Analysis of *Erwinia chrysanthemi* EC16 *pelE::uidA*, *pelL::uidA*, and *hrpN::uidA* Mutants Reveals Strain-Specific Atypical Regulation of the Hrp Type III Secretion System

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The plant pathogen *Erwinia chrysanthemi* produces a variety of factors that have been implicated in its ability to cause soft-rot diseases in various hosts. These include HrpN, a harpin secreted by the Hrp type III secretion system; PelE, one of several major pectate lyase isozymes secreted by the type II system; and PelL, one of several secondary Pels secreted by the type II system. We investigated these factors in *E. chrysanthemi* EC16 with respect to the effects of medium composition and growth phase on gene expression (as determined with *uidA* fusions and Northern analyses) and effects on virulence. *pelE* was induced by polygalacturonic acid, but *pelL* was not, and *hrpN* was expressed unexpectedly in nutrient-rich King's medium B and in minimal salts medium at neutral pH. In contrast, the effect of medium composition on *hrp* expression in *E. chrysanthemi* CUCPB1237 and 3937 was like that of many other phytopathogenic bacteria in being repressed in complex media and induced in acidic pH minimal medium. Northern blot analysis of *hrpN* and *hrpL* expression by the wild-type and *hrpL::ΩCm^r* and *hrpS::ΩCm^r* mutants revealed that *hrpN* expression was dependent on the HrpL alternative sigma factor, whose expression, in turn, was dependent on the HrpS putative σ^{54} enhancer binding protein. The expression of *pelE* and *hrpN* increased strongly in late logarithmic growth phase. To test the possible role of quorum sensing in this expression pattern, the *expI/expR* locus was cloned in *Escherichia coli* on the basis of its ability to direct production of acyl-homoserine lactone and then used to construct *expI* mutations in *pelE::uidA*, *pelL::uidA*, and *hrpN::uidA* *Erwinia chrysanthemi* strains. Mutation of *expI* had no apparent effect on the growth-phase-dependent expression of *hrpN* and *pelE*, or on the virulence of *E. chrysanthemi* in witloof chicory leaves. Overexpression of *hrpN* in *E. chrysanthemi*

resulted in approximately 50% reduction of lesion size on chicory leaves without an effect on infection initiation.

Erwinia chrysanthemi is a gram-negative phytopathogenic bacterium that has a broad host range and secretes an arsenal of plant cell wall-degrading enzymes (Barras et al. 1994). Most of these extracellular enzymes travel through the type II secretion pathway, which is encoded by the *out* genes (He et al. 1991; Reeves et al. 1993). Pectate lyases (Pels) are the most important virulence factors secreted by the Out pathway (Condemine et al. 1992; He et al. 1991; Salmond 1994). Pels degrade pectic polymers in primary cell walls and middle lamellae in many plants (Barras et al. 1994). The genes encoding the major Pel isozymes have been cloned and mutagenized, but none is essential for pathogenicity (Beaulieu et al. 1993; Ried and Collmer 1988). Among the four major Pels produced by *E. chrysanthemi* EC16, PelE is most important in causing soft rot of potato tuber (Barras et al. 1987; Keen and Tamaki 1986; Payne et al. 1987). In addition, a second set of Pels was found in infected plants or media containing plant extracts that had been inoculated with a mutant deficient in the major Pels (Beaulieu et al. 1993; Kelemu and Collmer 1993). A gene encoding one of these secondary Pels, *pelL*, was cloned from derivatives of *E. chrysanthemi* EC16 and 3937 (Alfano et al. 1995; Lojkowska et al. 1995). In strain 3937, *pelL* contributes demonstrably to virulence and is induced by either plant extracts or polygalacturonic acid (PGA) (Lojkowska et al. 1995). The *E. chrysanthemi* EC16 *pelL* is expressed in infected witloof chicory leaves (López-Solanilla et al. 2001). Additional *pel* genes (*pelZ* and *pelI*) were cloned from strain 3937 by screening PGA-inducible mutants and a genomic library of a known Pel-deficient mutant, respectively (Pissavin et al. 1996; Shevchik et al. 1997).

E. chrysanthemi also produces the *hrpN*-encoded harpin, a protein secreted by the Hrp type III-secretion system, which is capable of eliciting the hypersensitive response (HR) and, like many other gram-negative phytopathogenic bacteria, *E. chrysanthemi* can elicit the HR in tobacco leaves (observable with pectic enzyme-deficient mutants) (Bauer et al. 1994, 1995). The Hrp system is essential for HR elicitation in nonhost plants and pathogenicity in host plants by biotrophic gram-negative phytopathogenic bacteria (Alfano and Collmer 1997). *E. chrysanthemi* EC16 *hrpT* and *hrpN* mutants have reduced

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infectivity in witloof chicory leaves at low inoculum levels, suggesting that the Hrp system is involved in an early stage of pathogenesis (Bauer et al. 1994, 1995), and *E. chrysanthemi* 3937 *hrpG* and *hrcC* mutants are similarly impaired in lesion formation and growth in African violet leaves (Yang et al. 2002).

In biotrophic pathogens, such as *Pseudomonas syringae*, *Xanthomonas campestris*, *E. amylovora*, *Pantoea stewartii* subsp. *stewartii*, and *Ralstonia solanacearum*, *hrp* gene expression is controlled by diverse regulatory systems; however, in general, *hrp* genes are induced in acidic minimal media that mimic acidic apoplastic conditions and repressed in complex media (Alfano and Collmer 1997). In contrast, pectic enzyme production in the necrotrophic, soft-rot *Erwinia* spp. is induced by pectic substrates and plant extracts and is not repressed by complex media (Hugouvieux-Cotte-Pattat et al. 1996). Bacterial growth phase or culture density has a particularly large effect on pectic enzyme production in the soft-rot *Erwinia* spp. In *E. carotovora*, the production of an acyl-homoserine lactone (acyl-HSL) diffusible signal molecule (autoinducer) is essential for the production of the high levels of pectic enzymes that accompany the transition to stationary phase and also for bacterial virulence in susceptible plants (Jones et al. 1993; Pirhonen et al. 1993). The genes for autoinducer biosynthesis, *expI*, and an autoinducer-dependent transcriptional regulator, *expR*, have homologs in many bacteria with cell density or "quorum"-sensing systems (Fuqua et al. 1996; Pirhonen et al. 1993). Previous studies of *E. chrysanthemi* also suggested the operation of a quorum-sensing mechanism for the production of Pels (Hugouvieux-Cotte-Pattat et al. 1996). For example, overall Pel activity increases rapidly when cells reach early stationary phase, and expression of many *pel* genes is growth-phase dependent (Collmer et al. 1982; Hugouvieux-Cotte-Pattat et al. 1992; Lojkowska et al. 1995; Pissavin et al. 1996). However, mutations in *expI* and *expR* have little effect on pectic enzyme production or virulence in *E. chrysanthemi* 3937 (Nasser et al. 1998).

Studies of the molecular basis for *E. chrysanthemi* plant pathogenicity have focused on strains 3937 and EC16. Most work on pectic enzyme regulation and the secondary Pels has been done with strain 3937, which originally was isolated from African violet (*Saintpaulia ionantha*) (Hugouvieux-Cotte-Pattat et al. 1996). Regulation of the major Pels in strain EC16, which originally was isolated from chrysanthemum (*Chrysanthemum morifolium*), appears to be similar, as indicated by the failure of mutants deficient in enzymes that catabolize oligogalacturonates to be induced by PGA (Chatterjee et al. 1985) and by the higher expression of *pelE* promoter-Tn7-*lux* fusions in the presence of PGA and in late-growth-phase cultures (Gold et al. 1992). The structure and function of the major Pel isozymes has been studied in both strains, and EC16 differs from 3937 in producing only four major Pels because of a spontaneous deletion affecting one of the alkaline Pels (Tamaki et al. 1988). In contrast to research with pectic enzymes, work on the Hrp system has been done largely with strain EC16. Other differences in these strains have not been explored, although they might be expected because they belong to quite divergent subgroups within the species *E. chrysanthemi* (Dickey 1979; Nassar et al. 1994, 1996).

In this study, three representative *E. chrysanthemi* EC16 virulence factors, HrpN, PelL, and PelE, were investigated in terms of regulation by media composition and growth phase. PelL and PelE were chosen for this analysis because they have the highest pIs of the secondary and major Pels, respectively; alkaline Pel isozymes are most effective at macerating plant tissues, and the well-studied *pelE* provides a reference gene for comparative expression studies (Barras et al. 1987). The

unexpected expression of *hrpN* in rich media prompted us to investigate the role of the HrpL and HrpS regulatory proteins in *hrp* expression in *E. chrysanthemi*; and the observation that both *hrpN* and *pelE* were expressed most strongly in late-growth-phase cultures prompted us to clone, mutate, and test the regulatory role of the *expI* homologs in strain EC16.

RESULTS

Expression of the *E. chrysanthemi* AC4150 *hrpN*, *pelL*, and *pelE* genes during growth in media containing minimal salts or complex nutrients and polygalacturonate.

Investigation of the regulation of the three representative virulence determinants, PelE, PelL, and HrpN in *E. chrysanthemi*

Fig. 1. Expression of *Erwinia chrysanthemi* EC16 *hrpN::Tn5gusA1*, *pelL::uidA*, and *pelE::uidA* in response to polygalacturonate and of *hrpN::Tn5gusA1* and *hrpA::uidA* during growth in acidic pH (standard Hrp-inducing) minimal medium. **A**, Expression of *hrpN*, *pelL* and *pelE* in complex King's medium B (KB) with or without polygalacturonate (PGA) was determined by measuring the level of β -glucuronidase (GUS) activity in strains carrying the appropriate *uidA* transcriptional fusions. Bacterial cultures were started from overnight KB plates at an optical density at 600 nm (OD_{600}) of 0.1. GUS activity was determined at early stationary phase ($OD_{600} = 1.2$). **B**, Expression levels of *hrpN* and *hrpA uidA* fusions were measured at $OD_{600} = 1.2$ in KB and at $OD_{600} = 0.7$ in acidic pH Hrp-inducing medium (IM). The GUS specific activity (U) is pmol 4-methylumbelliferone produced per milliliter of culture per minute per OD_{600} . Values represent the mean and standard error from three replications.

themi AC4150 (an *Nal^r* derivative of EC16), was initiated by looking at two major factors expected to affect their production: the presence of PGA, which induces many *pel* genes in *E. chrysanthemi*, and nutrient-rich media, which represses *hrp* genes in diverse phytopathogens. The presence of PGA in King's medium B (KB) had little effect on the expression of *hrpN* and *pelL*, but it induced approximately 25-fold higher expression of *pelE* (Fig. 1A). The relatively strong expression of *hrpN* in nutrient-rich KB was unexpected and was investigated further by growing CUCPB5049 (*hrpN::Tn5gusA1*) in KB and an acidic pH minimal medium that is used to induce *hrp* expression in many phytopathogenic bacteria (Fig. 1B). Surprisingly, *hrpN* expression was higher in KB. To determine if genes encoding the Hrp secretion machinery were regulated like *hrpN*, we made a *uidA* transcriptional fusion to the *hrpA* operon and tested its expression in the two media. At least one conserved type III secretion system component, HrcJ, is encoded by the *hrpA* operon, and *hrpA* is predicted to encode the *E. chrysanthemi* Hrp pilus subunit (Rojas et al., in press). The expression of *hrpA*, like that of *hrpN*, was not repressed in rich media (Fig. 1B). However, the transcription level of *hrpA* was approximately 10-fold higher than that of *hrpN* in both culture conditions.

Expression of *hrpN* and *hrpL* in nutrient-rich media by *E. chrysanthemi* AC4150 but not by *E. carotovora* subsp. *carotovora* Ecc71.

To confirm the expression of *hrpN* in rich media and determine if *hrpL* was similarly expressed, we performed Northern blot analysis of wild-type *E. chrysanthemi* AC4150 cultures growing in KB and minimal medium, using *hrpN* and *hrpL* probes (Fig. 2). Because the *E. amylovora* and *P. stewartii* subsp. *stewartii* HrpL alternative sigma factors (ECF subfamily) activate operons preceded by Hrp box promoters (Frederick et al. 2001; Wei and Beer 1995), and the *E. chrysanthemi* EC16 *hrpN* gene is preceded by an Hrp box (Kim et al. 1998), we anticipated that *hrpN* activation would depend on *hrpL* and that these two genes would be similarly expressed in different culture conditions. Both *hrpN* and *hrpL* mRNA accumulated strongly in *E. chrysanthemi* during late log-phase growth in KB and neutral pH minimal salts medium supplemented with sucrose (Fig. 2). In contrast, *hrpN* and *hrpL* in *E. carotovora*

were expressed only in the minimal medium. These results corroborate those obtained for *hrpN* expression using *uidA* fusions, and they highlight the difference in the expression of *hrpL* in *E. chrysanthemi* and *E. carotovora*.

Expression of *E. chrysanthemi* AC4150 *hrpL* and *hrpN* in minimal salts medium is favored by neutral pH and sucrose as a carbon source.

We further explored the expression of *hrpL* and *hrpN* in different media. Here, it is important to note that the minimal medium used in the Figure 1B experiment had an acidic pH of 5.7, which has been shown to enhance *hrp* gene expression in other phytopathogens in the family *Enterobacteriaceae* and *P. syringae* (Huynh et al. 1989; Merighi et al. 2003; Wei et al. 1992). However, the minimal salts medium used in the Figure 2 experiment had a neutral pH, and it appeared to support strong expression of *hrpN* and *hrpL*. Therefore, in this experiment, we directly compared the expression of *E. chrysanthemi* *hrpL* and *hrpN* in minimal media at neutral and acidic pH and at neutral pH with different carbon sources (Fig. 3). We found that expression of both genes was strongest at neutral pH with sucrose as a carbon source. Acidic pH or the alternative carbon sources mannose, fructose, and glucose supported only weak expression.

Expression of *hrpN* is dependent on HrpL and HrpS.

To test the role of HrpL in *hrpN* regulation, we identified the *E. chrysanthemi* *hrpL* homolog by DNA sequence analysis of subclones of pCPP2156, which carries the complete EC16 *hrp* gene cluster (Ham et al. 1998). As in *E. amylovora*, the *E. chrysanthemi* *hrpL* is adjacent to *hrpV* (Rojas et al., in press). The *E. amylovora* and *E. chrysanthemi* HrpL proteins have

Fig. 2. Northern blot analysis of *hrpN* and *hrpL* transcript levels in *Erwinia chrysanthemi* AC4150 during growth in complex and neutral pH minimal media and of *hrpN* and *hrpL* transcripts in *E. carotovora* subsp. *carotovora* grown in the same media. Bacterial cultures were started at a Klett value of approximately 20 and grown in complex King's medium B (KB) or minimal-salts medium with sucrose (MM) and harvested at intervals for total RNA isolation. ³²P-labeled *hrpN*_{Ech}, *hrpL*_{Ech}, *hrpN*_{Ecc}, or *hrpL*_{Ecc} was used for Northern hybridization. The Klett values indicate culture turbidity at the time of sampling. The arrow shows the levels of total RNA as revealed by ethidium bromide staining of denatured agarose gel.

Fig. 3. Northern blot analysis of *hrpL* and *hrpN* transcript levels in *Erwinia chrysanthemi* AC4150 grown in neutral pH minimal-salts or acidic pH (standard Hrp-inducing) minimal-salts media supplemented with various carbon sources. Minimal-salts medium (MM), pH 7.5, and Hrp inducing medium (IM), pH 5.7, were supplemented with mannitol (Man), fructose (Fru), glucose (Glc), or sucrose (Suc), as indicated. The carbon sources were added to MM at a final concentration of 0.5% (wt/vol) and to IM at 20 mM. Klett values at the time of sampling are shown. The arrow shows the levels of total RNA as revealed by ethidium bromide staining of denatured agarose gel.

182 and 184 amino acids, respectively, and are 56% identical in amino acid sequence (BLASTP *E* value $2e-46$). Both *hrpL* genes are preceded by consensus sequences for σ^{54} -dependent promoters. To determine whether *E. chrysanthemi* *hrpN* expression was HrpL-dependent, we disrupted *hrpL* in *hrpN::uidA* strain CUCPB5049 by marker exchange of an allele containing an Ω Cm^r insertion to produce mutant CUCPB5117. Expression of *hrpN::Tn5gusA1*, as indicated by β -glucuronidase activity, was ninefold lower in CUCPB5117 than in CUCPB5049 during growth in both KB and neutral pH minimal salts sucrose medium (data not shown). Expression of *hrpL* in *E. amylovora* and *P. stewartii* subsp. *stewartii* is activated by HrpS, which is a σ^{54} enhancer binding protein of the NtrC family (Frederick et al. 1993; Merighi et al. 2003; Wei et al. 2000). To determine if *hrpL* and *hrpN* expression are dependent upon HrpS in *E. chrysanthemi* AC4150, we disrupted *hrpS* by marker exchange of an allele containing an Ω Cm^r insertion and produced mutant CUCPB5360. Both *hrpN* and *hrpL* expression were found to be dependent on HrpS (Fig. 4).

E. chrysanthemi CUCPB1237 and 3937 differ from AC4150 in expressing *hrpN* most strongly in acidic minimal medium.

To determine if the atypical regulation of *hrpN* that we observed with EC16 and its derivatives was characteristic of the species or strain-specific, we tested *E. chrysanthemi* strains CUCPB1237 and 3937, which represent two subdivisions in *E. chrysanthemi* that are distinct from the EC16 subdivision (Dickey 1979; Nassar et al. 1994), but whose pectic enzyme regulation appears to be similar to that of EC16 (Collmer et al. 1982; Robert-Baudouy et al. 2000). A direct comparison of *hrpL* and *hrpN* expression in AC4150 grown in KB, neutral pH minimal medium with sucrose, and acidic pH minimal medium confirmed the unusual regulation of the *hrp* genes in AC4150 (Fig. 5). Furthermore, prolonging the period of growth before or after shifting cultures to acidic pH minimal medium did not result in high levels of *hrpN* expression. In contrast, *E. chrysanthemi* strains CUCPB1237 and 3937 showed virtually no expression of *hrpL* or *hrpN* in KB and, instead, expressed these genes most strongly in standard acidic pH Hrp-inducing medium. Thus, the atypical regulation of *hrpN* appears to be unique to AC4150 and other derivatives of EC16.

Fig. 4. Northern blot analysis of *hrpN* and *hrpL* transcript levels in *Erwinia chrysanthemi* wild-type AC4150 and *hrpS* mutant CUCPB5360 grown in King's medium B or neutral pH minimal salts medium with sucrose. Bacteria were grown to the indicated Klett culture turbidity levels before sampling. The arrow shows the levels of total RNA as revealed by ethidium bromide staining of denatured agarose gel.

Increased expression of *hrpN* reduces the virulence of *E. chrysanthemi* AC4150 in chicory leaves.

The atypical regulation of *hrpN* in AC4150 suggested the possibility that Hrp regulation may not be coupled to pathogenesis, and altering levels of *hrpN* expression would have little effect on virulence. To test this notion, pCPP2172, in which *hrpN* is expressed from the *lac* promoter of vector pBluescript IKS(−) (Bauer et al. 1995), was electroporated into AC4150. Overproduction of HrpN by the resultant *E. chrysanthemi* AC4150(pCPP2172) was confirmed by Western blotting with anti-HrpN antibodies (data not shown). Interestingly, overexpression of *hrpN* resulted in approximately 50% reduction in lesion size on chicory leaves but did not have an effect on initiation of infection (Table 1).

Cloning and characterization of the *E. chrysanthemi* AC4150 *expl* and *expR* genes and mutagenesis of *expl*.

In the course of the previous experiments, we observed that *hrpN* and *pelE* were expressed most strongly at the end of logarithmic-phase growth (e.g., Fig. 2), which raises the possibility that they may be subject to quorum-sensing regulation by acyl-HSL. Therefore, we cloned and mutated the EC16 *expl* gene. The cloning was done with a cosmid library of *E. chrysanthemi* EC16 genomic DNA in pLAFR5, which was screened for production of acyl-HSL with the indicator strain *Escherichia coli* VJS533(pHV200I) (Chatterjee et al. 1995), as detailed below. A BLAST search revealed that the amino acid sequence of an open reading frame (ORF) directing acyl-HSL production was 95% identical with EchI (Swiss Prot accession number: Q46968) from *Erwinia chrysanthemi* strain NCPPB 1066 and 91% identical with ExpI (Nasser et al. 1998) from *E.*

Fig. 5. Northern blot analysis of *hrpL* and *hrpN* transcript levels in *Erwinia chrysanthemi* strains AC4150, CUCPB1237, and 3937 grown in King's medium B (KB), neutral pH minimal-salts with sucrose medium (MM), and acidic pH (standard Hrp-inducing) minimal medium (IM). The samples loaded in lanes 3, 7, and 10 were grown in KB to a Klett value of 100 and then transferred to IM medium and incubated for an additional 3 h. In lane 4, the culture was incubated in IM for 5 h before sampling; in lane 5, the culture was grown to a Klett value of 300 in KB and then incubated for 5 h in IM before sampling. Klett values at the time of sampling are shown. The arrow shows the levels of total RNA as revealed by ethidium bromide staining of denatured agarose gel.

chrysanthemi strain 3937. Following the precedence for the model strain 3937, we designated the EC16 homolog as *expI*. Further sequencing of the *expI* region by primer walking revealed another ORF which was 92% identical with EchR (Swiss Prot accession number Q46967) from *E. chrysanthemi* strain NCPPB 1066 and 91% identical with ExpR (Nasser et al. 1998) from *E. chrysanthemi* strain 3937. Because of its location neighboring *expI* and sequence identity with homologs, we designated this gene as *expR*. The *expI* and *expR* genes are convergently transcribed, showing a 34-bp overlap at their 3' ends, which is similar to *esaI* and *esaR* in *P. stewartii* subsp. *stewartii* (data not shown) (Beck von Bodman and Farrand 1995).

pCPP2351, which contains an 8.8-kb fragment carrying mini-Tn5Cm in *expI* (subcloned from pCPP2350), was used for marker-exchange mutagenesis of *expI* in *E. chrysanthemi* AC4150 and produced the following *expI* derivatives: CUCPB5084 (otherwise wild type), CUCPB5085 (*hrpN::gusA1*), CUCPB5086 (*pelE::uidA*), and CUCPB5087 (*pelL::uidA*). All of these *expI* derivatives lost production of acyl-HSL detectable by *Agrobacterium tumefaciens* A136(pCF218)(pCF372) (Fig. 6) (CUCPB5084 data not shown).

Effects of *expI* mutation on the virulence of *E. chrysanthemi* and the growth-phase-dependent expression of *hrpN*, *pelL*, and *pelE*.

The effect on virulence of the *expI* mutation in CUCPB5084 was tested in chicory leaves in comparison with the parental wild-type strain AC4150 (Table 2). The *expI* mutant showed the same level of virulence as AC4150, based on either frequency of lesion initiation or extent of lesion development (Table 2). We also investigated the expression of *hrpN*, *pelL*, and *pelE* during the course of bacterial growth in KB containing 0.5% PGA, a medium supporting the expression of all three genes, to see if their expression is dependent on growth phase and *expI*. In AC4150, the expression of *hrpN* and *pelE*

increased rapidly in early stationary phase but that of *pelL* did not (Fig. 7). The *expI* mutation neither abolished the growth-phase-dependent expression of *hrpN* and *pelE*, nor changed the expression level of any of the three genes tested, indicating that the acyl-HSL produced by *expI* is not involved in the regulation of these potential virulence factors under the conditions tested (Fig. 7).

DISCUSSION

The virulence of bacterial phytopathogens like *E. chrysanthemi* is based on multiple factors. Some, like those encoded by the *cbs*, *sap*, *msr*, *suf*, *sodA*, and *ind* genes, appear to aid in tolerating nutritional stresses and innate immune defenses in plants (El Hassouni et al. 1999; Lopez-Solanilla et al. 1998; López-Solanilla et al. 2001; Masclaux and Expert 1995; Nachin et al. 2001; Reverchon et al. 2002; Santos et al. 2001). Others, like HecA, have a role in adhesion to plant surfaces (Rojas et al. 2002). However, some virulence factors are likely to have a more aggressive role in attacking the plant cell wall and possibly redirecting plant metabolism. PelE, PelL, and HrpN are in the latter category. We analyzed the expression of these genes in response to four factors: presence of PGA, medium composition, culture growth phase, and acyl-HSL deficiency. This enabled us to identify aspects of virulence factor regulation in *E. chrysanthemi* EC16 that are different from that in the model strain 3937 and in other phytopathogenic bacteria. The discussion of our findings will focus on the failure of *pelL* to be induced by PGA in culture, the *expI*-independent expression of *hrpN* and *pelE* in late logarithmic growth, and the strain-specific atypical regulation of the Hrp system in EC16.

The expression of *pelL* in culture was very high compared with that of *pelE* or *hrpN*, which is consistent with the strong expression of *pelL* by AC4150 derivative CUCPB5081 in infected chicory (López-Solanilla et al. 2001). In chicory leaves, AC4150 derivatives are reduced 2.5-fold in *pelL* expression when the major *pel* genes are deleted (López-Solanilla et al. 2001), which suggests that fragments released from plant cell walls may induce further *pelL* expression. However, we found that the expression of *pelL* (in striking contrast to *pelE*) was not increased by the presence of PGA in either KB (Fig. 1) or neutral pH minimal medium (data not shown).

The *pelL* in strain 3937 is regulated differently than the *pelL* in AC4150, in that the former is induced by PGA in culture (Lojkowska et al. 1995). One factor that may account for this difference is the presence of P2 phage gene homologs (*Y*, *K*, and *lysB*) 5' of the *pelL* ORF in both AC4150 and 3937 and sequence diversity in the near upstream region of *pelL* (data not shown). Notably, the putative promoter and regulatory sites are different in the two strains. The *pelL* upstream region of EC16 shares with 3937 only one inverted repeat that could be a candidate operator, whereas 3937 has another inverted repeat and one direct tandem repeat (Lojkowska et al. 1995).

Table 1. Effect of HrpN overexpression in *Erwinia chrysanthemi* EC16 derivatives on virulence in chicory leaves

Strain	No. of lesions per 21 inoculations ^a	Size of lesions (mm ²)
AC4150	5	191.6 ± 66.3
AC4150 (pCPP2172) HrpN ⁺⁺	4	117.3 ± 14.3 ^b

^a Bacterial cells were applied (5×10^5 per inoculation) and lesions were measured at 48 h after inoculation.

^b Different from AC4150 (t test, $P < 0.01$).

Fig. 6. Impaired ability of *expI* mutant derivatives of *Erwinia chrysanthemi* AC4150 to stimulate expression of *lacZ* from a *traI::lacZ* transcriptional fusion in *Agrobacterium tumefaciens* A136(pCF218)(pCF372). The genotypes of *E. chrysanthemi* strains are: CUCPB5049 (*hrpN::Tn5gusA1*), CUCPB5085 (*hrpN::Tn5gusA1*, *expI*), CUCPB5065 (*pelE::uidA-nptII*), CUCPB5086 (*pelE::uidA-nptII*, *expI*), CUCPB5081 (*pelL::uidA-nptII*), and CUCPB5087 (*pelL::uidA-nptII*, *expI*). Cell-free supernatant (1 μ l) from each *E. chrysanthemi* overnight culture was dropped on a lawn of *A. tumefaciens* A136(pCF218)(pCF372), which had been grown for 12 h at 37°C on an LM plate amended with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, and then photographed after 12 h of further incubation.

Table 2. Effect of acyl-homoserine lactone production on the ability of *Erwinia chrysanthemi* EC16 derivatives to produce lesions in chicory leaves

Strain (relevant phenotype)	No. of lesions per 20 inoculations ^a	Size of lesions (mm ²) ^b
AC4150 (wild type)	13	120.9 ± 69.1
CUCPB5084 (Exp ⁻)	15	126.0 ± 61.1

^a Each chicory leaf was inoculated with 5.0×10^5 CFU; lesions were indicated by browning and maceration around the site of inoculation measured 48 h after inoculation.

^b Values are the product of the length and width of the macerated, necrotic area.

pelL of 3937 was reported to be expressed at a higher level in either a *pecS* (Lojkowska et al. 1995) or a *pecT* mutant background (Surgey et al. 1996). Both PecS and PecT are known as negative regulators of *pel* genes (Praillet et al. 1996; Reverchon et al. 1993; Surgey et al. 1996). High and less regulated expression of the *pelL* of EC16 might result from the loss of a binding site for one of these negative regulators.

The growth-phase-dependent elevated expression of the *hrpN* and *pelE* genes that we observed is consistent with previous reports involving the *E. carotovora* subsp. *carotovora* *hrpN* and *E. chrysanthemi* major *pel* genes, including the strain EC16 *pelE* gene (Chatterjee et al. 1985; Collmer and Bateman 1982; Gold et al. 1992; Hugouvieux-Cotte-Pattat et al. 1986; Ji et al. 1987; Mukherjee et al. 1997). The growth-phase-dependent expression of pectic enzymes also has been observed in *E. carotovora*, and in strains SCRI193 and SCC3193 it is dependent on *expI* homologs (Jones et al. 1993; Pirhonen et al. 1993). However, in *E. chrysanthemi* 3937, mutation of *expI* has no effect on virulence or production of alkaline Pels (Nasser et al. 1998). Our data indicate that the lack of a virulence phenotype for *expI* mutations is not unique to strain 3937 and appears to be characteristic of the species *E. chrysanthemi*.

Among alternative explanations for the *expI*-independent increase in the expression of *pelE* and *hrpN* in late log-phase is the action of an alternative quorum-sensing autoinducer, known as AI-2. This second autoinducer was discovered in *Vibrio harveyi* and is the product of LuxS action on S-ribosyl-

homocysteine (Schauder et al. 2001). *V. harveyi* produces light in response to either AI-1 (acyl-HSL) or AI-2, but *luxN* mutant BB170 responds only to AI-2. BB170 has been exploited to develop an assay for AI-2 production by test bacteria (Bassler et al. 1997). *Escherichia coli*, *Salmonella typhimurium*, *V. cholerae*, and *Enterococcus faecalis* have been shown by this assay to produce AI-2 (Schauder et al. 2001). *Erwinia chrysanthemi* AC4150 (wild type) and CUCPB5084 (*expI*::mini-Tn5Cm derivative of AC4150) also were found by this assay to produce AI-2 during log-phase growth in KB (B. L. Bassler, unpublished). Identification and mutagenesis of any *luxS* homolog in *E. chrysanthemi* will be required to determine if AI-2 has a role in the regulation of *hrpN*, *pelE*, and virulence in *E. chrysanthemi*.

The most notable feature of *E. chrysanthemi* AC4150 *hrpN* gene regulation was the high level of expression in nutrient-rich KB and in neutral pH minimal salts medium supplemented with sucrose rather than in an acidic pH minimal medium like that used to maximize *hrp* gene expression in most other phytopathogens (Hutcheson 1997; Huynh et al. 1989; Wei et al. 1992). *hrpA*, another component of the Hrp system, showed the same expression pattern as *hrpN*. Sucrose previously had been noted to promote the expression of *hrp* genes in *P. syringae* and *X. campestris* pv. *vesicatoria* (Huynh et al. 1989; Schulte and Bonas 1992).

Expression of *hrpN* in *E. chrysanthemi* was dependent on HrpL, as it is in *P. stewartii* subsp. *stewartii*, *E. amylovora*, and *E. carotovora* subsp. *carotovora* (Chatterjee et al. 2002;

Fig. 7. Kinetics of *hrpN*, *pelL*, and *pelE* expression in wild-type or *expI* *Erwinia chrysanthemi* AC4150 over the time course of culture growth. Filled and open circles correspond to bacterial growth and β -glucuronidase (GUS) activity, respectively. Each *E. chrysanthemi* strain was grown in King's medium B supplemented with 0.5% polygalacturonic acid at 25°C. GUS specific activity (U) indicates pmol 4-methylumbelliferone produced per milliliter of culture per minute per optical density at 600 nm (OD_{600}). Values represent the mean and standard error from three replications.

Frederick et al. 2001; Wei and Beer 1995), which suggests that factors acting above HrpL in the regulatory hierarchy determine the levels of Hrp expression. For example, HrpS, HrpX, and HrpY homologs are known to act in this role in *E. amylovora*, *E. carotovora* subsp. *carotovora*, and *P. stewartii* subsp. *stewartii* (Chatterjee et al. 2002; Merighi et al. 2003; Wei et al. 2000). This model was supported by Northern blot data showing that *hrpL* mRNA accumulates as strongly in complex as in minimal media. In contrast, in *E. carotovora*, *hrpL* is expressed only in minimal medium. The latter observation is consistent with the report that *hrpN* expression in *E. carotovora* is strongly favored by acidic pH minimal medium, and it indicates that the atypical regulation of the *E. chrysanthemi* AC4150 Hrp system is not a general property of soft-rot *Erwinia* spp.

Remarkably, AC4150 is atypical even among *E. chrysanthemi* strains in its regulation of *hrp* gene expression, because strains 3937 and CUCPB1237 expressed *hrpN* most strongly in acidic

minimal medium. Unlike the regulatory regions preceding the *pelL* genes in AC4150 and 3937, those preceding the *hrpL* and *hrpS* genes in these two strains do not contain phage sequences (data not shown). The *hrpL* genes in both strains are preceded by identical σ^{54} promoter sequences (–12 CTGGCA and –24 TTGCT), but promoter sequences upstream of *hrpS* have not been defined. However, comparison of the first 150 bp upstream of the two regulatory genes in AC4150 and 3937 revealed the region upstream of *hrpS* to be more divergent (44% identity) than the region upstream of *hrpL* (80% identity). This supports the notion that factors acting above HrpL in the regulatory hierarchy are responsible for the atypical regulation of the *E. chrysanthemi* AC4150 Hrp system.

We expected that *hrp* genes would be strongly expressed early in culture growth because of evidence that the Hrp system is involved in an early stage of infection (Bauer et al. 1994). However, expression of *hrpN* increased most strongly in early stationary phase, at the same time that *pelE* expression

Table 3. Bacterial strains and plasmids used in this work

Designation	Relevant characteristics and use ^a	Reference or source
<i>Escherichia coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (f80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i> , Nx ^r	Life Technologies
SM10 λ pir	SM10 lysogenized with λ -pir for mobilizing pUT::mini-Tn5Cm, Km ^r	
Top10	Sm ^r	Invitrogen
VJS 533	<i>ara</i> Δ (<i>lac-proAB</i>) <i>rpsL</i> ϕ 80 <i>lacZ</i> Δ M15 <i>recA56</i>	Chatterjee et al. 1995
<i>Erwinia chrysanthemi</i>		
AC4150	Nx ^r derivative of <i>E. chrysanthemi</i> EC16, isolated from <i>Chrysanthemum morifolium</i>	Chatterjee et al. 1983
3937	Wild-type strain isolated from <i>Saintpaulia ionantha</i>	Hugouvieux-Cotte-Pattat and Robert-Baudouy 1985
CUCPB1237	Wild-type strain isolated from <i>Dianthus carophyllus</i>	Collmer and Bateman 1981
CUCPB5047	Δ (<i>pelA/pelE</i>) Δ (<i>pelB/pelC</i>)::28-bp Δ (<i>pelX</i>) Δ 4bp <i>pehX</i> :: Ω Cm ^r	Alfano et al. 1995
CUCPB5049	<i>hrpN</i> ::Tn5 <i>gusA1</i> derivative of AC4150	Bauer et al. 1995
CUCPB5065	<i>pelE</i> :: <i>uidA-nptII</i> derivative of AC4150	This work
CUCPB5081	<i>pelL</i> :: <i>uidA-nptII</i> derivative of AC4150	This work
CUCPB5082	<i>pelL</i> :: <i>uidA-nptII</i> derivative of CUCPB5047	This work
CUCPB5084	<i>expI</i> ::mini-Tn5Cm derivative of AC4150	This work
CUCPB5085	<i>expI</i> ::mini-Tn5Cm derivative of CUCPB5049	This work
CUCPB5086	<i>expI</i> ::mini-Tn5Cm derivative of CUCPB5065	This work
CUCPB5087	<i>expI</i> ::mini-Tn5Cm derivative of CUCPB5081	This work
CUCPB5100	<i>hrpA</i> :: <i>uidA-nptII</i> derivative of AC4150	This work
CUCPB5117	<i>hrpL</i> :: Ω Cm ^r derivative of CUCPB5049	This work
CUCPB5360	<i>hrpS</i> :: Ω Cm ^r derivative of AC4150	This work
<i>Erwinia carotovora</i> subsp. <i>carotovora</i> 71	Wild type	Mukherjee et al. 1997
<i>Agrobacterium tumefaciens</i>		
A136(pCF218)(pCF372)	A test strain for acyl-homoserine lactone production, Sp ^r , Tc ^r	More et al. 1996
Plasmids		
pAKC345	Cosmid clone that contains <i>expI</i> and <i>expR</i> in pLAFR5, Tc ^r	This work
pBluescriptIISK(+)	Cloning vector, Ap ^r	Stratagene
pBluescriptIISK(-)	Cloning vector, Ap ^r	Stratagene
pBCKS(-)	Cloning vector, Cm ^r	Stratagene
pCPP2172	3.1-kb <i>PstI</i> clone in pBluescriptIISK(-) that overexpresses <i>hrpN</i>	Bauer et al. 1995
pCPP2505	10.5 kb- <i>BglII</i> fragment from pCPP2156 (Ham et al. 1998) in pBluescriptIISK(+), which contains <i>hrpL</i> and part of <i>hrpJ</i>	This work
pCPP2277	<i>uidA-nptII</i> cassette in pBluescriptIISK(+)	This work
pCPP2350	<i>expI</i> ::mini-Tn5Cm in pAKC345	
pCPP2351	8.8-kb <i>PstI/ClaI</i> subclone from pCPP2350 that contains <i>expI</i> ::mini-Tn5Cm in pBluescriptIISK(-)	This work
pCPP2360	4.3-kb <i>BamHI/BglII</i> clone in pUC18 that contains <i>hrpA</i> operon	This work
pCPP2441	6.4-kb <i>BamHI</i> clone in pBluescriptIISK(+) that contains <i>pelL</i>	This work
pCPP2442	<i>pelL</i> :: <i>uidA-nptII</i> construct generated from pCPP2441	This work
pCPP2443	<i>pelE</i> :: <i>uidA-nptII</i> construct in pBCKS(-)	This work
pCPP2444	<i>hrpA</i> :: <i>uidA-nptII</i> construct generated from pCPP2360	This work
pCPP2504	6.1-kb <i>BglII</i> fragment from pCPP2156 (Ham et al. 1998) in pBluescriptIISK(+), which contains <i>hrpS</i>	
pHV200I	Frameshift mutation of <i>luxI</i> in pHV200, Ap ^r	Rojas et al. 2003
pPEL74	8.2-kb <i>PstI</i> fragment from pPEL7 carrying <i>pelA</i> and <i>pelE</i> , Tc ^r	Chatterjee et al. 1995
pPEL748	Plasmid clone that overproduces <i>PelE</i> , Ap ^r	Keen and Tamaki 1986
pUT::mini-Tn5Cm	Mini-Tn5 transposon with Cm ^r on suicide plasmid pGP704 derivative for transposon mutagenesis, Ap ^r	Keen and Tamaki 1986
		de Lorenzo et al. 1990

^a Kanamycin (Km), chloramphenicol (Cm), streptomycin (Sm), spectinomycin (Sp), tetracycline (Tc), and ampicillin (Ap).

increased (Fig. 7). Furthermore, as discussed above, *hrpN_{Ecc}* expression in *E. carotovora* subsp. *carotovora* also has been observed to rapidly increase in early stationary phase, indicating that growth-phase-dependent expression of the *hrp* genes is not a unique feature of *E. chrysanthemi* (Mukherjee et al. 1997). The elevated expression of the *E. chrysanthemi* EC16 *hrpN* in media that is rich in nutrients or has a neutral pH suggests that the Hrp system may be strongly expressed in the later stages of infection when more host nutrients would be available and the pH of the infection site is higher (Nachin and Barras 2000).

Interestingly, constitutive expression of plasmid-borne *hrpN* caused a reduction in the maceration virulence of *E. chrysanthemi* AC4150. The most likely explanation is that overproduced HrpN elicits plant defenses, thereby interfering with disease development. Such an activation of defenses is consistent with the ability of harpins to elicit the HR and the observation that the exogenous application of *E. amylovora* or *P. syringae* harpins to various plants induces systemic acquired resistance (Strobel et al. 1996; Wei and Beer 1996). However, it also is possible that overproduction of HrpN in the bacterial cell somehow interferes with the Hrp system, as HrpZ overexpression does in *P. syringae* (Alfano et al. 1996). Perhaps most surprising is that the Hrp systems of EC16 and 3937 are regulated so differently even though they appear to make similar contributions to virulence. Future work may reveal whether this apparent aberration in EC16 reflects an adaptation to preferred hosts, a difference in the function of Hrp effectors in this strain, or some other aspect of the multifactorial pathogenesis of *E. chrysanthemi*.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and DNA manipulation techniques.

Bacterial strains and plasmids used in this study are listed in Table 3. *E. chrysanthemi* strains were routinely grown in KB (King et al. 1954) at 30°C. For gene expression tests, *E. chrysanthemi* strains were grown in either M63 neutral pH minimal medium, typically with sucrose as a carbon source (Miller 1992), acidic pH Hrp minimal medium with fructose as a carbon source (Huynh et al. 1989), or KB at 25°C. *Escherichia coli* and *A. tumefaciens* strains were grown routinely in LM medium (Hanahan 1983) at 37°C but *E. coli* (pCPP2441) was selected and grown on solid M9 medium amended with glucose as a carbon source (M9_{glc}) (Miller 1992). The following antibiotics were used in selective media: ampicillin (Ap), 100 µg/ml; chloramphenicol (Cm), 20 µg/ml; kanamycin (Km), 50 µg/ml; spectinomycin (Sp), 50 µg/ml; streptomycin (Sm), 25 µg/ml; and tetracycline (Tc), 10 µg/ml. DNA manipulations were performed with standard methods (Sambrook et al. 1989), except DNA fragment isolation for subcloning was done with the Prep-a-Gene kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and DNA probes for Southern blot were made with PRIME-IT II (Stratagene, La Jolla, CA, U.S.A.) following the instructions of the manufacturers. Electroporation was performed with the Gene Pulser (Bio-Rad Laboratories) set to 400 Ω and 2.5 kV for *Erwinia chrysanthemi*.

Construction of *pelE::uidA*, *pelL::uidA*, and *hrpA::uidA*.

pelE::uidA was constructed as follows. pPL74, which carries *pelE* (Keen and Tamaki 1986), was digested with *Kpn*I and *Not*I, and a 2.3-kb fragment containing *pelE* was ligated to pBCKS(-) (Stratagene), cut with the same set of restriction enzymes, and subsequently transformed into *Escherichia coli* DH5α. Transformants were selected on LM media containing Cm, and the selected construct was confirmed by Pel activity

on pectate semisolid agar medium (Starr et al. 1977) and by restriction digestion analysis. This pBCKS(-):*pelE* construct was digested with *Mlu*I and blunted with Klenow enzyme (Stratagene), followed by ligation with a *uidA-nptII* cassette, which had been isolated previously by *Xba*I/*Xho*I digestions from pCPP2277. Ligated DNA was transformed into *E. coli* DH5α, and transformants were selected on LM plates containing Cm, Km, and 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside (X-Gluc). The construct was confirmed for the proper orientation of *uidA-nptII* by restriction digestion analysis and designated pCPP2443.

To construct *pelL::uidA*, an *Erwinia chrysanthemi* EC16 cosmid library was screened by colony hybridization with ³²P-labeled 3.5- and 5.0-kb DNA fragments generated by *Xba*I/*Xho*I digestions of a 8.5-kb partial *Sau*3AI digested clone in pBluescriptIISK(+) (Stratagene) which contains the *pelL* region. Cosmid DNA isolated from a positive colony was digested with several different restriction enzymes and analyzed by further Southern blot analysis with the same DNA probes. *Bam*HI digestion yielded a single positive band. This 6.4-kb DNA fragment was ligated to *Bam*HI-digested pBluescript II SK(+). This subclone, named pCPP2441, was selected on M9_{glc} instead of LM because of the previously observed toxic effect of *pelL* clones on *Escherichia coli* (Alfano et al. 1995). *E. coli* DH5α (pCPP2441) did not grow in either LM or M9_{glc} broth; hence, bacteria were streaked on an M9_{glc} plate, grown overnight, recovered with an inoculation loop, and resuspended in M9_{glc} for plasmid preparations. Restriction mapping of pCPP2441 indicated that there are two *A*/III sites in the insert, one of which should be in the coding region of *pelL*, based on the *pelL* sequence (Alfano et al. 1995). pCPP2441 was partially digested with *A*/III and blunt-ended with Klenow enzyme, and the 9.4-kb fragment was isolated and ligated to the *uidA-nptII* cassette in pCPP2277. Ligated DNA was transformed into *E. coli* DH5α, and the appropriate construct was selected based on restriction mapping, then confirmed by DNA sequencing from the *uidA-nptII* cassette with the primer, 5'-AACGCGCTTTCCACCAA-3'. This *pelL::uidA* construct was named pCPP2442.

To construct *hrpA::uidA*, pCPP2360, which contains the *hrpA* operon of *Erwinia chrysanthemi* (J. H. Ham, unpublished data) was digested with *Sac*II, which cuts in the middle and upstream of the 5' end of the *hrpA* operon, followed by blunt-ending with T4 polymerase. A 5.3-kb DNA fragment that lacks the 3' half of the *hrpA* operon was ligated to the *uidA-nptII* cassette in pCPP2277 and transformed into *Escherichia coli* DH5α. A derivative with the *uidA-nptII* cassette in the right orientation was identified by restriction digestion analysis, confirmed by DNA sequencing with the same primer used, and designated pCPP2444. Plasmids carrying these *uidA* fusions then were used to construct mutations in *Erwinia chrysanthemi* by marker exchange, as described below.

Construction of *hrpL* disruption mutant.

pCPP2505, which contains the *E. chrysanthemi* EC16 *hrpL* gene in a 10.5-kb *Bg*/III fragment in pBluescriptIISK(+), was digested with *Bsu*36I, which cuts once inside *hrpL*, blunt ended with T4 DNA polymerase, and ligated to a blunt-ended ΩCm^r fragment. The resulting plasmid, pCPP3067, was used to transform CUCPB5049 (*hrpN*::Tn5*gusA1*) by electroporation, and *hrpL*::ΩCm^r was introduced into the chromosome by marker exchange (Roeder and Collmer 1985) to produce mutant CUCPB5117.

Construction of *hrpS* disruption mutant.

pCPP2504 containing *hrpS* in a 6.1-kb *Bg*/III fragment in pBluescriptIISK(+) was digested with *Aat*II, which cuts once

inside *hrpS*, blunt-ended with T4 DNA polymerase (New England Biolabs, Boston, MA, U.S.A.), and ligated to a *Sma*I-digested and purified Ω^{Sp} cassette. Spectinomycin-resistant clones containing the Ω^{Sp} cassette inserted in *hrpS* were isolated and used to electroporate *E. chrysanthemi* wild-type AC4150. Transformants obtained after electroporation were used for marker exchange (Roeder and Collmer 1985) to produce CUCPB5360.

RNA isolation and Northern blot analyses.

Bacterial cultures were started at a Klett value of approximately 20 and grown in KB or minimal-salts medium plus sucrose (0.5%, wt/vol) at 28°C. Cultures were harvested at desired intervals for total RNA isolation. 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 and 5, are included to show that equal amounts of total RNA were loaded in each well. ^{32}P -labeled *hrpN_{Ech}*, *hrpL_{Ech}*, *hrpN_{Ecc}*, or *hrpL_{Ecc}* were used for Northern hybridization. Prehybridization (6 h at 65°C) and hybridization (18 h at 65°C) were performed in hybridization 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 denatured salmon sperm DNA at 100 µg/ml). After hybridization, membranes were washed at 65°C in 2× SSC, 0.5% SDS for 30 min; 1× SSC, 0.5% SDS for 30 min, and 0.5× SSC, 0.5% SDS, for 30 min, then examined by autoradiography with an X-ray film (−80°C).

Cloning *expI* and making an *expI::mini-Tn5Cm* construct.

A cosmid library of *E. chrysanthemi* EC16 in pLAFR5 was screened for production of acyl-HSL as previously described (Chatterjee et al. 1995). pAKC345, a cosmid clone that produces *E. chrysanthemi* acyl-HSL, was randomly mutagenized with mini-Tn5Cm as previously described (Ham et al. 1998), except *Escherichia coli* Top10 (Sm^r) (Invitrogen, Carlsbad, CA, U.S.A.) was used as a recipient cell for the pool of pAKC345::mini-Tn5Cm plasmids. Several colonies of *E. coli* Top10(pAKC345::mini-Tn5Cm) were randomly selected and tested for random mini-Tn5Cm insertion by digestion with several restriction enzymes. Bacterial colonies of *E. coli* Top10(pAKC345::mini-Tn5Cm) were grown overnight in LM broth and centrifuged at 12,000 × *g* for 10 min. Each supernatant (1 µl) was dropped on top of a bacterial colony of *A. tumefaciens* A136(pCF218)(pCF372), the test strain for acyl-HSL type autoinducer synthesis (More et al. 1996), which initially had been grown for 12 h on LM agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The treated bacteria were incubated a further 12 h at 37°C. Of 200 *E. coli* Top10(pAKC345::mini-Tn5Cm) colonies, 5 did not change the color of the test strain in LM plates containing X-Gal when their culture fluids were applied. One of these was chosen for further work and the mutant plasmid named pCPP2350. An 8.8-kb *Pst*I/*Cl*aI digested fragment carrying mini-Tn5Cm from pCPP2350 was subcloned into pBluescript II KS(−) and named pCPP2351. pCPP2351 was used for both sequencing *expI/expR* and marker-exchange mutagenesis of *expI* in *Erwinia chrysanthemi* strains. All *expI* derivatives were confirmed for the loss of function in acyl-HSL production with either *A. tumefaciens* A136(pCF218)(pCF372) or *Escherichia coli* VJS533(pHV200I).

Sequencing of *expI*, *expR*, and *hrpL*.

Primers designed from both ends of mini-Tn5Cm, 5'-AGAT CTGATCAAGAGACAG-3' and 5'-CCGTGTGTATAAGAGTC AG-3', were used to initiate DNA sequencing of *expI*, and fur-

ther sequencing for *expI* and *expR* was performed by primer walking. *hrpL* was sequenced from pCPP2505 initiated with a universal primer for pBSIISK(+) and extended by primer walking. Sequences have been deposited in GenBank under accession numbers AF448800 (*expI* and *expR*) and AF448202 (*hrpL*).

Primers, DNA sequencing, and DNA sequence analysis.

Oligonucleotide synthesis and DNA sequencing were performed at the Cornell Biotechnology Center. DNA sequence data were managed and analyzed with the DNASTar program (DNASTar, Madison, WI).

Marker-exchange mutagenesis of *Erwinia chrysanthemi* strains.

pCPP2442, pCPP2443, pCPP2444, and pCPP2351 were transformed into *Erwinia chrysanthemi* strains by electroporation, and transformants were grown overnight in KB broth containing either Km (for *uidA-nptII* inserted constructs) or Cm (for pCPP2351). Each culture (50 µl) was transferred to 2.5 ml of phosphate-deficient medium (Roeder and Collmer 1985), and overnight-grown cultures were diluted and spread on KB plates containing Cm or Km. Colonies resistant to these antibiotics were replica plated on KB plates containing Ap for the identification of Ap^r colonies. The resulting mutants are listed in Table 3.

β-Glucuronidase assays.

Gus assays were performed using 4-methylumbelliferyl β-D-glucuronide (MUG) and a TKO 100 mini-fluorometer (Hoeffer Scientific Instruments, San Francisco). β-Glucuronidase-specific activity was determined as pmol 4-methylumbelliferone produced per milliliter of culture per minute (optical density at 600 nm) by standard methods (Miller 1992).

Plant assays.

Previously described procedures were used to assay HR elicitation activity in tobacco (*Nicotiana tabacum* L. cv. Xanthi) leaves (Bauer et al. 1994) and virulence in chicory leaves (Bauer et al. 1994).

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