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SHORT COMMUNICATION

The *Erwinia amylovora* PhoPQ system is involved in resistance to antimicrobial peptide and suppresses gene expression of two novel type III secretion systems

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Summary

The PhoPQ system is a pleiotropic two-component signal transduction system that controls many pathogenic properties in several mammalian and plant pathogens. Three different cues have been demonstrated to activate the PhoPQ system including a mild acidic pH, antimicrobial peptides, and low Mg^{2+}. In this study, our results showed that *phoPQ* mutants were more resistant to strong acidic conditions (pH 4.5 or 5) than that of the wild-type (WT) strain, suggesting that this system in *Erwinia amylovora* may negatively regulate acid resistance gene expression. Furthermore, the PhoPQ system negatively regulated gene expression of two novel type III secretion systems in *E. amylovora*. These results are in contrast to those reported for the PhoPQ system in *Salmonella* and *Xanthomonas*, where it positively regulates type III secretion system and acid resistance. In addition, survival of *phoPQ* mutants was about 10-fold lower than that of WT when treated with cecropin A at pH 5.5, suggesting that the PhoPQ system renders the pathogen more resistant to cecropin A.

Introduction

The two-component signal transduction system is the most prevalent signal transduction mechanism in prokaryotes mediating bacterial responses to environmental stimuli (Hoch 2000; Hoch and Silhavy 1995). One such sensing system is the PhoPQ system, which controls various virulence mechanisms including resistance to antimicrobial peptides (AMPs) (Bader et al. 2005; Fields et al. 1989; Gunn and Miller 1996; Miller et al. 1990; Soncini et al. 1996). The PhoPQ system constitutes the first regulatory system that uses extra-cellular Mg^{2+} as a primary signal (Garcia Vescovi et al. 1996).

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Other signals sensed by PhoQ include mild acidic pH and AMPs (Bader et al. 2005; Brodsky and Gunn 2005; Garcia Vescovi et al. 1996; Prost et al. 2007).

The PhoPQ system is the master regulator of virulence genes in *Salmonella* and other Gram-negative bacteria (Groisman, 2001). The PhoPQ is repressed *in vitro* containing 5 mM or greater Mg^{2+} and activated in low micromolar concentrations of Mg^{2+} (Garcia Vescovi et al. 1996; Gunn and Richards 2007). On the other hand, *Salmonella* survives host phagocytosis to promote disease during systemic infection. Within the phagosome, the PhoPQ system promotes bacterial resistance to a variety of antimicrobial factors including AMPs and acidic pH. It has been reported that, inside the *Salmonella*-containing vacuoles, the Mg^{2+} concentration is estimated at 1 mM, a repressing concentration (Prost et al. 2007). Under this condition, the PhoPQ system can be activated by sublethal concentrations of host AMPs (Bader et al. 2005). It has recently been demonstrated by Prost et al. (2007) that the PhoPQ system is maximally activated by pH 5.5. This activation is independent of the Mg^{2+} concentration, but is additive with AMPs. Moreover, it has been reported that *Escherichia coli* can withstand an acidic pH of 2.5 or below if the cell is in a stationary phase (Castanie-Cornet et al. 1999).

Plant apoplast, a major barrier for plant pathogenic bacteria to cause disease, is also acidic. Generally, the pH of a plant apoplast ranges between 4.5 and 6.5 (Grignon and Sentenac 1991) The PhoPQ of *Dickeya dadanti* (formerly *Erwinia chrysanthemi*) strain 3937, a plant enterobacterium that causes soft rot disease on a wide range of plant hosts, has been shown to be involved in virulence and resistance to AMPs, and in regulating expression of pectate lyase in response to both pH and Mg^{2+} (Haque and Tsuyumu 2005; Llama-Palacios et al. 2003, 2005). The PhoPQ system in *Xanthomonas oryzae pv. oryzae*, an important pathogen causing rice blight disease, promotes type III secretion (T3SS) gene expression and is required for virulence (Lee et al. 2008). However, the role of PhoPQ system in *Erwinia amylovora*, an enterobacterium causing fire blight disease of apples and pears, is still unknown.

In a previous study, we have generated *phoPQ* mutants of *E. amylovora* and demonstrated that mutations in *phoPQ* did not result in reduction of virulence and amylovoran biosynthesis (Zhao et al. 2009b). In this study, we further characterized *phoPQ* mutants in response to different stress conditions including acidic pH and AMPs. Our results showed that while the *phoPQ* mutants were more sensitive to AMPs, the *phoPQ* mutants were more resistant to strong acidic conditions (pH 4.5 or 5) than that of wild-type (WT) strain. We also found that the PhoPQ system negatively regulate two novel T3SS gene expression, though the role of these type III secretion systems is still not clear (Zhao et al. 2009a). These results are in contrast to the results reported for the PhoPQ system in *Salmonella* and *Xanthomonas*, where it promotes type III secretion system gene expression and positively regulates acid resistance.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) medium was routinely used for culturing *E. amylovora*. When necessary, the following antibiotics were added to the medium: kanamycin 20 $\mu g\ ml^{-1}$ and ampicillin 100 $\mu g\ ml^{-1}$. For most circumstances, bacteria were grown at 28 °C in LB medium or modified basal medium A (MBMA) (3 g KH_2PO_4, 7 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 2 ml glycerol, 0.5 g citric acid in one liter) and supplemented with different concentrations of $MgSO_4$ (10 or 250 μM or 10 mM) (Torriani 1960). Citric acid monohydrate was added instead of sodium citrate to buffer in the low pH ranges. The optical density of bacterial culture was measured with a spectrophotometer at 600 nm.

DNA manipulation and sequence analysis

Plasmid DNA purification, PCR amplification of genes, isolation of fragments from agarose gels, cloning and restriction enzyme digestion, and T4 DNA ligation were performed using standard molecular procedures (Sambrook and Russell 2001). DNA sequencing was performed at the Keck Center for Functional and Comparative Genomics at University of Illinois at Urbana-Champaign. Sequence management and contigs assembly were conducted using Sequencher 4.9 software. Database searches were conducted using the BLAST programs at NCBI (www.ncbi.nlm.nih.gov/BLAST) (Altschul et al. 1997).

Complementation of *phoPQ* mutants

To complement the *phoPQ* mutants, primer pairs PQ1–PQ2 were used to amplify 2.898 kb fragment from *E. amylovora* WT strain containing the flanking sequences of the *phoPQ* operon with its own promoter sequence. The amplified PCR product was cloned into the pGEM T-easy vector through A-T ligation. The ligated product was transformed into

Table 1. Bacterial strains, plasmids, and primers used in this study.

Strains, plasmids or primers	Relevant characters or sequences (5′–3′)[a]	Reference or source
E. amylovora		
Ea1189	Wild-type, isolated from apple	Burse et al. 2004
Z2324Δ*phoP*	*phoP::Kan;* Kan^r-insertional mutant of *phoP* of Ea1189, Kan^r	Zhao et al. 2009b
Z2323Δ*phoQ*	*phoQ::Kan;* Kan^r-insertional mutant of *phoQ* of Ea1189, Kan^r	Zhao et al. 2009b
Z2324-23Δ*phoPQ*	*phoPQ::Kan;* Kan^r-insertional mutant of *phoPQ* of Ea1189, Kan^r	Zhao et al. 2009b
E. coli		
DH10B	F$^-$*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*Δ139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ - *rps*L (Str^R) *nup*G	Invitrogen
Plasmids		
pGEM® T-easy	Ap^r, PCR cloning vector	Promega
pFPV25	Ap^R, GFP based promoter trap vector containing a promoter-less *gfpmut3a* gene	Valdivia and Falkow 1997
pSN2	A 2.898 kb PCR fragment containing *PhoPQ* operon in pGEM T-easy vector	This study
pYsaE3	774 bp EcoRI-BamHI DNA fragment containing promoter sequence of *ysaE2* gene in pFPV25	This study
pYsaF3	774 bp EcoRI-BamHI DNA fragment containing promoter sequence of *ysaF3* gene in pFPV25	This study
pYsaE2	618 bp EcoRI-BamHI DNA fragment containing promoter sequence of *ysaE1* gene in pFPV25	This study
pYsaF2	618 bp EcoRI-BamHI DNA fragment containing promoter sequence of *ysaF2* gene in pFPV25	This study
Primers[a]		
PQ1	AAACTCTGAAGGGAATCTTGGTC	
PQ2	GATATTCCATGATGGTTTCCTGA	
ysaE3F	CCGGAATTCCCAAACATTGGACCAAACAGTAT (*Eco*RI)	
ysaE3R	CGCGGATCCGCGATCAGTACGGATTGTTTTAG (*Bam*HI)	
ysaF3F	CCGGAATTCGCGATCAGTACGGATTGTTTTAG (*Eco*RI)	
ysaF3R	CGCGGATCCCCAAACATTGGACCAAACAGTAT (*Bam*HI)	
ysaE2F	CCGGAATTCCATATGGTTGCATCGTCCTG (*Eco*RI)	
ysaE2R	CGCGGATCCCCCATAGCGCCTCATATGTT (*Bam*HI)	
ysaF2F	CCGGAATTCCCCATAGCGCCTCATATGTT (*Eco*RI)	
ysaF2R	CGCGGATCCCATATGGTTGCATCGTCCTG (*Bam*HI)	

Underlined nucleotides are restriction sites added and the restriction enzymes are indicated at the end of primer.
[a]Kan^r, Ap^r=kanamycin and ampicillin resistance, respectively.

E. coli cells. The final plasmid containing the *phoPQ* operon was designated as pSN2 and was then introduced into *phoPQ* mutants by electroporation. Transformants were selected on LB plates with Ap and Km. The genotype of the plasmid was confirmed by enzymatic digestion and sequencing.

Construction of promoter-GFP fusions for gene expression assays

For gene expression assays, flanking sequences of the *ysaE3*/*ysaF3* and *ysaE2*/*ysaF2* ORFs were used to design primers to amplify DNA fragments (Zhao et al. 2009a). The primer pairs ysaE3F–ysaE3R (ysaF3F–ysaF3R) and ysaE2F–ysaE2R (ysaF2F–ysaF2R) with restriction sites were used to amplify 774 and 618 bp DNA fragments from *E. amylovora* WT strain, containing promoter sequences of *ysaE3*/*ysaF3* and *ysaE2*/*ysaFH2* genes, respectively. PCR products and the promoter trapping vector pFPV25 were both digested with *BamH*I and *EcoR*I. The resulting fragments were gel-purified, ligated together, and cloned upstream of the promoter-less *gfp* gene. The final plasmids were designated as pYsaE3, pYsaF3, pYsaE2, and pYsaF2 for the four genes, and were

confirmed by restriction enzyme digestion and sequencing.

GFP reporter gene assay by flow cytometry

The BD FACSCanto flow cytometer was used to monitor the GFP intensity of WT and mutant strains containing the corresponding promoter-GFP construct (Wang et al. 2009; Zhao et al. 2009b). Strains containing the promoter-GFP fusion plasmids were grown in LB overnight, harvested, and resuspended in water. Bacterial suspensions were re-inoculated in MBMA broth or LB broth and grown at 28 °C with shaking for 24 h. Bacterial cultures were then harvested by centrifugation, washed once with PBS, and then resuspended in PBS for flow cytometry assay. Flow cytometry was performed using a BD LSRII 10 parameter multilaser analyzer (BD Bioscience, San Jose, CA).

Data were collected for a total of 100,000 events and statistically analyzed by gating using flow cytometry software FCS Express V3 (De Novo Software, Los Angeles, CA). A geometric mean was calculated for each sample. Each treatment was performed in triplicate and each experiment was repeated three times.

Antimicrobial peptide killing assay

To test *E. amylovora* WT, *phoPQ* mutants and complementation strains for AMP resistance, strains were grown to mid-log phase in MBMA with 10 μM Mg^{2+} at pH 5.5 or 7.0 and collected by centrifugation. The pellets were resuspended in 200 μl PBS and serial 10-fold dilutions of bacterial suspensions were made on microtiter plates. Equal volumes of cecropin A dissolved in respective media were added to 100 μl bacterial suspensions with final concentration of 0.25 μg ml^{-1}. The bacterial suspensions were incubated at room temperature for 1 h. After the treatment, survived cells were counted by plating on LB media with or without antibiotics. Percentage survival was calculated by dividing AMP treated versus untreated. The experiments were repeated at least three times.

Results

The PhoPQ system renders *E. amylovora* more susceptible to acidic pH

In order to dissect how *E. amylovora* PhoPQ system responds to acidic pH, we first determined bacterial growth of *E. amylovora* WT strain under different pH and Mg^{2+} concentrations. It has been reported that bacterial cells grown in micromolar concentrations of Mg^{2+} results in significant growth inhibition (Garcia Vescovi et al. 1996; Groisman 2001). Indeed, bacterial growth of *E. amylovora* WT strain was normal when the magnesium concentration was 250 μM or above at pH 7 (Figure 1A). However, growth of *E. amylovora* WT strain was significantly reduced after 24 h if the Mg^{2+} concentration was less than 250 μM (Figure 1A). Next, Mg^{2+} concentration of 250 μM was selected to test how pH affects bacterial growth. As shown in Figure 1B, *E. amylovora* WT strain showed normal growth at pH 5.5 and above, moderate growth at pH 5.0, slight growth at pH 4.5 and no growth at all at pH 4 in MBMA broth with 250 μM Mg^{2+} concentration. These results suggest that both pH and Mg^{2+} concentrations affect the growth of *E. amylovora* WT *in vitro*. Interestingly, both pH 5.5

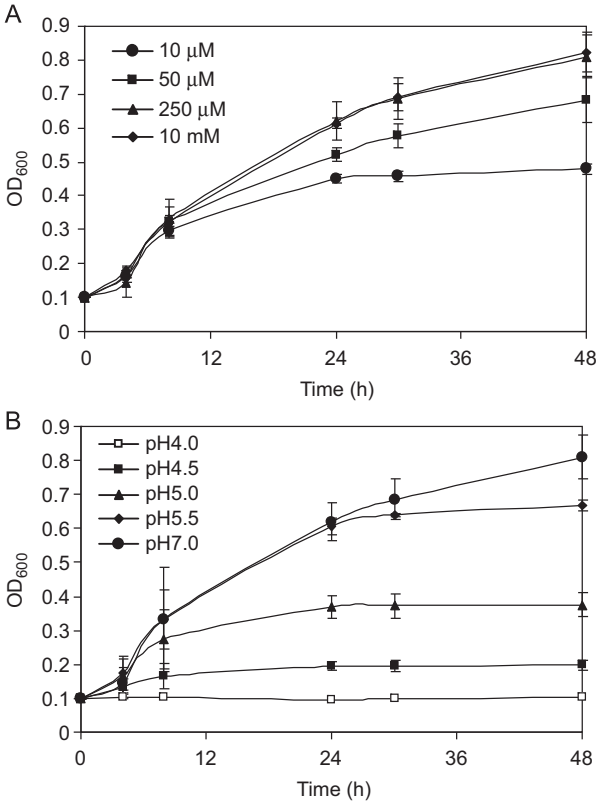

Figure 1. Effects of pH and Mg^{2+} concentration on bacterial growth. (A) Bacterial growth of *E. amylovora* WT strain in MBMA at pH 7.0 with different Mg^{2+} concentrations. (B) Bacterial growth of *E. amylovora* WT strain in MBMA with 250 μM Mg^{2+} concentration at different pH. The growth of bacteria was measured at 0, 4, 8, 24, 30 and 48 h post inoculation with initial concentration adjusted to OD_{600}=0.1. Data points represent the means of three replicates ± standard deviations.

and 250 μM Mg^{2+} concentrations are signals for the PhoPQ system.

Based on these results, we tested how different pH affects the growth of *phoPQ* mutants at 250 μM Mg^{2+} concentration. Bacterial growth for *phoP*, *phoQ*, and *phoPQ* mutants was similar to that of WT at pH 5.5 and 7.0 (Figure 2C and D). However, bacterial growth of *phoPQ* mutants at pH 4.5 and 5 was significantly higher than that of WT (Figure 2A and B). The complemented strains of *phoPQ* mutants restored the phenotype of the WT strain and had similar growth at pH 4.5 or 5 (data not shown). We also counted the bacterial number for WT and *phoPQ* mutants by dilution plating at pH 4.5. At 24 h, bacterial number for *phoPQ* mutants was from 1.9 to 3.4×10^8 CFU/ml; whereas the number for the WT strain was around 1×10^7 CFU/ml, suggesting that bacterial cells for the mutants were more than 10-fold higher than that of the WT. These results indicate that mutation in *phoPQ* renders the pathogen more resistance to strong acidic (pH 4.5 or 5) conditions.

The PhoPQ system suppresses gene expression of two novel type III secretion systems

Two novel T3SS pathogenicity island (PAI: PAI2 and PAI3) have been recently identified in *E. amylovora* (Bocsanczy et al. 2008; Zhao et al. 2009a). *In silico* analysis of the promoters in PAI2 and PAI3 indicated that genes encoded in both PAI2 and PAI3 are probably transcribed in three operons (namely Ysa, Prg, and Ysp) under the control of *ysaE* (*invF*), *ysaF* (*prgH*), and *sycB* promoters, respectively (Figure 3A; Zhao et al. 2009a). Previous studies have demonstrated that the AraC-like transcription regulator YsaE (InvF) in *Yersinia* Ysa (*Salmonella* SPI-1) plays a major role in regulating expression of genes encoding secreted proteins such as YspBCD (Aguirre et al. 2006; Walker and Miller 2004). It is interesting to note that the PhoPQ system is a master regulator of virulence genes in *Salmonella* including YsaE (InvF) and the needle complex membrane protein YsaF (PrgH). *In silico*

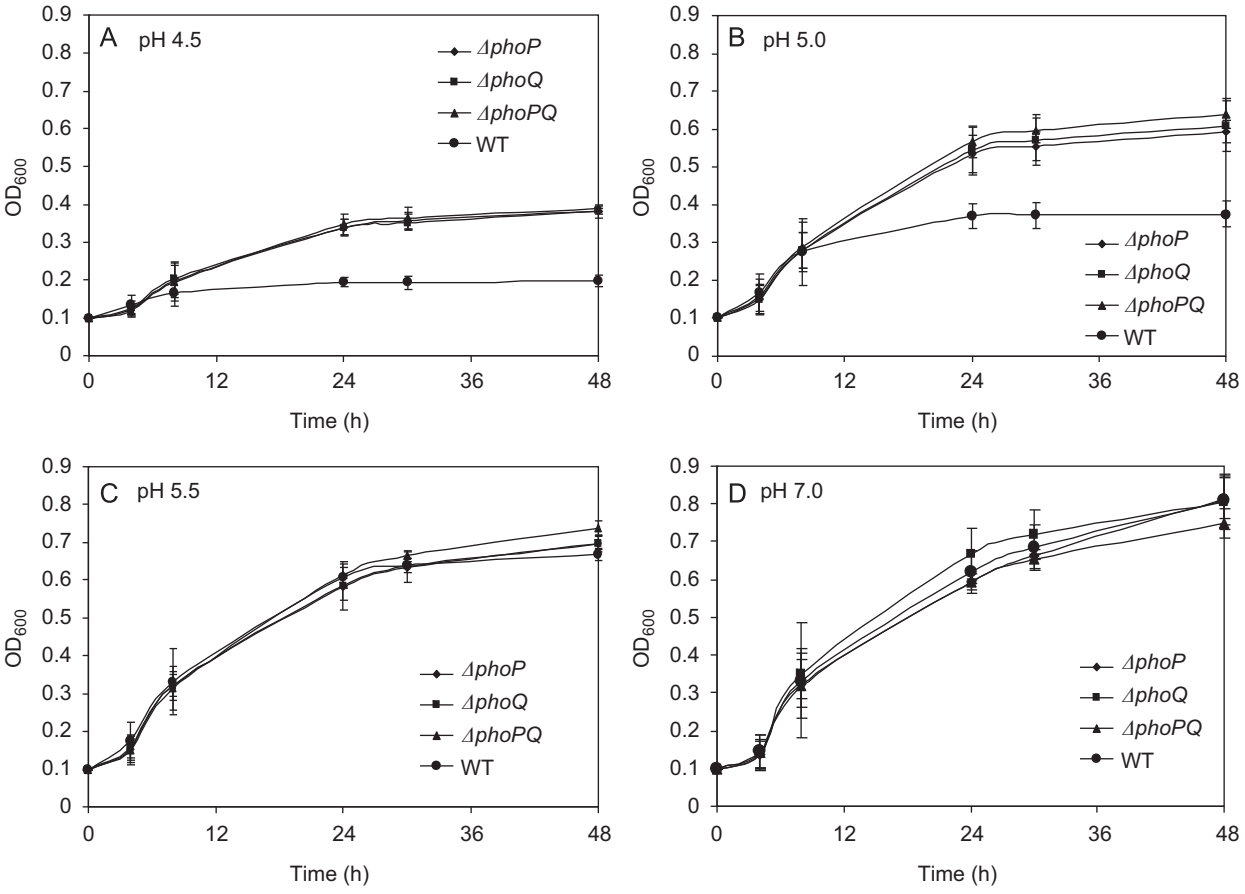

Figure 2. Effect of pH on bacterial growth of *phoPQ* mutants. The growth of the bacteria was measured at 0, 4, 8, 24, 30 and 48 h post inoculation with initial concentration adjusted to OD_{600}=0.1. Data points represent the means of three replicates ± standard deviation. (A) pH 4.5; (B) pH 5.0; (C) pH 5.5 and (D) pH 7.0.

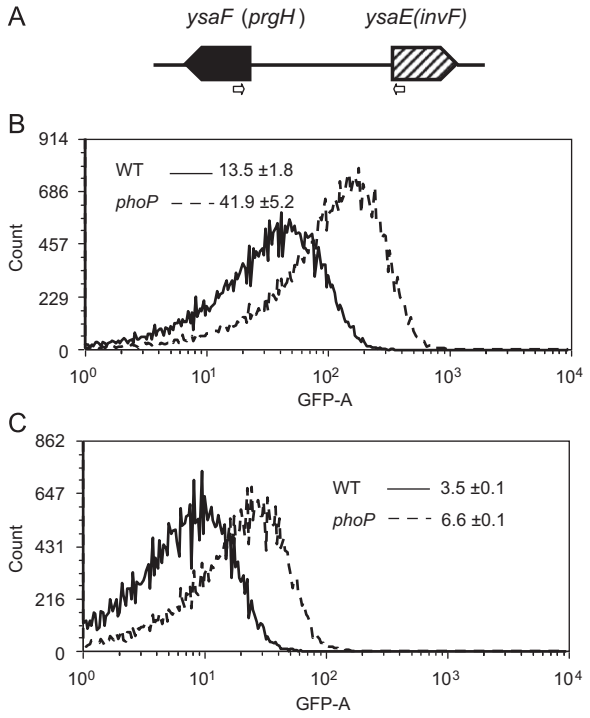

Figure 3. Gene expression of the *ysaE3 and ysaF3* genes in WT and *phoP* mutant. (A) Schematic map for the *ysaE* and *ysaF* gene organization in PAI2 and PAI3 of *E. amylovora* (Bocsanczy et al. 2008; Zhao et al. 2009a). Empty arrows indicate the positions for promoter cloning. (B) *ysaE3*, (C) *ysaF3*; GFP intensity in WT strain and *phoP* mutant containing the promoter-GFP fusion plasmids was measured by flow cytometry. The experiment was repeated at least three times and similar results were obtained. Numbers represent GFP intensity means of three replicates ± standard deviations. Count: number of bacterial cells; GFP-A: green fluorescence protein absorbance.

analysis of the promoter sequence of *ysaE* and *ysaF* in both PAI2 and PAI3 revealed that the promoters of *ysaE* and *ysaF* contain similar sequences as that of the so-called PhoP-box ((T/G)GTTT(A/T)), indicating that *ysaE* and *ysaF* may also be regulated by the PhoPQ system in *E. amylovora* (Figure 3A).

Using a GFP reporter system, we found that both *ysaF3* and *ysaE3* genes were expressed about two fold higher in *phoP* mutant background than those of WT strain in rich medium at 28 °C (Figure 3B and C). Similarly, the GFP intensity (geometric mean) for *ysaE2* and *ysaF2* promoters in *PhoP* mutant was 4.7 ± 0.36 and 3.0 ± 0.37; whereas the GFP intensity (geometric mean) in WT strain was 3.2 ± 0.17 and 1.9 ± 0.16, respectively (the GFP value of vector control is about 1.50). GFP expression of the four genes was not detectable in minimal medium or at low temperature (20 °C) (data not shown). These results indicate that the PhoPQ system is a repressor for both PAI3 and PAI2 genes. These results also indicate that the *ysaE3* had the highest expression level among these four genes; and the expression level of PAI3 genes (*ysaE3*/*ysaF3*) was higher than those of PAI2 genes (*ysaE2*/*ysaF2*) in both WT and *phoP* mutant background. These results suggest that PAI3 and PAI2 have differential gene expression patterns *in vitro*.

The PhoPQ system confers *E. amylovora* more resistance to cecropin A

The PhoPQ system has been shown to regulate resistance to AMPs (Garcia Vescovi et al. 1996; Guo et al. 1998; Miller et al. 1990). To determine whether the PhoPQ system in *E. amylovora* is also responsible for AMP resistance, AMP-mediated killing assay was performed for WT and *phoPQ* mutant strains treated with cecropin A at pH 5.5 or 7 with low Mg^{2+} (10 μM). Cecropin A is a 37 amino-acid linear peptide, which displays a powerful lytic activity against many Gram-negative bacteria including *E. amylovora* (Andreu et al. 1992).

After 1 h exposure to 0.25 μg ml^{-1} of cecropin A at pH 5.5, the WT strain had the survival rate of 14.4%, and the survival rate for *phoPQ* mutants was from 0.35% to 0.4% (Figure 4). When the bacteria were grown at pH 7.0, both WT and mutant strains showed increased resistance to cecropin A. The WT strain had the survival rate of 34.5%, whereas *phoPQ* mutants had a survival rate ranging from 2.7% to 13.5% (Figure 4). The complemented strains (*phoPQ*$^+$) partially restored the phenotype of the

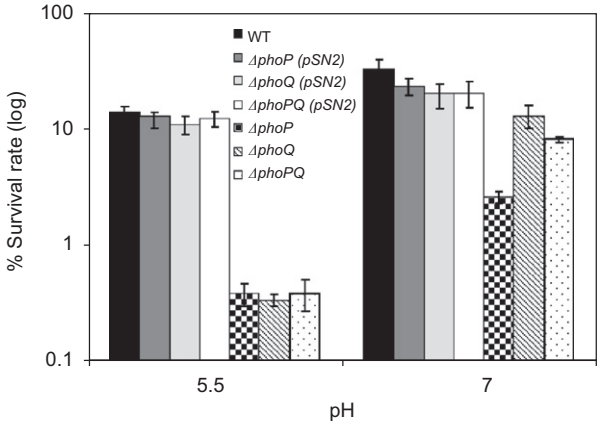

Figure 4. Susceptibility of *E. amylovora* WT, *phoPQ* mutants and complemented strains to cecropin A at low Mg^{2+} (10 μM). Bacterial strains were grown overnight in MBMA and treated with equal volume of cecropin A 0.5 μg ml^{-1} for 1 h. Bacterial cells that survived were counted by dilution plating on LB plates. Data points represent the means of three replicates ± standard deviations.

WT strain in both cases (Figure 4). These results indicate that the PhoPQ system confers the pathogen resistance to cecropin A. Since both the *phoPQ* mutants and WT strain showed increased resistance to cecropin A at neutral pH than at acidic pH, indicating that acidic pH suppresses this resistance. Furthermore, the impact of cecropin A on *phoP* mutant was much stronger than that of the *phoQ* and *phoPQ* mutants at pH 7 (Figure 4). It is possible that other systems may also be involved in the resistance to cecropin A, but act as a suppressor for resistance to cecropin A at acidic pH.

Discussion

Orthologs of PhoPQ system exist in both mammalian and plant pathogens including *Salmonella* spp., *E. coli*, *Yersinia* spp., *X. oryzae*, and *D. dadanti*. It has been demonstrated that this regulatory system is a master regulator of virulence genes in *Salmonella* (Groisman 2001). Activation of the PhoPQ system is necessary for intramacrophage survival, resistance to acid pH and AMPs (Aguirre et al. 2006). In this study, our results indicate that the conserved PhoPQ system in *E. amylovora* renders the pathogen more susceptible to strong acid conditions and suppresses two novel T3SS gene expression, suggesting that this system might act as a negative regulator for those genes involved in acid resistance and T3SS. These results are novel and in contrast with those reported in *Salmonella*, *Dickeya*, *Xanthomonas* and other bacteria. These results also suggest that the PhoPQ system, on one hand, may have maintained basic functions such as resistance to AMPs in response to acidic pH, and on the other hand, may also have evolved new functionalities for adaptation to different niches in different bacterial pathogens.

In *D. dadanti*, the *phoQ* mutant is unable to grow at acidic pH 5.5, while growing normally at neutral pH (Llama-Palacios et al. 2003, 2005). The *phoP* mutant of *Yersinia* shows reduced ability to grow in macrophage cell cultures and under conditions of both low pH and oxidative stress (Oyston et al. 2000). In *Salmonella*, *phoP* mutant is defective in macrophage intracellular survival and reduces resistance to acid pH (Bearson et al. 1998). In *Xanthomonas*, mutation in *phoP* but not *phoQ* eliminates tolerance to acidic conditions (Lee et al. 2008). In contrast to these reports, our results indicate that the PhoPQ system renders *E. amylovora* more susceptible to acidic conditions. The possible reason for this discrepancy is that the PhoPQ system in *E. amylovora* may act as a repressor for acid resistance gene expression.

Like many other Gram-negative plant pathogenic bacteria, *E. amylovora* encodes a T3SS (PAI1) that functions in the delivery of effector proteins directly into host plant cells to cause disease (Oh and Beer 2005). The two recently identified PAI2 and PAI3 have exactly the same gene organization as known T3SS PAIs such as SSR-1 and SSR-2 of *Sodalis glossinidius*, SPI-1 of *Salmonella enterica*, and Ysa of *Yersinia enterocolitica* (Dale et al. 2001; Zhao et al. 2009a). The SSR-1 is required for *S. glossinidius* to enter insect cells *in vitro* and establish infection *in vivo* in its natural host, the tsetse fly (Dale et al. 2001; Dale and Moran 2006). A recent study has reported that a similar T3SS (TTSS-2 with same gene organization as SSR-1) exists in *Pantoea stewartii* and is required for persistence in its flea beetle vector (Correa et al. 2008). This suggests that PAI2 and PAI3 of *E. amylovora* may have similar functions in insect interactions, though no evidence so far exists to prove that. More study is needed to clarify this hypothesis.

The PhoPQ system has been shown to be required for virulence in insects. Deletion of the *phoP* results in complete loss of virulence in insects when infected with *Photorhabdus luminescens* (Derzelle et al. 2004). Furthermore, the PhoPQ system in *Salmonella* and *Xanthomonas* promotes type III secretion system gene expression and is required for virulence (Aguirre et al. 2006; Groisman 2001; Lee et al. 2008). However, our data demonstrate that the PhoPQ system in *E. amylovora* is not required for virulence in plants (Zhao et al. 2009b), but represses gene expressions of PAI2 and PAI3, indicating that the PhoPQ system may be involved in interaction with insect vectors. In addition, PAI3 and PAI2 genes in *E. amylovora* are expressed at high temperature and in rich medium conditions, which is unusual for plant pathogenic bacteria, as plant-related T3SS genes (such as PAI1) of *E. amylovora* are normally expressed only under conditions such as minimal medium and relatively low temperature (20 °C) (Oh and Beer 2005; Zhao et al. 2009a). It is, for the first time, that we have demonstrated that T3SS genes in plant pathogenic bacteria are expressed in rich medium at high temperature (28 °C). Furthermore, it is noteworthy that SSR-1 and SSR-2 have distinct temporal patterns of gene expression in *S. glossinidius* (Dale et al. 2001; Dale and Moran 2006). Our results showed that PAI2 and PAI3 in *E. amylovora* also had differential gene expression patterns *in vitro*. These findings may further provide clues for future characterization of these novel type III secretion systems in *E. amylovora*.

The ubiquitous occurrence of cationic AMPs in nature suggests that these compounds are a

formidable host defense against microbial infection. In *Salmonella*, the PhoPQ system activates transcription of *pag* (PhoP-activated genes) genes within macrophages necessary for resistance to AMPs, and AMPs and acidic pH activates this system in an additive way (Bader et al. 2005; Prost et al. 2007). In addition, tolerance to AMPs is weakened in both *phoP* and *phoQ* knockout strains in *Xanthomonas* (Lee et al. 2008). In this study, our results demonstrated that the PhoPQ system confers the pathogen resistance to cecropin A. Our results also indicate that both WT and *phoPQ* mutants showed increased resistance to cecropin A at neutral pH, suggesting that resistance to cecropin A is not only dependent on the PhoPQ system, but may also depend on unknown systems that respond to acidic pH. One candidate system is the PmrAB system that responds to acidic pH and may confer resistance to AMPs (Perez and Groisman 2007, 2009). It will be interesting to investigate whether there are cross-talks between different two-component systems in *E. amylovora*.

In summary, we have genetically characterized the PhoPQ orthologue in *E. amylovora*. Though the *E. amylovora* PhoPQ system shares similarity with their counterparts in other bacteria in resistance to AMPs; our results clearly demonstrate that this system functions differently in regulating genes responsible for acid resistance and type III secretion. These results suggest that bacteria with different lifestyle may have developed different regulatory systems in response to environmental cues to regulate gene expression and survival. Further investigations such as determining the functions of PAI2 and PAI3 during insect interactions, identifying genes regulated by the PhoPQ system, and how the PhoPQ interacts with other systems such as the PmrAB system in *E. amylovora*, are needed to better understand the function of the PhoPQ system in the survival of *E. amylovora*. It is possible that the PhoPQ system may act as part of a regulatory network that governs *E. amylovora* in a wide variety of cellular functions, thus enhancing its survival under changing environments or different hosts such as insects (Groisman and Mouslim 2006; Zwir et al. 2005).

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