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Purified HrpA of *Pseudomonas syringae* pv. *tomato* DC3000 reassembles into pili

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Abstract ***Pseudomonas syringae* pv. *tomato* DC3000 produces Hrp pili under inducing in vitro conditions. A preparation of partially purified extracellular filaments contains HrpA, flagellin and some minor contaminants. HrpA was separated from the major contaminant, the flagellin, by gel filtration to a fraction containing HrpA as well as its three N-terminally truncated forms. These were further separated by two steps of reversed phase chromatography. HrpA and its degradation products were each shown to reassemble into filament structures after denaturation and renaturation showing that HrpA alone is sufficient for formation of filament structures.**

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Key words: Type III secretion; HrpA; Hrp pili; Plant-microbe interaction; *Pseudomonas syringae*

1. Introduction

Pseudomonas syringae is a foliar plant pathogenic bacterial species divided into over 40 pathovars causing disease in various plant hosts. Prior to development of disease symptoms, which in most cases are chlorotic and/or necrotic lesions, strains of *P. syringae* multiply on leaf surfaces. During this epiphytic phase pathogenic populations can reach relatively high densities without apparent disease symptoms. However, higher epiphytic populations of a pathogen correlate positively with the likelihood of disease outbreak (reviewed in [1]). Currently, the nature of the exact trigger in the transition from the epiphytic phase to the development of the disease is unknown.

P. syringae hrp genes (hypersensitive reaction and pathogenicity) are required for the pathogenic interaction with plants. *hrp* genes were first isolated on the basis of the loss of two functions: pathogenicity on host plants and recognition by non-host plants (resistant host plants or non-host plant species), manifested by the so-called hypersensitive reaction (HR), an induced localized cell death around the site of infection (reviewed in [2–4]). *hrp* genes of strain *P. syringae* pv. *syringae* 61 reside in one cluster (*hrp/hrm* cluster) of 26 coding regions [5]. Sequence analysis of the coding regions together with experimental evidence has led to the hypothesis that many of the *hrp* gene products are components of a type III secretion pathway, the Hrp pathway [2,3,6]. Nine of these components are conserved among *Pseudomonas* and the Gram-negative plant pathogenic genera *Erwinia*, *Ralstonia*, and *Xanthomonas*. In addition, all of these conserved components have homologues in type III secretion pathways involved in secretion of proteinaceous pathogenicity determinants of animal pathogenic bacteria such as *Yersinia*. Eight out of nine proteins also show homology to proteins involved in flagellar biogenesis of *Salmonella typhimurium* and *Bacillus subtilis* [6,7].

The prokaryotic type III secretion pathway is a *sec*-independent means for translocation of proteins from the cytoplasm to the extracellular milieu (reviewed in [8]). Translocated proteins have no cleavable N-terminal signal sequence [9], and it is suggested that there is no periplasmic phase during the secretion. Although no consensus sequence can be found in the N-terminus of proteins secreted through type III secretion pathway, the N-terminal part of a protein is required for the secretion [10,11]. In some cases, the first N-terminal methionine is missing from the extracellular form of the protein [12–14]. Proteins secreted through the Hrp pathway include the so-called hairpin, encoded by *hrpZ* in *P. syringae* and *hrpN* in *Erwinia amylovora*, PopA in *Ralstonia* (formerly *Pseudomonas*) *solanacearum*, and HrpA in *P. syringae* pv. *tomato* DC3000 [13–16] as well as several other, partially characterized proteins with unknown function [17]. In addition, the Hrp secretion pathway is required for the phenotypic expression of many bacterial avirulence genes (*avr*) in plants (reviewed in [18]), and for the suppression of papilla formation in the initial stages of compatible *Xanthomonas campestris* pv. *vesicatoria*-pepper interactions [19].

Pili, fimbriae, and flagella are extracellular proteinaceous filament structures. They are formed of subunit proteins, the number of which ranges from 500–1000 in pili and fimbriae to 20 000 in flagella. Most of the filament consists of the major subunit protein. Minor subunit proteins often function as adhesins and can be localized either to the tip of the filament or scattered along the whole filament [20,21]. In conjugation, pili are essential for successful mating-pair formation [22]. Recently, it was shown that the *virB* gene region of *Agrobacterium* Ti-plasmid is associated with production of pili that are involved in direct transfer of T-DNA from bacteria to the host plant, a phenomenon considered to be conjugation between bacterial and eukaryotic cells [23,24].

We have shown that under certain in vitro growth conditions *P. syringae* pv. *tomato* DC3000 produces pili consisting mainly of HrpA [14]. HrpA is a hydrophilic 11 kDa protein encoded by the first gene in the *hrpZ* operon of *P. syringae* pv. *tomato* DC3000 [25]. A chromosomal, non-polar mutation in the *hrpA* gene abolishes pathogenicity on host plants, induction of HR on non-host plants, and pilus production, all of which are restored by complementation *in trans*. These pili are suggested to be involved in the delivery of avirulence gene products directly into the plant cell [14], as also proposed

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for some *X. campestris* avirulence proteins [26], and in a manner analogous to *Agrobacterium* infection. To show the intrinsic capability of HrpA in forming a filament structure, we purified HrpA under denaturing conditions and showed that, after renaturation, HrpA reassembles into filament structures.

2. Materials and methods

2.1. Bacterial strains and growth media

For the isolation of Hrp pili, *P. syringae* pv. *tomato* DC3000 wild type was used. King's medium B broth [27] supplemented with rifampicin (75 μg/ml) was used to grow the inoculum. Minimal medium agar plates [28], supplemented with 10 mM fructose as carbon source, were inoculated with the liquid culture and grown for 2–3 days at room temperature (21–23°C).

2.2. Filament isolation

Hrp pili were partially purified as described by Roine et al. [14]. Briefly, extracellular filaments detached from the bacterial surface were pelleted and subjected to ultracentrifugation in a 10–60% (w/w) sucrose gradient. The resuspended pellet containing the Hrp pili together with some flagella was pelleted again to remove the remaining sucrose and the pellet was resuspended in 6 M urea in 50 mM Tris-HCl pH 7.5 for gel filtration.

2.3. HrpA purification

The dissolved pellet was next chromatographed on a Superdex-75 column (1×30 cm) equilibrated in 6 M urea, 50 mM Tris-HCl pH 7.5 at a flow rate of 0.5 ml/min. 0.5 ml fractions were collected and 20 μl aliquots of each fraction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [29].

Fractions containing Hrp pili were pooled and further purified by reversed phase high performance liquid chromatography (RP-HPLC) on a 0.21×15 cm Poros RII column (PerSeptive Biosystems, MA, USA) by elution with a linear gradient of acetonitrile (3–100% in 15 min) in 0.1% TFA. Chromatography was performed at a flow rate of 1 ml/min, and the proteins were detected by UV absorbance at 214 nm.

Fractions obtained from POROS RII column were dried in a vacuum centrifuge and resuspended in 0.1% TFA in water. Samples were further purified by RP-HPLC in a 0.46×5 cm TSK (Tosoh Corporation, Tokyo, Japan) TMS-250 (C-1) column by elution with linear gradient of acetonitrile (25–60% in 60 min) in 0.1% TFA. Chromatography was performed at a flow rate of 200 μl/min and the proteins were detected by UV absorbance at 214 nm. The chromatographic apparatus consisted of Applied Biosystems 140 B solvent delivery system, LKB2151 detector and Merck-Hitachi chromato-integrator.

2.4. Mass spectrometry

To determine the molecular masses of HrpA and its degradation products, MALDI-TOF MS was performed in the delayed-extraction mode with a BIFLEX mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany), using a 337 nm nitrogen laser. A thin-layer matrix preparation with saturated α-cyano-4-hydroxycinnamic acid in acetone was used [30]. 0.5 μl of matrix was deposited on a stainless steel target plate, allowed to dry, after which a 0.5 μl sample was added on the top of the matrix spot. External calibration was performed with insulin (human, Sigma) and cytochrome *c* (horse heart, Sigma).

2.5. Protein sequencing

An aliquot corresponding to approximately 50 pmol of protein was taken from the protein fractions obtained from the RP-HPLC. Samples were concentrated to 30 μl in a vacuum centrifuge and subjected to Edman degradation using a Procise 494 A sequencer (Applied Biosystems, Perkin Elmer, CA USA).

Alternatively, protein samples were separated in 15% SDS-PAGE followed by electroblotting onto ProBlott-PVDF membrane in 10 mM CAPS (3-cyclohexylamino-1-propane-sulfonic acid) pH 11 containing 10% methanol [31]. Proteins were visualized by Coomassie brilliant blue staining, and the bands of interest were cut out and loaded to the sequencer.

2.6. Protein renaturation and analysis

For renaturation and reassembly studies, purified HrpA and its fragments were dried in a vacuum centrifuge and denatured by dissolving in 6 M guanidine hydrochloride in 50 mM Tris-HCl pH 7.0. Renaturation was performed by dialysis against 50 mM Tris-HCl pH 7.0 at 4°C followed by concentration in a vacuum centrifuge. Assembled filaments were separated from the monomeric HrpA by centrifugation through a 10% sucrose cushion (in 200 mM Tris-HCl pH 7.0) at approximately 133 000×*g* (Beckman Airfuge A-100/18 rotor) for 1 h. Approximately 10–15 μg of sample was used for each run. After the run, monomeric HrpA was collected from the top of the tube, precipitated with trichloroacetic acid (TCA) and the precipitate was resuspended in 20 μl of Laemmli sample buffer. Pelleted proteins, including the filaments, were suspended in 20 μl of the same buffer.

2.7. Electron microscopy

A drop of the sample was applied to a copper grid coated with Pioloform and carbon, and stained with 1% potassium phosphotungstic acid (KPT) adjusted to pH 6.5. The grids were examined with a Jeol JEM-1200EX transmission electron microscope (TEM) at an operating voltage of 60 kV.

3. Results

3.1. HrpA purification and analysis

Hrp pili were isolated as described by Roine et al. [14]. As Hrp pili appear in aggregates, centrifugation of the extracellular filaments in the sucrose gradient resulted in a pellet containing the Hrp pili and some contaminating flagella. Depending on the batch of purification, SDS-PAGE analysis of the pellet fraction showed one or several additional contaminating proteins in addition to the HrpA and the flagellin.

To further purify HrpA, the protein pellet obtained in the last centrifugation step was dissolved in 6 M urea in 50 mM Tris-HCl pH 7.5 and subjected to gel filtration (Fig. 1). The 11 kDa HrpA eluted at around 22 min and could be well separated from the 32 kDa flagellin, which eluted around 14 min. Fractions containing HrpA were further purified by reversed phase perfusion chromatography using a Poros RII

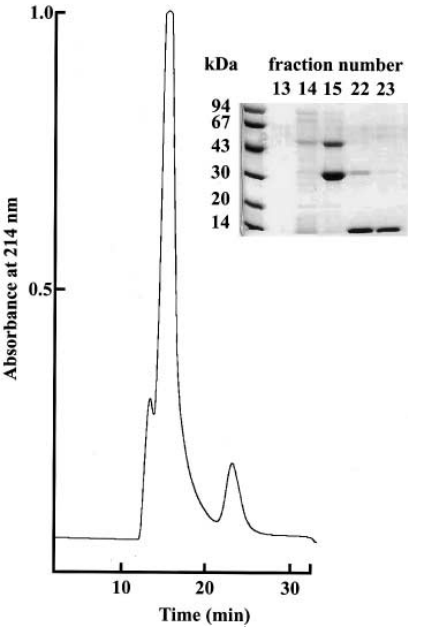

Fig. 1. A chromatogram of a representative run to separate HrpA from flagellin by gel filtration on a Superdex-75 column. 1 min fractions were collected and selected fractions were subjected to SDS-PAGE analysis as shown in the inset.

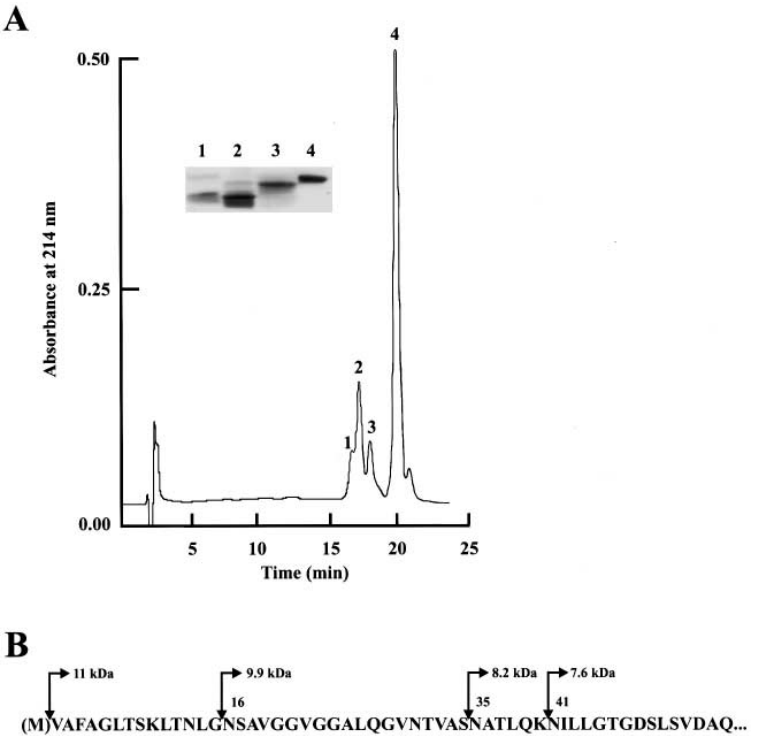

Fig. 2. Purification of HrpA by reversed phase chromatography on a C1 column. A: Elution profile of the HrpA fraction obtained from PO-ROS RII. The peak numbers correspond to the lanes in the SDS-PAGE analysis of these fractions as shown in the inset. B: The N-terminal sequence of the HrpA showing the cleavage sites and the molecular masses of the corresponding cleavage products.

column. This resulted in removal of the rest of contaminating flagellin and salts. According to the SDS-PAGE analysis, the HrpA fraction appeared to contain several proteins around the expected size of HrpA. To identify these components, proteins were transferred to PVDF membrane and subjected to N-terminal sequencing. All components were found to originate from HrpA.

To study the reassembly of the different forms of HrpA

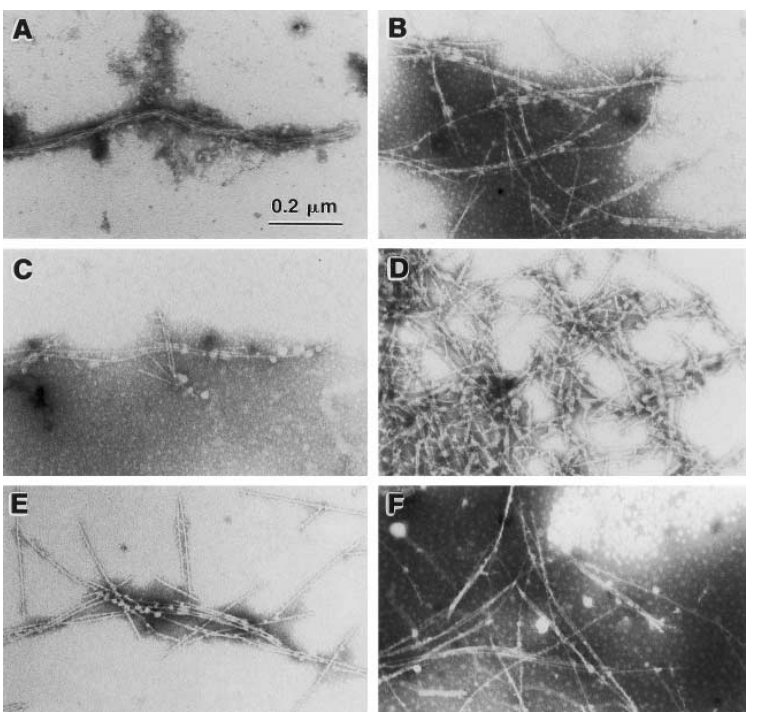

Fig. 3. Transmission electron micrographs of the renatured preparations of purified HrpA and its degradation products in the order of 8.2 kDa, 7.6 kDa, 9.9 kDa, and the 11 kDa full length HrpA (A–D), filaments formed by the mixture of proteins (E), and the filaments in the bacterial preparation (F).

individually, HrpA forms were separated by C1 reversed phase chromatography and SDS-PAGE analysis with results as shown in Fig. 2. Sequence analysis of the proteins gave N-terminal sequences NATLQKNILL, NILLGTGDSL, NSAVGGVGGA and VAFAGLTSKL (peaks 1–4), showing that the first three proteins are degradation products of the 11 kDa full length HrpA cleaved at positions N35, N41 and N16, respectively (Fig. 2B). MALDI-TOF mass analysis of the different HrpA forms gave molecular masses of 8244, 7588, 9884, and 11 270. The calculated masses of the corresponding forms assuming that the C-terminus is intact are 8242, 7587, 9882 and 11 256, which is in good agreement with the obtained results. This indicate that the cleaved proteins are processed only at their N-terminus. The N-terminal sequence of the full length HrpA shows that only the first methionine is removed in the secreted form as reported before [14].

3.2. *Renaturation and filament formation of full length HrpA and its degradation products*

HrpA protein and its degradation products were tested for the ability to assemble into filaments after the denaturation and renaturation steps as described in Section 2. Analysis of the renatured, concentrated negative stained samples by TEM showed that the full length HrpA and all of its degradation products were able to reassemble into filaments (Fig. 3A–D). The appearance of these filaments was somewhat variable when compared to the filaments in bacterial preparations (Fig. 3F), possibly as a result of the in vitro reassembly process. Mixtures of the full length protein and its degradation products (0.7 μg/μl, in approximate amounts of 60%, 15%, 15% and 10% of the full length protein, 9.9 kDa, 8.2 kDa and 7.6 kDa products, respectively) also formed filaments (Fig. 3E).

Since the fraction containing the 8.2 kDa product as the major constituent also contained some of the full length protein (Fig. 2A), it is possible that filaments were formed by the full length protein. The 7.6 kDa fragment, however, was forming filaments. This was shown by centrifugation through a 10% sucrose cushion (data not shown) which separates the filaments that can be pelleted from the monomers that cannot. This confirms that also the shorter fragments of HrpA can form filaments and that the N-terminus of HrpA is not essential for filament formation in vitro.

4. Discussion

Dissociation, denaturation and renaturation has been used to purify the major subunit proteins of extracellular filaments such as pili and flagella, and to show the intrinsic nature of a protein in forming filament structures [32–35]. The same approach was used in this study to confirm this ability for the *P. syringae* pv. *tomato* DC3000 HrpA protein.

After partial purification of HrpA filaments, some of the HrpA protein was found to have been degraded into smaller products. All the degradation products isolated and analyzed in this work result from cleavage at the N-terminal side of an asparagine residue suggesting a narrow specificity of the cleaving enzyme. Moreover, HrpA contains nine asparagine residues, but cleavage was observed at only three of them. In all these three cases, and in none of the others, a threonine is located three residues upstream of the asparagine, suggesting that these two residues may be required for cleavage (Fig. 2B). N-terminal degradation has also been shown for the PopA protein of *Ralstonia solanacearum* [13]. In *Ralstonia*, however, specificity in cleavage was not observed.

It is known that an N-terminal region is essential for the secretion of a protein through the type III secretion pathway in animal pathogenic bacteria [10,11]. Although it is not known whether this is true for the proteins secreted through the Hrp pathway, it seems likely that N-terminal part of the protein is essential also in this case since the majority of the extracellular HrpA is full length. This suggests that processing of HrpA is extracellular as also hypothesized for PopA [13]. In our study, however, all attempts to show extracellular proteinase activity on some common substrates or on HrpA failed (E. Roine, unpublished). Several reasons may account for this, such as a low amount of enzyme and/or activity linked with secretion. The observed processing may also be due to laboratory conditions with no biological significance for the Hrp system. Arlat et al. [13], however, suggested that PopA is cleaved to generate a more hydrophilic molecule, which would be more active inside the plant tissue. Indeed, in the assay conditions used, the smallest degradation product of PopA, PopA3, was more active in inducing the HR than PopA itself. Also, an N-terminal deletion product of *P. syringae* pv. *syringae* HrpZ was found to be more active in inducing HR than the full length protein, although the hydrophilicity of these proteins was not shown [16]. The degradation products of HrpA are more hydrophilic than the full length protein (i.e. elute earlier from the RP-HPLC). As HrpA is not an elicitor of HR, the activity in planta cannot be measured. Since all of these studies have been conducted in laboratory conditions, the biological significance of processing of HrpA and other proteins mentioned remains to be determined.

Renaturation studies show that also the smallest degradation product can form filaments suggesting that the C-terminal part of the protein is sufficient for the pilus formation. Dissociation/reassociation treatment has not always resulted in restoration of the function: *Pseudomonas aeruginosa* type IV pili subunits reassembled into thicker filaments were not capable of acting as phage PO4 receptors unlike the pili produced by bacteria [33]. In appearance the HrpA filaments varied somewhat from preparation to preparation. Since there are no assays to test the biological function of the HrpA filaments as such, the relationship between the appearance and the biological function remains to be determined. Our results confirm that HrpA alone, and the degradation products of it, are capable of forming the filament earlier designated the Hrp pilus [14]. Whether additional minor proteins are essential for the biological function of the pilus in planta requires further studies.

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