Original Article

PhoP-PhoQ Two-Component System Required for Colonization Leading to Virulence of *Dickeya dadantii* 3937 *in planta*

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Previously, we have reported that the PhoP-PhoQ two-component system (TCS) is essential for synthesis of pectate lyase, resistance to antimicrobial peptide and accumulation of acetyl-coenzyme A in *Dickeya dadantii* 3937. In this study, marker-exchange mutagenesis of *phoP* and *phoQ* of *D. dadantii* 3937 resulted in loss of virulence in Chinese cabbage and a severe reduction of bacterial population in infected area and its surrounding healthy tissue was also seen. Inoculation of bacteria grown and re-suspended in medium containing low concentration of Mg^{2+} resulted in reduced virulence and significantly reduced bacterial population in wild type while these mutants showed higher virulence and higher bacterial populations at high Mg^{2+} condition. Since Mg^{2+} concentration was detected to be very low (14.6 ± 4.23/100 g) in the apoplastic fluids of Chinese cabbage thus, the PhoP-PhoQ two-component system of *D. dadantii* 3937 is compromised virulence, at least in part, due to colonization of bacteria *in planta* responding to Mg^{2+}.

Keywords: Virulence, Bacterial population, Magnesium, *Dickeya dadantii*

Introduction

Dickeya dadantii (formerly *Erwinia chrysanthemi*, *Pectobacterium chrysanthemi*) is a necrotrophic, intracellular, plant pathogenic enterobacterium that induces soft rot diseases on a great variety of plants worldwide by secreting mainly multiple plant cell wall-degrading enzymes including pectinases, cellulases and proteases[1]. Additional factors that contribute to its pathogenicity are exopolysaccharides (EPS)[2], lypopolysantharides (LPS)[3], siderophore-mediated iron transport systems[4], Hrp system[5], resistance to antimicrobial peptides[6] and motility[7]. The precise roles of these virulence factors and regulation of their synthesis is often complex and responds to numerous environmental and specific signals.

Two-component system (TCS) is widespread signal transduction devices in bacteria that enable to elicit an adaptive response to environmental stimuli mainly, through changes in gene expression. Basically, TCS comprise a membrane-associated sensor histidine kinase and a cytoplasmic response regulator. Upon sensing specific signals, the histidine kinase autophosphorylates the conserved histidine residue of the kinase domain, and the phosphoryl group is subsequently transferred to the invariant aspartate residue of the cognate response regulator. Regulation is through modulation of the phosphorylation status of the response regulator protein, thereby controlling its affinity for DNA motifs present in the promoter region of target genes[8]. Currently more than 4,000 TCSs have been identified in 145 sequenced bacterial genomes[9]. Recently, *D. dadantii* strain 3937 genome sequence has been completed and predicted to contain genes that encode 30 TCSs (personal communication with Dr. JD Glasner, University of Wisconsin, USA). Among them, only PhoP-PhoQ (PhoP is a response regulatory protein and PhoQ is a sensor kinase) TCS is partially studied[6,10-11].

The PhoP-PhoQ TCS has been found in many Gram-negative bacteria[12], and its primary function seems to be control of physiological adaptation to Mg^{2+} availability[13]. This system has been extensively studied in *Salmonella enterica* serovar Typhimurium, in which it regulates expression of ≈3% of the genome, many of which are essential for growth in low Mg^{2+} conditions[14], survival in macrophages[15], epithelial cell invasion[16], pH[17], antimicrobial peptide resistance[18-19] and virulence[20]. Previously, we reported that the PhoP-PhoQ two-component system (TCS) is essential for virulence, pectate lyase synthesis, resistant to cationic antimicrobial peptide and accumulation of acetyl-coenzyme A[6,10]. In this article, we show that the PhoP-PhoQ TCS played an important role in virulence at least in part, due to colonization of *D. dadantii* 3937 *in planta*.

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Materials and Methods

Bacterial strains, plasmids, growth conditions and antibiotics

Dickeya dadantii 3937 (wild type) and its derivative strains MH70 (km cassette inserted *phoP* mutant), MH72 (km cassette inserted *phoQ* mutant), MP-1 (pPL85 transformed into *phoP* mutant) and MQ-1(pPL85 transformed into *phoQ* mutant) and the plasmid pPL85 (*phoP-phoQ* with its promoter region cloned into low copy broad host-range vector, pLAFR3) used in this study were described previously[6]. The strains were routinely grown in yeast extract-peptone (YP) medium (1% of peptone, 0.5% of yeast extract, pH 6.8) at 27ºC. M63 minimal medium (per litre) contains the following chemicals: 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 $FeSO_4.7H_2O$, 0.2% of glycerol, 10 mM or 10 mM of $MgSO_4.7H_2O$, and 2 g of thiamine hydrochloride. Milli-Q (Millipore SA 67120, Molsheim, France) water was used to prepare all kinds of media. When required, antibiotics were added at the following final concentrations: nalidixic acid at 50 mg/ml, kanamycin at 50 mg/ml, and tetracycline at 12.5 mg/ml. The optical density of the bacterial culture was measured by Bactomonitor BACT-500 (Intertech, Tokyo, Japan) at 660 nm.

Virulence and bacterial population in planta

Virulence tests on leaf of Chinese cabbage were carried out as follows: a wound of about 5 mm in length was made with a sterile scalpel and 10 ml of 1×10^4 cfu/ml bacterial suspensions in sterile milli-Q water grown in M63 glycerol minimal medium was placed on the wound. The inoculated plant piece was then placed on a moisten filter paper in a plastic container, wrapped and incubated at 27ºC. Chinese cabbage leaves were inoculated at five locations with *D. dadantii* 3937, *phoP, phoQ*, MP-1 and MQ-1 strains, respectively. Soft rot symptoms were observed periodically. Bacterial populations attained in Chinese cabbage were estimated as follows: bacteria were inoculated in Chinese cabbage was described above. One gram of macerated and non-macerated tissue (0-1 cm from the macerated Chinese cabbage) was collected after 20 h incubation, ground and homogenized in M63 minimal medium. Viable counts were determined by dilution plating on YP plates with appropriate antibiotics.

Electron microscopy and motility assay

Bacterial cells were negatively stained using 1% phosphotungstic acid pH 7.0 (TAAB, Berkshire, England) on collodion-coated 200-mesh grids (Nisshin EM, Tokyo). Samples were visualized using a transmission electron microscope (H-7500; Hitachi, Tokyo). Motility of the bacteria in liquid culture was examined under a phase-contrast microscope (Olympus B $\times$ 41). Two microlitre of suspensions (ca.10^7 cfu/ml) in milli-Q water from overnight culture grown on M63 minimal medium containing 10 mM or 10 mM Mg^{2+} were deposited in semisolid M63 minimal agar (0.3%) medium containing 10 mM or 10 mM Mg^{2+}. The plate was incubated at 27°C. The diameter of the bacterial growth halo generated 3 days after incubation by each strain was compared.

Determination of magnesium content from leaves of Chinese cabbage

Total Mg^{2+} content was determined as follows: 200 g of fresh leaves of Chinese cabbage was chopped, soaked in 200 ml of milli-Q water and autoclaved at 121°C for 5 min. The resulting aqueous layer was filtered through a double layer of cheesecloth then centrifuged at 10,000x g for 15 min. The resulting supernatant was collected and was re-centrifuged. The supernatant was collected and freeze at -80°C overnight then dry by freeze dryer (Eyela, Freeze dryer, FDU-830, Japan). Mg^{2+} content in apoplastic fluids of leaves of Chinese cabbage was determined by vacuum infiltration method previously described[21]. In brief, fresh leaves of Chinese cabbage were carefully washed with Milli-Q ice-cold water, air dried then weighed. Hundred grams of leaves of Chinese cabbage was soaked about 200 ml of Milli-Q water than infiltrated under vacuum pressure for 10 min. The sample was freeze at -80°C overnight and dry by freeze dryer (Eyela, Freeze dryer, FDU-830, Japan). Magnesium content (total and apoplastic) was measured after dry powder dissolved in Milli-Q water with a Polarized Zeeman, Atomic Absorption Spectophotometer, Hitachi Z-6000.

Isolation of RNA and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Strains were grown in M63 minimal medium containing 10 mM (low) or 10 mM (high) Mg^{2+} until early stationary phase (OD_{660} at 1.0) then total RNA was isolated using a QIAGEN RNeasy Plant Mini RNA isolation kit essentially as described by manufacturer (QIAGEN, Hilden, Germany). RNA was quantified using a NanoDroP ND-100 spectrophotometer (NanoDroP Technologies, Wilmington, DE). RT-PCR was performed using an mRNA-selective PCR kit (Ver. 1.1, Takara, Japan), essentially according to the manufacture's instructions. The primers used in the study listed in Table 1. One microgram of total RNA was used as the template in a total reaction volume of 50 ml. To estimate the total amount of RNA, 16S-rDNA was used as the standard. After adding one-fifth volume of loading buffer (0.25% of bromophenol blue, 0.25% of xylene cyanol, 30% glycerol, 5 mM EDTA-NaOH, pH 8.0) into each sample, 20 ml of each sample was applied on a 0.8% agarose gel.

Table 1. *List of reverse transcriptase-polymerase chain reaction RT-PCR primers used in this study*

Name of primer	Primer sequence (5´-3´)
flhD-forward	GGGTACCTCTGAATTGCTCAAGCAC
flhD-reverse	GCCCTTTTCTTAGGCAAGCCTTCT
fliA-forward	ACGCTTACAGGTTCGTCT
fliA-reverse	GAAGAATCTGGGATACTCC
fliC-forward	GACCGTACTGCAATCCAGC
fliC-reverse	CTGGAAGCGGTTCAGAGT
16S-rDNA-forward	AGAGGATGACCAGCCACACT[10]
16S-rDNA-reverse	AGGTGTAGCGGTGAAATGCG[10]

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Statistical analysis

Mean values and standard errors were calculated for each experiment. The difference between the strains assessed for statistical significance using a paired Student's *t*-test.

Results

Virulence of the phoP and phoQ mutants

The degree of maceration following inoculation of the mutant *phoP* or *phoQ* on Chinese cabbage (Figure 1) was drastically reduced compared to the wild type strain. Wild type macerated the tissue extensively, whereas the *phoP* mutant moderately, and *phoQ* mutant macerated it to lesser extent 20 h after inoculation suggesting that an alternate sensor kinase may activate PhoP and/or PhoQ may activate additional response regulators or PhoPQ may not be mutually exclusive. However, complementation of these mutants with pPL85 resulted in recovery of virulence to the wild type level (Figure 1). Thus, the PhoP-PhoQ TCS was confirmed to play an important role in the virulence of *D. dadantii* 3937.
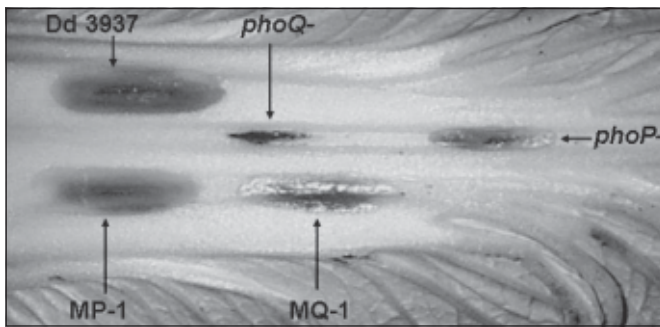

Figure 1. *Virulence test 20 h after inoculation with Dickeya dadantii (Dd) 3937, phoP, phoQ, MP-1 and MQ-1 strains. Bacterial suspensions (10 μl) adjust to OD$_{660}$ at 1.0 (1.0 x 10^4 cfu/ml) were inoculated onto the wounds made with a sterile scalpel on a leaf of Chinese cabbage and incubated in a moist chamber at 27°C. Five independent experiments showed similar results.*

PhoP-PhoQ TCS is required for colonization of bacteria in planta

The bacterial populations of the wild type, phoP and phoQ mutants in the same amount of macerated tissue of Chinese cabbage were indistinguishable (Table 2). Interestingly, when we estimated bacterial population in infected area and its surrounding (0-1 cm from infected area) healthy tissue of Chinese cabbage leaves, bacterial populations were detected to be significantly lower in both *phoP* and *phoQ* mutants with respect to the wild-type (Table 2) suggesting that motility may be considered to play an important role in spreading of bacteria in healthy plant tissue that results in larger macerated region.

Table 2. *Bacterial populations in macerated and its surrounding (non-macerated) tissue of Chinese cabbage*

Bacterial strain	CFU in 1 g of macerated tissue	Total CFU in whole macerated tissue	CFU in 1 g of non-macerated tissue
Wild type	213 ± 45 (x 10^7)	654 ± 76 (x 10^7)	179 ± 39 (x 10^6)
phoP mutant	237 ± 64 (x 10^7)	321 ± 54 (x 10^6)	94 ± 24 (x 10^6)
phoQ mutant	221 ± 42 (x 10^7)	265 ± 49 (x 10^6)	76 ± 31 (x 10^6)

PhoP-PhoQ TCS is required for bacterial motility

Under electron microscopic observation revealed that normal flagellation in the wild type, *phoP* and *phoQ*-mutants when bacteria were grown YP medium until OD$_{660}$ at 0.6 (Figure 2). In the present study, both the *phoP* and *phoQ* as well as wild-type strain were grown overnight in M63 minimal medium containing 10 μ*M* or 10 m*M* Mg^{2+} and the cultures were examined for motility under phase-contrast microscope. Interestingly, wild-type strain showed rapid swimming than those of *phoP* and *phoQ* responding to 10 μ*M* Mg^{2+} (data not shown). On the other hand, both the *phoP* and *phoQ* mutants exhibited rapid swimming than that of the wild-type strain responding to 10 m*M* Mg^{2+} (data not shown). The strains were further tested for motility on M63 minimal medium agar (0.3%) plates containing 10 μ*M* or 10 m*M* Mg^{2+}. The motility haloes formed by the *phoP* and *phoQ* mutants were significantly smaller than wild type in plates containing 10 μ*M* Mg^{2+} (Figure 3). On the contrary, after 72 h of inoculation, both the mutants were significantly migrated through the media compared to the wild-type strain (Figure 3). Wild type motility was restored when *phoP* and *phoQ* mutants were transformed with pPL85 plasmid (data not shown). It should be pointed out that none of the strains was impaired in growth in YP and M63 minimal medium supplemented with 10 μ*M* or 10 m*M* Mg^{2+} when grown in shake culture at 27°C demonstrated that increased motility is not due to increase ability to grow in liquid culture (data not shown). Thus, PhoP-PhoQ TCS appears to be regulated gene(s) involved in motility of *D. dadantii* 3937 responding to Mg^{2+} in plant host environment.

Wild type phoP mutant phoQ mutant

Figure 2. *Transmission electron microscopy of negatively stained wild type, phoP and phoQ mutant. Cells were grown to OD$_{660}$ at 0.6 at 27°C in YP medium.*
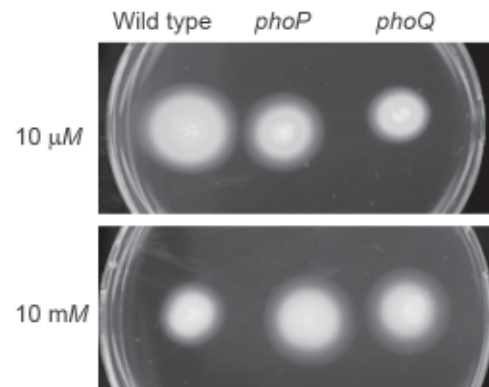

Figure 3. *Motility of wild type, phoP and phoQ strain after 72 h incubation at 27°C in semi-sold M63 minimal medium containing low (10 μM) and high (10 mM) concentrations of MgSO$_4$.7H$_2$O. A 2 μl of 1 x 10^5 cfu/ml bacteria were spotted onto plate.*

The PhoP-PhoQ controls the expression of genes required for flagellum synthesis

Curiously, we performed RT-PCR to determine whether increase motility might result, in part, from an increase in flagellar synthesis. For this experiment, total RNA was extracted from wild type, *phoP* and *phoQ* mutants, grown in M63 glycerol minimal medium supplemented with 10 μ*M* or 10 m*M* Mg^{2+} respectively (Figure 4). At low Mg^{2+} condition, synthesis of *flhD* (a master regulator primarily for expression of middle and some late genes) and *fliC* (a flagellar component called flagellin) transcripts were slightly elevated in wild type background. At high Mg^{2+} condition, *flhD, fliA,* and *fliC* were markedly upregulated in both *phoP* and *phoQ* backgrounds than the wild type background. Thus, *flhD*, *fliA*, and *fliC* expression might, therefore, be one of the factors responsible for the increase in motility.
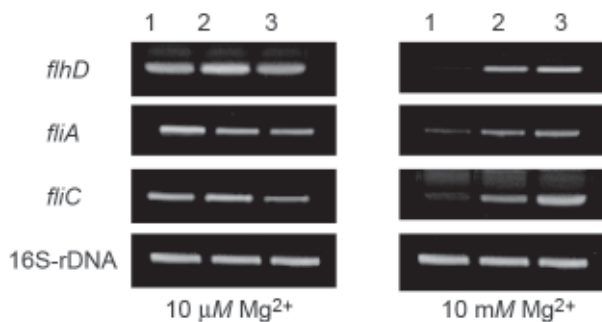

Figure 4. *Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of levels of expression of flagellum-related genes of wild type (Lane 1), phoP (Lane 2) and phoQ (Lane 3). Bacteria were grown in M63 minimal medium containing indicated levels of Mg^{2+} until early stationary phase (OD_{660} at 1.0) then total RNA was harvested and analyzed.*

Magnesium content in Chinese cabbage leaves

Apoplastic fluids and total magnesium in healthy Chinese cabbage leaves was analyzed by atomic absorption spectrophotometer with regard to the magnesium content. Magnesium content in apoplastic fluids was estimated to be $14.6 \pm 4.23/100$ g of leaves of Chinese cabbage. Thus, low concentration of magnesium in apoplastic fluids at least seems to be involved in spreading and multiplication of bacteria *in planta*. The total magnesium content was detected to be 34.3 ± 5.6 mg/100 g in Chinese cabbage leaves. Thus, when bacterial cells extensively macerated parenchymatous tissues of Chinese cabbage leaves, high levels of Mg^{2+} may be become available due to cell lysis, which may be repressed bacterial populations in wild type thereby reduced virulence.

Effect of magnesium concentrations on tissue maceration and bacterial population

Following inoculation of bacteria grown in M63 minimal medium supplemented with a low (10 μ*M*) concentration of Mg^{2+}, the degree of maceration and number of bacteria by the wild type *D. dadantii* 3937 strain was significantly higher than that of the mutants (Figure 5A and 5B). The degree of maceration and number of bacteria with wild type was dramatically reduced compared to that with the mutant strains (Figure 5A and 5B). Thus, virulence appears to be correlated with the number of bacterial population *in planta* responding to Mg^{2+}.
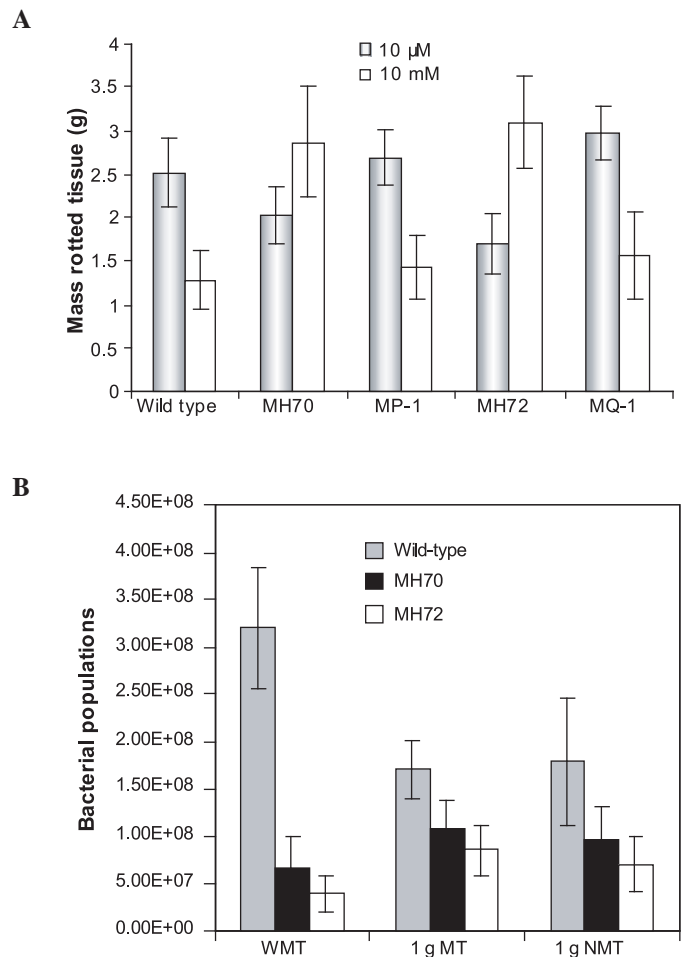

Figure 5. *Effect of Mg^{2+} on maceration of Chinese cabbage (A) and bacterial populations (B). Maceration and bacterial populations were determined 20 h after inoculation. WMT = Whole macerate tissue; 1 g MT = 1 g Macerated tissue (1 g MT); 1 g NMT = 1 g Non-macerated tissue; Wild type = Dickeya dadantii 3937 (wild type); MH70 = Strain with km cassette inserted phoP mutant; MH72 = Strain with km cassette inserted phoQ mutant.*

Discussion

In this article, we show that PhoP-PhoQ two-component system (TCS) of *Dickeya dadantii* 3937 contributes to virulence due to reduced bacterial population and migration of bacteria *in planta* (Figure 1 and Table 2). Under low Mg^{2+} condition, wild type confirmed extensively migrated in semi-solid agar medium, however, at high Mg^{2+} condition, both the mutants, repressed this phenotype (Figure 3). It showed be pointed out that *D. dadantii* 3937 resides in the intracellular fluids and so far is not known to enter plant cells. As we found that the concentration of

Mg^{2+} in apoplastic fluids of Chinese cabbage leaves is very low, thus, low concentrations of Mg^{2+} might be induced the PhoP-PhoQ system leading to virulence.

High bacterial motility at low Mg^{2+} and low motility at high Mg^{2+} (Figure 3) suggest inverse regulation between bacterial motility and PhoP-PhoQ system in *D. dadantii* 3937. These results also demonstrate that bacteria might display motility when *phoPQ* genes are induced, which is likely to be the case before plant infection where Mg^{2+} concentration might be low and switch off the motility when *phoPQ* genes are repressed (at high Mg^{2+}), which is the case in the plant after infection. However, specific *in planta* assays would be needed for further demonstration of this hypothesis.

We observed that the bacterial populations in non-macerated tissue of Chinese cabbage leaves were significantly higher in the wild type than those of the mutants (Table 2). Thus, bacterial motility may be considered to play an important role in migration of bacteria in healthy plant tissue leading to virulence of *D. dadantii* 3937 which is controlled by the PhoP-PhoQ TCS in response to low Mg^{2+} in plant apoplastic fluids. Other plant pathogens such as *Erwinia carotovorum* subsp. *carotovorum*[22-23], *E. carotovorum* subsp. *atrosepticum*[24] and *Ralstonia solanacearum*[25] also need motility to invade host plants efficiently. Tans-Kersten *et al.*[25] showed that swimming motility of *R. solanacearum* is regulated by FlhDC and the plant host environment. It has been shown that the *fliC* and *fliD* of *E. carotovorum* subsp. *carotovorum* are essential for motility and virulence[22-23]. Accordingly, *fliD*, *fliA* and *fliC* are controlled by the PhoP-PhoQ TCS responding to Mg^{2+} (Figure 4). Motility and virulence have often been associated in animal pathogenic bacteria. In *Salmonella*, motility and flagellin expression are coregulated by low pH and are dependent on activity of the *phoPQ* pathway, which directly or indirectly negatively regulates transcription of the flagellin gene *fliC*[26]. Thus, migration of bacteria and bacterial populations *in planta* are played an important role in virulence of *D. dadantii* 3937.

Acknowledgement

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