only the *pel* gene cluster is responsible for the EPS production in the early stage of biofilm formation, whereas the *psl* gene cluster remains unfunctional probably because of the genomic island integration.

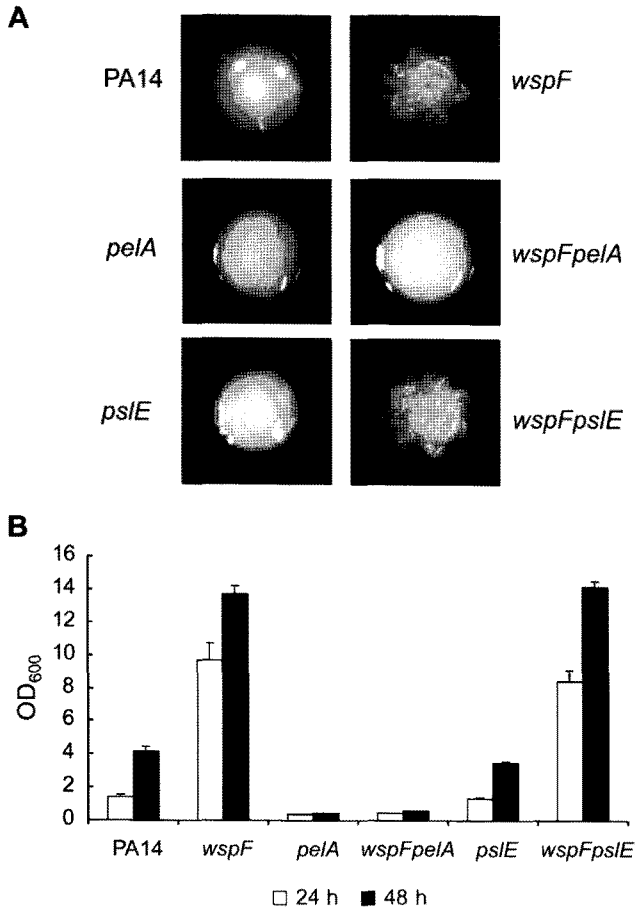


**Fig. 2.** Effect of EPS production on colony morphology and surface adherence of the *wspF* mutant.
**A**. Photographs of the colony derived from single cells (PA14, *wspF*, *pelA*, *pslE*, *wspFpelA*, and *wspFpslE*) that had been grown overnight on LB agar plates at room temperature. **B**. The crystal violet assay was used to measure solid surface adherence at 30°C for 24 h (empty bar) and 48 h (filled bar) as described in Materials and Methods.

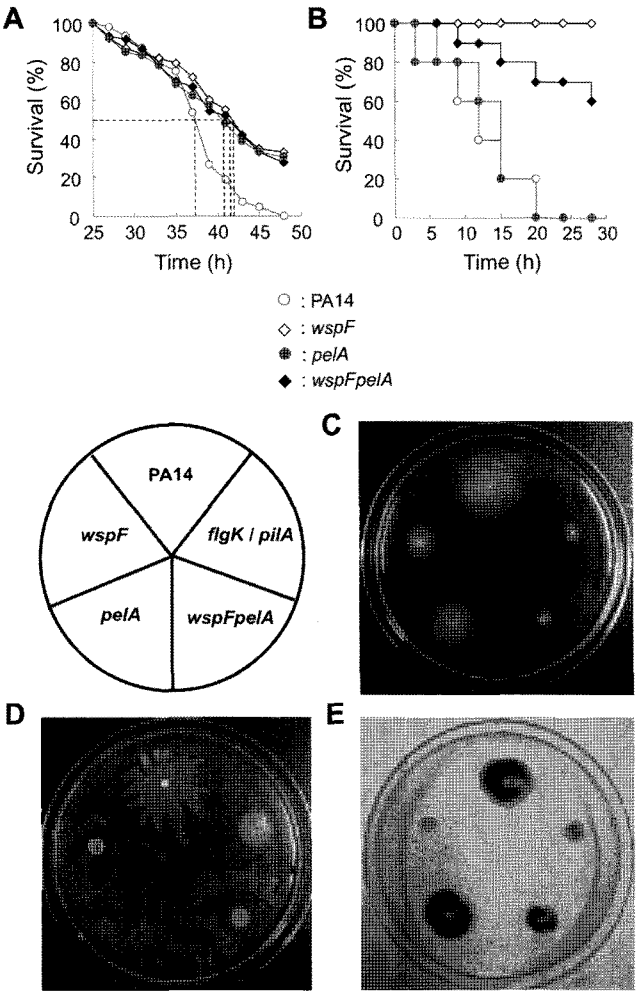


**Fig. 3.** Effect of PEL production on the reduced virulence and motilities of the *wspF* mutant.
**A** and **B**. The virulence of the *wspFpelA* double mutant. Mortality curves were determined as in Fig. 1. Simbols: ○, PA14; ◇, *wspF*; ●, *pelA*; ◆, *wspFpelA*. The values are the averages of five replicate experiments for *D. melanogaster* (**A**) and three independent experiments for mouse (**B**) infections. The dotted lines in **A** represent the time required to reach 50% mortality. **C**, **D**, and **E**. The motilities of the *wspFpelA* double mutant. The same bacteria as in **A** were inoculated at the 12, 2, 5, 7, and 10 o'clock directions of each motility plate as designated. One of the two mutant bacteria (*flgK*/*pilA*) was included as the negative control: a flagella-deficient mutant (*flgK*) for swimming (**C**) and swarming (**D**) motilities; a type IV pilus-deficient mutant (*pilA*) for twitching motility (**E**).

### The *wspF* Mutant Does Not Require *pel* Gene for Virulence Attenuation and Reduced Swarming and Swimming Motilities

We tested whether the *wspF* mutant requires the PEL synthesis for its virulence attenuation, by examining the virulence phenotypes of the *wspFpelA* double mutant. As shown in Figs. 3A and 3B, the *pelA* mutant that completely lacks the PEL polysaccharide is not virulence-attenuated at all in both *D. melanogaster* and mouse infections, indicating that PEL EPS is not a virulence determinant, at least in our infection conditions. Furthermore, the *wspFpelA* double mutant that also completely lacks the PEL was still attenuated in virulence, although it is evident that the virulence attenuation of *wspFpelA* in the mouse infection was not completely restored (Fig. 3B). This indicates the minor contribution by PEL overproduction to the virulence attenuation of the *wspF* mutant, at least in the mouse infection. As a result, this demonstrates that PEL EPS *per se* is most likely not involved in the virulence of *P. aeruginosa*, although the PEL hyperproduction in the *wspF* mutant could contribute to its full virulence attenuation, probably by affecting motilities. This speculation is based on the finding that the *pelA* mutant displayed enhanced swarming motility and the excessive PEL production facilitated bacterial adhesion to the surface, which might decrease the movement on the surface [3].

Thus, we needed to examine all the motilities of the *wspFpelA* double mutant. We observed that the *pelA*

mutant showed markedly increased swarming as observed previously, but slightly decreased swimming motilities (Figs. 3C and 3D), whereas the twitching of the *pelA* mutant was almost the same as that of the wild type (Fig. 3E). Taken together, we suggest that the PEL production affects swimming, and, to the larger extent, swarming motilities of *P. aeruginosa* in an opposite way. Even though the effect of PEL polysaccharide on twitching motility is not apparent, the twitching defect of the *wspF* mutant was partially recovered in the *wspFpelA* double mutant (Fig. 3E), indicating that EPS overproduction could in part contribute to the reduced twitching motility of the *wspF* mutant. The genetic study here, therefore, can be summarized as follows, in regards to the effect of PEL polysaccharide on *P. aeruginosa* motilities: the twitching motility is not associated with PEL in the wild type, but the full twitching defect of the *wspF* mutant requires the PEL synthesis; the swimming is not completely defective in the *wspF* mutant and the PEL synthesis is required for optimal swimming both in the wild-type and *wspF* mutant bacteria; the PEL synthesis is inhibitory to the swarming motility in the wild type, but not required for the defective swarming in the *wspF* mutant.

## Discussion

The *P. aeruginosa* Wsp chemosensory system is known to regulate the level of c-di-GMP in the PAO1 strain [17]. The elevated level of c-di-GMP by the *wspF* mutation or by the WspR overexpression leads to the hyperproduction of the EPS matrix in PAO1 and PA14 strains [17, 26]. To the best of our knowledge, it has been reported that three genetic determinants produce EPS in *P. aeruginosa* strains; *alg*, *psl*, and *pel* genes. Wozniak *et al.* [46] suggested that alginate (ALG) is not involved in biofilm formation *in vitro*, although it has some protective function from host immune responses in a chronic infection situation [29]. Instead, ALG is generally considered as the EPS involved in mucoid conversion that occurs months or years after initial colonization in most CF patients [1, 12]. In contrast, both PSL and PEL polysaccharides are involved in the surface adherence that mainly occurs during the initial stage of biofilm formation *in vitro*, suggesting that PSL and/or PEL are the major EPS materials involved in the early phase of *P. aeruginosa* biofilm growth.

The PA14 strain, however, lacks the *pslABCD* region by the integration of a potential pathogenicity island that we have identified (data not shown). Based on the microarray-based study of genome make-ups of 19 *P. aeruginosa* strains [45] and our PCR detection of the *pslA* gene in the 34 non-rugose *P. aeruginosa* strains of our culture collection [14, 16], all the strains tested except for PA14 contains the *pslA* region (data not shown). Furthermore, we here functionally verified that the *psl* gene is not required for the hyperadherence of the *wspF* mutant, although we did not directly measure the production of PSL polysaccharide. As a result, we confirmed that PEL might be the only early EPS in PA14, which is hyperproduced in the *wspF* mutant, as in the cells overexpressing WspR and other diguanylate cyclases [26].

The virulence attenuation of the *wspF* mutant is interesting, in that the *wspF* gene is listed in the nonsynonymous mutations that are favored in chronic infections of a real human patient [40]. Which is more critical for the *wspF* virulence attenuation, the c-di-GMP elevation or the PEL hyperproduction or both, in the case of acute infections? To answer this question, we needed to create a strain that overproduces PEL polysaccharide, even at a lower c-di-GMP level. However, the PEL production was not increased merely by increasing the *pel* gene expression, because PEL production requires c-di-GMP-mediated posttranscriptional regulation of the PelD protein by direct binding [26]. Based on the genetic evidence presented in this study, we demonstrate that the PEL is dispensable for *P. aeruginosa* pathogenesis and that the PEL hyperproduction in the *wspF* mutant is not the major contributor to its attenuated virulence and impaired motilities, although it is critical for the hyperadherence and wrinkly appearance. This and the observation that the impaired motilities in swimming and/or swarming rather than in twitching might coincide with the virulence attenuation of the *wspF* mutant, indicate that PEL production-mediated changes in adherence and motility might be independently modulated by c-di-GMP and thus appropriate motility regulation involving c-di-GMP may be important for virulence in acute infections. Since net motility and chemotaxis affect the infectivity of *Vibrio cholerae* [2], we presume that the altered chemotactic and/or flagellar behaviors in the *wspF* mutant of *P. aeruginosa* might be attributed to its virulence attenuation, which is associated with c-di-GMP, but not with PEL production. This genetic dismantling of the effect of c-di-GMP on PEL production and motilities and/or virulence must await further characterizations at the molecular level in the near future.

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