Inactivation of the *sapA* to *sapF* Locus of *Erwinia chrysanthemi* Reveals Common Features in Plant and Animal Bacterial Pathogenesis

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We investigated the role in pathogenesis of bacterial resistance to plant antimicrobial peptides. The *sapA* to *sapF* (for sensitive to antimicrobial peptides) operon from the pathogenic bacterium *Erwinia chrysanthemi* has been characterized. It has five open reading frames that are closely related (71% overall amino acid identity) and are in the same order as those of the *sapA* to *sapF* operon from *Salmonella typhimurium*. An *E. chrysanthemi sap* mutant strain was constructed by marker exchange. This mutant was more sensitive than was the wild type to wheat α-thionin and to snakin-1, which is the most abundant antimicrobial peptide from potato tubers. This mutant was also less virulent than was the wild-type strain in potato tubers: lesion area was 37% that of the control, and growth rate was two orders of magnitude lower. These results indicate that the interaction of antimicrobial peptides from the host with the *sapA* to *sapF* operon from the pathogen plays a similar role in animal and in plant bacterial pathogenesis.

INTRODUCTION

Antimicrobial peptides are essential effectors of innate immunity, a nonspecific defense mechanism that seems to be shared by animals and plants (reviewed in García-Olmedo et al., 1992, 1995; Gabay, 1994; Boman, 1995; Hoffman, 1995; Broekaert et al., 1997; Shewry and Lucas, 1997). Peptide families identified in animals include both linear and disulfide-folded types (Gabay, 1994; Ganz and Lehrer, 1994; Boman, 1995; Hoffman, 1995), whereas only disulfide-containing antimicrobial peptides have been found in plants (García-Olmedo et al., 1992, 1995; Broekaert et al., 1997; Shewry and Lucas, 1997). Among the cysteine-rich plant peptides that have been shown to be active in vitro against pathogens are thionins, hevein- and knottin-like peptides, lipid transfer proteins (LTPs), and defensins (reviewed in Broekaert et al., 1997; Shewry and Lucas, 1997) as well as snakins (Moreno, 1995; A. Segura, M. Moreno, F. Madueño, A. Molina, and F. García-Olmedo, manuscript in preparation). The expression patterns of these peptides have been found to be compatible with a defense role, and enhanced tolerance to different pathogens has been observed through transgenic overexpression of genes encoding some of them, such as thionins (Carmona et al., 1993; Epple et al., 1997), defensins (Terras et al., 1995), or LTPs (Molina and García-Olmedo, 1997).

The role of antimicrobial peptides in animal innate immunity has been highlighted by the observation of increased susceptibility to infection in Drosophila mutants affected in their synthesis (Lemaitre et al., 1996) and by the fact that certain human disorders characterized by recurrent infections are associated with a lack of a particular class of antimicrobial peptides (defensins) in blood phagocytes (Ganz et al., 1988), but no such evidence is available with respect to plant peptides because appropriate plant mutants with decreased peptide levels have not yet been obtained. An alternative line of evidence about the role of antimicrobial peptides is based in the production of peptide-sensitive mutants of a pathogen. The hypothesis that peptides are involved in defense would be supported by a decrease of virulence in this type of mutant. Indeed, in the animal pathogen *Salmonella typhimurium*, both rough lipopolysaccharide (LPS) mutants and *sapA* to *sapF* (*sap* stands for sensitive to antimicrobial peptides) mutants, which show increased sensitivity to antimicrobial peptides, have reduced virulence, suggesting that resistance to host peptides has a direct role in *Salmonella* pathogenesis (Macias et al., 1990; Groisman et al., 1992; Parra-Lopez et al., 1993). Similarly, we found previously that thionin- and LTP-sensitive mutants of *Ralstonia* (*Pseudomonas*) *solanacearum* were altered in their LPS structure and were avirulent in tobacco (Titarenko et al., 1997).

Analysis of *sap* mutants from *S. typhimurium* has led to the discovery of genes *sapA* to *sapF*. These are required for peptide resistance and for virulence (Parra-Lopez et al., 1993). These genes are organized in a single operon and exhibit sequence similarity with ABC transporters described in

prokaryotes and eukaryotes. The proposed mechanism of action for the Sap system includes binding of the periplasmic component SapA to the antimicrobial peptide, followed by peptide transport to the cytoplasm, where peptide degradation and/or activation of resistance determinants occurs.

We have now investigated the *sap* operon in *Erwinia chrysanthemi*, which is an economically important phytopathogenic bacterium that causes soft rot diseases in a wide range of crops (Perombelon and Kelman, 1980). Active virulence mechanisms are known to contribute to the pathogenesis of this bacterium. The most important are the secretion of hydrolytic enzymes that attack the pectic fraction of the plant cell wall (Collmer and Keen, 1986) and the induction of plant necrosis elicited by *hrp* (for hypersenstive response and pathogenicity) gene products (Bauer et al., 1994). However, little is known about the passive virulence mechanisms that enable *E. chrysanthemi* to resist the action of antimicrobial agents from the plant host. Although *S. typhimurium* and *E. chrysanthemi* have very different pathogenic behavior, the fact that they are phylogenetically related and that antimicrobial peptides occur in their respective animal and plant hosts led us to investigate the possible role of the Sap system in plant-pathogen interactions. We report here that *sap* genes are structurally conserved in *E. chrysanthemi* with respect to *S. typhimurium* and that their inactivation by insertion mutagenesis makes the bacterium more sensitive to certain plant antimicrobial peptides and less virulent to plants. These data suggest that the SapA to SapF resistance mechanism plays a similar role in plant and animal pathogenesis.

RESULTS

Cloning and Analysis of the *sap* Locus of *E. chrysanthemi*

To investigate the presence of a *sap* operon homolog in *E. chrysanthemi*, we analyzed genomic DNA from the AC4150 strain of this bacterium by DNA gel blotting. A fragment from plasmid pEG6179 was used as a probe (Parra-Lopez et al., 1993). pEG6179 contains most of the *S. typhimurium sap* operon. Two fragments that hybridized with this probe were cloned in the λ ZAPII vector and subcloned in pBluescript SK− (pB103, 6.5-kb fragment; pB104, 1.7-kb fragment). To obtain a clone containing the complete *sapA* to *sapF* operon, we made an *E. chrysanthemi* genomic library in the vector λ FIX II and screened it with probes obtained from inserts of plasmids pB103 and pB104. A positive phage was isolated, and the EcoRI-EcoRI 8.2-kb fragment, containing the whole operon, was subcloned in pBluescript SK− (Figure 1, pB105).

The nucleotide sequences of the inserts in plasmids pB103 and pB104 were determined (EMBL accession number AJ222649) and found to contain five open reading frames that were homologs of the five *sap* genes described for *S. typhimurium* by Parra-Lopez et al. (1993). The deduced amino acid sequences aligned with the corresponding ones from *S. typhimurium* products, showing that the *sap* operon has the same structure in the two species. The average identity of the amino acid sequences of the homologous genes from the two species was 71%, which justified the designation of the *E. chrysanthemi* locus as *sap* and the corresponding open reading frames as *sapA* to *sapF*. Figure 1 shows a genetic and physical map of the *sap* region from *E. chrysanthemi*. Parra-Lopez et al. (1993) noted the occurrence of homologous *sap* sequences in *E. herbicola*. As shown in Figure 2, we have found sequences homologous to *sap* in seven out of eight species of *Erwinia* tested by DNA gel blot hybridization, using a 964-bp PstI fragment internal to *sapA* from pB103 as a probe.

Insertional Inactivation of the *sap* Locus in *E. chrysanthemi*

To ascertain the involvement of the *sapA* to *sapF* operon from *E. chrysanthemi* in resistance to antibiotic peptides, we mutated this locus. A region containing part of the *sapA* sequence was replaced by the Ω interposon (Prentki and Krisch, 1984) and then marker exchanged into the *E. chrysanthemi* chromosome (see Figure 1). Out of several recombinants (data not shown), one mutant strain, named BT105, was selected for further analysis. To investigate the effect of the *sap* mutation on outer membrane permeability, the susceptibility of BT105 to lysozyme, erythromycin, and rifampicin was assayed, and no difference with respect to the wild type was found (data not shown). Furthermore, no difference in the mutant with respect to the wild type was found for the

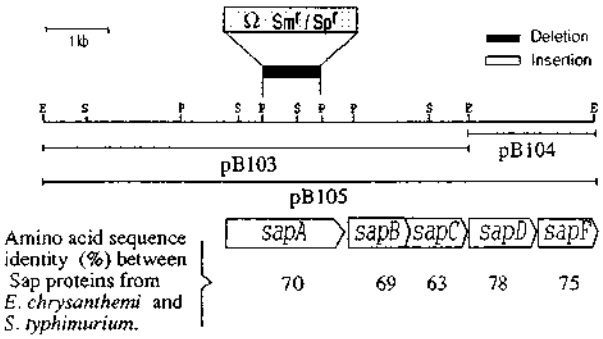

Figure 1. Genetic and Physical Map of the *sap* Region from *E. chrysanthemi*.

The deletion in *sapA* and insertion of the Ω interposon used for the construction of mutant BT105 are represented by bars. Binary alignments of the corresponding *E. chrysanthemi*– and *S. typhimurium*–deduced proteins were performed, and the identity percentage is indicated. E, EcoRI; P, PstI; S, SacII; Sm^r, streptomycin resistance; Sp^r, spectinomycin resistance.

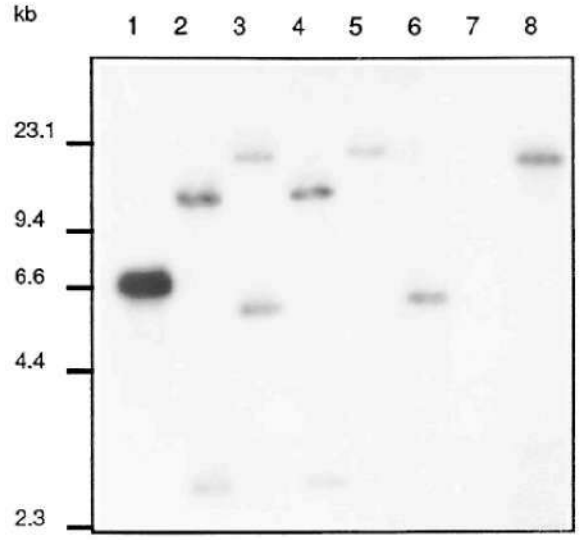

Figure 2. Distribution of *sap* Sequences among *Erwinia* spp.

Gel blot hybridization analysis was performed using total chromosomal DNA digested with EcoRI and probed with the labeled 964-bp PstI fragment internal to the *sap* operon (Figure 1), as described in Methods. Lane 1, *E. chrysanthemi* AC4150; lane 2, *E. carotovora* subsp *carotovora* NCPPB312; lane 3, *E. stewartii* 2994; lane 4, *E. c.* subsp *carotovora* ATCC15713; lane 5, *E. rhapontici* 1025; lane 6, *E. lupinicola* 3299; lane 7, *E. nigrifluens* 3026; and lane 8, *E. rubrifaciens* 3032. Length standards are given at left in kilobases.

following characteristics: growth rate, colony size and morphology, cell size and appearance, and production of pectic enzymes (data not shown).

Sensitivity of the BT105 mutant to antimicrobial peptides was compared with that of the wild type. Inhibition tests were performed in vitro with two peptides from potato tubers, namely, defensin-Pth1 and snakin-1, α-thionin from wheat endosperm, and salmon protamine. As shown in Figure 3, the mutant had an increased sensitivity for snakin-1 and α-thionin but not for defensin-Pth1 or protamine.

Mutant BT105 was transformed with pB105, which contains the whole *sapA* to *sapF* operon, and the complemented strain recovered the resistance to snakin-1 and α-thionin to wild-type levels.

Reduced Virulence of the *sap* Mutant

To investigate the possible effect on virulence of decreased resistance in vitro to antimicrobial peptides, potato tubers were chosen because typically they are affected by this pathogen and contain up to 0.3 mmol/kg fresh weight of snakin-1 (Moreno, 1995; A. Segura, M. Moreno, F. Madueño, A. Molina, and F. García-Olmedo, manuscript in preparation). The *sap* mutant showed increased sensitivity in vitro to snakin-1. In each of three independent experiments, potato tubers were pair inoculated with a suspension containing the wild-type or the mutant strain. Necrotic areas of the developed lesions were measured in all of the tubers after 48 hr. The data shown in Table 1 were subjected to statistical analysis. The average necrotic area of lesions produced by the mutant was 37% that of the wild type, and this difference was statistically significant. A typical result is shown in Figures 4A and 4D.

The effect of added peptide on lesion size was investigated by addition of purified snakin-1 (0, 70, and 140 μM) or α-thionin (0, 100, and 200 μM) to the inoculum (Figure 4). The intermediate concentration abolished the symptoms in the mutant but not in the wild type (Figures 4B and 4E). These concentrations were lower than those required for in

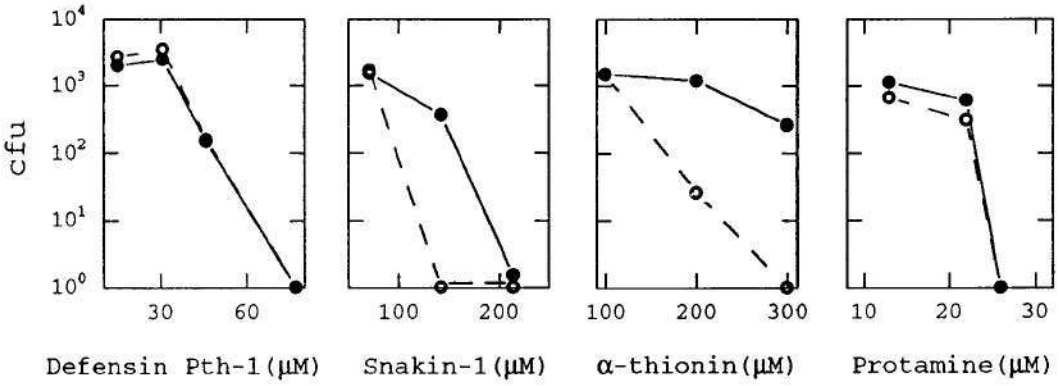

Figure 3. Susceptibility of *E. chrysanthemi* AC4150 and Mutant BT105 to Different Antimicrobial Proteins.

Bacteria and peptides were incubated at 30°C for 6 hr, diluted, and plated in King's B agar plates (King et al., 1954). Bacterial survival was measured by counting colony-forming units (cfu). The results are for a typical experiment from three independent trials, and the magnitude of standard errors was smaller than are the symbols in all cases. Closed circles, *E. chrysanthemi* AC4150; open circles, mutant BT105.

Table 1. Effects of Δ(*sap* A)::Ω Mutation on the Virulence of *E. chrysanthemi* on Potato Tubers

Experiment Number[a]	Size of Lesion (cm^2, mean ±SE)[b]	
	AC4150	BT105 (Sap^-)
1	1.28 ± 0.12	0.73 ± 0.11[c]
2	0.92 ± 0.13	0.34 ± 0.08[c]
3	0.79 ± 0.12	0.30 ± 0.08[c]

[a] In each experiment, 20 potato tubers were pair inoculated at two locations with 5×10^5 cells of wild-type and mutant strains; lesions were indicated by browning and maceration around the site of inoculation, as shown in Figure 4.
[b] Values are the product of the length and width of the necrotic area.
[c] Differences between parental and mutant strains are significant, according to the Student's *t* test ($P < 0.001$).

vitro inhibition of the mutant (Figure 3). The highest concentration abolished symptoms in the mutant and in the wild type (Figures 4C and 4F). These results are consistent with an additive effect of the incorporated peptide and those originally present in the plant tissue.

Growth rates in the plant of the wild type and the mutant were also determined by inoculation in potato tuber discs, as shown in Figure 5A. The population of the BT105 mutant strain was two orders of magnitude below that of the wild type along all of the time intervals. Figure 5B shows that browning of the inoculated discs correlated well with the estimated sizes of the bacterial populations.

DISCUSSION

Mutation of the *sap* Operon Supports the Involvement of Plant Antimicrobial Peptides in Defense

In animal pathogenesis, it has been shown that a pathogen must overcome inhibitors from the host to be able to proliferate in it. Thus, LPS and *sap* mutants of the bacterium *S. typhimurium*, which are more sensitive to antimicrobial peptides than is the wild type, have decreased or no virulence (Macías et al., 1990; Groisman et al., 1992; Parra-Lopez et al., 1993). More recently, a correlation between in vitro peptide sensitivity and virulence has been found in the analysis of mutants and complemented strains of the *rfaF* gene, which encodes a heptose transferase involved in LPS biosynthesis in the plant pathogen *R. solanacearum* (Titarenko et al., 1997). However, indirect effects of the mutation in the *rfaF* gene could not be completely excluded as responsible for the changes in virulence. This type of effect is less likely to occur in the case of the *sap* mutation reported here, because we have failed to find any additional effects of this mutation other than differential peptide sensitivity. Our results are consistent with a significant role of the Sap system in *E. chrysanthemi* virulence, which is similar to what has been observed in animal pathogenesis. This finding further supports the hypothesis that antimicrobial peptides are part of a primitive defense system that predates the evolutionary separation of plants and animals.

The ability of the Sap system to discriminate among different types of plant antimicrobial peptides raises the possibility that the specificity of the interaction between plant and pathogen might depend not only on the recognition of avirulence factors by the products of plant resistance genes but also on the recognition of predominant plant antimicrobial peptides by the pathogen resistance system. Indeed, the significant differences in virulence observed in the assays with potato tubers are consistent with the snakin-1 peptide being a key determinant of the interaction. In a screening of

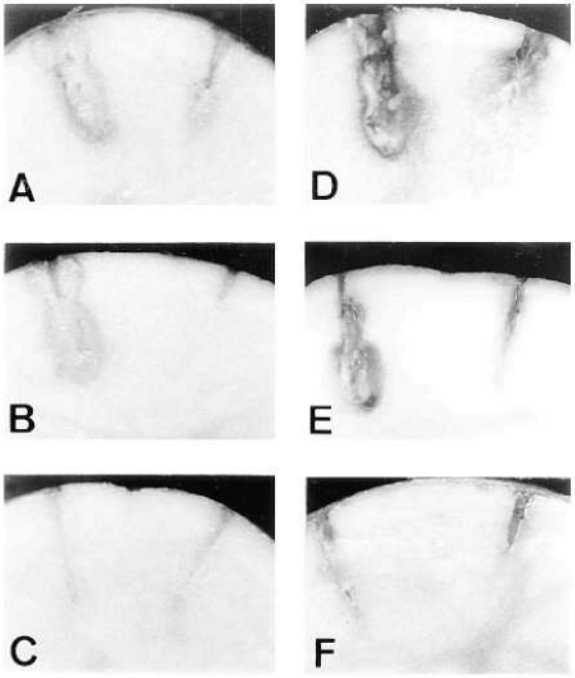

Figure 4. Soft-Rot Symptoms Produced by *E. chrysanthemi* AC4150 and Mutant BT105 on Potato Tubers.

Each tuber was pair inoculated (AC4150 at left and BT105 at right) three times with 50 μL of a suspension containing 5×10^5 cells plus the peptide.
(A) and **(D)** Control.
(B) Thionin (100 μM).
(C) Thionin (200 μM).
(E) Snakin (70 μM).
(F) Snakin (140 μM).

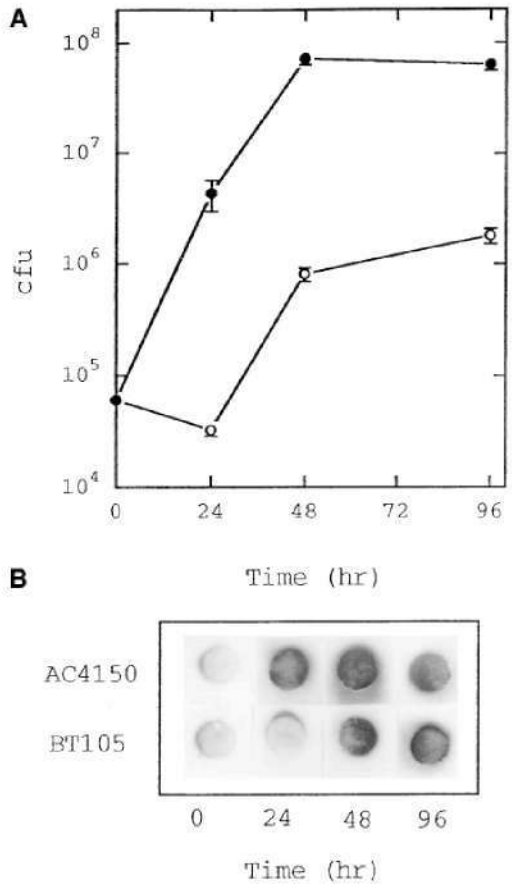

Figure 5. Growth of *E. chrysanthemi* AC4150 and Mutant BT105 on Potato Tuber Discs.

After inoculation of the discs with 5×10^4 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 (closed circles) and mutant BT105 (open circles) on potato discs at different times. Bars represent SE.

(B) Browning of potato discs at different times, inoculated as in **(A)**.

antibacterial peptides of potato tubers, snakin-1 was the peptide found at the highest concentration (0.3 mmol/kg fresh weight) (Moreno, 1995; A. Segura, M. Moreno, F. Madueño, A. Molina, and F. García-Olmedo, manuscript in preparation). This in vivo concentration is within the range of peptide concentrations in which differential inhibition of mutant BT105 was found in the in vitro assays. In contrast, the concentration of defensin Pth-1, the second most abundant antimicrobial peptide in potato tuber, was 100-fold lower (Moreno et al., 1994). Intraspecific and interspecific variations in peptide composition in plants could contribute significantly to the determination of host specificity and range in combination with observed natural differences in peptide sensitivity among strains of a given pathogen (Molina and García-Olmedo, 1993). Furthermore, at least part of the reported differences of virulence among different strains of *E. chrysanthemi* when confronted with different hosts (Dickey, 1978; Boccara et al., 1991) could be related to variation in plant peptide composition and/or changes of peptide specificity of the *sap* operon among the different pathogen strains.

The quantitative importance of the *sapA* to *sapF* operon contribution to virulence can be ascertained by comparing the effects of its mutation with those of *pelE* and *hrp* genes. Thus, in the standard potato tuber virulence assay of *E. chrysanthemi*, the *sap* mutant produced lesions that were ~37% the size of those of the wild type, whereas a mutant lacking PelE, the most important pectolytic enzyme for maceration, has been reported to retain 50% of wild-type virulence (Payne et al., 1987).

There is ample evidence that *Erwinia* spp elicit the full array of defense reactions in the plant (Davis and Ausubel, 1989; Castresana et al., 1990; Palva et al., 1992; Yang et al., 1992; Bauer et al., 1994). Nevertheless, *E. chrysanthemi* causes disease in a large number of different hosts, which suggests that this bacterium has evolved efficient mechanisms to withstand the host's defense mechanisms and that these mechanisms are important for pathogenicity. The Sap system is one of them.

Several recent reports have shown that animal and bacterial pathogens share common pathogenic strategies, namely, that some of the above-mentioned *hrp* genes from phytopathogenic bacteria are homologs to certain components of the type III secretion system found in *Yersinia*, *Shigella*, and *Salmonella* spp (Van Gijsegem et al., 1993). The *hrp* genes play a key role in pathogenicity through their ability to deliver virulence proteins into the animal or plant host cells (Alfano and Collmer, 1996). According to the results reported here, the Sap system is also a common feature in animal and plant bacterial pathogenesis.

Conservation and Specificity of the *sapA* to *sapF* Operon

The conservation of gene order and the sequence similarity of the *sapA* to *sapF* operon from *E. chrysanthemi* with respect to that from *S. typhimurium* are consistent with the evolutionary relatedness of the two bacterial species. However, functional divergence has probably occurred, as shown by the different behavior of the two versions of the operon with respect to protamine: mutation of the operon in the animal pathogen determined differential sensitivity to protamine and melittin but not to other peptides, such as magainin-2 and defensin NP-1 (Groisman et al., 1992; Parra-Lopez et al., 1993), whereas that reported here for the plant pathogen affects sensitivity to snakin-1 and α-thionin but not to protamine and defensin-Pth1. This suggests that coevolution of

the defense mechanism with the predominant pathogen(s) of the host may have occurred.

Our data show that the *sapA* to *sapF* operon is responsible for specific resistance to given antimicrobial peptides and not to a general one. The model for *sap* operon function in *S. typhimurium* proposes that the first step of the defense mechanism consists of the binding of the periplasmic component SapA to the antimicrobial peptide (Parra-Lopez et al., 1993). This binding could provide the observed specificity with respect to the different peptides. However, specificity could also reside on any of the other proposed steps of the Sap mechanism or even depend on other genetic factors outside of the *sap* operon.

In contrast to the mechanism of resistance to antimicrobial peptides associated with the LPS (Macías et al., 1990; Groisman, 1994; Titarenko et al., 1997), the Sap mechanism is not associated with changes in outer membrane permeability because susceptibility to lisozyme, erythromycin, and rifampicin was unchanged in the mutant. This mechanism seems to have little or no involvement in other cellular processes, as determined by the lack of pleiotropic effects of the mutation on properties such as growth rate in liquid medium, colony size and morphology, cell size and appearance, and production of pectic enzymes. Our data show that the Sap system confers specific resistance to certain antimicrobial peptides and that this feature makes an important contribution to bacterial virulence. Determining the Sap mechanism of resistance should help us to understand the basis for its specificity and its importance in different plant hosts.

METHODS

Microbiological Methods, and DNA Manipulation and Sequencing

Bacterial strains and plasmids used in this work are described in Table 2. Strains of *Escherichia coli* were cultivated at 37°C in Luria-Bertani medium. Strains of *Erwinia chrysanthemi* were cultivated at 30°C in nutrient broth (Difco) or King's B medium (King et al., 1954). Antibiotics were added to the media at the following concentrations: ampicillin, 100 μg/mL; spectinomycin, 25 μg/mL; and streptomycin, 125 μg/mL (for multicopy plasmid resistance) or 10 μg/mL (for chromosomal resistance). Marker exchange in *E. chrysanthemi* was performed as described by Roeder and Collmer (1985). A genomic library of *E. chrysanthemi* was constructed in λ ZAP II and in λ FIX II (Stratagene, La Jolla, CA). Phagemid pBluescript SK− (Stratagene) was used for subcloning. Standard molecular cloning techniques employed in this study (small- and large-scale plasmid and genomic DNA purification, restriction enzyme digestion [Pharmacia], agarose gel electrophoresis, DNA subcloning [Stratagene], blot and hybridization and colony screening by hybridization [Amersham]) were performed as described by Sambrook et al. (1989) and following the manufacturers' indications. DNA sequencing of both strands was done by the chain

Table 2. Bacterial Strains and Plasmids Used in This Study

Designation	Relevant Characteristics	Source or Reference
E. coli		
DH5α	*supE44 ΔlacU169 (ø80 lacZM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1983)
SOLR™	*e14—(mcrA) Δ (mcrCB-hsdSMR-mrr) 171 sbcC recB recJ umuC::*Tn*5(kan^r) uvrC lac gyrA96 relA1 thi-1 endA1 F′ (F′proAB,lacIZΔM15) Su⁻* (nonsuppressing)	Stratagene
XL1-Blue MRF′	*Δ (mcrA) 183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* ([*F′proAB,lacIZ ΔM15*] Tn*10* [*tet^r*])	Stratagene
E. chrysanthemi		
AC4150	Wild-type strain	Chatterjee et al. (1983)
BT105	Δ(*sap* A)::ΩSp^r/Sm^r derivative of AC4150	This work
Plasmids and phages		
pBluescript II SK−	Amp^r	Stratagene
pB103	pBluescript II carrying AC4150 *sapA* to *sapC* genes	This work
pB104	pBluescript II carrying AC4150 *sapD* to *sapF* genes	This work
pB105	pBluescript II carrying AC4150 *sapA* to *sapF* genes	This work
λ ZAP II	Phage vector	Stratagene
λ FIX II	Phage vector	Stratagene

termination method on double-stranded DNA templates, using an Abiprism Dye Terminator cycle sequencing kit (Perkin-Elmer) in a 377 DNA Sequencer (Perkin-Elmer). Sequence alignments were performed at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), using the Blast network service (Atschul et al., 1990).

Antimicrobial Peptide Purification

Thionin was purified from wheat flour, as described by Ponz et al. (1982). Defensin-Pth1 and snakin-1 were purified from potato tuber as follows: 20 g of frozen material was ground to powder in liquid nitrogen, using a mortar and pestle, and extracted once with 80 mL of buffer (0.1 M Tris-HCl, 10 mM EDTA, pH 7.5) and twice with 80 mL of H_2O. The resulting pellet was then extracted with 50 mL of 1.5 M LiCl at 4°C for 1 hr, and the extract was dialyzed against 5 liters of water using a Spectra/Por 6 (molecular weight cutoff, 3000; Spectrum, Laguna Hills, CA) membrane and freeze dried. The extract was subjected to reverse-phase HPLC, as described by Molina et al. (1993). Collected peaks were analyzed by SDS-PAGE in preformed gradient gels (4 to 20%; Bio-Rad). Protamines were purchased from Sigma.

Susceptibility and Virulence Assays

Susceptibility to antimicrobial peptides was assayed as follows: Log phase cells grown in nutrient broth were diluted to 10^5 colony-forming units per mL in 0.3 × nutrient broth, and 10 μL of the diluted material was placed in an Eppendorf tube and added to the appropiate amount of peptide dissolved in the same solution to reach the desired concentration. Cells and peptides were incubated for 6 hr at 30°C with shaking, and then a portion of each sample was diluted and plated on nutrient broth agar plates to assess bacterial viability.

Virulence of *E. chrysanthemi* AC4150 wild type and BT105 mutant was assayed by inoculating 50 μL of a suspension containing 5×10^5 bacteria in a potato tuber, cv Jaerla, purchased in a local supermarket. The bacteria were contained in a plastic tip, which was inserted at a constant depth of 1.5 cm. Two inoculations (wild-type and mutant strains) were made in each tuber. Potatoes were left at 30°C with 100% relative humidity for 48 hr. After this time, tubers were sliced at the inoculation point, and the damage was estimated by measuring the affected area. Differences between the wild type and the mutant were assessed statistically using a paired Student's *t* test. To monitor bacterial growth in potato tuber, 50 μL of a bacterial suspension containing 10^4 bacteria was inoculated in 1-cm in diameter potato discs. Discs were incubated at 30°C and high humidity, and were 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.

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