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Mini review

Hrp pilus – reaching through the plant cell wall

Martin Romantschuk, Elina Roine and Suvi Taira
University of Helsinki, Department of Biosciences, Division of General Microbiology, P.O. Box 56
(Viikinkaari 9C), FIN-00014 University of Helsinki, Finland (Phone: +358 919159219; Fax: +358 919159262;
E-mail: Martin.Romantschuk@Helsinki.fi)

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Abstract

A number of Gram negative pathogenic bacteria are equipped with a macromolecule translocation machinery that enables them to inject virulence determinants directly into the cells of their eukaryotic hosts. For plant pathogens, the plant cell wall represents an extra challenge for such injection. The ability to overcome this barrier has turned out to be so beneficial that two non-homologous injection systems, type III and type IV secretion machineries, have evolved. A morphologically similar pilus structure is associated with both of these systems. In this review, the direct protein translocation by type III (Hrp) secretion machinery is discussed with focus on the function of Hrp pilus. Comparisons between the Hrp system and the analogous translocation of T-DNA by *Agrobacterium* type IV secretion machinery are made and the differences between type III systems of plant and animal pathogens are discussed. Alternative hypothetical models for the mechanistic mode of action of the pilus are presented.

Introduction

The interaction of biotrophic and even some necrotrophic bacteria with the target plant is very complex and involves the delivery of proteinaceous signals into the plant cell. The result of this interaction depends on the host–pathogen combination. In plant pathogenesis the outcome may be disease; a situation where the bacteria multiply in the plant tissues causing disease symptoms without being halted by the plants defence. Alternatively, the plant may develop the hypersensitive reaction (HR), which is a rapid defence response characteristic of challenge with potentially pathogenic bacteria and leads to elimination of the bacteria (for a review see Leach and White, 1996). Ample evidence, although mainly indirect, implies that the decision on the outcome is based on recognition between specific bacterial proteins, and their receptors in the plant, and occurs inside the plant cell cytoplasm or in the nucleus. How the bacterial effector proteins are delivered from the bacteria into the plant cell has remained an open question for a long time. Steps towards answering the question came with the sequence analysis of bacterial *hrp* genes necessary for both HR elicitation and pathogenesis. Several *hrp* genes were found to be homologous to the genes encoding components of type III secretion machinery. Type III secretion has turned out to be a widespread mechanism in Gram negative bacteria and several recent reviews are available (Alfano and Collmer, 1997; Hueck, 1998; He, 1998). The DNA-sequence of the type III secretion gene cluster has partly or totally been determined for a growing number of animal pathogens, such as *Salmonella*, *Shigella*, *Yersinia*, and plant pathogens, such as *Pseudomonas*, *Xanthomonas*, *Ralstonia*, *Erwinia* (reviewed by Hueck, 1998). Homologues are found also in some non-pathogenic plant associated bacteria, such as *Rhizobium* (Viprey et al., 1998) and *P. fluorescens* (Rainey, 1999).

The plant pathogenic bacteria employing the type III secretion system are extracellular pathogens and, in contrast to the animal pathogens, they must translocate their effector proteins through an extra barrier, the plant cell wall. In *P. syringae*, a long pilus structure associated with the Hrp secretion system is the main candidate for being the physical link directing the protein traffic between the bacterium and the plant cell interior (Roine et al., 1997a). Structural and functional analogies can be drawn to a non-homologous translocation system, the T-DNA transfer by *Agrobacterium tumefaciens* into plant cells. In this review, we will focus on the structures involved in the type III secretion dependent protein translocation process. By comparing homologous and analogous systems, taking into account what is presently known, we will hypothesize over how this protein transport might be accomplished.

Protein secretion and translocation in pathogenesis

The cell wall of Gram negative bacteria is a multilayered diffusion barrier which is not penetrable for macromolecules such as proteins. Since many of the bacterial pathogenesis-associated proteins function outside the bacterial cell, the proteins have to be translocated out of the cell to the external medium or directly into target cells. At least four distinct secretion pathways (types I, II, III, and IV) have been identified in Gram negative plant pathogenic bacteria (see Fath and Kolter, 1993; Pugsley, 1993; Hueck, 1998; and Christie, 1997 respectively). All of these secretion systems are employed in translocation of virulence proteins in plant pathogens (reviewed by Salmond, 1994). Extracellular proteases and cell wall degrading enzymes of e.g. *Erwinia* utilize the type I and type II pathways (general secretion pathway). Protein secretion through these two pathways results in release of proteins into the extracellular milieu. In contrast, type III and IV pathways are also able to translocate macromolecules into the eucaryotic host cells, apparently without an extracellular stage. The type III pathway can translocate effector proteins directly into the cell cytoplasm (reviewed by He, 1998) and the type IV pathway of *Agrobacterium* translocates T-DNA-protein complexes directly into the plant cells (reviewed by Burns, 1999; Zupan et al., 1998).

The four secretion pathways share little homology with each other, although they are used for analogous functions. The closest homologues to the type IV pathway are certain conjugation machineries that perform interbacterial DNA transfer. By this homology, and mechanistic similarities in DNA transfer, the type IV system can be considered a special modification of conjugation (reviewed in Christie, 1997). In the type III secretion pathway, an outer membrane protein YscC/HrcC is homologous to secretin proteins associated with type II secretion dependent protein release, and extrusion of filamentous phages from the bacterial cell, and it forms a pore in the outer membrane (reviewed in Hobbs and Mattick, 1993). The closest gene homologues for the innermost (inner membrane associated) components of the type III secretion machinery are found among genes needed for flagellar assembly. The flagellar apparatus can be considered a genuine secretion pathway since flagellin monomers travel to the exterior through the already formed flagellar structure. Recently, the flagellar apparatus has been suggested to belong to the type III secretion family (Macnab, 1999). Interestingly, in addition to flagellar components, secretion of a virulence factor, the YlpA phospholipase of *Y. enterocolitica*, has been shown to be dependent on the flagellar secretion machinery (Young et al., 1999).

Pili and translocation

Type III and type IV translocation associated pili

Pilus structures have been associated with the protein translocating type III (Figure 1) and IV pathways and they appear to be essential components of the secretion machineries. In both cases, they are believed to form a bridge between the bacterial and host cells during translocation. Transmission of T-DNA, and pathogenesis of *Agrobacterium* are dependent on the formation of pili, where VirB2 is the major pilin subunit (Lai and Kado, 1998; Lai et al., 2000). Correspondingly, in *P. syringae*, *hrpA* encodes the major pilin subunit and is indispensable for protein secretion and pathogenesis (Roine et al., 1997; Taira et al., 1999; Wei et al., 2000). Although the pilins of these plant pathogens share no similarity, they are both used to form a long and flexuous filament structure. The physical barrier for translocation of virulence determinants, formed by the Gram negative bacterial cell wall and the target plant cell wall, is the same for both *Agrobacterium* and *Pseudomonas*. Despite the lack of gene homology, the technical solutions for the translocation seem to be the same.

Hrp pilus and other type III secretion appendages

Animal pathogens *Salmonella* and *E. coli* that use type III secretion for pathogenic interaction, do not seem to

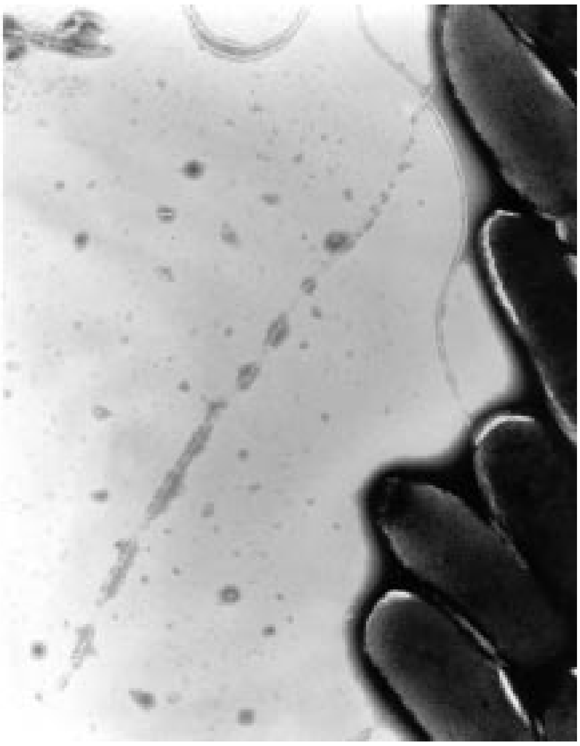

Figure 1. Hrp pilus labelled with HrpA antiserum and gold. Transmission electron microscope picture of *P. syringae* DC3000 grown in Hrp inducing conditions. Several micrometers long pilus structure labeled with HrpA antiserum can be distinguished from the thicker flagella also expressed by DC3000. Picture provided by Ian Brown, Wye college, U.K.

rely on several microns long slender extracellular structures like the Hrp pili – instead shorter, up to 1 μm long and about 50 nm thick appendages have been observed (Ginocchio et al., 1994; Ebel et al., 1998; Knutton et al., 1998). The expression of these appendages appears to be transient and they seem to retract or detach from the bacterial cells immediately after the type III secretion dependent morphological changes of the host cells become visible. The transient nature of the type III pilus expression has been reported also in the plant pathogen *Ralstonia solanacearum* (Van Gijsegem et al., 2000), where the pili seem to detach from bacteria grown *in vitro* after logarithmic growth phase. The inner components of the type III secretion apparatus form so called secretons or needle structures, spanning the bacterial inner and outer membrane. These structures have been isolated from animal pathogens *Salmonella* (Kubori et al., 1998) and *Shigella* (Blocker et al., 1999). The pilus-like appendages were not present in the secretion preparations indicating a loose connection between the pilus and the core secretion apparatus. Attempts to isolate morphologically similar secretion structures from *Pseudomonas syringae* have not been successful.

Although there is as yet no direct evidence that the type III secretion-associated appendages of animal pathogens serve an equal or comparable function to Hrp pili, it is tempting to suggest this to be the case, and that the observed morphological differences of these structures reflect the differences in host cell surface. An elongated thin pilus would have evolved to enable penetration of the porous plant cell wall. Indirectly supporting this notion is the fact that cell wall degrading enzymes of for example plant pathogenic *Pseudomonas* strains play only a minor role if any, in pathogenesis (Bauer and Collmer, 1997). Bacterial cells can be seen in electron micrographs in plants to be physically separated from the plant cell membrane by the intact plant cell wall even during ongoing infection (Bestwick et al., 1998; I. Brown and J. Mansfield, Wye College, U.K., personal communication).

Hrp pilins are variable

The pilin gene is the least conserved part of the type III secretion machinery – the identity between pilins of, for example, *P. syringae* pathovars *tomato* and *glycinea* is only about 30%. It would be tempting to suggest that the high variability of the pilins reflects the different host specificity of the pathovars, but this does not fit with observations regarding the HR (see below). The variability may simply be due to the exposed location of the protein. Exposed structures are ideal receptors for bacteriophages (Romantschuk and Bamford, 1985), and evolutionary pressure towards the pilin variation could rise from avoidance of phages rather than ability to invade a certain plant race or species.

The Hrp pilus of *P. syringae* DC3000 is so far the best characterized type III secretion associated pilus. The major pilin subunit, HrpA, is a 113 amino acid long linear protein that is structurally similar to several other pilin proteins (Roine et al., 1997a). The pilins for the *Agrobacterium* T-pilus and conjugal pili are also small but in contrast to HrpA they are cyclic peptides (Eisenbrandt et al., 1999). When isolated, HrpA was found as four different sized proteins processed amino terminally at three specific sites (Roine et al., 1997b). Both the full length and the processed pilins can autoassemble into pilus-like filaments (Roine et al., 1997b), and mutational analysis of the pilin protein has shown that the cleavable amino terminal part is not needed for the pilus function during infection (Taira et al., 1999). Interestingly, the most conserved part of the *P. syringae* pv. *tomato* and pv. *glycinea* pilins is this dispensable amino terminus. The *hrpA* gene of *Erwinia amylovora* is shorter than those of *P. syringae* strains (Kim et al., 1997), and the weak homology between them is found in the carboxy terminus of the *P. syringae* genes, indicating that *Erwinia* lacks the amino terminus found dispensable in *P. syringae*. Mutational analysis of the *P. syringae hrpA* gene suggests that the domains needed for pilus assembly are located in the carboxy terminus (Taira et al., 1999).

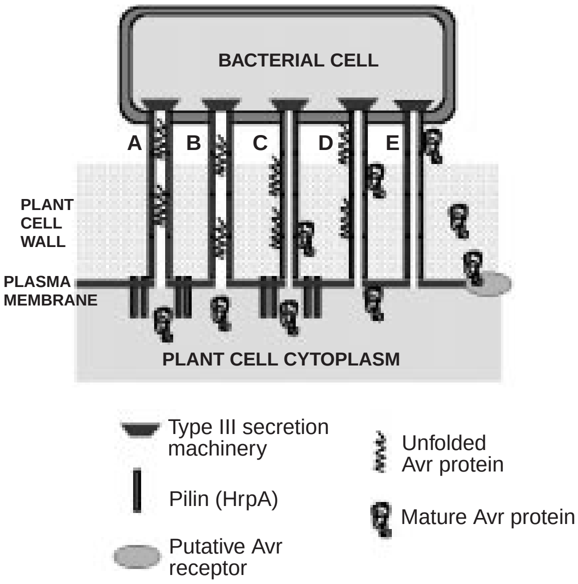

Figure 2. Schematic representation of hypothetical models for the Hrp pilus functional mechanism. The bacterial cell is seen lined up against a section of the plant cell surface. The cell membranes are separated by the plant cell wall matrix, but the bacterium is still able to translocate gene products into the plant cell interior. A–E represent proposed mechanisms for involvement of the pilus in the translocation process. A: The pilus is assembled at the base and disassembled at the plant membrane – the Avr protein is translocated inside the moving pilus and released into the plant cell; B: The pilus is stationary (assembled at the base or the tip) and functions as a pipeline for the Avr protein, which might be pumped through the tube; C: As A, but the Avr proteins are bound to the outside of the pilus; D: As B but with the Avr on the outside. Here we have no suggestion for how the movement of the Avr towards the plant membrane takes place; E: The pilus is merely a docking devise and the Avr proteins are secreted to the medium and separately taken up by the plant. In this review mainly cases A–D are discussed.

The role of Hrp pilus in translocation

If avirulence/virulence determining effector proteins are transported from the bacterial cell into the cytoplasm of the target plant cell, and assuming that this transport occurs directly from cell to cell and not via an extracellular intermediate, the question remains: how does this translocation take place? Here we will discuss hypothetical mechanistic alternatives to protein translocation from the perspective of the pilus, and relate the process to what we know about systems that may be functionally similar.

Docking device or guide?

The first open question is whether the Hrp pilus is merely an adhesion device or if it is directly involved in protein translocation (Figure 2A–D vs. E). At present, the best characterized pilus-employing macromolecule translocation system is the conjugation apparatus, reviewed by Firth et al. (1996). In the favoured conjugation model, the pilus is thought only to initiate the translocation process by binding the recipient cell and retracting in order to bring the mating cells into close contact, after which a junction is formed between the interacting cells, enabling DNA transport to occur. However, there is also evidence for transfer between cells that are not in surface contact (Harrington and Rogerson, 1990), suggesting a more direct role for the pilus. In the animal pathogen *Shigella*, close contact with the target cell is critical for protein translocation (Blocker et al., 1999) and also in *E. coli* (EPEC strains) the appearance of the type III appendage is, as with conjugation, a pre-requisite for intimate contact between the bacterium and the host (Knutton et al., 1998). Here, formation of a membrane fusion between the pathogens and their host cells would be theoretically possible. For plant pathogens, the problem of traversing the plant cell wall still remains, and forming a membrane fusion through the wall seems highly unlikely. The only structure observed so far that could span the wall is the Hrp pilus. Hence, although far from proven, the working hypothesis is that the main function of the pilus is in directing the Avr proteins to their target, i.e. that the pilus indeed plays an active role in protein translocation.

Plant cell receptors for the pilus?

In order to form any type of cell-to-cell link, the pilus has to make contact with the recipient cell, and this type of interaction has always been observed to require a receptor on the recipient cell surface. Most of the classical adhesion fimbriae use polysaccharides exposed on the host cell surface, but proteins also function as bacterial receptors (reviewed in Westerlund and Korhonen, 1993). A pilus receptor has not been identified for any of the type III machineries, neither for plant nor animal pathogens. Many plant pathogenic *hrp* harboring bacteria elicit the HR in a wide variety of plants. Thus, if the HR indicates successful protein delivery, the plant cell receptor should be present in all plants able to respond to the invader. The pilus receptor on the plant cell should hence be highly conserved. In *E. coli*, Intimin and Tir (transported intimin receptor) are associated with type III secretion, but function separately from the type III pilus. Both proteins are encoded by the bacterium and Tir is a type III secreted protein that is targetted to the host cell membrane (for a review see DeVinney et al., 1999). The idea of the bacterium inserting its own receptor into the host should be kept in mind in the quest for the pilus receptor. This notion opens up the new question of whether the bacterially encoded receptor itself requires a receptor to be able to associate with the target cell, or whether it inserts non-specifically into the target membrane.

A conveyor or a tube?

If the Hrp pilus is indeed directing the proteins into the plant cell, the second open question is whether it is a tube enclosing the proteins being transported, or are the Avr/Vir proteins translocated on the outside of the pilus (Figure 2A/B vs. C/D)? If Avr proteins travel attached to the outside of the pilus it would infer that the plant cell wall is yielding enough to allow relatively large folded proteins to pass. Harpins (HrpZ and/or HrpW) have been suggested to be plant cell wall destabilizing enzymes, which could loosen the wall structure (Charkowski et al., 1998) and facilitate passage of the pilus.

By homology with the flagellar system, the tube theory would seem more plausible. In flagellar assembly the flagellin subunits travel through the hollow basal body and flagellum structures, and the flagellar assembly occurs at the tip of the growing organelle (Macnab, 1992). Structural analysis of the flagellar filament has revealed a 3 nm axial channel (Morgan et al., 1995), large enough to accommodate and allow movement of an extended protein, but probably not a folded one. In comparison, the conjugation pilus with an outer diameter of 8-10 nm has a central lumen of 2 nm (Ippen-Ihler and Minkley, 1986; Marvin and Folkhard, 1986). The outer diameter of the *P. syringae* Hrp pilus is about 8 nm (Roine et al., 1997a), and if it is analogously helically assembled, the central lumen could also be around 2 nm in diameter.

To drag or to pump?

Yet another mechanistically interesting question is the movement of the Avr proteins in relation of the pilus structure (Figure 2A/C vs. B/D). If we assume that the Hrp pilus grows through the plant cell wall, by being pushed by forces generated upon assembly of the filament, avirulence proteins could also be included in the filament, perhaps being embedded inside the pilus, and they would thus be pushed through the plant cell wall. When the tip of the pilus reaches the plant cell membrane, the pilins would dissolve into the membrane and the pilus would thus continue to be in motion carrying the Avr protein through the membrane releasing it into the cytoplasm. Mechanistic analogies to this model can be found by examining the entry of filamentous phages, such as f1 through the *E. coli* host cell wall. Attachment of the phage to the pilus tip causes retraction of the pilus. Upon pilus disassembly the pilins re-enter a pool in the bacterial membrane (reviewed by Firth et al., 1996; Jacobson, 1972) bringing the phage particle to the cell wall. In further steps viral capsid proteins are inserted into the membrane and uncover the viral nucleic acid which is released into the cytoplasm (Click and Webster, 1998). In discussing the filamentous phage – *hrp/avr* analogy several assumptions must be made: (a) the pilus can disassemble and the components can be integrated into a recipient membrane (such as the plant cell plasma membrane) during pilus growth, and (b) the pilus-embedded macromolecule can be a protein (Avr) instead of a nucleic acid. This model postulates that small amounts of the pilin should be found in the plant cell membrane as a result of Avr transfer.

Alternatively, the proteins might also travel through a static pilus and thus move in relation to the pilus, in analogy with the flagellin being translocated through the flagellum. The force to drive such movement may be generated at the bacterial cell wall, if the proteins are pushed forward by later translated units. The energy metabolism of the type III secretion is compatible with this model. Only one protein resembling an ATPase has been found in *hrp* gene clusters. The general type II secretion also involves only one putative energy providing protein presumably required for 'pumping' proteins to the outside. The VirB2 pilin-encoding locus of *Agrobacterium* contains three putative energy producing proteins for transfer of T-DNA and also several ATPases are associated with the conjugal transfer of DNA. The energy requirements of these pili seem to be similar to the system used by *Pseudomonas aeruginosa* type IV pilus, where two extra ATPases are needed for pilus retraction and reassembly (reviewed by Alm and Mattick, 1995). Hence, by energy requirements, the type III secretion resembles the general secretion pathway rather than retractile pili, but a constantly growing pilus (see above) lacking the retraction function is not ruled out. However, the difference in supply of ATPases suggests a less dynamic role of the pilus in translocation, in comparision with conjugal- and T-pili.

Pilus assembly and regulation of the secretion

Hrp pilus assembly is still an open question, but once resolved, it will shed light on the secretion process as well. Pili and fimbriae that have been studied in sufficient detail have been shown to grow from the base so that pilin monomers are added one by one to form a helical array being pushed out through the bacterial cell wall (Hultgren et al., 1991; Ippen-Ihler and Minkley, 1986). If the pilus grows from the base like the fimbriae or the conjugation pilus, the translocated proteins could enter the hollow structure while the pilus is growing. If the pilus grows from the tip like the flagellum, the expression and/or translocation of the Avr proteins should be regulated so as not to enter the pilus before the circumstances are right (such as the pilus reaching its target).

Flagellar gene expression is controlled by the assembly and growth of the flagellar apparatus in such a way, that genes needed in later assembled structures are transcribed only after the earlier ones are ready (Hughes et al., 1993). Also in the *hrp* cluster, indications of such controlled expression exist. In mutants deficient in formation of the inner pore through the cytoplasmic membrane, the intracellular amount of harpin (HrpZ) does not increase, whereas in an outer membrane pore (*hrcC*) mutant the total amount of cell bound HrpZ does increase, but the protein accumulates in the periplasm (Charkowski et al., 1997). Hence, apparently the intracytoplasmic level of the secreted proteins is regulated. Recently, the Hrp pilin has also been suggested to have a regulatory role in gene expression (Wei et al., 2000). By comparing to other complex bacterial structures characterized in sufficient detail (ribosomes, bacteriophages etc), it is clear that not only relative amounts of protein and other components but also timing of the appearance of these components is controlled. There is no reason to assume the hrp/type III protein delivery system would not be subjected to a same kind of regulation.

Concluding remarks

Determining the actual steps of the infection process and pathogenesis on a cellular or molecular level is a demanding task in plant–microbe interactions. In part this is due to lack of convenient tools, such as monolayer cell cultures, and simply due to the structure of the plant cell wall. For animal pathogenic bacteria, the type III protein translocation causes drastic histological changes in the recipient animal cell, often leading to the internalization of the bacterium. In contrast, during early interaction of Hrp-secreting plant pathogens with their hosts gross changes are not seen in either of the participating cells. Hence, the protein traffic occurs between cells physically separated by the plant cell wall. In both types of pathogen–host interactions the delivery apparatus is optimized to achieve the same goal, to deliver effector proteins into the host. In plant–bacterial interaction the requirement for longer distance delivery is reflected in the Hrp pilus morphology.

In spite of the fact that type III secretion has been in the scientific spotlight during the last decade, the basic nature of the translocation process is still obscure. The leading hypothesis of the pilus being the physical conduit between the bacterium and the host is still merely an educated guess, being indirectly supported by what is known about the homologous flagellar transport system. The unresolved questions we are facing in type III protein translocation are the same that remain unanswered in the study of bacterial conjugation, where there is still a dispute about the molecular function of the conjugal pilus in the DNA translocation process. This may reflect the lack of proper tools for studying such transient/dynamic processes. Here seemingly different interactions have been compared, in order to find common denominators and underlying common mechanisms. The new hypotheses which have emerged can be tested by further experiments and highlight the numerous dynamic processes performed by bacteria.

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