Relative Effects on Virulence of Mutations in the *sap*, *pel*, and *hrp* Loci of *Erwinia chrysanthemi*

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We constructed strains of *Erwinia chrysanthemi* EC16 with multiple mutations involving three virulence systems in this bacterium, namely *pel* (coding for the major pectate lyases *pelABCE*), *hrp* (hypersensitive response and pathogenicity), and *sap* (sensitivity to antimicrobial peptides). The relative effects on virulence of those mutations have been analyzed on potato tubers and chicory leaves. In potato tubers, the *sap* mutation (BT105) had a greater effect in the reduction of the virulence than the *pel* (CUCPB5006) and *hrp* (CUCPB5039) mutations. This reduction was similar to that observed in the *pel–hrp* double mutant (CUCPB5037). The analysis of the strains affected in Pel–Sap (BT106), Hrp–Sap (BT107), and Pel–Hrp–Sap (BT108) suggested that the effects of these mutations are additive. In chicory leaves, the mutation in the *sap* locus appeared to have a greater effect than in potato tubers. The competitive indices of strains BT105, UM1005 (Pel$^-$), CUCPB5039, and CUCPB5037 have been estimated in vivo and in vitro. These results indicate that the mutation in the *hrp* locus can be complemented in vivo by coinfection, whereas the mutations in *pel* and *sap* cannot.

Erwinia chrysanthemi causes soft-rot diseases, which are economically important in a wide range of crops (Boccara et al. 1991; Dickey 1979). The pathogenic behavior of this bacterium is characterized by a rapid necrosis of parenchymatous tissues, a wide host range, and the absence of a clear-cut compatible–incompatible reactions with its hosts (Alfano and Collmer 1996), which is very different from other necrogenic bacteria such as *Pseudomonas* and *Xanthomonas* spp. Soft-rot symptoms are caused fundamentally by pectic enzymes that degrade the middle lamellae and the primary cell wall of plant cells (Barras et al. 1994; Bateman and Basham 1976). In *E. chrysanthemi* EC16, the genes encoding the four major isozymes of pectate lyase (Pel/*pelABCE* genes) have been characterized (Keen et al. 1984; Tamaki et al. 1988). Their role in pathogenesis was demonstrated by the observation that the virulence of a mutant deleted in the four genes was significantly reduced (Ried and Collmer 1988). This mutant retained partial maceration ability as a result of the production of a second set of plant-inducible Pel isozymes (Kelemu and Collmer 1993). Similarly, strain 3937 produces five Pel isozymes and the *pelABCDE* mutant also retains residual maceration activity (Beaulieu et al. 1993). The different effects of individual *pel* (coding for the major pectate lyases *pelABCE*) mutations in the virulence on different hosts suggest that Pel isozymes may collectively contribute to the wide host range of *E. chrysanthemi* (Beaulieu et al. 1993). The expression of the pectinolytic activity is modulated by a complex regulatory network that responds to a wide range of environmental signals (Hugouvieux-Cotte-Pattat et al. 1992). Iron availability controls *pel* gene expression and iron transport functions, which represent another determinant of phytopathogenicity of *E. chrysanthemi* 3937 (Franza et al. 1999; Masclaux et al. 1996; Sauvage and Expert 1994). Recently it has been shown that the expression of certain genes in plant-associated bacteria responds to population density through the production of quorum-sensing signals (Cha et al. 1998) and that the transcription of *pel* genes of *E. chrysanthemi* responds to these signals (Nasser et al. 1998).

E. chrysanthemi is able to elicit a hypersensitive response that is independent of host range but normally is obscured by the pectolytic maceration (Bauer et al. 1994). The Hrp type III secretion system is considered to play a central role in bacterial pathogenicity through the delivery of proteins inside the plant cell (Alfano and Collmer 1997; Ham et al. 1998). Some of the *hrp* (hypersensitive response and pathogenicity) genes are conserved in several animal pathogenic bacteria such as *Yersinia*, *Shigella*, and *Salmonella* spp. (Galan and Collmer 1999; Van Gijsegem et al. 1993) and have been renamed *hrc* (hypersensitive reaction and conserved) (Bogdanove et al. 1996). The *hrp* mutants of *E. chrysanthemi* EC16 are reduced in their ability to incite infection in witloof chicory leaves but retain most of their maceration activity (Bauer et al. 1994). Thus, the rapid symptoms produced by *E. chrysanthemi* can be attributed to a macerative necrosis that is dependent on pectic enzymes and to a hypersensitive necrosis that is dependent on the *hrp* genes.

We recently characterized the *sap* (sensitivity to antimicrobial peptides) operon in *E. chrysanthemi* (López-Solanilla et al. 1998). This system is similar to that found in *Salmonella typhimurium* (Parra-Lopez et al. 1993) and probably constitutes a detoxification mechanism that enables the bacteria to withstand the action of antimicrobial peptides from their

hosts. The *sap* mutant of *E. chrysanthemi* is impaired in its virulence in potato tubers, where the size of the necrotic lesions caused by the mutant were 37% of that caused by the wild type (López-Solanilla et al. 1998).

It is generally accepted that the above-mentioned mechanisms (Pel, Hrp, and Sap) have an important role in the virulence of *E. chrysanthemi*. The main objectives of this work are to ascertain the relative contribution of these different virulence determinants on different hosts and to investigate how these mechanisms act during the development of the infection. The construction and analysis of mutants affected in one to three of these mechanisms enabled us to conclude that i) the mutation in the Sap system has the greater effect in virulence in potato and in chicory leaves; ii) the effects of these mutations on virulence are additive; and iii) the determination of competitive indices suggests that the mutation in the Hrp system can be complemented in planta by coinfection, whereas mutations in Sap and Pel cannot.

RESULTS

Construction of multiple mutations in *pel*, *hrp* and *sap* loci.

To analyze the contribution of the *sap* locus to the virulence of *E. chrysanthemi* with respect to other known virulence determinants, namely the production of pectic enzymes (PelABCE) and the induction of hypersensitive necrosis (Hrp) (Bauer et al. 1994), the sap locus was inactivated in mutant strains affected in these loci. A Δ(sap A)::ΩSpr–Smr mutation was marker exchanged into the PelABCE$^-$ mutant (CUCPB5006), the Hrp$^-$ mutant (CUCPB5039), and the PelABCE$^-$–Hrp$^-$ double mutant (CUCPB5037) to construct BT106, BT107, and BT108, respectively. The insertion of the Ω interposon in the chromosome replacing the *sapA* sequence was confirmed by Southern blot hybridization with DNA from the Ω interposon or different fragments of pB103 corresponding to the *sapA* sequence (data not shown) as a probe. No differences in the mutant strains with respect to corresponding parental strains was found for growth rate, colony size and morphology, cell size, and appearance. The ability of all the strains to produce pectic enzymes in culture was confirmed with a spectrophotometric assay (see below). As expected, the strains deleted in the major Pels only retained residual pectolytic activity, whereas the other strains showed the same level of enzymatic activity as the wild type (data not shown). All of the strains used in this work are summarized in Table 1.

Virulence of the mutant strains on potato tubers.

The virulence of the wild-type strain (AC4150) and the single- (BT105, CUCPB5006, and CUCPB5039), double- (CUCPB5037, BT106, and BT107), and triple-mutation (BT108) strains were compared with the experimental design described below (Fig. 1). The average necrotic area of the lesions produced by the *sap* mutant was smaller than that produced by the *pel* or *hrp* mutant. When the Sap system was inactivated in the *pel* and *hrp* mutant backgrounds (Pel$^-$Sap$^-$ and Hrp$^-$Sap$^-$, respectively), the double-mutant strains produced a necrotic area smaller than those of the corresponding parental strains (Fig. 1). The *pel–hrp–sap* mutant strain produced the smallest necrotic area but still retained a residual virulence (Fig. 1). In addition, the pathogenicity of each strain was assayed by the estimation of the bacterial population growth on potato discs (Fig. 2). These results correlated with the estimated necrotic area produced by each strain.

Virulence on chicory leaves.

The ability to infect witloof chicory leaves also was assayed in order to compare the magnitude of the effect of Sap inactivation on virulence with those of the effects reported for the *pel* or *hrp* mutant strains (Bauer et al. 1994). Inocula (5×10^5 cells) of the wild-type (AC4150) or the *sap* mutant (BT105) were infiltrated on individual leaves, and the frequency of developed lesions was recorded. In these assay conditions, the wild type produced lesions in 50% of the leaves, whereas the mutant completely failed to develop any lesion (Fig. 3).

The assay was modified by increasing the level of inoculum (see below) in order to compare the virulence of the *sap* mutant strain (BT105) with the other mutant strains. The data presented in Table 2 indicate that the lesions produced by the *sap* mutant (BT105) were smaller than those produced by the wild-type (AC4150) and *hrp* mutant strain (CUCPB5039), whereas there was no significant difference between the size of the necrotic areas produced by the *sap* and the *pel* mutant (CUCPB5006). The necrotic area produced by the *pel–hrp*

Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics	Source or reference
Escherichia coli		
DH5α	*supE44 lac U169* (ø80 *lac ZM15 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan 1983
Erwinia chrysanthemi		
AC4150	Wild-type strain	Chatterjee et al. 1983
BT105	Δ(*sapA*)::ΩSp^r–Sm^r derivative of AC4150	López-Solanilla et al. 1998
UM1005	Δ(*pelB pelC*)::28 bp Δ(*pelA pelE*)::*nptI* derivative of AC4150	Ried and Collmer 1988
CUCPB5006	Δ(*pelB pelC*)::28 bp Δ(*pelA pelE*) derivative of AC4150	He and Collmer 1990
CUCPB5039	*hrp-1*::Tn*10* mini-kan derivative of AC4150	Bauer et al. 1994
CUCPB5037	*hrp-1*::Tn*10* mini-kan derivative of CUCPB5006	Bauer et al. 1994
BT106	Δ(*sapA*)::ΩSp^r–Sm^r derivative of CUCPB5006	This work
BT107	Δ(*sapA*)::ΩSp^r–Sm^r derivative of CUCPB5039	This work
BT108	Δ(*sapA*)::ΩSp^r–Sm^r derivative of CUCPB5037	This work
CUCPB5081	*pelL*::*uidA–nptII* derivative of AC4150	J. Hyun Ham, *unpublished*
BT115	Δ(*sapA*)::ΩSp^r–Sm^r derivative of CUCPB5081	This work
CUCPB5082	pel L::*uidA–nptII* Δ(*pelB pelC*)::28 bp Δ(*pelA pelE*) *pehx*::*cat19* derivative of AC4150	J. Hyun Ham, *unpublished*
BT116	Δ(*sapA*)::ΩSp^r–Sm^r derivative of CUCPB5082	This work
Plasmids		
pB103	pBluescript II carrying AC4150 *sapA* to *sapC* genes	López-Solanilla et al. 1998

double mutant (CUCPB5037) was not significantly different from that of the *sap* mutant. When the strains mutated in the Pel or Hrp systems were compared with the mutant strains of the Sap system in these backgrounds, the double mutants (*pel–sap*, BT106 and *hrp–sap*, BT107) produced lesions of approximately 50% of those produced by the parental strains. The triple mutant *pel–hrp–sap* also produced a smaller necrotic area than the double mutant *pel–hrp*. In addition, the results presented in Table 2 indicate that the percentage of inoculated leaves displaying lesions after inoculation is lower in those strains harboring the sap mutation.

sap mutation and the expression of plant-inducible pectate lyase PelL.

To ascertain that the *sap* mutation does not affect the expression of plant-inducible pectic enzymes, *sap* mutations were introduced into strains harboring GUS fusions in the plant-inducible gene *pelL* in the wild type background and in the strain lacking the major pectate lyases (PelABCE$^-$) and exo-poly-α-D-galacturonidase (PehX$^-$). Strain BT115 was obtained by marker exchange of a Δ(*sapA*)::ΩSpr–Smr mutation into the *pelL*::*uidA–nptII* strain (CUCPB5081). Strain BT116 was constructed by marker exchange of a Δ(*sapA*)::ΩSpr–Smr mutation into *pelL*::*uidA–nptII* Δ*pelABCE pehX*::*cat19* strain (CUCPB5082). The insertion of the Ω interposon in the chromosome replacing the *sapA* sequence was confirmed by Southern blot hybridization with DNA from the Ω interposon or different fragments of pB103 corresponding to the *sapA* sequence (data not shown) as a probe. The characteristics of these strains are summarized in Table 1.

Chicory discs were infected with 1.2×10^6 CFU of BT115, BT116, CUCPB5081, CUCPB5082, and the wild-type strain as a control. After 24 h at 28°C, each disc was ground in 1 ml of 50 mM $NaPO_4$ (pH 7) and 1 mM EDTA, and the glucuronidase activity was determined. Figure 4 shows that the specific GUS activity of each *sap* mutant strain is not significantly different from that of the corresponding parental strain. As expected, the wild type showed residual GUS activity. Interestingly the specific GUS activity of the CUCPB5082 strain was approximately one-third of that of the CUCPB5081 strain. These results indicate that the *sap* mutation has no effect on the expression of *pelL*, but the deletion of *pelABCE* and *pehX* has a significant effect on the expression of this gene.

Competitive indices of the mutant strains.

To determine if there are in planta complementation phenomena or any interference when the two populations have to incite the infection at the same time, we investigated the behavior of the mutant strains when coinoculated with the wild type. Chicory leaves were inoculated with 1.2×10^6 CFU of mixed inocula (1:1) of the wild-type strain and each of the mutant strains (*sap*, *pel*, *hrp*, or *pel–hrp*). After 24 h, bacteria were recovered from the tissue and viable cells from each population were determined by dilution plating on selective media.

The results from these experiments and those obtained from the in vitro experiments are shown in Table 3. The results are expressed as indices of competition of the mutant strains relative to the wild-type strain. The in vivo competitive indices obtained for *sap* (BT105), *pel* (UM1005), and *pel–hrp* (CUCPB5037) mutants were significantly different from those obtained in vitro and indicated that these mutants are impaired in their ability to multiply in the tissue relative to the wild-type strain. To further investigate this phenomenon, we estimated the in vivo competitive index for the *pel* mutant when a culture filtrate from the wild type containing 0.1 units of total Pel activity was added to the inoculum. The exogenous addition of pectic enzymes restored the competitive index of the *pel* mutant to 0.95.

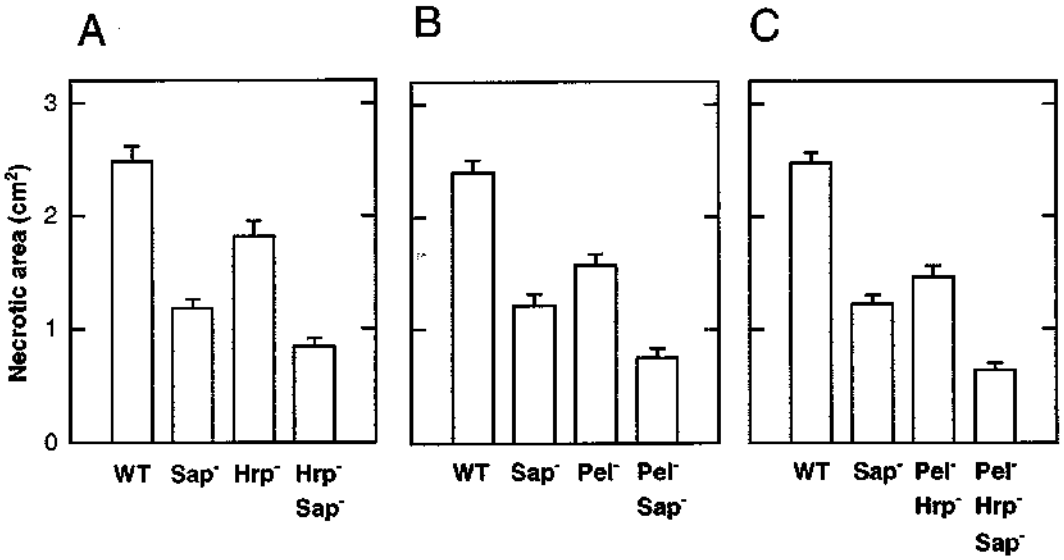

Fig. 1. Virulence on potato tubers. Comparison of the virulence of *Erwinia chrysanthemi* AC4150 wild-type and mutant strains on potato tubers. Three different experiments were performed with 40 potato tubers in each experiment. Each potato tuber was inoculated with four different strains. Inocula combinations: **A**, wild-type and *sap*, *pel*, and *pel–sap* mutant strains; **B**, wild-type and *sap*, *hrp*, and *hrp–sap* mutant strains; and **C**, wild-type and *sap*, *pel–hrp*, and *pel–hrp–sap* mutant strains. Necrotic area was determined after 48 h of incubation at 30°C with high humidity. Errors bars indicate standard errors. Mean differences are significant in all cases at the 95% confidence level, according to the least significant difference procedure.

The in vivo index obtained for the *hrp* mutant (CUCPB5039), however, was not significantly different from the in vitro index, indicating that this strain and the wild type were able to multiply equally in the tissue following mixed inoculation.

DISCUSSION

Our understanding of the pathogenesis mechanisms of *E. chrysanthemi* is still incomplete. This bacterium successfully infects a large number of different plants, implying that it must be able to grow in very different plant niches and withstand several inducible and constitutive defense barriers. *E. chrysanthemi* pathogenicity was formerly attributed mostly to the production and secretion of pectic enzymes (Barras et al. 1994) and to the siderophore-mediated iron utilization (Sauvage and Expert 1994). This view has begun to change in recent years with the discovery of other virulence determinants such as Hrp (Bauer et al. 1994), Sap (López-Solanilla et al. 1998), or the methionine sulfoxide reductase antioxidant mechanism (Hassouni et al. 1999), among others. Moreover, the existence of additional pathogenicity determinants cannot be ruled out.

The analysis of the mutants affected in these three well-known virulence determinants on potato tubers showed that the mutation in the Sap system had a greater effect on the virulence in this tissue than did the mutations in the Pel or Hrp systems. Actually, the reduction of the necrotic area produced by the *sap* mutant was similar to that produced by the double *pel–hrp* mutant. This result was unexpected and suggests that antimicrobial peptides constitute a significant barrier for *E. chrysanthemi* infection in this tissue. It also should be noted that the mutations in the Hrp system produced a reduction of the size of necrotic lesion in potato, whereas in chicory leaves this mutant showed a reduction in infection frequency but not in the size of the necrotic lesion (Bauer et al. 1994). In the double and triple mutants, the effect of introducing a new mutation is additive, which suggests that the three different mechanisms operate in an independent way.

The Sap system shows specificity against certain antimicrobial peptides (López-Solanilla et al. 1998). Because different plant tissues differ in the quantity and type of antimicrobial peptides (García-Olmedo et al. 1998), it can be presumed that the effects of the *sap* mutation also will vary in different hosts. In chicory leaves, the *sap* mutant was unable to cause lesions in the standardized assay (see below and Fig. 3). In comparison, the *pel* mutant incited lesions that were 37% of those produced by the wild type (Bauer et al. 1994) in the same assay conditions. This result suggests that antimicrobial peptides, which have not yet been investigated in chicory, constitute a greater defense barrier than they do in potato tubers for the *sap* mutant. The inability of the *sap* mutant to incite infection in chicory forced us to modify the conditions of this assay in order to test the mutants affected in the different virulence mechanisms. With the use of a higher inoculum, the *sap* mutation continued to cause a significant reduction in virulence with respect to the wild-type and *hrp* mutant strains. The difference in virulence with respect to the *pel* mutant strain bordered in statistical significance. When the effect of the *sap* mutation in the Pel^- and Hrp^- background was analyzed, it was observed that the reduction in virulence was about 50% in both cases, which suggests, as it did in the potato tuber experiments, that the three systems have an independent mode of action. Because the effect of the *sap* mutation on virulence appeared to be greater in chicory leaves than in potato tubers, it will be interesting to analyze the effect of *sap* mutation in a larger number of hosts.

We used the estimation of competitive indices from coinoculation experiments to analyze whether the effect on

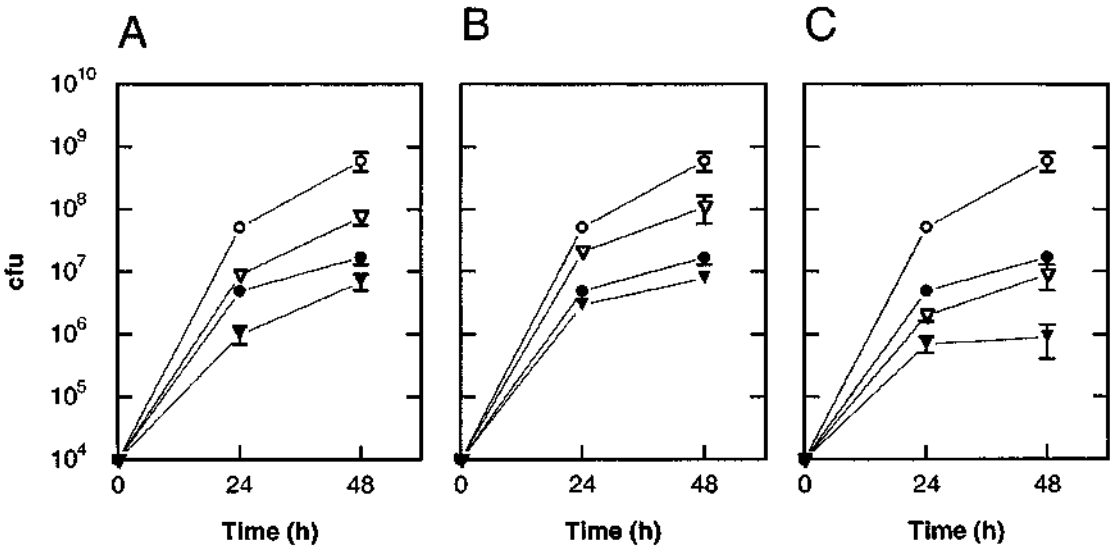

Fig. 2. Growth of *Erwinia chrysanthemi* wild-type and mutant strains on potato tuber discs. After inoculation of the discs with 10^4 cells, bacterial populations were estimated at different times by grinding the tissue and plating appropriate dilutions in King's B agar plates (King et al. 1954). **A,** Bacterial population of *E. chrysanthemi* AC4150 (open circles) and *sap* (closed circles), *pel* (open triangles) and *pel–sap* mutants (closed triangles). **B,** Bacterial population of *E. chrysanthemi* AC4150 (open circles) and *sap* (closed circles), *hrp* (open triangles) and *hrp–sap* mutants (closed triangles). **C,** Bacterial population of *E. chrysanthemi* AC4150 (open circles) and *sap* (closed circles), *pel–hrp* (open triangles) and *pel–hrp–sap* mutants (closed triangles). Bars represent standard errors.

virulence of these mutations could be complemented in planta by the wild-type strain. The competitive index is defined as the output ratio of mutant to wild-type bacteria divided by the input ratio of mutant to wild-type bacteria. This index has been used widely in animal systems to analyze mutants with a diminished ability to colonize host tissues (Freter et al. 1981; Heithoff 1999; Taylor et al. 1987). When the competitive index is significantly less than 1.0 in vivo but not in vitro, it can be considered that the mutation has an effect on the ability to colonize the tissue and cannot be complemented by coinfection. The coinoculation of the *sap* mutant and the wild type resulted in an in vivo competitive index of 0.13. This agrees with the proposed function for the Sap system because it was not expected that such a bacterial protective mechanism against antimicrobial peptides could be complemented by the presence of a wild-type bacterium. In the case of the *hrp* mutant, the in vivo competitive index was not statistically different from 1.0. The proposed function of the Hrp system is the delivery of bacterial effector proteins inside the host cell, which may have the final effect of changing the apoplast conditions in a way that is favorable to the bacteria. If this model is correct, it can be expected that the mutant cells benefit from this mechanism as much as the wild-type cells. In contrast, the finding that the *pel* mutation cannot be complemented in planta by coinfection was rather unexpected. Pectic enzymes act extracellularly and presumably release nutrients from plant cells. Thus, it is difficult to explain why the mutant cells are unable to benefit from the pectolytic activity of the wild-type cells. A possible explanation of these results relies on the fact that the action of pectic enzymes in planta could be limited to a small area surrounding the bacterium. Our finding that the competitive index of the *pel* mutant increases with the addition of pectic enzymes points to this conclusion.

Our data favor the view that the virulence of *E. chrysanthemi* EC16 depends on multiple factors and that the Sap system makes a more important contribution than does the PelABCE isozymes in the analyzed tissues. To gain a deeper understanding of the pathogenic process, it is necessary to know which other virulence determinants are involved, to study the spatial and temporal mode of action of the virulence determinants during the infection process, and to learn more about how bacterial cells counteract plant defense mechanisms.

Fig. 3. Virulence on chicory leaves. Response of witloof chicory leaves to *Erwinia chrysanthemi* AC4150 and *sap* mutant BT105. Witloof chicory leaves were inoculated at small wounds with 5×10^5 cells and photographed after incubation for 72 h in a moist chamber.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

Bacterial strains and plasmids used in this work are described in Table 1. Strains of *Escherichia coli* were cultivated at 37°C in Luria-Bertani medium. Strains of *E. chrysanthemi* were cultivated at 30°C in nutrient broth (NB) (Difco, Detroit, MI, U.S.A.) or King's B media (King et al. 1954). Antibiotics were added to the media at the following concentrations per ml: 100 μg of ampicillin, 25 μg of spectinomycin, and 125 (for multicopy plasmid resistance) or 10 μg (for chromosomal resistance) of streptomycin. Standard pectate semisolid agar medium

Table 2. Virulence on chicory leaves

Strains in pairwise tests	No. of lesions per 10 inoculations	Size of lesions (mm^2, mean ± SE)[a]
AC4150	10	97 ± 14[b]
BT105(Sap⁻)	6	24 ± 4
CUCPB5006(Pel⁻)	10	92 ± 47
BT105(Sap⁻)	5	46 ± 15
CUCPB5039(HR⁻)	9	71 ± 12
BT105(Sap⁻)	6	39 ± 10[b]
CUCPB5037(Pel⁻HR⁻)	7	60 ± 24
BT105(Sap⁻)	5	46 ± 8
CUCPB5006(Pel⁻)	10	54 ± 9[b]
BT107(Pel⁻Sap⁻)	6	17 ± 5
CUCPB5039(HR⁻)	10	85 ± 17[b]
BT108(HR⁻Sap⁻)	5	24 ± 8
CUCPB5037(Pel⁻HR⁻)	10	41 ± 3[b]
BT109(Pel⁻HR⁻Sap⁻)	5	22 ± 5

[a] Each chicory leaf was inoculated at two locations (Fig. 3) with 1.2 × 106 cells of the indicated strains. Each test involved 10 leaves. Values are the product of the length and width of the necrotic area. Differences in the values for BT105 are a result of the batch variability in chicory leaves.

[b] Differences between the two compared strains are significant ($P < 0.05$), according to the Student's *t* test.

Table 3. Indices of competition between mutant and wild-type strains

Strain	Relevant genotype	In vivo CI[a]	In vitro CI
BT105	Δ(*sap*A)::ΩSp^r–Sm^r	0.13[b]	1.80
UM1005	Δ(*pelB pelC*)::28 bp Δ(*pelA pelE*)::*nptI*	0.19[b]	1.75
CUCPB5039	*hrp-1*::Tn*10* mini-kan	1.28	1.85
CUCPB5037	Δ(*pelB pelC*)::28 bp Δ(*pelA pelE*) *hrp-1*::Tn*10* mini-kan	0.45[b]	1.50

[a] Competitive index is defined as the output ratio of mutant to wild-type bacteria divided by the input ratio of mutant to wild-type bacteria AC4150. Each result is the average of the CIs from six inoculations on chicory leaves.

[b] The in vivo competitive index differ ($P < 0.05$) from the in vitro CI for this strain according to the Student's *t* test.

was prepared, as described by Starr et al. (1977), with polygalacturonic acid (Sigma Chemical, St. Louis, MO, U.S.A.).

General DNA manipulation.

Construction of the pB103 derivative with a replacement of part of the *sapA* gene by the Ω interposon (Prentki and Krisch 1984) has been described elsewhere (López-Solanilla et al. 1998). Small- and large-scale plasmid and genomic DNA purification, restriction enzyme digestion, agarose gel electrophoresis, DNA subcloning, blot and hybridization, and colony screening by hybridization were performed as described by Sambrook et al. (1989) and following the manufacturer's instructions (Amersham Pharmacia Biotech, Little Chalfont, U.K., and Stratagene, La Jolla, CA, U.S.A.).

Marker-exchange mutagenesis of *E. chrysanthemi* strains.

Marker exchange in *E. chrysanthemi* was performed as described by Roeder and Collmer (1985). The pB103 derivative was transformed into *E. chrysanthemi* CUCPB5006, CUCPB5039, CUCPB5037, CUCPB5081, and CUCPB5082 by electroporation by an Electro Cell Manipulator ECM 600 (BTX, San Diego, CA, U.S.A.). Marker-exchanged recombinants were identified by picking colonies in NB-spectinomycin and streptomycin agar medium with and without ampicillin. Genomic DNA from selected mutants (spectinomycin, streptomycin resistant, and ampicillin sensitive) were subsequently analyzed by Southern blot hybridization. One mutant out of several recombinants from each parental strain was selected for further analysis (data not shown).

Enzymatic assays.

Culture filtrates containing a mixture of extracellular pectic enzymes were obtained as previously described (Miguel et al. 2000). The enzymatic assay of pectic enzymes was produced in culture on the basis of the monitoring the increase of absorbance at 232 nm as a result of the 4,5-unsaturated reaction products. This assay was performed as described by Collmer et al. (1988). GUS activity was measured by the spectrophotometric assay, described by Wilson et al (1992).

Virulence assays.

Potato tubers (cv. Jaerla) and heads of witloof chicory were purchased from a local supermarket. The cells from an overnight NB liquid medium culture were washed with 10 mM $MgCl_2$ by centrifugation and resuspended in an appropriate volume of the same buffer to obtain the desired inoculum concentration. Potato tubers were inoculated with 50 µl of a suspension containing 5×10^5 bacteria by inserting a plastic micropipettor tip at a constant depth of 1.5 cm. Three different experiments were performed, with 40 potato tubers in each experiment. Each potato tuber was inoculated with four different strains. In the first experiment, the inocula combinations were wild-type, *sap*, *pel*, and *pel–sap* mutant strains. In the second experiment, the combinations were wild-type, *sap*, *hrp*, and *hrp–sap* mutant strains. Finally, in the third experiment, the combinations were wild-type, *sap*, *pel–hrp*, and *pel–hrp–sap* mutant strains. This experimental design minimized the effect of the variability among individual potato tubers. Potatoes were left at 30°C in 100% relative humidity for 48 h. Afterward, the tubers were sliced at the inoculation point and damage was estimated by measuring the macerated area. Statistical analysis of the data of each experiment was performed by the respective analysis of variance with the Statgraphics Plus 3.1 (Statistical Graphics, Englewood Cliffs, NJ, U.S.A.) program. Necrotic area means produced by each mutant and the wild type were compared pairwise with Fisher's least significant difference procedure. To monitor bacterial growth in potato discs, 10 µl of a bacterial suspension containing 10^4 bacteria was inoculated in 1-cm potato discs. Discs were incubated at 30°C and high humidity, recovered at different times, and ground with a tissue homogenizer in 500 µl of 10 mM $MgCl_2$. Bacterial colony-forming units in the homogenate were determined by dilution plating. Virulence assays on witloof chicory leaves were performed, as described by Bauer et al. (1994), to compare the wild-type and *sap* mutant strains. This assay was modified by increasing the level of inoculum in order to compare the virulence of the *sap* mutant with that of the other mutant strains. The inoculum selected for these experiments was 1.2×10^6 bacteria per inoculation point because in these conditions, 50% of the leaves were infected by the *sap* mutant. Each chicory leaf was inoculated at two locations, and ten leaves were inoculated with each pair of compared strains. Chicory leaves were incubated for 72 h in a moist chamber at 30°C. The difference between each parental and mutant strains were assessed statistically with a paired Student's *t* test.

Competition assays.

We used the competitive index defined as the change in the population ratio of two strains after grown together under experimental conditions (Freter et al. 1981; Taylor et al. 1987). In vitro competition was determined by growth at 37°C for 24 h in 10 ml of NB liquid medium from a starting density of 1.2×10^6 CFU/ml. In vivo competition was determined by growth on chicory leaves inoculated with 1.2×10^6 CFU. The bacteria from the tissue were recovered 24 h later. The input

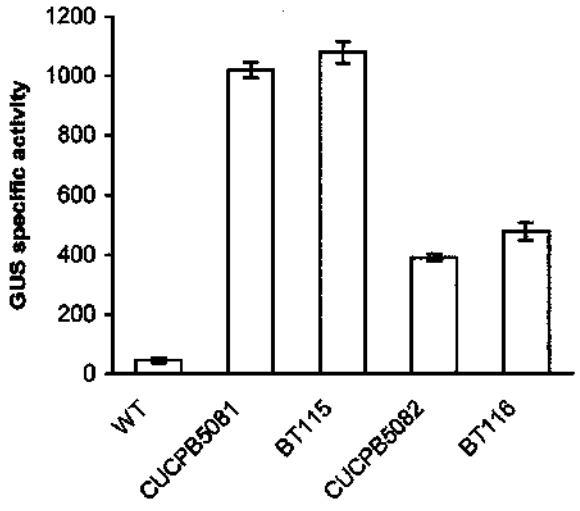

Fig. 4. Expression of *pelL::uidA* gene fusions after inoculation of chicory discs. The wild-type strain and four different mutants containing a fusion between the *pelL* genes and reporter gene *uidA* were inoculated in chicory discs. GUS activity was determined after 24 h. Specific activity of GUS is expressed as the nmol of *p*-nitrophenol liberated per 10^6 CFU. Error bars indicate standard deviation calculated from three different experiments.

ratio was approximately 1.0 in both types of competition experiments, and competitive indices were corrected for the input ratio. Viable cell counts and the ratio of the two strains were determined by plating dilutions onto NB agar containing nalidixic acid, kanamycin, or spectinomycin–streptomycin to identify the different strains.

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