Harpin mediates cell aggregation in Erwinia chrysanthemi 3937

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Harpin Mediates Cell Aggregation in \textit{Erwinia chrysanthemi} 3937

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The enterobacterial plant pathogen \textit{Erwinia chrysanthemi} 3937 displays aggregative behavior manifested in the formation of a cohesive mat (pellicle) at the air-liquid interface. Pellicle formation in 3937 requires a functional type III secretion system (T3SS) (23). A similar phenomenon has been observed in \textit{Escherichia coli}, where a degenerate T3SS is required for both virulence and bacterial aggregation (10). However, based on experiments with another plant pathogen, \textit{Ralstonia solanacearum}, it does not appear that the T3SS of plant pathogens contributes to bacterial adhesion to host cells (20).

It is tempting to speculate that a T3SS substrate functions as an adhesin that promotes bacterial aggregation. This was inferred from the observation that the addition of protease K into the pellicle-inducing medium, SOBG medium (23), prevents pellicle formation but not bacterial growth (Fig. 1A), suggesting that the aggregative factor is extracellular protein. Pellicle cultures were grown essentially as described by Yap et al. (23). Bacterial strains were grown overnight in SOBG medium and subcultured into the same medium at a 1:100 dilution. Cultures were incubated without shaking at 25°C, and pellicle formation was visualized after 3 days. Appropriate antibiotics were added at the following concentrations: kanamycin, 50 \textmu g/ml; carbenicillin, 50 \textmu g/ml.

One known T3SS-secreted protein in \textit{E. chrysanthemi} 3937 is the harpin protein encoded by \textit{hrpN}. Using information from the genome sequence (8), we deleted the 3937 \textit{hrpN} gene by crossover PCR-assisted allelic-exchange mutagenesis (17, 22). The \textit{ΔhrpN} mutant, WPP122, was unable to form a pellicle. Pellicle formation was restored by providing \textit{hrpN} on a plasmid. Notably, pellicles were never observed in WPP122 carrying the vector control pCPP50 (Fig. 1B). This suggests that \textit{HrpN}\textsubscript{3937} serves as an aggregative factor and possibly contributes to adhesion in the plant host.

Most harpin proteins share little sequence homology, but they are all acidic, glycine-rich proteins that lack cysteine, and they can elicit the hypersensitive response (HR) when purified and infiltrated into leaf tissue (3, 4). Yang et al. (22) demonstrated that \textit{hrpN} makes a small contribution to virulence in strain 3937, but its function in pathogenesis is unclear. The HR elicitor activity of harpins is not confined to a single region. For example, nonoverlapping N- and C-terminal fragments of \textit{Pseudomonas syringae} HrpZ elicit the HR in tobacco leaves (2, 9).

In \textit{Xanthomonas axonopodis}, however, only the extreme N terminus of HpaG is necessary for elicitor activity (11). By analogy, we hypothesized that portions of \textit{HrpN}\textsubscript{3937} are sufficient for cell aggregation in \textit{E. chrysanthemi} 3937.

To identify the regions conferring aggregative activity upon \textit{HrpN}\textsubscript{3937}, a series of deletions in \textit{hrpN} were constructed using the primers listed in Tables 1 and 2. Internal deletions were made by crossover PCR (14). Since a stretch of heterologous nucleotides has to be included in the two internal primers as the adapter to link the two PCR fragments together in the second round of amplification, a 20-bp linker (\textsuperscript{5′}-GGTATC AACGCGAGTACGC-\textsuperscript{3′}), which encodes GINAEY, was, by necessity, introduced into each construct. The PCR products were cloned into plasmid pCPP50 (5), and DNA sequencing was performed to ensure that the reading frames were correct. Most of the constructs retain the first 50 amino acids since numerous T3SS-secreted proteins have been found to contain an N-terminal secretion signal (18). The production of truncated \textit{HrpN} derivatives by various plasmids was confirmed by immunoblot analysis with anti-\textit{HrpN} EC16 antibody (Fig. 1C).

For these experiments, 1-ml samples of 2-day-old SOBG cultures were harvested, the cell pellets were boiled in 100 \textmu l of 1× Laemmli buffer (12), and the whole-cell lysate was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Anti-\textit{HrpN}\textsubscript{EC16} antibody that had been preabsorbed with total protein from \textit{E. coli} DH5\textsuperscript{α} and an Immuno-Star AP goat anti-rabbit immunoglobulin G (IgG) chemiluminescence kit (Bio-Rad, Hercules, CA) were used for detection of \textit{HrpN} derivatives. Both full-length \textit{HrpN}\textsubscript{3937} (estimated to be 34 kDa) and truncated derivatives exhibited slower electrophoretic mobilities on SDS-polyacrylamide gel electrophoresis gels than expected on the basis of their sequence-deduced sizes (Fig. 1C). A similar observation was reported for \textit{P. syringae} HrpW (6), and both cases of slower mobilities may be due to inefficient binding of SDS to the acidic harpins (15).
TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
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<tr>
<td>E. coli DH5α</td>
<td><em>supE44 ΔlacU169 (d80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</em></td>
<td>Clontech</td>
</tr>
<tr>
<td>E. chrysanthemi 3937</td>
<td>Wild type, <em>Saintpaulia</em> (African violet) isolate</td>
<td>13</td>
</tr>
<tr>
<td>EC16</td>
<td><em>Chrysanthemum morifolium</em> isolate</td>
<td>7</td>
</tr>
<tr>
<td>WPP98</td>
<td><em>hrcJ-kan Km</em>; 3937 derivative</td>
<td>23</td>
</tr>
<tr>
<td>WPP122</td>
<td>ΔhrpN:kan Km*; 3937 derivative</td>
<td>22; this work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCPP50</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;; pNII&lt;sup&gt;13&lt;/sup&gt;-A2-based expression vector</td>
<td>5</td>
</tr>
<tr>
<td>p50HrpN</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;; 1.25-kb full-length hrp&lt;sup&gt;N&lt;/sup&gt; was PCR amplified with primers P0303 and P0304 and cloned into the SpeI and HindIII sites of pCPP50</td>
<td>This work</td>
</tr>
<tr>
<td>p50HrpN&lt;sub&gt;Δ2-43&lt;/sub&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;; 1.15-kb hrp&lt;sup&gt;N&lt;/sup&gt; was PCR amplified with primers P0303, P0304, and cloned into the SpeI and HindIII sites of pCPP50</td>
<td>This work</td>
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<tr>
<td>p50HrpN&lt;sub&gt;Δ50-117&lt;/sub&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;; 0.98-kb hrp&lt;sup&gt;N&lt;/sup&gt; was PCR amplified with primers P0303, P0304, and cloned into the SpeI and HindIII sites of pCPP50</td>
<td>This work</td>
</tr>
<tr>
<td>p50HrpN&lt;sub&gt;Δ117-197&lt;/sub&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;; 0.85-kb hrp&lt;sup&gt;N&lt;/sup&gt; was PCR amplified with primers P0303, P0304, and cloned into the SpeI and HindIII sites of pCPP50</td>
<td>This work</td>
</tr>
<tr>
<td>pCPP2174</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;; 0.9-kb hrp&lt;sup&gt;N&lt;/sup&gt; was PCR amplified with primers P0303 and P039 and cloned into the SpeI and HindIII sites of pCPP50</td>
<td>This work</td>
</tr>
<tr>
<td>pMA1</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;; 1.8-kb HindIII hrp&lt;sup&gt;N&lt;/sup&gt; of <em>P. stewartii</em> subsp. <em>stewartii</em> in pBluescript M13&lt;sup&gt;+&lt;/sup&gt;</td>
<td>21</td>
</tr>
<tr>
<td>pCPP1084</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;; 1.3-kb HindIII hrp&lt;sup&gt;N&lt;/sup&gt; of <em>E. amylovora</em> in pBluescript SK</td>
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</table>

FIG. 1. Pellicle formation in *E. chrysanthemi* 3937- and 3937 ΔhrpN-expressing hrpN fragments. Pellicles are a type of biofilm that forms at the air-liquid interface. In 3937, pellicles are genetically distinct from the biofilm that forms at the surface-air interface (23). (A) Exogenous addition of proteinase K (Invitrogen, Carlsbad, CA) inhibited pellicle formation in 3937. Amounts of proteinase K added to SOBG medium equal 0 μg/ml, 125 μg/ml, 250 μg/ml, and 500 μg/ml, from left to right. (B) The ΔhrpN mutant failed to form a pellicle; pellicle formation was restored by providing the parental hrpN in trans. Tube 1, wild type; tube 2, WPP122; tube 3, WPP122(pCPP50) (vector control); tube 4, WPP122(p50HrpN). (C) Immunodetection of full-length and truncated HrpN proteins with rabbit polyclonal anti-HrpNEC16. Lane 1, wild-type *E. chrysanthemi* 3937; lane 2, WPP122; lane 3, WPP122(p50HrpN); lane 4, WPP122(p50HrpN<sub>Δ2-43</sub>); lane 5, WPP122(p50HrpN<sub>Δ50-117</sub>); lane 6, WPP122(p50HrpN<sub>Δ117-197</sub>); lane 7, WPP122(p50HrpN<sub>Δ118-342</sub>); lane 8, WPP122(p50HrpN<sub>Δ230-342</sub>); lane 9, WPP122(p50HrpN<sub>Δ230-342</sub>). (D) Diagram of truncated HrpN derivatives and other harpin proteins used to test their ability to restore pellicle formation to the ΔhrpN mutant. The C terminus of HrpN<sub>3937</sub> was dispensable in promoting cell aggregation. The aggregative activities of each construct in a WPP122 background are rated positive (+) or negative (−) in the right column. The plasmids pCPP2174, pMA1, and pCPP1084 carry the HrpN-encoding genes from *E. chrysanthemi* EC16 (4), *P. stewartii* subsp. *stewartii* (1), and *E. amylovora* (21), respectively. a.a., amino acids.
The cell aggregation phenotypes of each hrpN derivative expressed in WPP122, the strain 3937 hrpN deletion mutant, are depicted in Fig. 1D. None of the constructs expressing hrpN with internal deletions restored pellicle formation to WPP122. The first N-terminal 118 amino acids alone were not sufficient to direct pellicle formation (Fig. 1D). Thus, HrpN3937 requires the N-terminal region comprising amino acids 1 to 229 for pellicle formation, and only a portion of the C terminus is dispensable. Surprisingly, production of a protein with a small deletion from residues 2 to 43 was not observed in WPP122 or E. coli DH5α (Fig. 1C). We reisolated plasmid p50HrpN_{3937-2-43} from SOBG cultures of both hosts and sequenced the plasmid DNA but found no frameshift or other obvious reason for the lack of protein production (data not shown). We later learned that the presence of a glycine residue in the second position of a protein sequence reduces or eliminates protein translation in some cases (16). Thus, the inability of this construct to promote aggregation may be due to the deletion strategy resulting in a glycine as the second residue in this construct.

The sequence identities of HrpN 3937 with the harpins of E. amylovora strain 321 (GenBank accession no. AAC31644); Pst SS104, HrpN of P. stewartii subsp. stewartii strain SS104 (GenBank accession no. AAG01466); Ech EC16, HrpN of E. chrysanthemi EC16 (GenBank accession no. AAC31978); Ech 3937, HrpN of E. chrysanthemi 3937 (https://asap.ahabs.wisc.edu/annotation/php/query_features.php [Feature ID 20784]). N-terminal regions of harpins are highly variable compared to the C terminus. The conserved C-terminal region (underlined) represents the regions of HrpN_{3937} dispensable for aggregative activity.

### TABLE 2. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5'-3')</th>
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<tr>
<td>P0303</td>
<td>TGTCGGCATGGTTCGACTAGTGG</td>
<td>SpeI</td>
</tr>
<tr>
<td>P0304</td>
<td>GTTTCCGAAGCCTTGCGAGCAACC</td>
<td>HindIII</td>
</tr>
<tr>
<td>P0319</td>
<td>TCAGCAAGCCACAAGCTTC</td>
<td>HindIII</td>
</tr>
<tr>
<td>P0324</td>
<td>GCGTACTCTGGCTTGATACCCAGTATCGATGGTCCGGG</td>
<td></td>
</tr>
<tr>
<td>P0325</td>
<td>GGTATCAGCGAGAGAGGCGGAGCAGACAGCATGACAGCAACC</td>
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</tr>
<tr>
<td>P0326</td>
<td>GGTATCAGCGAGAGAGGCGGAGCAGACAGCATGACAGCAACC</td>
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</tr>
<tr>
<td>P0356</td>
<td>GCGTACTCTGGCTTGATACCCAGTATCGATGGTCCGGG</td>
<td></td>
</tr>
<tr>
<td>P0413</td>
<td>GCGTACTCTGGCTTGATACCTGATATTTGTTTCTCTAT</td>
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<tr>
<td>P0414</td>
<td>GGTATCAGCGAGAGAGGCGGAGCAGACAGCATGACAGCAACC</td>
<td></td>
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</tbody>
</table>

* Enzyme restriction sites are underlined.
chrysanthemi EC16, *P. stewartii* subsp. *stewartii* (HrpNPst), and *E. amylovora* (HrpNEam) are 82% (279/340 residues), 43% (142/333 residues), and 41% (151/360 residues), respectively (Fig. 2). To test whether these heterologous *hrpN* genes are able to restore pellicle formation to strain WPP122, the *E. chrysanthemi* 3937 *hrpN* mutant, plasmids pCPP2174 (4), pCPP1084 (21), and pMA1 (1) were electroporated into WPP122, and the transformants were tested for cell aggregation. The closely related HrpNEC16 was capable of mediating pellicle formation, while the other harpins, which are most similar to HrpN3937 in the C-terminal region, were not (Fig. 1D and 2). Thus, the *hrpN* genes from *E. chrysanthemi* 3937, *E. amylovora*, and *P. stewartii* do not appear to be functionally interchangeable. Although *E. chrysanthemi* EC16 can confer pellicle formation on the 3937 Δ*hrpN* mutant, EC16 does not form pellicles in SOBG medium (23), even though it produces HrpN in this medium (data not shown). We hypothesized that this could be due to differential localization of HrpN by these two strains or because a second factor required for aggregation is not produced by EC16.

Since harpins are secreted outside of bacterial cells via the T3SS, we examined whether HrpN3937 was freely released from the cells or remained cell associated after secretion. Notably, HrpN_Eam is surface localized (21) and HrpN_Pst is predominantly secreted into the culture medium (1). We used anti-HrpN_EC16 antibodies and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR) to determine the localization of HrpN3937 in unpermeabilized cells by epifluorescence microscopy. Bacterial cells harvested from 2-day-old SOBG cultures were fixed with 4% paraformaldehyde in 3× phosphate-buffered saline (pH 7.4) and smeared onto poly-L-lysine-coated slides (Electron Microscopy Sciences, Hatfield, PA). Cells were then probed with anti-HrpN_EC16 antibody diluted in blocking solution (1× phosphate-buffered saline supplemented with 2% bovine serum albumin), followed by FITC-conjugated secondary anti-rabbit IgG antibody (Molecular Probes) at a final concentration of 5 μg/ml.

All *E. chrysanthemi* 3937 cells observed in the phase-contrast image were fluorescent (Fig. 3A and D), while only occasional fluorescence was detected in the *hrpN* mutant (Fig. 3B and E), and it was not associated with the bacterial cells. HrpN3937 appears to be cell surface associated and might serve as an intercellular aggregative factor. This localization is dependent on a functional T3SS; a secretion-deficient *hrcJ* mutant, WPP98, which is unable to form pellicles, was not fluorescent (Fig. 3C and F).

EC16, which produces a HrpN capable of restoring pellicle formation to a strain 3937 *hrpN* mutant, does not form pellicles in SOBG medium. We hypothesized that this was due to a lack of production or a lack of association of HrpN_EC16 on the cell surface. However, EC16 produces a cell surface-localized HrpN in King’s B and SOBG media (data not shown). The EC16 HrpN produced in King’s B medium was sensitive to proteinase K, further evidence that the EC16 protein is localized to the outside of the bacterial cell and that localization of HrpN to the outside of the bacterial cell is not sufficient for aggregation (data not shown). Similarly, we have found that strain 3937 produces HrpN at 36°C even though no pellicle forms at this temperature (23).

This suggests that cell-cell adhesion is mediated by contact between HrpN and another extracellular molecule, which may not be produced by EC16, rather than HrpN-HrpN interac-
Cellulose, which is a major constituent of both the 3937 pellicle and plant cell walls, is not a likely candidate since a strain 3937 cellulose synthase mutant is still able to form bacterial aggregates (23). Therefore, the basis of the interaction between HrpN and other surface molecules on bacterial cells and host cells remains to be discovered. Our data also suggest that this form of cell-cell adhesion plays at most a small role in plant-bacterium interactions since a 3937 hpn mutant makes only a subtle contribution to virulence (22).

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