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Identification of PhoP Regulon Members in *Xanthomonas oryzae* pv. *oryzae* PXO99A

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Abstract. *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a causal agent of bacterial leaf blight disease in rice. The two-component regulatory system (TCS) of *Xoo*, PhoP-PhoQ, was previously shown to be negatively regulated by RaxR-RaxH, another TCS. RaxR-RaxH senses population cell density and modulates the activity of Ax21, a small secreted bacterial protein recognized by a rice resistance protein, *XA21*. In this work, whole-genome microarray analysis was performed to identify members of the PhoP regulon under Ca^{2+}-limited conditions. This analysis revealed that PhoP governs broad cellular pathways including the stress defense response, cation transportation, the regulatory system, and bacterial virulence. This study also suggests a putative regulatory loop between PhoP-PhoQ and RaxR-RaxH.

Keywords: Microarray analysis, *phoP*, *Xanthomonas oryzae* pv. *oryzae* PXO99A, Ax21

1. Introduction

Bacteria have developed numerous mechanisms to adapt themselves for successful colonization in either limited-nutrient niches or hosts that are laden with various toxic substances. Most phytopathogenic bacteria colonize and multiply in the apoplast, where conditions are quite acidic pH condition due to abundance of organic acids, such as citric and malic acids [1]. To cope with this stress, bacteria have evolved a mechanism that is the two-component regulatory systems (TCSs). TCSs are simply composed of histidine kinases (HKs) and response regulators (RRs). After sensing to the environmental stimuli, the HK phosphorylates the cognate RR, which then regulates the expression of target genes [2].

Xa21, the first cloned *R* gene in rice, encodes a receptor-like kinase protein with a predicted extracellular, leucine-rich-repeat (LRR) domain, a transmembrane domain and a cytoplasm-located kinase domain [3]. Rice lines carrying *Xa21* are able to mount an effective defense response to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the causal agent of bacterial leaf blight disease in rice, strains producing Ax21 molecule (Activator of XA21-mediated immunity), but not to strains lacking Ax21 activity. We previously identified eight *Xoo* genes required for Ax21 activity *(rax)*. *Xoo* strains carrying mutations in any of the these genes no longer, trigger XA21-mediated immunity [4]. *raxA*, *raxB* and *raxC* encode components of a type I secretions system (TOSS), *raxST*, *raxP* and *raxQ* encode enzymes involved in sulfation on Ax21, and *raxR* and *raxH* encode a TCS [4]. Subsequent studies identified a second TCS, called *phoP* and *phoQ* that is also required for Ax21 activity, and that is negatively regulated by RaxR [5]. Also *phoP* is required for *Xoo* virulence and expression of *hrp* (hypersensitive reaction and pathogenicity) genes [5].

Recently we successfully identified Ax21 molecule as a small secreted protein and characterized its biological function as a quorum sensing factor [6]. However, little is known about the mechanism that controls Ax21 production and how Ax21 signal is transferred to control bacterial phenotypes. TCSs sense the

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change of intracellular and extracellular stimuli and transfer the signal to activate the bacterial responses by changing the gene expression pattern. We hypothesize that PhoP, similar to RaxR, either functions as a activator of *ax21* expression or other genes that control Ax21 activity. Here we performed microarray analysis to identify PhoP-regulated genes by comparing the transcriptional profiles of wild-type *Xoo* (strain PXO99A) and a *phoP* knockout mutant. The knowledge from this study provides better understanding in the mechanism how *Xoo* establish Ax21 activity.

2. Materials and Methods

2.1. Bacterial Strains, Growth Conditions, and Media

Xanthomonas oryzae pv. *oryzae* strain PXO99A was used for all experiments. Cells were grown at 28 ^{o}C with shaking at 200 rpm, in nutrient-rich PS broth until the OD_{600} was equal to 0.2, washed twice, and then immediately transferred into modified M9 minimal media that contained Ca^{2+} at 10 μM for 24 hrs. Cells were harvested by centrifugation at 8,000 rpm, for 5 mins and then were used as material for RNA preparation immediately. Three separate bacterial cultures were prepared and served as three biological replicates for each set of experiments.

2.2. RNA Preparation and Microarray Analysis

RNA from *Xoo* PXO99A and the *Xoo phoP* knockout mutant [5] cultured in low Ca^{2+} were isolated by following the protocol described previously [7]. Fluorescent-labelled cDNA were synthesized using SuperScript^{TM}III Indirect cDNA Labelling System (Invitrogen) by following the protocol described previously [7]. 50 pMol of each labelled cDNA samples were hybridized onto *Xo* microarray slides. After combining results from three biological replicates, genes were assigned to be differentially regulated if they exhibit at least 1.8 fold-change and has False Discovery Rate (FDR) < 5%. All of the microarray data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) under accession number GSE 12099.

2.3. Validation of Expression Patterns of Candidate Genes Using Quantitative RT-PCR

For quantitative RT-PCR (Q-RT-PCR), the same RNA samples as were used in array analysis were used as templates for Q-RT-PCR. To generate second-strand cDNA, the SuperScript^{TM} III First-Strand synthesis system for RT-PCR kit was used (Invitrogen, CA, USA) by following the protocol described previously [7]. The level of gene expression was normalized to the 16sRNA.

3. Results and Discussion

3.1. Overview of Microarray Analysis

PhoP-PhoQ is a TCS that regulates numerous cellular activities, including virulence, and adaptation to Mg^{2+}-limiting environments. Low Mg^{2+} concentration is typical environment present in the plant apoplast and has been shown to promote PhoP-dependent transcription [8]. In our previous work, a microarray representing the whole genome of *Xoo* was constructed [7]. In this study, we set experiments to identify PhoP-regulated genes by comparing the transcriptional profiles of wild-type *Xoo* and a *phoP* knockout mutant in low levels of Ca^{2+} (because *Xoo phoP* expression is inhibited at a high Ca^{2+} concentration (10 mM) [5]). The PhoP-regulated genes were identified as described in the method, resulting in a total 214 genes. Among all PhoP-regulated genes, 137 genes were down-regulated (PhoP-activated) while 77 genes were up-regulated (PhoP-repressed) in the *phoP* knockout mutant. Each PhoP-regulated genes were assigned roles according to the TIGR role terms as available at Comprehensive Microbial Resource (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi). The PhoP-up-regulated genes encode primarily hypothetical proteins (16.88%), mobile and extrachromosomal element functions (12.99%), or proteins involved in cellular processes (10.38%); most of PhoP-down-regulated genes encode hypothetical proteins (18.98%) or proteins associated with the cell envelope (11.68%), protein fate (8.0%), regulatory functions (8.0%), or transport and binding (8.0%) (Fig. 1).

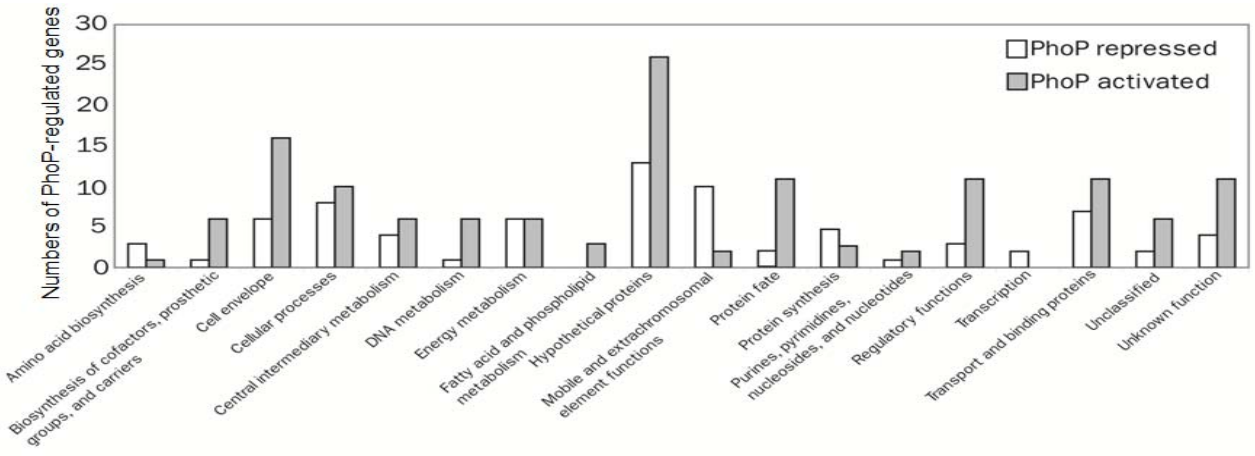

Fig. 1: Eighteen functional categories of PhoP-regulated genes were catagorized according to TIGR Comprehensive Microbial Resouces.

To validate the array data, we performed Q-RT-PCR with 6 genes that identified by microarray analysis. Results indicated that the magnitudes of the fold changes obtained using Q-RT-PCR and the array hybridizations were different, but the trends observed in fold changes were the same (Fig. 2).

Gene	Microarray	Q-RT-PCR	Primers sequences
PXO_02671	12.04	4.26	FW 5' GACCACAGCGTTGGGAAC 3'/ RV 5' GCTCGCCATCTTCATTCACT 3'
PXO_02640	-3.86	-2.36	FW 5' AAGGAAGAGCCCAAGCATTT 3'/ RV 5' CGATGGAAGCTGCCGTAAC 3'
PXO_02432	-2.41	-4.88	FW 5' CCATCCAGATCCTTTCCTCA 3'/ RV 5' TGTTGTTGGGATCGTTGAAA 3'
PXO_04812	2.39	4.14	FW 5' GATCGACACCTCACTGTCCA 3'/ RV 5' GGCAGGAGCTCTTCTGAGAC 3'
PXO_01320	5.43	5.05	FW 5' ATCTTGCTCACCATCGCTTT 3'/ RV 5' CCCAATTGTTGTCGCATTC 3'
PXO_04572	4.11	3.20	FW 5' GGAAGCTACGCTGTGTTGGT 3'/ RV 5' GTAGCCGAGCAGCTTCACC 3'

Fig. 2: Validation of selected PhoP-regulated genes using Q-RT-PCR. Expression levels of six genes were compared in *Xoo* wild-type PXO99A and in the *Xoo phoP* knockout mutant. Fold change of gene expression quantitated by microarray and Q-RT-PCR were shown (+ = gene expression was up-regulated, - = gene expression was down-regulated in the *phoP* knockout mutant). Primers used in Q-RT-PCR of each gene are shown in the last column.

3.2. Cation Transport System

PhoP protein directly transcriptional activates the expression of two of Mg^{2+} transporters present in *S. typhimurium*, MgtA and MgtB, when the bacterium experiences low Mg^{2+} environments [9]. Our microarray analysis showed that *phoP* positively regulated *mgtE* (PXO_02891) expression for 2.2 fold. In addition, expression of *feoB* (PXO_00098; a component of the free iron (II) transporter system) is also down-regulated in the *phoP* knockout mutant that are cultured in nutrient-rich media (unpublished data). Interestingly, microarray analysis indicated that expression of *iucA* (PXO_01928), the product of which is involved in metal ion acquisition and siderophore biosynthesis, was suppressed for 2 fold by PhoP.

3.3. Stress-Protective Functions

PhoP has been shown to be important for tolerance to organic acid stress and oxidative stress [10]. The *phoP* knockout mutants in *Salmonella typhimurium* are hypersensitive to oxidative stress-dependent Fe(II)-mediated killing [11]. Although expression of *sodM* (PXO_00389) and *catB* (PXO_02830) did not change in the array experiment, the expression of these genes was affected by Ca^{2+} (unpublished data). Another adaptive responses to survive in stressful conditions is induction of heat shock protein expression. Heat shock proteins play roles in repairing and preventing damage caused by an accumulation of unfolded proteins resulting from the environmental stress [12]. Microarray analysis showed that the major cold shock proteins encoded by *cspA* (PXO_04812 and PXO_00399) and another cold shock gene, *scoF* (PXO_01731), were up-regulated for 2.40 and 2.30 fold respectively. Elevation of the ScoF-1 transcript level is shown to associated with both transcriptional activation and post-transcriptional mRNA stability [13]. The fact that PhoP enhanced expression of various stress-responsive genes, this should be accounted for the increased

sensitivity of the *phoP* mutant to the unfavourable conditions. Therefore, the expression of PhoP-dependent stress-defensive genes likely enables bacteria to survive under stress conditions in the host.

3.4. Regulatory Functions

Once bacteria encounter their host and sense certain signals, e.g. temperature or pH, a cascade of signaling pathways is activated. One single regulatory element controls the expression of downstream genes, including secondary effectors, that assist their survival within a particular niche. ColR-ColS, a TCS, was originally identified as an important component of the root-colonizing ability of *Pseudomonas fluorescens* WCS365 [14]. It later was demonstrated to enhance the bacterial tolerance against stress conditions, i.e. heavy metal toxins, and phenol, via regulation of membrane functionality [15]. In this study, we identified 14 PhoP-regulated genes (e.g. *colR* (PXO_02303), *regR* (PXO_00797)) that encode proteins putatively assigned roles related to regulatory functions, suggesting that the PhoP has pleiotropic effects to control multiple bacterial responses to the various stimuli.

3.5. Virulence and Pathogenicity

The PhoP/PhoQ system was reported to be an important factor for pathogenesis in bacteria [16]. In our previous study, we found that *hrpG*, *hrpX*, and *hrpA* expression were decreased in the *phoP* knockout mutant under low Ca^{2+} concentration [5]. In this study, we observed down-regulation of *slyA* (PXO_03534) for 2 fold in the *phoP* knockout mutant. The SlyA of *Salmonella enterica* is a member of the MarR family of transcription regulators and is required for virulence and survival in macrophages [17]. The direct regulation between PhoP and SlyA that are crucial for *S. enterica* virulence, was observed as a result of investigation of the PhoP box in the *slyA* promoter [17].

As the integrity of the bacterial cytoplasm is protected by membranes, bacteria have adopted a variety of methods for transportation of molecules produced in bacteria to the surrounding environment, including their hosts. Bacteria require the mechanism to take up nutrients, and transport toxin across their membranes for the ability to cause disease. We observed the down-regulation of *tatA* (PXO_03840) and *tatC* (PXO_03838) for 2.3 and 2.35 fold. The Tat pathway targets folded proteins and secretes those proteins across the inner membrane [18]. In many bacteria, a wide range of substrates are secreted using the Tat pathway and as a result, *tat* mutants often show pleiotropic phenotypes. Recently, analysis of the Tat secretome [19] demonstrated that seventy proteins were secreted through the Tat secretion system, including AcvB-like protein (PXO_00416), which was observed to be activated (5.4 fold) by PhoP in our experiment.

3.6. A Putative Regulatory Loop between PhoP-Q and RaxR-H

Because *phoP* is up-regulated in a *raxR* knockout mutant, and the *raxR* knockout mutant does not completely eliminate Ax21 activity, we hypothesized that PhoP might modulate Ax21 activity [5]. When inoculate on *Xa21* transgenic rice, a *phoQ* knockout showed reduction of Ax21 activity. Contrarily, a *phoP* knockout mutant exhibited no change in Ax21 activity. Also, the *phoP* knockout mutant had reduced growth on wild-type rice. Studies of these *phoP* knockout phenotypes suggests that the absence of *phoP* either did not affect Ax21 activity or reduced the overall virulence of this strain [5]. In this study, array analysis showed that *raxA*, *raxB*, and *raxST* expression was down-regulated in the *phoP* knockout and this result was confirmed with RT-PCR (Fig. 3). These observations serve as evidence to support the hypothesis that *phoP* regulates Ax21 activity, partly through regulation of *raxA*, *raxB*, and *raxST* expression.

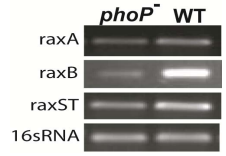

Fig. 3: Comparison of *raxA*, *raxB*, and *raxST* expression in PXO99A and the *phoP* knockout mutant using semiquantitative RT-PCR. The 16sRNA gene was used as a control for both samples.

3.7. Final Remarks

The PhoP-PhoQ has been shown to sense environmental cues and effectors, which in turn, trigger gene expression to increase bacterial survival under stressful conditions or in hosts. Genomic-scale transcription

profiling can reveal a global view of the PhoP regulons that associated to the cation transportation, stress defense mechanisms, other regulatory systems, and bacterial virulence. The array analysis suggests a putative regulatory function of PhoP to be a regulator of *Xoo* Ax21 activity through the activation of *raxA*, *raxB*, and *raxST* genes. The overall expression PhoP regulon is a sum of the signals that are the status of nutrient, pH, cation, and Ax21-dependent population density. Further expression analysis to investigate the direct regulation of *ax21* by PhoP is aimed to be achieves to provide insights of the mechanisms how PhoP regulates Ax21 activity and triggers the response to the environmental stress.

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