Role of PhoP-PhoQ Two-Component System in Biofilm Formation of the Phytopathogen *Dickeya dadantii* Strain 3937

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Abstract

To elucidate the role of PhoP-PhoQ two-component system in biofilm formation by the phytopathogen *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) strain 3937, we used marker-exchanged mutants deficient in both *phoP* and *phoQ*. A biofilm-inducing medium, salt-optimized broth plus glycerol (SOBG) which contains salt-optimized broth (SOB) plus 2% of glycerol, supported biofilm formation by *D. dadantii* strain 3937 to a greater extent than either M63 glycerol minimal medium or yeast extract peptone (YP) medium or Luria-Bertani (LB) medium. In addition, magnesium greatly induced biofilm formation. It was found that both the *phoP* and *phoQ* mutants exhibited enhanced ability to form a biofilm on the surface of the glass test tube as compared to the wild-type strain in SOBG medium containing high concentration of magnesium. In addition, under high magnesium condition, both the mutants produced elevated level of exopolysaccharide. Accordingly, genes responsible for exopolysaccharide production (*weaP, wza* and *wzc*) were derepressed in the mutants. These results suggest that the PhoP-PhoQ two-component system may regulate formation of biofilm, at least in part, by transcriptional control of genes responsible for exopolysaccharide biosynthesis of *D. dadantii* strain 3937. Since biofilm-associated bacteria showed more tolerance to acidic pH and high osmotic stress, it is apparent that the formation of biofilm may be an important factor for the survival of *D. dadantii* strain 3937 in unfavorable environment.

Keywords: PhoP-PhoQ two-component system, magnesium, biofilm formation, exopolysaccharide production, environmental stress, and *Dickeya dadantii* strain 3937

1. Introduction

Biofilm is a bacterial community encased in an extracellular matrix, which is predominantly composed of polysaccharides, in which the bacteria are imbedded (Costerton *et al*., 1995; Denese *et al*., 2000). Development of biofilm is a multistep process. In general, development pathway proceeds from the initial attachment and immobilization of bacteria to a solid surface followed by the formation of the cell cluster or micro-colony and ultimately by a three-dimensional architecture (Costerton *et al*., 1995). Bacterial biofilms have a great significance in medical, industrial and environmental settings, for examples; bacteria in biofilms are more resistance to adverse environmental stresses such as desiccation, extreme temperature, antimicrobial agents and chlorination (Costerton *et al*., 1995 and 1999; Solano *et al*., 2002; Scher *et al*., 2005). Several factors have been suggested to account for biofilm resistance, for examples, slow growth, the presence of an exopolysaccharide matrix that can slow the diffusion of chemicals or inactivate antimicrobial agents, and phenotypic changes in bacteria resulting in resistance occurring within the biofilm environment (Scher *et al*., 2005). The biofilm forming ability of bacteria promotes host colonization and is required for virulence as shown for several animal and plant pathogenic

bacteria (Verhoelf, 1997; Marques *et al*., 2002; Walker *et al*., 2004).

Recent molecular and genetic studies have identified several genes whose products are thought to be important for biofilm formation. For examples, biofilm formation is impaired by mutations in genes involved in flagellar or twitching-mediated motility, synthesis of exopolysaccharides, quorum sensing, outer membrane adhesins as well as global regulators of gene expression (Davies *et al*., 1993; Pratt and Kolter, 1998; O'Toole *et al*., 2000; Solano *et al*., 2002; Prouty and Gunn, 2003). However, no information is available about the formation of biofilm and its regulation in *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) strain 3937, which is a brute-force, necrotrophic phytopathogen that macerates the plant cell wall of a wide array of dicotyledons, causing soft-rot disease by secreting multiple virulence factors (Toth *et al*., 2003).

Signal transduction across biological membranes is central to the ability of cells to integrate and process environmental information. In bacteria, signaling across membranes is most often accomplished by use of so called two-component systems. The PhoP-PhoQ system is a classical two-component system, where PhoP is a response regulatory protein that is necessary to transcribe more than 40 genes and PhoQ is a sensor protein that possesses both autophosphorylation and phosphatase activities in response to magnesium and calcium in animal pathogen *Salmonella* (Miller and Mekalanos, 1990; Garcia-Vescovi *et al*., 1996). It was earlier reported that the homologue of PhoP-PhoQ in *D. dadantii* strain 3937 is required for virulence, hyperinduction of pectate lyase synthesis, resistance to antimicrobial peptide, accumulation of acetyl-coenzyme A, polygalacturonase production, and motility (Haque and Tsuyumu, 2005; Haque *et al*., 2005; Haque *et al*., 2006; Haque *et al*., 2008). In the present article, we show that the PhoP-PhoQ two-component

regulatory system is required for biofilm formation in *D. dadantii* strain 3937.

2. Materials and Methods
2.1. Bacterial strains and culture media
D. dadantii 3937 (wild type) and its derivative strains MH70 (km cassette inserted *phoP* mutant), and MH72 (km cassette inserted *phoQ* mutant) used in this study were described previously (Haque and Tsuyumu, 2005). The growth medium SOBG (salt-optimized broth plus glycerol) consisted of SOB plus 2% of glycerol (per liter, 20 g of tryptone, 5 g of yeast extract, 0.5 g of NaCl, 2.5 g of $MgSO_4$, 0.186 g of KCl, and 50 ml of 40% glycerol) was used to induce biofilm formation. To study the effect of magnesium on induction of biofilm formation, *D. dadantii* strain was grown at room temperature in SOBG medium supplemented with 0, 0.1, 1.0, 2.5 or 10 mM of $MgSO4.7H_20$. The strain was also grown in yeast extract-peptone (YP) medium (1% of peptone, 0.5% of yeast extract, pH 6.8), Luria-Bertani (LB) medium (1% of tryptone, 0.5% of yeast extract, 0.5% NaCl, pH 7.0), and M63 glycerol minimal medium (per liter, 2.5 g of NaCl, 3 g of KH_2PO_4, 7 g of K_2HPO_4, 2 g of $(NH_4)_2SO_4$, 0.5 mg of $FeSO_4$, 1 g of $MgSO_4$, 2 g of thiamine hydrochloride, and 0.2% (wt/vol) of glycerol. When required, antibiotics were added at the following final concentrations: nalidixic acid at 50 μg/ml, kanamycin at 50 μg/ml, and tetracycline at 12.5 μg/ml. The optical density of the bacterial culture was measured by Bactomonitor BACT-500 (Intertech, Tokyo, Japan) at 660 nm.

2.2. Biofilm formation assay
Biofilm formation was assayed by the ability of cells to adhere to the glass test tubes, as described previously (O'Toole and Kolter, 1998a) with few modifications. Early stationary growth phase cells were diluted (1:10) in fresh SOBG medium and incubated at 27ºC without shaking at least 72 h. Then the cultures were

removed from the test tubes and rinsed 2-3 times with sterile distilled water to remove loosely associated cells. A 1% of crystal violet (CV) solution (about 5.5 ml) was added to each glass test tube (dye stains the cells but not the glass surface) and kept at room temperature for 30 min then rinsed 2-3 times with sterile distilled water. At this time, the biofilm was visible as purple rings formed at the interface between the air and liquid medium. For quantitative analysis, about 2 ml of 95% ethanol was added to each CV-stained glass test tube then vortex vigorously to solubilize the cells. The biofilm production was measured at 600 nm by spectrophotometer (Spectronic, New York, USA).

2.3. Phase contrast microscopy

After staining of the attached bacterial cells with crystal violet (CV), the glass test tube was examined using a phase-contrast microscope (Olympus BX51, Tokyo, Japan). Images were captured using viewfinder (Pixera, version 3.0.1).

2.4. Quantification of exopolysaccharide

To estimate exopolysaccharide production, strains were grown in SOBG at 27ºC with shaking at 180 rpm until OD_{660} at 1.0. One ml of culture was centrifuged at 40,000 rpm for 10 min at 4ºC. The resultant supernatant was mixed with three volume of chilled 95% ethanol and centrifuged at 100,000 rpm for 20 min at 4ºC to precipitate the exopolysaccharide. The supernatant was discarded and after drying, the pellet was resuspended in distilled water. To quantify the purified exopolysaccharide, phenol-sulfuric acid method described by Hodge and Hofreiter (1962) was used.

2.5. Real-time quantitative-PCR

Wild type, *phoP* and *phoQ* mutants were grown in SOBG medium containing high concentrations of magnesium (10 mM) at early stationary phase (OD_{660} at 1.0), then total RNA was isolated using a QIAGEN RNeasy RNA isolation kit as described by the manufacturer (QIAGEN Hilden, Germany). RNA was quantified using a NanoDroP ND-100 spectrophotometer (NanoDroP Technologies, Wilmington, DE). Real-time PCR was performed in an MX3000p Multiplex quantitative PCR system (Stratagene) using the SYBR Premix EX.*Tag* RT-PCR kit (TaKaRa, Japan). The transcripts of *weaP*, *wza* and *wzc* were amplified using primers described in Table 1. The 16S rRNA gene was selected as control of experiment as it is constitutively expressed. The amount of RNA was expressed as the n-fold difference relative to the control gene ($2^{-\Delta CT}$, where ΔCT represents the difference in threshold cycle between the target and control genes). The final value is represented as the mean and standard deviation of three independent experiments.

2.6. Survival assays

After biofilm formation (about 72 h after incubation) by *D. dadantii* strain 3937, culture was removed from glass test tube, and then rinsed 2-3 times with sterile distilled water to remove loosely associated bacteria from surface of the glass test tube. To remove biofilm associated bacteria from surface of the glass test tube about 5.5 ml of sterile distilled water was added in each test tube then vortex. Cells were then centrifuged and resuspended in sterile distilled water to a final concentration of ca 10^7 colony forming unit (CFU)/ml. Logarithmic- and stationary- phase cultures of wild type was obtained by growing cells in YP to optical density of 0.6 and 1.5, respectively under shaking condition. Cells were then centrifuged and resuspended in sterile distilled water to a final concentration of ca 10^7 CFU/ml. To test susceptibility to acidic pH (4.0), cells were resuspended in 1 ml of sterile citrate-phosphate buffer, pH 4.0. Aliquots were taken at 1 h, serially diluted and plated. Testing for osmotic stress resistance was similarly carried out by incubating cells in the presence of 1 M NaCl. All experiments were carried out at least three times.

Table 1. List of primers used in this study

Name of primer	Primer sequence (5´------3´)	Product length
weaP-forward	GTTGCAACGTCAGGTGAA	275 bp
weaP-reverse	TAATGACGAGGGAACCGA	---------
wza-forward	GTCAGCAACCTATCTCCA	327 bp
wza-reverse	CCATCTGGTTCATCCCTT	---------
wzc-forward	GAAGCTGAGATCTCCAAG	227 bp
wzc-reverse	CTAGCCTTACTGATGCTC	---------
16S-rRNA-forward	AGAGGATGACCAGCCACACT	Haque *et al.*, 2005
16S-rRNA-reverse	AGGTGTAGCGGTGAAATGCG	Haque *et al.*, 2005

3. Results and Discussion

3.1. *Biofilm formation by D. dadantii strain 3937 in various growth conditions*

The ability of *D. dadantii* strain 3937 to form biofilm on different abiotic surfaces such as polyvinyl chloride (PVC), polystyrene and glass test tube were examined. Polyvinyl chloride and glass test tube surface were found suitable for biofilm formation (data not shown). We investigated biofilm formation in various growth media at 27ºC (Fig. 1). The biofilm was visualized as a purple ring of CV-stained cells that formed at the interface between the air and the medium of a static liquid culture. *D. dadantii* strain 3937 formed biofilms in various growth media to a different extent. After 72 h incubation, higher and thicker biofilms were built up in SOBG medium and it could be formed biofilms in M63 glycerol minimal medium comparable to those formed on SOBG. However, a few biofilms were detected on YP and LB medium. Nutritional status of the media play an important role in biofilm formation in several other bacteria, for examples, *Salmonella* spp. produce thicker and denser biofilms in nutrient-limited medium, while *Pseudomonas fluorescens* produces better biofilms in nutrient rich medium (Song and Leff, 2006).

Next, we examined the ability of different divalent cations such as Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+} and Ba^{2+} and monovalent cations such as Na^+ and K^+ on biofilm formation using 1 mM of their corresponding chloride salts. It was observed that Mg^{2+} greatly induced biofilm formation whereas Ca^{2+}, Mn^{2+} and Zn^{2+} enhanced biofilm formation only slightly but Cu^{2+}, Ba^{2+}, Na^+ or K^+ failed to promote biofilm formation (data not shown). Moreover, the influence of different concentration of Mg^{2+} (0, 0.1, 1.0, 2.5 and 10 mM) on biofilm formation was investigated in SOBG medium at pH 7.0 (Fig. 2). The maximal biofilm formation was observed in standard SOBG medium, where 2.5 mM Mg^{2+} was used. The minimum concentration of Mg^{2+} that showed significant and positive effect for biofilm formation was 0.1 mM. However, biofilm formation was decreased when 10 mM Mg^{2+} was used than that in the medium containing 2.5 mM Mg^{2+}. However, the media without Mg^{2+}, biofilm was not formed. Interestingly, when 2.5 mM Mg^{2+} was added to this culture, biofilm formation was recovered dramatically after 48 h incubation period (data not shown). It is worthy to note that biofilm formation was thoroughly inhibited when grown in the presence of 2.5 mM Mg^{2+} together with 0.5 M EDTA or more (data not shown). This result suggests that biofilm inhibition of *D. dadantii* 3937 by EDTA is due to chelating of Mg^{2+}. The growth rate of *D. dadantii* strain 3937 was indistinguishable with respect to the concentrations (0 to 10 mM) of Mg^{2+} (data not shown). Thus, lower level of biofilm formation by *D. dadantii* strain 3937 in YP or in LB medium may be, at least in part, due to lack of magnesium in these media.
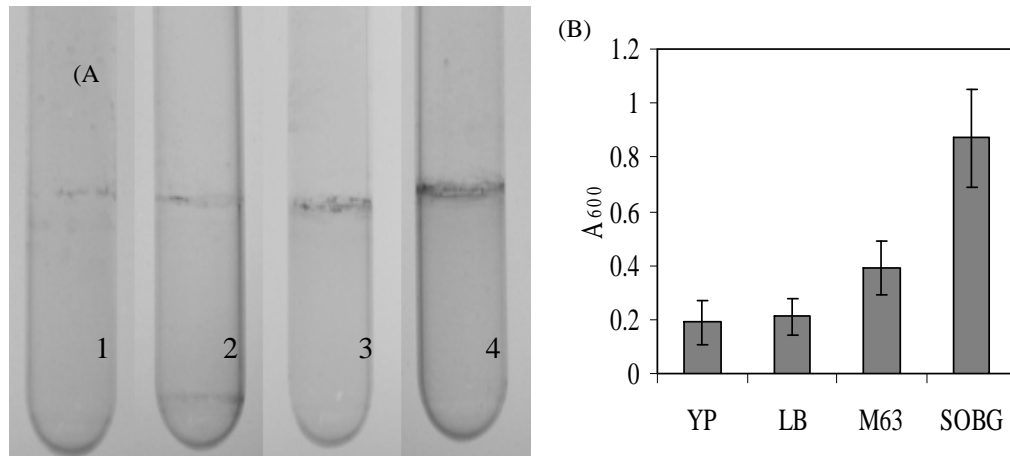
Fig. 1. Effect of different media on biofilm formation by *D. dadantii* strain 3937 in glass test tube (1 = YP, 2 = LB, 3 = M63 glycerol minimal medium and 4 = SOBG). The extent of biofilm formation after 72 h of incubation in the indicated media (A) and quantification of biofilms as the absorbance at 600 nm (B). Data are expressed as the mean of five independent experiments. Error bars indicate the standard deviation (±SD).
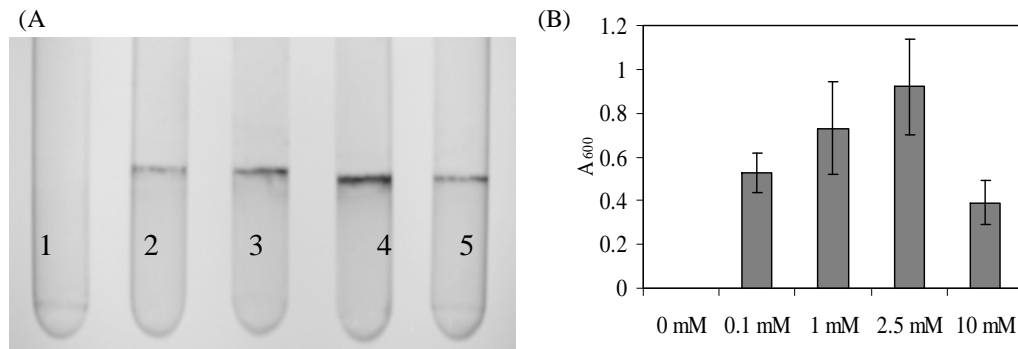

Fig. 2. Effect of different concentrations of magnesium (MgSO$_4$.7H$_2$O) on biofilm formation of *D. dadantii* strain 3937 in glass test tube (1 = 0 mM, 2 = 0.1 mM, 3 = 1.0 mM, 4 = 2.5 mM and 5 = 10 mM). Biofilms were stained with crystal violet 72 h after incubation (A). To quantify, biofilms are solubilized in 95% ethanol then optical density of the resultant solution was measured at 600 (B). Data are expressed as the mean of five independent experiments. Error bars indicate the standard deviation (±SD).

It has been demonstrated that divalent cations including magnesium and calcium influence biofilm formation directly through their effect on electrostatic interactions and indirectly via physiology-dependent attachment processes by acting as important cellular cations and enzyme cofactors (Fletcher, 1998). Moreover, electrostatic interactions contribute to biofilm cohesion, and cations are significant cross linkers of the biofilm matrix because they contribute to the integrity and stability of the outer membranes of the bacteria and properties of the lipopolysaccharides (Geesey *et al*., 2000). Furthermore, Costerton and associates (1995) have been reported that high concentration of magnesium might contribute to an increase in

exopolysaccharide production and biofilm stabilization. They also stated that magnesium increased the abundance of attached cells, possibly by reducing the repulsive force between the negatively charged bacterial and substratum surfaces and between negative groups on the polysaccharides, which constitute the structure of biofilms.

3.2 The PhoP-PhoQ two-component regulatory system repressed biofilm formation

Previously, we have shown that multiple virulence-associated traits are repressed by the PhoP-PhoQ two-component system of *D. dadantii* strain 3937 in response to high concentration of magnesium (Haque and Tsuyumu, 2005; Haque *et al*., 2005; Haque *et al*., 2006; Haque *et al*., 2008). In this study, both the mutants lacking functional *phoP* and *phoQ* were formed thicker and denser biofilms in SOBG medium containing high concentration of magnesium (10 mM) compare to that by wild-type strain in the surface of glass test tube (Fig. 3A). When biofilm formed on the surface of the glass test tube was examined under phase-contrast microscopy, both the mutants of *phoP* and *phoQ* formed large clusters of the cells closely adhered with each other covering almost the entire surface of the glass. In contrast, wild-type strain formed small clusters of cells as a scattered pattern leaving wide regions empty (Fig. 3B). However, no obvious difference was observed in the number of attached cells between the *phoP* and *phoQ* mutants. When formation of biofilm was quantified, wild-type strain showed about the one third of its absorbance as compare to that by the mutants of *phoP* and *phoQ* (Fig. 3C). Thus, PhoP-PhoQ two-component regulatory system seems to negatively regulate biofilm formation in *D. dadantii* strain 3937 in response to high concentration of magnesium. In *Salmonella*, high concentration of magnesium stimulated the PhoQ phosphatase activity that dephosphorylates phospho-PhoP. This dephophorylation then represses the transcription of PhoP-regulated genes and derepresses PhoP-repressed genes (Castelli *et al*., 2000). In this study, biofilm formation was repressed by the PhoP-PhoQ two-component system in response to high concentration of magnesium, suggesting that high concentration of magnesium might interact with the periplasmic domain of PhoQ shifting the balance toward a phosphatase-dominant state of the sensor protein. Under this condition PhoP would become dephosphorylated, and the expression of the PhoP-activated genes would be shut down. This/these gene(s) may lead to *D. dadantii* strain 3937 to repressed biofilm formation.

3.3. PhoP-PhoQ two-component system upregulates exopolysaccharide production and genes responsible for exopolysaccharide biosynthesis

It has been reported that exopolysaccharide play an important role in biofilm formation in different animal pathogenic enterobacteria such as *Salmonella typhimurium* and *Escherichia coli* (Ross *et al*., 1991; Solano *et al*., 2002; Danese *et al*., 2000). To examine, if *phoP* and *phoQ* has any effect on exopolysaccharide production, the mutants of *phoP* and *phoQ* as well as wild-type strain were grown in SOBG medium containing high concentration of magnesium until early stationary phase (OD_{660} at 1.0) then exopolysaccharide production was determined. Production of expolysaccharide by the mutants of *phoP* and *phoQ* was 0.93 ± 0.15 mg/ml and 1.12 ± 0.22 mg/ml, respectively whereas exopolysaccharide production by the wild-type strain was 0.61 ± 0.12 mg/ml. In order to identify gene(s) responsible for exopolysaccharide production that may be regulated by *phoP* and *phoQ*, the transcriptional profile of *D. dadantii* 3937 mutant strains lacking functional *phoP* and *phoQ* were compared with that of the wild-type strain in SOBG medium containing high concentration of magnesium by real time quantitative polymerase chain reaction (Fig. 4). The transcript levels of *weaP*, *wza* and *wzc* were upregulated in the *phoP* background than that of the wild type. Similar upregulation was also observed in *phoQ* mutant background (data not shown).
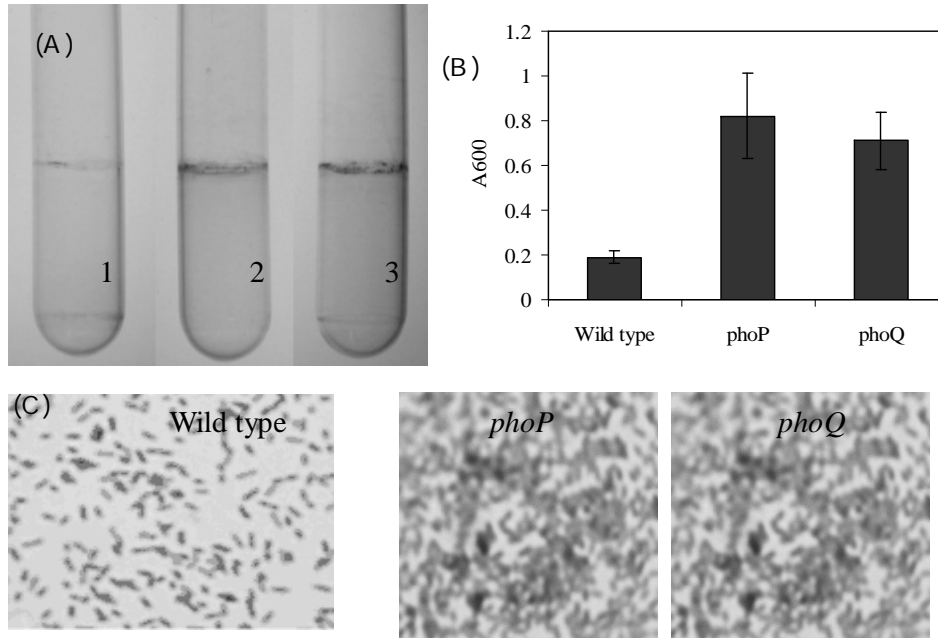
Fig. 3. Biofilms formed by different strains (1 = wild type, 2 = *phoP* mutant and 3 = *phoQ* mutant) in glass test tube (A), quantification (B) and visual observation of biofilms by phase contrast microscopy after 72 h incubation (C).

Motility has been shown to be involved in biofilm formation (O'Toole and Kolter, 1998b; Pratt and Kolter, 1998; Choy *et al*., 2004). In case of *P. fluorescens*, the flagellum itself was reported to play a direct role in adhesion (Lawrence *et al*., 1987). However, Hossain and Tsuyumu (2006) observed that not only the aflagellate mutant (Δ*fliC*) but also the paralyzed mutant (Δ*motA*) were equally reduced in their ability to form a biofilm, suggesting that functional flagella rather than nonfunctional flagella, are involved in biofilm formation. Moreover, mutation of gene involved in exopolysaccharide production has been shown to inability to form a biofilm (Danese *et al*., 2000; O'Toole *et al*., 2000). Recently, we reported that genes responsible for flagellum biosynthesis (*fliD, fliC and fliA*) are transcriptionally controlled by the PhoP-PhoQ two-component system in responding to magnesium (Haque *et al*., 2008). In this study, exopolysaccharide production and its genes (*weaP, wza* and *wzc)* are controlled by the PhoP-PhoQ two-component system (Fig. 4). These results, together with our previous study, suggest that the PhoP-PhoQ two-component system may be regulated formation of biofilm by transcriptional regulation of genes responsible for exopolysaccharide production in addition to genes responsible for bacterial motility of *D. dadantii* strain 3937.

3.4. Role of biofilm cells in virulence and survival in unfavorable environment

It has been reported that biofilm cells are resistant to various environmental stresses (Scher *et al*., 2005; Solano *et al*., 2002; Elkins *et al*., 1999). To determine whether biofilms cells of *D. dadantii* strain 3937 showed increased resistance to logarithmic- and stationary-phase growing cells, survival assays were carried out in acidic pH (4.0) and high osmotic stress because when *D. dadantii* strain 3937 infect a plant, it confronts an acidic pH in the intercellular apoplastic fluid.
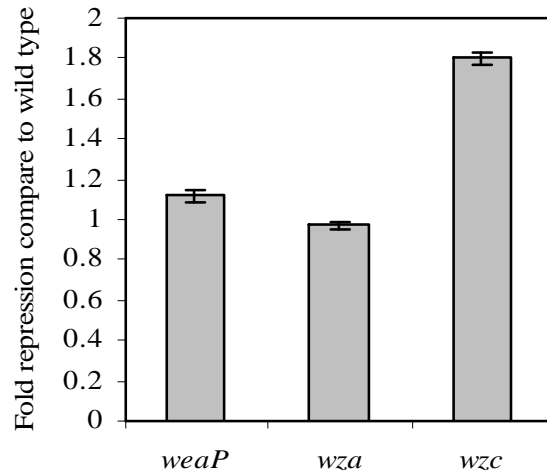
Fig. 4. Analyses of genes responsible for exopolysaccharide production (EPS) by real time quantitative polymerase chain reaction. Expressed as the ratio of the specific gene, expression level in the *phoP* mutant background compared to that in the wild type normalized to the level of expression of the 16S rRNA gene.
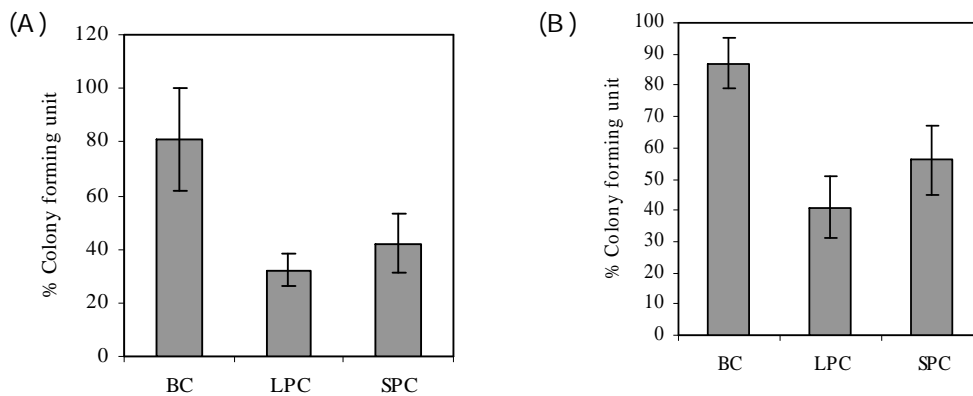

Fig. 5. Effect of acidic pH, 4.0 (A) and high osmolarity (1 M NaCl) on susceptibility to biofilm cells (BC), logarithmic phase cells (LPC) and stationary phase cells (SPC) of *D. dadantii* strain 3937. For susceptibility tests, the number of colony forming units (CFU) were compared with standard YP and setting at 100%. All experiments were carried out at least three times.

However, disruption of cells after maceration probably provokes an increase in the osmolarity of the infected plant tissue. It was observed that cells in the biofilm are more tolerant to acidic pH and high osmotic stress as compare to logarithmic- and stationary-phase cells (Fig. 5).

When virulence of biofilm cells compared with logarithmic- and stationary-phase growing cells, no difference was observed (data not shown). Thus, formation of biofilm may be an important factor for the survival of *D. dadantii* strain 3937 in natural environment.

4. Conclusions

PhoP-PhoQ two-component system repressed biofilm formation in the phytopathogen *D. dadantii* strain 3937 in response to high concentration of magnesium. Accordingly, exopolysaccharide production and genes responsible for exopolysaccharide production are markedly upregulated in both *phoP* and *phoQ* mutant backgrounds than the parental strain. These results, together with our previous study, suggest that the PhoP-PhoQ two-component system may regulate formation of biofilm by transcriptional control of genes responsible for exopolysaccharide production in addition to genes responsible for bacterial motility of *D. dadantii* strain 3937 in responding to magnesium. In addition, biofilm cells showed more tolerant to acidic pH and high osmolarity, thus, formation of biofilm at least may protect *D. dadantii* strain 3937 from various adverse environmental cues.

References

Castelli, M. E., Garcia-Vescovi, E. and Soncini, F. C. 2000. The phosphatase activity is the target for Mg^{2+} regulation of the sensor protein PhoQ in *Salmonella*. *Journal of Biological Chemistry*, 30: 22948-22954.

Choy, W. K., Zhou, L., Syn, C. K., Zhang, L. H. and Swarup, S. 2004. MorA defines a new class of regulators affecting flagellar development and biofilm formation in adverse *Pseudomonas* species. *Journal of Bacteriology*, 186: 7221-7228.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R. and Lappin-Scott, H. M. 1995. Microbial biofilms. *Annual Review of Microbiology*, 49: 711-745.

Costerton, J. W., Stewart, P. S. and Greenberg, E. P. 1999. Bacterial biofilms: a common cause of persistent infections. *Science*, 284: 1318-1322.

Danese, P. N., Pratt, L. A. and Kolter, R. 2000. Exopolysaccharide production is required for development of *Echerichia coli* K-12 biofilm architecture. *Journal of Bacteriology*, 182: 3593-3596.

Davies, D. G., Chakrabarty, A. M. and Geesey, G. G. 1993. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology*, 59: 1181-1186.

Elkins, J. G., Hassett, D. J., Stewart, P. S., Schweizer, H. P. and McDermott, T. R. 1999. Protective role of catalase in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide. *Applied and Environmental Microbiology*, 65: 4594-4600.

Fletcher, M. 1988. Attachment of *Pseudomonas fluorescens* to glass and influence of electrolytes on bacterium-substratum separation distance. *Journal of Bacteriology*, 170: 2027-2030.

Garcia-Vescovi, E., Soncini, F. C. and Groisman, E. A. 1996. Mg^{2+} as an extracellular signal: environmental regulation of *Salmonella* virulence. *Cell*, 84: 165-174.

Geesey, G. G., Wigglesworth-Cooksey, B. and Cooksey, K. E. 2000. Influence of calcium and other cations on surface adhesion of bacteria and diatoms: a review. *Biofouling*, 15: 195-205.

Haque, M. M., Yamazaki, A. and Tsuyumu, S. 2005. Virulence, accumulation of acetyl-coenzyme A, and pectate lyase synthesis are controlled by PhoP-PhoQ two-component regulatory system responding to organic acids of *Erwinia chrysanthemi* 3937. *Journal of General Plant Pathology*, 71: 133-138.

Haque, M. M. and Tsuyumu, S. 2005. Virulence, resistance to magainin II and expression of pectate lyase are controlled by the PhoP-PhoQ two-component regulatory system responding to pH and magnesium in *Erwinia chrysanthemi* 3937. *Journal of General Plant Pathology*, 71: 47-53.

Haque, M. M., Gomes, I. and Tsuyumu, S. 2006. Functional analysis of the PhoP-PhoQ two-component system in *Erwinia chrysanthemi*

3937. *Bangladesh Journal of Microbiology*, 23: 38-42.

Haque, M. M., Nahar, K., Rahim, M. A., Gomes, I. and Tsuyumu, S. 2008. PhoP-PhoQ two-component system required for colonization leading to virulence of *Dickeya dadantii 3937 in planta*. *Bangladesh Journal of Microbiology*, 25: 36-40.

Hodge, J. E. and Hofreiter, B. T. 1962. Determination of reducing sugars and carbohydrates. In: Whistler, R. L., and M. L. Wolfrom (ED). *Methods in Carbohydrate Chemistry*. Academic Press, New York. p. 388.

Hossain, M. M. and Tsuyumu, S. 2006. Flagella-mediated motility is required for biofilm formation by *Erwinia carotovora* subsp. *carotovora*. *Journal of General Plant Pathology*, 72: 34-39.

Lawrence, J. R, Delaquis, P. J., Korber, D. R. and Caldwell, S. E. 1987. Behavior of *Pseudomonas fluorescens* within the hydrodynamic boundary layers of surface microenvironments. *Microbial Ecology*, 14: 1-14.

Marques, L. L. R., Ceri, H., Manfio, G. P., Reid, D. M. and Olson, M. E. 2002. Characterization of biofilm formation by *Xylella fastidiosa* in vitro. *Plant Disease*, 86: 633-638.

Miller, S. I. and Mekalanos, J. J. 1990. Constitutive expression of the *phoP* regulon attenuates *Salmonella* virulence and survival within macrophages. *Journal of Bacteriology*, 172: 2485-2490.

O'Toole, G. A. and Kolter, R. 1998a. Initiation of biofilm formation in *Psudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis. *Molecular Microbiology*, 28: 449-461.

O'Toole, G. A. and Kolter, R. 1998b. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molecular Microbiology*, 30: 295-304.

O'Toole, G. A., Gibbs, K. A., Hager, P. W., Phibbs, P. V. Jr. and Kolter, R. 2000. The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by *Pseudomonas aeruginosa*. *Journal of Bacteriology*, 182: 425-431.

Pratt, L. A. and Kolter, R. 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Molecular Microbiology*. 30: 285-293.

Prouty, A. M. and Gunn, J. S. 2003. Comparative analysis of *Salmonella enterica* Serovar Typhimurium biofilm formation on gallstones and on glass. *Infection and Immunity*, 71: 7154-7158.

Ross, P., Mayer, R. and Benziman, M. 1991. Cellulose biosynthesis and function in bacteria. *Microbiology Review*, 55: 279-281.

Scher, K, Römling, U. and Yaron, S. 2005. Effect of heat, acidification, and chlorination on *Salmonella enterica* serovar Typhimurium cells in a biofilm formed at the air-liquid interface. *Applied and Environmental Microbiology*, 71: 1163-1168.

Solano, C., Garcia, B., Valle, J., Berasain, C., Ghigo, J. M., Gamazo, C. and Lasa, I. 2002. Genetic analysis of *Salmonella enteritidis* biofilm formation: critical role of cellulose. *Molecular Microbiology*, 43: 793-808.

Song, B. and Leff, L. G. 2006. Influence of magnesium ions on biofilm formation by *Pseudomonas fluorescens*. *Microbiological Research*, 161: 355-361.

Toth, I. K., Bell, K., Holeva, M. C. and Birch, P. R. J. 2003. Soft rot erwiniae: from genes to genomes. *Molecular Plant Pathology*, 4: 17-30.

Verhoef, J. 1997. Host defense against infection. In: Crossley K. B., G. L. Archer (ED). *The Staphylococci in Human Disease*. Churchill Livingstone, NY pp 213-232.

Walker, T. S., Bais, H. P., Deziel, E., Schweizer, H. P., Rahme, L. G., Fall, R. and Vivanco, J. M. 2004. *Pseudomonas aeruginosa*-plant root interactions: Pathogenicity, biofilm formation, and root exudation. *Plant Physiology*, 134: 320-331.