Regulation of the Type III Secretion System in Phytopathogenic Bacteria

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The type III secretion system (TTSS) is a specialized protein secretion machinery used by numerous gram-negative bacterial pathogens of animals and plants to deliver effector proteins directly into the host cells. In plant-pathogenic bacteria, genes encoding the TTSS were discovered as hypersensitive response and pathogenicity (hrp) genes, because mutation of these genes typically disrupts the bacterial ability to cause diseases on host plants and to elicit hypersensitive response on nonhost plants. The hrp genes and the type III effector genes (collectively called TTSS genes hereafter) are repressed in nutrient-rich media but induced when bacteria are infiltrated into plants or incubated in nutrient-deficient inducing media. Multiple regulatory components have been identified in the plant-pathogenic bacteria regulating TTSS genes under various conditions. In Ralstonia solanacearum, several signal transduction components essential for the induction of TTSS genes in plants are dispensable for the induction in inducing medium. In addition to the inducing signals, recent studies indicated the presence of negative signals in the plant regulating the Pseudomonas syringae TTSS genes. Thus, the levels of TTSS gene expression in plants likely are determined by the interactions of multiple signal transduction pathways. Studies of the hrp regulons indicated that TTSS genes are coordinately regulated with a number of non-TTSS genes.

Numerous gram-negative bacterial pathogens of plants and animals rely on the type III secretion system (TTSS) to invade their hosts (Galan and Collmer 1999). The TTSS is a syringe needle-like structure consisting of inner and outer membrane rings and a protruding filament called hrp pilus in the plant pathogens (Kubori et al. 1998; Marlovits et al. 2004; Roine et al. 1997; Van Gijsegem et al. 2000; Weber et al. 2005). The hrp pilus functions as a conduit to guide the translocation of the type III effectors to the interior of the host cells (Jin and He 2001; Li et al. 2002). The TTSS is encoded by a cluster of approximately 20 hypersensitive response and pathogenicity (hrp) genes that are organized into several operons on either the chromosome or plasmid of plant-pathogenic bacteria (Arnold et al. 2003). The hrp genes clusters often are flanked by several type III effectors and other types of virulence-related genes; together, these genes form the pathogenicity island that is defined from the rest of the genome by tRNA or mobile elements (Alfano et al. 2000; Arnold et al. 2003). Additional type III effector genes are scattered in the rest of the bacterial genomes either in clusters or singly (Arnold et al. 2003). The expression of TTSS genes is coordinately regulated by various host and environmental factors (Arlat et al. 1992; Rahme et al. 1992; Schulte and Bonas 1992; Wei et al. 1992; Xiao et al. 1992). According to the hrp operon structures and the regulatory systems controlling TTSS gene expression, the hrp genes of the plant bacterial pathogens are divided into two main groups. The hrp genes of Erwinia spp., Pantoea stewartii, and Pseudomonas syringae are in group I, and those of Xanthomonas spp. and Ralstonia solanacearum are in group II (Alfano and Collmer 1997). Multiple signal transduction components have been identified for both groups of the hrp genes (Fig. 1A and B). This review will highlight hrp gene regulation in these phytopathogenic bacteria.

The regulatory system for group I hrp genes.

Group I hrp genes in Erwinia spp., Pantoea stewartii, and P. syringae are regulated by HrpL, a member of the ECF family of alternative sigma factors (Frederick et al. 2001; Wei and Beer 1995; Xiao et al. 1994). The HrpL proteins of these bacteria are highly conserved in amino acid sequences and all recognize a consensus sequence (GGAACC-N15/16-CCACNNA) named the “hrp box” in the promoters of the hrp operons and type III effectors (Fouts et al. 2002; Frederick et al. 2001; Wei and Beer 1995). The hrp box motif contains two conserved modules. Recent studies in Pantoea agglomerans pv. gypsophilae indicated that the hrp box consensus can be divided into crucial and noncrucial nucleotides. The five nucleotides (GGAAC) in the upstream module and the three nucleotides (ACNNA) in the downstream module are crucial, whereas other consensus and adjacent nonconsensus nucleotides exert a significant effect on the promoter’s strength (Nissan et al. 2005). The hrp box consensus sequence has served as an important genomic marker for the identification of candidate type III effector genes of the group I bacteria via computational analysis (Fouts et al. 2002; Zwiesler-Vollick et al. 2002). In addition, several functional genomic approaches have been developed to identify the type III effector genes in Pseudomonas syringae strains based on their dependence on hrpL for induction (Chang et al. 2005; Fouts et al. 2002; Lan et al. 2006; Losada et al. 2004). It was shown recently that HrpL directly binds the hrp box motif in gel shift assay but, surprisingly, mutations of the consensus nucleotides in the downstream module that eliminate the promoter activity do not affect the HrpL-binding (Nissan et al. 2005).

Consistent with the role as the transcriptional activator of TTSS genes, hrpL itself also is induced under the hrp-inducing
rimerization is believed to be crucial for the transcriptional activity of \( hrpL \) (Hutcheson et al. 2001). \( hrpS \) alone is capable of activating the \( hrpL \) gene in \emph{P. syringae} but only at a very low level, and the maximal induction of \( hrpL \) requires both \( hrpR \) and \( hrpS \) (Bretz et al. 2002; Grimm et al. 1995; Hutcheson et al. 2001). Both \( HrpR \) and \( HrpS \) contain an enhancer-binding domain and a motif that interacts with the \( \sigma^{54} \)-RNA polymerase holoenzyme. Consistently, transcription of \( hrpL \) is under the control of a \( \sigma^{54} \)-dependent promoter in an alternate \( \sigma \) factor RpoN-dependent manner (Chatterjee et al. 2002; Hendrickson et al. 2000). It was hypothesized that, under inducing conditions, \( HrpR \) and \( HrpS \) form a heterodimer on the \( hrpL \) promoter to stimulate the transcription of \( hrpL \) by interacting with the RpoN-RNA polymerase holoenzyme (Hutcheson et al. 2001). Additional regulators of \( hrpL \) were reported in \emph{Erwinia carotovora} subsp. \emph{carotovora} that include the RsmA/RsmB RNA-mediated post-transcriptional system and genes affecting the RsmA/rsmB production (Chatterjee et

**Fig. 1.** Models of type III secretion system (TTSS) gene regulation in phytopathogenic bacteria. The numbers indicate the major signaling events. **A**, Group I bacteria. 1, The hrpXY operon in \emph{Erwinia} spp. and \emph{Pantoea stewartii} is transcribed and translated into HrpX and HrpY proteins. 2, Signal in inducing medium (IM) triggers autophosphorylation of HrpX. 3, HrpX phosphorlylates HrpY. 4, HrpY activates the transcription of \( rpoD \) in \emph{Erwinia} spp., \emph{Pantoea stewartii}, and \emph{P. syringae}. 7 and 7', \( HrpS \) is repressed by HrpV via protein–protein interaction. 8 and 8', HrpG interacts with HrpV and derepresses HrpS. 9, HrpS and HrpR proteins form heterodimer in \emph{P. syringae}. 10, HrpR protein is degraded by Lon protease in \emph{P. syringae}. 11, HrpR and HrpS in \emph{P. syringae} and \emph{Pantoea stewartii} associate with RpoN in the \( hrpL \) promoter to activate \( hrpL \) expression. 12, \( hrpL \) RNA is degraded by RsmA-dependent mechanism. 13, \emph{rsmB} RNA inhibits the function of RsmA. 14, HrpL recognizes the hrp box promoter and activates the transcription of hrp regulons. 15, HrpA acts upstream of \( hrpRS \) transcription to stimulate TTSS gene expression. **B**, Group II bacteria. 1, Phl and PhrR proteins are encoded by the \( phlR \) operon. 2, PhrR protein is located in the bacterial inner membrane. 3, PhrA protein located in the outer membrane perceives the plant cell wall signal and transfers the signal to PhrR. 4, PhrR activates Phl. 5, Phl activates the transcription of \( phlH \). 6, PhlH activates the transcription of \( hrpG \). 7, PhlA represses \( hrpG \) at the post-transcriptional level. 8, HrgA activates the transcription of \( hrpX \) in \emph{Xanthomonas} spp. and \( hrpB \) in \emph{Ralstonia solanacearum}. 9, HrpX and HrpB activate the PIP/hrpII box promoters. Components of group II bacteria in the shaded area are required for \( hrp \) gene activation in both inducing medium and plants. Components in the unshaded area are \emph{R. solanacearum} genes specifically required for \( hrp \) gene activation in plants. IM indicates unknown signal in inducing medium.
The regulatory system for group II hrp genes.

Most group II hrp operons are activated by an AraC-like activator, which was designated HrpB in *R. solanacearum* (Gemin et al. 1992) and HrpX in *Xanthomonas* spp. (Kamdar et al. 1993; Wengelnik and Bonas 1996). The HrpX and HrpB proteins are highly similar at the amino acid level. The promoters of *hrp* operons and type III effector genes regulated by HrpX and HrbP often carry a conserved motif termed plant-inducible promoter (PIP-box) (TTGCC-N15-TTCGC) in *Xanthomonas* spp. (Buttner and Bonas 2002) and hrpb-II-box (TTCG-N16-TTCG) in *R. solanacearum* (Cunnac et al. 2004a). Individual mutations of the PIP/hrpII-box consensus nucleotides generally reduce the promoter activity; however, alterations of the space between the two conserved modules abolish the promoter induction (Cunnac et al. 2004a; Tsuge et al. 2005). A search of the *Xanthomonas* spp. and *R. solanacearum* genome sequence with this consensus sequence identified a large number of candidate type III effector genes (Cunnac et al. 2004b; da Silva et al. 2002; Thieme et al. 2005). Some of these genes were identified as the *hrpX/hrpB*-induced genes by microarray, promoter trap assay, and cDNA differential display (Mukaihara et al. 2004; Noel et al. 2001; Occhialini et al. 2005). Nonetheless, it should be pointed out that several well-
characterized type III effector genes in Xanthomonas spp., including avrBs1 and members of the avrBs3 family, are constitutively expressed and do not have a PIP-box in their promoters. Even the promoters of some HrpX/HrpB-regulated effector genes do not have the PIP/HRP-II box and, thus, probably are regulated indirectly by HrpX/HrpB (Buttner and Bonner 2002; Occhialini et al. 2005).

The hrpX and hrpB genes are activated by the HrpG proteins in Xanthomonas spp. and R. solanacearum (Brito et al. 1999; Wengenilk et al. 1996). The HrpG proteins in Xanthomonas spp. and R. solanacearum share >40% amino acid identity. HrpG belongs to the OmpR family of the two-component response regulators, with an N-terminal receiver domain and a C-terminal DNA-binding motif (Brito et al. 1999; Wengenilk et al. 1996). Using the yeast two-hybrid analysis, HrpG of Xanthomonas axonopodis pv. citri was found to interact with a two-component system sensor kinase and a few other bacterial proteins of unknown function (Alegría et al. 2004); it remains to be determined whether this sensor kinase has the ability to phosphorylate HrpG of its receiver domain and whether phosphorylation of HrpG is important to its regulatory activity. Nonetheless, three point mutations in the HrpG protein of X. campestris pv. vesicatoria render the protein constitutively active even under TTSS repressive conditions (Wengenilk et al. 1999), suggesting that conformational change of the HrpG protein likely plays an important role in TTSS gene regulation.

HrpG in R. solanacearum is a converging point of signal transduction pathways mediating TTSS gene induction (Brito et al. 2002). hrgP is constitutively expressed in complete and minimal media but induced by a plant-specific signal (Brito et al. 1999). Four signal transduction components acting upstream of hrgP to mediate the hrg gene induction specifically in plants are discussed in the following section. It has been identified recently that PhcA is a negative regulator of the HrpG protein in complete medium, probably via a post-transcriptional mechanism (Genin et al. 2005). PhcA is a LysR family transcriptional regulator that coordinates the expression of multiple virulence factors such as exopolysaccharides, various plant cell-wall-degrading enzymes, quorum sensing, and bacterial motility (Schell 2000). The phcA mutation does not affect the transcription of hrgP but elevates the expression of TTSS genes in the complete medium (Genin et al. 2005).

Regulation of TTSS genes by host and environmental signals.

Host sensing is critical for a successful infection by bacterial pathogens. Upon sensing the host, pathogenic bacteria reprogram their transcription, which results in coordinated activation of TTSS genes (Brencic and Wimans 2005). Animal bacterial TTSS genes are activated upon contact with host cells (Francis et al. 2002). In Yersinia pseudotuberculosis, the contact with HeLa cells is believed to trigger the release of LcrQ, a type III secreted protein that negatively regulates TTSS gene expression (Pettersson et al. 1996). This TTSS-dependent feedback regulation also operates the contact-induction of TTSS genes in Y. enterocolitica (Stainier et al. 1997).

Several studies elegantly demonstrated that the R. solanacearum TTSS genes are induced when the bacterium is in contact with the plant cell (Aldon et al. 2000). Unlike animal bacteria, the contact-dependent induction of type III genes in Ralstonia spp. does not require the type III pilus. Instead, it is mediated by PrhA, a membrane protein with significant similarities with siderophore receptors (Marenda et al. 1998). The prhA mutation disrupts the contact-dependent type III gene induction but not the induction by the minimal medium (Marenda et al. 1998), suggesting that distinct signals are perceived from host cell and culture medium. The plant signal perceived by PrhA is likely a nondiffusible plant wall component that is resistant to protease and heat treatments (Aldon et al. 2000); however, the chemical nature of the signal remains unknown. Additional components acting downstream of PrhA are PrhR, Phl, PrhJ, HrpG, and HrpB that function in a sequential order (Brito et al. 1999, 2002). HrpG and HrpB are required for induction of TTSS genes in both minimum medium and plants, whereas PrhR, Phl, and PrhJ are required specifically for the induction by plant cells. PhrR and Phh are in the same operon and encode a transmembrane protein and a sigma factor of the ECF family, respectively (Brito et al. 2002). PrhJ is a LuxR/UhrA family transcription activator (Brito et al. 1999). PrhR and Phh act upstream of PrhJ and, together, the three proteins are required for the induction of hrgP which, in turn, activates hrgB in response to host cell contact. According to Brito and associates (2002), a plant cell-wall-derived signal, likely a macromolecule, is perceived by PrhA. PrhA subsequently transfers the signal to PrhR, which sequentially activates the transcription factor genes prhl, prhj, hrgP, and hrgB. The prhA, prhJ, prhl, and prhR mutants all are affected in pathogenicity in Arabidopsis and hypersensitive response (HR) in resistant plants (Brito et al. 1999, 2002; Marenda et al. 1998). In particular, the prhJ mutant is completely nonpathogenic in plants. Together, the results demonstrate that host-cell sensing by R. solanacearum is fundamental to pathogenesis.

The involvement of plant-specific signals in inducing TTSS genes is likely true for other bacteria as well. For example, it was reported that induction of the P. syringae pv. phaseolicola hrpL gene in planta is much greater compared with the induction by the inducing medium, suggesting the presence of a plant-derived signal (Rahme et al. 1992). In addition, mutants of a conserved hypothetical protein have been isolated recently in two pathovars of P. syringae that completely abolish the hrg promoter induction in plant but only marginally affect the hrg promoter induction in the inducing medium. The mutants displayed significantly reduced pathogenicity on their host plants, bean and tomato, and the HR-inducing activity on the nonhost tobacco plants (Y. Xiao and X. Tang, unpublished results). Similarly, a heat-stable compound derived from a plant suspension culture was reported to induce TTSS genes of X. campestris pv. vesicatoria (Schulte and Bonas 1992).

Certain environmental conditions, including temperature, medium composition, and pH, also affect TTSS gene expression (Arlat et al. 1992; Huynh et al. 1989; Rahme et al. 1992; van Dijk et al. 1999; Wei et al. 1992). TTSS genes of plant bacteria are suppressed by rich media but induced by minimal synthetic media. The inhibitory activity of rich media is exerted by high pH and osmolarity and complex carbon and nitrogen nutrient sources. TTSS-inducing media are acidic, low osmotic, nutritionally poor, and contain certain sugars as the carbon source. The optimal expression of TTSS genes can be obtained when the P. syringae bacteria are grown at 20 to 30°C (van Dijk et al. 1999). These environmental conditions are thought to simulate the physiological environment encountered by bacteria during infection. The factors influencing TTSS gene expression in the inducing medium differ somewhat between different pathogens, which may reflect the differences between the apoplastic conditions of different host species. For example, the nature of carbon source plays a critical role for TTSS gene activation, and fructose and sucrose are the best inducers of the P. syringae TTSS genes among the carbon sources tested (Huynh et al. 1989). Minimal medium supplemented with mannitol induced the avrB gene in P. syringae pv. glycinea almost as well as did the medium supplemented with fructose (Huynh et al. 1989); however, mannitol is unable to induce TTSS genes in P. syringae pv. phaseolicola strain NPS3121 (Y. Xiao and X. Tang, unpublished results). Differ-
ential sugar effects also are established for *R. solanacearum* (Arlat et al. 1992). It is possible that in planta induction of TTSS genes requires both plant-specific signals and suitable physiological and chemical conditions in the plant apoplast.

In a recent study attempting to identify host signals involved in TTSS gene regulation, an *Arabidopsis attl* loss-of-function mutant was isolated that supports significantly higher induction of TTSS genes than did the wild-type plant (Xiao et al. 2004). This observation suggests the existence of plant-associated signals that negatively regulate TTSS genes. *ATT1* encodes a cytochrome P450 monoxygenase catalyzing fatty acid hydroxylation. In plants, hydroxylated fatty acids form extracellular polyesters that are major constituents of cutin, which envelopes the aerial part of the plant. The cutin content in *att1* mutant is reduced to 30% of the wild-type level, and the composition of cutin monomers also is largely altered in the *att1* mutant compared with the wild-type plant, indicating that *ATT1* plays an important role in biosynthesis of cutin monomers. Certain commercially available fatty acids, such as 9,10-epoxy stearic acid, and plant cutin extracts can specifically suppress the induction of TTSS genes (Xiao et al. 2004). Taken together, certain cutin monomer species may act as negative signals to regulate TTSS genes in planta. The negative regulation of TTSS genes by cutin-related signals may be important to epiphytic fitness of *P. syringae* bacteria that repress TTSS genes during epiphytic growth on the leaf surface (Xiao et al. 2004). How the cutin-related negative signal is perceived by *P. syringae* remains to be elucidated.

The coregulation of TTSS and other virulence-related genes.

In addition to TTSS genes, in vivo expression technology assays in *P. syringae*, *R. solanacearum*, and *Erwinia* spp. have identified a large number of non-TTSS genes that also are induced in the plant (Boch et al. 2002; Brown and Allen 2004; Yang et al. 2004), indicating coregulation of TTSS genes with other biological processes during the bacterial interaction with plants. The regulatory mechanisms for many of the non-TTSS genes remain unclear. However, a number of the non-TTSS genes are induced by the hrp regulators such as *hrpL*, *hrpB*, *hrpG*, and *hrpX*. Co-induction of these genes with TTSS genes likely enables the bacteria to cope with various stress factors encountered during the interaction with the plant and exploit the host nutrients to promote growth.

It has been shown in *P. syringae* pv. *tomato* DC3000 strain that the *iaaL*, *cfa1*, and *cfa6* genes are induced by *hrpL* (Chang et al. 2005; Fouts et al. 2002; Lan et al. 2006). *iaaL* encodes indoleacetate-lysine ligase capable of producing IAA conjugates, an inactive form of the phytohormone auxin. *iaaL* has an “hrp box” motif in its promoter. DC3000 is capable of producing auxin (Glickmann et al. 1998) that may interfere with an “hrp box” motif in its promoter. *DC3000* is capable of producing IAA conjugates, which has a putative *hrp box* promoter and likely are induced by *hrpL* (Chang et al. 2005). Two hemagglutinin-like protein genes and two transposase genes also were shown to be *hrpB*-induced by microarray (Occhiolini et al. 2005). A few of the *hrpB* upregulated non-TTSS genes identified by microarray also were identified by the *hrpB*-dependent promoter trap assays (Mukaihara et al. 2004). A significant number of the *hrpB*-activated non-TTSS genes have the *hrpII-box* in their promoters. Similarly, a number of *hrpG/hrpX*-induced non-TTSS genes were identified by cDNA differential display in *X. campestris* pv. *vesicatoria* (Noel et al. 2001). It was reported recently that a gene encoding a protein secreted by the general secretion pathway in *X. oryzae* pv. *oryzae* has a “PIP box” promoter and is *hrpX*-activated (Furutani et al. 2004).

Perspectives.

The studies of bacterial TTSS gene regulation in the previous years enabled the construction of signal transduction pathways immediately upstream of the *hrp* effector genes. Although additional regulatory genes have been identified regulating TTSS genes in various plant bacteria, the connection of these regulatory components with the downstream pathways remains obscure for most of these genes. The key puzzles for TTSS gene regulation are the identity and nature of the host and environmental signals regulating TTSS gene expression, the bacterial sensory proteins perceiving the signals, and the links between signal sensors and TTSS gene expression. The possible cross-talk between TTSS gene expression and other biochemical processes in bacteria also remains to be exploited.

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LITERATURE CITED


