

HecA, a member of a class of adhesins produced by diverse pathogenic bacteria, contributes to the attachment, aggregation, epidermal cell killing, and virulence phenotypes of *Erwinia chrysanthemi* EC16 on *Nicotiana clevelandii* seedlings

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Edited by Eugene W. Nester, University of Washington, Seattle, WA, and approved August 1, 2002 (received for review June 14, 2002)

Erwinia chrysanthemi is representative of a broad class of bacterial pathogens that are capable of inducing necrosis in plants. The *E. chrysanthemi* EC16 *hecA* gene predicts a 3,850-aa member of the *Bordetella pertussis* filamentous hemagglutinin family of adhesins. A *hecA::Tn7* mutant was reduced in virulence on *Nicotiana clevelandii* seedlings after inoculation without wounding. Epifluorescence and confocal laser-scanning microscopy observations of *hecA* and wild-type cells expressing the green fluorescent protein revealed that the mutant is reduced in its ability to attach and then form aggregates on leaves and to cause an aggregate-associated killing of epidermal cells. Cell killing also depended on production of the major pectate lyase isozymes and the type II, but not the type III, secretion pathway in *E. chrysanthemi*. HecA homologs were found in bacterial pathogens of plants and animals and appear to be unique to pathogens and universal in necrogenic plant pathogens. Phylogenetic comparison of the conserved two-partner secretion domains in the proteins and the 16S rRNA sequences in respective bacteria revealed the two datasets to be fundamentally incongruent, suggesting horizontal acquisition of these genes. Furthermore, *hecA* and its two homologs in *Yersinia pestis* had a G+C content that was 10% higher than that of their genomes and similar to that of plant pathogenic *Ralstonia*, *Xylella*, and *Pseudomonas* spp. Our data suggest that filamentous hemagglutinin-like adhesins are broadly important virulence factors in both plant and animal pathogens.

Bacterial attachment to host tissues by various adhesins is a first step in the pathogenesis of many animal pathogens, but a role for attachment in plant pathogenesis is less clear. The exception is the tumorigenic pathogen *Agrobacterium tumefaciens*, whose attachment to plant tissues before transfer of T-DNA (the portion of the tumor-inducing Ti plasmid that is transferred to plant cells) involves a Ca²⁺-dependent adhesin (1), a repertoire of proteins encoded by *att* (attachment) genes (2–4), exo- and capsular polysaccharides (5, 6), and bacterial cellulose fibrils (5). In contrast, the role of attachment and adhesins in virulence is unclear for the more prevalent necrogenic (rather than tumorigenic) plant pathogens. These bacteria colonize the surface and intercellular spaces of plants and attack with various combinations of virulence effector proteins injected by type III secretion systems, extracellular pectic enzymes, and low molecular-weight toxins.

The necrogenic pathogens have been reported to produce a variety of potential adhesins, including fimbriae by *Erwinia rhapsodica*, *Erwinia carotovora*, *Pseudomonas syringae* (7), *Xanthomonas campestris* (8), and *Ralstonia solanacearum* (9), type IV pili by *P. syringae* (10), and adhesive factors such as lipopolysaccharide by *R. solanacearum* (11). Attachment to leaf surfaces by type IV pili slightly promotes the epiphytic fitness of *P. syringae* (12), and the related process of self aggregation is also promoted in *P. syringae* by type IV pili and in *X. campestris* by FimA fimbriae (8). The recent report that the *Xylella fastidiosa* (13) and *R. solanacearum* (14)

genomes encode multiple candidate adhesins, most notably the 14 homologs in *R. solanacearum* of the *Bordetella pertussis* filamentous hemagglutinin (FHA), indicates that the potential role of adhesins in plant pathogenesis warrants closer scrutiny.

Much more is known about the adhesins produced by animal pathogens. These are usually proteins that are assembled in a structure, such as a pilus or fimbriae, or are surface exposed (afimbrial adhesins). The afimbrial adhesins are anchored directly in the outer membrane with the adhesin domain exposed to the extracellular surface (15). One of the afimbrial adhesins from *B. pertussis*, FHA, has been extensively characterized because of its large size (220 kDa) and the possibility of its use for vaccination against whooping cough (16). FHA is secreted in a Sec-dependent manner by the two-partner secretion system, which involves an outer membrane-associated accessory protein (FhaC “partner” protein) interacting with a conserved FHA N-proximal secretion domain. FHA is proteolytically processed after secretion, has multiple substrate-binding domains and proapoptotic activity, and promotes bacterial aggregation (17).

The plant pathogens *Erwinia chrysanthemi* and *E. carotovora* also encode FHA homologs (18, 19). *E. chrysanthemi* and *E. carotovora* cause soft-rot diseases on a wide variety of plants and are representative of a major subgroup of necrogenic pathogens that rapidly kill and macerate seedlings or mature fleshy tissues. The adhesin-encoding *hecA* gene was partially sequenced because of its genomic proximity to genes encoding the type III secretion (*hrp/hrc*) genes in *E. chrysanthemi* EC16 (18). The *E. chrysanthemi* Hrp system contributes to the infectivity of strain EC16 on witloof chicory leaves and strain 3937 on African violet (20, 21), although the main virulence factors in soft-rot disease are the multiple pectate lyase isozymes encoded by *pel* genes (22) and secreted via the *out* gene-encoded type II secretion system (23).

The pectolytic attack of susceptible tissues by *E. carotovora* is thought to be activated by *N*-acyl-homoserine lactone-mediated induction of pectic enzyme genes that occurs when bacterial populations in infection sites achieve “quorum” densities (24, 25). Although the quorum-sensing system in *E. chrysanthemi* appears more complex, the dynamics of pectic enzyme production and pathogenesis appear similar (26). *E. chrysanthemi* strain 3937 produces five major Pel isozymes and several secondary isozymes,

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: Hec, hemolysin-like protein in *E. chrysanthemi*; FHA, filamentous hemagglutinin.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF501263).

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and this multiplicity may contribute to the wide host range of the bacterium, as suggested by the varying contributions of the major Pels to cumulative virulence in different hosts (27). Other factors that may contribute to virulence in different hosts include siderophores and exopolysaccharide (systemic invasion of Saint-paulia), the Sap system (resistance to antimicrobial peptides in potato), and methionine sulfoxide reductase for repair of oxidative stress (macerated lesions in chicory leaves) (28–31).

Established virulence assays for soft-rot *Erwinia* spp. are based on the size of macerated lesions and/or numbers of plants locally or systemically diseased after artificial wound inoculations or on the numbers of axenic tobacco seedlings macerated after spray inoculation (27, 32). With the advent of *E. chrysanthemi* (www.ahabs.wisc.edu/~pernalab/erwinia/index.html) and *E. carotovora* (www.sanger.ac.uk/Projects/Microbes/) genomics projects, new assays are needed that provide additional phenotypes for virulence factors and a more detailed view of pathogenesis, particularly the early events. Green fluorescent protein (GFP) from *Aequorea victoria* has been used for this purpose with other plant pathogens, such as *R. solanacearum* (33). However, a previous attempt to use GFP in *E. carotovora* subsp. *atroseptica* had limited success because the level of fluorescence was not suitable for observing bacterial movement in *planta*, and GFP subsequently has not been used with soft-rot *Erwinia* spp.

In this study, we report that (i) pGFP-TIR (34), unlike many other available GFP variants, produces sufficient fluorescence for use with *E. chrysanthemi* in *planta*; (ii) virulence on unwounded *Nicotiana clevelandii* seedlings is strongly reduced in a *hecA* mutant and in mutants deficient in the major Pel isozymes; (iii) the *hecA* mutant is reduced in its ability to attach to and aggregate on leaves; (iv) the *hecA* mutant is similarly unable to cause an early, localized, pectic enzyme-dependent killing of *N. clevelandii* epidermal cells that is associated with bacterial aggregates; and (v) analyses of the complete sequence of HecA, the N-terminal (secretion) domain of several HecA-related proteins, and the associated bacterial rRNA sequences suggest that these adhesin genes have been horizontally acquired.

Materials and Methods

Bacterial Strains and Plasmids. *E. chrysanthemi* wild-type strains (35, 36) and the mutants CUCPB5006 (PelABCE⁻) (37), CUCPB5026 (Out⁻) (37), and CUCPB5040 (Hrp⁻) (20) have been described previously. *E. chrysanthemi* strains were grown at 30°C in King's medium B (KB) supplemented with 20 µg/ml of nalidixic acid, 50 µg/ml of kanamycin, 10 µg/ml of gentamycin, and 100 µg/ml of ampicillin. Overnight cultures were washed twice with KB broth and adjusted to an OD₆₀₀ of 0.3. Plasmids were isolated from *E. coli* DH5α following standard procedures (38). To obtain plasmid pCPP2394 containing the *hecA* and *hecB* genes, pCPP2157 (39) was digested with *Bgl*III, and the 14-kb fragment was purified by using the Prep-a-Gene kit (Bio-Rad) and ligated to pBluescript SK(-) (Stratagene). To obtain plasmid pCPP3095 containing *hecA*, pCPP2157 was digested with *Not*I, and the 10-kb fragment was purified and ligated to pBluescript SK(-). Plasmids were introduced into *E. chrysanthemi* strains by electroporation by using a Gene Pulser (Bio-Rad).

DNA Sequencing. The sequence of the *hecA* region was obtained by primer walking. DNA sequencing was performed with an ABI 3700 DNA sequencer (Applied Biosystems) at the Cornell University BioResource Center. Sequences were assembled by using SEQMAN 5.03 (DNASTar, Madison, WI). The GenBank accession no. for *hecA* is AF501263.

***hecA* Mutagenesis.** pCPP3095 was mutagenized with Tn7 by using the Genome Priming System (New England Biolabs). The location of the transposon insertions was determined by sequencing outward of the transposon by using Tn7 primers gpsS and gpsN, provided by

the manufacturer. Six plasmid clones containing transposon insertions in *hecA* gene were introduced into the chromosome of wild-type *E. chrysanthemi* strain AC4150 (nalidixic acid resistant derivative of EC16) by marker exchange, following standard procedures (40).

Plant Bioassays. *N. clevelandii* seeds were grown on agar medium following previously described procedures (32) with some modifications. *N. clevelandii* seeds were surface sterilized and planted in 24-well tissue culture plates (Costar) on Murashige and Skoog media (GIBCO/BRL). Planted seeds were grown in a growth chamber at 25°C for 4 weeks with a photoperiod of 12 h per day. Four-week-old *N. clevelandii* seedlings were inoculated with bacterial suspensions prepared as described above. Inoculation was done by pipetting 50 µl of suspension to the apical meristem followed by incubation at 25°C. Maceration was scored at 24 and 48 h after inoculation.

Identification of an Available GFP Variant That Functions in *E. chrysanthemi* EC16. Several plasmids expressing fluorescent proteins were tested in *E. chrysanthemi*: pGFP, pGFPuv, pEGFP, and pEYFP (CLONTECH) produced no detectable fluorescence in AC4150; pTB93G and pTB93F (41), produced fluorescence in only 1% of the transformants. pGFPmut3.1 (CLONTECH) (42) made AC4150 fluorescent, but it reduced bacterial viability and virulence, and single-copy integration into the genome via insertion into a mini-Tn5 transposon did not correct this problem. pDsRed1 (CLONTECH) (43) also failed to fluoresce in AC4150, although it did in *E. chrysanthemi* strain 3937. In contrast, pGFP-TIR (34) was fluorescent in both AC4150 and 3937 and did not abolish virulence.

Epifluorescence and Confocal Laser-Scanning Microscopy. Four-week-old *N. clevelandii* leaves were detached and dipped in suspensions containing GFP-labeled bacteria and incubated for 1, 2, 4, and 8 h in a moist chamber. Bacterial behavior on *N. clevelandii* leaves was initially observed and described by using an Olympus (New Hyde Park, NY) BL-FLA microscope equipped with a FITC filter. To further compare the location of bacterial cells relative to the leaf tissue, we used a confocal laser-scanning microscope (Bio-Rad MRC600) equipped with two filter cubes (excitation 488, 568/dichroic 560/emission 522 DF35) at the Microscopy and Imaging Facility at Cornell University. Inoculated *N. clevelandii* leaves were observed at different time points, and the leaf epidermis was used to fix the starting point for the scanning. Scanning was done to 20 µm below the epidermis into the mesophyll cells and 40 µm above the epidermis. Images taken in the red and green channels were merged by using CONFOCAL ASSISTANT 4.02 (Bio-Rad).

Attachment, Aggregation, and Cell Death Assays. *N. clevelandii* leaves were detached from 4-week-old plants and placed in a moist chamber. Leaf surfaces were inoculated with 10 µl of bacterial suspension containing 1 × 10⁸ cfu per ml. Inoculated leaves were incubated for 1, 2, 4, and 8 h. After each incubation time, leaves were rinsed in 1 ml of H₂O and vortexed at maximum speed for 20 sec. Bacterial cells that remained attached to the leaf surface were counted by using epifluorescence microscopy. Individual bacterial cells on the leaf epidermis were counted in 10 randomly selected microscopic fields from three leaves per bacterial strain. In assays of potential inhibitors, AC4150 was incubated for 30 min with chloramphenicol (20 µg/µl) at room temperature or proteinase K (100 µg/µl) on ice before inoculation on detached *N. clevelandii* leaves. Adhesion and aggregation were scored at 4 and 8 h postinoculation, respectively. To assess aggregation in colonies grown in culture, bacterial strains were grown overnight on King's medium B agar, then a loopful of cells was spread in 50 µl of 1× PBS on a microscope slide and observed immediately by bright-field microscopy with a ×100 objective lens as described previously (44). For plant cell death assay, detached *N. clevelandii* leaves were

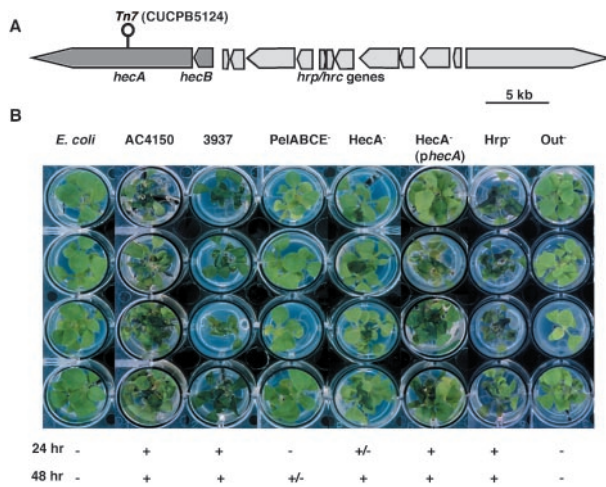


Fig. 1. Physical map of the *hecA* mutation and reduced virulence phenotype of the *hecA* mutant in *N. clelandii* seedlings. (A) The *hecA* and *hecB* genes are adjacent to the *hrp/hrc* cluster in *E. chrysanthemi* AC4150. (B) The virulence of *E. chrysanthemi* is partially reduced by deleting the major *pel* genes or mutating *hecA*. *N. clelandii* seedlings were inoculated with *E. coli* DH5 α , *E. chrysanthemi* wild-type strains AC4150 and 3937, and AC4150 mutant derivatives CUCPB5006 (Δ peIABCE), CUCPB5124 (*hecA*::Tn7), CUCPB5124 carrying *hecA*⁺ pCPP2394, CUCPB5040 (*hrcT*::Tn10), and CUCPB5026 (*out*::TnPhoA). Seedlings were photographed 24 h after inoculation and scored for disease development at 24 and 48 h (-, no leaves macerated; \pm , at least one but not all leaves macerated on each replicate seedling; +, most or all leaves in all replicates macerated).

inoculated with bacterial suspensions as described above and incubated for 8 h. Then leaves were stained on a microscope slide with 50 μ l of propidium iodide (100 μ g/ml). After incubation for 10 min, the leaves were rinsed with water and observed under confocal laser-scanning microscopy.

HecA Protein Sequence and Phylogenetic Analyses. The entire *hecA* sequence was used to search the GenBank nonredundant database by using the BLASTX program (45). Significant similarity (E values $\leq 1 \times 10^{-18}$) was found to regions at the N and C termini of putative adhesins of the hemagglutinin/hemolysin type represented by the FHA from *B. pertussis*. The large size of these proteins, the presence of multiple copies in some genomes, and the potential redundancy for submissions of partial protein sequences led us to restrict the data set for the analysis to the well characterized *B. pertussis* FHA and proteins found in fully sequenced genomes and larger than 1,200 aa. The previously identified secretion domain located at the N terminus (46) was shown to be conserved and was chosen to align the proteins found in plant pathogenic bacteria. Alignment was done by using the CLUSTALW method (47) with Gonnet protein weight matrix (48) from MEGALIGN (DNASTar). To determine whether the relative similarities of these proteins correlate with the phylogeny of the bacteria, a test of congruence between the secretion domain from all plant and animal pathogens and a 16S rRNA data set was conducted. The 16S rRNA sequences from the same set of fully sequenced genomes were recovered from GenBank and aligned by the CLUSTALW method by using the PAM250 matrix (49) from MEGALIGN (DNASTar). The 16S rRNA alignment was combined with the secretion domain alignment, and the homogeneity partition test of Farris was performed (50).

Results

***E. chrysanthemi* Mutant CUCPB5124 Carries a Tn7 Insertion in the 11.5-kb *hecA* ORF.** The *hecA* gene is downstream of *hecB* and is linked to a functional cluster of *hrp/hrc* genes in *E. chrysanthemi* AC4150 (18), as shown in Fig. 1A. The *hecA* gene was sequenced and mutagenized with Tn7 in *E. coli*. Several *hecA*::Tn7 insertions

were marker exchanged into AC4150. In mutant CUCPB5124, the *hecA* ORF is disrupted by Tn7 insertion at nucleotide 7686.

***hecA* Mutation Reduces *E. chrysanthemi* Virulence in *N. clelandii* Seedlings.** To detect any virulence phenotype for *hecA* mutants, we inoculated various test plants with 1×10^8 cfu per ml of AC4150 and CUCPB5124. We wound inoculated potato tuber slices and chicory leaves and measured the resulting weight of macerated tissue (potato) and size of necrotic lesion (chicory leaf) but could detect no phenotype for the mutant. We then surface inoculated axenic seedlings of *Nicotiana tabacum* cv Samsun, *N. tabacum* cv Xanthi, *Nicotiana benthamiana*, and *N. clelandii*. Only *N. clelandii* showed a clearly differential response 24 h after inoculation between wild-type AC4150 and its *hecA* mutant derivative (Fig. 1B): wild-type strains AC4150 and 3937 caused maceration and total collapse of *N. clelandii* seedlings within 24 h, whereas all *hecA* mutants showed reduced virulence and produced water-soaked lesions in only a few individual leaves at this time. However, seedlings completely succumbed to *hecA* mutants by 48 h after inoculation, and the wild-type phenotype was restored in *hecA* mutants with a plasmid construct harboring the *hecA* and *hecB* genes (pCPP2394) *in trans*. The *hecA* mutant CUCPB5124 was chosen for further study. We also assayed on *N. clelandii* seedlings CUCPB5040 (Hrp⁻), CUCPB5006 (PeIABCE⁻), and CUCPB5026 (Out⁻) (Fig. 1B). The Hrp⁻ mutant was indistinguishable from the wild type, the Out⁻ mutant caused no disease during the 48-h assay period, and the PeIABCE⁻ mutant was like the HecA⁻ mutant in showing partially reduced virulence.

Labeling *E. chrysanthemi* with GFP Reveals That HecA Enables the Bacterium to Attach to the Surface of *N. clelandii* Leaves. The reduction in virulence associated with the *hecA* mutation suggested that *hecA* may mediate an attachment event in pathogenesis. We examined the behavior of *E. chrysanthemi* AC4150 and CUCPB5124 on the surface of *N. clelandii* seedling leaves by epifluorescence microscopy by using bacteria carrying pGFP-TIR (34). As early as 1 h after inoculating seedling leaves, GFP-labeled wild-type strain AC4150 and *hecA*-complemented strain CUCPB5124(pCPP2394) began to attach to the leaf surface, whereas the *hecA* mutant strain CUCPB5124 remained planktonic. Inducing bulk flow in water, by pressing on the coverslip, accentuated the planktonic and anchored and immobilized phenotypes, which are presented in the timeline in Fig. 2A. To document with static images the inability of the *hecA* mutant to attach to the leaf surface, we used confocal laser-scanning microscopy to selectively view those bacteria that were at the plane of the leaf surface rather than in the volume of water further above the leaf. As shown in Fig. 2B, many more AC4150 and CUCPB5124(pCPP2394) than CUCPB5124 cells were localized (immobilized) to the plane of the leaf surface 4 h after inoculation.

To quantify the role of HecA in attachment, we used epifluorescence microscopy to count the number of GFP-labeled bacteria that remained attached after vigorous washing of the inoculated leaves at different time points (Fig. 2C). This treatment demonstrated that wild-type AC4150 and complementing mutant CUCPB5124(pCPP2394) attach tightly to the plant surface, and that attachment is HecA dependent and increases over time. These results also confirmed the utility of using the 4-h postinoculation time point for additional studies of factors affecting attachment. We also tested for the ability of *E. chrysanthemi* to attach to the surface of polystyrene and glass by using a standard assay for biofilm formation (51). In contrast to leaves, neither polystyrene nor glass retained bacteria after washing (data not shown).

HecA Is Also Involved in Aggregation. The attachment studies also revealed that 4 h after inoculation, bacteria began to form aggregates that eventually became large clumps of cells immobilized on

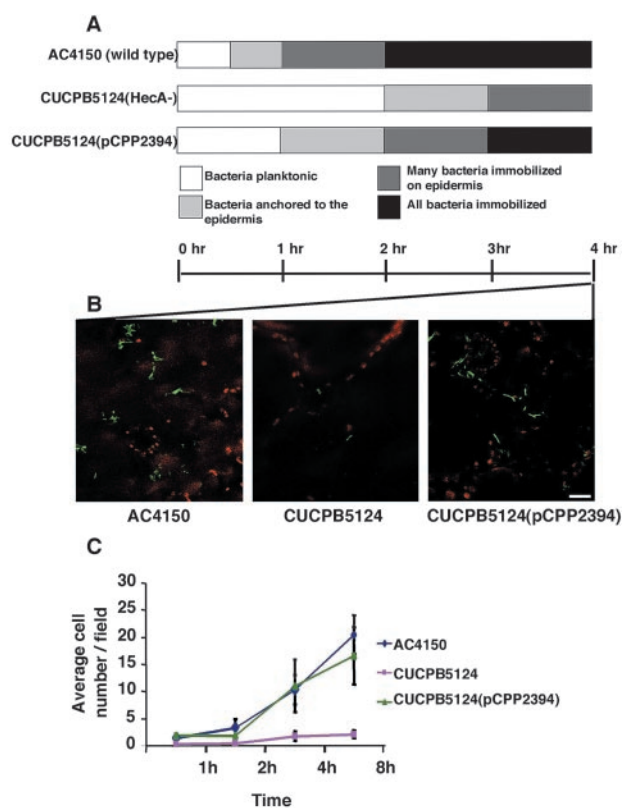


Fig. 2. HecA-dependent adhesion of GFP-labeled *E. chrysanthemi* to *N. cleavelandii* seedlings. (A) Epifluorescence microscopy was used to determine the time course of bacterial anchoring (bacteria movement in flowing water was limited and “anchored” to a point on the leaf) and immobilization (bacteria lie flat on the surface of leaf and are completely immobile in flowing water). (B) Confocal laser-scanning microscopy was used to observe the relative numbers of bacteria at the plane of the epidermal surface 4 h after inoculation. Stacked image composites show green bacteria within the first 40 μm above the epidermis and red chloroplast autofluorescence in the underlying mesophyll. Note that CUCPB5124 cells were distributed throughout the film of water (≈ 1 mm thick) above the leaf and were not concentrated on the surface. (Bar = 20 μm .) (C) In leaf-washing experiments, inoculated leaves were vortexed in a large volume of water at the indicated times; the relative numbers of bacteria remaining on the leaf surface were determined by epifluorescence microscopy. The mean and standard deviations are shown.

the leaf surface. These appeared to develop primarily through recruitment of planktonic cells by freshly attached cells. That is, attachment preceded aggregation, and large aggregates were not observed to be planktonic. However, small groups of planktonic cells (typically two to four) could be seen to move in flowing water as if they were tethered together end to end. Aggregates were observed occasionally in stomates, but they formed predominantly on the surface of the leaf, and 8 h after inoculation there was no evidence that bacteria had invaded the mesophyll.

The formation of aggregates on the surface of the leaves depended on HecA, and the maximum difference between AC4150 and CUCPB5124 in this phenotype was observed 8 h after inoculation. Fig. 3A shows aggregates of AC4150 and CUCPB5124(pCPP2394) on the surface of *N. cleavelandii* seedling leaves that are visible by confocal laser-scanning microscopy. The occurrence of small groups of planktonic bacteria, tethered end to end, also depended on HecA. HecA-dependent aggregation also was observed in bacterial colonies on agar medium after transfer of bacteria to glass slides. Fig. 3B shows bright-field microscopy images of AC4150 and CUCPB5124(pCPP2394) cells that are aggregated, typically as arrays of parallel cells. The *hecA* mutant CUCPB5124

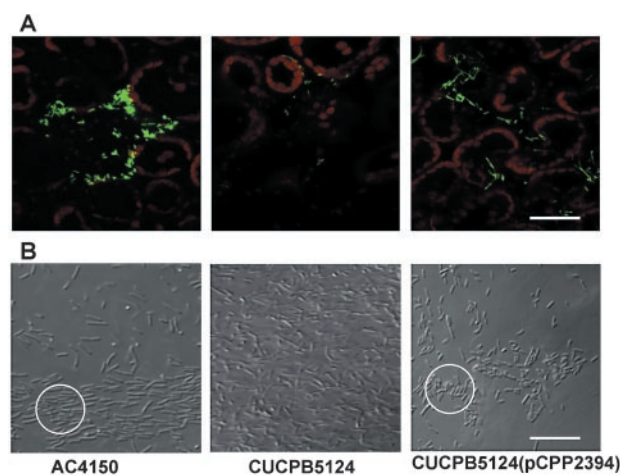


Fig. 3. HecA-dependent aggregation of GFP-labeled *E. chrysanthemi* on the surface of *N. cleavelandii* seedling leaves and during growth on agar medium. (A) Detached leaves from *N. cleavelandii* seedlings were inoculated with AC4150 (wild type), CUCPB5124 (*hecA::Tn7*), and CUCPB5124 carrying *hecA*⁺ pCPP2394. Leaves were observed at 8 h postinoculation by using confocal laser-scanning microscopy to scan 20 μm above and below the epidermal surface for green bacteria and autofluorescent red chloroplasts. (Bar = 20 μm .) (B) Bacteria were scraped from growing colonies on agar medium and observed immediately by bright-field microscopy. Note the parallel arrays of bacteria in the circles with AC4150 and CUCPB5124(pCPP2394). CUCPB5124 does not aggregate in such arrays, and the bacteria appear more diffuse in bright-field microscopy because of the greater mobility of unclustered cells. (Bar = 10 μm .)

cells appeared strikingly different in bright-field microscopy, because almost all cells moved independently in the water.

Inhibition of Adhesion and Aggregation. To determine whether the HecA-dependent adhesion of *E. chrysanthemi* to *N. cleavelandii* seedling leaves and aggregation depended on *de novo* protein synthesis and extracellular protein, we treated the bacteria with either chloramphenicol or proteinase K just before inoculation (Table 1). We then observed the GFP-labeled bacteria at 4 and 8 h. Incubation of the bacteria with chloramphenicol did not inhibit attachment but did inhibit aggregation, whereas proteinase K prevented both attachment and aggregation. We also tested the attachment and aggregation phenotypes of CUCPB5040 (a Hrp⁻ mutant blocked in the type III protein secretion pathway) and CUCPB5026 (an Out⁻ mutant blocked in the type II protein secretion pathway). Neither mutant was altered in adhesion or aggregation.

Cell Death. When observing *E. chrysanthemi* AC4150 aggregates on the surface of *N. cleavelandii* seedling leaves by bright-field micros-

Table 1. Effects of different mutations and treatments on *E. chrysanthemi* adhesion, aggregation, and epidermal cell killing on *N. cleavelandii* seedling leaves

Strain or treatment	Adhesion, 4 h	Aggregation, 8 h	Epidermal cell death, 8 h
AC4150	+	+	+
CUCPB5124 (<i>HecA</i> ⁻)	-	-	-
CUCPB5026 (<i>Out</i> ⁻)	+	+	-
CUCPB5006 (<i>PeIABCE</i> ⁻)	+	+	-
CUCPB5040 (<i>Hrp</i> ⁻)	+	+	+
AC4150 + Cm (20 $\mu\text{g}/\mu\text{l}$)	+	-	ND
AC4150 + proteinase K (100 $\mu\text{g}/\mu\text{l}$)	-	-	ND

+, All the microscopic fields examined showed tested phenotypes. -, None of the microscopic fields examined showed tested phenotypes. ND, not done.

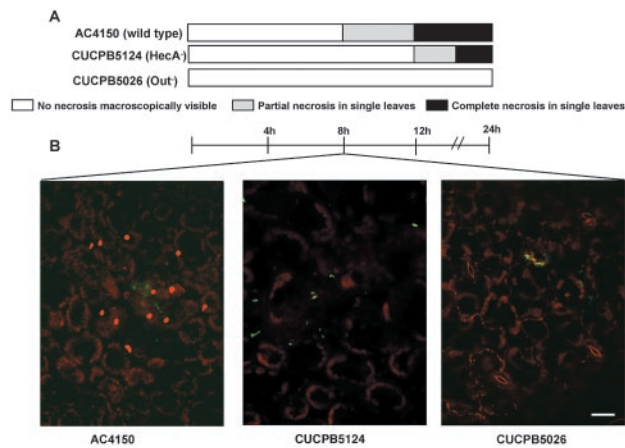


Fig. 4. Reduced killing of *N. clelandii* seedlings and leaf epidermal cells by *E. chrysanthemi* HecA⁻ and Out⁻ mutants. (A) The time course of macroscopically observable necrosis in seedlings was determined after inoculation as described in Fig. 1. (B) The killing of *N. clelandii* epidermal cells by AC4150 was demonstrated by staining with propidium iodide. Detached leaves were dipped in bacterial suspensions of GFP-labeled AC4150 (wild type), CUCPB5124 (*hecA::Tn7*), and CUCPB5026 (*out::TnPhoA*) and incubated for 8 h. Leaves then were stained with propidium iodide and observed under confocal laser-scanning microscopy. Stacked images penetrating 40 μm into the leaf show background autofluorescence of chloroplasts in the underlying mesophyll and the bright fluorescence of propidium iodide-stained nuclei in epidermal cells. (Bar = 50 μm .)

copy, we noted a localized translucence in the underlying tissue. To determine the viability of epidermal cells under these aggregates, we used propidium iodide, which accumulates in the nuclei of cells with compromised cytoplasmic membranes. Fig. 4A summarizes the relative development of macroscopically visible necrosis in *N. clelandii* seedlings inoculated with AC4150, CUCPB5124 (HecA⁻), and CUCPB5026 (Out⁻). Using confocal laser-scanning microscopy, we observed the appearance of clusters of dead epidermal cells ≈ 8 h after inoculation with AC4150 (Fig. 4B). These clusters were associated with aggregates of wild-type bacteria and were not observed 8 h after inoculation with either CUCPB5124 or CUCPB5026. The failure of Out⁻ mutant CUCPB5026 to cause macroscopic necrosis and maceration is likely because of the inability of the bacterium to secrete pectic enzymes. Consistent with this interpretation, CUCPB5006 (PelABCE⁻) did not elicit clusters of propidium iodide-stained epidermal cells at 8 h postinoculation (Table 1). In contrast, CUCPB5040 (Hrp⁻) retained the wild-type cell killing phenotype. These observations indicate that this early killing of epidermal cells depends on HecA and pectic enzymes but is independent of the Hrp system.

hecA Encodes a Protein with Homology to FHA-Related Adhesins in Animal and Plant Bacterial Pathogens. The *hecA* ORF is 11.5 kb in size and predicts a 3,850-aa protein. Fig. 5 shows the signal peptide sequence as predicted by SIGNALP (www.cbs.dtu.dk/services/SignalP/), repetitive sequences found by using Statistical Analysis of Protein Sequences (www.ch.embnet.org/software/SAPS-form.html), and other relevant structural features such as Arg-Gly-Asp motifs and Gly-rich regions. The overall structure of all of the FHA-like adhesins found by BLASTX search (Table 2, which is published as supporting information on the PNAS web site) is similar, so we assumed that the entire proteins are homologous in these taxa. Comparison of the N-proximal 110 aa revealed that the two-partner secretion domain noted in HecA (46) is conserved in all of the HecA homologs recently reported in other plant pathogens (13, 14). The congruency test between the secretion domains and 16S rRNA sequences found the two datasets to be fundamentally incongruent ($P = 0.001$). Thus, the phylogenetic history of

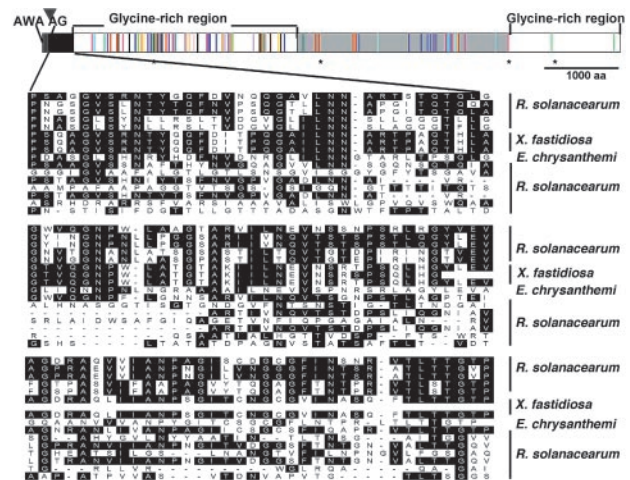


Fig. 5. Structural features of *E. chrysanthemi* HecA. Putative domains of the protein are depicted with gray indicating the N-terminal signal peptide (with cleavage site), black indicating the conserved two-partner secretion domain, asterisks indicating Arg-Gly-Asp motifs, and vertical lines indicating 25 different repeats. The two-partner secretion domains of FHA-like adhesins found in plant pathogens are aligned, and conserved residues are highlighted. An expanded version of this figure, including GenBank accession nos., is published as Fig. 6 in the supporting information on the PNAS web site, www.pnas.org.

HecA and HecA-related proteins does not correlate with the phylogeny of the bacterial species.

Discussion

The soft-rot diseases caused by *E. chrysanthemi* and *E. carotovora* represent the “common cold” of plants in their ubiquity and greater dependence on environmental factors rather than host genotype. Although dynamic aspects of the interaction appear to determine the development of these diseases, little is known of the underlying events, especially those occurring early in pathogenesis. Here, we have found that HecA, a member of a class of adhesins important in several animal pathogens, is important in the virulence of *E. chrysanthemi* on *N. clelandii* seedlings. Closer inspection of the pathogenic defect in *hecA* mutant CUCPB5124 revealed three events, attachment, aggregation, and epidermal cell killing, which occur in sequence before the appearance of disease symptoms and that appear to represent the earliest steps in *E. chrysanthemi* pathogenesis on this host. These events and the structure of HecA invite further consideration of the initiation of the pectolytic attack on plant tissues, the multiple activities of HecA, similarities between plant and animal pathogens, and the evolution of FHA-like adhesins among pathogens.

The importance of pectic enzymes in the active maceration phase of *E. chrysanthemi* pathogenesis seems certain and is supported here by the loss in pathogenicity of mutant CUCPB5026 (which cannot secrete pectic enzymes) and by the delayed virulence of mutant CUCPB5006 (which can produce only the “secondary” Pel isozymes). How pectic enzymes are deployed at the initiation of the pectolytic attack is less clear. Induction of pectic enzyme production by *E. carotovora* in potato appears to be a social event depending on a quorum of bacteria in the infection site. In contrast, the parasitic benefits of pectic enzyme production for a mixed population of *E. chrysanthemi* wild-type and nonpectolytic mutants in chicory appear to go only to those individuals that produce the enzymes (52). Using propidium iodide, we determined here that the first *N. clelandii* seedling cells to die were epidermal cells associated with aggregates of immobilized bacteria. The formation of these aggregates depended on HecA, and the epidermal killing in turn depended on both aggregation and production of the major Pel

isozymes. These observations raise two new questions. First, is aggregation needed for epidermal cell killing because it promotes quorum-dependent *pel* induction or because it promotes more focused Pel activity? Second, is HecA uniquely important when inoculum is applied to the surface of a susceptible host rather than to a wound?

HecA and related proteins found in other plant pathogenic bacteria are highly diverse. However, they share structural features of FHA-like adhesins, including large size, the presence of an N-terminal signal peptide, a 110-aa N-proximal two-partner secretion domain, Gly-rich regions, repetitive sequences, and multiple Arg-Gly-Asp motifs. Furthermore, the function of HecA is independent of Hrp and Out systems, suggesting that the *hecB* gene, located upstream of *hecA*, should encode a functional TpsB transporter family (46) to aid in HecA secretion. HecA contains neither the heparin-nor the carbohydrate-binding domain found in FHA. We do not know yet whether HecA is like FHA in being proteolytically cleaved on secretion, or whether it has any independent biological activity on host cells (although it is not sufficient to kill epidermal cells). The effects of chloramphenicol and proteinase K pretreatment on *E. chrysanthemi* interactions with *N. clelandii* seedlings are consistent with the *hecA*-dependent attachment ability being conferred by a constitutively expressed extracellular protein whose continued production is required for bacterial aggregation. We observed KdGR-like binding sequences upstream of *hecA*, but expression of a *uidA* insertion under the control of these *hecA* upstream sequences was not induced by polygalacturonic acid, and *hecA* appears to be expressed constitutively (data not shown).

The factors known so far to contribute to the virulence of both plant and animal pathogens include regulators, protein secretion systems, and stress tolerance mechanisms that operate within the pathogen (53). Extracellular virulence factors that target hosts of both kingdoms are less common. Examples have been found in the multikingdom opportunistic pathogen *P. aeruginosa* and in a small subset of the effector proteins secreted by the type III

systems of different plant and animal pathogens (53, 54). Our data suggest that FHA-like adhesins represent a class of external virulence factors that are broadly important in both plant and animal pathogens. Several things are noteworthy here: (i) The diverse plant pathogens *E. chrysanthemi*, *R. solanacearum* (root-infecting wilt pathogen with a type III secretion system), *X. fastidiosa* (insect vectored xylem-limited pathogen lacking a type III secretion system), and the most recently sequenced *Xanthomonas axonopodis* pv *citri* and *X. campestris* pv *campestris* (55) all encode at least one FHA homolog, suggesting such proteins may be universal among necrogenic (but not tumorigenic) plant pathogens. (ii) The 19 proteins most closely related to HecA are all from pathogens, further supporting a role for these proteins in host interactions. (iii) The distribution of similarities among these proteins suggests that the encoding genes have been acquired horizontally.

Further support for the horizontal acquisition of FHA-like adhesins is found with HecA and the two adhesins in *Y. pestis*. The *Y. pestis* adhesins are the proteins that are most similar to HecA despite differences in host kingdom, and the G+C content of the encoding genes is more than 10% higher than that of the respective genomes for *E. chrysanthemi* and *Y. pestis*. This finding suggests that these adhesins were horizontally acquired relatively recently from another pathogen, such as *P. aeruginosa*, with a higher % G+C genome. In contrast, the G+C content of the genes encoding the other adhesins in Table 2 was approximately the same as the respective genomes. In summary, the ubiquity of FHA-like adhesins among plant pathogens suggests that these proteins provide good starting points for future investigations of early events in the development of many bacterial plant diseases.

We thank Pablo Rodriguez-Palenzuela for help in testing the efficacy of GFP variants in *E. chrysanthemi*, Adela Ramos and Ryan Rapp for useful suggestions, and Terrence P. Delaney for assistance with epifluorescence microscopy. This work was supported by U.S. Department of Agriculture National Research Initiative Competitive Grants Program Grant 97-35303-4488 (A.C.) and National Science Foundation Division of Environmental Biology Grant 0089483 (J.J.D.).

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