Regulation of *Erwinia carotovora* *hrpL*_{Ecc} (*sigma-L*_{Ecc}), Which Encodes an Extracytoplasmic Function Subfamily of Sigma Factor Required for Expression of the HRP Regulon

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In *Erwinia carotovora* subsp. *carotovora* (Ecc) strain 71 (Ecc71), HrpL_{Ecc}, an alternate sigma factor of the extracytoplasmic function subfamily, plays a central role in the expression of the *hrp* (hypersensitive reaction and pathogenicity) regulon. We document here that sigma-54 (RpoN) is required for full expression of *hrpL*_{Ecc} and that HrpS, in conjunction with sigma-54, activates *hrpL*_{Ecc} transcription. We also made the novel observation that integration host factor is required for the activation of the *hrpL*_{Ecc} promoter. Our findings reveal that the RsmA/*rsmB* RNA-mediated post-transcriptional system, known to control extracellular enzyme and harpin production, affects *hrpL*_{Ecc} expression as well. For example, *hrpL*_{Ecc} RNA levels are barely detected in an RsmB⁻ strain. Conversely, *hrpL*_{Ecc} mRNA levels are much higher in RsmA⁻ bacteria than in the RsmA⁺ parent. This effect is due to RsmA-promoted decay of *hrpL*_{Ecc} RNA. Moreover, the following regulators known to control the production of either RsmA, *rsmB* RNA, or both also affect *hrpL*_{Ecc} expression: GacA (response regulator of a two-component system), KdgR (an IclI type repressor), HexA (a LysR type repressor), RsmC (a putative transcriptional adapter). Based upon the data now available for Ecc and extrapolating from the evidence in other systems, we propose a tentative model that depicts the Hrp regulatory system of Ecc and explains the basis for coregulation of extracellular enzyme production and expression of the Hrp regulon.

Many plant pathogenic bacteria use the type III secretion system to deliver virulence factors and effectors, such as harpins, avirulence gene (*avr*) products, and disease-specific (*dsp*) gene products, into host cells (Alfano et al. 2000; Collmer et al. 2000; Galan and Collmer 1999; He 1998; Staskawicz et al. 2001). Studies with several *Erwinia* and *Pseudomonas* species have disclosed that the alternate sigma factor, HrpL (Sigma L), activates the expression of *hrp* (hypersensitive response [HR] and pathogenicity) genes, including those required for the type III secretion machinery as well as *dsp* and *avr* genes (Collmer et al. 2000; Frederick et al. 2001; Mor et al. 2001; Wei and Beer 1995; Wei et al. 2000; Xiao et al. 1994). Recent results of whole genome searches have extended this inventory to many genes that are known to be or are presumed to be controlled by HrpL in *Pseudomonas syringae* pathovars *tomato* and *maculicola* (Fouts et al. 2002; Guttman

et al. 2002). These findings point to a critical role of this alternate sigma factor in bacteria-plant interactions. Consistent with this view are the observations that, in *Erwinia carotovora* subsp. *carotovora*, HrpL_{Ecc} is required for the expression of *hrp* genes responsible for the type III secretion system as well as the production of harpin and is required for symptom production in *Arabidopsis thaliana* (Chatterjee et al. 2002).

In *Erwinia amylovora* (Wei et al. 2000) and *Pseudomonas syringae* (Hendrickson et al. 2000; Hutcheson et al. 2001), HrpL expression is controlled in response to various signals or cues by regulatory cascades comprising two component systems, NtrC-like activators (i.e., HrpS proteins) and alternate sigma factors (i.e., RpoN homologs). The data presented here confirm that HrpS and RpoN are required for the activation of *hrpL*_{Ecc} transcription in Ecc. Moreover, we have found that the promoter region of *hrpL*_{Ecc} contains a putative integration host factor (IHF) binding site. Studies with a well-characterized *Escherichia coli* HimA⁻ mutant (HimA, the α -subunit of IHF) and cloned Ecc *himA*⁺ DNA demonstrate for the first time that, in addition to HrpS_{Ecc} and sigma-54, IHF is required for the activation of the *hrpL*_{Ecc} promoter.

Ecc and most other soft-rotting *Erwinia* species are unusual in that the wild-type RsmA⁺ strains fail to elicit the typical HR, due to low level of expression of *hrpN*_{Ecc}, which encodes the inducer of the HR (Cui et al. 1996; Mukherjee et al. 1997). RsmA is a small RNA-binding protein that promotes RNA decay in Ecc and other enterobacteria (Chatterjee et al. 1995; Cui et al. 1995; Romeo 1998). *rsmB* RNA, on the other hand, functions as a positive regulator, presumably by sequestering RsmA (Liu et al. 1998; Romeo 1998). Our finding that the stability of *hrpN*_{Ecc} mRNA was not adversely affected by RsmA was unexpected and prompted us to consider the possibility that RsmA affects *hrpL*_{Ecc}, which in turn, causes low level of *hrpN*_{Ecc} transcription in the wild-type RsmA⁺ bacteria. The data presented here establish that this is indeed the case.

In a recent study, we found that the two-component system comprising GacS, a putative histidine sensor kinase, and GacA, a presumed cognate response regulator (Heeb and Haas 2001), controls the production of harpin and extracellular enzymes in Ecc by modulating transcription of *rsmB*, a component of the post-transcriptional system (Cui et al. 2001). GacA also controls the expression of *rsmZ* (a homolog of *rsmB*) in *Pseudomonas fluorescens* (Heeb et al. 2002). The GacS/GacA regulatory system, which has been conserved in many prokaryotes, affects many different traits (Heeb and Haas 2001). In *Pseudomonas syringae* pv. *syringae*, the GacS/GacA system controls toxin, protease, and symptom production in plant hosts but apparently not the elicitation of the HR on the nonhost to-

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AAGCGACGGCGAACCTTGCTGCATCAGCAGATCGCGCAGGCGGCTCAGCTGCTGATCGAG -598
 CGTACTTTGCGACGGATTTTCCAGCATCCTGAACAGTTCTGTCAATCCGTTTC -538
 GACATTGGCGGTGCGCGCGCGTCCGGCTGCTGGGCGATGTGGCGCCGATTACGCGATTT -478
 GCTTCTTCGCTCAGCTTGCTCGCCAAATGCCATCGCCACTTCTTCCATCGAGGTGGATAA -418
 CGGCGAACCGGGGAGGCATGGTGCGAACCTGTCTGCTGCACCGCGGCCTGTGGCACGGG -358
 ATCGTCATCCACGACGACTGGGCGCGGAGGCGCTGCTGGAGGAAGGACAGTCGGCAT -298
 TTTGATCATGATGTCATCCTGAATAAATCGGCTACGTGTCGTGGTGTGAACGTGCAGCGG -238
 TATGAATGTATTGCTGTGTGGACGCAGAAAAGGATGGGTTCCCCGACGTGAAGTCATCGG -178
 AGCAGGCCGAATTGATGGCGGATTGAATTAAATGTCGTTTTACTGATTGCAAGAACTTG -118
 CAATTCACGCCGCTGACGTTGGCAACAAGTGGCGTGATAAAAAGCAAAGTTAAAAATAA -58
 CTGATTAAAAATCAGTGTGATAAAAATTATTCGGCTGGCACAAGGCTTGCCTCTCCGT +3
 TTTTGCCAAATGGGGCACAACAACGGAGAGTCAGCAAATGGAAATGTCTACCCTGAAACA +63
 CATCGAACAGACCCCTTCGCTTTACCCAGCGTTATCTGTCGATTGGGAACAGGTGTTTCG +123
 I E Q T P S L Y P A L S V D W E Q V F R

A

B

Fig. 1. A, Nucleotide sequence of upstream DNA of *Ecc71 hrpL_{Ecc}*. The transcriptional start site is indicated with an asterisk. Two putative σ^{54} promoters are marked. The putative integration host factor binding site is double underlined. Three direct repeats are triple underlined; one repeat is somewhat imperfect since, out of five, one base (G) replaces T. Part of the putative amino acid sequence is also shown. 1 = pAKC1077 (-654 nt to +74 nt); 2 = pAKC1078 (-545 nt to +74 nt); 3 = pAKC1079 (-434 nt to +74 nt); 4 = pAKC1080 (-267 nt to +74 nt); 5 = pAKC1081 (-190 nt to +74 nt); 6 = pAKC1082 (-164 nt to +74 nt) and 7 = pAKC1083 (-136 nt to +74 nt). **B**, β -galactosidase activities of *E. coli* MC4100/pBluescriptSK⁺ or MC4100/pAKC1076 (HrpS_{Ecc}⁺) with pMP220, pAKC1077, pAKC1078, pAKC1079, pAKC1080, pAKC1081, pAKC1082, or pAKC1083, respectively. Bacterial cultures were grown at 28°C in minimal salts medium plus glucose (0.5%), thiamine (5 mM), ampicillin, and tetracycline to a Klett value of approximately 180 and harvested for assays.

bacco leaves (Willis et al. 1990). This contrasts with the effects of GacS/GacA on *hrpN_{Ecc}* in *Ecc* (Cui et al. 2001). Hence, it was of interest to examine the influence of GacS/GacA on *hrpL* expression and to determine if the GacS/GacA effects were mediated via *rsmB*.

One of the puzzling aspects of the global regulation of extracellular proteins in *Ecc* has been the coregulation of extracellular pectinase, cellulase, and protease production with the Hrp regulon. This coregulation was noted with several regulators, including RsmC (Cui et al. 1999), HexA (Mukherjee et al. 2000; Harris et al. 1998), and KdgR (Hyytiäinen et al. 2001; Liu et al. 1999). Our recent findings have established that these regulators actually act via the *rsmA-rsmB* system. Thus, it is most likely that the effects of these regulators on *hrpL_{Ecc}* expression as detailed below are also mediated via this post-transcriptional system known to coregulate extracellular enzyme and harpin production in *Ecc*.

The findings reported here disclose that *hrpL_{Ecc}* is subject to two types of regulation. HrpS, RpoN, and IHF regulate transcription. Post-transcriptional regulation is mediated by RsmA and *rsmB* RNA and various other regulators, such as GacA, RsmC, HexA, and KdgR that affect *hrpL_{Ecc}* expression by modulating the levels of either RsmA, *rsmB* RNA, or both. Thus, the production of the Hrp-specific sigma factor in *Ecc* is controlled by highly integrated regulatory networks that ensure that *hrp* genes and genes for putative effectors are expressed in response to various signals and environmental conditions and the physiological state of the bacterial cell.

RESULTS

RpoN (sigma-54) and HrpS are required for the expression of *hrpL_{Ecc}*.

The presence of two potential sigma-54 binding sites located upstream of the *hrpL_{Ecc}* transcriptional start site (Fig. 1A) suggested that sigma-54 may be required for the expression of *hrpL_{Ecc}*. To test this possibility, we transferred the *HrpL_{Ecc}*⁺ HrpS_{Ecc}⁺ plasmid pAKC921 or the cloning vector pLAFR5 into an *E. coli* RpoN⁻ mutant TH1 and the RpoN⁺ *E. coli* MC4100. Northern blot analysis (Fig. 2) revealed that MC4100 carrying pAKC921 produced high levels of *hrpL_{Ecc}* transcript (Fig. 2, lane 3); by contrast, the RpoN⁻ strain TH1 carrying pAKC921 did not produce detectable levels of *hrpL_{Ecc}* transcript (Fig. 2, lane 4). These results demonstrate that sigma-54 is required for the expression of *hrpL_{Ecc}*. As would be expected, MC4100 or

TH1 carrying pLAFR5 did not yield any signal with the *hrpL_{Ecc}* probe (Fig. 2, lanes 1 and 2).

To test the effect of *hrpS* on *hrpL_{Ecc}* expression, total RNA samples were obtained from *E. coli* RpoN⁺ strain MC4100 and RpoN⁻ strain TH1 carrying pAKC1075 (*HrpL_{Ecc}*⁺) and pBluescript SK⁺ or pAKC1075 and pAKC1076 (*HrpS_{Ecc}*⁺) and subjected to Northern blot analysis (Fig. 2, lanes 5 to 8). In *E. coli* MC4100 or TH1 carrying pAKC1075, *hrpL_{Ecc}* transcript was not detected in the absence of the *hrpS_{Ecc}*⁺ plasmid (Fig. 2, lanes 5 and 6). While the RpoN⁺ strain MC4100 carrying pAKC1075 and pAKC1076 produced high level of *hrpL_{Ecc}* transcript (Fig. 2, lane 7), the transcript was not detected in the RpoN⁻ strain TH1 carrying these plasmids (Fig. 2, lane 8). These results indicate that both RpoN and HrpS_{Ecc} are required for the expression of *hrpL_{Ecc}*.

In RpoN-activated promoters, upstream activator sequences (Kustu et al. 1989) have been detected where activators are presumed to bind, causing DNA bending (e.g., Perez-Martin and de Lorenzo 1997; Perez-Martin et al. 1994). To localize such a region in *hrpL_{Ecc}*, MC4100 carrying pBluescriptSK⁺ or pAKC1076 (*HrpS_{Ecc}*⁺) plus various segments of *hrpL_{Ecc}* upstream regions (-654 nt to +74 nt, -545 nt to +74 nt, -434 nt to +74 nt, -267 nt to +74 nt, -190 nt to +74 nt, -164 nt to +74 nt, and -136 nt to +74 nt) in pMP220 were assayed for β -galactosidase activities. The data (Fig. 1B) show that a DNA region spanning -190 nt to -164 nt is required for HrpS_{Ecc}-mediated activation of *hrpL_{Ecc}* transcription.

IHF is required for *hrpL_{Ecc}* expression.

As indicated in Fig. 1A, there is a potential IHF binding site located at -130 nt to -118 nt, which is close to the putative HrpS_{Ecc} binding site. To assess the physiological relevance of this sequence, we transferred the *HrpL_{Ecc}*⁺ plasmid (pAKC1084) and the HrpS_{Ecc}⁺ plasmid (pAKC1076) into IHF⁺ *E. coli* MC4100 and IHF⁻ (*HimA*⁻) *E. coli* HP4110. The data in Figure 3 reveal that the *hrpL_{Ecc}* transcript level was high in IHF⁺ bacteria, whereas in the IHF⁻ bacteria, the *hrpL_{Ecc}* transcript was barely detected (Fig. 3, lanes 3 and 4). This pattern was reproducible in several repeat experiments. Furthermore, the introduction of pAKC1085 (*himA*⁺ DNA of *Ecc*71) into the *E. coli* IHF⁻ mutant carrying pAKC1084 and pAKC1076 stimulated *hrpL_{Ecc}* transcription (Fig. 3, lane 8). In addition, MC4100 carrying pAKC1076 plus pAKC1084 plus pCL1921 (vector) produced lower levels of *hrpL_{Ecc}* transcript

Fig. 2. Northern blot analysis of *hrpL_{Ecc}* mRNA in *E. coli* MC4100 carrying pLAFR5 (vector, lane 1), its RpoN⁻ mutant, TH1, carrying pLAFR5 (lane 2), MC4100 carrying pAKC921 (*HrpL_{Ecc}*⁺, *HrpS_{Ecc}*⁺, lane 3), TH1 carrying pAKC921 (lane 4), MC4100 carrying pBluescriptSK⁺ (vector) and pAKC1075 (*HrpL_{Ecc}*⁺, lane 5), TH1 carrying pBluescriptSK⁺ and pAKC1075 (lane 6), MC4100 carrying pAKC1076 (*HrpS_{Ecc}*⁺) and pAKC1075 (lane 7), and TH1 carrying pAKC1076 and pAKC1075 (lane 8). Bacteria were grown at 28°C in minimal salts medium plus glucose (0.5%, wt/vol), thiamine (5 mM), and drugs to a Klett value of approximately 150 for total RNA isolation. Each lane contained 10 μ g of total RNA. The arrow shows the levels of total RNA as revealed by ethidium bromide staining of denatured agarose gel.

Fig. 3. Effects of HimA on transcript levels of *hrpL_{Ecc}*. Bacteria were grown at 28°C in minimal salts medium plus glucose (0.5%, wt/vol), thiamine (5 mM), and antibiotics to a Klett value of approximately 150 for total RNA isolation. Lanes 1 and 2, *E. coli* MC4100 or HP4110 (*HimA*⁻ mutant of MC4100) carrying pRK415Gm^r + pBluescriptSK⁺ (vectors); lanes 3 and 4, MC4100 or HP4110 carrying pAKC1076 (*HrpS_{Ecc}*⁺) + pAKC1084 (*HrpL_{Ecc}*⁺), respectively; lane 5, MC4100 carrying pAKC1076 + pAKC1084 + pCL1921 (vector); lane 6, MC4100 carrying pAKC1076 + pAKC1084 + pAKC1085 (*HimA_{Ecc}*⁺); lane 7, HP4110 carrying pAKC1076 + pAKC1084; and Lane 8, HP4110 carrying pAKC1076 + pAKC1084 + pAKC1085. Each lane contained 10 μ g of total RNA. The arrow shows the levels of total RNA as revealed by ethidium bromide staining of denatured agarose gel.

than MC4100 carrying pAKC1076 plus pAKC1084 plus pAKC1085 (Fig. 3, lanes 5 and 6). The higher level of transcripts in lane 6 than in lane 5 is due to multiple copies of Ecc *himA*⁺ DNA.

Effects of RsmA and *rsmB* RNA on *hrpL*_{Ecc} expression.

Our previous studies demonstrated that *hrpN*_{Ecc}, which encodes the inducer of HR in Ecc, is negatively controlled by the global regulatory gene, *rsmA* (Cui et al. 1996; Mukherjee et al. 1997). Studies in Ecc (Chatterjee et al. 2001) as well as in *Erwinia amylovora* (Wei and Beer 1995; Wei et al. 2000), *Erwinia chrysanthemi* (Ham 1999) and *Pseudomonas syringae* (Xiao et al. 1994) have revealed that HrpL controls the expression of *hrpN*. These observations collectively suggested that RsmA actually controls *hrpL*_{Ecc}, and the effects on *hrpN*_{Ecc} transcript levels in RsmA⁺ and RsmA⁻ bacteria were due to differential expression of *hrpL*_{Ecc}. The results of Northern blot analysis (Fig. 4) revealed that the levels of *hrpL*_{Ecc} and *hrpN*_{Ecc} mRNA were lower in Ecc71 (RsmA⁺; Fig. 4, column 1) compared to that in AC5071 (RsmA⁻; Fig. 4, column 3).

rsmB RNA acts as a positive regulator by neutralizing the effects of RsmA (Liu et al. 1998; Ma et al. 2001). This finding taken along with the results presented above predicted that, in an RsmA⁺ *rsmB*⁻ strain, *hrpL*_{Ecc} expression would be severely reduced. Indeed, the data in Figure 4 (column 2) reveal that *hrpL*_{Ecc} transcripts are barely detectable in a strain producing RsmA but no *rsmB* RNA.

RsmA promotes decay of *hrpL*_{Ecc} mRNA.

The lower level of *hrpL*_{Ecc} mRNA in the RsmA⁺ parent than in its RsmA⁻ mutant was presumed to result from RsmA-promoted decay of *hrpL*_{Ecc} RNA. To test this possibility, we compared the stability of *hrpL*_{Ecc} RNA and *hrpN*_{Ecc} RNA in wild-type Ecc AC5047 and RsmA⁻ mutant AC5070. The results (Fig. 5) show that *hrpN*_{Ecc} mRNA stability was comparable in AC5047 and AC5070. By contrast, *hrpL*_{Ecc} mRNA was more stable in RsmA⁻ bacteria than in the RsmA⁺ wild-type strain.

Other global regulators also control *hrpL*_{Ecc} expression.

Effect of GacA. *hrpL*_{Ecc} and *hrpN*_{Ecc} transcripts were significantly lower in the RsmA⁻GacA⁻ double mutant (AC5087)

than in its parent, AC5071 (RsmA⁻ GacA⁺) (Fig. 6A). We should note that *hrpL*_{Ecc} transcript level was extremely low in Ecc wild-type strain AH2 and its GacS⁻ mutant AH2552 (data not shown), thereby precluding any definitive conclusions regarding the effects of the putative histidine sensor kinase GacS. However, extrapolating from our previous observations of the effects of GacS on *hrpN*_{Ecc} expression (Cui et al. 2001) and the predicted regulatory roles of GacS and GacA (Heeb and Haas 2001), we suggest that the two components work in conjunction to regulate *hrp* gene expression.

The GacA effect is mediated mostly by modulating *rsmB* RNA production, and the increased levels of *rsmB* RNA, in turn, positively control expression of the target genes (Cui et al. 2001; Heeb et al. 2002). In light of the effect of GacA on *hrpL*_{Ecc} expression, it was of interest to see if the response was channeled via *rsmB* as well. To test this possibility, we transferred a *plac-rsmB* plasmid (pAKC1049), a *rsmB*⁺ plasmid (pAKC264), the cloning vectors (pCL1920 or pSF6), or the GacA⁺ plasmid (pAKC1055) into the Ecc71 GacA⁻ mutant AC5056 and determined the levels of *hrpL*_{Ecc} RNA. The data (Fig. 6B) show that *hrpL*_{Ecc} transcript was absent in the mutant carrying pSF6, and the RNA level was very low in AC5056 carrying pAKC264 (Fig. 6B, lanes 3 and 4). We attribute this finding to a very low level of expression of *rsmB* in pAKC264 in the absence of GacA. In AC5056 carrying pAKC1049, we detected a high level of *hrpL*_{Ecc} transcript (Fig. 6B, lane 2); in fact, the level produced by this construct was higher than that in a mutant carrying the GacA⁺ plasmid (Fig. 6B, lane 6). We ascribe this difference to activation of expression of multiple copies of *rsmB* by the *lac* promoter, which, as expected, is functional in the absence of GacA. In separate experiments (Fig. 6C), we have determined that the RsmB⁻ GacA⁺ mutant does not produce detectable levels of *hrpL*_{Ecc} RNA (Fig. 6C, lanes 1 and 3), and this effect is relieved by providing *rsmB*⁺ DNA (pAKC1049; Fig. 6C, lane 2) but not by providing multiple copies of *gacA*⁺ DNA (pAKC1055; Fig. 6C, lane 4). These results establish that the positive effect of GacA on *hrpL*_{Ecc} expression is mediated via *rsmB*, and that GacA is located higher up in the *rsmB* RNA-RsmA regulatory hierarchy.

Effects of KdgR, HexA, and RsmC. In Ecc71, *rsmA* and *rsmB* are differentially regulated by HexA and RsmC; *rsmA* expres-

Fig. 4. Effects of *rsmB* and RsmA on the transcript levels of *hrpL*_{Ecc} and *hrpN*_{Ecc}. Column 1, Ecc71 (wild-type parent); column 2, AC5088 (*rsmB*⁻); column 3, AC5071 (RsmA⁻). Bacteria were grown at 28°C in minimal salts medium plus sucrose (0.5%, wt/vol) to a Klett value of approximately 150 for total RNA extraction. Each lane contained 10 µg of total RNA. X-ray film was exposed at -80°C for approximately 18 h. The arrow shows the levels of total RNA as revealed by ethidium bromide staining of denatured agarose gel.

Fig. 5. Stability of *hrpL*_{Ecc} and *hrpN*_{Ecc} transcripts in Ecc strain AC5047 and its RsmA⁻ derivative, AC5070. After hybridization and washing, blots were exposed to X-ray film at -80°C for 18 h and developed. Column 1 to 6, AC5047 at 0, 2.5, 5, 7.5, 10, and 12.5 min after rifampicin addition, respectively. Column 7 to 12, AC5070 at 0, 2.5, 5, 7.5, 10, and 12.5 min after rifampicin addition, respectively. For columns 1 to 6, each lane contained 20 µg of total RNA, and for columns 7 to 12, each lane contained 7 µg of total RNA. The arrows show the levels of total RNA as revealed by ethidium bromide staining of denatured agarose gel.

sion is positively controlled, whereas *rsmB* transcription is negatively regulated (Cui et al. 1999; Mukherjee et al. 2000). Moreover, in this strain, KdgR represses *rsmB* transcription (Liu et al. 1999) but has little or no effect on *rsmA* expression (A. Chatterjee, unpublished data). The findings described above suggested that mutants deficient in KdgR, HexA, and RsmC would overproduce *hrpL_{Ecc}* transcripts. Indeed, this prediction is supported by the results given in Figure 7. However, it should be noted that the deficiency of HexA and RsmC (Fig. 7, lanes 3 and 4) has more pronounced effect than the inactivation of KdgR (Fig. 7, lane 2). We attribute this differential effect to dual action of HexA and RsmC on expression of both *rsmA* and *rsmB* and the effect of KdgR on *rsmB* transcription but not RsmA production (A. Chatterjee, unpublished data; Cui et al. 1999; Liu et al. 1999; Mukherjee et al. 2000).

Fig. 6. A, Effect of GacA on levels of *hrpL_{Ecc}* and *hrpN_{Ecc}* transcripts. Column 1, AC5071 (GacA⁺, RsmA⁻) and column 2, AC5087 (GacA⁻, RsmA⁻). B, Northern blot analysis of *hrpL_{Ecc}* mRNA in AC5056 (GacA⁻) carrying pCL1920 (vector, lane 1), pAKC1049 (*plac-rsmB*, lane 2), pSF6 (vector, lane 3), pAKC264 (*rsmB*⁺, lane 4), pRK415 (vector, lane 5), and pAKC1055 (*gacA*⁺, lane 6). C, Northern blot analysis of *hrpL_{Ecc}* mRNA in AC5088 (RsmB⁻) carrying pCL1920 (vector, lane 1), pAKC1049 (*plac-rsmB*, lane 2), pRK415 (vector, lane 3), and pAKC1055 (*gacA*⁺, lane 4). Total RNAs were extracted from bacteria grown at 28°C in minimal salts medium plus sucrose (0.5%, wt/vol) and drugs to a Klett value of approximately 150. Each lane contained 10 µg of total RNA. The arrows show the levels of total RNA as revealed by ethidium bromide staining of denatured agarose gel.

DISCUSSION

As in *Erwinia amylovora* (Kim and Beer 2000; Kim et al. 1997; Wei and Beer 1995; Wei et al. 2000; Wei et al. 1992), *Pantoea stewartii* (Frederick et al. 1993, 2001), *Erwinia chrysanthemi* (Bauer et al. 1994, 1995; Ham 1999; Kim et al. 1998) and *Erwinia herbicola* pv. *gypsophylae* (Mor et al. 2001), the Hrp cluster of Ecc, contains the genes for harpin, the type III secretion system, and several Hrp regulators, such as HrpX_{Ecc}, HrpY_{Ecc}, HrpS_{Ecc}, and HrpL_{Ecc} (Chatterjee et al. 2001; Rantakari et al. 2001), and HrpL_{Ecc} plays a central regulatory role (Chatterjee et al. 2000). Extrapolating from what is known in other systems and in Ecc, we propose a tentative model for the regulation of *hrpL_{Ecc}* (Fig. 8). The proposed scheme postulates the presence of at least two complementary pathways responding to plant and environmental signals; one pathway entails Hrp specific regulatory components and the other involves global regulators, GacS and GacA. It is presumed that HrpX_{Ecc}, a putative sensor kinase, responds to Hrp-specific signals such as starvation, osmolarity, and possibly yet unknown factors. The activated form of HrpX_{Ecc} then modifies HrpY_{Ecc}, a response regulator, which in turn stimulates HrpS_{Ecc} transcription. HrpS_{Ecc} in conjunction with RpoN and IHF activates the *hrpL_{Ecc}* promoter. HrpL_{Ecc} is required for the expression of genes for the type III secretion system and most, if not all, proteins secreted by this pathway. The regulatory hierarchy as depicted in the model awaits verification but seems logical based upon what is now known in other systems (Bauer et al. 1994; Hutcheson et al. 2001; Wei and Beer 1995; Wei et al. 2000; Xiao et al. 1994).

As documented here, expression of the Hrp regulon is controlled by the global system comprising GacS and GacA, members of a classical two-component system. The following series of events are presumed to occur. GacS, the putative sensor kinase, responds to cell density, growth phase, and various yet unrecognized environmental conditions and autophosphorylates. It then phosphorylates GacA, and the activated form of GacA stimulates transcription of *rsmB*. Moreover, HexA, a LysR type regulator, and RsmC, a putative transcriptional adapter, play a dual role, i.e., they positively control RsmA production and inhibit *rsmB* RNA production. KdgR, suppresses *rsmB* RNA production but not RsmA levels (Fig. 8).

Fig. 7. Effects of KdgR, HexA, and RsmC on the transcript levels of *hrpL_{Ecc}* and *hrpN_{Ecc}*. Column 1, Ecc71 (wild type); column 2, AC5073 (KdgR⁻); column 3, AC5076 (HexA⁻); and column 4, AC5053 (RsmC⁻). Bacteria were grown at 28°C in minimal salts medium plus sucrose (0.5%, wt/vol) to a Klett value of approximately 150 for total RNA extraction. Each lane contained 10 µg of total RNA. X-ray film was exposed at -80°C for approximately 18 h. The arrows show the levels of total RNA as revealed by ethidium bromide staining of denatured agarose gel.

The cumulative actions of these regulators determine the pool size of free RsmA and RsmA-*rsmB* RNA ribonucleoprotein complex. Free RsmA is believed to be responsible for the decay of *hrpL*_{Ecc} mRNA. In the absence of HrpL_{Ecc}, the genes for the downstream components of the Hrp regulon remain silent. RsmA and *rsmB* RNA also control extracellular enzyme production in Ecc (Chatterjee et al. 1995; Cui et al. 1995; Liu et al. 1998). These observations, therefore, identify RsmA and *rsmB* RNA as common regulatory components controlling the expression of the Hrp regulon and extracellular enzyme genes.

By utilizing well-characterized *E. coli* RpoN⁻ and RpoN⁺ strains, we have established that RpoN is required for *hrpL*_{Ecc} transcription. It is now generally accepted that for most sigma-54 controlled genes, activators (repressors) interact cooperatively with the sigma factor and RNA polymerase to activate transcription (Perez-Martin et al. 1994; Shingler 1996). For HrpL regulons of *Pseudomonas syringae* pv. *syringae* (Hendrickson et al. 2000; Hutcheson et al. 2001) and *Erwinia amylovora* (Wei et al. 2000), there is substantial evidence that HrpS functions in this capacity. Thus, a compelling case can be made that HrpS functions as an enhancer binding protein. Indeed, sequence comparisons have revealed high homology of HrpS_{Ecc} with members of the NtrC family of regulators that act in conjunction with RpoN to activate transcription (data not shown). Again, by using *E. coli* as the surrogate host, we have documented that HrpS_{Ecc} is essential and that HrpS_{Ecc} acts in conjunction with RpoN to activate *hrpL*_{Ecc} expression. The sequences spanning -190 nt to -164 nt, required for the manifestation of the HrpS_{Ecc} effect (Fig. 1B), may represent upstream activator sequences (Kustu et al. 1989). Although details of the interaction of HrpS_{Ecc} with this DNA region await elucidation, it seems likely that binding of HrpS_{Ecc} to this DNA could cause

conformational changes leading to more efficient transcription initiation by RNA polymerase-sigma54 holoenzyme. While this model remains to be tested, it is apparent that the findings reported here, those of Wei and associates (2000) with *Erwinia amylovora*, and those of Hutcheson and associates (2001) for *Pseudomonas syringae* pv. *syringae* support the idea that regulatory events controlling *hrpL* transcription have generally been conserved in these plant pathogenic bacteria.

A novel finding was the effect of IHF on *hrpL*_{Ecc} expression. IHF is known to act mostly as a positive regulator, although there are a few instances in which it has been shown to act as a negative regulator (Goosen and van de Putte 1995; Wassem et al. 2000). In the case of *hrpL*_{Ecc}, the putative IHF binding site is strategically located close to the putative HrpS_{Ecc} binding region. Following the generally accepted model for IHF action (Goosen and van de Putte, 1995), we postulate that IHF binding could lead to bent DNA that would prompt the HrpS_{Ecc}-Sigma 54 complex to contact the Sigma 54 promoter and to activate it. According to this scenario, IHF binding should result in the activation of *hrpL*_{Ecc} transcription, as is indicated by the data presented here. Within the putative *hrpL* regulatory regions of *Erwinia amylovora*, *Pantoea stewartii*, *Erwinia herbicola* pv. *gypsophila*, and *Erwinia chrysanthemi* we have detected sequences closely resembling the consensus IHF binding site [(5' WATCAANNNTTTR 3'; W = A or T, R = A or G, and N = any base). Thus, it is quite likely that, like Ecc71, *hrpL* expression in these bacteria is positively controlled by IHF as well. The IHF effect raises several critical issues, including the pool size of IHF in Ecc and other *Erwinia* species under various environmental and cultural conditions, relative affinities of IHF for HrpS_{Ecc}-free *hrpL*_{Ecc} DNA and HrpS_{Ecc}-bound *hrpL*_{Ecc} DNA, and configuration of IHF bound DNA. We have now ini-

Fig. 8. A speculative model depicting the regulatory events leading to the expression of *hrp* genes in *Erwinia carotovora* subsp. *carotovora* responsible for the type III secretion system and production of harpin, effector proteins, and putative *hrp* pili.

tiated studies to understand molecular interactions between IHF, HrpS, and RpoN in the context of *hrpL_{Ecc}* transcription.

Our findings demonstrate that RsmA plays a very critical role by affecting the stability of *hrpL_{Ecc}* mRNA. In fact, it is now apparent that the inability of the wild-type Ecc strains to elicit the HR is mostly, if not solely, due to the negative effect of RsmA on *hrpL_{Ecc}* transcripts. While stability of *hrpN_{Ecc}* in RsmA⁺ and RsmA⁻ bacteria is very similar, *hrpL_{Ecc}* transcripts are less stable in RsmA⁺ bacteria than in RsmA⁻ bacteria. These observations suggest specificity in RsmA-promoted decay of RNA species in Ecc. We have also detected a lack of RsmA effect on the levels of transcript of *hexA*, *rpoS*, and *recA* (data not shown). Such findings, although somewhat limited in scope, support the notion that certain RNA species of Ecc are refractile to RsmA action. It is possible that structural characteristics of the RsmA-RNA complex may prevent accelerated decay of certain RNA species. The high levels of *rsmB* RNA in RsmA⁺ bacteria than in RsmA⁻ bacteria support this possibility (Chatterjee et al., unpublished data).

A number of the extracytoplasmic function (ECF) subfamily of sigma factors is subject to modulation by anti-sigma factors

(Hughes 1998). However thus far, there is no evidence for an anti-HrpL. Instead, the available data strongly suggest that RpoN, HrpS, and IHF control *hrpL* expression in *Erwinia* species. Furthermore, *hrpS* expression itself is most likely controlled by HrpX and HrpY, members of the two-component system. Additionally, as documented here, *hrpL_{Ecc}* is subject to post-transcriptional regulation mediated by RsmA and *rsmB* RNA. These observations demonstrate that Ecc and other *Erwinia* species use an elaborate and highly sophisticated scheme (Fig. 8) for controlling the genes for harpin, the type III secretion system, and various proteins that are secreted via this pathway.

MATERIALS AND METHODS

Most of the experiments presented in this manuscript were performed at least three times, and the results were reproducible.

Bacterial strains, plasmids, and media.

Bacterial strains and plasmids are described in Table 1. The strains carrying drug resistance markers were maintained on

Table 1. Bacterial strains and plasmids used in this study

Strain/Plasmids	Relevant characteristics	Source/ Reference
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>		
Ecc71	Wild type	Zink et al. 1984
AC5047	Nal ^r derivative of AC5006	Chatterjee et al. 1995
AC5070	RsmA ⁻ derivative of AC5047	Chatterjee et al. 1995
AC5071	RsmA ⁻ derivative of Ecc71, Km ^r	Mukherjee et al. 1997
AC5053	RsmC ⁻ derivative of Ecc71, Km ^r	Cui et al. 1999
AC5056	GacA ⁻ derivative of Ecc71, Gm ^r	Cui et al. 2001
AC5073	KdgR ⁻ derivative of Ecc71, Sp ^r	Liu et al. 1999
AC5076	HexA ⁻ derivative of Ecc71, Sp ^r	Mukherjee et al. 2000
AC5087	GacA ⁻ derivative of AC5071 Gm ^r , Km ^r	This work
AC5088	RsmB ⁻ derivative of Ecc71, Cm ^r	Lab collection
AH2	Wild type	Frederick et al. 1997
AH2552	RpfA ⁻ (= GacS ⁻) derivative of AH2	Frederick et al. 1997
<i>Escherichia coli</i>		
DH5 α	ϕ 80lacZ Δ M15 Δ (lacZYA-argF), U169 hsdR17 recA1 endA1 thi-1	Gibco BRL
MC4100	araD139 Δ (lacIPOZYA)U169 recA1 thi-1 Str ^r	Liu et al. 1994
HP4110	HimA ⁻ derivative of MC4100, Tc ^r	Lab collection
TH1	Δ rpoN2518, endA1, thi-1, hsdR17, supE44, Δ lacU169	Oguiza and Buck 1997
pBluescript KS+	Ap ^r	Stratagene
pBluescript-SK+	Ap ^r	Stratagene
pCL1920	Sp ^r , Sm ^r	Lerner and Inouye 1990
pCL1921	Sp ^r , Sm ^r	Lerner and Inouye 1990
pLAFR5	Tc ^r	Keen et al. 1988
pLG339	Tc ^r , Km ^r	Stoker et al. 1982
pMP220	Tc ^r , promoter-probe vector	Spaink et al. 1987
pRK415	Tc ^r	Keen et al. 1988
pRK415Gm ^r	Gm ^r , Gm ^r cassette inserted into pRK415	Lab collection
pSF6	Sp ^r	Selvaraj et al. 1984
pRK2013	Mob ⁺ , Tra ⁺ , Km ^r	Figurski and Helinski 1979
pAKC264	Sp ^r , <i>rsmB</i> ⁺ DNA in pSF6	Murata et al. 1991
pAKC870	Km ^r , Tc ^r , RsmA ⁻ DNA in pLAFR5	Chatterjee et al. 1995
pAKC921	Tc ^r , pLAFR5 containing Hrp regulators (<i>hrpL_{Ecc}</i> , <i>hrpS_{Ecc}</i> , and <i>hrpXY</i>) and <i>hrpN_{Ecc}</i> from genomic library of Ecc71	Cui et al. 1996
pAKC924	Ap ^r , <i>hrpN_{Ecc}</i> in pBluescriptSK+	Cui et al. 1996
pAKC1049	Sp ^r , <i>plac-rsmB</i> in pCL1920	Ma et al. 2001
pAKC1055	Tc ^r , <i>gacA</i> ⁺ DNA in pRK415	Cui et al. 2001
pAKC1071	Ap ^r , <i>hrpL_{Ecc}</i> ⁺ , 4.0 kb <i>EcoRI</i> fragment of pAKC921 in pBluescript SK+	This work
pAKC1075	Km ^r , <i>hrpL_{Ecc}</i> ⁺ , 4.0-kb <i>EcoRI</i> fragment of pAKC1071 in pLG339.	This work
pAKC1076	Ap ^r , 2.0 kb <i>BglII</i> - <i>Clal</i> <i>hrpS_{Ecc}</i> ⁺ fragment from pAKC921 cloned in pBluescript SK+	This work
pAKC1077	Tc ^r , -654 ~ +74 of <i>hrpL_{Ecc}</i> cloned in pMP220	This work
pAKC1078	Tc ^r , -545 ~ +74 of <i>hrpL_{Ecc}</i> cloned in pMP220	This work
pAKC1079	Tc ^r , -434 ~ +74 of <i>hrpL_{Ecc}</i> cloned in pMP220	This work
pAKC1080	Tc ^r , -267 ~ +74 of <i>hrpL_{Ecc}</i> cloned in pMP220	This work
pAKC1081	Tc ^r , -190 ~ +74 of <i>hrpL_{Ecc}</i> cloned in pMP220	This work
pAKC1082	Tc ^r , -164 ~ +74 of <i>hrpL_{Ecc}</i> cloned in pMP220	This work
pAKC1083	Tc ^r , -136 ~ +74 of <i>hrpL_{Ecc}</i> cloned in pMP220	This work
pAKC1084	Tc ^r , 4.0-kb <i>EcoRI</i> fragment of pAKC1072 in pRK415Gm ^r	This work
pAKC1085	Sp ^r , HimA _{Ecc} ⁺ DNA cloned in pCL1921	Lab collection

Luria-Bertani (LB) agar containing appropriate antibiotics. The wild-type strains were maintained on LB agar. Recipes of LB medium, King's B medium, minimal salts medium, and minimal salts medium plus celery extract have been described (Chatterjee et al. 1995; Murata et al. 1994). When required, antibiotics were supplemented as follows (μg per ml): ampicillin (Ap), 100; kanamycin (Km), 50; spectinomycin (Sp), 50; and tetracycline (Tc), 10. Media were solidified by the addition of 1.5 % agar.

DNA techniques.

Standard procedures were used in the isolation of plasmid and chromosomal DNAs, electroporation, restriction endonuclease digests, gel electrophoresis, and DNA ligation (Sambrook et al. 1989). Southern blot analysis was carried out as described by Cui and associates (1995). PCR was performed as described by Liu and associates (1998). Restriction and modifying enzymes were obtained from Promega (Madison, WI, U.S.A.). The Primer-a-Gene DNA labeling system from Promega was used for labeling DNA. Nucleotide sequences were determined at the DNA Core Facility of University of Missouri–Columbia, U.S.A.

RNA preparation and Northern hybridization experiments.

Total RNA was obtained from *Erwinia carotovora* subsp. *carotovora* constructs grown in minimal salts medium plus sucrose (0.5%, wt/vol) at 28°C to a Klett value of approximately 150 units. *E. coli* strains were grown in minimal salts medium plus thiamine (5 mM), glucose (0.5%, wt/vol), and drugs at 28°C to a Klett value of approximately 150 units for total RNA extraction.

To determine the stability of *hrpL_{Ecc}* and *hrpN_{Ecc}* mRNA in RsmA⁺ and RsmA[−] strains, cultures of AC5047 and AC5070 were grown in minimal salts medium plus sucrose to a Klett value of 150, at which point rifampicin (0.2 mg/ml) was added to block further initiation. Culture samples (8 ml each) were then withdrawn at various time points into tubes containing 5 ml ice-cold water, and total RNA was extracted.

The concentration of RNA samples was spectrophotometrically detected and normalized for each sample. Pictures of ethidium bromide-stained total RNAs in denatured agarose gels, indicated by arrows in Figures 2, 3, 4, 5, and 6, are included to show that equal amounts of total RNA were loaded in each well.

The probes used in this work were the 287-bp *BglIII-EcoRV* fragment of *hrpL_{Ecc}* from pAKC1071 and the 779-bp *EcoRV-SmaI* fragment of *hrpN_{Ecc}* from pAKC924 (Cui et al. 1996). DNA probes were labeled with γ -³²P[dATP] by random-priming according to the manufacturer's instructions (Promega). Prehybridization (6 h at 65°C) and hybridization (18 h at 65°C) were performed in prehybridization buffer (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 2× Denhardt's, 0.1% sodium dodecyl sulfate (SDS), and 100 μg of denatured salmon sperm DNA per ml). After hybridization, membranes were washed twice for 20 min each at 50°C in 2× SSC and 0.5% SDS and then were washed for 30 min at 65°C in 0.5× SSC and 0.5% SDS and examined by autoradiography with X-ray film (Kodak Rochester, NY, U.S.A.).

Deletion analysis of *hrpL_{Ecc}* upstream DNA.

Primers were designed for PCR amplification of the fragments corresponding to nucleotides of −654 ~ +74, −545 ~ +74, −434 ~ +74, −267 ~ +74, −190 ~ +74, −164 ~ +74, and −136 ~ +74 from the putative transcriptional start site of *hrpL_{Ecc}*. The 5'-end primers were tagged with *BglIII*, and the primers for the 3'-end were tagged with *EcoRI*. Fragments were amplified by PCR from pAKC1071, digested by *BglIII-EcoRI*, and cloned

into pMP220 to yield plasmids pAKC1077, pAKC1078, pAKC1079, pAKC1080, pAKC1081, pAKC1082, and pAKC1083, respectively. These plasmids were transferred into *E. coli* MC4100 carrying pBluescriptSK⁺ or pAKC1076 (*HrpS_{Ecc}*⁺). Bacterial constructs were grown in minimal salts medium plus thiamine (5 mM), glucose (5%, wt/vol), ampicillin, and tetracycline at 28°C to a Klett value of 150 and harvested for β -galactosidase activities. The β -galactosidase assays were carried out according to Miller (1972).

Construction of RsmA[−]GacA[−] mutants.

To isolate a RsmA[−]GacA[−] mutant, the RsmA[−] plasmid pAKC870 (Chatterjee et al. 1995) was transferred into AC5056 (GacA[−] mutant of Ecc, Table 1) with the helper plasmid pRK2013. Transconjugants were selected on minimal salts agar containing sucrose and kanamycin. Km^rGm^rTc^s isolates were tested for the absence of *rsmA* RNA by Northern blot analysis.

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LITERATURE CITED

- Alfano, J. R., Charkowski A. O., Deng, W.-L., Badel, J. L., Petnicki-Ocwieja, T., van Dijk, K., and Collmer, A. 2000. The *Pseudomonas syringae* Hrp pathogenicity island has a tripartite mosaic structure composed of a cluster of type III secretion genes bounded by exchangeable effector loci that contribute to parasitic fitness and pathogenicity in plants. *Proc. Natl. Acad. Sci. U.S.A.* 97:4856-4861.
- 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_{Ecc}: An elicitor of the hypersensitive response that contributes to soft-rot pathogenesis. *Mol. Plant-Microbe Interact.* 8:484-491.
- 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., Cui, Y., Liu, Y., and Chatterjee, A. K. 2000. Molecular characterization of *Erwinia carotovora* *hrpL*, which encodes an alternate sigma factor. Pages 237-239 in: *Plant Pathogenic Bacteria*. S. H. de Boer, ed. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Chatterjee, A., Cui, Y., Chaudhuri, S., and Chatterjee, A. K. 2002. Identification of regulators of *hrpL* genes of *Erwinia carotovora* ssp. *carotovora* and characterization of HrpL_{Ecc} (Sigma L_{Ecc}), an alternative sigma factor. *Mol. Plant Pathol.* 3(1): In press.
- Collmer, A., Alfano, J. R., Abderson, D. M., Badel, J. L., Deng, W.-L., Fouts, D. E., Rehm, A. H., Rojas, C. M., Schneewind, O., and van Dijk, K. 2000. Hrp (type III) protein secretion and the virulence of *Pseudomonas syringae* and *Erwinia chrysanthemi*. Pages 65-70 in: *Biology of Plant-Microbe Interactions*, Vol. 2. P. J. G. M. de Wit, T. Bisseling, and W. Stiekema, eds. International Society for Molecular Plant-Microbe Interactions, St. Paul, MN, U.S.A.
- 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.
- Cui, Y., Mukherjee, A., Dumenyo, C. K., Liu, Y., and Chatterjee, A. K.

1999. *rsmC* of the soft-rotting bacterium, *Erwinia carotovora* subsp. *carotovora*, negatively controls extracellular enzyme and harpin_{Ecc} production and virulence by modulating the levels of regulatory RNA (*rsmB*) and RNA binding protein (RsmA). *J. Bacteriol.* 181:6042-6052.
- Cui, Y., Chatterjee, A., and Chatterjee, A. K. 2001. Effects of the two-component system comprising GacA and GacS of *Erwinia carotovora* subsp. *carotovora* on the production of global regulatory *rsmB* RNA, extracellular enzymes, and harpin_{Ecc}. *Mol. Plant-Microbe Interact.* 14:516-526.
- Figurski D. H., and Helinski, D. R. 1979. Replication of an origin-containing derivative of plasmid RK2 depend on a plasmid function provided in trans. *Proc. Natl. Acad. Sci. U.S.A.* 76:1648-1652.
- Fouts, D. E., Abramovitch, R. B., Alfano, J. R., Baldo, A. M., Buell, C. R., Cartinhour, S., Chatterjee, A. K., D'Ascenzo, M., Gwinn, M. L., Lazarowitz, S. G., Lin, N. C., Martin, G. B., Rehm, A. H., Schneider, D. J., van Dijk, K., Tang, X. Y., and Collmer, A. 2002. Genomewide identification of *Pseudomonas syringae* pv. tomato DC3000 promoters controlled by the HrpL alternative sigma factor. *Proc. Natl. Acad. Sci. U.S.A.* 99:2275-2280.
- Frederick R. D., Chiu, J., Bennetzen, J. L., and Handa, A. K. 1997. Identification of a pathogenicity locus, *rpfA*, in *Erwinia carotovora* subsp. *carotovora* that encodes a two-component sensor-regulator protein. *Mol. Plant-Microbe Interact.* 10:407-415.
- Frederick, R. D., Majerczak, D. R., and Coplin, D. L. 1993. *Erwinia stewartii* WtsS, a positive regulator of pathogenicity gene expression, is similar to *Pseudomonas syringae* pv. *phaseolicola* HrpS. *Mol. Microbiol.* 9:477-485.
- Frederick R. D., Ahmad, M., Majerczak, D. R., Arroyo-Rodriguez, A. S., Manulis, S., and Coplin, D. L. 2001. Genetic organization of the *Pantoea stewartii* subsp. *stewartii* *hrp* gene cluster and sequence analysis of the *hrpA*, *hrpC*, *hrpN*, and *wtsE* operons. *Mol. Plant-Microbe Interact.* 14:1213-1222.
- Galan J. E., and Collmer, A. 1999. Type III secretion machines: Bacterial devices for protein delivery into host cells. *Science* 284:1322-1328.
- Goosen, N., and van de Putte, P. 1995. The regulation of transcription initiation by integration host factor. *Mol. Microbiol.* 16:1-7.
- Guttman, D. S., Vinatzer, B. A., Sarkar, S. F., Ranall, M. V., Kettler, G., Greenberg, J. T. 2002. A functional screen for the type III (Hrp) secretome of the plant pathogen *Pseudomonas syringae*. *Science* 295:1722-1726.
- Ham, J. H. 1999. Extracellular proteins, secretion pathways, and the virulence of *Erwinia chrysanthemi*. Ph.D. thesis. University of Cornell, Ithaca, NY.
- Harris, S. J., Shih, Y.-L., Bentley, S. D., and Salmond, G. P. C. 1998. The *hexA* gene of *Erwinia carotovora* encodes a LysR homologue and regulates motility and the expression of multiple virulence determinants. *Mol. Microbiol.* 28:705-717.
- He, S. Y. 1998. Type III protein secretion systems in plant and animal pathogenic bacteria. *Annu. Rev. Phytopathol.* 36:363-392.
- Heeb, S., and Haas, D. 2001. Regulatory roles of the GacS/GacA two-component system in plant-associated and other gram-negative bacteria. *Mol. Plant-Microbe Interact.* 14:1351-1363.
- Heeb, S., Blumer, C., and Haas, D. 2002. Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. *J. Bacteriol.* 184:1046-1056.
- Hendrickson, E. L., Guevera, P., and Ausubel, F. M. 2000. The alternative sigma factor RpoN is required for *hrp* activity in *Pseudomonas syringae* pv. *Maculicola* and acts at the level of *hrpL* transcription. *J. Bacteriol.* 182:3508-3516.
- Hughes, K. T. 1998. The anti-sigma factors. *Annu. Rev. Microbiol.* 52:231-286.
- Hutcheson S. W., Bretz, J., Sussan, T., Jin, S., Pak, K. 2001. Enhancer-binding proteins HrpR and HrpS interact to regulate *hrp*-encoded type III protein secretion in *Pseudomonas syringae* strains. *J. Bacteriol.* 183:5589-5598.
- Hyttiäinen H., Montesano, M., and Palva, E. T. 2001. Global regulators ExpA (GacA) and KdgR modulate extracellular enzyme gene expression through the RsmA-*rsmB* system in *Erwinia carotovora* subsp. *carotovora*. *Mol. Plant-Microbe Interact.* 14:931-938.
- 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.
- Kim, J. F., and Beer, S. V. 2000. *hrp* Genes and harpins of *Erwinia amylovora*: A decade of discovery. Pages 144-162 in: Fire Blight. J. L. Vanneste, ed. CABI Publishing, Hamilton, New Zealand.
- Kim, J. F., Wei, Z. M., Beer, S. V. 1997. The *hrpA* and *hrpC* operons of *Erwinia amylovora* encode components of a type III pathway that secretes harpin. *J. Bacteriol.* 179:1690-1697.
- Kim, J. F., Ham, J. H., Bauer, D. W., Collmer, A., and Beer, S. V. 1998. The *hrpC* and *hrpN* operons of *Erwinia chrysanthemi* EC16 are flanked by *plcA* and homologs of hemolysin/adhesin genes and accompanying activator/transporter genes. *Mol. Plant-Microbe Interact.* 11:563-567.
- Kustu, S., Santero, E., Keener, J., Popham, D., and Weiss, D. 1989. Expression of σ^{54} (*ntfA*)-dependent genes is probably united by a common mechanism. *Microbiol. Rev.* 53:367-376.
- Lerner, C. G., and Inouye, M. 1990. Low copy number plasmids for regulated low-level expression of cloned genes in *Escherichia coli* with blue/white insert screening capability. *Nucleic Acids Res.* 18:4631.
- Liu, Y., Chatterjee, A., and Chatterjee, A. K. 1994. Nucleotide sequence, organization and expression of *rdgA* and *rdgB* genes that regulate pectin lyase production in the plant pathogenic bacterium *Erwinia carotovora* subsp. *carotovora* in response to DNA damaging agents. *Mol. Microbiol.* 14:999-1010.
- Liu, Y., Cui, Y., Mukherjee, A., and Chatterjee, A. K. 1998. Characterization of a novel RNA regulator of *Erwinia carotovora* subsp. *carotovora* that controls production of extracellular enzymes and secondary metabolites. *Mol. Microbiol.* 29:219-234.
- Liu, Y., Jiang, G.-Q., Cui, Y., Mukherjee, A., Ma, W.-L., and Chatterjee, A. K. 1999. *kdgR_{Ecc}* negatively regulates genes for pectinases, cellulase, protease, harpin_{Ecc}, and a global RNA regulator in *Erwinia carotovora* subsp. *carotovora*. *J. Bacteriol.* 181:2411-2422.
- Ma, W., Cui, Y., Liu, Y., Dumenyo, C. K., Mukherjee, A., and Chatterjee, A. K. 2001. Molecular characterization of global regulatory RNA species that control pathogenicity factors in *Erwinia amylovora* and *Erwinia herbicola* pv. *gypsophylae*. *J. Bacteriol.* 183:1870-1880.
- Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Mor, H., Manulis, S., Zuck, M., Nizan, R., Coplin, D. L., and Barash, I. 2001. Genetic organization of the *hrp* gene cluster and *dspAE/BF* operon in *Erwinia herbicola* pv. *gypsophylae*. *Mol. Plant-Microbe Interact.* 14:431-436.
- Mukherjee, A., Cui, Y., Liu, Y., and Chatterjee, A. K. 1997. Molecular characterization and expression of the *Erwinia carotovora* *hrpN_{Ecc}* gene, which encodes an elicitor of the hypersensitive reaction. *Mol. Plant-Microbe Interact.* 10:462-471.
- Mukherjee, A., Cui, Y., Ma, W.-L., Liu, Y., and Chatterjee, A. K. 2000. *hexA* of *Erwinia carotovora* subsp. *carotovora* strain Ecc71 negatively regulates production of RpoS and *rsmB* RNA, a global regulator of extracellular proteins, plant virulence and the quorum sensing signal, *N*-(3-oxohexanoyl)-L-homoserine lactone. *Env. Microbiol.* 2:203-215.
- 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.
- Murata, H., Chatterjee, A., Liu, Y., and Chatterjee, A. K. 1994. Regulation of the production of extracellular pectinase, cellulase, and protease in the soft rot bacterium *Erwinia carotovora* subsp. *carotovora*: Evidence that *aepH* of *E. carotovora* subsp. *carotovora* 71 activates gene expression in *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica*, and *Escherichia coli*. *Appl. Environ. Microbiol.* 60:3150-3159.
- Oguiza, J. A., and Buck, M. 1997. DNA binding domain mutants of sigma-N(σ^N , σ^{54}) defective between closed and stable open promoter complex formation. *Mol. Microbiol.* 26:655-664.
- Perez-Martin, J., and de Lorenzo, V. 1997. Clues and consequences of DNA bending on transcription. *Annu. Rev. Microbiol.* 51:593-628.
- Perez-Martin, J., Rojo, F., and de Lorenzo, V. 1994. Promoters responsive to DNA bending: A common theme in prokaryotic gene expression. *Microbiol. Rev.* 58:268-290.
- Plano, G. V., Day, J. B., and Ferracci, F. 2001. Type III export: New uses for an old pathway. *Mol. Microbiol.* 40:284-293.
- Rantakari, A., Virtaharju, O., Vähämiko, S., Taira, S., Palva, E. T., Saarilahti, H., and Romantschuk, M. 2001. Type III secretion contributes to the pathogenesis of the soft-rot pathogen *Erwinia carotovora*: Partial characterization of the *hrp* gene cluster. *Mol. Plant-Microbe Interact.* 14:962-968.
- Romeo, T. 1998. Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB. *Mol. Microbiol.* 29:1321-1330.
- 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, U.S.A.
- Selvaraj, G., Fong, Y. C., and Iyer, V. N. 1984. A portable DNA sequence carrying the cohesive site (*cos*) of bacteriophage λ and the *mob* (mobilization) region of the broad-host-range plasmid PK2: A module for the construction of new cosmids. *Gene* 32:235-241.
- Shingler, V. 1996. Signal sensing by σ^{54} -dependent regulators: Derepres-

- sion as a control mechanism. *Mol. Microbiol.* 19:409-416.
- 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 pRL1JI. *Plant. Mol. Biol.* 9:27-39.
- Staskawicz, B. J., Mudgett, M. B., Dangl, J. L., and Galan, J. E. 2001. Common and contrasting themes of plant and animal diseases. *Science* 292:2285-2289.
- Stoker, N. G., Fairweather, N. F., and Spratt, B. G. 1982. Versatile low-copy-number plasmid vector for cloning in *Escherichia coli*. *Gene* 18:335-341.
- Wassem, R., de Souza, E. M., Yates, M. G., de O. Perosa, F., and Buck, M. 2000. Two roles for integration host factor at an enhancer-dependent *nifA* promoter. *Mol. Microbiol.* 35:756-764.
- Wei, Z. M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer A., and Beer, S. V. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* 257:85-88.
- Wei, Z. M., and Beer, S. V. 1995. *hrpL* activates *Erwinia amylovora hrp* gene transcription and is a member of the ECF subfamily of sigma factors. *J. Bacteriol.* 177:6201-6210.
- Wei Z. M., Kim J. F., and Beer, S. V. 2000. Regulation of *hrp* genes and type III protein secretion in *Erwinia amylovora* by HrpX/HrpY, a novel two-component system, and HrpS. *Mol. Plant-Microbe Interact.* 13:1251-1262.
- Willis, D. K., Hrabak, E. M., Rich, J. J., Barta, T. M., Lindow, S. E., and Panopoulos, N. J. 1990. Isolation and characterization of a *Pseudomonas syringae* pv. *syringae* mutant deficient in lesion formation on bean. *Mol. Plant-Microbe Interact.* 3:149-156.
- Wu, Y., and Datta, P. 1992. Integration host factor is required for positive regulation of the *tdc* operon of *Escherichia coli*. *J. Bacteriol.* 174:233-240.
- Xiao Y., Heu, S. G., Jinseong, Y., Lu, Y., and Hutcheson, S. W. 1994. Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *pseudomonas syringae* pv *syringae* pss61 *hrp* and *hrmA* genes. *J. Bacteriol.* 176:1025-1036.
- 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.