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REVIEW

Two-component signal transduction systems and regulation of virulence factors in *Xanthomonas*: a perspective

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Abstract Two-component signal transduction systems (TCSTSs), consisting of a histidine kinase and a response regulator, play a critical role in regulating virulence gene expression in Gram-negative phytopathogenic bacteria *Xanthomonas* spp.. To date, 12 TCSTS genes have been identified, accounting for approximately 10% of the TCSTS genes in each genome that have been experimentally identified to be related to pathogenesis. These TCSTSs modulate the expression of a number of virulence factors through diverse molecular mechanisms such as interacting with DNA, protein-binding and involvement in second messenger metabolism, which generates a high level of regulatory versatility. Here we summarize the current knowledge in this field and discuss the emerging themes and remaining questions that are important in deciphering the signaling network of TCSTSs in *Xanthomonas*.

Keywords *Xanthomonas*, two-component signal transduction system, virulence factor

1 Introduction

After invading host tissues or cells, the life style of bacterial pathogens undergoes a critical transition from the free-living to the biotrophic or necrotrophic state. This transition requires bacteria to promptly adapt to substantial environmental changes and threats from host immune responses. For survival, bacteria have evolved subtle cellular machineries to sense environmental stimuli and respond accordingly by modulating their gene expression in an appropriate spatiotemporal manner. Since the discovery of the first two-component signal transduction system (TCSTS) from *Escherichia coli* in 1986 (Ninfa and Magasanik, 1986; Nixon et al., 1986), it has been clearly recognized that TCSTS is the dominant molecular mechanism by which bacteria sense and respond to outside stimuli (Stock et al., 2000). As shown in Fig. 1, the prototype of the TCSTS consists of a membrane-bound sensor histidine kinase (HK) and a cytoplasmic response regulator (RR). After detecting a specific stimulus with its input domain, the dimeric HK can be autophosphorylated on a conserved His residue within its transmitter domain, followed by the transfer of the phosphoryl group onto a conserved Asp residue within the N-terminal receiver domain of the cognate RR. In due course, the activated RR carries out adaptive regulation of downstream gene expression through its C-terminal output domain, which can function as a transcription factor (Stock et al., 2000; Goulian, 2010). TCSTSs regulate almost all physiological processes of bacteria, such as nutrition assimilation, cell motility, chemotaxis, biofilm formation, quorum-sensing and virulence. Besides eubacteria, TCSTSs have also been identified in archae, slime mold, fungi and plants. However, it has been found that animal genomes do not encode this signaling system, indicating that TCSTSs may serve as potential targets for developing novel antimicrobial chemicals (Gotoh et al., 2010).

The genus *Xanthomonas* consists of a group of single-cell, single-flagellum, obligately aerobic, Gram-negative bacteria. These bacteria can live in diverse environments such as soil, water, and even human blood (Swings and Civerolo, 1993). Furthermore, almost all bacteria of *Xanthomonas* are plant pathogens. Among them, three strains have long been used as model organisms in studying plant-bacteria interactions, including *X. campestris* pathovar. *campestris* (*Xcc*), the causal agent of black rot disease of crucifers; *X. campestris* pv. *vesicatoria* (*Xcv*), the causal agent of bacterial spot disease of tomato and sweet pepper; and *X. oryzae* pv. *oryzae* (*Xoo*), the causal

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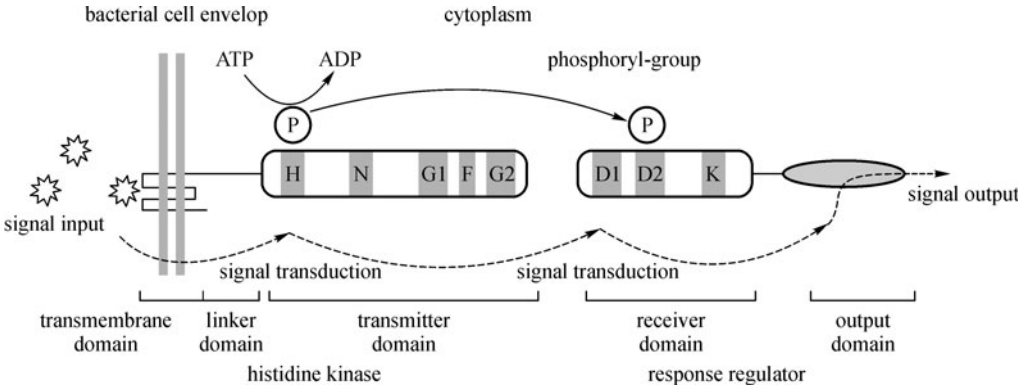

Fig. 1 A schematic illustration of mechanism of the two-component signal transduction system. The direction of phosphoryl-group transfer is depicted by solid line and the direction of signal transduction is depicted by a dashed line. P in the circular frame represents the phosphoryl group. H, N, G1, F and G2 represent typical conserved motifs in the histidine kinase sensor. D1, D2, and K represent typical conserved motifs in the response regulator. Definition of these motifs is according to a previous study (Parkinson and Kofoid, 1992).

agent of bacterial blight of rice. During the last three decades, great efforts have been made to understand the genetic basis of *Xanthomonas* pathogenesis. These have resulted in the identification of a number of virulence factors and virulence-associated biological pathways, such as biosynthesis of exopolysaccharides (EPS), lipopolysaccharides (LPS), extracellular enzymes, type I, II and III secretion systems, iron transport systems, detoxification systems and housekeeping pathways responsible for the synthesis of purines and aromatic amino acids (Qian et al., 2005; He and Zhang, 2008; Büttner and Bonas, 2010). As anticipated, TCSTSs of *Xanthomonas* have been shown to control and coordinate these complex cascades and in this review we summarize progress in the recent study of these TCSTSs and discuss several important unanswered questions in this field.

2 Overview of TCSTSs in the genomes of *Xanthomonas*

To date, 14 complete *Xanthomonas* genome sequences have been deposited in public databases, corresponding to: three strains of *Xcc*; three strains of *Xoo*; two strains of *X. fuscans* pv. *aurantifolii* (*Xfa*, causing cankers on *Citrus* spp.); a strain of *Xcv*; a strain of *X. oryzae* pv. *oryzicola* (*Xoc*, causing rice bacteria stripe); a strain of *X. axonopodis* pv. *citri* (*Xac*, causing bacterial canker in citrus); a strain of *X. albilineans* (*Xal*, causing leaf scald in sugarcane); a strain of *X. campestris* pv. *vasculorum* (*Xcva*, causing gumming disease in sugarcane); and a strain of *X. campestris* pv. *musacearum* (*Xcm*, causing bacterial wilt in banana). TCSTSs of the eight strains have been systematically annotated by bioinformatic analysis (Qian et al., 2008a; Barakat et al., 2009). Each *Xanthomonas* genome encodes a large number of TCSTS genes which comprise approximately 3% of the nucleotide sequences of these genomes (Table 1), higher than the estimated average number (about 2%) of other bacteria (Galperin, 2005). All these TCSTSs are encoded by bacterial chromosomes and none of them has been identified in plasmids (Qian et al., 2008a; Barakat et al., 2009). Among them, it is noticeable that the numbers of TCSTSs encoded by *Xoo* genomes are remarkably smaller than those of the other species. Further comparative analysis has revealed that a substantial process of gene loss has occurred during *Xoo* genomic evolution (Qian et al., 2008a). This reduction strongly suggests that TCSTS regulation in *Xoo* underwent a certain

Table 1 Histidine kinases and response regulators encoded by the genomes of *Xanthomonas*

genome	genome size/mb	orthodox histidine kinase	hybrid histidine kinase	response regulator	total
Xcc 8004	5.15	32	20	54	106
Xcc ATCC 33913	5.08	32	20	54	106
Xcc B100	5.08	32	20	54	106
Xoo PXO99	5.24	32	13	58	104
Xoo KACC10331	4.94	28	12	52	92
Xoo MAFF311013	4.94	28	15	50	93
Xcv 85-10	5.18	37	23	61	121
Xac 306	5.18	35	21	58	114

amount of reprogramming or reorganization, most probably caused by accelerated evolution along with the recent domestication of their host plant, cultivated rice (*Oryza sativa*).

The putative TCSTS proteins of *Xanthomonas* exhibit high levels of diversity in their secondary structures (Qian et al., 2008a). For HKs, apart from a few subfamilies detected in Archea (Grebe and Stock, 1999), the genomes of *Xanthomonas* encode almost all known substructural groups of bacterial HKs that have been categorized based on the organization of their phosphorylation sites (H-boxes). RRs of *Xanthomonas* are classified according to the characteristics of their output domains. Although the biological functions of the majority of these RRs remain unknown, this classification indicates that they can modulate downstream factors by means of (1) DNA-binding; (2) RNA-binding; (3) protein-protein interaction, and (4) enzymatic activity such as catalysis of the turnover of the cellular secondary messenger bis-(3'5')-cyclic di-guanosine monophosphate (c-di-GMP) (Qian et al., 2008a).

About 70 TCSTS genes are shared by all studied genomes of *Xanthomonas*, constituting a "core set" of TCSTSs. Due to functional constraints, these shared proteins have been conserved by accumulating point mutations at a slow rate, suggesting they carry out similar biological functions. However, comparative genomic analysis has revealed that other TCSTS genes have been subject to extensive genetic recombinations, such as gene fusion or fission, gain or loss, location rearrangement and gene duplication (Qian et al., 2008a). Among them, hybrid histidine kinases (HyHKs), that is, HKs which contain additional receiver (REC) domains, seem to have specific evolutionary properties. Compared with the orthodox HK and RR genes, HyHK genes have higher levels of DNA polymorphisms and evolutionary rates measured by nonsynonymous substitution *vs* synonymous substitution rates (Ka-Ks), indicating they have been quite active during evolution. Since HyHKs contain additional phosphorylation sites and they usually transfer signals via a series of multiple steps called phosphorelay (Raghavan and Groisman, 2010), rapid evolution of these genes will undoubtedly add to the complexity of cell signaling and promote the potential of bacteria in adapting to environmental changes.

3 TCSTSs regulating expression of virulence factors in *Xanthomonas* spp.

To date, 12 TCSTS proteins have been identified that are related to virulence regulation in *Xanthomonas* spp., including RpfC-RpfG, RavS-RavR, HrpG, VgrS-VgrR (also named ColS-ColR), VemR, RaxH-RaxR, and PhoQ-PhoP. Domain compositions of these proteins are shown in Fig. 2.

3.1 The RpfC-RpfG system: sensing the quorum sensing signal (DSF) to regulate the virulence gene expression

The RpfC-RpfG system was the first and best-documented TCSTS to be identified in *Xanthomonas*. The *rpf* (regulation of pathogenicity factors) gene cluster was identified about 20 years ago by sequencing a recombinant plasmid that was capable of restoring pathogenicity deficiency after being transformed into an *Xcc* mutant (Tang et al., 1991). It contains nine genes named *rpfA*, *B*, *F*, *C*, *H*, *G* and *rpfD*, *E*, and *I*. Among them, RpfC is a HyHK containing five transmembrane regions, an additional receiver domain and an Hpt (histidine-containing phosphotransfer) domain besides conserved phosphoracceptor and ATPase domains (Slater et al., 2000) (Fig. 2). RpfG is an unusual response regulator which uses an HD-GYP domain as its output region (Fig. 2). The HD-GYP domain of RpfG was confirmed to have phosphodiesterase activity capable of degrading the bacterial second messenger c-di-GMP into pGpG or GTP (Ryan et al., 2006). Mutations in either of the two genes significantly attenuate bacterial virulence and pilus-dependent cell motility (Slater et al., 2000).

RpfC-RpfG has been shown to regulate the biosynthesis of a number of virulence factors during quorum-sensing, a cell-cell communication phenomenon whereby a single bacterial cell can sense and respond to the population density through detection of the concentration of signaling molecules (Ng and Bassler, 2009). The molecular basis of signal transduction of quorum-sensing has been well studied in the last two decades. *Xcc* has also evolved cell-cell communication systems to regulate its gene expression and cell behavior. Two molecules, one called diffusible signal factor (DSF) and the other called diffusible factor (DF), take part in quorum-sensing in *Xcc* (He and Zhang, 2008). Of them, the structure of DSF has been resolved and shown to be cis-11-methyl-dodecenoic acid, an α, β-unsaturated fatty acid (Wang et al., 2004). *rpfC*, *rpfG* and *rpfF* inactivation mutants form aggregates when growing in L medium, and addition of DSF restores the dispersed planktonic state of the *rpfF* mutant (Dow et al., 2003). This result clearly demonstrates that RpfC-RpfG is involved in biofilm development, which is regulated in a population-dependent manner. In addition, the RpfC-RpfG system has long been observed to positively modulate the biosynthesis of exocellular polysaccharides (EPS) and extracellular enzymes, such as proteases, endoglucanase, endomannase, amylases and cellulases (Dow and Daniels, 1994). It has been proposed that the perception of the DSF signal by RpfC triggers this process. Experimental evidence has shown that this regulation requires the phosphorylation of both RpfC and RpfG, and RpfG has been proven to be an obligatory component for the activation of these virulence factors (Dow, 2008). Since deletion of the HD-GYP domain of RpfG results in a decrease of EPS and extracellular enzymes, it appears that c-di-GMP turnover is important in

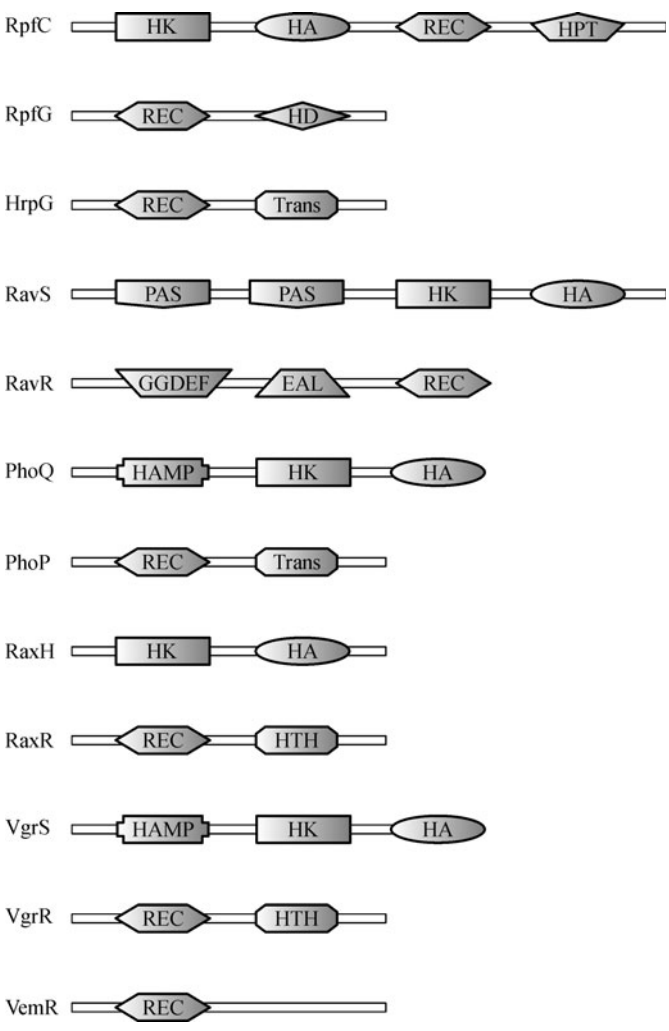

Fig. 2 Domain organization of the virulence and avirulence regulatory histidine kinases and response regulators in *Xanthomonas* spp.. The names of the domains are according to those of the pfam database. HK (HisKA, pfam acc. No.: PF07730); HA (HATPase_c, PF02518); REC (Response_reg, PF00072); HPT (PF01672); HD (PF1966); Trans (Trans_reg, PF00486); PAS (PF00989); GGDEF (PF00990); EAL (PF00563) and HAMP (PF00672).

controlling these virulence factors. Studies have shown that a global transcriptional regulator, the cAMP receptor protein (CRP)-like protein (Clp), which is highly similar to the CRP of *E. coli*, is downstream of the RpfC-RpfG system (He et al., 2007). Overexpression of Clp in *rpfG* or *rpfF* mutants can restore the deficiency in EPS and extracellular enzyme production to wild-type levels. Recent studies have demonstrated that Clp is a c-di-GMP effector (Leduc and Roberts, 2009; Tao et al., 2010). After binding with c-di-GMP, Clp undergoes an allosteric conformational change followed by dissociation from its target gene promoter (*engXCC*), supporting the notion that the binding of c-di-GMP represses Clp activity (Tao et al., 2010). It has been proposed that after receiving the DSF signal, RpfC activates RpfG via phosphorylation and the latter degrades c-di-GMP, which leads to the increase of free Clp molecules in cells and promotes the transcription of downstream virulence genes (Tao et al., 2010).

Genome-scale transcriptional analysis has revealed that DSF controls the expression of at least 165 genes. Besides EPS and extracellular enzyme-coding genes, it also regulates the expression of genes responsible for iron uptake, flagellum synthesis, tricarboxylic acid (TCA) cycle, aerobic respiration and fatty acid metabolism (He et al., 2007). Most intriguingly, DSF has been found to play a critical role in cell-cell signaling of other microbes, including the Gram-negative bacterium *Pseudomonas aeruginosa*, the Gram-positive bacterium *Mycobacterium* spp. and the fungus *Candida albicans* (Wang et al., 2004). These observations indicate that DSF-like chemicals participate in cross-kingdom signaling.

Besides sensing the DSF signal then regulating virulence factors via protein phosphorylation, it has been confirmed that the receiver domain of RpfC alone negatively modulates the biosynthesis of DSF itself (He et al., 2006). Inactivation of *rpfC* leads to the overproduction

of DSF; this repression of RpfC is independent of protein phosphorylation and does not require RpfG (Slater et al., 2000; He et al., 2006), indicating that the HyHK RpfC acts as both a sensor and a regulator in controlling downstream genes. In *Xcc*, the biosynthesis of DSF involves RpfF, a putative enoyl-CoA hydratase, and RpfB, a putative long-chain fatty acyl CoA ligase (Wang et al., 2004). Both far-western blotting and coimmunoprecipitation have shown that RpfC can bind RpfF, suggesting that this binding may result in repression of RpfF activity (He et al., 2006). In addition, when growing *in planta*, *Xcc* is capable of reverting stomatal closure induced by abscisic acid (ABA), lipopolysaccharides and bacterial infection. *rpfC* and *rpfF* mutants lack this ability, implying that DSF signaling is required to modulate the plant's innate immunity system (Gudesblat et al., 2009). The current working model is that when the bacterial population reaches a high level, RpfC is autophosphorylated, dissociates from RpfF and loses its ability to repress RpfF activity. However, further biochemical evidence is needed to support this mechanism.

In order to identify the auxiliary factors involved in RpfC-RpfG signaling, Andrade et al. (2006) performed a yeast two-hybrid screen for proteins interacting with RpfC and RpfG of *Xac*. Interestingly, nine GGDEF domain-containing proteins were found to bind RpfG *in vitro* (Andrade et al., 2006). Because the GGDEF domain has diguanylate cyclase activity and is responsible for the synthesis of c-di-GMP, it was proposed that the interaction between GGDEF and HD-GYP domains may be critical in fine-tuning c-di-GMP turnover. Recently, Ryan et al. employed fluorescence resonance energy transfer (FRET) to confirm that direct physical interactions exist between RpfG and two GGDEF domain-containing proteins, XC_0249 and XC_0420, in *Xcc* (Ryan et al., 2010). Substitution of amino acid residues within the GYP motif of RpfG, rather than the HD motif, prevents all these interactions, indicating that the GYP motif is essential for interactions between the GGDEF and HD-GYP domains. In addition, in DSF-deficient *rpfF* mutants, the FRET signal is undetectable but can be significantly restored after exogenous DSF is added to the medium (Ryan et al., 2010). These results strongly support the notion that the DSF signal is essential for interactions between these proteins. Unlike the single gene mutants, a double mutant of *XC_0420* and *XC_0249* exhibits a substantial decrease in cell motility (swimming) and virulence, phenotypes also observed in the *rpfG* mutant (Ryan et al., 2010). These recent findings indicate that the bacteria employ subtle mechanisms to coordinate c-di-GMP regulation of different physiological pathways, an area discussed later in this review.

It is worth noting that although the *rpf* gene cluster is conserved in bacteria of *Xanthomonadaceae*, substantial differences have been found in *rpf* gene regulation and DSF perception. For example, in *Xylella fastidiosa*, a close-relative of *Xanthomonas* spp., the *rpfF* mutant is hypervirulent, rather than virulence-deficient, against a grape host plant. The DSF of *X. fastidiosa* has been characterized as a 12-methyl-tetradecanoic acid, a structural analog of DSF in *Xcc* (Chatterjee et al., 2008; Dow, 2008). Furthermore, an *rpfC* mutant of *X. fastidiosa* is deficient in transmission from a vector insect (blue-green sharpshooters) into a host plant, indicating that it is critical in host alteration. In *X. oryzae* pv. *oryzae*, an *rpfF* mutant overproduces siderophores required for iron acquisition and grows slowly under low iron conditions (Tang *et al.*, 1996). Based on these observations, it appears that functional differentiation of RpfC-RpfG orthologs occurs in different bacteria of *Xanthomonadaceae*, which may result from coevolution of pathogenic bacteria and their host plants.

3.2 RavS-RavR system: controlling c-di-GMP turnover to regulate virulence gene expression

Similar to the RpfC-RpfG system, the RavS-RavR (regulation of adaptation and virulence) system is also a TCSTS that is associated with c-di-GMP turnover. In fact, before genomic sequences became available, a partial sequence of *ravR* of *Xcc* 8004 was reported in 1990. Deletion of this gene, however, did not result in deficiency in bacterial virulence (Osbourn et al., 1990). However, two research groups independently showed that in two other *Xcc* strains, *Xcc* ATCC 33913 and *Xcc* CN1, knockout of *ravR* orthologs led to attenuation of virulence, suggesting that strain-specific differences exist in the regulatory function of RavS-RavR (Qian et al., 2008b; He et al., 2009). RavS is an HK that contains two PAS domains in the N-terminal sensor region (Fig. 2), suggesting it may be involved in sensing signals from light, oxygen or redox potential (Hefti et al., 2004). Mutations in the second PAS domain have been shown to affect the function of RavS. RavR is an RR containing both GGDEF and EAL domains that act as a diguanylate cyclase and a phosphodiesterase, respectively. Specifically, the GGDEF and EAL domains of RavR are localized in the N-terminal region, which is quite different from other RRs where these domains are usually arranged in the C-terminal region (Qian et al., 2008b). The deficiency in production of EPS and extracellular protease is restored in *ravR* mutants expressing either the GGDEF-EAL domain or the EAL domain alone. In addition, high performance liquid chromatography (HPLC) analysis has shown that while both the GGDEF-EAL and the EAL domains catalyze degradation of c-di-GMP into GTP, the GGDEF domain does not contain detectable diguanylate cyclase activity. Taken together, this genetic and biochemical evidence indicates that RavR acts as a phosphodiesterase via its EAL domain (He et al., 2009). Microarray analysis further revealed that RavS-RavR affects the transcription of at least 245 genes. Among them, the transcript level of the global regulator

Clp decreased by 1.8 fold in the *ravR* mutant relative to the wild-type, and *in trans* complementation of *clp* in *ravR* mutant restored the EPS and extracellular enzyme production. Moreover, comparison of RavS-RavR and Clp regulons showed 172 out of 245 genes in common, indicating that RavS-RavR regulates downstream virulence factors through Clp (He et al., 2009).

Since RpfC-RpfG and RavS-RavR TCSTSs are responsible for degrading c-di-GMP, regulating biosynthesis of EPS and extracellular enzymes, and both carry out functions through the same downstream regulator (Clp), it is intriguing how these c-di-GMP dependent regulatory cascades maintain specificity and crosstalk during pathogenesis. In fact, signal sequestration among different pathways is a general problem since bacterial genomes usually encode a number of GGDEF, EAL and HD-GYP domain-containing proteins that are involved in c-di-GMP turnover (Tamayo et al., 2007). Although there is no study so far to address this problem specifically in *Xanthomonas* spp., evidence has accumulated to guide the future direction of investigation. (1) RpfC-RpfG and RavS-RavR seem to sense different stimuli, since deletion of *ravS-ravR* neither affected DSF biosynthesis nor led to form cell aggregation as RpfC-RpfG did (He et al., 2009). This result suggests that RavS-RavR is not involved in perception of the DSF signal and thus the expression of RpfC-RpfG and RavS-RavR regulons may be controlled by different environmental cues. (2) By green fluorescent protein (GFP) fusion assay, RpfC and RpfG were found to be localized predominantly at the cellular poles. However, in the background of the *rpfF* mutant, RpfG was localized throughout the cell (Ryan et al., 2010). To date, the cellular localization of RavS-RavR is unclear. As demonstrated in *Caulobacter crecentus*, a model bacterium in studying cell cycle, the principal regulatory PleD (which contains a GGDEF domain), the phosphodiesterase TipF and the c-di-GMP effector protein PopA were dynamically localized in different cell poles during cell division (Paul et al., 2008), suggesting that differential localization of c-di-GMP associated proteins provides a mechanism to sequester unwanted signal crosstalk, possibly by altering local c-di-GMP concentration. (3) The activity of RpfC-RpfG and RavS-RavR may be regulated by interactions with different auxiliary factors. As mentioned above, the binding of RpfC-RpfG with GGDEF-containing proteins plays an important role in regulating DSF-dependent factors (Ryan et al., 2010). If RavS-RavR does not interact with the same auxiliary proteins, this would also provide a potential functional or spatial mechanism to sequester different signal cascades.

3.3 HrpG system: a core regulator of virulence factor secretion through T3SS and T2SS

Pathogenic bacteria employ type III secretion systems (T3SSs) to deliver multiple effectors into host cells to induce, suppress or interfere with the immune responses of the host (Alfano and Collmer, 2004). In phytopathogenic bacteria, the *hrp* (hypersensive response and pathogenicity) gene cluster, consisting of more than 20 genes that are organized into several transcriptional units, is responsible for the T3SS assembly and secretion of effectors (Büttner and Bonas, 2010). Inactivation of the *hrp* genes usually causes deficiency in virulence and failure to elicit programmed cell death, referred to as a hypersensitive response (HR) on the nonhost plant. In *Xanthomonas*, it has been shown that HrpG, an OmpR-family RR with a C-terminal helix-turn-helix domain as the output region (Fig. 2), is the central protein regulating the expression of *hrp* genes (Wengelnik et al., 1996). Phosphorylated HrpG is indispensable for the transcription activation of an AraC-family transcription factor, HrpX. After being activated, HrpX regulates the expression of most, but not all, *hrp* genes, by interacting with a conserved *cis*-regulatory element named as PIP box (plant inducible promoter, consensus sequence TTCGC-N_{15}-TTCGC) within their promoter regions (Wengelnik and Bonas, 1996). A constitutively expressed GntR-family transcription factor, *trh*, has been identified in *Xoo*. Inactivation of *trh* decreases, but does not completely ablate, the expression of *hrpG*, *hrpX* and *hrp* genes when bacteria grow in T3SS-inducing minimum medium or *in planta*, indicating that *trh* is an upstream factor controlling HrpG regulon (Tsuge et al., 2006). However, mutants of *trh* have no detectable deficiency in virulence, suggesting that multiple regulatory pathways regulate the expression of HrpG (Tsuge et al., 2006). In addition, in *Xoo,* the zinc uptake regulator Zur has been shown to be required for eliciting HR reaction and indirectly modulating the transcription of a number of *hrp* genes via activation of *hrpX* (but not include *hrpG*) (Huang et al., 2009). Up to the present time, the cognate HK of HrpG remains unknown, and genome-scale approaches, such as the phosphotransfer profiling method developed based on kinetic preference between cognate HK and RR pair (Skerker et al., 2005), are required for future investigation in this context.

The bacteria of *Xanthomonas* encode two type II secretion systems (T2SS, including Xps and Xcs systems) which pump out plant cell-wall degrading enzymes (da Silva et al., 2002; Qian et al., 2005). Evidence accumulated to date indicates that signal crosstalk may occur between T2SS and T3SS. For example, an HrpG-dependent, MarR-family transcription factor was identified by both cDNA-AFLP analysis and genome-scale mutagenesis (Noël et al., 2001; Qian et al., 2005). Further investigation identified this gene as *hpaR* (hypersensitive response and pathogenicity-associated regulator) and showed that deletion of *hpaR* caused virulence attenuation and deficiency in eliciting a hypersensitive response. However, although the transcription of *hpaR* is dependent on the expression of *hrpG* and *hrpX*, this gene is not likely to affect the expression of *hrp* genes since inactivation of *hpaR* had no effect on expression of these genes (Wei et al., 2007).

Rather, enzymatic activities of the extracellular proteases (but not other extracellular enzymes such as amylase and endoglucanase) in an *hpaR* mutant were substantially higher than those of wild-type, indicating that HpaR is a specific suppressor of extracellular proteases (Wei et al., 2007). In *Xcc*, two T2SS (Xps)-dependent polygalacturonases (PghAxc and PghBxc) that are responsible for degrading pectic polymers of host plants were found to have perfect PIP boxes in their promoter regions. As anticipated, the expression of these two genes is regulated by HrpG, HrpX and Clp, indicating that the specific interaction between HrpX and PIP box plays an important role during signal crosstalk between T2SS and T3SS (Wang et al., 2008). Such HrpG-dependent T2SS-secreted enzymes were also identified in other species of *Xanthomonas*, including 11 extracellular enzymes in *Xac* and a cysteine protease in *Xoo* (Furutani et al., 2004; Yamazaki et al., 2008). Very recently, Szczesny et al. (2010) systematically characterized extracellular enzymes in *Xcv* and found that translocation of T3SS-dependent effectors into host plants is affected by T2SS. Whereas expression of the T2SS machinery genes *xpsE* and *xpsF* is enhanced, an extracellular xylanase (*xynC*) is down-regulated by HrpG and HrpX (Szczesny et al., 2010). Taken together, the crosstalk between T2SS and T3SS indicates that the two secretion systems are tightly coordinated. It will be interesting to determine how HrpG and HrpX activate or repress different T2SS-associated genes to obtain more adaptive fitness during bacterial pathogenesis.

3.4 VgrS-VgrR and VemR systems: two novel identified virulence-associated TCSTSs

VgrS-VgrR (also named as ColS-ColR) is a TCSTS identified in *Xcc* by two independent studies (Qian et al., 2008b; Zhang et al., 2008). Two TCSTSs are annotated as ColS-ColR in the genome, one of which in *Xcc* has different functions in regulating virulence factors while the other, in bacteria of *Pseudomonas*, controls phenol tolerance and colonization of plant roots (Dekkers et al., 1998; Hõrak et al., 2004). To avoid confusion, we have recommended renaming this system as VgrS-VgrR (virulence and growth regulator) (Qian et al., 2008b). VgrS is an HK containing a HAMP domain, and VgrR is an OmpR-family transcription factor (Fig. 2). Inactivation of these two genes causes a decrease in virulence, EPS production and growth in medium or *in planta* (Qian et al., 2008b). VgrS-VgrR is involved in eliciting HR reaction on the nonhost pepper plant, and expression of the *hrcC* and *hrcE* operons is positively regulated by VgrS-VgrR (Zhang et al., 2008). The involvement of VgrS-VgrR in modulating EPS production and *hrp* gene expression indicates that this system may interact with both pathogen triggered immunity (PTI) and effector triggered immunity (ETI) of host plants. Moreover, evolutionary analysis has revealed that the orthologs of VgrR and the signal transduction region of VgrS are highly conserved in the bacteria *Xanthomonas*. Intriguingly, the signal input domain of $VgrR_{Xoo}$ exhibits a substantially higher level of nonsynonymous amino acid substitution *vs* synonymous substitution rate (9.1 times the average level), indicating that this region has undergone substantial positive selection during bacterial speciation (Qian et al., 2008a). The rapid evolution of this sensing region implies that signal perception via the receptor VgrS may be quite different among *Xoo* and its close-relative bacteria *Xanthononas*. Consequently, the VgrS-VgrR system may represent a specific evolutionary case in which the mode of signal perception, rather than the transcription factor-DNA binding pattern as in other bacteria, changed during evolution (Perez et al., 2009). This would allow the modified TCSTS to recognize novel stimuli without affecting downstream signal transduction. We speculate that this kind of evolutionary pattern would significantly promote bacterial ability with a minimum addition of genetic load.

Recently an RR named VemR (virulence, EPS synthesis and mobility regulator) was identified (Tao and He, 2010). Deletion of this gene caused a substantial decrease in virulence, production of EPS and swimming ability in TYGS medium. *vemR* is also an "orphan" RR since no HK genes are located in the vicinity of its locus. Since its protein product does not have an output domain,VemR belongs to stand-alone RRs which may regulate downstream factors via protein-protein interactions. A *fleQ* gene encoding a putative c-di-GMP receptor is located near the *vemR*. Double mutants of *vemR* and *fleQ* showed a restored level of EPS production and cell motility, indicating that *fleQ* might be a repressor upstream of *vemR* (Tao and He, 2010). However, more genetic and biochemical evidences are needed to investigate the relationship between the two proteins.

3.5 RaxH-RaxR and PhoQ-PhoP systems: modulating the activity of the avirulence factor Ax21

In general, two kinds of plant innate immune responses have been defined in previous studies. One is referred to as pathogen-associated molecular pattern (PAMP) triggered immunity (PTI), where basal defensive response can be elicited by the pattern recognition receptor (PRR) of plant cells after sensing conserved pathogenic elicitors, such as flagellin, glucan, LPS and the elongation factor EF-TU. The other is called ETI, where a specific plant resistance protein (R-protein) recognizes a corresponding bacterial effector and then induces immune responses (Jones and Dangl, 2006). Interestingly, the first successfully-cloned resistance gene of the host plant of *Xanthomonas*, *Xa21*, appears to be involved in both PTI and ETI. *Xa21* is derived from the wild rice (*Oryza longistaminata*) and encodes a typical receptor kinase that is similar to PRR of animals, such as Toll-like receptor (TLR) (Song et al.,

1995). Initially, *Xa21* was regarded as a typical R gene since resistance occurred only when a rice cultivar containing *Xa21* interacted with an *Xoo* strain containing a corresponding avirulence gene (*avrXa21*), which is definitely according to the classical "gene-for-gene" relationship (Flor, 1974). However, after *avrXa21* (now named as *ax21*) was cloned (Lee et al., 2009), it was intriguing to find that unlike traditionally-identified bacterial effectors, Ax21 is secreted by a type I secretion system (T1SS), rather than by T3SS. Furthermore, this 194 amino acid-length protein is widely expressed by bacteria of *Xanthomonas*, rather than being specific to a limited group of *Xoo* strains (Lee et al., 2009), suggesting that Ax21 has properties of both an effector and a PAMP. Therefore, the dual attributes of Ax21 strongly support that the definition of PTI and ETI cannot be strictly separated.

In *Xoo*, a TCSTS (RaxH-RaxR) and six additional *rax* (required for AvrXa21 activity, including *raxA, raxB, raxC, raxP, raxQ* and *raxST*) genes were identified to be related to Ax21 (Burdman et al., 2004; da Silva et al., 2004). Among them, *raxA, raxB and raxC* encode a type I secretion system that secretes Ax21, and *raxP*, *raxQ* and *raxST* are involved in sulfation of a Tyr^{22} site within a 17 aa invariant peptide of Ax21 (Lee et al., 2009). The sulfation is critical to Ax21 activity in inducing disease resistance, indicating that post-translational modification plays an important role in invoking molecular recognitions between hosts and bacteria. Among these *rax* genes, inactivation of *raxH* or *raxR* causes a partial decrease in Ax21 activity, and this TCSTS may regulate the expression of the *raxSTAB* operon (Burdman et al., 2004). In addition, the expression of *rax* genes is population density-dependent and overexpression of *raxR* has been found to disrupt this trend (Lee et al., 2006). These results suggest that the RaxH-RaxR system is important in regulating the posttranslational modification of Ax21 and density-dependent expression of *rax* genes.

Besides RaxH-RaxR, the PhoQ-PhoP system of *Xoo* is also associated with the Ax21 activity. PhoQ-PhoP is a well-studied virulence regulatory TCSTS in enterobacteria, such as *Samolella enterica* and *E. coli* (Prost and Miller, 2008). In *Xoo*, the expression of PhoQ and PhoP is suppressed by RaxH-RaxR, and plant inoculation has shown that a mutation in *phoQ*, but not *phoP*, resulted in a slight decrease in Ax21 activity (Lee et al., 2008). It is not clear whether RaxH-RaxR and PhoQ-PhoP are sufficient to regulate Ax21 activity. Furthermore, PhoQ-PhoP of *Xoo* is required for full virulence of *Xoo* strains which do not encode Ax21, indicating that it also modulates the expression of other unidentified virulence factors. Similar to enterobacteria, this system can sense signals from Ca^{2+}, Mg^{2+}, acidic conditions and antimicrobial peptides (Lee et al., 2008). The deficiency of virulence in the *phoP* mutant can be partially restored by constitutive expression of *hrpG*, suggesting that HrpG is a downstream factor in PhoQ-PhoP regulon (Lee et al., 2008). In addition, it is noticeable that the orthologs of PhoQ-PhoP in *Xcc* are XCC3942 and XCC3943. In a systematic mutational analysis, the insertional mutants of XCC3942 and XCC3943 could not be generated, indicating that this TCSTS has an essential role in *Xcc* (Qian et al., 2008b). As a result, the question of whether the biological functions of orthologous PhoQ-PhoP systems are altered during microevolution needs to be clarified.

4 Concluding remarks and perspective

As discussed in this review, each genome of *Xanthomonas* spp. encodes approximately 100 TCSTS genes to sense and respond to environmental or intracellular stimuli (Qian et al., 2008a; Barakat et al., 2009). Among them, 12 genes have been experimentally identified to have roles in regulating various virulence and avirulence factors. Although genome-scale mutagenesis methods have been employed to screen for virulence regulatory TCSTS genes, the possibility that other genes not discussed here also contribute to virulence cannot be excluded. Firstly, while highly conserved in DNA sequences, orthologs of TCSTS genes may have quite different functions in regulating downstream gene expression, as revealed by RavS-RavR orthologs of *Xcc* 8004 and *Xcc* ATCC 33913 (Qian et al., 2008b), and RpfC-RpfG orthologs among *Xcc*, *Xoo* and *Xylella fastidiosa* (Dow, 2008). These results imply that microevolution of the TCSTS gene functions and their regulatory roles within the different genetic contexts are attractive topics for future study. Secondly, most of the virulence-associated TCSTS genes were identified by measuring the disease symptoms after wound inoculation on host plants. However, successful infection is a complicated process that includes bacterial colonization, entrance or penetration, survival, proliferation and transmission. Each process requires interactions with different host-derived factors and corresponding reprograming of gene regulation. It is necessary therefore to establish more sensitive pathological models to dissect these processes and elucidate the regulatory roles of TCSTSs. As a consequence, the currently identified TCSTSs can be regarded as a "core set" which modulates pathogenesis of *Xanthomonas* spp., as shown in Fig. 3.

For TCSTSs, the nature of the signals or ligands sensed by histidine kinases, and the manner in which histidine kinases detect stimuli, are general questions in this field (Goulian, 2010). Even for the most well-documented system, EnvZ-OmpR, which governs expression of the outer-membrane porins in *E. coli*, the signals monitored by EnvZ remain unknown (Gao and Stock, 2009). To date, only several signal perceptions have been clearly elucidated for bacterial TCSTSs, e.g., the enzymatic activities of LOV (light, oxygen, or voltage) domain-containing histidine kinases in *Brucella melitensis* can be induced by irradiation with blue light, requiring the formation of a covalent flavin cysteinyl adduct between flavin mono-

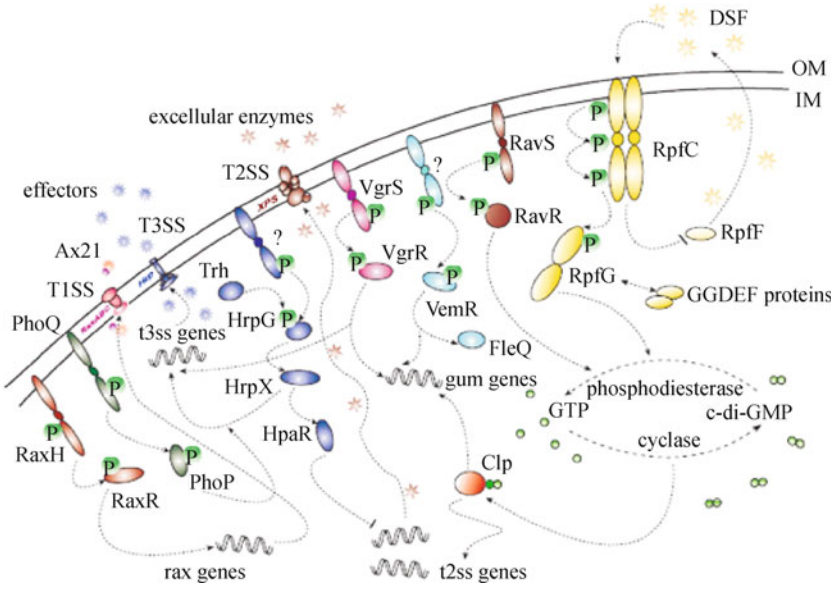

Fig. 3 Network of the two-component signal transduction systems in regulating virulence and avirulence factor expressions in *Xanthomonas* spp.. Different colors are applied to different signaling cascades. The RpfC-RpfG system (yellow) positively controls extracellular enzyme and exocellular polysaccharides (EPS) biosynthesis, and negatively regulates diffusible signal factor (DSF) synthesis. The RavS-RavR system (brown) controls EPS biosynthesis by affecting c-di-GMP turnover. VemR (light blue) is probably associated with FleQ. VgrS-VgrR system (pink) regulates EPS biosynthesis and transcription of the *hrp* genes. HrpG (blue) is the central regulator of type III secretion system (T3SS) and has relationship to the type II secretion system (T2SS). The RaxH-RaxR (light brown) and PhoQ-PhoP (green) systems are involved in expression and modification of the avirulence factor Ax21. OM: outer-membrane; IM: inner-membrane; T1SS: type I secretion system. P in the circular frame indicates the phosphoryl group. Names of the proteins and genes are according to the main text.

nucleotide (FMN) and a cysteine residue in LOV proteins (Swartz et al., 2007). In *Xanthomonas*, there is experimental evidence that virulence regulatory TCSTSs have the ability to sense certain environmental stimuli. For example, RpfC-RpfG may sense the DSF signal and PhoQ-PhoP may monitor concentrations of divalent ions such as Mg^{2+} and Ca^{2+} (Lee et al., 2008; Ryan et al., 2010). However, direct biochemical evidence is needed to support these hypotheses. Future studies will identify the signal perception mechanism coupled with protein phosphorylation and dephosphorylation reactions of these TCSTSs, especially when bacteria grow *in planta*.

The central mechanism of TCSTS regulation involves the maintenance of the appropriate amount of the active phosphorylated RR, which is achieved through two opposite biochemical pathways catalyzed by specific kinases and phosphatases, respectively (Stock et al., 2000). Usually the phosphatase activity is carried out by an additional auxiliary phosphatase, such as the CheZ of the CheA-CheY system, or by the histidine kinase itself, which also has the ability to dephosphorylate the RR (Silversmith, 2010). In *Xanthomonas*, all of the identified virulence-associated HKs belong to the structural I group, rather than the CheA-like, structural II group (Qian et al., 2008a), indicating that they have the enzymatic activities of kinase, phosphotransferase and phosphatase. However, to date there has been no study to address the question of how these HKs are regulated by specific input signals and how they dephosphorylate the cognate RRs. Future studies in this area will elucidate biochemical details of the phosphorylation-dephosphorylation process in regulating expression of virulence factors.

As mentioned above, virulence factors of *Xanthomonas* are not exclusively controlled by a single TCSTS. For example, EPS biosynthesis of *Xcc* is spontaneously modulated by RpfC-RpfG, RavS-RavR, VgrS-VgrR and VemR, and deletion of any of these systems substantially decreases EPS production. This prompts the question of how these TCSTSs are cross-regulated during bacterial infection, since illegitimate cross-phosphorylations from HKs to noncognate RRs are usually strictly prevented in cells by diverse molecular mechanisms (Laub and Goulian, 2007). One possibility of cross-regulation is that some TCSTSs have a cumulative effect on the expression of virulence factors by controlling the same regulator. An example is the interaction between RpfC-RpfG and RavS-RavR systems, during which Clp and c-di-GMP appear to act as signaling switches in controlling the transcription of the *gum* gene cluster (He et al., 2009). Furthermore, as revealed in *P. aeruginosa*, an HK RetS can physically interact with another HK GacS, and subsequently interfere with the phosphorylation of the latter (Goodman et al.,

2009). The direct interaction between heterologous HKs adds an additional level of regulation by integrating different inputs into a signaling cascade, and it will be interesting to determine whether TCSTSs in *Xanthomonas* also have this kind of regulation.

In conclusion, the TCSTSs of *Xanthomonas* spp. represent a fascinating model in which a number of signaling proteins constitute a signaling network to regulate virulence factor expression. Although many details remain to be investigated in the near future, available evidence indicates that signaling cascades within this molecular network are not only strictly programmed during pathogenesis, but have also evolved rapidly during the microevolution of bacterial strains. Further studies will undoubtedly shed light on how pathogenic bacteria sense exogenous signals and integrate information from various biochemical pathways, enabling them to successfully survive within different ecological niches.

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