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HrpW of *Erwinia amylovora*, a new Hrp-secreted protein

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Abstract ***Erwinia amylovora*** **strain CFBP1430 secretes a protein called HrpW in a Hrp-dependent manner. HrpW was detected in culture supernatant of the wild-type strain grown on solid inducing *hrp* medium. This protein shares structural similarities with elicitors of the hypersensitive response such as HrpN of *Erwinia amylovora* and PopA of *Ralstonia solanacearum*. Furthermore, the C-terminal region of HrpW is homologous to class III pectate lyases. An *hrpW* mutant is as aggressive as the wild-type strain on pear and apple seedlings. It elicits the hypersensitive response on tobacco at a lower concentration than the wild-type strain.**

Key words: Type III secretion; HrpW; Harpin; Pectate lyase; *Erwinia amylovora*

1. Introduction

Erwinia amylovora is the bacterial causal agent of fire blight on most of the Maloideae such as pear trees and apple trees [1]. With *Pseudomonas syringae*, *Ralstonia solanacearum* and *Xanthomonas campestris*, it is a model for studying the *hrp* (hypersensitive response and pathogenicity) gene cluster. This cluster is required both for elicitation of the hypersensitive response (HR) [2], which is a rapid and localized cell death restricted to the inoculation site on non-host plants, and for pathogenicity on host plants (for review see [3]).

Sequence analysis has revealed that many predicted Hrp proteins share similarities with components of the type III secretion system involved in secretion of virulence proteins in animal pathogenic bacteria [4]. These homologies suggest a role for the Hrp system in the secretion of virulence determinants in plant pathogenic bacteria. To understand plant-bacterial interactions, an important challenge is to identify factors that traverse the Hrp apparatus.

Harpins, the first proteins shown to be secreted in a Hrp-dependent manner, were isolated on the basis of their capacity to induce a HR on tobacco. Named HrpN (44 kDa) in *E. amylovora* [5,6], HrpZ (45 kDa) in *P. syringae* [7] and PopA (38 kDa) in *R. solanacearum* [8], these elicitor proteins are heat stable, hydrophilic, glycine-rich and lack cysteine. Mutants blocked in their biosynthesis are fully pathogenic in *R. solanacearum* [8] and slightly aggressive in *E. amylovora* [9]. Other secreted proteins have subsequently been described. In *P. syringae* pv. *tomato* DC3000, at least three additional proteins are known to be secreted [10]. One of them, HrpA, is a 10 kDa protein involved in the formation of a pilus-like structure and is essential for disease or HR elicitation [11]. In addition to HrpN, *E. amylovora* secretes through the Hrp secretion pathway an essential pathogenicity determinant [12]. It is a 198 kDa protein called DspA which is functionally equivalent to AvrE of *P. syringae* pv. *tomato* [13].

In this paper, we present a new Hrp-secreted protein of *E. amylovora* called HrpW. HrpW is structurally similar to HR elicitors such as HrpN of *E. amylovora* and PopA of *R. solanacearum* and homologous to class III pectate lyases. A mutant blocked in HrpW biosynthesis is not impaired in pathogenicity on pear and apple seedlings and presents an increased ability to induce a HR on tobacco.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains and plasmids are listed in the text. *Escherichia coli* DH5α was used for standard DNA manipulations and *E. coli* K38 [14] for harpin overexpression.

E. coli and *E. amylovora* were routinely grown in Luria-Bertani broth (LB) medium and M9 minimal medium [15] at 37°C and 30°C respectively. When necessary, the following antibiotics were added to the medium: 50 μg/ml ampicillin, 10 μg/ml chloramphenicol, 20 μg/ml kanamycin, 20 μg/ml rifampicin.

2.2. Preparation of culture supernatants

Supernatants of culture plated on M9 minimal medium supplemented with galactose 0.2% and nicotinic acid 0.02% were prepared and concentrated as previously described [12].

2.3. Preparation of a polyclonal HrpN antiserum

To raise a polyclonal antibody against HrpN, the 1.2 kb *Hin*cII-*Hin*dIII fragment, which harbors the *hrpN* gene, was cloned into pT7.7. The resulting plasmid, named pMAB64, was electroporated into strain K38 pGp1-2 and the T7 RNA polymerase was induced as described by Tabor [14]. The cells were harvested, resuspended in 2 ml of phosphate buffer (pH 7, 10 mM), boiled for 10 min and centrifuged. 10 μl of the supernatant was analyzed on an 8% SDS-polyacrylamide gel to test for the presence of HrpN. The remainder was used for rabbit subcutaneous immunization (three injections with 30 μg of protein each at intervals of 2 weeks). The antiserum was collected 6 days after the third injection.

2.4. Analytical procedures

20 μl of culture supernatant obtained from 5×10^{11} induced cells was separated on SDS-polyacrylamide gel and proteins were detected by rapid silver staining [15].

HrpN antiserum was used at a dilution of 1/5000. Antibody binding was detected with goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase.

20 μl of culture supernatant obtained from 5×10^{12} induced cells was electrofocused in an ultrathin 10% polyacrylamide gel (pH gradient 3–10) followed by an overlay with PGA agar gel and staining of pectate lyase activity with 1% Cetavlon [16].

2.5. DNA sequence analysis

pSG9 (a pUC18 derivative plasmid carrying the 0.7 kb *Hin*dIII fragment) which contains the beginning of *hrpW* had already been sequenced [12]. pMAB37 (a pUC18 derivative plasmid carrying the 2.5 kb *Hin*dIII fragment), which contains the end of *hrpW*, was sequenced on both strands by the dideoxy chain termination procedure.

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The continuity of the sequence between the pMAB37 insert and the pSG9 insert was confirmed by partial sequencing of pMAB7 (a pUC18 derivative plasmid carrying the 4.3 kb *Sma*I fragment) around the *Hin*dIII site. DNA and deduced protein sequences were analyzed by the Genetic Computer Group (GCG) package. The XNU and SEG sequence pre-filters were used for comparison searches. The nucleotide sequence of *hrpW* has been deposited in the EMBL data base under accession number Y13831.

2.6. Enzyme assay

Pectate lyase activity of 100 μl of culture supernatant obtained from 10^{13} induced cells was determined by monitoring spectrophotometrically the formation of unsaturated products from PGA at 235 nm. The assay mixture consisted of 0.1 M Tris-HCl (pH 9.2), 0.6 mM $CaCl_2$ and 0.5 g/l of PGA.

2.7. Maceration tests

Slices were cut from commercial Bintje potato tubers. 50 μl of culture supernatant obtained with 10^{13} induced cells was placed on each slice. Samples were incubated at 30°C and examined after 24 and 48 h.

2.8. Plant tests

Pathogenicity tests were performed on apple and pear seedlings as previously described [17]. Hypersensitive responses were tested on tobacco cv. Xanthi as described by Klement [2].

2.9. Electrolyte leakage determination

Tobacco cv. Xanthii leaf disks (5 mm diameter) were vacuum-infiltrated with a bacterial suspension in assay medium (0.5 mM MES, 0.5 mM $CaCl_2$, pH 6). Disks were blotted dry for 30 min and incubated in vials with assay medium under light conditions, at 25°C, with continuous stirring. Three repetitions of six disks in 2.5 ml of medium per treatment were carried out. Conductivity probes (Tacussel XE 120) were permanently immersed in each vial and connected to a computer monitored conductivimeter. Measurements were performed every 2 h for 30 h. Each experiment was repeated at least twice.

3. Results

3.1. Detection of Hrp-secreted proteins

In the range of 200 to 66 kDa, the only Hrp-secreted protein identified was the 198 kDa protein, DspA [12]. In order to detect Hrp-secreted proteins smaller than 66 kDa, culture supernatants of the wild-type strain CFBP1430 [18] and Hrp secretion mutant PMV6023 [12] were prepared and their protein contents were analyzed on a 10% SDS-polyacrylamide gel which allowed the separation of 70 kDa to 31 kDa proteins. Two proteins with apparent molecular weights of 44 kDa and 54 kDa were detected in culture supernatant of the wild-type strain whereas they were not found in culture supernatant of the Hrp secretion mutant PMV6023 (Fig. 1).

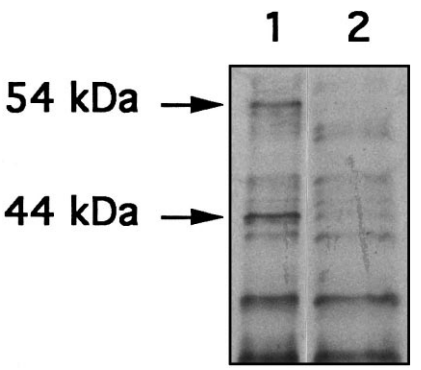

Fig. 1. Detection of two Hrp-secreted proteins (shown by arrows) in the range of 70 to 31 kDa. Supernatants of the wild-type strain (lane 1) and *hrcV* mutant PMV6023 (lane 2) were prepared and analyzed on a 10% SDS-polyacrylamide gel. Proteins were visualized by silver staining.

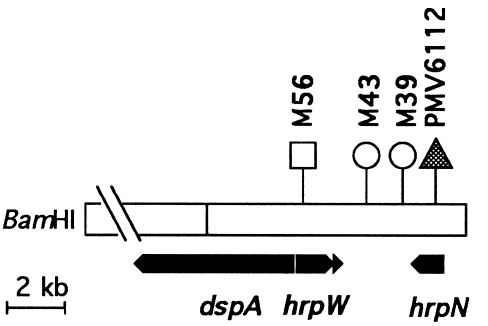

Fig. 2. Schematic location of mutant insertions. The location of *Bam*HI sites, Tn*3*-gus (circle), Mu*d*I1734 (square), *uidA*:Km cassette (flag) and Mu*d*IIPR13 (triangle) insertions is indicated. Mutations leading to a fully pathogenic phenotype are indicated by open symbols and those leading to a reduction in aggressiveness are indicated by gray symbols. The large black arrows represent ORFs.

The harpin of *E. amylovora*, HrpN, previously shown to be secreted in a Hrp-dependent manner in culture supernatant of the wild-type strain, is a 44 kDa protein [5,6]. To check that the detected 44 kDa protein was HrpN, culture supernatant of the *hrpN* mutant PMV6112 [9] (Fig. 2) was prepared and its protein content was analyzed in the same conditions as below. The 44 kDa protein was not detected in culture supernatant of the *hrpN* mutant PMV6112 (Fig. 3A, lane 2). An immunoblot analysis performed with a HrpN antiserum on culture supernatants of the wild-type strain and the mutants PMV6112 and PMV6023 confirmed that the 44 kDa protein was HrpN (Fig. 3B, lanes 1, 2 and 4). Therefore, in addition to DspA and HrpN, a 54 kDa protein appears to be secreted through the Hrp secretion pathway.

3.2. Identification of the 54 kDa Hrp-secreted protein

Partial sequencing of the chromosomal region located between the *hrpN* and *dspA* genes has revealed the beginning of an open reading frame (ORF) that belongs to the HrpL regulon and whose product shows homology with HrpN and PopA [12]. These observations suggest that this region encodes Hrp-secreted proteins. To test if this region could encode the 54 kDa protein, culture supernatants of mutants M39, M43 and M56 bearing transposon insertions in this region (Fig. 2) were prepared and their protein content was analyzed. The 54 kDa protein was still observed in mutant M39 and M43 supernatants (data not shown). In mutant M56 supernatant, the 54 kDa protein was not detected (Fig. 3A, lane 3).

DNA sequence analysis of the region encompassing mutation M56 was performed. This region contains two ORFs organized in an operon. Upstream of the first ORF, a HrpL promoter was identified (GGAACC-N_{16}-CACTC). Downstream of the second ORF, the terminator search program of the GCG package detected a palindromic sequence typical of a rho-independent terminator. The location of Mu*d*I1734 insertion present in mutant M56 was determined. This insertion was located inside the first ORF. The first ORF is 1345 bp long and is expected to encode a polypeptide of 447 amino acids with a molecular weight of 45.3 kDa and an isoelectric point of 4.51. The second ORF is 333 bp long and is expected to encode a polypeptide of 111 amino acids with a molecular

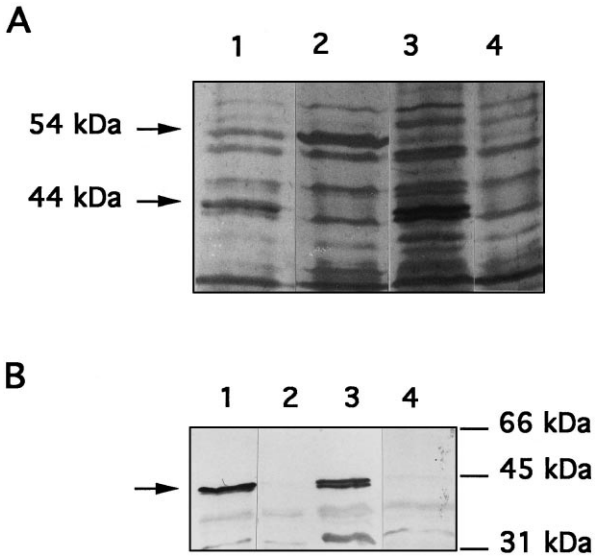

Fig. 3. Supernatants of the wild-type strain (lane 1), *hrpN* mutant PMV6112 (lane 2), *hrpW* mutant M56 (lane 3) and *hrcV* mutant PMV6023 (lane 4) were prepared. A: Proteins were analyzed on a 10% SDS-polyacrylamide gel and visualized by silver staining. B: Immunoblot analysis was performed with HrpN antiserum (dilution 1/5000). Secreted proteins are shown by arrows.

weight of 12.7 kDa and an isoelectric point of 5.62. Therefore, according to its molecular weight, the 54 kDa protein is certainly the product of the first ORF.

3.3. The 54 kDa protein is homologous to harpin and pectate lyase

Comparison of the first ORF of the operon with sequences in the database shows that the first 414 nucleotides were identical to the beginning of a partially sequenced ORF, named *hrpW*, in *E. amylovora* strain Ea321 (accession number U97504). Therefore, the first ORF of the operon was called *hrpW*.

A comparison search with translated sequences in the database revealed a similarity with PopA of *R. solanacearum* (23% identity, 48% similarity) and HrpN of *E. amylovora* (25% identity, 46% similarity). These similarities covered the whole length of the proteins and were based on their high glycine content since they disappeared when a filter masking out glycine residues was used. Despite these structural similarities, an immunoblot analysis performed with the HrpN antiserum on wild-type strain culture supernatant did not detect HrpW (Fig. 3B, lane 1). Surprisingly, in culture supernatant of the *hrpW* mutant M56, the HrpN antiserum identified HrpN as a doublet which suggests the accumulation of a HrpN degradation product in this culture supernatant (Fig. 3B, lane 3).

The C-terminal region of HrpW (amino acids 259–447) was found to be homologous to YvpA, the deduced protein of the *yvpA* gene of *Bacillus subtilis* whose function is unknown [19]. It also showed homology with a lower degree of similarity to PelA, PelB, PelC and PelD, four class III pectate lyases of the fungus *Nectria haematococca* [20–23], and PelI, a class III pectate lyase of the bacterium *Erwinia chrysanthemi* [24] (Fig. 4). Pectate lyase activity was looked for in culture supernatant of the wild-type strain. Neither enzymatic assay on

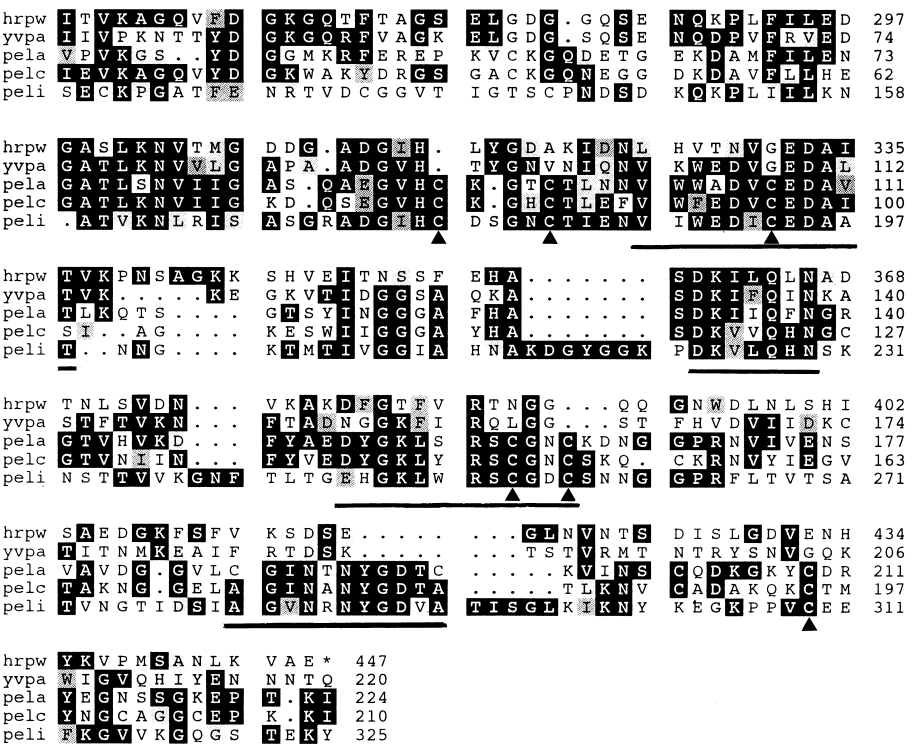

Fig. 4. Alignment of regions of HrpW (hrpw) of *E. amylovora*, YvpA (yvpa) of *B. subtilis*, PelA (pela) and PelC (pelc) of *N. haematococca* and PelI (peli) of *E. chrysanthemi*. Residues identical (similar) in at least three of the proteins are represented by letters on a black (gray) background. The numbers at the right indicate the position of the residues within the protein. Lines and triangles indicate respectively the four conserved regions and the six conserved cysteine residues in class III pectate lyases [24].

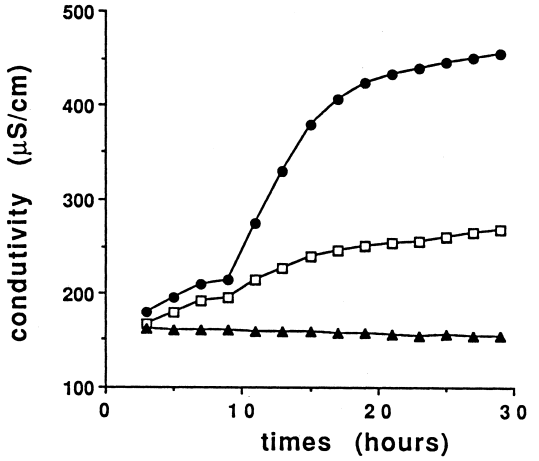

Fig. 5. Effect of the wild-type strain CFBP1430 (white square), the *hrpW* mutant M56 (black circle) or water (black triangle) on electrolyte leakage from tobacco disks. Disks were infiltrated with bacterial suspensions of 3×10^8 cells/ml. Values are means of three replicates.

culture supernatants nor specific staining after electrofocusing separation of culture supernatant proteins could detect any pectate lyase activity. Ability to macerate potato tubers was also tested with culture supernatant of wild-type strain. No maceration effect could be observed.

3.4. Role of HrpW in HR elicitation and virulence

To determine if HrpW plays a role in virulence or in HR elicitation, suspensions of different concentrations of the wild-type strain and the *hrpW* mutant M56 were inoculated on apple and pear seedlings and infiltrated into tobacco leaves.

Bacterial inoculation on pear and apple seedlings showed no differences between the wild-type strain and *hrpW* mutant M56: when the inoculum was lower than 10^4 cells/ml, neither the wild-type strain nor the *hrpW* mutant M56 was able to induce disease. The two strains were equally able to induce fire blight symptoms when the inoculum was greater than 10^4 cells/ml.

When infiltrated onto tobacco leaves, *hrpW* mutant M56 elicited a HR at a lower concentration than the wild-type strain. In four independent experiments, the minimal inoculum concentration necessary to induce a confluent HR was between 6×10^7 and 2.5×10^8 cells/ml for the wild-type strain and was two- or eight-fold lower for the *hrpW* mutant M56. To confirm these results, electrolyte leakage from tobacco leaf disks, after incubation with wild-type strain or *hrpW* mutant M56, was measured. The ability to elicit a HR on leaves correlated with the ability to induce electrolyte leakage from leaf disks. Furthermore, the latter test has been shown to be more sensitive than the former [25,26]. When infiltrated with a wild-type strain bacterial suspension of 3×10^8 cells/ml, the conductivity of the medium slowly increased during the 30 h of the experiment to 260 µS/cm. The electrolyte leakage induced in the same conditions by the *hrpW* mutant M56 increased much more rapidly: 20 h after the beginning of the experiment, the conductivity of the medium reached a plateau and stabilized at 450 µS/cm until the end of the measurement (Fig. 5). Therefore, the *hrpW* mutant M56 has an increased ability compared to the wild-type strain to elicit HR and electrolyte leakage on tobacco.

4. Discussion

In this paper, we present a new protein, HrpW, secreted by the Hrp secretion apparatus. This protein of 54 kDa is detected in culture supernatant of strain CFBP1430 of *E. amylovora* in the same way as DspA and HrpN. These latter two secreted proteins play a major role in the disease process since *hrpN* and *dspA* mutants are respectively severely affected and blocked in their pathogenicity [9,12]. In contrast, *hrpW* mutants inoculated on pear and apple seedlings are as aggressive as the wild-type strain, showing that HrpW is not a major virulence factor in the interaction between *E. amylovora* and pear and apple trees.

The ORF following *hrpW* is predicted to encode a small protein (12.7 kDa) with an acidic isoelectric point (5.62). These features suggest that it could function as a chaperone for HrpW since specific chaperones described for type III secreted proteins present the same characteristics (for a review see [27]).

The C-terminal region of HrpW is homologous to class III pectate lyases. The bacterial pectate lyases that have been identified so far are secreted through the type II secretion pathway [28]. In contrast, HrpW does not possess a signal peptide and is secreted through the type III secretion pathway. This difference shows that structurally related proteins evolved to be secreted through different routes. We did not manage to detect any pectate lyase activity in culture supernatant of the wild-type strain. Thus, pectate lyase activity, if any, should be weak. This is expected since *E. amylovora* does not induce maceration symptoms on its hosts. A weak maceration activity was observed for PelA, a major pectate lyase of *E. chrysanthemi* which, like Hrp W, has an acidic isoelectric point [27]. Although the catalytic site of class III pectate lyases is unknown, four blocks of amino acids and six conserved cysteine residues have been described in all known class III pectate lyases (Fig. 4) [24]. In HrpW and its closest homolog, YvpA, these signatures are degenerated or absent. Remarkably, HrpW and YvpA lack cysteine. These differences in signature might reflect the different secretion pathway used by those proteins and/or the absence of a class III pectate lyase activity.

HrpW has structural similarities, based on its high glycine content and its lack of cysteine, with HrpN of *E. amylovora* and PopA of *R. solanacearum*. Both HrpN and PopA have been shown to elicit HR. Does HrpW have any role in HR elicitation? If any, the *hrp* mutant would be expected to present a reduced ability to elicit HR on tobacco. However, since the *hrpW* mutant still secretes HrpN, a major HR elicitor, the decrease would be expected to be subtle. Surprisingly, the *hrpW* mutant elicits a HR on tobacco leaves at a lower concentration than the wild-type strain. This is confirmed by the higher electrolyte leakage induced by the *hrpW* mutant. Therefore, in an unknown way, HrpW acts as a negative effector of the HR mechanisms. Furthermore, we observed an accumulation of a HrpN degradation product in culture supernatant of the *hrpW* mutant. One can ask whether this HrpN degradation product is a stronger HR inducer than HrpN and whether it is responsible for the increased HR ability of the *hrpW* mutant.

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