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The *Pseudomonas syringae* Hrp Regulation and Secretion System Controls the Production and Secretion of Multiple Extracellular Proteins

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***Pseudomonas syringae* pv. *tomato* DC3000 produces seven to eight major extracellular proteins (EXPs) in a minimal medium inducing *hrp* genes. Using a polyclonal antibody against DC3000 EXPs, we have determined that the production and secretion of five EXPs (EXP-60, EXP-45, EXP-43, EXP-22, and EXP-10) are under the control of the Hrp regulation and secretion system.**

Plant-pathogenic bacteria are intercellular pathogens, and they do not invade host cells. All signal exchanges between plant cells and bacteria occur in (or through) the plant apoplast. The ability of necrogenic plant-pathogenic bacteria (e.g., *Pseudomonas syringae*) to elicit the hypersensitive response (HR) in resistant plants and to cause disease in susceptible host plants is controlled by *hrp* genes (3, 10, 23). Many *hrp* genes (such as *P. syringae hrpH*) encode proteins that are components of a type III bacterial protein secretion pathway (the Hrp secretion pathway) (2, 5, 12, 13, 16, 24). Other *hrp* genes, such as *P. syringae hrpS*, are involved in the regulation of *hrp* genes and *avr* genes (9, 25, 26).

For the past several years, we have been characterizing *P. syringae* extracellular proteins (EXPs) traversing the Hrp secretion pathway. Using a cloning approach based on HR-eliciting activity, we previously identified an extracellular Hrp protein (HrpZ; formerly harpin$_{Pss}$) (11). HrpZ was shown to elicit HR (11), systemic acquired resistance (22), and plant defense-related genes (8) when infiltrated into the apoplasts of several plants. HrpZ appears to be the only Hrp protein that possesses HR-eliciting activity and is secreted in culture (11). However, nonpolar *hrpZ* deletion mutants still elicit HR, albeit to a lesser extent (1), suggesting the involvement of other factors in eliciting HR. One such HR-eliciting factor is *P. syringae* pv. *glycinea* AvrB. AvrB was recently shown to elicit an *RPM1*-dependent HR in *Arabidopsis thaliana* in a Hrp-secretion-pathway-dependent manner (7, 17). Interestingly, expressing the *avrB* gene directly in the plant cell alleviates the dependence of AvrB on the Hrp secretion pathway, suggesting that AvrB may also traverse the Hrp secretion pathway, but, unlike HrpZ, AvrB is likely secreted into the plant cytoplasm to trigger a genotype-specific HR (7).

To search for additional *hrp*-controlled EXPs that are not likely to be identified by an approach based on HR-eliciting activity (e.g., those putatively involved in sensing plant-bacterial contact in the plant apoplast), we have recently used a *hrp*-inducing medium (11, 14) to discover five *P. syringae* pv. *tomato* DC3000 EXPs (EXP-60 [i.e., the 60-kDa EXP], EXP-45, EXP-43, EXP-22, and EXP-10) that are secreted only in *hrp*-inducing medium (19). In this paper, we report the *hrp* gene-dependent production and secretion of EXP-60, EXP-45, EXP-43, and EXP-22. Characterization of EXP-10 (HrpA, a major structural component of a *hrp*-dependent pilus) is reported elsewhere (19).

To facilitate characterization of DC3000 EXPs, we cloned the *P. syringae* pv. *tomato* DC3000 *hrp* gene cluster and raised a polyclonal antibody against the DC3000 EXPs. The *P. syringae* pv. *tomato* DC3000 *hrp* gene cluster was cloned in two plasmids (pDC3000-1 and pDC3000-2) by using pUCP19 as the cloning vector (21). By hybridization with individual ^{32}P-labeled *P. syringae* pv. *syringae* 61 *hrp* gene probes, we have determined that pDC3000-1 contains a 10.9-kb insert that carries the *hrpH*, *hrpZ*, and *hrpS*/*R* operons. pDC3000-2 contains a 16.7-kb insert that carries the *hrpL*, *hrpU*, and *hrpJ* operons. *hrpH* and *hrpS* mutants were constructed by standard marker-exchange mutagenesis with Tn*5Cm* (4).

A polyclonal rabbit antibody was prepared against a mixture of DC3000 EXPs as follows. DC3000 was first incubated in King's B broth (15) until the optical density at 600 nm reached 0.6 to 0.8. Bacteria were collected by centrifugation and resuspended in the original volume of the *hrp*-inducing medium (11, 14) and incubated with shaking (250 rpm) at 21 to 23°C for 24 h. Bacteria were pelleted by centrifugation at 15,000 × *g* for 1 h. The medium fraction was concentrated 50-fold with Centricon concentrators (Amicon; molecular weight cutoff of 10 kDa) at 4°C. The concentrated DC3000 EXP solution was desalted by dialyzing it against 500 volumes of phosphate-buffered saline (pH 7.0) overnight at 4°C. Each of two New Zealand White rabbits was injected twice, 2 weeks apart, with 0.5 ml of concentrated EXP preparation (1 mg of protein per ml) mixed with 0.5 ml of Freund incomplete adjuvant. The third injection was performed in the same way 1 week after the second injection. One week after the third injection, approximately 50 ml of antiserum was collected, passed through a 0.22-μm-pore-size filter, and stored at −70°C. The crude antiserum was extensively absorbed with *Escherichia coli* DH5α and DC3000 cell lysates before use. DC3000 cells for antibody preabsorption were grown in King's B broth, which represses the expression of *hrp* genes so that the antibodies reacting with Hrp-controlled EXPs are selectively preserved. DH5α cells were not expected to contain any antigens related to DC3000 Hrp-controlled EXPs. The preabsorbed antibody reacted well with EXP-60, EXP-45, EXP-36, and EXP-21 but relatively poorly with EXP-50, EXP-43, EXP-22, and EXP-10, presumably because of the low antigenicities and/or low concentrations of the latter EXPs in the medium (Fig. 1, lane 1). The

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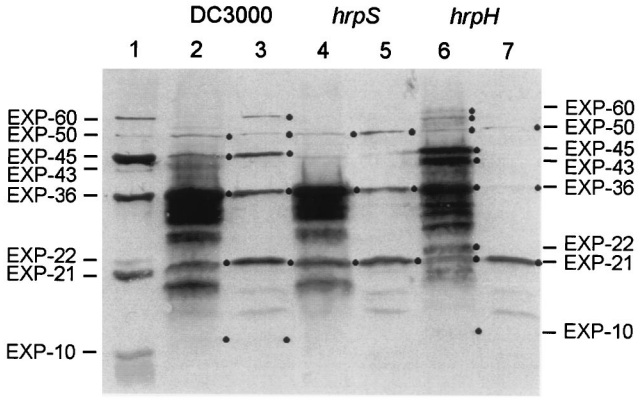

FIG. 1. Immunoblot analysis of the cellular location of *P. syringae* pv. *tomato* DC3000 EXPs. DC3000 and *hrpS* and *hrpH* mutants were grown with shaking at 30°C overnight in *hrp*-inducing medium to optical densities at 600 nm of 0.8 to 1.0. Cells were separated from the culture medium by centrifugation, washed twice in 5 mM $MgCl_2$, and resuspended in 1/10 of the original volume of 5 mM $MgCl_2$. The culture medium, devoid of bacteria after passing through a 0.22-μm-pore-size filter was concentrated 10-fold with an Amicon Centricon-3 concentrator. Ten microliters of cell suspensions (lanes 2, 4, and 6) and concentrated (10-fold) culture medium fractions (lanes 3, 5, and 7) of DC3000 and *hrpS* and *hrpH* mutants were electrophoresed in a SDS–15% polyacrylamide gel according to a protocol provided by Hoefer Scientific Instruments; this was followed by immunoblotting with a polyclonal antibody against DC3000 EXPs. Lane 1 was loaded with a DC3000 EXP sample concentrated 50-fold to permit visualization of all major EXPs. Because of higher salt and polysaccharide concentrations in the sample, some of the EXPs (EXP-22, EXP-21, and EXP-10) appeared to migrate faster in this lane. Other lanes were loaded with EXP samples concentrated only 10-fold to avoid the detection of too many cellular proteins. A goat anti-rabbit immunoglobulin G antibody conjugated with alkaline phosphatase was used as a second antibody. Dots on the right of bands indicate major EXPs.

antibody also reacted with several cell-associated proteins (Fig. 1, lanes 2, 4, and 6), but the presence of these proteins did not interfere with the detection of major EXPs (Fig. 1, dots). Because this antibody reacted with not only the EXPs but also several cell-bound proteins, identification of proteins with the antibody was based on a number of considerations: the total number of intracellular and extracellular proteins detected by the antibody in each bacterium, the molecular weights of these proteins, and the appearance and location of these proteins in DC3000, and in *hrpH* and *hrpS* mutants.

As shown in Fig. 1 (lanes 1 and 3), DC3000 produced and secreted all detectable EXPs. EXP-43 and EXP-22 were less abundant and were visualized only when the medium fraction was concentrated 50-fold (Fig. 1, lane 1). In the *hrpS* mutant, EXP-60, EXP-45, EXP-43, EXP-22, and EXP-10 were not produced, whereas the production and secretion of EXP-50, EXP-36, and EXP-21 were not affected (Fig. 1, lanes 4 and 5). EXP-43 and EXP-22 were not detected even when the medium fraction was concentrated 50-fold (data not shown). The *hrpH* mutant produced EXP-60, EXP-45, EXP-43, EXP-22, and EXP-10 but did not secrete them into the medium (Fig. 1, lanes 6 and 7); instead, these EXPs accumulated in the cell (Fig. 1, lane 6). Interestingly, at least one additional protein 56 kDa in size showed up in the cell fraction of the *hrpH* mutant. This protein could be the truncated product of EXP-60. Alternatively, it could be an additional *hrp*-controlled extracellular protein that normally would escape detection by Coomassie staining or by antibody binding because of its low concentration in the medium. In the *hrpH* mutant EXP-56 may have accumulated to higher levels and therefore became detectable on an immunoblot.

To determine the identities of DC3000 EXPs, we used a polyclonal rabbit antibody against purified *P. syringae* pv. *syringae* 61 $HrpZ_{Pss}$ protein (11) to identify DC3000 $HrpZ_{Pst}$. EXPs of strain 61 and DC3000 grown in *hrp*-inducing broth were concentrated 10-fold and separated on a sodium dodecyl sulfate (SDS)–15% polyacrylamide gel (Fig. 2A). Western blot (immunoblot) analysis with the $HrpZ_{Pss}$ antibody identified EXP-45 as the DC3000 $HrpZ_{Pst}$ protein (Fig. 2B). The presence of HrpZ (a typical Hrp-controlled EXP) in the DC3000 EXP mixture demonstrates that the approach of using *hrp*-inducing medium is appropriate for identification of Hrp-controlled EXPs of DC3000. We next determined the amino-terminal sequences of several abundant EXPs (Fig. 3). The N-terminal sequence of EXP-45 matches the predicted N-ter-

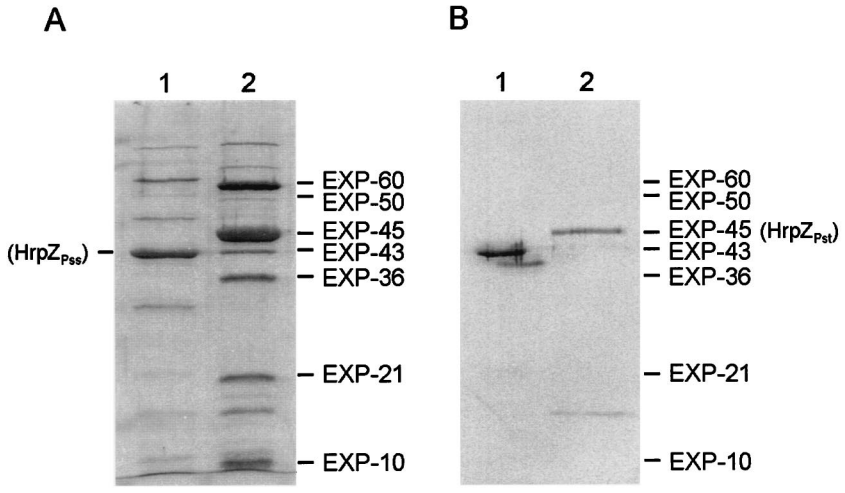

FIG. 2. Identification of *P. syringae* pv. *tomato* DC3000 $HrpZ_{Pst}$. Bacteria were grown in a *hrp*-inducing medium (11, 14) at 30°C overnight to an optical density at 600 nm of 0.8 to 1.0. Bacteria were separated from the culture medium by centrifugation. The culture medium, devoid of bacteria after passing through a 0.22-μm-pore-size filter was concentrated 10-fold with an Amicon Centricon-3 concentrator. Ten-microliter protein samples were boiled in SDS-polyacrylamide gel electrophoresis loading buffer and electrophoresed in two identically prepared SDS–15% polyacrylamide gels according to a protocol provided by Hoefer Scientific Instruments. One gel (A) was stained with 0.025% Coomassie blue R-250 to visualize the EXPs of *P. syringae* pv. *syringae* 61 (lane 1) and *P. syringae* pv. *tomato* DC3000 (lane 2). The other gel (B) was immunoblotted with a rabbit polyclonal antibody against *P. syringae* pv. *syringae* 61 $HrpZ_{Pss}$ to identify DC3000 $HrpZ_{Pst}$.

EXP-60	MRIGITPRPQQ
EXP-45	MQALNSISSLQTSASLFPVVLNAD
EXP-21	AQPNTMTLNGYA

FIG. 3. Amino-terminal sequences of *P. syringae* pv. *tomato* DC3000 EXPs. A highly concentrated DC3000 EXP preparation was loaded onto a preparative SDS–15% polyacrylamide gel electrophoresis gel. Separated proteins were electrophoretically transferred to a polyvinylidene fluoride membrane. Individual protein bands visualized after staining with 0.025% Coomassie blue R-250 were cut out, and the amino-terminal sequences were analyzed in the University of Kentucky Macromolecule Structure Analysis Facility.

minal sequence of *hrpZ* (18), confirming that EXP-45 is DC3000 $HrpZ_{Pst}$. The N termini of EXP-60 and EXP-21 were determined, but they show no similarities to any protein sequences deposited in the current sequence databases (Fig. 3). The amino-terminal sequences of EXP-36 (flagellin) and EXP-10 (HrpA) are reported in a separate paper (19). The N-terminal sequences of EXP-45 (HrpZ) and EXP-60 start with Met, which would be expected for proteins traversing the type III, Sec-independent secretion apparatus (20); the N-terminal sequence of EXP-21 does not start with Met, suggesting that EXP-21 may have a precursor bearing a signal peptide that is cleaved once the protein travels through the type II, Sec-dependent pathway (20).

The Hrp-controlled EXPs identified in this study could have several functions during plant-*P. syringae* interactions. First, some EXPs may be involved in sensing plant-bacterial contact in the plant apoplast. In *Yersinia* spp., an extracellular protein (YopN) secreted via the Ysc secretion pathway (a type III secretion pathway) was shown to be involved in detecting the lack of calcium in vitro and host-bacterial contact in vivo, which are required for the expression of *yop* genes in vitro and in vivo, respectively (6, 19a). Second, EXPs like HrpZ are candidate virulence factors that may make plant cells release nutrients required for bacteria to grow. Finally, some EXPs may be involved in the translocation of a subset of bacteria virulence factors (proteins and/or low-molecular-weight compounds) into the plant cell. This idea is consistent with recent observations that the AvrB protein of *P. syringae* elicited a genotype-specific HR when produced in *Arabidopsis* cells containing the corresponding *RPM1* resistance gene (7) and that *P. syringae* produces a *hrp*-dependent pilus (the Hrp pilus) (19). It appears that the type III secretion pathway conserved among necrosis-causing plant-pathogenic bacteria may consist of three parts: a protein complex in the bacterial envelope, the Hrp pilus, and another possible protein complex in the plant plasmalemma. The extracellular part of the Hrp secretion pathway, including the Hrp pilus and the putative complex in the plant plasmalemma, may be made up of some of the Hrp-controlled EXPs identified in this study. It is also possible that some of the EXPs observed in *hrp*-inducing medium in vitro may be secreted into the plant cells in vivo. Lack of plant-bacterial contact and assembly of a complete inter-plant-bacterial secretion apparatus in the absence of plant cells may have resulted in the release to the culture medium of proteins destined for the plant cell.

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