Molecular Characterization and Expression of the *Erwinia carotovora* *hrpN*_{Ecc} Gene, Which Encodes an Elicitor of the Hypersensitive Reaction

Asita Mukherjee, Yaya Cui, Yang Liu, and Arun K. Chatterjee

Department of Plant Pathology, University of Missouri, Columbia 65211 U.S.A.

Received 24 December 1996. Accepted 5 March 1997.

The nucleotide sequence of *hrpN*_{Ecc} DNA, cloned from *Erwinia carotovora* subsp. *carotovora* strain Ecc71, reveals a coding region of 1,068 bp which matches the size of *hrpN*_{Ecc} transcripts. *hrpN*_{Ecc} is predicted to encode a glycine-rich protein of approximately 36 kDa. Like the elicitors of the hypersensitive reaction (HR) produced by *E. chrysanthemi* (Harpin_{Ech}) and *E. amylovora* (Harpin_{Ea}), the deduced 36-kDa protein does not possess a typical signal sequence, but it contains a putative membrane-spanning domain. In *Escherichia coli* strains overexpressing *hrpN*_{Ecc}, the 36-kDa protein has been identified as the *hrpN*_{Ecc} product by Western blot analysis using anti-Harpin_{Ech} antibodies. The 36-kDa protein fractionated from *E. coli* elicits the HR in tobacco leaves. Moreover, a HrpN[−] and RsmA[−] double mutant (RsmA = regulator of secondary metabolites) does not produce this 36-kDa protein or elicit the HR, although this strain, like the RsmA[−] and HrpN⁺ bacteria, overproduces extracellular enzymes and macerates celery petioles. These observations demonstrate that *hrpN*_{Ecc} encodes the elicitor of the HR, designated Harpin_{Ecc}. The levels of *hrpN*_{Ecc} transcripts are affected in both RsmA⁺ and RsmA[−] strains by media composition and carbon sources, although the mRNA levels are substantially higher in the RsmA[−] strains. The expression of *hrpN*_{Ecc} in Ecc71 is cell density dependent and is activated by the quorum-sensing signal, *N*-(3-oxohexanoyl)-L-homoserine lactone (OHL). By contrast, *hrpN*_{Ecc} expression in an RsmA[−] strain is independent of cell density, and substantial expression occurs in the absence of OHL. The effects of cultural conditions and the occurrence of putative *cis*-acting sequences, such as consensus σ^{54} promoters and an *hrp* promoter upstream of the transcriptional start site, indicate that the production of Harpin_{Ecc} in wild-type RsmA⁺ *E. carotovora* subsp. *carotovora* is tightly regulated. These observations, taken along with the finding that the HR is caused by RsmA[−] mutants but not by RsmA⁺ strains (Cui et al., 1996, Mol. Plant-Microbe Interact. 9:565-573), strongly support the idea that the inability of the wild-type pectolytic *E. carotovora* subsp. *carotovora* to elicit the HR is due to the lack of a significant level of Harpin_{Ecc} production.

Additional keywords: growth phase regulation; *N*-acyl homoserine lactone; RsmA effect; soft-rotting *Erwinia*

Many plant-pathogenic bacteria elicit a rapid localized response in nonhost plants, generally known as the hypersensitive reaction (HR) (Alfano and Collmer 1996; Bonas 1994; Willis et al. 1991). A typical HR in tobacco leaves is characterized by the rapid collapse of leaf tissue followed by necrosis of the collapsed area. Among gram-negative bacteria, *Agrobacterium* spp. and soft-rotting *Erwinia* are unusual in that they do not elicit a typical HR when infiltrated into the leaves of nonhost plants. However, in a recent report we documented that RsmA[−] mutants (RsmA = regulator of secondary metabolites) of the soft-rotting bacterium *E. carotovora* subsp. *carotovora*, like most other gram-negative bacteria, cause a typical HR in tobacco leaves (Cui et al. 1996). A gene designated *hrpN*_{Ecc} was cloned from the wild-type strain Ecc71, and the putative product was found to have significant homology with the HR elicitors (Harpins) produced by *E. amylovora* (Wei et al. 1992b) and *E. chrysanthemi* (Bauer et al. 1995). We proposed that *hrpN*_{Ecc} encodes an elicitor of the HR and that the elicitation of the HR by the RsmA[−] mutants, but not by their RsmA⁺ parents, was due to higher levels of *hrpN*_{Ecc} expression. Since the RsmA[−] mutants also overproduce extracellular enzymes such as pectate lyase, polygalacturonase, cellulase, and protease, the possibility remained that the rapid limited necrosis in tobacco leaves was elicited by one or more secreted enzymes. We have now eliminated this possibility by constructing HrpN[−] derivatives of RsmA[−] strains by marker exchange and by demonstrating that such strains, like the RsmA[−] and HrpN⁺ strains, produce the full complement of extracellular enzymes but do not elicit the HR in tobacco leaves. In addition, we have identified the product of *hrpN*_{Ecc} as Harpin_{Ecc}, a 36-kDa glycine-rich protein with elicitor activity. We have also examined several parameters that control *hrpN*_{Ecc} expression. The results support the hypothesis that *hrpN*_{Ecc} expression is tightly regulated in *E. carotovora* subsp. *carotovora* by a two-tier regulatory system, i.e., by factors that control transcription as well as by RsmA, which modulates the levels of transcripts, presumably by a posttranscriptional mechanism.

RESULTS

Structure of *hrpN*_{Ecc}

The *hrpN*_{Ecc} gene (Fig. 1) is predicted to encode a protein (Harpin_{Ecc}) of 356 amino acid residues, having significant similarities with the HrpN proteins (Harpins) of *E. amylovora*

Corresponding author: Arun K. Chatterjee; Phone: 573-882-1892; Fax: 573-882-0588; E-mail: ACHATTERJEE@PSU.MISSOURI.EDU

(Ea) and *E. chrysanthemi* (Ech) (Cui et al. 1996) as well as *E. stewartii* (Es) (Ahmad et al. 1996; D. Coplin, *personal communication*). Like the other Harpins, Harpin_{Ecc} lacks cysteine and is rich in glycine, especially in the N-terminal region, where 48 residues out of the first 151 are glycine (Fig. 1). From the deduced amino acid sequence of Harpin_{Ecc}, the N-terminal region does not contain a putative signal peptide sequence. Instead, in the Harpin_{Ecc} sequence from amino acid residue 54 to residue 145 there are four α -helices, each consisting of 21 residues (Fig. 1). These sequences could form a transmem-

<i>EcoRI</i>	
GAATTCCGACAATCAGCATGACTTTCTCACGGCGCTGGAAACGGCGCCGGC	-350
CGCTTATTGGACAGTACAACCGGGCGTCACGCTGTGGTTTACGGCAGTGCC	-299
UAS	
AGCGCGGCGAGCGACACTCACCTTAACGCTGGCTCCCGCCGACATTATCC	-248
CGGAATGCTCGCGCAAAATTTTACAACGCGCTTTCTGGAGGCTGACCGGCT	-197
UAS	
GTTTGCCATTAGCCCTGAGCCTGGATGAACAGCAATCTCGGTTACATCG	-146
GGCATTATCCACATTGACTGATTCAGTTGCGCAATCGACGAATGTGGCG	-95
UAS	
TTTGGCGGCTCTGCGCGCGCTGAACCGTGAAGATCATGCGTTTTCGT	-44
hrp promoter	+1
GTGTACGATCGGAATCGAGCAGCAAGAAATCACTTAATGGGGAGTGTC	+8
ACAATCACGCGGGCTGACCGCACATCATTTGGCGCTTTCATAACCCATTAC	+59
RBS	
TCATTGAGGAAACATTATGCTTAATCTCTTGGTGGCGGAGCTTCTCTGCA	+110
M L N S L G G G A S L Q	12
AATCAGCATCAAAGCGGGCGGTAAACGGGTTTATTTCCCTCTCAGTCTTC	+161
I T I K A G G N G G L F P S Q S S	29
ACAAAATGGCGGATCGCCGTCGCGAGTCAGCGTTTGGTGGCCAGCGTAGCAA	+212
Q N G G S P S Q S A F G G Q R S N	46
<i>EcoRV</i>	
CATCGCAGAACTGTCCGATATCATGACGACCATGATGTTTCATGGGCAG	+263
I A E Q L S D I M T T M M F M G S	63
CATGATGGGCGGCGCATGAGCGGTGGCGGTTTGGGTAGCAGTCT	+314
M M G G G M S G G L G G L G S S L	80
GGGCGGACTCGGAGGTGGTCTACTGGGCGGTGGTCTGGGGCGGCTCGG	+365
G G L G G G L L G G G L G G G L G	97
CAGAGCGCTTGGCAGCGGTTGGGAAGTGGCTCGGTGGTGGATTGGGCGG	+416
S S L G S G L G S A L G G G L G G	114
CGCGCTGGGTCGGGTATGAATGCCATGAATCCATCGGCCATGATGGGCAG	+467
A L G A G M N A M N P S A M M G S	131
<i>BglIII</i>	
CCTGCTGTTTACGCGCGTGAAGATCTGCTGGGCGGCGGCATGTCACAGCA	+518
L L F S A L E D L L G G G M S Q Q	148
GCAAGTGGGGTTTTCGGCAACAAACAGCGCTCGTCCGCGGAATTTCTGC	+569
Q G G L F G N K Q P S S P E I S A	165
CTATACCAAGGCGTTAAACGATGCGCTGTCCGCAATTTTGGGCAACGGCCT	+620
Y T Q G V N D A L S A I L G N G L	182
GAGCCAGACGAAAGGCAACGTCACCGCTGCAATTGGGTAATAACGGTCT	+671
S Q T K G Q T S P L Q L G N N G L	199
GCAAGCCTTACGCGGCGCGGTGCGTTTAATCAACTGGGCGAGCAGCTGGG	+722
Q G L S G A G A F N Q L G S T L G	216
GATGAGCGTTGGGCAAAAAGCGGTTTGCAGGAGTTGAACAACATCAGTAC	+773
M S V G Q K A G L Q E L N N I S T	233
GCACAACGACGCCGCGGTTATTTCTGTCGATAAAGAAGATCGCGGAAT	+824
H N D S P T R Y F V D K E D R G M	250
GGCGAAAGAGATTGGTCACTTTATGATCAATATCCTGAAGTCTTCGGCAA	+875
A K E I G Q F M D Q Y P E V F G K	267
GGCCGAATACGAAAGACAATGCGCAGACGGCGAAACAGGAGGATAATC	+926
A E Y Q K D N W Q T A K Q E D K S	284
CTGGGCTAAAGCGCTGAGCAAGCGGATGACGACGGTATGACCAAGGCGAG	+977
W A K A L S K P D D D G M T K G S	301
CATGGATAAATTCATGAAGCGGTTGGCATGATCAAGAGTCCGATACGGGG	+1028
M D K F M K A V G M I K S A I R G	318
TGATACTGGCAACACTAATCTGAGTGTCTGTTGGCAACGGCGGCGCTCGT	+1079
D T G N T N L S A R G N G G A S L	335
GGGTATTGATGACGCGATGATCGGCGACCGTATCGTCAATATGGGGTTGAA	+1130
G I D A A M I G D R I V N M G L K	352
GAACTCTCCAGCTAAACGGAAGTTGAGGAGAGCAGGCGGAAGTCTTGCT	+1181
K L S S *	356
CTCTGTTTAAATTGCAAGAAACTATTTCACTATTAATCAATTGATTGG	+1232
CCTGTTTTCAGCCCTTCTTCGCACTATTGAGTTTCACTTCGC	+1283

Fig. 1. Nucleotide and deduced amino acid sequences of *hrpN_{Ecc}*. The guanine residue at the transcriptional start site (+1) and the putative ribosome binding site (RBS) are indicated in boldface. The putative *cis*-acting regulatory sequences are indicated as follows: *hrp* promoter and σ^{54} promoters by underlines, with their consensus sequences in boxes, and the upstream activating sequences (UAS) of the σ^{54} promoters by double lines. The numbers on the right refer to the positions of the nucleotides and the amino acid residues for each respective line. Some restriction endonuclease sites are also shown. The amino acid sequences of four putative membrane-associated helices spanning amino acid residues 54 to 145 are underlined. The putative cell attachment tripeptide sequence at residues 317 to 319 is shown in a box.

Fig. 2. Primer extension analysis of *hrpN_{Ecc}* mRNA. Lane 1 represents an RNA sample from the RsmA⁻ mutant AC5070 grown in minimal salts medium containing sucrose. The nucleotides on the left refer to the nucleotide sequence beyond the transcriptional start site. The asterisk denotes the guanine residue at which transcription was initiated.

Fig. 3. A, Overproduction of HrpN_{Ecc} in *Escherichia coli* strain JM109-(DE3). Lane 1, lysate of JM109(DE3) carrying the vector pT7-7; lane 2, lysate of JM109(DE3) carrying pAKC925. Each lane contained 4.5 μ g of protein. The arrow indicates the overproduced 36-kDa HrpN_{Ecc}. B, Western blotting assay of HrpN_{Ecc} produced in *E. coli* using anti-HrpN_{Ech} antibodies. Lane 1, lysate of JM109(DE3) carrying the vector pT7-7; lane 2, lysate of JM109(DE3) carrying pAKC925. Each lane contained 0.4 μ g of protein.

brane domain. It is noteworthy that a cell attachment sequence, the RGD tripeptide, is localized at residues 317 to 319 of HrpN_{Ecc} (Fig. 1). Such tripeptides have been found to play crucial roles in receptor interaction and cell adhesion of several proteins (D'Souza et al. 1991). In the corresponding positions in HrpN_{Ea}, HrpN_{Ech}, and HrpN_{Es}, AGD tripeptides (with a neutral alanine residue) are present instead of the RGD tripeptide (with its basic arginine residue).

The detection of *hrpN_{Ecc}* transcripts of approximately 1,100 bases (Cui et al. 1996) indicates that *hrpN_{Ecc}* consists of a monocistronic operon. In order to localize the promoter region of *hrpN_{Ecc}*, we identified its transcriptional start site at the guanine residue preceding the presumed translational start site by 75 nucleotides (Figs. 1 and 2). The -10 and -35 consensus hexanucleotide sequences corresponding to the sigma-70 promoter are not detected within the DNA region upstream of this transcriptional start site. Instead, there is an *hrp* promoter 6 bp before the transcriptional start site (Fig. 1). The consensus sequences for putative sigma-54 promoters and the cognate upstream activating sequences (Arsene et al. 1996; Morett and Segovia 1993; Pérez-Martin et al. 1994) are also present further upstream of the transcriptional start site (Fig. 1). A putative ribosome-binding site [-GAGGAA-] is localized 5 bp upstream of the start codon (Fig. 1). However, no

rho-independent terminator was found within the sequences downstream of the *hrpN_{Ecc}* coding region.

Identification of the *hrpN_{Ecc}* product as the elicitor of the HR.

The coding region of the *hrpN_{Ecc}* gene was amplified by polymerase chain reaction (PCR) and cloned into the T7 expression vector, pT7-7, to produce the plasmid pAKC925. Following IPTG (isopropyl-β-D-thiogalactopyranoside) induction, *Escherichia coli* JM109(DE3) carrying pAKC925 produced a major 36-kDa polypeptide as inclusion bodies (Fig. 3A), which specifically reacted with anti-Harpin_{Ech} antibodies (Fig. 3B); no other polypeptide present in the lysate of *E. coli* JM109(DE3) carrying pT7-7 was recognized by anti-Harpin_{Ech} antibodies (Fig. 3A and B). The inclusion bodies, fractionated by the protocol of Bauer et al. (1995), were substantially (about 80%) enriched with the 36-kDa protein (data not shown). Various dilutions of this preparation were introduced into tobacco leaves. The symptoms of typical HR resulted from the introduction of this preparation at concentrations of 200 µg/ml or higher (Fig. 4). Thus, the overproduced polypeptide had a molecular mass matching that of the predicted HrpN_{Ecc} product; it reacted with the Harpin_{Ech} antibodies; and most significantly, the preparation enriched with this protein elicited the HR. Therefore, we concluded that the 36-kDa polypeptide is Harpin_{Ecc}, an elicitor of the HR produced by *E. carotovora* subsp. *carotovora*.

To genetically confirm this conclusion, we constructed HrpN⁻ derivatives of the RsmA⁻ mutant, AC5071, by replac-

Fig. 4. Elicitation of the hypersensitive reaction by HrpN_{Ecc} in a tobacco leaf. Leaf panels A through E were infiltrated with suspensions of partially purified HrpN_{Ecc} at 500, 350, 200, 100, and 50 µg/ml, respectively, in 5 mM morpholinoethanesulfonic acid (MES), pH 6.5. Leaf panel F was infiltrated with the lysate of *Escherichia coli* JM109(DE3) carrying vector pT7-7 at a protein concentration of 350 µg/ml. Leaf panel G was infiltrated with 5 mM MES, pH 6.5. Leaf panel H was infiltrated with cells of AC5070 (RsmA⁻, HrpN_{Ecc}⁺) at a concentration of 2×10^8 cfu/ml in 5 mM MES, pH 6.5.

Fig. 5. Immunoblot analysis of Harpin_{Ecc} produced by *Erwinia carotovora* subsp. *carotovora* RsmA⁻ strain AC5071 (lane 1) and its HrpN⁻ derivative AC5081 (lane 2). Each lane contained 20 µg of protein.

ing the *hrpN_{Ecc}⁺* allele with *hrpN_{Ecc}-omega(spc)* DNA. Immunoblot analysis (Fig. 5) showed that Harpin_{Ecc} was produced by the RsmA⁻ HrpN⁺ strain (AC5071) but not by its HrpN⁻ derivative (AC5081). Similar results were obtained with HrpN⁺ Ecc71 and its HrpN⁻ derivative, except that the level of Harpin_{Ecc} in Ecc71 was much lower than in AC5071 (data not shown). To establish that Harpin_{Ecc} is responsible for the HR, we infiltrated tobacco leaves with cell suspensions of strains AC5071 (RsmA⁻, HrpN⁺) and AC5081 (RsmA⁻, HrpN⁻). *Pseudomonas syringae* pv. *tomato* (DC3000) was used as the positive control. The RsmA⁻ HrpN⁻ double mutant did not induce the HR (Fig. 6). By contrast, the parent strain AC5071, like DC3000, produced typical HR symptoms within 24 h after infiltration.

Extracellular enzyme production and pathogenicity.

The data shown in Fig. 7 reveal that the amounts of pectate lyase, polygalacturonase, cellulase, and protease produced by AC5081 (RsmA⁻, HrpN⁻) in salts-yeast extract-glycerol medium containing celery extract were very similar to the levels produced by AC5071 (RsmA⁻, HrpN⁺). These bacterial strains caused extensive maceration of celery petioles (Fig. 8), and the extent of maceration was similar with various inoculum

levels (data not shown). The disruption of *hrpN_{Ecc}* in RsmA⁺ bacteria also had no discernible effect on the maceration of celery petioles. Thus, in these assays, the inactivation of *hrpN_{Ecc}* did not compromise extracellular enzyme production or tissue maceration by *E. carotovora* subsp. *carotovora*.

Effects of media composition on the expression of *hrpN_{Ecc}*.

E. carotovora subsp. *carotovora* strain Ecc71 and its RsmA⁻ derivative AC5071 were grown in Luria-Bertani (LB) broth or in a minimal salts medium supplemented with various sugars. Total RNAs were extracted and analyzed by Northern blot

Fig. 6. Effect of disruption of *hrpN_{Ecc}* on the elicitation of the hypersensitive reaction by *Erwinia carotovora* subsp. *carotovora*. AC5071 and its HrpN⁻ derivative AC5081 were grown on Luria-Bertani agar overnight at 28°C, and cells were suspended in water. Leaf segments were infiltrated with bacterial cells (2×10^8 cfu/ml). A, Water; B, *Pseudomonas syringae* pv. *tomato* DC3000; C, AC5071 (RsmA⁻, HrpN⁺); D, AC5081 (RsmA⁻, HrpN⁻).

Fig. 7. Agarose plate assays of pectate lyase (Pel), polygalacturonase (Peh), protease (Prt), and cellulase (Cel) activities of *Erwinia carotovora* subsp. *carotovora* RsmA⁻ strain AC5071 (A) and its HrpN⁻ mutant AC5081(B). Bacteria were grown in salts-yeast extract-glycerol plus celery extract medium to saturation at 28°C. Culture supernatants were diluted fivefold in 10 mM Tris-HCl buffer (pH 7.5), and 20 µl of the diluted samples was used for the assays.

Fig. 8. Plant tissue maceration by *Erwinia carotovora* subsp. *carotovora* AC5071 (A) and its HrpN⁻ mutant AC5081 (B). About 2×10^8 cells were injected into this celery petiole at each inoculation site. The inoculated petiole was incubated in a moist chamber at 25°C for 24 h.

hybridization using an internal fragment of *hrpN_{Ecc}* as the probe. The data shown in Figure 9 allow the following conclusions. (i) The transcript levels were consistently higher in the RsmA⁺ strain (lanes 8 to 12) than in the RsmA[−] bacterium (lanes 2 to 6). (ii) The levels of transcripts were markedly influenced by the carbon source: the highest levels were found with sucrose (lanes 6 and 12), followed by glucose (lanes 5 and 11), fructose (lanes 3 and 9), and mannitol (lanes 4 and 10). (iii) *hrpN_{Ecc}* expression was severely inhibited by glycerol (lanes 2 and 8), especially in the RsmA⁺ bacteria. (iv) *hrpN_{Ecc}* transcripts were not detected in RsmA⁺ and RsmA[−] bacteria grown in the complex (LB) medium (lanes 1 and 7).

To quantify *hrpN_{Ecc}* expression, the plasmid carrying an *hrpN_{Ecc}-lacZ* fusion (pAKC928) (Table 1; see Materials and Methods) was transferred into the RsmA⁺ strain AC5006 and the RsmA[−] strain AC5070. The bacteria were grown in various media to a Klett value of 200, and β -galactosidase activity was assayed. The data (Fig. 10) essentially confirmed the results of transcript assays in that higher levels of β -galactosidase were found in cultures grown in minimal salts medium containing sucrose than in those grown in minimal salts medium containing glycerol or LB. Also, the levels of β -galactosidase were higher in AC5070 carrying pAKC928 than in AC5006 containing the plasmid, except in LB, in which β -galactosidase activity was essentially undetectable in both constructs. Since the 474 bp of DNA upstream of the translational start site of *hrpN_{Ecc}* responded to various regulatory parameters (see below), we concluded that it contained all or most of the potential regulatory sequences.

Effect of cell density.

For a quantitative analysis of the effect of cell density on *hrpN_{Ecc}* expression, we transferred pAKC928 into strains AC5047 and AC5070. The constructs were grown in minimal salts medium containing sucrose; samples were removed when the cultures reached 50, 100, 150, and 200 Klett units (Fig. 11A) and assayed for β -galactosidase activity. In the RsmA⁺ strain AC5047, the rate of β -galactosidase synthesis was considerably lower during exponential growth at cell densities of 50 to 150 Klett units but increased to a higher rate at 200 Klett units when the culture entered a postexponential growth phase (Fig. 11B). By contrast, β -galactosidase was produced at a constant rate by the RsmA[−] strain AC5070 at these cell densities (Fig. 11A and B). Northern blot analysis of *hrpN_{Ecc}* transcripts essentially confirmed these results (data not shown). Thus, *hrpN_{Ecc}* expression is cell density dependent in the RsmA⁺ strain but not in RsmA[−] bacteria.

Fig. 9. Northern blot analysis of *hrpN_{Ecc}* mRNA of *Erwinia carotovora* subsp. *carotovora* RsmA⁺ strain Ecc71 and its RsmA[−] derivative AC5071 grown in Luria-Bertani medium (LB) or minimal salts medium (MM) containing different carbon sources. Lanes 1–6, RNA samples from Ecc71; each lane contained 20 μ g of total RNA. Lanes 7–12, RNA samples from AC5071; each lane contained 10 μ g of total RNA. RNA of cells from LB (lanes 1 and 7), MM + glycerol (lanes 2 and 8), MM + fructose (lanes 3 and 9), MM + mannitol (lanes 4 and 10), MM + glucose (lanes 5 and 11), and MM + sucrose (lanes 6 and 12).

Effect of *N*-(3-oxohexanoyl)-L-homoserine lactone.

Since *hrpN_{Ecc}* expression is affected by cell density in the RsmA⁺ strains (see above) and since *N*-(3-oxohexanoyl)-L-homoserine lactone (OHL) is known to function as a cell-density-sensing signal in *E. carotovora* subsp. *carotovora* (Bainton et al. 1992; Jones et al. 1993; Pirhonen et al. 1993) and various other bacteria (Fuqua et al. 1994; Salmond et al. 1995), we tested the effect of OHL deficiency on the expression of *hrpN_{Ecc}*. For this we grew Ecc71 and AC5070 and their OHL-deficient derivatives, AC5094 and AC5090, respectively, in minimal salts medium containing sucrose to the late exponential stage (i.e., to a Klett value of 200), extracted the total RNAs, and assayed for levels of *hrpN_{Ecc}* mRNAs by Northern analysis (Fig. 12). The levels of transcripts were much lower in the OHL[−] RsmA⁺ strain, AC5094, than in its OHL⁺ RsmA⁺ parent, Ecc71. By contrast, substantial levels of transcripts were present in the OHL[−] RsmA[−] strain, AC5090, although the levels of transcripts were somewhat higher in the OHL⁺ RsmA[−] parent, AC5070 (Fig. 12). To quantify the effect of OHL deficiency, pAKC928 was transferred into AC5047 and its OHL[−] derivative, AC5091. The bacteria were grown in minimal salts medium containing sucrose to a Klett value of 200 and then assayed for β -galactosidase activity. AC5047 carrying pAKC928 produced 410 Miller units of the enzyme. By contrast, AC5091 carrying pAKC928 produced 97 Miller units of β -galactosidase. These data collectively demonstrate that expression of *hrpN_{Ecc}* in the RsmA⁺ bacteria is substantially impaired in the absence of the quorum-sensing signal.

DISCUSSION

In this study we have established by the following criteria that *hrpN_{Ecc}* encodes a 36-kDa protein (Harpin_{Ecc}) which is responsible for the elicitation of the HR by RsmA[−] mutants of *E. carotovora* subsp. *carotovora* (Cui et al. 1996). (i) This protein is present in bacteria carrying the *hrpN_{Ecc}*⁺ allele but not in bacteria carrying the *hrpN_{Ecc}-omega* DNA. (ii) The RsmA[−] HarpN⁺ strain, but not its HarpN[−] derivative, elicits the HR. (iii) An *E. coli* cellular fraction enriched with Harpin_{Ecc}

Fig. 10. Effect of media composition on the expression of *hrpN_{Ecc}* in RsmA⁺ strain AC5006 and RsmA[−] strain AC5070. Bacteria carrying pAKC928, a plasmid harboring the *hrpN_{Ecc}-lacZ* fusion (Table 1), were grown in appropriate media at 28°C to 200 Klett units, and the cultures were assayed for β -galactosidase activity.

causes the typical HR in tobacco leaves. (iv) The deduced Harpin_{Ecc} has sequence homology with the other *Erwinia* Harpins, such as those produced by *E. amylovora*, *E. chrysanthemi*, and *E. stewartii*. Additional evidence of this homology is that the proteins produced by *E. chrysanthemi* and Ecc71 share common epitopes, as indicated by the cross reaction of Harpin_{Ecc} with anti-Harpin_{Ech} antibodies.

Despite the limited amount of data now available on Harpin structure, it is becoming apparent that the *Erwinia* Harpins represent a class of elicitors distinct from those of *P. syringae*. For example, none of the *Erwinia* Harpins has greater than 10% identity with the *P. syringae* Harpins. This contrasts with identities of 30% or more between the *Erwinia* Harpins. In addition, while the *Erwinia* *hrpN* genes are organized as monocistronic operons, those of *P. syringae* are the components of polycistronic operons. Another difference lies in the apparent redundancy in elicitors. For example, disruptions of *hrpZ_{ps}* do not completely eliminate the capacity of *P. syringae* to elicit the HR (Alfano et al. 1996; Collmer et al. 1996). Similarly, inactivation of *popA* does not affect the elicitation of the HR by *P. solanacearum* (Arlat et al. 1994). By contrast, *Erwinia* species carrying inactivated *hrpN* alleles fail to elicit the typical HR in tobacco leaves (Bauer and Beer 1991, 1994; this report).

The expression of *hrpN_{Ecc}* in *E. carotovora* subsp. *carotovora* strain 71 appears to be cell density dependent, with maximal expression occurring during late exponential growth (Fig.

11). Thus, the temporal expression of *hrpN_{Ecc}* is very similar to the expression of the genes for various extracellular enzymes, including *pel* (pectate lyase), *peh* (polygalacturonase), and *cel* (cellulase). Since previous studies have shown that the cell-density-dependent expression of the *pel*, *peh*, and *cel* genes in *E. carotovora* subsp. *carotovora* requires the quorum-sensing signal, OHL (Chatterjee et al. 1995; Cui et al. 1995; Jones et al. 1993; Pirhonen et al. 1993), we considered the possibility that *hrpN_{Ecc}* expression in RsmA⁺ bacteria may also

Fig. 12. Effect of deficiency of *N*-(3-oxohexanoyl)-L-homoserine lactone (OHL) on the expression of *hrpN_{Ecc}*. Bacteria were grown to a Klett value of 200 at 28°C in minimal salts medium containing sucrose. Total RNAs were isolated and subjected to Northern blot analysis. Lane 1, Ecc71 (OHL⁺); lane 2, AC5094 (OHL⁻ derivative of Ecc71); lane 3, AC5070 (RsmA⁻, OHL⁺); lane 4, AC5090 (OHL⁻ derivative of AC5070). Lanes 1 and 2 contained 20 µg of total RNA, and lanes 3 and 4 contained 10 µg.

Fig. 11. Cell-density-dependent expression of an *hrpN_{Ecc}-lacZ* gene fusion (pAKC928) in *Erwinia carotovora* subsp. *carotovora* strains AC5047 (RsmA⁺) and AC5070 (RsmA⁻). Bacteria carrying pAKC928 were grown at 28°C in minimal salts medium containing sucrose and tetracycline. **A**, Culture turbidities at desired intervals. **B**, β-Galactosidase activity of samples of cultures at 50, 100, 150, and 200 Klett units.

be controlled by OHL. Indeed, our findings (Fig. 12) show that *hrpN_{Ecc}* expression was substantially lower in an OHL⁻ bacterium than in the OHL⁺ parent. Thus, *hrpN_{Ecc}* expression in Ecc71 appears to respond to the diffusible signal and metabolic conditions that affect extracellular enzyme production. The rationale for controlling Harpin_{Ecc} as well as extracellular enzyme production by OHL is at present unclear. However, we should emphasize that in the absence of additional information, especially regarding the regulation of OHL production and the mechanism of signal transduction, caution should be exercised in interpreting the findings of effects of cell density and the quorum-sensing signal. It is conceivable that the conditions in the in vitro culture may not truly reflect the conditions prevailing in tissues of host and nonhost plants, where *hrpN_{Ecc}* may respond to hitherto unrecognized signals.

Transcription of *hrpN_{Ecc}* is markedly influenced by cultural conditions such as complex and minimal salts media and carbon sources. The finding that *hrpN_{Ecc}* transcripts are most abundant in a minimal salts medium with sucrose as the carbon source is particularly striking, since these conditions mimic the environment bacteria are presumed to encounter in plant tissues. Also, the cultural conditions that stimulate the levels of *hrpN_{Ecc}* transcripts are generally similar to those known to activate *hrp* expression in *E. amylovora* (Wei et al. 1992a), *P. syringae* (Hutcheson et al. 1994; Rahme et al. 1991; Xiao et al. 1992), and *Xanthomonas campestris* pv. *vesicatoria* (Fenselau and Bonas 1995; Schulte and Bonas 1992a,b). We should, however, point out that RsmA⁻ bacteria generally respond to these cultural conditions like RsmA⁺ strains, except that *hrpN_{Ecc}* expression is invariably higher under every growth condition (Figs. 9 and 10). A noteworthy exception is the absence of *hrpN_{Ecc}* transcripts in both RsmA⁺ and RsmA⁻ bacteria grown in LB medium. These findings, taken along with the presence of *cis*-acting sequences presumed to affect promoter activation (see above and Fig. 1) and the occurrence of transcriptional activators in *E. amylovora*, *Pseudomonas*, and *Xanthomonas* species (Genin et al. 1992; Grimm et al. 1995; Wei and Beer 1995; Wengelnik and Bonas 1996; Wengelnik et al. 1996; Xiao et al. 1994; see also Bonas 1994 and Hutcheson et al. 1994 and the references cited therein), suggest that *hrpN_{Ecc}* expression in *E. carotovora* is modulated at two levels: (i) transcription is controlled by a complex regulatory system in response to various cultural or environmental conditions, and (ii) a posttranscriptional control is mediated by RsmA (Cui et al. 1995, 1996; this report), presumably by affecting the stability of *hrpN_{Ecc}* mRNA.

The findings with *E. carotovora* subsp. *carotovora* (Chatterjee et al. 1995; Cui et al. 1996) now provide us with some clues regarding the inability of these soft-rotting bacteria to elicit the HR. A hallmark of these pathogens is their propensity to produce a large array of extracellular enzymes in massive quantities that can cause extensive damage to the plant cell wall and cell membrane (Barras et al. 1994). If this effect is overwhelming, as generally is the case, the bacteria probably do not need to deploy Harpins. In *E. carotovora* and other soft-rotting *Erwinia*, Harpins may function as ancillary factors that are needed only in certain host-pathogen interactions (see, for example, Bauer et al. 1994, 1995). In the course of evolution, the *hrp* genes, especially *hrpN* of *E. carotovora*, may have been subjected to very stringent regulation to ensure that they are expressed only under specific conditions. This idea is

clearly supported by the finding that while all *E. carotovora* subspecies possess *hrpN*, the expression of these genes is markedly influenced by bacterial genotype as well as cultural conditions (our unpublished data). However, once *hrpN_{Ecc}* expression is partially derepressed, as in the RsmA⁻ bacteria, they cause the HR in spite of their ability to produce the pectolytic enzymes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

Bacterial strains and plasmids are described in Table 1. The strains carrying antibiotic markers were maintained on LB agar containing appropriate antibiotics. The wild-type *Erwinia* and *Pseudomonas* strains were maintained on LB agar.

The compositions of LB medium, minimal salts medium, nutrient gelatin agar, polygalacturonate–yeast extract agar, and salts–yeast extract–glycerol plus celery extract medium have been described elsewhere (Barras et al. 1987; Chatterjee 1980; Murata et al. 1991). To test the effects of carbon sources on *hrpN_{Ecc}* expression, bacteria were grown in the minimal salts medium supplemented with sucrose, glucose, fructose, mannitol, or glycerol at 0.5% (w/v). When required, antibiotics and drugs were supplemented as follows: ampicillin (Ap), 100 µg/ml; kanamycin (Km), 50 µg/ml; nalidixic acid (Nal), 50 µg/ml; spectinomycin (Spc), 50 µg/ml; and tetracycline (Tc), 10 µg/ml. Media were solidified by the addition of 1.5% agar.

The compositions of agarose media for semiquantitative assays of enzymatic activities were described by Chatterjee et al. (1995).

DNA techniques.

Standard procedures were used in the isolation of plasmid and chromosomal DNAs, transformation, restriction endonuclease digests, gel electrophoresis, and DNA ligation (Sambrook et al. 1989). Restriction and modifying enzymes were obtained from Promega Biotec (Madison, WI). The Primer-a-Gene DNA labeling system of Promega Biotec was used for labeling DNA. The PC/GENE package (Intelligenetics Inc., Mountain View, CA) was used to analyze the DNA and protein sequences.

RNA isolation and Northern blot analyses.

Bacterial cultures were grown at 28°C in LB or minimal salts medium supplemented with different carbon sources. Unless stated otherwise, cells were collected when cultures had reached approximately 150 Klett units. The procedures for RNA isolation and Northern blot analysis have been described elsewhere (Chatterjee et al. 1991; Liu et al. 1993). A 779-bp *EcoRV*–*SmaI* fragment from pAKC924 (Table 1), containing a 776-bp segment of *hrpN_{Ecc}* from nucleotide +235 to nucleotide +1,011 (Fig. 1), was used as the probe. The *SmaI* restriction site in pAKC924 was derived from the polylinker of pAKC-922.

Identification of the transcriptional start site.

For RNA isolation, *E. carotovora* subsp. *carotovora* strain AC5070 was grown in minimal salts medium supplemented with sucrose to approximately 200 Klett units at 28°C. The ³²P-labeled oligonucleotide primer (5'-TGATCGTGATTG-CAGAGAAGC-3') complementary to the base positions +121

to +100 of *hrpN_{Ecc}* (Fig. 1) was annealed to the RNA, and primer extension was carried out by the use of the kit from Promega Biotech. The extension product was run in an 8% acrylamide-urea sequencing gel in parallel with a DNA ladder obtained from the use of the same primer and pAKC923 (Table 1) as the template DNA.

Overproduction and identification of HrpN_{Ecc}.

The coding region of *hrpN_{Ecc}* was amplified by PCR using 5'-GCCGTATGCTTAATTCTCTTGGTGGCG-3' as the 5' primer, 5'-GCAAGCTTCCGTTTACGTGGAGAGCTTCT-3' as the 3' primer, and pAKC923 as the template DNA. PCR was carried out as follows: one cycle at 94°C for 5 min, 55°C for 2 min, and 72°C for 3 min; 25 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min; and one cycle at 94°C for 1 min, 55°C for 2 min, and 72°C for 7 min. PCR products were digested with *Nde*I and *Hind*III and cloned into the expression plasmid pT7-7 to produce pAKC925. *E. coli* strain JM109(DE3) carrying pAKC925 and JM109(DE3) carrying pT7-7 were grown at 37°C in 250 ml of LB medium containing ampicillin. The T7 promoter was activated with 1 mM IPTG when the cultures reached an A₆₀₀ value of 0.7. After an additional 6 h of incubation, cells were collected by centrifugation (4,000 × *g* for 10 min at 4°C), resuspended in 3 ml of TESP buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride [PMSF])

and sonicated in a Braunsonic 1510 (B. Braun Biotech Inc., Allentown, PA) at 100-W output. The samples were centrifuged at 15,000 × *g* for 15 min at 4°C, and the pellets, which contained the bulk of the Harpin_{Ecc} as inclusion bodies, were collected, washed twice with 9 volumes of the lysis buffer (the same as TESP but containing 0.5% Triton X-100 and 10 mM EDTA instead of 1 mM EDTA), and resuspended in 2 ml of TESP. Double-strength sodium dodecyl sulfate (SDS) loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 2% glycerol) was added, and the samples were boiled for 5 min. Proteins were fractionated by (0.1%) SDS-(12%) polyacrylamide gel electrophoresis and examined by staining with Coomassie brilliant blue or by Western blot analysis. For the latter, proteins were transferred to NitroBind nitrocellulose membrane (Micron Separations, Inc., Westboro, MA), probed with anti-Harpin_{Ecc} antibodies (Bauer et al. 1995), and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase (Promega Biotech).

Harpin_{Ecc} was subsequently fractionated according to the method of Bauer et al. (1995). Briefly, the pellet was taken up in TESP buffer (2 ml), dissolved by the addition of 2.5 ml of 8 M guanidine-HCl in TESP buffer, and then diluted with 5.0 ml of water. The protein solution, contained in spectraPor dialysis tubing (molecular weight cutoff 12,000 to 14,000 Da), was dialyzed at 4°C against 2.0 liters of 5 mM morpholinoethane-

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>		
71	Wild type	Zink et al. 1984
AC5006	Lac ⁻ mutant of Ecc71	Murata et al. 1990
AC5047	Nal ^r derivative of AC5006	Chatterjee et al. 1995
AC5070	RsmA ⁻ mini-Tn5-Km mutant of AC5047, Km ^r , Nal ^r	Chatterjee et al. 1995
AC5071	RsmA ⁻ derivative of Ecc71, Km ^r	Lab collection
AC5081	RsmA ⁻ HrpN ⁻ derivative of AC5071 by marker exchange with pAKC927, Km ^r , Spc ^r	This study
AC5082	HrpN ⁻ derivative of Ecc71 by marker exchange with pAKC927, Spc ^r	This study
AC5090	Ohl ⁻ derivative of AC5070, Km ^r , Nal ^r , Spc ^r	Chatterjee et al. 1995
AC5091	Ohl ⁻ derivative of AC5047, Nal ^r , Spc ^r	Chatterjee et al. 1995
AC5094	Ohl ⁻ derivative of Ecc71, Km ^r	Lab collection
<i>Pseudomonas syringae</i> pv. <i>tomato</i>		
DC3000	Wild type	Cuppels 1986
<i>Escherichia coli</i>		
DH5α	φ80lacZ ΔM15 Δ(lacZYA-argF) U169 hsdR17 recA1 endA1 thi-1	BRL
JM109(DE3)	endA1 recA1 gyrA96 hsdR17 supE44 relA1 thi Δ(lac-pro) F' [traD36 proAB ⁺ lacI ^q lacZΔM15] λc1857 ind1 Sam7 lacUV5-T7 gene 1	Promega
Plasmids		
pAKC921	pLARF5 containing <i>hrpN_{Ecc}</i> from genomic library of Ecc71, Tc ^r	Cui et al. 1996
pAKC922	pLARF5 containing <i>hrpN_{Ecc}</i> from genomic library of Ecc71, Tc ^r	Cui et al. 1996
pAKC923	4.0-kb <i>Eco</i> RI fragment of pAKC921 containing <i>hrpN_{Ecc}</i> cloned in pSK ⁺ , Ap ^r	Cui et al. 1996
pAKC924	1.4-kb <i>Eco</i> RI fragment of pAKC922 containing <i>hrpN_{Ecc}</i> cloned in pSK ⁺ , Ap ^r	Cui et al. 1996
pAKC925	Polymerase chain reaction product of <i>hrpN_{Ecc}</i> cloned in pT7-7, Ap ^r	This study
pAKC926	pAKC 924 containing an omega-Spc cassette at the <i>Bgl</i> II site of <i>hrpN_{Ecc}</i> , Ap ^r , Spc ^r	This study
pAKC927	3.4-kb <i>Eco</i> RI fragment of pAKC926 containing <i>hrpN_{Ecc}</i> -omega in pRK415, Spc ^r , Tc ^r	This study
pAKC928	<i>hrpN_{Ecc}</i> -lacZ, 633-bp <i>Eco</i> RI- <i>Eco</i> RV fragment of pAKC924 cloned in pMP220, Tc ^r	This study
pHP45Ω	Source of Spc ^r omega fragment	Prentki and Krisch 1984
pMP220	Promoter probe vector, Tc ^r	Spaink et al. 1987
pRK415	Tc ^r	Keen et al. 1988
pRK2013	Mob ⁺ , Tra ⁺ , Km ^r	Figurski and Helinski 1979
pBluescript-II SK ⁺	Ap ^r	Stratagene
pT7-7	T7 promoter expression vector, Ap ^r	Tabor and Richardson 1985

sulfonic acid (MES), pH 6.5, containing 0.05 mM PMSF. The precipitate that formed during dialysis was collected by centrifugation ($4,300 \times g$ for 15 min at 4°C). The pellet was washed twice with 10 ml of 5 mM MES, pH 6.5, containing 0.1 mM PMSF, and then resuspended in the same buffer. For HR tests the sample was sonicated to produce a fine suspension of Harpin_{Ecc}. Protein concentration was determined by the bicinchoninic acid (Pierce Co., Rockford, IL) method with bovine serum albumen as a standard.

For the detection of Harpin_{Ecc} in *E. carotovora* subsp. *carotovora*, strains Ecc71 and AC5071 and their HrpN⁻ derivatives AC5082 and AC5081, respectively, were grown in minimal salts medium plus sucrose at 28°C for 24 h. The bacterial cells were collected by centrifugation ($4,000 \times g$ for 10 min at 4°C), resuspended in the lysis buffer, and sonicated. Cell lysates were used for Western blot analysis as described above. The protein concentration of the cell lysates was determined by the bicinchoninic acid method.

Construction of HrpN⁻ strains by marker exchange.

The plasmid pAKC927 (Table 1), in which *hrpN*_{Ecc} was inactivated by the insertion of an Omega-Spc cassette at the *Bgl*II site (Fig. 1), was transferred into AC5071 and Ecc71 by means of the helper plasmid pRK2013. Transconjugants were selected on minimal salts agar plus sucrose supplemented with Spc. Isolates that were Spc^r and Tc^s were tested for elicitation of the HR in tobacco leaves. The marker exchange was confirmed by Southern blot hybridization. AC5081 and AC5082, the HrpN⁻ derivatives of AC5071 and Ecc71, respectively, were selected for further studies.

Construction of a low-copy *hrpN*_{Ecc}-*lacZ* fusion plasmid and β -galactosidase assay.

For quantitative estimation of *hrpN*_{Ecc} expression, a low-copy *hrpN*_{Ecc}-*lacZ* plasmid, pAKC928, was constructed by cloning the 633-bp *Eco*RI-*Eco*RV fragment (Fig. 1) into the promoter probe vector pMP220 (Table 1).

The β -galactosidase assays were carried out according to Miller (1972).

Assays for HR and pathogenicity.

Erwinia strains and *P. syringae* pv. *tomato* DC3000 were grown on LB agar overnight at 28°C. Cells were suspended in water and used for the HR and pathogenicity assays as previously described (Cui et al. 1996; Mukherjee et al. 1996; Murata et al. 1991).

ACKNOWLEDGMENTS

This work was supported by the Food for the 21st Century Program of the University of Missouri and by the National Science Foundation (grant DMB-94-19403). This article is journal series 12,583 of the Missouri Agricultural experiment station. We thank Alan Collmer for anti-Harpin_{Ech} antibodies and J. E. Schoelz for reviewing the manuscript.

LITERATURE CITED

Ahmad, M., Majerczak, D. R., and Coplin, D. L. 1996 Harpin is not necessary for the pathogenicity of *Erwinia stewartii* on maize. *Phytopathology* 86:S77.
 Alfano, J. R., Bauer, D. W., Milos, T. M., and Collmer, A. 1996. Analysis of the role of the *Pseudomonas syringae* pv. *syringae* HrpZ harpin in elicitation of the hypersensitive response in tobacco using func-

tionally non-polar *hrpZ* deletion mutations, truncated HrpZ fragments, and *hrmA* mutations. *Mol. Microbiol.* 19:715-728.
 Alfano, J. R., and Collmer, A. 1996. Bacterial pathogens in plants: Life up against the wall. *Plant Cell* 8:1684-1698.
 Arlat, M., Van Gijsegem, F., Huet, J. C., Pernollet, J. C., and Boucher, C. A. 1994. PopA1, a protein which induces a hypersensitivity-like response on specific *Petunia* genotypes, is secreted via the Hrp pathway of *Pseudomonas solanacearum*. *EMBO J.* 13:543-553.
 Arsene, F., Kaminski, P. A., and Elmerich, C. 1996. Modulation of NifA activity by P_{II} in *Azospirillum brasilense*: Evidence for a regulatory role of the NifA N-terminal domain. *J. Bacteriol.* 178:4830-4838.
 Bainton, N. J., Bycroft, B. W., Chhabra, S. R., Stead, P., Gledhill, L., Hill, P. J., Rees, C. E. D., Winson, M. K., Salmond, G. P. C., Stewart, G. S. A. B., and Williams, P. 1992. A general role for the *lux* auto-inducer in bacterial cell signalling: Control of antibiotic biosynthesis in *Erwinia*. *Gene* 116:87-91.
 Barras, F., Thurn, K. K., and Chatterjee, A. K. 1987. Resolution of four pectate lyase structural genes of *Erwinia chrysanthemi* (EC16) and characterization of the enzymes produced in *Escherichia coli*. *Mol. Gen. Genet.* 209:319-325.
 Barras, F., van Gijsegem, F., and Chatterjee, A. K. 1994. Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. *Annu. Rev. Phytopathol.* 32:201-234.
 Bauer, D. W., and Beer, S. V. 1991. Further characterization of an *hrp* gene cluster of *Erwinia amylovora*. *Mol. Plant-Microbe Interact.* 4:493-499.
 Bauer, D. W., Bogdanove, A. J., Beer, S. V., and Collmer, A. 1994. *Erwinia chrysanthemi* *hrp* genes and their involvement in soft rot pathogenesis and elicitation of the hypersensitive response. *Mol. Plant-Microbe Interact.* 7:573-581.
 Bauer, D. W., Wei, Z.-M., Beer, S. V., and Collmer, A. 1995. *Erwinia chrysanthemi* harpin_{Ech}: An elicitor of the hypersensitive response that contributes to soft-rot pathogenesis. *Mol. Plant-Microbe Interact.* 8:484-491.
 Bonas, U. 1994. *Hrp* genes of phytopathogenic bacteria. Pages 79-98 in: *Current Topics in Microbiology and Immunology*. Vol. 192, *Bacterial Pathogenesis of Plants and Animals: Molecular and Cellular Mechanisms*. J. L. Dangl, ed. Springer-Verlag, Berlin.
 Chatterjee, A. K. 1980. Acceptance by *Erwinia* spp. of R plasmid R68.45 and its ability to mobilize the chromosome of *Erwinia chrysanthemi*. *J. Bacteriol.* 142:111-119.
 Chatterjee, A., Cui, Y., Liu, Y., Dumenyo, C. K., and Chatterjee, A. K. 1995. Inactivation of *rsmA* leads to overproduction of extracellular pectinases, cellulases, and proteases in *Erwinia carotovora* subsp. *carotovora* in the absence of the starvation/cell density sensing signal, *N*-(3-oxohexanoyl)-L-homoserine lactone. *Appl. Environ. Microbiol.* 61:1959-1967.
 Chatterjee, A., McEvoy, J. L., Chambost, J. P., Blasco, F., and Chatterjee, A. K. 1991. Nucleotide sequence and molecular characterization of *pnlA*, the structural gene for damage-inducible pectin lyase of *Erwinia carotovora* subsp. *carotovora* 71. *J. Bacteriol.* 173:1765-1769.
 Cui, Y., Chatterjee, A., Liu, Y., Dumenyo, C. K., and Chatterjee, A. K. 1995. Identification of a global repressor gene, *rsmA*, of *Erwinia carotovora* subsp. *carotovora* that controls extracellular enzymes, *N*-(3-oxohexanoyl)-L-homoserine lactone, and pathogenicity in soft-rotting *Erwinia* spp. *J. Bacteriol.* 177: 5108-5115.
 Cui, Y., Madi, L., Mukherjee, A., Dumenyo, C. K., and Chatterjee, A. K. 1996. The *RsmA*⁻ mutants of *Erwinia carotovora* subsp. *carotovora* strain Ecc71 overexpress *hrpN*_{Ecc} and elicit a hypersensitive reaction-like response in tobacco leaves. *Mol. Plant-Microbe Interact.* 9:565-573.
 Collmer, A., Alfano, J. R., Bauer, D. W., Preston, G. M., Loniello, A. O., Conlin, A., Ham, J. H., Huang, H.-C., Gopalan, S., and He, S. Y. 1996. Secreted proteins, secretion pathways, and the plant pathogenicity of *Erwinia chrysanthemi* and *Pseudomonas syringae*. Pages 159-164 in: *Biology of Plant-Microbe Interactions*. G. Stacey, B. Mullin, and P. M. Gresshoff, eds. International Society for Molecular Plant-Microbe Interactions, St. Paul, MN.
 Cuppels, D. A. 1986. Generation and characterization of Tn5 insertion mutations in *Pseudomonas syringae* pv. *tomato*. *Appl. Environ. Microbiol.* 51:323-327.
 D'Souza, S. E., Ginsberg, M. H., and Plow, E. F. 1991. Arginyl-glycyl-aspartic acid (RGD): A cell adhesion motif. *Trends Biochem. Sci.* 16: 246-250.
 Fenselau, S., and Bonas, U. 1995. Sequence and expression analysis of

- the *hrpB* pathogenicity operon of *Xanthomonas campestris* pv. *vesicatoria* which encodes eight proteins with similarity to components of the Hrp, Ysc, Spa, and Fli secretion systems. *Mol. Plant-Microbe Interact.* 8:845-854.
- Figurski, D. H., and Helinski, D. R. 1979. Replication of an origin-containing derivative of a plasmid RK2 depend on a plasmid function provided in trans. *Proc. Natl. Acad. Sci. USA* 76:1648-1652.
- Fuqua, W. C., Winans, S. C., and Greenberg, E. P. 1994. Quorum sensing in bacteria: The LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* 176:269-275.
- Genin, S., Gough, C. L., Zischek, C., and Boucher, C. A. 1992. Evidence that the *hrpB* gene encodes a positive regulator of pathogenicity genes from *Pseudomonas solanacearum*. *Mol. Microbiol.* 6:3065-3076.
- Grimm, C., Aufsatz, W., and Panopoulos, N. J. 1995. The *hrpRS* locus of *Pseudomonas syringae* pv. *phaseolicola* constitutes a complex regulatory unit. *Mol. Microbiol.* 15:155-165.
- Hutcheson, S. W., Heu, S., Huang, H.-C., Li, T.-H., Lu, Y., Xiao, Y. 1994. The *hrp* genes of *Pseudomonas syringae*: An evaluation of their role in determining non-host resistance. Pages 203-223 in: *Biotechnology and Plant Protection*. D. D. Bills and S.-D. Kung, eds. World Scientific Publishing, Teaneck, NJ.
- Jones, B., Yu, B., Bainton, Birdsall, M., Bycroft, B. W., Chhabra, S. R., Cox, A. J. R., Golby, P., Reeves, P. J., Stephens, S., Winson, M. K., Salmond, G. P. C., Stewart, G. S. A. B., and Williams, P. 1993. The *lux* autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. *EMBO J.* 12:2477-2482.
- Keen, N. T., Tamaki, S., Kobayashi, D., and Trollinger, D. 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene* 70:191-197.
- Liu, Y., Murata H., Chatterjee, A., and Chatterjee, A. K. 1993. Characterization of a novel regulatory gene *aepA* that controls extracellular enzyme production in the phytopathogenic bacterium *Erwinia carotovora* subsp. *carotovora*. *Mol. Plant-Microbe Interact.* 6:299-308.
- Miller, J. H. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Morett, E., and Segovia, L. 1993. The σ^{54} bacterial enhancer-binding protein family: Mechanism of action and phylogenetic relationship of their functional domains. *J. Bacteriol.* 175:6067-6074.
- Mukherjee, A., Cui, Y., Liu, Y., Dumenyo, C. K., and Chatterjee, A. K. 1996. Global regulation in *Erwinia* species by *Erwinia carotovora* *rsmA*, a homologue of *Escherichia coli* *csrA*: Repression of secondary metabolites, pathogenicity and hypersensitive reaction. *Microbiology* 142:427-434.
- Murata, H., Fons, M., Chatterjee, A., Collmer, A., and Chatterjee, A. K. 1990. Characterization of transposon insertion *Out*⁻ mutants of *Erwinia carotovora* subsp. *carotovora* defective in enzyme export and of a DNA segment that complements *out* mutations in *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica*, and *Erwinia chrysanthemi*. *J. Bacteriol.* 172:2970-2978.
- Murata, H., McEvoy, J. L., Chatterjee, A., Collmer, A., and Chatterjee, A. K. 1991. Molecular cloning of an *aepA* gene that activates production of extracellular pectolytic, cellulolytic, and proteolytic enzymes in *Erwinia carotovora* subsp. *carotovora*. *Mol. Plant-Microbe Interact.* 4:239-246.
- Pérez-Martin, J., Rojo, F., and de Lorenzo, V. 1994. Promoters responsive to DNA bending: a common theme in prokaryotic gene expression. *Microbiol. Rev.* 52:268-290.
- Pirhonen, M., Flego, D., Heikinheimo, R., and Palva, E. T. 1993. A small diffusible signal molecular is responsible for the global control of virulence and exoenzyme production in the plant pathogen, *Erwinia carotovora*. *EMBO J.* 12:2467-2476.
- Prentki, P., and Krusch, H. M. 1984. *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* 29:303-313.
- Rahme, L. G., Mindrinos, M. N., and Panopoulos, N. J. 1991. Genetic and transcriptional organization of the *hrp* cluster of *Pseudomonas syringae* pv. *phaseolicola*. *J. Bacteriol.* 173:575-586.
- Salmond, G. P. C., Bycroft, B. W., Stewart, G. S. A. B., and Williams, P. 1995. The bacterial 'enigma': Cracking the code of cell-cell communication. *Mol. Microbiol.* 16:615-624.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schulte, R., and Bonas, U. 1992a. Expression of the *Xanthomonas campestris* pv. *vesicatoria* *hrp* gene cluster, which determines pathogenicity and hypersensitivity on pepper and tomato, is plant inducible. *J. Bacteriol.* 174:815-823.
- Schulte, R., and Bonas, U. 1992b. A *Xanthomonas* pathogenicity locus is induced by sucrose and sulfur-containing amino acids. *Plant Cell* 4:79-86.
- Spaink, H. P., Okker, R. J. H., Wijffelman, C. A., Pees, E., and Lugtenberg, B. J. J. 1987. Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL 1 J1. *Plant Mol. Biol.* 9:27-39.
- Tabor, S., and Richardson, C. C. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. USA* 82:1074-1078.
- Wei, Z.-M., and Beer, S. V. 1995. *hrpL* activates *Erwinia amylovora* *hrp* gene transcription and is a member of the ECF subfamily of σ factors. *J. Bacteriol.* 177:6201-6210.
- Wei, Z.-M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A., and Beer, S. V. 1992b. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* 257:85-88.
- Wei, Z.-M., Sneath, B. J., and Beer, S. V. 1992a. Expression of *Erwinia amylovora* *hrp* genes in response to environmental stimuli. *J. Bacteriol.* 174:1875-1882.
- Wengelnik, K., and Bonas, U. 1996. HrpX_V, an AraC-type regulator, activates expression of five of the six loci in the *hrp* cluster of *Xanthomonas campestris* pv. *vesicatoria*. *J. Bacteriol.* 178:3462-3469.
- Wengelnik, K., Van den Ackerveken, G., and Bonas, U. 1996. HrpG, a key *hrp* regulatory protein of *Xanthomonas campestris* pv. *vesicatoria* is homologous to two-component response regulators. *Mol. Plant-Microbe Interact.* 9:704-712.
- Willis, D. K., Rich, J. J., and Hrabak, E. M. 1991. *hrp* genes of phytopathogenic bacteria. *Mol. Plant-Microbe Interact.* 4:132-138.
- Xiao, Y., Heu, S., Yi, J., Lu, Y. and Hutcheson, S. W. 1994. Identification of a putative alternate sigma factor and characterization of a multi-component regulatory cascade controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* and *hrmA* genes. *J. Bacteriol.* 176:1025-1036.
- Xiao, Y., Lu, Y., Heu, S., and Hutcheson, S. W. 1992. Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 *hrp* cluster. *J. Bacteriol.* 174:1734-1741.
- Zink, R. T., Kemble, R. J., and Chatterjee, A. K. 1984. Transposon Tn5 mutagenesis in *Erwinia carotovora* subsp. *carotovora* and *Erwinia carotovora* subsp. *atroseptica*. *J. Bacteriol.* 157:809-814.