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Harpin Mediates Cell Aggregation in *Erwinia chrysanthemi* 3937

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The hypersensitive response elicitor harpin (HrpN) of soft rot pathogen *Erwinia chrysanthemi* strains 3937 and EC16 is secreted via the type III secretion system and remains cell surface bound. Strain 3937 HrpN is essential for cell aggregation, but the C-terminal one-third of the protein is not required for aggregative activity.

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 adhesin 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 Δ*hrpN* mutant, WPP122, was unable to form a pellicle. Pellicle formation was restored by providing *hrpN* on a plasmid. Notably, pellicles were never observed in WPP122 carrying the vector control pCPP50 (Fig. 1B). This suggests that $HrpN_{3937}$ 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 *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 $HrpN_{3937}$ are sufficient for cell aggregation in *E. chrysanthemi* 3937.

To identify the regions conferring aggregative activity upon $HrpN_{3937}$, 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′-GGTATC AACGCAGAGTACGC-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× Laemmli buffer (12), and the whole-cell lysate was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Anti-$HrpN_{EC16}$ 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 $HrpN_{3937}$ (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 *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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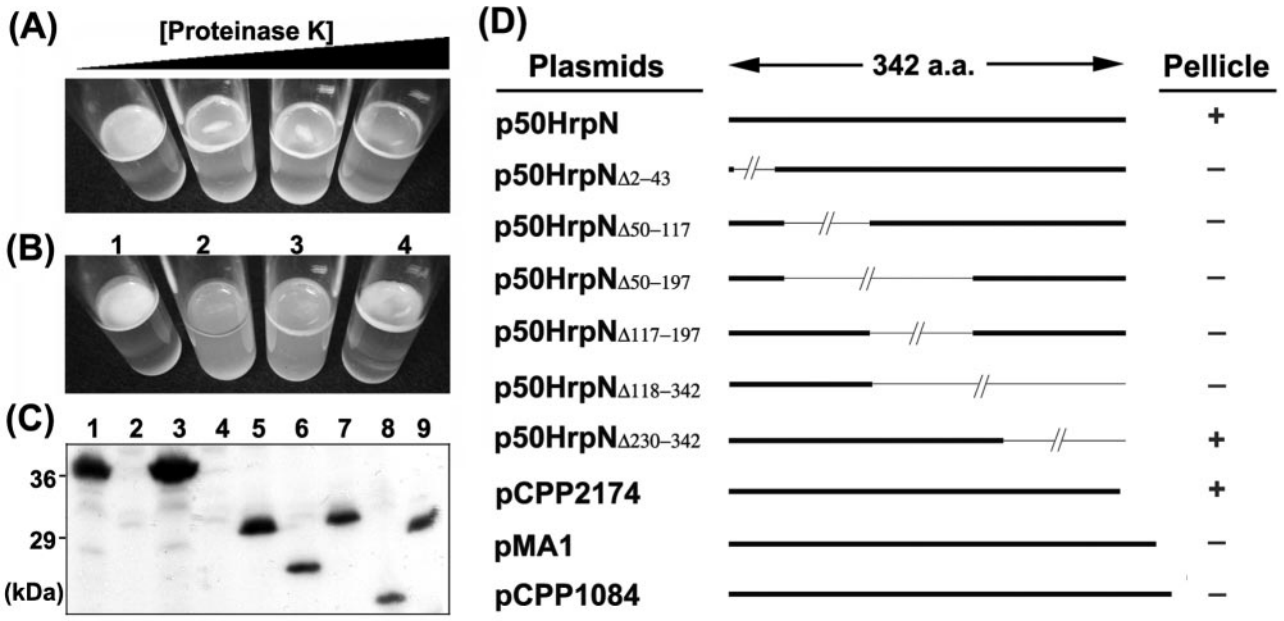

FIG. 1. Pellicle formation in *E. chrysanthemi* 3937- and 3937 Δ*hrpN*-expressing *hrpN* fragments. Pellicles are a type of biofilm that forms at the air-liquid interface. In 3937, pellicles are genetically distinct from the biofilm that forms at the surface-liquid-air interface (23). (A) Exogenous addition 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 Δ*hrpN* 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-$HrpN_{EC16}$. Lane 1, wild-type *E. chrysanthemi* 3937; lane 2, WPP122; lane 3, WPP122(p50HrpN); lane 4, WPP122(p50$HrpN_{\Delta 2-43}$); lane 5, WPP122(p50$HrpN_{\Delta 50-117}$); lane 6, WPP122(p50$HrpN_{\Delta 50-197}$); lane 7, WPP122(p50$HrpN_{\Delta 117-197}$); lane 8, WPP122(p50$HrpN_{\Delta 118-342}$); lane 9, WPP122(p50$HrpN_{\Delta 230-342}$). (D) Diagram of truncated HrpN derivatives and other harpin proteins used to test their ability to restore pellicle formation to the Δ*hrpN* mutant. The C terminus of $HrpN_{3937}$ 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.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli DH5α	*supE44 ΔlacU169* (ϕ*80lacZΔM15*) *hsdR17 recA1endA1 gyrA96 thi-1 relA1*	Clontech
E. chrysanthemi		
3937	Wild type, *Saintpaulia* (African violet) isolate	13
EC16	*Chrysanthemum morifolium* isolate	7
WPP98	*hrcJ*::*kan* Km^r; 3937 derivative	23
WPP122	Δ*hrpN*::*kan* Km^r; 3937 derivative	22; this work
Plasmids		
pCPP50	Ap^r; $pINIII^{113}$-A2-based expression vector	5
p50HrpN	Ap^r; 1.25-kb full-length $hrpN_{3937}$ was PCR amplified with primers P0303 and P0304 and cloned into the SpeI and HindIII sites of pCPP50	This work
p50$HrpN_{\Delta 2-43}$	Ap^r; 1.15-kb $hrpN_{3937}$ was crossover PCR amplified with primers P0303, P413, P0414, and P0304 and cloned into the SpeI and HindIII sites of pCPP50	This work
p50$HrpN_{\Delta 50-117}$	Ap^r; 1.06-kb $hrpN_{3937}$ was crossover PCR amplified with primers P0303, P304, P0324, and P0325 and cloned into the SpeI and HindIII sites of pCPP50	This work
p50$HrpN_{\Delta 50-197}$	Ap^r; 0.8-kb $hrpN_{3937}$ was crossover PCR amplified with primers P0303, P304, P0324, and P0326 and cloned into the SpeI and HindIII sites of pCPP50	This work
p50$HrpN_{\Delta 117-197}$	Ap^r; 1.04-kb $hrpN_{3937}$ was crossover PCR amplified with primers P0303, P304, P0326, and P0356 and cloned into the SpeI and HindIII sites of pCPP50	This work
p50$HrpN_{\Delta 118-342}$	Ap^r; 0.58-kb $hrpN_{3937}$ was PCR amplified with primers P0303 and P356, blunt-ended, digested with SpeI, and cloned into the SpeI and SmaI sites of pCPP50	This work
p50$HrpN_{\Delta 230-342}$	Ap^r; 0.9-kb $hrpN_{3937}$ was PCR amplified with primers P0303 and P319 and cloned into the SpeI and HindIII sites of pCPP50	This work
pCPP2174	Ap^r; 1.0-kb $hrpN_{EC16}$ cloned into the NcoI-HindIII sites of pSE280	4
pCPP1084	Ap^r; 1.3-kb HindIII *hrpN* of *E. amylovora* in pBluescript M13+	21
pMA1	Ap^r; 1.8-kb HindIII *hrpN* of *P. stewartii* subsp. *stewartii* in pBluescript SK	1

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′→3′)[a]	Restriction site
P0303	TGTCGGCATGGTTCGACTAGTGG	SpeI
P0304	GTTTCCGAAGCTTCGGGCAACCGC	HindIII
P0319	TCGCCAGGCCACAAGCTTC	HindIII
P0324	GCGTACTCTGCGTTGATACCCAGCTTATCGATGGTGCCGC	
P0325	GGTATCAACGCAGAGTACGCGGGCCATGACACCGTGACCA	
P0326	GGTATCAACGCAGAGTACGCCGCCATCGGTATGGGCGTT	
P0356	GCGTACTCTGCGTTGATACCGGTCACGGTGTCATGGCCCA	
P0413	GCGTACTCTGCGTTGATACCTGCATAATTTCGTTTCCTCAT	
P0414	GGTATCAACGCAGAGTACGCGCGGCACCATCGATAAGCTG	

[a] Enzyme restriction sites are underlined.

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, $HrpN_{3937}$ 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 $p50HrpN_{\Delta 2\text{-}43}$ 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.*

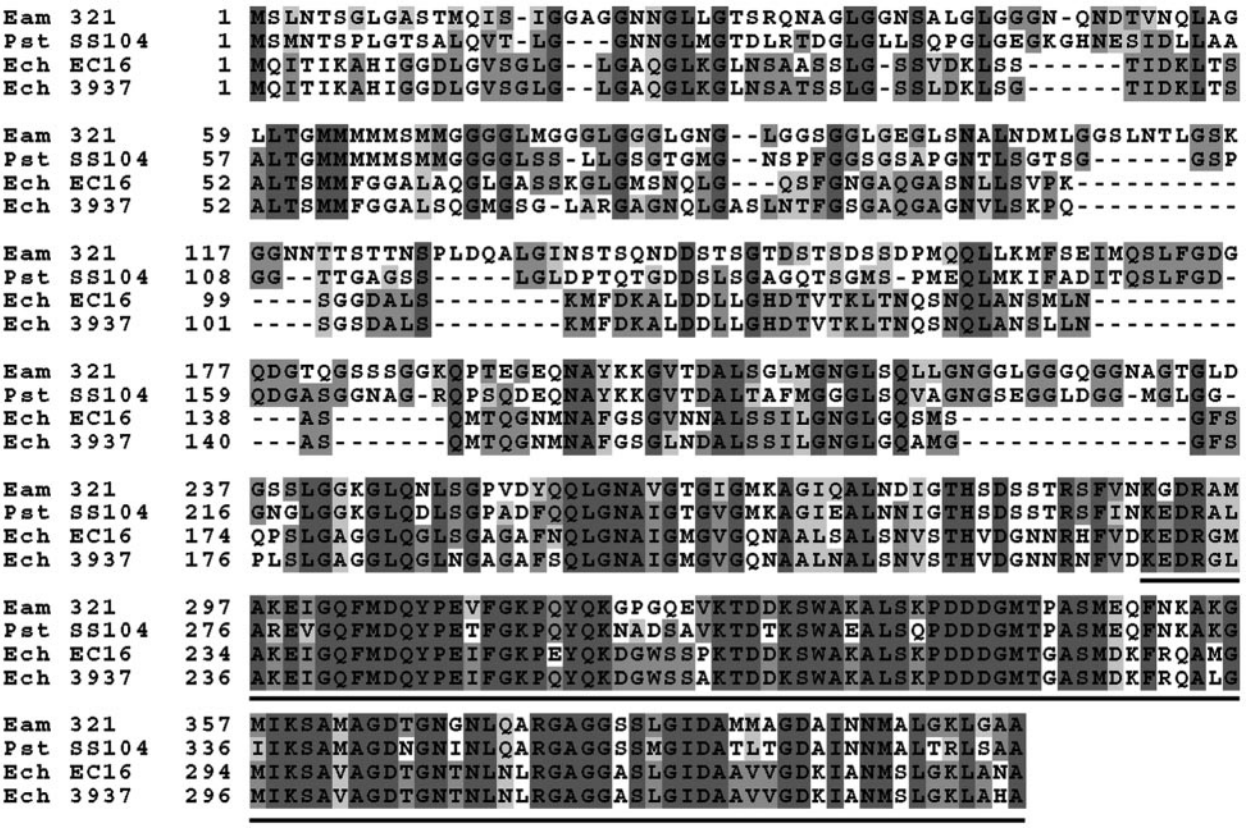

FIG. 2. Multiple protein sequence alignment of harpins used to complement the Δ*hrpN* mutant of strain 3937. The alignment was performed by the CLUSTAL W method (19) with default parameters. Amino acid sequences are indicated as follows: Eam 321, HrpN of *E. amylovora* strain 321 (GenBank accession no. 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.

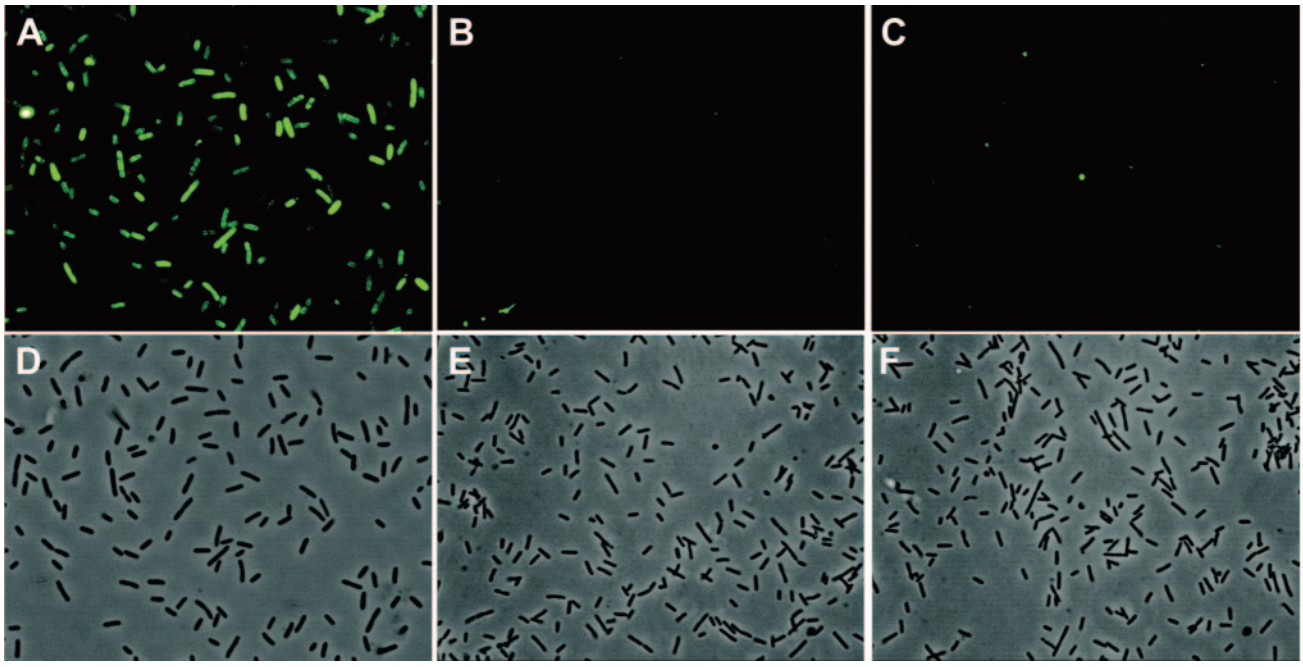

FIG. 3. Indirect immunofluorescence micrographs showing cell surface-associated HrpN in *E. chrysanthemi* 3937. Fluorescence images (A to C) were acquired using an Olympus BHT2 electron microscope (Olympus America, Inc., Melville, N.Y.) with a green fluorescent protein filter set and recorded with a MagnaFireSP charge-coupled-device camera (Optronics, Goleta, Calif.) and Image Pro Plus software (MediaCybernetics, Silver Spring, MD); the same fields corresponding to each FITC image were taken with phase-contrast microscopy (D to F). (A and D) Wild-type 3937; (B and E) WPP122, a Δ*hrpN* mutant; (C and F) WPP98, an *hrcJ*::Km mutant.

chrysanthemi EC16, *P. stewartii* subsp. *stewartii* ($HrpN_{Pst}$), and *E. amylovora* ($HrpN_{Eam}$) 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 $HrpN_{EC16}$ was capable of mediating pellicle formation, while the other harpins, which are most similar to $HrpN_{3937}$ 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 $HrpN_{3937}$ 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 $HrpN_{3937}$ 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. $HrpN_{3937}$ 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 $HrpN_{3937}$ 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 *hrpN* mutant makes only a subtle contribution to virulence (22).

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