PecS and PecT Coregulate the Synthesis of HrpN and Pectate Lyases, Two Virulence Determinants in *Erwinia chrysanthemi* 3937

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Erwinia chrysanthemi strain 3937 is a necrotrophic bacterial plant pathogen. Pectinolytic enzymes and, in particular, pectate lyases play a key role in soft rot symptoms; however, the efficient colonization of plants by *E. chrysanthemi* requires additional factors. These factors include HrpN (harpin), a heat-stable, glycine-rich hydrophilic protein, which is secreted by the type III secretion system. We investigated the expression of *hrpN* in *E. chrysanthemi* 3937 in various environmental conditions and different regulatory backgrounds. Using *lacZ* fusions, *hrpN* expression was markedly influenced by the carbon source, osmolarity, growth phase, and growth substrate. *hrpN* was repressed when pectinolysis started and negatively regulated by the repressors of pectate lyase synthesis, PecS and PecT. Primer extension data and in vitro DNA–protein interaction experiments support a model whereby PecS represses *hrpN* expression by binding to the *hrpN* regulatory region and inhibiting transcript elongation. The results suggest coordinated regulation of HrpN and pectate lyases by PecS and PecT. A putative model of the synthesis of these two virulence factors in *E. chrysanthemi* during pathogenesis is presented.

Additional keyword: hypersensitive response.

The pectinolytic *Erwinia* spp. are soft-rotting, gram-negative bacteria that cause severe disease in a wide range of plant species, including many crops of economical importance. They recently have been classified in the resuscitated genus *Pectobacterium* (Hauben et al. 1998), but the nomenclature has not yet been widely accepted by plant pathologists (Pérombelon 2002). Plant tissue maceration by the soft rot *Erwinia* spp. is due to the disintegration of the plant cell wall by extracellular enzymes, among which pectinases play a predominant role (Barras et al. 1994; Hugouvieux-Cotte-Pattat et al. 1996). Pectinases are secreted by a type II system encoded by the *out* genes (Condemine et al. 1992; Reeves et al. 1993). The pecti-

nases of *E. chrysanthemi* strain 3937 have been investigated by several groups. Five major endo-pectate lyases (Pels) were identified by isoelectrofocusing overlay gels (Bertheau et al. 1984), and the five corresponding *pel* genes (*pelA* to *pelE*) were cloned (Reverchon et al. 1987; Hugouvieux-Cotte-Pattat and Robert-Baudouy 1992). Expression in planta of the *pel* genes was studied mostly in *Saintpaulia ionantha* using *pel::uidA* fusions. *pelD* was shown to be the first and highest expressed *pel* gene, whereas *pelC*, *pelB*, and *pelE* were moderately expressed subsequently to *pelD* (Masclaux et al. 1996). Other factors are important for *E. chrysanthemi* strain 3937 pathogenicity, such as siderophores (Expert 1999), lipopolysaccharides (Schoonejans et al. 1987), exopolysaccharides (Condemine et al. 1999), or proteins involved in resistance against plant defense mechanisms (El Hassouni et al. 1999; Favey et al. 1995; Lopez-Sollania et al. 1998; Reverchon et al. 2002; Santos et al. 2001).

Production of virulence factors, particularly the Pels, is tightly regulated and responds to several environmental conditions and metabolic stimuli, including growth phase, catabolic repression, pectin or plant extract, temperature, osmolarity, pH, and iron (Expert 1999; Hugouvieux-Cotte-Pattat et al. 1996). Several regulators modulating the expression of virulence genes in *E. chrysanthemi* have been previously characterized (Franza et al. 1999; Nasser and Reverchon 2002; Nasser et al. 1992, 1998; Nomura et al. 1998; Praillet et al. 1996; Reverchon et al. 1991, 1994, 1997; Surgey et al. 1996). The induction of *pel* gene expression by pectic compounds is mediated by KdgR (Nasser et al. 1994; Reverchon et al. 1991). The induction by plant extracts and iron starvation is mediated by Pir (Nomura et al. 1998) and Fur (Franza et al. 1999), respectively. ExpR is a quorum-sensing regulator that participates in the modulation of *pel* gene expression in response to *N*-acyl-homoserine lactones (acyl-HSLs) generated by the ExpI synthase (Nasser et al. 1998).

An *hrp* locus also contributes to *Erwinia* pathogenicity (Alfano and Collmer 1997). The *hrp* genes encode proteins that constitute the type III secretion system required for pathogenicity in host plants and elicitation of the defensive hypersensitive response (HR) in nonhost plants. The HR is characterized by rapid, localized cell death at the site of infection. This cell death response is associated with the induction of defense-related genes, which play important roles in containing pathogen propagation (Dangl and Jones 2001; Scheel 1998). A characteristic feature of an HR is a sustained increase of reactive oxygen species (ROS). The harpin protein (HrpN) is one of the proteins translocated by the Hrp secretion system (Alfano

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et al. 1996). Harpin-induced cell death as well as generation of ROS has been reported for several plants (Boccardo et al. 2001; Desikan et al. 1998; Krause and Durner 2004; Wei et al. 1992; Xie and Chen 2000). *E. chrysanthemi* also produces a harpin protein (HrpN_{Ech}), and *hrpN_{Ech}* mutants display reduced virulence (Bauer et al. 1995; Yang et al. 2002). In contrast to the *pel* genes, little is known about the control of *hrpN_{Ech}* gene expression. Recently, the expression of *hrpN_{Ech}* was shown to be dependent on the alternative sigma factor HrpL and is growth-phase regulated (Ham et al. 2004). However, the growth-phase regulation of *hrpN_{Ech}* is independent of the ExpI-ExpR system (Ham et al. 2004). Here, we report the results of investigations of *hrpN_{Ech}* expression in strain *E. chrysanthemi* 3937 during different conditions and stages of growth, including the carbon source, osmolarity, growth-phase, and the nature of the growth medium.

RESULTS

hrpN from strain 3937 is weakly expressed in rich and minimal salts media and is surface induced.

Plasmid pCPN2, containing the *hrpN* gene, was introduced into *Escherichia coli* POI1734 strain for miniMu transposon mutagenesis. Different miniMu insertions were analyzed by restriction mapping. Constructs displaying a transcriptional *hrpN_{Ech}::lacZ* organization were sequenced with a Mu end primer (5'-CCGAATAATCCAATGTCC-3') and the pRV85 insertion was retained. The insertion in pRV85 was located at nucleotide 744 from the *hrpN* start codon. β -Galactosidase synthesis by *E. coli* strain NM522, transformed by the construct pRV85, confirmed that the corresponding MudI1734 insertion resulted in a *lacZ* fusion. The construction was marker exchanged into the *Erwinia chrysanthemi* A350 chromosome (strain A350 is a *lacZ* derivative of the 3937 strain recently sequenced). The generated strain was called A4353 (Table 1).

HrpN is known to be involved in the early stage of infection (Bauer et al. 1994); therefore, the expression of *hrpN_{Ech}::lacZ* was examined for modulation by various environmental conditions similar to those encountered in the intercellular spaces of plant tissues, including liquid minimal salts medium (M63 and M9) supplemented with glycerol or various sugars (glucose, fructose, arabinose, and sucrose), liquid-rich Luria broth (LB) medium, and solid agar plate media. The results led to the following conclusions: i) the expression level was low in liquid minimal salts medium containing glycerol (Fig. 1A), as well as in LB medium (4.2 Miller units for LB versus 4.6 Miller units for M63 plus glycerol); ii) the level of expression was influenced by the carbon source, the highest levels being achieved with sucrose, followed by glucose and fructose (Fig. 1A); and iii) the expression level was increased at least fivefold when bacteria were grown on solid medium, regardless of the medium (Fig. 1B; data not shown for LB and M63 plus glycerol or sucrose media). Based on the results, the minimal salts medium supplemented with glucose was selected for the subsequent experiments on *hrpN_{Ech}* expression.

We further investigated the effect of growth phase and osmolarity of the growth medium on the expression of *hrpN_{Ech}*. Media with low (M63/2) or high (M63 plus 100 to 300 mM KCl or NaCl) osmolarity reduced the *hrpN_{Ech}* expression (Fig. 1C). It is important to note that the level of the *hrpN_{Ech}* expression modulation by osmolarity depends on the nature of the carbon source used. Indeed, we observed only a twofold decrease in the *hrpN_{Ech}* expression between growth in M63 compared with M63 plus 300 mM NaCl in the presence of glycerol as the carbon source (data not shown), instead of a fivefold decrease in the presence of glucose (Fig. 1C) or sucrose (data not shown).

The data revealed that the expression level of *hrpN_{Ech}* also was dependent on growth phase, the maximal expression occurring during late exponential growth (Fig. 2). However as

Table 1. Bacterial strains, plasmids, and phages used in this work

Strain	Relevant characteristics and use ^a	Reference or source
<i>Escherichia coli</i>		
NM522	$\Delta(lac-proAB) thi hsd-5 supE (F' proAB+ lacIq lacZ\Delta M15)$	Stratagene
POI1734	$F^- araD139\Delta lacU169 rpsL thi$ with Mu dI1734	Castilho et al. 1984
<i>Erwinia chrysanthemi</i>		
3937	Wild-type strain isolated from <i>Saintpaulia ionantha</i>	Kotoujansky et al. 1982
A350	<i>lmrT(con) lacZ2</i>	Hugouvieux-Cotte-Pattat and Robert-Baudouy 1985
A1880	<i>lmrT(con) lacZ2 pelC::uidA Km</i>	Hugouvieux-Cotte-Pattat et al. 1992
A1798	<i>lmrT(con) lacZ2 pelD::uidA Km</i>	
A3719	<i>lmrT(con) lacZ2 kdgR::miniTn5-Sm</i>	Laboratory collection
A1524	<i>lmrT(con) lacZ2 pecS::MudIIPR13</i>	Reverchon et al. 1994
A2148	<i>lmrT(con) lacZ2 pecT::uidA Cm</i>	Surgey et al. 1996
A3810	<i>lmrT(con) lacZ2 pir::Cm</i>	Laboratory collection
A2757	<i>lmrT(con) lacZ2 expl::Cm</i>	Nasser et al. 1998
A3009	<i>lmrT(con) lacZ2 expR::Cm</i>	Nasser et al. 1998
A4353	<i>lmrT(con) lacZ2 hrpN::lacZ</i>	This work
A4354	<i>lmrT(con) lacZ2 pecS::MudIIPR13 hrpN::lacZ</i>	This work
A4355	<i>lmrT(con) lacZ2 pecT::uidA Cm hrpN::lacZ</i>	This work
A4356	<i>lmrT(con) lacZ2 kdgR::miniTn5-Sm hrpN::lacZ</i>	This work
A4357	<i>lmrT(con) lacZ2 pir::Cm hrpN::lacZ</i>	This work
A4358	<i>lmrT(con) lacZ2 expR::Cm hrpN::lacZ</i>	This work
A4359	<i>lmrT(con) lacZ2 expl::Cm hrpN::lacZ</i>	This work
Plasmids		
pGEM-T	Cloning vector, Ap ^r <i>lacZ'</i>	Promega
pWN3013	pGEM-T with the 424-bp fragment containing the <i>hrpN</i> regulatory region	This work
pCPN2	pUC18 with a 4.2-kb fragment containing the <i>hrpN</i> gene	Yang et al. 2002
pRV85	pCPN2 with MudI1734 phage generating a <i>lacZ::hrpN</i> fusion	This work
Phages		
PhiEC2	General transducing phage of <i>Erwinia chrysanthemi</i>	Résibois et al. 1982
MudI1734	miniMu <i>lacZ</i> transcription fusion phage (<i>lacZ</i> -Km)	Castilho et al. 1984

^a Genotype symbols are according to Berlyn (1998). *lmrT(con)* indicates that the transport system encoded by the gene *lmrT*, which mediates the entry of lactose, melibiose, and raffinose into the cells, is constitutively expressed. *lacZ'* indicates that the 3' end of this gene is truncated. Km = Kanamycin, Cm = chloramphenicol, Ap = ampicillin, and Sm = streptomycin.

previously reported (Ham et al. 2004), the grow-phase-dependent elevated expression of *hrpN* is independent of the quorum-sensing signal *N*-(3-oxo-hexanoyl)-L-homoserine lactone (OHL) produced by *expI* (data not shown).

hrpN and *pel* genes are differently expressed in planta.

To elucidate whether the regulatory effect of environmental or physiological conditions on *pel* and *hrpN_{Ech}* gene expression observed under laboratory conditions had physiological relevance during plant infection by *E. chrysanthemi*, we analyzed the in planta expression of *hrpN_{Ech}*, *pelD*, and *pelC* genes. We first studied the virulence of an *hrpN_{Ech}* mutant in witloof chicory leaves. After inoculation of the bacterial suspension (10^6 CFU), the progression of infection was followed by a daily measurement of the macerated surface areas. The growth rate of the mutant and wild-type strains was similar for the first 72 h after inoculation (data not shown). We observed in the case of the A350 strain, 24 h after inoculation, a consistent maceration of most of the inoculated leaves, whereas there was a delay in the appearance of symptoms in the *hrpN_{Ech}* mutant.

However, 72 h post inoculation, the macerated surfaces were almost identical for the two strains (Table 2). Thus, we observed in witloof chicory leaves what previously has been observed in *Saintpaulia* plants (Yang et al. 2002), a delay in symptom development with the *hrpN_{Ech}* mutant. We then studied the expression of *hrpN_{Ech}::lacZ* at different times after the infection of witloof chicory leaves. We observed a four- to fivefold-induction of *hrpN_{Ech}* expression in planta within less than 8 h after inoculation, reaching a maximum after 24 h, then decreasing (Fig. 3) and disappearing after 72 h (not shown). In parallel, we followed the expression of *pelC::uidA* and *pelD::uidA* reporters. Expression of these two *pel* genes increased shortly after infection and was maintained at a relatively high level after 48 h (Fig. 3). Consistently, Pel activity, which could not be detected earlier than 24 h after infection, increased over time (Fig. 3). These results show that, during infection, *hrpN_{Ech}* is expressed transiently; the strong expression observed in the first steps of infection matched the previous evidence that the Hrp system was involved in the early stage of infection (Bauer et al. 1994). Moreover, the in planta expression pattern of

Fig. 1. Expression of *Erwinia chrysanthemi hrpN_{Ech}::lacZ* in response **A**, to different carbon sources, **B**, to the media composition, and **C**, to osmolarity during growth in minimal salts M63 medium. Glucose was used as carbon source in B and C, and growth assays in A and C were performed in liquid medium. Bacteria A4353 (*ps* = parental strain; *PecS*⁺, *PecT*⁺), A4354 (*PecS*[−]), and A4355 (*PecT*[−]) were grown overnight (stationary phase) in appropriate media at 30°C, and the cultures were assayed for β -galactosidase activity. Results are expressed in Miller units; the presented values for strains A4353, A4354, and A4355 were subtracted from the residual values obtained in the parental counterparts A350, the *pecS* mutant A1524, and the *pecT* mutant A2148, respectively. Each value represents the mean of three experiments. Bars indicate the standard deviation. Results similar to those presented in B were obtained when rich Luria broth medium was used instead of minimal medium.

hrpN_{Ech} was consistent with the involvement of HrpN in the stimulation of ROS production, which has been shown to occur as early as 3 h after infection of *Saintpaulia* plants by *E. chrysanthemi* (Santos et al. 2001).

PecS and PecT repress *hrpN*.

Pectinolysis in *E. chrysanthemi* requires several regulators (Hugouvieux-Cotte-Pattat et al. 1996; Nasser and Reverchon 2002). Transduction with phage phiEC2 was used to transfer the *hrpN_{Ech}::lacZ* fusion into different genetic backgrounds in order to explore the relationship between *hrpN_{Ech}* expression and pectinolysis. Although a mutation in the *kdgR*, *expR*, or *pir* backgrounds had no effect on *hrpN_{Ech}* transcription (data not shown), *hrpN_{Ech}* expression increased 4- to 14-fold and 2.5- to 5-fold in the *pecS* and *pecT* mutants, respectively, depending on the growth phase (Fig. 2). These results establish that PecS and PecT repress *hrpN_{Ech}* expression. The *hrpN_{Ech}* expression modulations by the stated environmental or physiological conditions are conserved in the *pecS* and *pecT* backgrounds (Figs. 1 and 2); thus, the signals modulating the action of these two regulators on *hrpN_{Ech}* expression are not elucidated.

To test whether transcriptional regulation of *hrpN* is achieved by a direct binding of PecS to the promoter region, in

vitro DNA-protein interactions were performed. We recently succeeded in determining the PecS DNA binding consensus (C₋₁₁G₋₁₀A₋₉N₋₈W₋₇T₋₆C₋₅G₋₄T₋₃A₋₂)T₋₁A₀T₁ (T₂A₃C₄G₅A₆N₇N₈N₉C₁₀G₁₁) (Rouanet et al. 2004). A sequence displaying a significant identity (58%) with the defined consensus was identified in the *hrpN_{Ech}* translation initiation region (Fig. 4). The binding of PecS to the *hrpN_{Ech}* regulatory region (PhrpN) first was measured by band-shift assays. PecS could interact specifically with PhrpN (-323 to +101 versus the translation initiation codon ATG) (Fig. 4A). The apparent dissociation constant (*K_d*) was 25 nM. This finding, therefore, suggests that PecS is capable of directly regulating the expression of the *hrpN_{Ech}* gene. For PecT, we were not able to determine a consensus sequence, because this protein aggregates when over-produced (Castillo et al. 1998). Thus, characterization of the PecT mechanism on *hrpN_{Ech}* was not further analyzed.

To place the PecS binding site at PhrpN into context, it was necessary to attempt to establish the *hrpN_{Ech}* transcription start point. Primer extension analysis with RNA from the end of exponential phase cultures (optical density [OD] = 0.7) of the *E. chrysanthemi* parental strain A350, or its *pecS* and *pecT* derivatives, revealed that *hrpN_{Ech}* transcription was initiated at 91 bases upstream of the translation start (Fig. 4B). In addition to

Fig. 2. Cell-density-dependent expression of an *hrpN_{Ech}::lacZ* fusion in *Erwinia chrysanthemi* A4353, A4354, and A4355. Bacteria were grown at 30°C in liquid minimal salts M63 medium containing glucose, and 1-ml samples were taken every hour. Results are expressed in Miller units; the presented values for A4353, A4354, and A4355 were subtracted from the residual values obtained in the parental counterparts A350, the *pecS* mutant A1524, and the *pecT* mutant A2148, respectively. Each value represents the mean of three experiments. Bars indicate the standard deviation.

the discussed transcription initiation site, a second band 11 bp shorter also was observed. The relevance of this band, which might be the result of incomplete elongation or an alternative transcription initiation site of *hrpN*, has still to be elucidated.

Table 2. Representative disease lesions caused by the *lacZ* *Erwinia chrysanthemi* A350 and its *hrpN* derivative on witloof chicory leaves

Strain	Inf./inoc. ^b	Size of maceration (cm ²) ^a	
		24 h	72 h
A350 (parental strain)	23/25	3.2 ± 0.8	83 ± 25
A350 <i>hrpN::lacZ</i> (A4353)	22/25	1.6 ± 0.4	81 ± 31

^a Values are the product of the length and width of the macerated area.
^b Number of leaves infected/inoculated. Bacterial suspension (2 µl, 5 × 10⁸ CFU/ml) was inoculated, without wounding, and lesions were measured at 24 and 72 h after inoculation.

Consistent with the previous gene fusion studies, a more abundant accumulation of *hrpN_{Ech}* transcripts (nine- and threefold) was observed in the *pecS* and *pecT* backgrounds, respectively, compared with the parental strain. Seven bases further upstream, there were potential HrpL alternative sigma factor (ECF sub-family) consensus elements TCACTC(N₁₆)GGAACC (Frederick et al. 2001; Wei and Beer 1995). This reinforced the feature of a requirement of HrpL for *hrpN_{Ech}* expression (Ham et al. 2004; Yap et al. 2005) as previously reported in *Pantoea stewartii* subsp. *stewartii*, *E. amylovora*, and *E. chrysanthemi* EC16 (Frederick et al. 2001; Ham et al. 2004; Wei and Beer 1995). Accordingly, the *hrpN_{Ech}* transcription start point mapped here is located in an appropriate context.

The regions of the DNA segment that interact with PecS were identified by DNase I footprinting analysis (Fig. 4C and D). At a PecS concentration of 20 nM, only an increased inten-

Fig. 3. Expression of *Erwinia chrysanthemi* A350 *hrpN_{Ech}::lacZ* (A4353), *pelC::uidA* (A1880), and *pelD::uidA* (A1798) in chicory leaves. Bacteria were inoculated into witloof chicory leaves and the inoculated and macerated area was collected, cells were counted, and β-galactosidase, β-glucuronidase, and pectate lyase activities were measured. The β-galactosidase activities presented represent the difference between the values of the *hrpN_{Ech}::lacZ* fusion and the residual activities obtained in the parental strain A350. β-galactosidase activities are expressed in Miller units, β-glucuronidase-specific activity is expressed as nanomoles of *p*-nitrophenol produced per minute per milligram of bacterial dry weight, pectate lyase-specific activity is expressed as nanomoles of unsaturated product liberated per minute per milligram of bacterial dry weight. Each value represents the mean of three experiments. Bars indicate the standard deviation.

sity of certain digested bands (+67, +68, +71, +80, and +123) was observed, compared with the profile of the control digestion performed in the absence of PecS. Increasing the PecS concentration up to 50 nM resulted in the appearance of a strong protected area extending from +80 to +114, relative to the transcription initiation site, and of two regions located apart from the protected area (+81 to +64 and +115 to +128) with a modified intensity in several digested DNA fragments (increased or decreased). Further increasing the PecS concentration up to 250 nM did not significantly modify the digestion pattern compared with that obtained in the presence of 50 nM of PecS. It is important to note that the strongly protected region contains the PecS consensus sequence. Overall, these results indicate that PecS directly represses the *hrpN_{Ech}* gene expression by interacting with the predicted binding site.

DISCUSSION

Coordination of virulence factor synthesis is central to pathogenicity. We found that, in *E. chrysanthemi*, the synthesis of two functions essential for pathogenicity, HrpN_{Ech} and Pels, are regu-

lated in a coordinated manner by the PecS and PecT regulators. Moreover, we showed that expression of *hrpN* is modulated by the medium composition, growth phase, and osmolarity.

The most notable feature of *E. chrysanthemi* 3937 *hrpN_{Ech}* gene regulation by environmental conditions was the high level of expression when bacteria are grown in a minimal salts medium containing sucrose or glucose as the carbon source and when they are in contact with the solid surface. These findings are particularly relevant because these two conditions are presumed to mimic the environment bacteria encounter in plant tissue. Similar regulation of *hrpN* expression previously was observed for various pathogens, including *E. amylovora* (Wei et al. 1992) and *Pseudomonas syringae* (Hutcheson 1997). These two signals (presence of sucrose or glucose and contact with solid surface) could be important in modulating HrpN synthesis during plant infection by *E. chrysanthemi*. The hypothesis of *hrpN* induction by plant cell surface is supported by recent results establishing that cuticle lipid composition modulates type III gene expression in *P. syringae* (Xiao et al. 2004). We also pointed out that *hrpN_{Ech}* expression is reduced by low and high osmolarities. Plant tissues usually display a

Fig. 4. Effect of the *pecS* and *pecT* mutations on the *hrpN_{Ech}* transcripts and analysis of PecS binding on the *hrpN_{Ech}* regulatory region. In vitro DNA-protein interaction experiments were performed with a 465-bp DNA fragment containing the *hrpN* operator/promoter region PhrpN (424-bp, -323 to +101, versus the translation initiation codon ATG). **A**, Band-shift assay. Lane 1, no protein; lanes 2 to 4, DNA with 20, 50, and 250 nM PecS, respectively. The position of free DNA (F) and the PecS-DNA complexes (C) are indicated. **B**, Identification of the transcriptional start site of *Erwinia chrysanthemi* A350 *hrpN_{Ech}* by primer extension experiments. The reactions were performed with 10 µg of RNA extracted from the parental strain A350 (lane 2), its *pecS* derivative A1524 (lane 1), and its *pecT* derivative A2148 (lane 3). DNA sequencing ladders were generated with the same primer (lanes A, C, G, and T). The retained transcriptional start site is indicated by an asterisk. **C**, DNase I footprinting analysis on the top strand of the regulatory region. Lane 1, no protein; lanes 2 to 4, DNA with 20, 50, and 250 nM PecS, respectively. The arrowheads indicate hypersensitivities induced by binding of PecS on PhrpN. The regions protected and modified by PecS are indicated on the right by the thick and dashed lines, respectively. The sequences are numbered with respect to the transcriptional start site of *hrpN_{Ech}*. **D**, Sequence of the *hrpN_{Ech}* regulatory region. The regions protected and modified by PecS are indicated as in C. The start point of transcription is indicated by an arrow, and the sequences showing significant similarity with the PecS DNA-binding consensus (C₋₁G₋₁₀A₋₉N₋₈W₋₇T₋₆C₋₅G₋₄T₋₃A₋₂T₋₁A₀T₁(T₂A₃C₄G₅A₆N₇N₈N₉C₁₀G₁₁)) (Rouanet et al. 2004) are boxed. The potential *hrp* alternative sigma factor HrpL binding elements (Wei and Beer 1995; Frederick et al. 2001), as well as the translation initiation codon ATG, are in bold characters.

relatively high osmolarity (Pérombelon 2002) but water availability in plant varies during infection, essentially because cell lysis in macerated tissues leads to a modification in water distribution between the different cellular compartments (wall, intercellular spaces, cytoplasm, and vacuole) (Pérombelon 2002). Thus, osmolarity might contribute to the modulation of *hrpN_{Ech}* expression during pathogenesis. The high osmolarity encountered in the plant tissues at the beginning of the infection may prevent an early elevated expression of *hrpN_{Ech}* during the state of latency of the bacteria.

Our results partially agree with those recently published by Ham and associates (2004). Unlike strain EC16, strain 3937 poorly expresses *hrpN_{Ech}*. However, *hrpN_{Ech}* from strain 3937 was expressed similarly in LB-rich medium and in glycerol minimal salts media (4.2 Miller units versus 4.6 Miller units, respectively). Moreover, as previously reported in *E. carotovora* (Mukherjee et al. 1997), our investigations on *E. chrysanthemi* 3937 revealed a strong stimulation of the *hrpN_{Ech}* gene expression by sucrose and glucose, whereas *E. chrysanthemi* EC16 exhibited stimulation by sucrose only (Ham et al. 2004). These discrepancies could be explained by differences in experimental conditions or genotypic variation in the *E. chrysanthemi* strains, as previously suggested (Ham et al. 2004).

In synthetic media, the temporal expression of *hrpN_{Ech}* is similar to the expression of genes for various extracellular enzymes, including *pel* (Barras et al. 1994; Hugouvieux-Cotte-Pattat et al. 1992). The relevance of *HrpN_{Ech}* induction by the growth phase is, at present, unclear because previous work has suggested an involvement of *HrpN* in the early stage of infection (Bauer et al. 1994). Moreover, the results obtained in studies performed on synthetic media diverge from those obtained in planta, which showed a strong expression of *hrpN_{Ech}* during the first hours of infection (i.e., at low cellular density). The most probable explanation could reside in the fact that *hrpN_{Ech}* expression in planta is modulated by various signals and that those encountered in the first steps of infection, such as acidic pH (Ham et al. 2004) or contact with a solid surface, were the most relevant. However, it cannot be excluded that a late *hrpN_{Ech}* stimulation takes place in particular conditions not yet determined in planta. By contrast, the occurrence of *Pel* activity in planta globally fits the growth phase regulation observed in synthetic media. Thus, the decrease in *hrpN_{Ech}* expression is correlated with pectate lyase accumulation, indicative of co-regulatory circuits.

In vivo gene fusion data and transcript quantification experiments revealed that *PecS* and *PecT* repress *hrpN_{Ech}* expression.

Fig. 5. Schematic representation of the regulation of the synthesis of various virulence factors in *Erwinia chrysanthemi* at different stages of infection. The lanes with a bar and arrows represent a repression and activation mechanism, respectively; wide, thin, and dotted arrows under the genes indicate, strong, intermediate, and low transcription, respectively; 'a' and 'i' represent active and inactive forms of the regulators, respectively. During the first stages of infection, contact with the plant cell walls or sugar present in the intercellular spaces initiate the production of *HrpN_{Ech}*, whereas synthesis of Pels is strongly repressed by the repressors *PecS*, *KdgR*, and *PecT*. *HrpN_{Ech}*, by initiating plant cell lysis, would facilitate the release of nutrients, but it also induces the production of reactive oxygen species (ROS) by the host plant, which would, in turn, neutralize the *PecS* repressor (Reverchon et al. 2002). Concomitantly, the basal production of Pels allows the initiation of pectin degradation. In the intermediate stages of infection, a full inactivation of *PecS* by the increased amount of ROS, of *KdgR* by pectic inducers, and of *PecT* results in an increased synthesis of Pels, *HrpN*, and indigoidine. This significant production of the virulence factors allows a strong attack on the plant tissues at the inoculation site. In the advanced stages of infection, the ROS neutralization by indigoidine allows reactivation of *PecS*, which, in turn, represses *HrpN_{Ech}*, Pels, and indigoidine; however, the Pels that have been massively produced are sufficient to ensure bacteria propagation in the plant.

This feature reinforces the pleiotropic action of these two regulators on virulence factor production in *E. chrysanthemi* because previous observations have shown that PecS and PecT, in addition to the control of Pel production, also affect various other virulence-associated factors such as motility, production of cellulases, EPS synthesis, or production of indigoidine (Castillo and Reverchon 1997; Condemine et al. 1999; Reverchon et al. 1994, 2002; Surgey et al. 1996). A coregulation of the synthesis of HrpN_{Ech} and Pels by PecT and PecS was not documented in *E. carotovora*, where the link between these two virulence factors mainly is ensured by cellular density via the quorum-sensing signal OHHL, produced by the synthase ExpI, and via the RNA-binding protein RsmA (Mukherjee et al. 1997). The possibility, in *E. chrysanthemi*, of a nonidentified quorum-sensing system controlling Pel synthesis, as well as that of the Hrp system, cannot be disregarded. Furthermore, homologues of PecS and PecT have been found in *E. carotovora*, where they control the synthesis of Pels (Bell et al. 2002; Harris et al. 1998; Matsumoto et al. 2003). Equally, the involvement of these two regulators in the control of HrpN synthesis in *E. carotovora* cannot be excluded. In vitro DNA–protein interaction experiments allowed the identification of the *hrpN_{Ech}* DNA segment that specifically interacts with PecS. The PecS-protected region is between 80 to 114 bp upstream of the transcription initiation site. Binding of PecS on this region would result in the inhibition of the transcript elongation from the *hrpN_{Ech}* promoter.

By integrating these results with the data accumulated over recent years, we are able to propose a model to describe how the synthesis of Pels and HrpN_{Ech} might be regulated in *E. chrysanthemi* during infection. Upon entering the plant, the bacterium faces nutritional starvation with regard to iron (Neema et al. 1993) and other metabolites in the apoplast. HrpN_{Ech} and other factors secreted by the type III system might help in this initiation step. Indeed, HrpN from *P. syringae* has been shown to form ion-conducting pores in lipid bilayers which could facilitate the release of nutrients from the host plant as well as the introduction of bacterial virulence factors in the host cells (Lee et al. 2001). During the first steps of infection, contact with the plant cell walls or sugar present in the intercellular spaces could initiate the stimulation of HrpN_{Ech} production, whereas the *pel* genes mostly are repressed by the strong KdgR repressor, indicated by the relatively low amount of pectin-degradative products present in the plant intercellular spaces. HrpN_{Ech} also induces the plant defense reactions, in particular the production of ROS, which are believed to inactivate the PecS regulator (Reverchon et al. 2002). After a certain time, the basal production of Pels allows the initiation of pectin degradation (during the first 4 h after infection) (Figs. 3 and 5). During the early stages of infection (e.g., between 4 and 8 h after infection) (Figs. 3 and 5), inactivation of PecS by the increased amount of ROS has two consequences: stronger production of HrpN_{Ech} and of the radical scavenger pigment indigoidine. During the intermediate stage of infection (e.g., between 8 and 24 h after infection), a simultaneous inactivation of PecS by ROS, of KdgR by pectic inducers, and probably of PecT by an unknown signal, results in an increased production of HrpN_{Ech}, Pels, and indigoidine. This strong production of the co-regulated virulence factors allows a strong attack on the host plant and the development of soft rot symptoms at the inoculation site. Finally, in the advanced stages of infection (e.g., 48 h after infection) (Figs. 3 and 5), the ROS neutralization by indigoidine results in a reactivation of PecS, which in turn represses HrpN_{Ech}, Pels, and indigoidine synthesis. However, this effect on Pel synthesis is certainly less pronounced because the KdgR and probably

the PecT repressors are no longer active. At this stage of infection, the plant defense reactions are neutralized and the plant cell-wall-degrading enzymes massively produced are sufficient to ensure the plant invasion by the bacteria.

The sequencing of the *E. chrysanthemi* 3937 genome (University of Wisconsin, Madison, WI, U.S.A.) recently has opened new avenues for more accurate studies of the regulatory network in this bacterium and for more precise investigations regarding the relationship between the different virulence factors. Identification of the PecS and PecT regulons using global approaches, such as transcriptome analysis or in vivo expression technology, will provide new insights into the mechanisms used by *Erwinia* spp. to evade host defenses and cause disease.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture conditions, and DNA manipulation techniques.

Bacterial strains and plasmids used in this study are described in Table 1. *E. chrysanthemi* and *Escherichia coli* were grown at 30 and 37°C, respectively. The rich medium used was LB medium; we used M63 or M9 minimal salts medium (Miller 1972) supplemented with a carbon source (polygalacturonate at 0.4% [wt/vol] or glycerol at 0.2% [wt/vol]). To test the effects of carbon sources on *hrpN_{Ech}* expression, bacteria were grown in the minimal salts medium M63 supplemented with sucrose, glucose, fructose, arabinose, or glycerol at 0.4% (wt/vol). Media were solidified by the addition of 1.5% agar. When required, the antibiotics were as follows: ampicillin at 100 µg/ml, kanamycin and chloramphenicol at 50 µg/ml, or streptomycin at 20 µg/ml. DNA manipulations were performed using standard methods (Sambrook et al. 1989).

The plasmid pWN3013 was generated by cloning the *hrpN_{Ech}* regulatory region (424 bp, –323 to +101 relative to the translation initiation codon ATG) in the vector pGEM-T (Stratagene, La Jolla, CA, U.S.A.). The *hrpN_{Ech}* regulatory region was generated using polymerase chain reaction with primer *hrpND* (5'-GATGTGTTGATCCTCTGGTG-3') and primer *hrpNPext2* (5'-GGATGACGTAGCGGAGTTC-3') using genomic DNA from *Erwinia chrysanthemi* A350 as template. The primers *hrpND* and *hrpNPext2* were complementary to –323 to –304 and +83 to +101, respectively (relative to the *hrpN_{Ech}* translation initiation codon ATG).

Genetic techniques.

Mutagenesis with MudI1734 on plasmid pCPN2 carrying the *hrpN* gene was performed as described previously (Boccard et al. 1988). Localization of the site of insertion was determined precisely by sequencing with a Mu end primer (5'-CGAATAATCCAATGTCC-3'). Transduction with phage phiEC2 was performed as described by Résibois and associates (1984).

Protein and enzyme assays.

β-Galactosidase and β-glucuronidase assays were performed on cell extracts treated with toluene. β-Galactosidase activity was measured by monitoring the degradation of *o*-nitrophenyl-β-D-galactoside into *o*-nitrophenol that absorbs at 420 nm, as described by Miller (1972). β-Glucuronidase activity was measured by monitoring the degradation of *p*-nitrophenyl-β-D-glucuronide into *p*-nitrophenol that absorbs at 405 nm (Bardonnet and Blanco 1992). Specific activity for both enzymes is expressed as nanomoles of product liberated per minute per milligram (dry weight) of bacteria. For growth in synthetic medium, bacterial concentration was estimated by measuring turbidity at 600 nm given that an OD of 1.0 at 600 nm corresponds to

10⁹ bacteria/ml and to 0.47 mg of bacteria (dry weight) per milliliter. When grown on solid medium, the bacteria were first resuspended in NaCl 0.9% and an aliquot was used for assays.

To study the activities in planta, 2 × 10⁶ bacteria contained in 5 µl of M63 medium were deposited on a witloof chicory leaf, maintained during the experiment in a moist chamber at 28°C. Pieces of tissue were collected at set times and resuspended in 0.9% NaCl, bacterial populations were counted by plating on LB agar medium supplemented with appropriate antibiotic, and an aliquot was used for enzyme assays as described previously (Masclaux and Expert 1995). Pectate lyase specific activity in rotted tissues was determined by the degradation of polygalacturonate to unsaturated products that absorb at 230 nm (Moran et al. 1968).

PecS was overproduced and purified as described previously (Praillet et al. 1996).

Plant tests.

Witloof chicory leaves from the Mouffetard market (Paris), maintained in a moist atmosphere at 28°C, were inoculated, without wounding, with 2 µl of bacterial suspension (5 × 10⁸ CFU/ml). The surface of the maceration was recorded daily.

RNA isolation and primer extension analysis.

RNA extraction and normalization, as well as primer extension experiments, were performed essentially as previously described (Reverchon et al. 1997). The primer hrpNPext2 used for the specific detection of mRNA was 5' end labeled. The extension products were resolved on a 6% sequencing gel and visualized by autoradiography on Amersham MP film. The length of the transcripts was identified using the corresponding dideoxy sequencing reactions as a reference.

In vitro DNA-protein interaction.

Band-shift assay and DNase I footprinting were performed as described previously (Praillet et al. 1996). The regulatory region of the *hrpN* DNA fragment (PhrpN) was recovered from plasmid pWN3013 by a *Sall*-*SacII* digestion. The 465-bp DNA fragment obtained was further end labeled by filling up the *Sall* extremity in the presence of (α-³²P)dCTP (3000 Ci/mmol, Amersham Biosciences, Piscataway, NJ, U.S.A.) and the Klenow fragment of DNA polymerase. The labeled DNA was purified after electrophoresis on agarose gel using the Qiagen gel extraction kit (Qiagen, Chatsworth, CA, U.S.A.). The signals obtained were detected by autoradiography on Amersham MP film and quantified using ImageMaster TotalLab software (version 2.01; Amersham Biosciences). The apparent K_d was determined as described earlier (Nasser et al. 1994).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

University of Wisconsin-Madison, Department of Animal Health and Biomedical Sciences, Genome Evolution Laboratory *Erwinia chrysanthemi* 3937 genome webpage: asap.ahabs.wisc.edu