

Hrp-dependent Biotrophic Mechanism of Virulence: How Has It Evolved in Tumorigenic Bacteria?

I. Barash¹ and S. Manulis²

A mechanism of virulence mediated by *hrp*-genes is present in many Gram-negative bacterial pathogens. It involves delivery of effector proteins into host cells *via* the type III secretion system (TTSS) and the interaction of TTSS effectors with plant proteins. These interactions may either promote responses beneficial to the pathogen or trigger the hypersensitive response if an effector is recognized by corresponding resistance protein. *Pantoea agglomerans*, which is widespread in nature mainly as an epiphyte, has evolved into a *hrp*-dependent and host-specific tumorigenic pathogen by acquiring a plasmid containing a pathogenicity island (PAI). This PAI harbors a *hrp*-gene cluster, and genes encoding for TTSS effector proteins and biosynthesis of IAA and cytokinins. The results reviewed describe how the interplay between negative-acting and positive-acting TTSS effectors determines the transformation of *P. agglomerans* into two related pathovars. Furthermore, the PAI's structure supports the premise that these pathovars are recently evolved pathogens. Finally, the possible interaction between TTSS effectors and phytohormones for gall formation is proposed.

KEY WORDS: Type III secretion system; Type III effectors; biotroph; necrotroph; plasmid; *hrp*-gene cluster; galls; IAA; cytokinins; virulence; pathogenicity island; IS elements.

The strategies used by plant pathogens to invade and colonize host plants can be broadly divided into necrotrophic and biotrophic. In the necrotrophic strategy, as exemplified by soft rot bacteria such as *Erwinia carotovora*, the pathogens kill the host tissue in advance and feed on the contents, whereas in the biotrophic strategy, as exemplified by necrogenic bacteria such as *Pseudomonas syringae*, the pathogens multiply as a parasite on living cells and induce delayed symptoms. In order to multiply effectively within the host prior to symptoms appearance, biotrophic pathogens have to (a) suppress the basal host defenses that operate also in susceptible plants and (b) acquire sufficient nutrients from the neighboring living cells to support their growth. To achieve these goals, Gram-negative phytopathogenic bacteria, with the exception of *Agrobacterium* spp., have developed a sophisticated mechanism that allows them to deliver virulence effector proteins directly into the host cell *via* the type III secretion system (TTSS) (11). Once inside the host cell and depending upon the plant genotype, these TTSS effectors may become positive-acting determinants and initiate signaling pathways to promote responses beneficial to the pathogen. Alternatively, if these effectors are recognized by corresponding resistance (R) proteins, they act as negative determinants known as avirulence proteins (Avrs), and trigger a rapid localized programmed cells death (hypersensitive response, HR) at the infection site. Although suppression of basal or induced host defenses by several TTSS effectors

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¹Dept. of Plant Sciences, Tel-Aviv University, Ramat Aviv 69978, Israel. e-mail: isaaci@post.tau.ac.il

²Dept. of Plant Pathology, ARO, The Volcani Center, Bet Dagan 50250, Israel. e-mail: shulam@volcani.agri.gov.il

has been demonstrated, information on their other expected functions, namely, nutrient acquisition or release of bacteria to the organ surface, is either absent or very scanty (4).

The presence of *hrp* (HR and pathogenicity) and *hrc* (hr conserved) genes is essential for pathogenicity of most Gram-negative phytopathogenic bacteria. Mutations in *hrp/hrc* genes eliminate pathogenicity of the bacteria in susceptible plants and the ability to elicit HR in resistant or non-host plants (24). Furthermore, these genes are necessary for supporting bacterial multiplication within the host apoplast, the natural plant habitat of biotrophic bacteria. The *hrp/hrc* genes encode for the TTSS proteinaceous elicitors of HR in non-host plants (*i.e.*, harpins) and regulatory proteins. The latter proteins activate the genes encoding for the TTSS machinery and also the TTSS effectors, in response to the apoplast environment. The *hrp/hrc* genes are organized in large clusters comprising >20 genes that are highly conserved among phytopathogenic bacteria. A key feature of the *hrp/hrc*-gene-encoded TTSS is the Hrp pilus that serves as a conduit for mobilization of TTSS effectors into the host cell (15).

During the past two decades many *avr* genes have been cloned from phytopathogenic bacteria and have generally been found to be diverse in structure (22). The observations that *avr* genes are *hrp* regulated and function inside the host cell identified them as TTSS effectors (19). Various Avr proteins have been shown to function, albeit weakly, as virulence factors in hosts lacking the corresponding R genes. The recent whole genome sequencing of several phytopathogenic bacteria (see 4) allowed genomic mining of TTSS effectors through bioinformatics and functional genomic analysis and prompted their identification. The questions of what is the inventory of TTSS effectors by a given bacterium and how many of them are necessary for pathogenicity may soon be resolved (6). The virulence role of most intracellular TTSS effectors is not yet completely understood and neither is the exact nature of the R-Avr interaction that leads to HR response. However, recent studies indicate that the simple hypothesis from Flor's gene-for-gene theory (18), *i.e.*, that R proteins may act as receptors for Avr effector ligands, is not valid in most cases (36). Instead, a model was proposed (8) where R proteins act as guards to monitor the behavior of molecules that are targets of the TTSS effectors. Accordingly, the Avr products interact with and modify the virulence targets that are non-R cellular factors. The R protein perceives the altered status of the virulence target and induces a rapid defense response. Thus the R proteins actually act to accelerate the overall defense response (4,8). Recent reports (*e.g.* 25,26) strongly corroborate the guard hypothesis. The function of a few effectors has recently been demonstrated (17). Research on the *hrp*-dependent biotrophic mechanism of phytopathogenic bacteria has been continuously subjected to many critical reviews that are not cited here.

Until the mid 1990s, prior to our work on the tumorigenic pathogen *Pantoea agglomerans* (formerly *Erwinia herbicola*) pv. *gypsophylae*, the prevailing notion was that the *hrp*-dependent mechanism of virulence is exclusive to necrogenic but not tumorigenic bacterial pathogens. This idea was nourished from the reported absence of *hrp* genes from *Agrobacterium tumefaciens* (20) and the lack of reported *hrp* genes in the olive knot-forming pathogen *P. syringae* subsp. *savastanoi* (*Psav*) as well as the primary role of auxin and cytokinins reported for these gall-forming bacteria (32,41). Only recently, *hrp*-dependency was also reported in gall formation by *Psav* (37). In the present mini-review we shall briefly describe the current knowledge on the involvement of TTSS effectors in host specificity of *P. agglomerans* pv. *gypsophylae* and its related pathovar *P. agglomerans*

pv. betae. Furthermore, we shall provide support to the idea that *hrp*-dependent virulence mechanism has only lately been acquired by these two pathovars and may interact with indole-3-acetic acid (IAA) and cytokinin to elicit gall formation. A comprehensive review on gall formation by *P. agglomerans* was published 2 years ago (27) and only relevant and recent information will be described here.

Pantoea agglomerans is widespread in nature mainly as an epiphyte but also as an endophyte on many different plants. *P. agglomerans* *pv. gypsophila* (*Pag*) elicits galls on the crown only in gypsophila (7), whereas *P. agglomerans* *pv. betae* (*Pab*) causes galls on both beet and gypsophila (2). Both pathogens may cause moderate to severe economic losses to their respective hosts. When we started our studies of *Pag* and *Pab*, our basic question was what caused these pathogens to switch from commensal bacteria associated with many different plants into host-specific tumorigenic pathogens? Demonstrating that the pathogenicity of both pathovars is determined by an indigenous pathogenicity plasmid designated as pPATH_{*Pag*} and pPATH_{*Pab*} for *Pag* and *Pab*, respectively (28,29), has provided the key to understanding how these pathogens have evolved. Since IAA and cytokinins have been considered primary pathogenicity factors in the two known major tumorigenic bacteria *A. tumefaciens* and *Psav* (32), we concentrated first on elucidating the role of these phytohormones in gall formation by *Pag* (5,23). Simultaneous inactivation of the pathways for IAA and cytokinin biosynthesis in *Pag* substantially reduced gall size but did not eliminate gall initiation (30), suggesting that the primary virulence factors might be other than the phytohormones. A breakthrough in understanding the virulence of these pathogens was achieved by the identification of a functional *hrp* gene cluster on the pPATH_{*Pag*} that was mandatory for gall formation (31,34). As indicated earlier, these reports refuted the previously existing concept that tumorigenic bacteria lack the *hrp*-dependent virulence mechanism. The sequence of the pPATH_{*Pag*} (~140 kb) demonstrated the presence of a pathogenicity island (PAI) of approximately 75 kb. In addition to the *hrp*-gene cluster, this PAI accommodates genes encoding TTSS effectors, as well as a cluster of IAA and cytokinin biosynthetic genes (5,9,10,13,23,39). The presence of several copies of highly diverse IS elements on the PAI could signify an extensive horizontal gene transfer that presumably was instrumental in the PAI creation. Although the PAI in pPATH_{*Pab*} has not yet been extensively studied, it showed close similarity to pPATH_{*Pag*} (28).

One of the most intriguing questions is: how has the host specificity of *Pag* and *Pab* evolved? Our studies demonstrated clearly that these two related pathovars have acquired host specificity by the interplay between negative-acting and positive-acting TTSS effectors. The gene *pthG* was isolated from pPATH_{*Pag*}, but only remnants of its sequence were detected on the pPATH_{*Pab*} (9). PthG exhibits a dual function: it encodes a protein that acts as a virulence effector in gypsophila and as an Avr on multiple beet species (10). Mobilizing *pthG* into the beet pathovar *in trans* caused the latter to induce HR on beet, but to retain full pathogenicity on gypsophila and HR on tobacco. Moreover, marker exchange of a mutated *pthG* into *Pag* caused the latter to extend its host range and induce galls on beet while substantially reducing gall formation on gypsophila (9). Thus, PthG may be responsible for the inability of *Pag* to infect beet. In contrast to PthG, HsvG was characterized as a positive-acting TTSS effector that determines host specificity on gypsophila by both pathovars (38,39). *hsvG* was located on both pPATH_{*Pag*} and pPATH_{*Pab*}. Mutations in *hsvG* eliminated the ability of *Pag* and *Pab* to induce gall formation on gypsophila but still allowed full pathogenicity of *Pab* on beet. HsvG, which

has a molecular mass of 72 kDa, is composed of 672 amino acids with two consecutive direct repeats, RI=74 aa (359-453aa) and RII =71 aa (453-524aa), with 88% identity. Recently we have identified two *hsvG* homologs (74% aa identity), one on pPATH_{Pag} and the other on pPATH_{Pab}, that confer host specificity on beet (as opposed to *hsvG* specificity on gypsophila) (G. Nissan *et al.* unpublished). These homologs, designated as *hsvB*_{Pag} or *hsvB*_{Pab}, respectively, lack partially or completely the RI repeat. Deletion of RI in HsvG endows the latter with HsvB function and *vice versa*, insertion of an intact RI to HsvB caused it to function as HsvG. Studies are now in progress to determine whether the two repeats are equivalent in function. These findings are reminiscent of the AvrBs3 family of *Xanthomonas campestris* in which the number of amino acid repeats determines host specificity but mainly in the cultivar level (21). However, the number of the repeat domains in AvrBs3 protein is high and additional fundamental structural differences exist between HsvG/HsvB and AvrBs3. Further preliminary studies have suggested that HsvG and probably HsvB effectors possess a gene-activation domain and are suspected to act as DNA binding proteins and potential transcriptional effectors in the host plants (G. Nissan, unpublished results).

All the above-mentioned results led us to hypothesize that host-specific genes were instrumental in the evolution of the two pathovars. We hypothesize that the *hsvG* and *hsvB* genes have split off at an early stage of evolution, possibly through gene duplication (12) and allowed the creation of an ancestor strain pathogenic on beet and gypsophila. Recognition of a TTSS virulence effector (*i.e.*, PthG) as an Avr protein by the beet defense surveillance system resulted in an HR response on beet and generation of *Pag*, the current gypsophila pathovar. The beet pathovar, *Pab*, may have evolved from *Pag* by inactivation of *pthG* through partial deletion and modification (9) and thus evaded the HR response. The need to replace the virulence function of PthG and presumably the partial isolation of the two pathovar populations on two different hosts led to their divergent evolution. Thus, although both pathovars can elicit galls on gypsophila they may not necessarily carry identical genes for virulence on this host. This supposition is supported by different morphologies of galls induced by *Pag* and *Pab* on gypsophila (2).

The concept of PAIs has emerged to describe genomic regions of pathogens which carry virulence genes together with loci the presence of which strongly indicates horizontal gene transfer between species or even genera (14). Some of the major characteristics of PAI include: (i) a cluster of virulence genes; (ii) its presence in pathogenic but not in closely related nonpathogenic stains; (iii) a different G+C content from that of the host bacteria DNA; (iv) occupation of large chromosomal regions; and (v) the presence of (often cryptic) 'mobility' genes, namely, IS elements, transposases and integrases. The definition of PAIs originally included chromosomal location but the increasing volume of sequence data available for virulence plasmids, particularly in human-pathogenic bacteria, extended the definition also to plasmids (14). The PAIs on the pPATH_{Pag} or pPATH_{Pab} comply with the above-mentioned characteristics (27).

It appears that PAI structures strongly reflect different stages of evolution. While some PAIs are composed of compact genetic elements carrying a functionally related cluster of genes, other PAIs carry many cryptic genes, open reading frames (ORFs) of unrelated functions or pseudogenes (14). Over time, the sizes of successful PAIs tend to be reduced to include only genes required for key functions. The PAI in pPATH_{Pag} is characterized by lower GC content, as compared to the species, and harbors IS elements,

interspersed pseudogenes, various unrelated ORFs and remnants of known genes from different bacteria (28). This information as well as the relatively large size of the PAI and its location on a plasmid, strongly support the premise that it exists in its early stages of evolution. In contrast, the chromosomal *hrp* PAI of *P. syringae* provides an example of evolutionary advanced PAI (1). It has a compact tripartite mosaic structure composed of a cluster of TTSS effector genes bound by exchangeable effector and conserved effector loci that contribute to parasitic fitness and pathogenicity in plants. It is noteworthy that the difference in the evolutionary stages between the PAIs of *Pag* and *Pseudomonas* could have an impact on the contribution of TTSS effectors to virulence. The pPATH_{*Pag*}-born PAI accommodates five characterized and five putative TTSS effectors (27; and M. Panijel, unpublished). Mutations in all the five characterized TTSS effector genes either completely eliminated pathogenicity or substantially reduced gall formation. In contrast, *P. syringae* effector genes do not have a strong phenotype when mutated because they are redundant (6). We postulate that mutations in *Pag* genes significantly affect virulence because this is a recently emerged pathogen. *Pag* has not yet had the need to acquire other functionally redundant effectors by horizontal gene exchange.

The term macroevolution denotes evolutionary processes, which occur within a longer period of time and may lead to the formation of new species or pathotype (33). In contrast, microevolution, which generates new variants of a given species or pathotypes, may take only weeks or even days. We assume that both processes contributed to the creation of PAIs in *Pag* and *Pab*. The comparative analysis of the structure of PAI among different strains of each pathovar should provide an insight into the relative impact of macro- and microevolution in the transformation of *P. agglomerans* into a pathogen. Thus structural diversity within PAIs of different strains in the same pathovar could result from ongoing processes of rearrangements, deletions, etc., that are characteristic to microevolution. In contrast, stable PAIs may favor macroevolution as a dominant factor in shaping their structure. Moreover, the distribution of the PAI among pathogenic strains can be analyzed in the light of two hypotheses: (i) clonal multiplication of a given pathogenic strain and (ii) conjugative transfer of the plasmid among genetically diverse strains. Preliminary results suggest the presence of a genetically diverse population of *Pag* (D. Wienthal *et al.*, unpublished). This may favor conjugative transfer of pPATH_{*Pag*} and pPATH_{*Pab*} under natural conditions which has not yet been demonstrated.

How did *P. agglomerans* profit from its transformation into a biotrophic pathogen? By acquiring the *hrp*-dependent mechanism of virulence, clones of this commensal *bacterium* succeeded in improving their competitive ability by conquering new, exclusive ecological niches, namely, their specific host plants. Furthermore, they have gained the ability to generate galls by overproduction of IAA and production of cytokinins through genes residing on the pPATH. It may be postulated that the active cell division involved in the hyperplasia during gall development serves as a sink for nutrients mobilization into the infection sites.

The role of IAA and cytokinins in gall formation by *Pag* and *Pab* deserves some comment. The pPATH_{*Pag*} and pPATH_{*Pab*} harbor a cluster containing the genes encoding for IAA biosynthesis through the indole-3-acetamide (IAM) pathway and for isopentenyl transferase, the key enzyme for cytokinin biosynthesis (27). Genes for IAA biosynthesis *via* the indole-3-pyruvate (IPyA) pathway are located on the chromosome of pathogenic and nonpathogenic *P. agglomerans* strains. The differential contribution of IAM and of the

IPyA pathways for IAA biosynthesis to pathogenicity and epiphytic fitness, respectively, was demonstrated in *Pag* (30). Thus, inactivation of the IAM pathway substantially reduced gall formation but not epiphytic fitness, whereas inactivation of the IPyA pathway adversely affected epiphytic fitness but not pathogenicity. Moreover, cytokinins and IAA production *via* the IAM pathway were found to be mandatory for gall formation by *A. tumefaciens* (40) and *Psav* (16). In contrast to the foregoing bacteria, a triple *Pag* mutant in which the two pathways for IAA production and cytokinin biosynthesis were inactivated, caused substantial reduction in gall appearance but still did not eliminate gall initiation (30). Anatomical studies performed on the small galls produced by the triple *Pag* mutant showed characteristic features caused by IAA, suggesting its involvement in gall initiation (3). These results could imply that production of IAA and possibly cytokinins by additional bacterial pathways may take place under *in vivo* conditions. Alternatively, it can be postulated that TTSS effectors ‘injected’ by *Pag* into the host cell enhance mobilization of plant-synthesized phytohormones into the infection site or increase sensitivity of plant cells to phytohormones, as reported for the *tml* gene in *A. tumefaciens* (42), or both.

Many plant-associated bacteria synthesize IAA (35). While IAA produced by phytopathogenic bacteria, *via* the IAM pathway, has been implicated in the induction of plant tumors, it was not clear whether IAA synthesized *via* the IPyA pathway could also be involved in gall formation. A recent report has demonstrated that cranberry stem gall is induced by multiple opportunistic bacteria, including *P. agglomerans*, which produce IAA only through the IPyA pathway (40). Opportunist pathogens are unspecialized and attack plants under special predisposed conditions which might generally be difficult to reproduce by artificial inoculation. A prerequisite for stem gall production in cranberry is bacterial multiplication to a high population level (40). In comparison, the *hrp*-dependent biotrophic mechanism has endowed *Pag* and *Pab* with a clear survival advantage, namely, the ability to initiate disease on their host-specific plants without competition and with relatively minimal predisposing conditions using only a few bacterial cells for infection.

Since *Pag* and *Pab* may be regarded as recently evolved pathogens, they provide an excellent model for understanding the transformation of a commensal bacterium into a host-specific pathogen. Understanding such a phenomenon is important for gaining insight into how new variants of existing pathogens or new pathogens originate in nature. The origin of the two related PAIs that occupy part of the pPATH_{*Pag*} and pPATH_{*Pab*} and allow the conversion of *P. agglomerans* into *Pag* and *Pab* is not known. It is now assumed that bacteria may acquire pathogenicity not by slow adaptive evolution but by ‘quantum leaps’ that enable them to acquire complete virulence systems in the course of one or a few steps. Thus it is possible to assume that the PAI originally evolved in other bacteria and has been acquired *via* horizontal transfer by a plasmid of *P. agglomerans* to create the pPATH. The intimate association of *P. agglomerans* strains with plants as an epiphyte or endophyte could facilitate its conversion into a pathogen, since at least a few required virulence genes had presumably pre-existed. The presence of the PAI in genetically different populations of *Pag* could result from conjugative transfer of the pPATH_{*Pag*}. It remains to be determined whether such a transfer is still an ongoing process, as the Ti in *A. tumefaciens*, or has been lost. Host specificity at the species or genus level is most likely a complex phenomenon and in most cases may involve interactions among several genes. However, understanding the structure-activity relationship and the mode of action of individual effectors such as HsvG and HsvB is a prerequisite for understanding the mechanisms leading to host range determination.

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