

# The Hrp pilus of *Pseudomonas syringae* elongates from its tip and acts as a conduit for translocation of the effector protein HrpZ

Chun-Mei Li, Ian Brown<sup>1</sup>, John Mansfield<sup>1</sup>, Conrad Stevens<sup>1</sup>, Tristan Boureau, Martin Romantschuk and Suvi Taira<sup>2</sup>

Department of Biosciences, Division of General Microbiology, University of Helsinki, PO Box 56, FI-00014, University of Helsinki, Finland and <sup>1</sup>Department of Agricultural Sciences, Imperial College at Wye, University of London, Ashford, Kent TN25 5AH, UK

<sup>2</sup>Corresponding author  
e-mail: suvi.taira@helsinki.fi

C.-M.Li and I.Brown contributed equally to this work

**The type III secretion system (TTSS) is an essential requirement for the virulence of many Gram-negative bacteria infecting plants, animals and man. Pathogens use the TTSS to deliver effector proteins from the bacterial cytoplasm to the eukaryotic host cell, where the effectors subvert host defences. Plant pathogens have to translocate their effector proteins through the plant cell wall barrier. The best candidates for directing effector protein traffic are bacterial appendages attached to the membrane-bound components of the TTSS. We have investigated the protein secretion route in relation to the TTSS appendage, termed the Hrp pilus, of the plant pathogen *Pseudomonas syringae* pv. *tomato*. By pulse expression of proteins combined with immunoelectron microscopy, we show that the Hrp pilus elongates by the addition of HrpA pilin subunits at the distal end, and that the effector protein HrpZ is secreted only from the pilus tip. Our results indicate that both HrpA and HrpZ travel through the Hrp pilus, which functions as a conduit for the long-distance translocation of effector proteins.**  
*Keywords:* pathogenesis/pilus assembly/protein translocation/*Pseudomonas syringae*/type III secretion

## Introduction

The type III secretion system (TTSS) is a virulence mechanism shared by many unrelated Gram-negative bacteria that cause diseases in plants and animals (reviewed by Hueck, 1998). Diverse examples include *Yersinia pestis*, the cause of bubonic plague, and pathovars of *Pseudomonas syringae*, which attack several crop plants. The conservation of common virulence determinants in a wide range of bacteria infecting phylogenetically distant eukaryotic hosts is intriguing. The export system enables bacteria to deliver proteins, termed effectors, directly to the eukaryotic target cell (Galan and Collmer, 1999). In plant pathogenic bacteria, the components of the TTSS are encoded by a cluster of *hrp* genes. Mutations in *hrp* genes lead to pleiotropic loss of the ability to elicit the hypersensitive reaction (HR) in resistant plants and

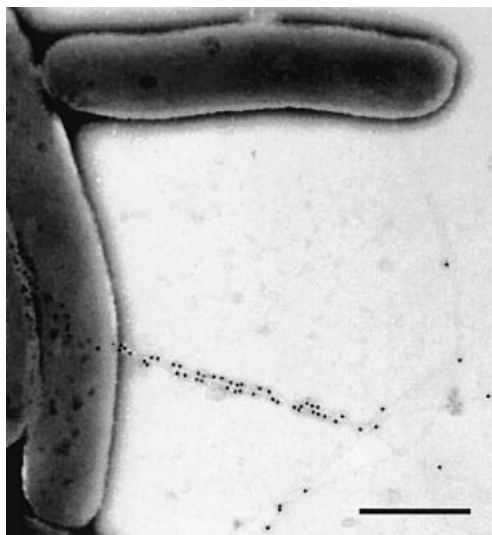
pathogenicity towards hosts (Alfano and Collmer, 1997). Two classes of effector proteins are secreted by the TTSS in plant pathogens: harpin proteins, such as HrpZ, which are readily exported *in vitro* and target cell membranes (Lee *et al.*, 2001a,b); and avirulence (Avr) and virulence (Vir) proteins, such as AvrPto and VirPphA, which are at best poorly secreted in culture, but are believed to be translocated directly to the plant cell cytoplasm (Jackson *et al.*, 1999; Jin *et al.*, 2001). Expression of *avr* genes in resistant plants activates the HR via interaction with the protein products of resistance (*R*) genes (Stevens *et al.*, 1998). The precise role of the effectors in plant pathogenesis is unknown. In the absence of matching *R* genes, both Avr and Vir proteins are thought, like effectors from animal pathogens, to suppress host defences by interaction with intracellular targets (Leach and White, 1996; Tsiamis *et al.*, 2000). The HrpZ harpin from pathovars of *P. syringae* has been demonstrated to bind to lipid bilayers and to form an ion-conducting pore *in vitro* (Lee *et al.*, 2001a).

The common components of the TTSS architecture found in both plant and animal pathogens are: a structure genetically homologous to the flagellar basal body, which traverses the bacterial inner membrane (Hueck, 1998; Kubori *et al.*, 1998); an outer membrane-puncturing pore composed of a protein belonging to the secretin family (Hobbs and Mattick, 1993); and a genetically and morphologically varying extracellular appendage (Ginocchio *et al.*, 1994; Roine *et al.*, 1997a; Ebel *et al.*, 1998; Knutton *et al.*, 1998; Kubori *et al.*, 1998; Blocker *et al.*, 1999; Hoiczky and Blobel, 2001). The Hrp pilus of the plant pathogen *P. syringae* pv. *tomato* DC3000 is the best characterized example of a TTSS-associated appendage (Roine *et al.*, 1997a,b; Taira *et al.*, 1999; Brown *et al.*, 2001). Mutations in the major pilin subunit gene *hrpA* lead to the Hrp minus phenotype and failure to secrete Avr and harpin proteins such as AvrPto and HrpW *in vitro* (Wei *et al.*, 2000). The Hrp pilus is, therefore, an indispensable component of the functional TTSS. The HrpA pilin is itself translocated to the bacterial surface by the internal components of the secretion apparatus.

The first TTSS appendage was found in *Salmonella typhimurium* by Ginocchio *et al.* (1994), who reported that surface appendages are formed upon contact with epithelial cells. Later, similar structures were observed on shiga toxin-producing *Escherichia coli*, and the appendages were associated with TTSSs (Ebel *et al.*, 1998). The Hrp pilus of *P. syringae* was the first TTSS appendage observed in plant pathogenic bacteria (Roine *et al.*, 1997a). Similar structures have now been reported in *Erwinia amylovora* (Jin *et al.*, 2001) and *Ralstonia solanacearum* (Van Gijsegem *et al.*, 2000). So-called needle complexes have been isolated from TTSS-expressing *Salmonella*, *Shigella*, *Yersinia* and *E. coli* (Kubori *et al.*,

1998; Blocker *et al.*, 1999; Hoiczky and Blobel, 2001; Sekiya *et al.*, 2001). Needle complexes are isolated type III secretion apparatuses that resemble morphologically the flagellar basal body with a needle-like extension attached at the distal end. Structures puncturing the bacterial inner membrane, the periplasm and the outer membrane of the bacterium were distinguished by electron microscopy. The needles observed in all four bacteria were thinner than the appendages observed by Ginocchio *et al.* (1994) and Ebel *et al.* (1998). In *E.coli*, a thick sheath-like structure was shown to be attached onto the core needle complex (Sekiya *et al.*, 2001). The filament resembled in size the appendage observed by Ebel *et al.* (1998), and indeed it was shown, by immunoelectron microscopy, to be the same structure composed of EspA protein. The EspA filament has been shown to be involved in long-distance protein translocation to eukaryotic cells, allowing protein transfer without direct cell-to-cell contact between *E.coli* and its host (Shaw *et al.*, 2001).

The Hrp pilus of *P.syringae* differs morphologically from the appendages found in animal pathogens. It is ~6–8 nm in diameter (Roine *et al.*, 1997a), thinner than the EspA filament, but similar to the needle of *Salmonella*. Importantly, the Hrp pilus can extend to a length of several micrometres (Brown *et al.*, 2001), whereas the needle of *Yersinia* is only 60–80 nm long (Hoiczky and Blobel, 2001). Whether the Hrp pilus is an extended version of the needle or a thinner variation of the EspA filament is not known. The genes encoding the pilin subunits are not conserved between plant pathogens and animal pathogens, albeit they all encode for small,  $\alpha$ -helical proteins (Ebel *et al.*, 1998; Delahay *et al.*, 1999). In all interactions studied, the pili/filaments/needles have been suggested to form a channel between the bacterium and its eukaryotic host, although direct evidence for this has not been found. However, the appendages have been shown to form physical links between bacteria and their animal host cell



**Fig. 1.** Immunogold localization of the FLAG epitope (20 nm gold particles) in a Hrp pilus composed entirely of FLAG-HrpA. The FLAG tag was inserted after amino acid 23 of HrpA and cloned into pDN18. The *hrpA* mutant of DC3000 harbouring the FLAG-HrpA-encoding pDN18 derivative was used to examine pilus production after 18 h incubation in Hrp-inducing minimal medium. Bar = 0.5  $\mu$ m.

(Ebel *et al.*, 1998), and to penetrate the plant cell wall (Brown *et al.*, 2001; Hu *et al.*, 2001).

Immunocytochemical studies have demonstrated that secreted harpins and Avr proteins are associated with the Hrp pilus both *in vitro* and *in vivo* (Brown *et al.*, 2001; Jin *et al.*, 2001). The patterns of immunogold labelling observed in the earlier work did not distinguish between the secretion of proteins from the base or tip of the appendage. We have addressed the question of the movement of proteins through the pilus by investigating the polarity of pilus elongation and the site of secretion of the HrpZ harpin. Our experiments involved placing both genes under the control of a strictly induced mercury-responsive promoter. Growing bacteria under conditions for pilus development allowed a small appendage to form; next, the addition of HgCl<sub>2</sub> induced production of either HrpA or HrpZ, which were identified by immunocytochemistry. By using this inducible system we were able to determine that the Hrp pilus of *P.syringae* pv. *tomato* elongates at the distal end and that the harpin protein HrpZ is secreted only from the pilus tip.

## Results

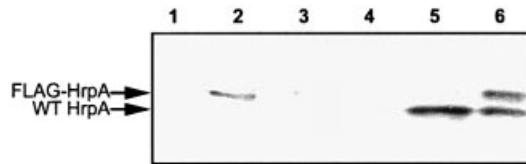
### FLAG tagging of the Hrp pilus

In order to distinguish newly expressed HrpA from the earlier synthesized pilins, we epitope tagged the HrpA pilin subunit. The C-terminal part of the HrpA pilin is responsible for pilus assembly, whereas the N-terminus tolerates insertions and large deletions without affecting pilus formation or function *in planta* (Taira *et al.*, 1999). A FLAG epitope was cloned at four different N-terminus-encoding sites in the *hrpA* gene. The cloning sites after codons 15, 23, 24 and 48 were linker insertion sites created earlier by scanning mutagenesis (Taira *et al.*, 1999). Complementation of *P.syringae* DC3000 *hrpA*<sup>-</sup> with pDN18 derivatives encoding the tagged pilins showed that FLAG insertion at all four sites permitted pilin secretion, pilus assembly and function *in planta* (data not shown). Immunocytochemistry with monoclonal anti-FLAG antiserum revealed that the epitope was exposed on the pilus surface when inserted after codons 23 (Figure 1) or 24, as recognized by immunogold labelling. The anti-FLAG antiserum did not label the pilus with a FLAG insertion after amino acid 48 of the pilin, and very few immunogold particles were observed on pili carrying the FLAG tag after amino acid 15. The pilin FLAG-tagged at amino acid 23 was chosen for further studies and is referred to as FLAG-HrpA in the following text.

### FLAG-HrpA produced under the control of a mercury-inducible promoter

In order to express FLAG-HrpA independently of the rest of the TTSS, we cloned the gene encoding FLAG-HrpA under the control of a mercury-inducible promoter (see Materials and methods). The fusion construct, termed pMerFLAGHrpA, was introduced to wild-type *P.syringae* DC3000 together with the *mer* repressor-harboring plasmid pTPT11 (Petänen *et al.*, 2001). Immunoblotting with anti-FLAG and anti-HrpA antisera showed that the expression of FLAG-HrpA was completely turned off in the absence of mercury and strongly induced by 100 nM HgCl<sub>2</sub>, both in the minimal medium, which induces all *hrp*

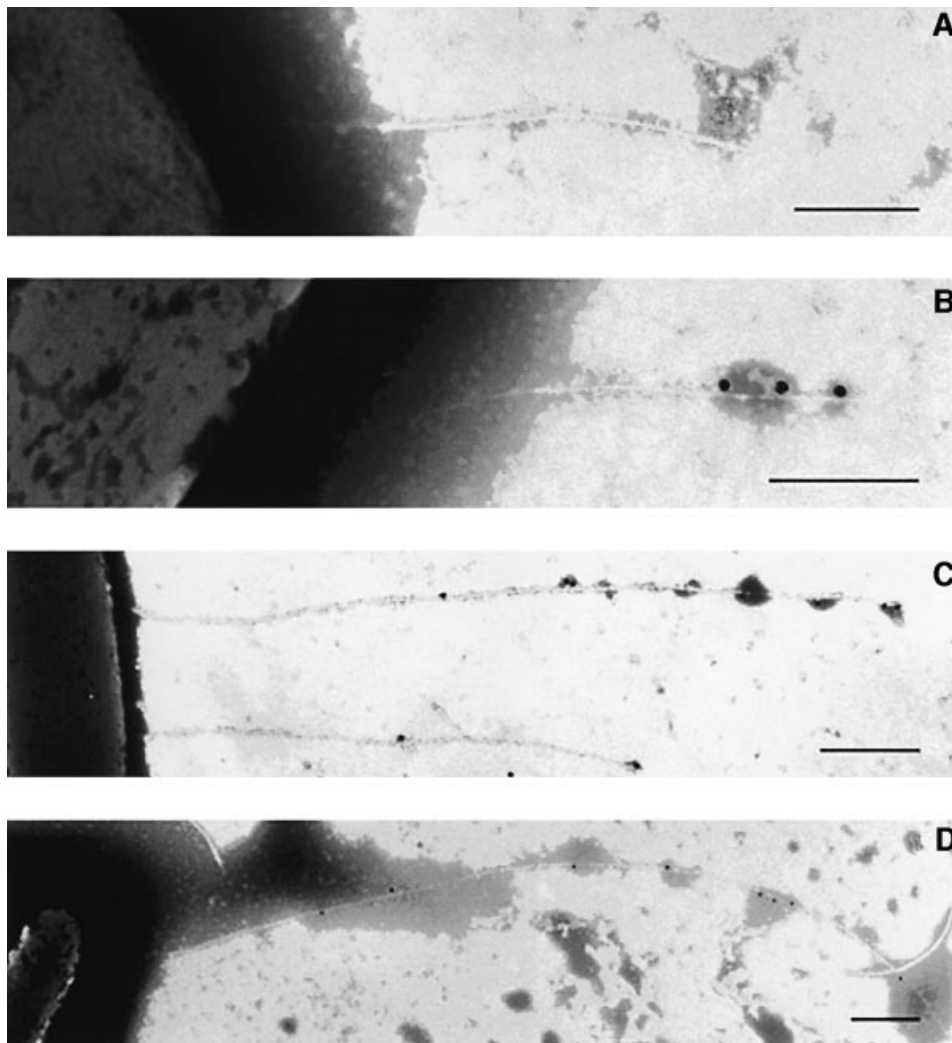
promoters (Huynh *et al.*, 1989), and in a rich medium, where *hrp* genes are normally silent. Wild-type DC3000 harbouring pMerFLAGHrpA expressed and secreted both FLAG-HrpA and wild-type HrpA encoded by the chromosomal *hrpA* locus when grown in minimal medium in the presence of 100 nM HgCl<sub>2</sub> (Figure 2).



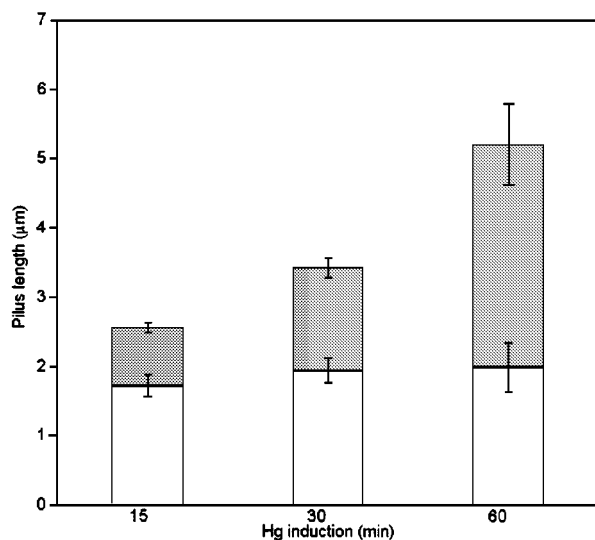
**Fig. 2.** Immunoblot showing production and secretion of FLAG-HrpA by DC3000 harbouring pMerFLAGHrpA with anti-HrpA antiserum. Bacteria were grown for 18 h before extraction of proteins, which were separated by 16% SDS-PAGE and probed with anti-HrpA antibodies. Lanes 1 and 2 are cellular fractions; lanes 3–6 are extracellular fractions. Lanes 1 and 3, King's B medium; lanes 2 and 4, King's B with HgCl<sub>2</sub>; lane 5, Hrp-inducing medium; lane 6, Hrp-inducing medium with HgCl<sub>2</sub>.

### Immunoelectron microscopy of the growing pilus

The development of Hrp pili was examined using modifications of the method devised by Brown *et al.* (2001), in which bacteria are grown on electron microscopy grids to avoid disrupting their fragile appendages. DC3000 (pMerFLAGHrpA) was grown for 8 h in *hrp*-inducing minimal medium before induction of FLAG-HrpA by addition of HgCl<sub>2</sub>. Samples were examined 15, 30 and 60 min after mercury induction. By the first time point, only 15 min after mercury induction, a few pilus-associated gold particles were observed at the distal end of the appendage (Figure 3B). At later time points, sites of immunogold labelling increased, but only at the distal end of the pilus (Figure 3C and D). The density of labelling of the Hrp pilus with anti-FLAG antibody was lower in the DC3000 wild-type background than in the *hrpA*<sup>-</sup> mutant (compare Figures 1 and 3). Such a reduced level of FLAG-HrpA incorporation would be expected if the pili produced were a mixture of wild-type and epitope-tagged HrpA, and indicates that there is no absolute selection in recruitment of HrpA or FLAG-HrpA for pilus assembly.



**Fig. 3.** Pili incorporating induced FLAG-HrpA are decorated by immunogold labelling (20 nm gold particles) of the FLAG epitope at the distal end of the appendage. Bacteria (DC3000 pMerFLAGHrpA) were grown for 8 h in minimal medium to allow pilus formation and then examined after the addition of HgCl<sub>2</sub> to induce FLAG-HrpA. (A) Pilus before addition of HgCl<sub>2</sub>; note the absence of immunogold label; (B–D) 15, 30 and 60 min, respectively, after FLAG-HrpA induction. Bars: (A) and (B) = 0.25  $\mu$ m; (C) and (D) = 0.5  $\mu$ m.



**Fig. 4.** Quantitative analysis of the development of the Hrp pilus. The length of the unlabelled proximal (open column) and immunogold-labelled distal (shaded column) portion of the Hrp pilus incorporating the FLAG-HrpA subunit was recorded from at least 30 pili at each time point after induction of FLAG-HrpA (means  $\pm$  SEM are given). Immunogold labelling was achieved using anti-FLAG monoclonal antibodies.

Quantitative analysis of the length of pilus containing incorporated FLAG-HrpA demonstrated the presence of an unlabelled basal (proximal) portion of the pilus and an expanding, FLAG-tagged distal region (Figure 4). The time course also allowed the rate of extension to be determined as  $\sim 50$  nm/min. Our results clearly demonstrated rapid growth of the pilus by the incorporation of HrpA subunits at the tip of the filament and suggest that HrpA monomers are translocated acropetally through the growing pilus. The mean numbers of particles of gold labels recorded per pilus 15, 30 and 60 min after induction were  $3.9 \pm 0.3$ ,  $5.1 \pm 0.9$  and  $8.7 \pm 0.7$ , respectively. An additional feature of the quantitative study was the finding that no short pili labelled all along their filament were observed. The absence of such appendages suggests that no new TTSS or pili were formed during the incubation periods after the addition of  $\text{HgCl}_2$  to induce FLAG-HrpA.

#### **HrpZ secretion occurs at the pilus tip**

We next addressed the possibility that effector proteins may also travel through the pilus. As Avr and Vir proteins are secreted *in vitro* with low efficiency, we used the harpin protein HrpZ as a model. We cloned the *hrpZ* gene from *P.syringae* pv. *phaseolicola* under the control of the *mer* promoter, as described in Materials and methods, and transferred the resulting plasmid, pMerHrpZ, into DC3000 $\Delta$ *hrpZ*. As the translocation of HrpZ was examined in an *hrpZ* deletion mutant, an epitope tag was unnecessary for this experiment, and secretion could easily be followed using polyclonal antiserum to HrpZ. DC3000 $\Delta$ *hrpZ*(pMerHrpZ) was grown on electron microscopy grids as above. When the mercury induction was performed 8 h after Hrp induction, newly made HrpZ was first detected at the tip of the pilus, and in time coated the distal portion of the growing appendage (Figure 5A and

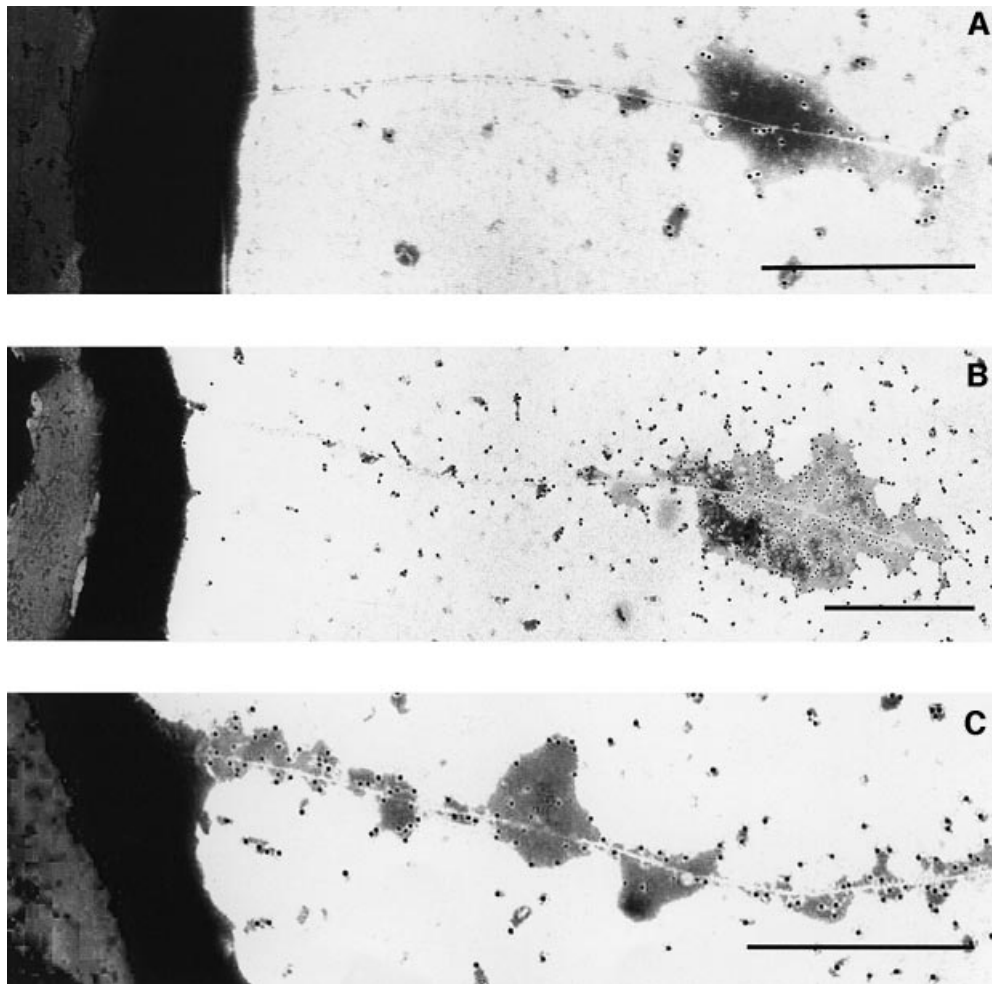
B). If bacteria were exposed to  $\text{HgCl}_2$  throughout the incubation, all parts of the emerging pilus were decorated by the HrpZ antiserum (Figure 5C). As observed with HrpA, a secretion route through the pilus was demonstrated.

## **Discussion**

The molecular mechanism by which TTSS appendages contribute to protein delivery has until now been elusive. The results of our pulse-expression experiments with *P.syringae* demonstrated that secreted proteins emerge in the extracellular space at the pilus tip. The simplest explanation of our immunocytochemical data is that the pilus functions as a conduit through which travel pilin subunits and TTSS-dependent effectors. In *P.syringae*, the long Hrp pilus potentially allows the long-distance delivery of proteins directly to the plant cell membrane by crossing the plant cell wall barrier (Brown *et al.*, 2001). The protein traffic from animal pathogens such as *Shigella* or *Salmonella* to their hosts occurs between membranes that are in close contact. Perhaps in consequence, the needle extensions observed in these bacteria are considerably shorter than the Hrp pili.

The Hrp pilus of *P.syringae* and the needles of animal pathogens have an external diameter of 6–8 nm (Roine *et al.*, 1997b; Kubori *et al.*, 1998; Blocker *et al.*, 1999; Brown *et al.*, 2001). The internal diameter of the *Yersinia* needle has been estimated to be 2 nm (Hoiczky and Blobel, 2001). Considering pilus dimensions, proteins using this secretion route would be expected to be at least partially unfolded for transport. Many of the effector proteins translocated by TTSSs have been shown to be dependent on chaperones, which keep the effector in a partially unfolded form in the bacterial cytoplasm (Stebbins and Galan, 2001). It is envisaged that, in *P.syringae*, the effectors would therefore enter the secretion channel unfolded, travel the remarkably long intra-pilus route across the plant cell wall, and take their active conformation at their destination following secretion. Such a scenario is probably the case for the Hrp pilin itself. Hrp pilin can auto-assemble to form filaments *in vitro* (Roine *et al.*, 1997b), and may therefore be able to fold and combine to form the pilus structure at the tip of the growing appendage. The mode of pilus extension also explains how the long and flexible pilus may wriggle through the porous cellulose matrix that comprises the plant cell wall. As growth occurs at the tip, pilus assembly must occur inside the plant cell wall rather than the structure being driven into the wall by basal extension, as proposed by Brown *et al.* (2001).

The next interesting question concerns the fate of the pilus when it reaches the plant cell membrane. It is possible that the emerging pilin monomers are dispersed within the lipid membrane. Alternatively, the pilus may puncture the membrane, as suggested for the *Yersinia* needle (Hoiczky and Blobel, 2001). Whether pilus assembly provides enough energy to force the pilus through the membrane or if a plant membrane protein is involved in this interaction is not known. HrpZ would be an excellent candidate to serve as a mediator in the pilus–membrane interaction, since it has been shown to insert in lipid membranes (Lee *et al.*, 2001a) and, as it is



**Fig. 5.** Immunogold localization of HrpZ in DC3000 $\Delta$ hrpZ harbouring pMerHrpZ, which allows mercury-driven HrpZ expression. (A and B) Emergence of HrpZ (immunogold label) at the tip of the pilus 30 and 60 min, respectively, after the addition of HgCl<sub>2</sub> to induce HrpZ following 8 h growth in minimal medium to allow pilus formation. (C) Labelling along the proximal base of the pilus if bacteria were grown continuously in minimal medium + HgCl<sub>2</sub> for 8 h. Bars = 0.5  $\mu$ m.

secreted at the pilus tip, it is at the right place at a high local concentration.

Clearly, the assembly of the Hrp pilus diverges from the basal-growth pattern of type I and type IV pili (Hultgren and Normark, 1991). Our result, showing distal extension of the Hrp pilus, indicates similarity with the bacterial flagellum, since newly synthesized flagellin monomers are also added to the tip of the filament (Emerson *et al.*, 1970). It has been suggested, based on protein homology (Macnab, 1999), that the flagellar apparatus is a member of the TTSS family. Our observation supports the idea of a common evolutionary origin of the flagellum and the TTSS by revealing functional and constructional similarities. The flagellum is a hollow structure (Namba *et al.*, 1989) through which flagellin monomers reach the distal end of the filament. Our observations indicate that the needle/pilus part of the TTSS is also a hollow tube, through which the secreted proteins travel to the tip.

Our experiments have confirmed the route for TTSS-dependent protein delivery. Most significantly, they raise fundamental questions for further study, including the energy requirements for movement through the long pilus,

and the possible need for regulation of protein traffic in the narrow and presumably crowded pilus tunnel.

## Materials and methods

### Strains and growth media

*Pseudomonas syringae* DC3000 (D.Cuppels, London, Ontario, Canada) and its *hrpA*<sup>-</sup> derivative (Roine *et al.*, 1997b) or *hrpZ* derivative (A.Collmer, Ithaca, NY) were used in all experiments. Hrp induction was carried out in minimal medium with fructose as the sole carbon source (Huynh *et al.*, 1989), and antibiotics used were rifampicin (75  $\mu$ g/l), tetracycline (10  $\mu$ g/l) and chloramphenicol (35  $\mu$ g/l). King's B (King *et al.*, 1954) was used as the Hrp-repressing medium.

### Immunoblotting

DC3000 harbouring pMerFLAGHrpA was grown overnight in King's B or Hrp-inducing medium in the presence or absence of 100 nM HgCl<sub>2</sub>. Cell density was adjusted spectrophotometrically to be the same in all samples. Cellular and extracellular fractions were separated by centrifugation at 16 000 *g* for 15 min. Supernatant representing secreted and detached pili, and cell pellet representing cell-associated pili, were boiled in sample buffer and an equal amount of each sample was loaded in the gel. The wild-type HrpA and FLAG-tagged HrpA were separated in 16% SDS-polyacrylamide gel, and transferred on PVDF membranes (Schleicher & Schuell, USA). HrpA antiserum (Roine *et al.*, 1997a), anti-

rabbit alkaline phosphatase conjugate (Sigma) and standard colour reaction were used to visualize the pili.

### FLAG tagging the *Hrp pilus*

A FLAG epitope-encoding linker was made by annealing oligonucleotides GGCCGCGACTACAAGGACGATGACGATAAG and GGCCCT-TATCGTCATCGTCTTGTAGTCGC together. The linker contains *NotI*-compatible cohesive ends. The FLAG linker was cloned into *NotI* sites in *hrpA* created earlier by linker scanning mutagenesis (Taira *et al.*, 1999).

### Expression of FLAG-HrpA and HrpZ under the control of a mercury-inducible promoter

The gene encoding FLAG-HrpA without its natural promoter was fused with the *mer* promoter of Tn21 (Misra *et al.*, 1984) at the transcription start point of *mer* and -42 bp upstream of the *hrpA* start codon (transcriptional start of *hrpA*), using standard PCR protocols. The oligonucleotides used were: (1) *hrpA* 3' region-specific GTACCTTCTAGAGGTAGCGGCC, introducing a *XbaI* site; (2) *mer* 5' promoter region-specific AATTAAGCTTCGAGCTAAGGCATAGC, introducing a *HindIII* site; (3) fusion oligonucleotide GAAGTA-AGGTACGCTATACCAAGCAATCACGCTG, containing sequence specific to the *mer* promoter immediately upstream of the transcription start site, and sequence specific to *hrpA* immediately after the transcription site; and (4) the complement of oligonucleotide 3. The *mer* promoter was amplified with oligonucleotides 2 and 4, and *hrpA* with oligonucleotides 1 and 3. The resulting PCR fragments were mixed and used as templates in PCR with oligonucleotides 1 and 2. The final PCR product was cloned into a broad host range plasmid pBBR1MCS (Kanter-Smolner *et al.*, 1994) as a *HindIII*-*XbaI* fragment. Since the fusion disrupts the *merR* promoter required for *mer* repressor production, plasmid pTPT11, which harbours the intact gene for the *mer* repressor, was used to provide the repressor *in trans*. For *mer* promoter-driven HrpZ, we PCR amplified the *mer* promoter and the entire *merR* gene with oligonucleotide 2 above and oligonucleotide TCGGATCCTTTTGAATTTGGATTG, which introduce *HindIII* and *BamHI* sites, respectively, and cloned the resulting fragment in pDN18. The gene encoding *P. syringae* pv. *phaseolicola* *hrpZ* was amplified with oligonucleotides AAGGAT-CCTACTTTGAGGAGGTTG and TTGAATTCTCCGT-CAGGCGG, which introduce *BamHI* and *EcoRI* sites, and the resulting fragment was cloned after the *mer* promoter in pDN18. In pMerHrpZ, 19 bp were included upstream of the *hrpZ* start codon, hence the ribosome-binding site of the original *mer* gene was replaced by the ribosome-binding site of *hrpZ*.

### Electron microscopy

Methods were essentially as described by Brown *et al.* (2001) apart from the following modifications. Carbon/formvar-coated gold grids (300 mesh) were floated carbon side down on 18 µl drops of bacterial suspension in Hrp-inducing minimal medium adjusted to an OD of 0.3 at 600 nm. For the pulse-expression experiments, grids were incubated at 20°C for 8 h when 2 µl of HgCl<sub>2</sub> stock solution were added to the drop, giving a final concentration of 100 nM. Grids were further incubated for 15, 30 and 60 min, after which they were fixed by addition of 80 µl of 2% formaldehyde and 0.5% glutaraldehyde in 50 mM sodium cacodylate buffer pH 7.2 to the 20 µl droplet. Grids were left to fix this way for 15 min before being floated on a fresh drop of fixative at 4°C overnight. Anti-FLAG M2 monoclonal antibody (Sigma, UK) was used at a concentration of 50 µg/ml. Grids were washed by passing them through six 20 µl drops, rather than in a flow of buffer from a wash bottle, in order to preserve the delicate pili. HrpZ was labelled with a rabbit polyclonal antibody as described previously (Brown *et al.*, 2001). Second antibodies were goat anti-mouse IgG-20 nm gold and goat anti-rabbit IgG-10 nm gold (British Biocell International, Cardiff, UK) diluted 1:50, and were used to visualize FLAG and HrpZ, respectively. Grids were negatively stained in 1% phosphotungstic acid/KOH at pH 6.5 for 10 s and were observed at an accelerating voltage of 75 kV in an Hitachi H-7000 transmission electron microscope.

Images were recorded of 30 pili for each time point for the quantitative analysis of pilus elongation using FLAG labelling. Only those pili that could be tracked back unambiguously to a bacterium were sampled. The lengths of the epitope-labelled tip and the unlabelled basal portion were measured from negatives and the numbers of gold particles recorded.

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## References

- Alfano, J.R. and Collmer, A. (1997) The type III (Hrp) secretion pathway of plant pathogenic bacteria: trafficking harpins, Avr proteins and death. *J. Bacteriol.*, **179**, 5655–5662.
- Blocker, A., Gounon, P., Larquet, E., Niebuhr, K., Cabiaux, V., Parsot, C. and Sansonetti, P. (1999) The tripartite type III secretion of *Shigella flexneri* inserts IpaB and IpaC into host membranes. *J. Cell Biol.*, **147**, 683–693.
- Brown, I.R., Mansfield, J.W., Taira, S., Roine, E. and Romantschuk, M. (2001) Immunocytochemical localization of HrpA and HrpZ supports a role for a transfer of effector proteins from *Pseudomonas syringae* pv. *tomato* across the plant cell wall. *Mol. Plant Microbe Interact.*, **14**, 394–404.
- Delahay, R.M., Knutton, S., Shaw, R.K., Hartland, E.L., Pallen, M.J. and Frankel, G. (1999) The coiled-coil domain of EspA is essential for the assembly of the type III secretion translocon on the surface of enteropathogenic *Escherichia coli*. *J. Biol. Chem.*, **274**, 35969–35974.
- Ebel, F., Podzadel, T., Rohde, M., Kresse, A.U., Kramer, S., Deibel, C., Guzman, C.A. and Chakraborty, T. (1998) Initial binding of Shiga toxin-producing *Escherichia coli* to host cells and subsequent induction of actin rearrangements depend on filamentous EspA-containing surface appendages. *Mol. Microbiol.*, **30**, 147–161.
- Emerson, S.U., Tokuyasu, K. and Simon, M. (1970) Bacterial flagella: polarity of elongation. *Science*, **169**, 190–192.
- Galan, J.E. and Collmer, A. (1999) Type III secretion machines: bacterial devices for protein delivery into host cells. *Science*, **284**, 1322–1328.
- Genocchio, C.C., Olmsted, S.B., Wells, C.L. and Galan, J.E. (1994) Contact with epithelial cells induces the formation of surface appendages on *Salmonella typhimurium*. *Cell*, **76**, 717–724.
- Hobbs, M. and Mattick, J.S. (1993) Common components in the assembly of type 4 fimbriae, DNA transfer systems, filamentous phage and protein-secretion apparatus: a general system for the formation of surface-associated protein complexes. *Mol. Microbiol.*, **10**, 233–243.
- Hoicyk, E. and Blobel, G. (2001) Polymerization of a single protein of the pathogen *Yersinia enterocolitica* into needles punctures eukaryotic cells. *Proc. Natl Acad. Sci. USA*, **98**, 4669–4674.
- Hu, W., Yuan, J., Jin, Q.-L., Hart, P. and He, S.Y. (2001) Immunogold labeling of Hrp pili of *Pseudomonas syringae* pv. *tomato* assembled in minimal medium and *in planta*. *Mol. Plant Microbe Interact.*, **14**, 234–241.
- Hueck, C.J. (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol. Mol. Biol. Rev.*, **62**, 379–433.
- Hultgren, S.J. and Normark, S. (1991) Biogenesis of the bacterial pilus. *Curr. Opin. Genet. Dev.*, **1**, 313–318.
- Huynh, T.V., Dahlbeck, D. and Staskawicz, B.J. (1989) Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. *Science*, **245**, 1374–1377.
- Jackson, R.J. *et al.* (1999) Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native plasmid in the bean pathogen *Pseudomonas syringae* pathovar *phaseolicola*. *Proc. Natl Acad. Sci. USA*, **96**, 10875–10880.
- Jin, Q., Hu, W., Brown, I., McGhee, G., Hart, P., Jones, A.L. and He, S.Y. (2001) Visualization of secreted Hrp and Avr proteins along the Hrp pilus during type III secretion in *Erwinia amylovora* and *Pseudomonas syringae*. *Mol. Microbiol.*, **40**, 1129–1139.
- Kanter-Smolner, G., Dahlqvist, A. and Sunnerhagen, P. (1994) pBBR1MCS: a broad-host-range cloning vector. *Biotechniques*, **16**, 800–802.
- King, E.O., Ward, M.K. and Raney, D.E. (1954) Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.*, **22**, 301–307.
- Knutton, S., Rosenshine, I., Pallen, M.J., Nisan, I., Neves, B.C., Bain, C., Wolff, C., Dougan, G. and Frankel, G. (1998) A novel EspA-associated

- surface organelle of enteropathogenic *Escherichia coli* involved in protein translocation into epithelial cells. *EMBO J.*, **17**, 2166–2176.
- Kubori,T., Matsushima,Y., Nakamura,D., Uralil,J., Lara-Tejero,M., Sukhan,A., Galan,J.E. and Aizawa,S.I. (1998) Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. *Science*, **280**, 602–605.
- Leach,J.E and White,F.F. (1996) Bacterial avirulence proteins. *Annu. Rev. Phytopathol.*, **34**, 153–179.
- Lee,J. *et al.* (2001a) HrpZ(Psph) from the plant pathogen *Pseudomonas syringae* pv. *phaseolicola* binds to lipid bilayers and forms an ion-conducting pore *in vitro*. *Proc. Natl Acad. Sci. USA*, **98**, 289–294.
- Lee,J., Klessig,D.F. and Nurnberger,T. (2001b) A harpin binding site in tobacco plasma membranes mediates activation of the pathogenesis-related gene HIN1 independent of extracellular calcium but dependent on mitogen-activated protein kinase activity. *Plant Cell*, **13**, 1079–1093.
- Macnab,R.M. (1999) The bacterial flagellum: reversible rotary propeller and type III export apparatus. *J. Bacteriol.*, **181**, 7149–7153.
- Misra,T.K., Brown,N.L., Fritzing,D., Pridmore,R., Barnes,W. and Silver,S. (1984) Mercuric ion-resistance operons of plasmid R100 and transposon Tn501: the beginning of the operon including the regulatory region and the first two structural genes. *Proc. Natl Acad. Sci. USA*, **81**, 5975–5979.
- Namba,K., Yamashita,I. and Vonderviszt,F. (1989) Structure of the core and the central channel of bacterial flagella. *Nature*, **342**, 648–654.
- Petänen,T., Virta,M., Karp,M. and Romantschuk,M. (2001) Construction and use of broad host range mercury and arsenite sensor plasmids in the soil bacterium *Pseudomonas fluorescens* OS8. *Microb. Ecol.*, **41**, 360–368.
- Roine,E., Wei,W., Yuan,J., Nurmiaho-Lassila,E.L., Kalkkinen,N., Romantschuk,M. and He,S.Y. (1997a) Hrp pilus: an hrp-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. *tomato* DC3000. *Proc. Natl Acad. Sci. USA*, **94**, 3459–3464.
- Roine,E., Saarinen,J., Kalkkinen,N. and Romantschuk,M. (1997b) Purified HrpA of *Pseudomonas syringae* pv. *tomato* DC3000 reassembles into pili. *FEBS Lett.*, **417**, 168–172.
- Sekiya,K., Ohishi,M., Ogino,T., Tamano,K., Sasakawa,C. and Abe,A. (2001) Supermolecular structure of the enteropathogenic *Escherichia coli* type III secretion system and its direct interaction with the EspA-sheath-like structure. *Proc. Natl Acad. Sci. USA*, **98**, 11638–11643.
- Shaw,R.K., Daniell,S., Ebel,F., Frankel,G. and Knutton,S. (2001) EspA filament-mediated protein translocation into red blood cells. *Cell. Microbiol.*, **3**, 213–222.
- Stebbins,C.E. and Galan,J.E. (2001) Maintenance of an unfolded polypeptide by a cognate chaperone in bacterial type III secretion. *Nature*, **414**, 77–81.
- Stevens,C., Bennett,M.A., Athanassopoulos,E., Tsiamis,G., Taylor,J.D. and Mansfield,J.W. (1998) Sequence variations in alleles of the avirulence gene *avrPphE.R2* from *Pseudomonas syringae* pv. *phaseolicola* lead to loss of recognition of the AvrPphE protein within bean cells and a gain in cultivar-specific virulence. *Mol. Microbiol.*, **29**, 165–177.
- Taira,S., Tuimala,J., Roine,E., Nurmiaho-Lassila,E.L., Savilahti,H. and Romantschuk,M. (1999) Mutational analysis of the *Pseudomonas syringae* pv. *tomato* *hrpA* gene encoding Hrp pilus subunit. *Mol. Microbiol.*, **34**, 737–744.
- Tsiamis,G. *et al.* (2000) Cultivar-specific avirulence and virulence functions assigned to *avrPphF* in *Pseudomonas syringae* pv. *phaseolicola*, the cause of bean halo-blight disease. *EMBO J.*, **19**, 3204–3214.
- Van Gijsegem,F., Vasse,J., Camus,J.-C., Marena,M. and Boucher,C. (2000) *Ralstonia solanacearum* produces Hrp-dependent pili that are required for PopA secretion but not for attachment of bacteria to plant cells. *Mol. Microbiol.*, **36**, 249–260.
- Wei,W., Plovanch-Jones,A., Deng,W.-L., Jin,Q.-L., Collmer,A., Huang,H.-C. and He,S.Y. (2000) The gene coding for the Hrp pilus structural protein is required for type III secretion of Hrp and Avr proteins in *Pseudomonas syringae* pv. *tomato*. *Proc. Natl Acad. Sci. USA*, **97**, 2247–2252.

## Note added in proof

After submission of this manuscript and in support of our findings, extrusion of effector protein AvrPto from the Hrp pilus tip of *Pseudomonas syringae* was described by Q.Jin and S.Y.He (*Science*, **294**, 2556–2558, 2001).

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