Harpin mediates cell aggregation in Erwinia chrysanthemi 3937

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Harpin Mediates Cell Aggregation in *Erwinia chrysanthemi* 3937

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The enterobacterial plant pathogen *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 *Escherichia coli*, where a degenerate T3SS is required for both virulence and bacterial aggregation (10). However, based on experiments with another plant pathogen, *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 adhesion that promotes bacterial aggregation. This was inferred from the observation that the addition of proteinase 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 μg/ml; carbenicillin, 50 μg/ml.

One known T3SS-secreted protein in *E. chrysanthemi* 3937 is the harpin protein encoded by hrpN. Using information from the genome sequence (8), we deleted the 3937 hrpN gene by crossover PCR-assisted allelic-exchange mutagenesis (17, 22). The ΔhrpN mutant, WPP122, was unable to form a pellicle. Pellicle formation was restored by providing hrpN on a plasmid. Notably, pellets were never observed in WPP122 carrying the vector control pCPP50 (Fig. 1B). This suggests that HrpN3937 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, glycin-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 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 *Pseudomonas syringae* HrpZ elicit the HR in tobacco leaves (2, 9). In *Xanthomonas axonopodis*, however, only the extreme N terminus of HpaG is necessary for elicitor activity (11). By analogy, we hypothesized that portions of HrpN3937 are sufficient for cell aggregation in *E. chrysanthemi* 3937.

To identify the regions conferring aggregative activity upon HrpN3937, a series of deletions in 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 (5′-GTTATC AAGCAGAGTGACGC-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 HrpN derivatives by various plasmids was confirmed by immunoblot analysis with anti-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 μl of 1× Laemmlli buffer (12), and the whole-cell lysate was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Anti-HrpN ER16 antibody that had been preabsorbed with total protein from *E. coli* DH5α and an Immuno-Star AP goat anti-rabbit immunoglobulin G (IgG) chemiluminescence kit (Bio-Rad, Hercules, CA) were used for detection of HrpN derivatives. Both full-length HrpN3937 (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 *P. syringae* HrpW (6), and both cases of slower mobilities may be due to inefficient binding of SDS to the acidic harpins (15).

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### 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>
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<tr>
<td><strong>Strains</strong></td>
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</tr>
<tr>
<td>E. coli DH5α</td>
<td>supE44 ΔlacU169 (Δ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>Clontech</td>
</tr>
<tr>
<td>E. chrysanthemi</td>
<td>Wild type, Saintpaulia (African violet) isolate</td>
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</tr>
<tr>
<td>EC16</td>
<td>Chrysanthemum monilfolium isolate</td>
<td>7</td>
</tr>
<tr>
<td>WPP98</td>
<td>ΔhrcJ::kan Km(^{\text{R}}); 3937 derivative</td>
<td>23</td>
</tr>
<tr>
<td>WPP122</td>
<td>ΔhrpN::kan Km(^{\text{R}}); 3937 derivative</td>
<td>22; this work</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
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<tr>
<td>pCPP50</td>
<td>Ap(^{+}); pLNII(^{13})-A2-based expression vector</td>
<td>5</td>
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<tr>
<td>p50HrpN</td>
<td>1.25-kb full-length hrcJ (^{3937}) was PCR amplified with primers P0303 and P0304 and cloned into the SpeI and HindIII sites of pCPP50</td>
<td>This work</td>
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<tr>
<td>p50HrpN(_{A2-43})</td>
<td>1.15-kb hrcJ (^{3937}) was PCR amplified with primers P0303, P413, P0414, and P0304 and cloned into the SpeI and HindIII sites of pCPP50</td>
<td>This work</td>
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<td>p50HrpN(_{A50-117})</td>
<td>1.06-kb hrcJ (^{3937}) was PCR amplified with primers P0303, P304, P0324, and P0325 and cloned into the SpeI and HindIII sites of pCPP50</td>
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<td>p50HrpN(_{A50-197})</td>
<td>0.8-kb hrcJ (^{3937}) was PCR amplified with primers P0303, P304, P0324, and P0326 and cloned into the SpeI and HindIII sites of pCPP50</td>
<td>This work</td>
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<tr>
<td>p50HrpN(_{A171-197})</td>
<td>1.04-kb hrcJ (^{3937}) was PCR amplified with primers P0303, P304, P0326, and P0356 and cloned into the SpeI and HindIII sites of pCPP50</td>
<td>This work</td>
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<tr>
<td>p50HrpN(_{A171-342})</td>
<td>0.58-kb hrcJ (^{3937}) was PCR amplified with primers P0303 and P356, blunt-ended, digested with SpeI, and cloned into the SpeI and Smal sites of pCPP50</td>
<td>This work</td>
</tr>
<tr>
<td>p50HrpN(_{A230-342})</td>
<td>0.9-kb hrcJ (^{3937}) was PCR amplified with primers P0303 and P319 and cloned into the SpeI and HindIII sites of pCPP50</td>
<td>This work</td>
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<tr>
<td>pCPP2174</td>
<td>Ap(^{+}); 1.0-kb hrcJ (^{EC16}) cloned into the NcoI-HindIII sites of pSE280</td>
<td>4</td>
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<td>pCPP1084</td>
<td>Ap(^{+}); 1.3-kb HindIII hrcJ of P. stewartii subsp. stewartii in pBluescript M13+ and pMA1</td>
<td>21</td>
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<tr>
<td>pMA1</td>
<td>Ap(^{+}); 1.8-kb HindIII hrcJ of P. stewartii subsp. stewartii in pBluescript SK</td>
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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 p50HrpN2-43 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 HrpN3937 with the harpins of E. amyllovora 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 HrpN3937 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>TGTCCGCGATGTTCTGGGACTAGTGG</td>
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</tr>
<tr>
<td>P0304</td>
<td>GTTTCGCGAAGCGCTTGGGCACGCCG</td>
<td>HindIII</td>
</tr>
<tr>
<td>P0319</td>
<td>CGTCGACGCCACCAAGTC</td>
<td>HindIII</td>
</tr>
<tr>
<td>P0323</td>
<td>GCCTACTCGTGCTTATCCAGCTTTATCGATGCAGCCG</td>
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<tr>
<td>P0325</td>
<td>GGTATACGCAGAGTACGGGCGACATGACCGTACCA</td>
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<tr>
<td>P0330</td>
<td>GGTATACGCAGAGTACGGGCGACATGACCGTACCA</td>
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<tr>
<td>P0413</td>
<td>GCCTACTCGTGCTTATCCAGCTTTATCGATGCAGCCG</td>
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</tr>
<tr>
<td>P0414</td>
<td>GGTATAATCAGGATACGGGCCACACTCGATAAGCTG</td>
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</table>

* Enzyme restriction sites are underlined.

FIG. 2. Multiple protein sequence alignment of harpins used to complement the ΔhrpN mutant of strain 3937. The alignment was performed by the CLUSTAL W method (19) with default parameters. Amino acid sequences are indicated as follows: Eam 321, HrpN of E. amyllovora 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 HrpN3937 dispensable for aggregative activity.
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 ΔhrpN 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-
tions. 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\textsuperscript{3937} and other surface molecules on bacterial aggregates is still to be discovered and remains to be discovered.

Support was provided by Hatch award 4605 from the College of Agricultural and Life Sciences at UW-Madison and by the University of Wisconsin—Madison Graduate School, and the National Science Foundation (award no. MCB-0211750). We thank Alan Collmer and Steve Beer of Cornell University for providing the HrpN antibodies and pCPP1084, respectively, and Dave Coplin of Ohio State University for providing pMA1. We also thank Nicole Perna of the University of Wisconsin for providing access to the annotated \textit{E. chrysanthemi} genome sequence.

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